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Article

The Small-Molecule Iron Transport Inhibitor Ferristatin/NSC306711 Promotes Degradation of the Transferrin Receptor

Lior Horonchik[1] and Marianne Wessling-Resnick[1,*]
[1]Department of Genetics and Complex Diseases, Harvard School of Public Health, Boston, MA 02115, USA
*Correspondence: wessling@hsph.harvard.edu
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SUMMARY

Iron delivery by transferrin (Tf) is accomplished through clathrin-mediated endocytosis of Tf receptors. The small molecule NSC306711 inhibits iron uptake from the Tf-TfR pathway. Here we show that the drug's mechanism of action is to induce internalization and degradation of unoccupied Tf receptors through an unexpected endocytic pathway. Unlike classical clathrin-mediated Tf receptor endocytosis, internalization promoted by NSC306711 is independent of clathrin and dynamin, and is sensitive to the cholesterol-depleting agents filipin and nystatin. The finding of this cholesterol-dependent Tf receptor internalization pathway through use of the small-molecule inhibitor sheds light on the pleiotropic nature of membrane trafficking dynamics and adds a complex dimension to our understanding of receptor regulation. Because of its unusual properties to inhibit iron uptake, we refer to NSC306711 as "ferristatin."

INTRODUCTION

Iron is an essential nutrient required for DNA synthesis, energy production, and normal cellular growth. Iron circulates in the plasma bound to transferrin (Tf) (Hentze et al., 2004). Most cells express Tf receptors on their surface to bind and internalize Tf by receptor-mediated endocytosis (Karin and Mintz, 1981; Klausner et al., 1983b). Elements of Tf-mediated iron delivery have been exploited to elucidate a mechanistic understanding of the endocytic process (Conner and Schmid, 2003; Kirchhausen, 2000; Ungewickell and Hinrichsen, 2007). This well-established pathway is initiated by the clustering of Tf receptors in clathrin-coated pits on the plasma membrane and subsequent internalization by clathrin-coated vesicles. After clathrin coat disassembly, the endocytic vesicles fuse with and transfer their contents to a compartment known as the early endosome. The endosome's acidic environment promotes the release of diferric iron from Tf. After reduction of Fe^{3+} to Fe^{2+}, ferrous iron is transported across the endosomal membrane to the cytosol (Ohgami et al., 2005), where it may participate in iron-dependent metabolism (e.g., heme biosynthesis) or become stored for future purpose (e.g., incorporated into ferritin) (Hentze et al., 2004). After the release of iron from Tf, apo-Tf remains bound to the Tf receptor and returns to the plasma membrane via recycling endosomes. At the cell surface, apo-Tf dissociates and is released for further rounds of iron delivery (Dautry-Varsat et al., 1983; Klausner et al., 1983a).

In a screen of the National Cancer Institute's Diversity Set, we previously discovered ten small-molecule inhibitors of iron uptake (Brown et al., 2004). One of these compounds, NSC306711, was found to block Tf-mediated iron uptake with IC_{50} ~20 μM. Here we show that NSC306711 targets the Tf receptor by inducing its internalization and degradation. This activity uncovered an unexpected endocytic pathway for Tf receptor internalization. Unlike the classical clathrin-mediated endocytic pathway, internalization of the Tf receptor induced by NSC306711 is independent of clathrin and dynamin-1. Instead, it is sensitive to filipin and nystatin, cholesterol-depleting agents that disrupt membrane trafficking by lipid rafts. Use of this small-molecule inhibitor sheds new light on the pleiotropic nature of membrane trafficking dynamics and adds a complex dimension to receptor regulation. Because of its unusual properties to inhibit iron uptake through a cholesterol-dependent pathway, we refer to NSC306711 as "ferristatin."

RESULTS

Ferristatin Inhibits Endocytosis of Tf

Ferristatin, or NSC306711 (Figure 1A), was found to inhibit iron transport in an earlier screen for small-molecule inhibitors of iron uptake (Brown et al., 2004). It is a polyaromatic compound that contains copper. As shown in Figure 1B, ferristatin not only blocks ^{55}Fe assimilation but also inhibits internalization of ^{125}I-labeled Tf, suggesting that the drug disrupts Tf internalization to acidic endocytic compartments where iron is released for cellular delivery (Dautry-Varsat et al., 1983; Klausner et al., 1983a). This idea is supported by fluorescence microscopy experiments showing that HeLa cells treated with ferristatin take up very little Alexa-red Tf (Figure 1C). Inhibition of Tf internalization is not due to a global effect on endocytosis, because uptake of FITC-dextran and DiI-LDL remain unaffected.

Ferristatin Induces the Degradation of Tf Receptor

To better understand how ferristatin perturbs uptake of Tf, ligand binding was measured for cells treated with or without ferristatin for 4 hr at 37°C. Specific cell-associated radioactivity was then determined at 4°C using [^{125}I]Tf (Figure 2A). The results of these experiments demonstrate that the presence of ferristatin does

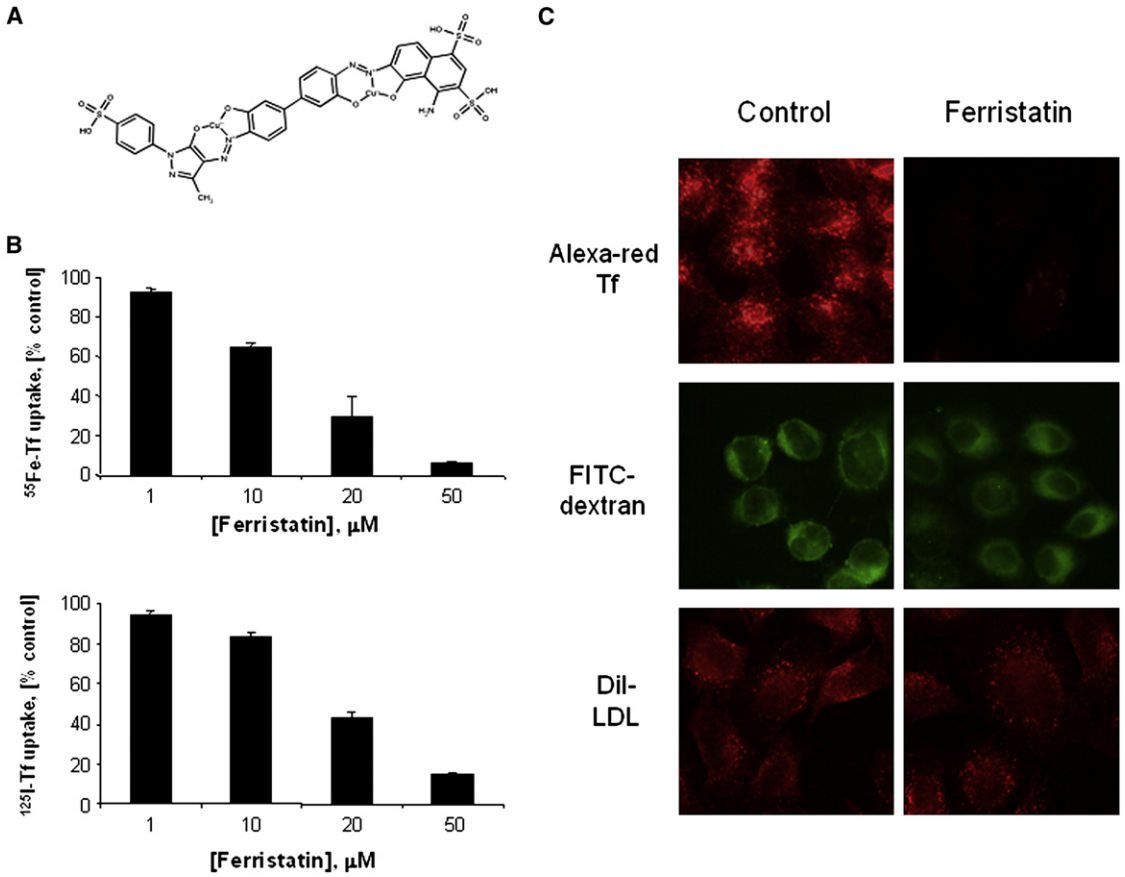

Figure 1. Inhibition of Tf Uptake by Ferristatin

(A) Structure of ferristatin (NSC306711).

(B) HeLa cells were incubated for 4 hr at 37°C in serum-free media containing 40 nM [^{55}Fe]Tf (upper) or [^{125}I]Tf (lower) and the indicated concentrations of ferristatin. After chilling on ice in the presence of unlabeled Tf to displace any surface-bound ligand, cells were washed and then suspended in lysis buffer, and the amount of [^{55}Fe]Tf or [^{125}I]Tf taken up was determined and normalized to cell protein. Shown are the means (±SEM) from two experiments performed in duplicate for [^{55}Fe]Tf uptake (n = 4) and in triplicate for [^{125}I]Tf uptake (n = 6). Identical results for [^{55}Fe]Tf uptake were obtained using ferristatin-treated HEK293T cells.

(C) HeLa cells were incubated for 4 hr at 37°C with or without 50 μM ferristatin, and then Alexa-red Tf, FITC-dextran, or DiI-LDL were added to the cells for an additional 20 min incubation. The cells were then placed on ice, rinsed, and fixed with 4% paraformaldehyde and mounted. Cells were examined by fluorescence microscopy (Nikon Eclipse E600, Zeiss) at a nominal magnification of 60×. Digital images were taken using a Spot Slider camera and Spot Advance software (RT Diagnostics).

not interfere with Tf binding to its receptor because without preincubation there was no loss of cell-surface binding in the presence of the drug compared to control (untreated) cells. However, the number of receptors on the cell surface was significantly reduced after a 4 hr preincubation with ferristatin. Western blot analysis revealed that after this incubation period, the total amount of Tf receptor protein was reduced by 60%–70% compared to cells treated with vehicle (DMSO) alone (Figure 2B). In contrast, LDL receptor levels were not affected, in agreement with the fluorescence microscopy results for DiI-LDL uptake (Figure 1D).

To determine the dose response for ferristatin's effects, western blot analysis was also carried out on cell lysates obtained after a 4 hr pretreatment at 37°C with increasing concentrations of the drug (Figure 2C). Loss of Tf receptors was dose dependent over the same concentration range observed to inhibit [^{55}Fe]Tf and [^{125}I]Tf uptake (Figure 1B). Identical results were obtained when the cells were treated with the same concentrations of ferristatin for 24 hr (data not shown). Time-course experiments indicated that >65% of the protein was lost within 4 hr of treatment with 50 μM ferristatin (Figure 2D). Because the Tf receptor's half-life is fairly long (14–18 hr) (Davies et al., 2003; Rutledge et al., 1991; Ward et al., 1982), the significant reduction in protein levels over this time frame is most likely due to enhanced receptor degradation rather than reduced biosynthesis. Consistent with this idea, downregulation of [^{125}I]Tf cell-surface binding in the presence of ferristatin occurred more rapidly than loss of receptor protein (Figure 2E). These combined results suggest that ferristatin induces degradation of cell-surface Tf receptors after their internalization, thereby reducing the number of receptors recycling back to the cell surface.

Both Lysosomal and Proteasomal Inhibitors Block Ferristatin-Induced Degradation

To confirm that the loss of Tf receptors arises due to increased degradation, cells were treated with the lysosomal inhibitors

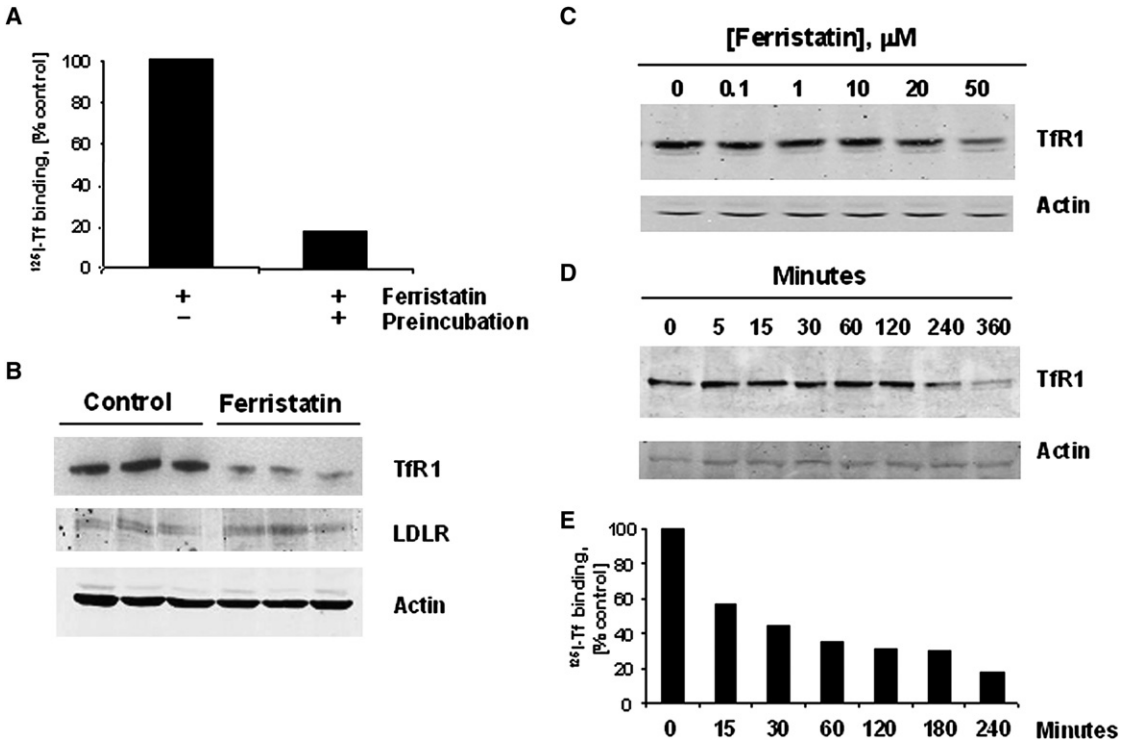

Figure 2. Ferristatin Induces the Degradation of Tf Receptor

(A) HeLa cells were incubated for 4 hr at 37°C with or without 50 μM ferristatin, and then both sets of cells were further incubated for 2 hr on ice with 50 μM ferristatin and 500 nM [^{125}I]Tf in the presence or absence of 5 μM unlabeled Tf. After washing, cell-associated radioactivity was determined, and the difference measured in the presence and absence of unlabeled Tf was calculated as specific cell-surface binding and normalized to control (treated with vehicle alone). The average of duplicate values is shown; similar results were obtained on three different occasions.

(B) HeLa cells were treated with or without 50 μM ferristatin for 4 hr at 37°C and cell lysates were prepared and electrophoresed on a 7.5% SDS-polyacrylamide gel. After transfer to nitrocellulose, immunoblotting was used to detect Tf receptor (top) or LDL receptor (middle) levels as described in the Experimental Procedures. Actin was also immunoblotted as a loading control (bottom).

(C) HeLa cells were incubated for 4 hr with increasing concentrations of ferristatin as shown. After washing, cell lysates were prepared and immunoblotted for Tf receptor levels.

(D) HeLa cells were treated with 50 μM ferristatin for the indicated times, and then lysates were prepared and immunoblotted to determine Tf receptor levels.

(E) HeLa cells were treated with or without 50 μM ferristatin at 37°C for the indicated times. Cell-surface [^{125}I]Tf binding was determined as described for (A). The average of duplicate values is shown; similar results were obtained on three different occasions.

bafilomycin A_1, leupeptin, or chymostatin, and then further incubated with or without ferristatin. Ferristatin-induced Tf receptor degradation was blocked by each of these three inhibitors (Figure 3A). Cells were also treated with two proteasome inhibitors, epoxomicin and MG132, prior to incubation with ferristatin. Cells treated with the proteasome inhibitors were also resistant to ferristatin-induced receptor degradation (Figure 3B). Thus, both lysosomal and proteosomal pathways appear to play a role in ferristatin-induced receptor degradation. To determine whether lysosome or proteasome inhibition interfered with internalization of Tf receptors, cells were again treated with chymostatin (to inhibit lysosomal protease activity) or epoxomicin (to block proteasomal activity), and then incubated with or without ferristatin and assayed for surface [^{125}I]Tf binding. The amount of Tf bound to the cell surface was reduced even though ferristatin-induced Tf receptor degradation was blocked by both inhibitors (Figures 3C and 3D). These combined observations indicate that steps sensitive to both lysosomal and proteosomal inhibitors occur after the receptor is internalized.

Characterization of Tf Receptor Trafficking in the Presence of Ferristatin

To explore whether Tf receptor internalization in the presence of ferristatin occurs by the classical clathrin-mediated endocytic pathway, we used siRNA to knock down clathrin expression (Huang et al., 2004). Fluorescence microscopy confirmed the reduction of clathrin levels in siRNA-treated cells and showed that uptake of Alexa-red Tf by HeLa cells was blocked, with pronounced surface staining observed in comparison to vehicle control (Figure 4A). However, in the presence of ferristatin, Tf receptors were still lost from these cells, with little Alexa-red Tf staining observed. Thus, the Tf receptor is internalized and becomes degraded in the presence of ferristatin by a clathrin-independent mechanism.

Dynamin is a GTPase known to participate in clathrin-mediated endocytosis (Conner and Schmid, 2003; Kirchhausen, 2000; Ungewickell and Hinrichsen, 2007). To study its role, cells were transfected with either wild-type (WT) or the dominant-negative K44A mutant of dynamin-1. Seventeen hours posttransfection,

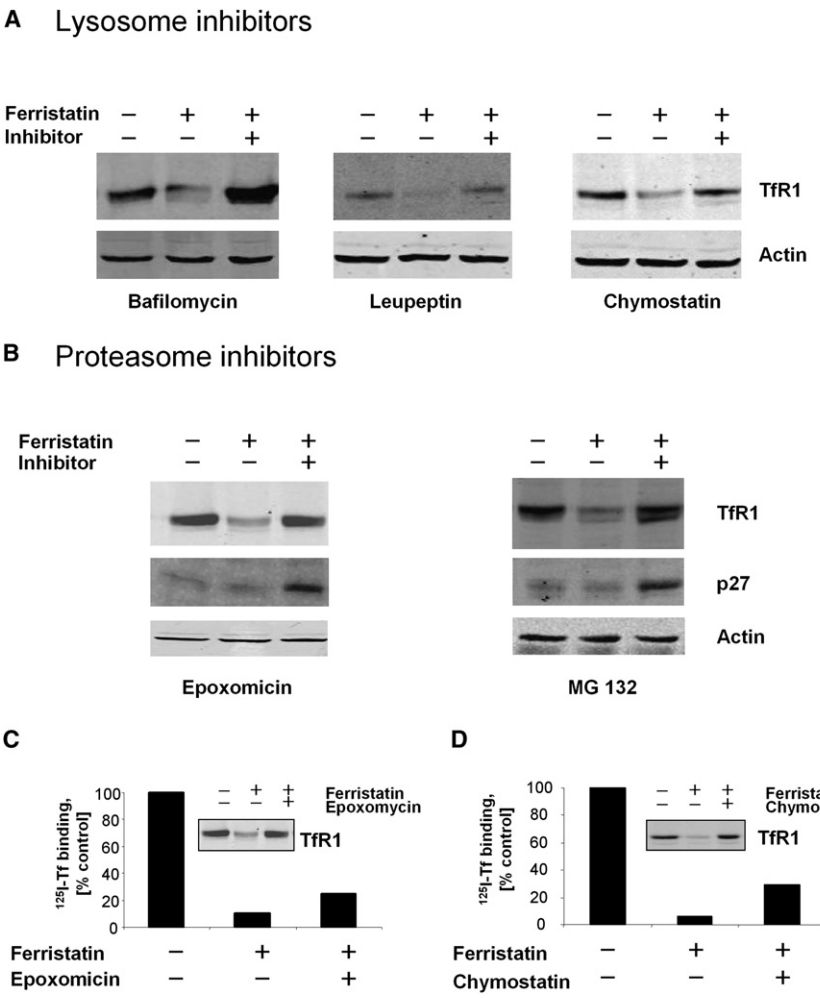

Figure 3. Lysosomal and Proteasomal Inhibitors Block Ferristatin-Induced Tf Receptor Degradation

(A) HeLa cells were treated overnight with the lysosomal inhibitors bafilomycin A_1 (100 nM), leupeptin (90 μg/ml), or chymostatin (10 μg/ml).

(B) HeLa cells were treated overnight with the proteasomal inhibitors epoxomicin (100 nM) and MG132 (20 μM). After preincubation with these inhibitors, the cells were washed and further incubated in fresh serum-free medium with or without the inhibitors and with or without 50 μM ferristatin as indicated. After a 4 hr incubation, cells were washed and lysed and the samples were immunoblotted to detect Tf receptor. Actin levels were also determined as a loading control. Immunoblots detecting p27 levels as a positive control for proteasome inhibition are included.

(C and D) HeLa cells were pretreated overnight with 100 nM epoxomicin (C) or 10 μg/ml chymostatin (D) and then further treated with or without 50 μM ferristatin for 4 hr as indicated. Cells were placed on ice and incubated for 2 hr with 500 nM [^{125}I]Tf in the presence or absence of 5 μM unlabeled Tf. After washing, cell-associated radioactivity was determined, and the difference measured in the presence and absence of unlabeled Tf was calculated. The average of duplicate values is shown; similar results were obtained on two different occasions. Cell lysates were also immunoblotted to determine Tf receptor levels (insets).

cells were treated with ferristatin and then incubated with Alexa-red Tf. Figure 4B shows that uptake of Alexa-red Tf was blocked, with most surface staining observed in cells transfected with the dominant-negative dynamin mutant; however, in the presence of ferristatin, very little Alexa-red Tf staining was observed, indicating that Tf receptors were lost from cells transfected with wild-type as well as the K44A mutant. The fact that loss of Tf receptors occurred in the presence of the dominant-negative mutant suggests that dynamin is not involved in the ferristatin-induced internalization pathway.

Alternate pathways for endocytic uptake that are independent of clathrin and dynamin involve lipid rafts and are known to be sensitive to cholesterol depletion (Brown, 2006; Kirkham and Parton, 2005; Parton and Richards, 2003). Therefore, the effects of filipin and nystatin, two cholesterol-binding agents, were studied. Western blot analysis showed that Tf receptor degradation induced by ferristatin was blocked by cholesterol depletion (Figure 5A). This effect correlated with the ability of nystatin to block the internalization of Tf receptors in the presence of ferristatin, as measured by cell-surface [^{125}I]Tf binding (Figure 5B). Ferristatin did not appear to have more generalized effects on the lipid raft endocytic pathway, because trafficking of cholera toxin subunit B was unaffected (Figure 5C). This nontoxic subunit enters cells by binding to the lipid raft ganglioside GM1 (Miller et al., 2004). Furthermore, ferristatin did not alter the stability of flotillin, a resident of the lipid raft pathway (Bickel et al., 1997), as shown by western blot analysis (Figure 5D). Because nystatin-sensitive endocytosis has been implicated in some investigations to involve the actin cytoskeleton (Chichili and Rodgers, 2007; Kirkham and Parton, 2005; Parton and Richards, 2003), the influence of cytochalasin B, latrunculin A, and jasplakinolide were studied. Cell-surface ligand binding and western blot analysis showed that none of these actin inhibitors blocked ferristatin-induced Tf receptor internalization or degradation (see Figure S1 available online).

DISCUSSION

As a small-molecule inhibitor, ferristatin (NSC306711) was previously shown to block iron assimilation from Tf (Brown et al., 2004). The results reported here reveal that ferristatin targets this pathway by inducing the internalization and subsequent degradation of cell-surface Tf receptors. Our characterization of ferristatin's mechanism of action has yielded insights about Tf receptor regulation and trafficking. Ferristatin appears to enhance endocytosis of Tf receptors by nystatin-sensitive internalization via lipid rafts. The fact that its activity is independent of clathrin and dynamin supports the idea that this alternative pathway is induced by ferristatin to mediate receptor endocytosis.

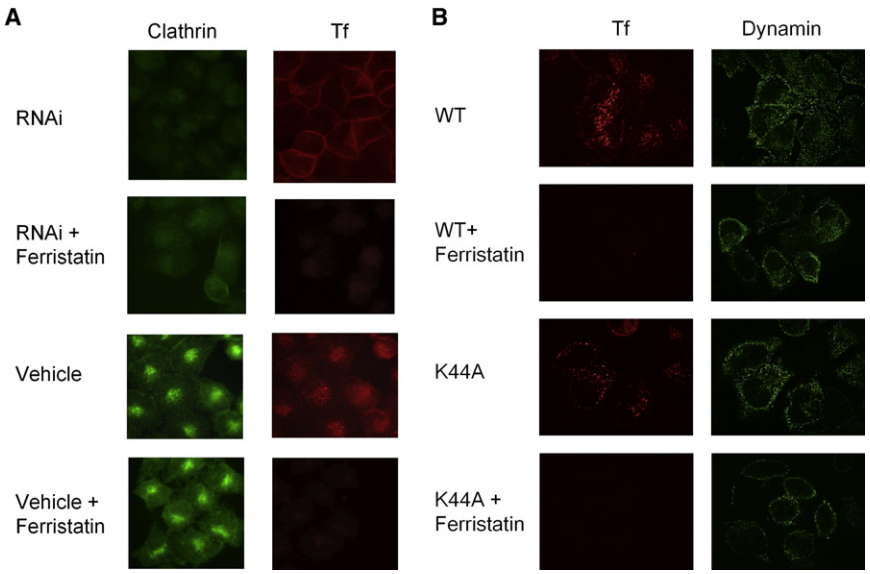

Figure 4. Ferristatin-Induced Tf Receptor Degradation Is Clathrin and Dynamin Independent

(A) HeLa cells were transfected with siRNA to knock down expression of clathrin as described in the Experimental Procedures. After incubation with 50 μM ferristatin for 4 hr at 37°C, Alexa-red Tf (5 μg/ml) was added for an additional 10 min. The cells were then rinsed with ice-cold PBS and fixed with 4% paraformaldehyde. After permeabilization with 0.1% Triton X-100, the cells were immunostained with a monoclonal anti-clathrin antibody (X22) and a secondary goat anti-mouse antibody conjugated to Alexa Fluor 488.

(B) HeLa cells were transfected with either wild-type (WT) or the dominant-negative K44A mutant of dynamin-1. After a 4 hr incubation with 50 μM ferristatin, Alexa-red Tf (5 μg/ml) was added to the cells for 20 min to monitor Tf uptake. The cells were then washed, fixed, and immunostained with mouse anti-dynamin antibody (Hudy1) using an Alexa Fluor 488-conjugated secondary antibody as described above. Cells were examined by confocal microscopy on a Nikon TE2000 inverted microscope at a nominal magnification of 100×. Digital images were taken using an Orca ER cooled CCD camera (Hamamatsu Photonics) and processed using Slidebook software (Intelligent Imaging Innovations).

Elements of Tf receptor endocytosis are well characterized (Karin and Mintz, 1981; Klausner et al., 1983a, 1983b; Dautry-Varsat et al., 1983), including functions associated with coated vesicle biogenesis involving clathrin and dynamin (Conner and Schmid, 2003; Kirchhausen, 2000; Ungewickell and Hinrichsen, 2007). Unfortunately, few studies have explored alternative pathways for Tf receptor trafficking that are known to regulate receptor uptake in other systems. For example, high concentrations of epidermal growth factor (EGF) promote internalization of its receptor through a clathrin-independent pathway involving lipid rafts; this pathway has also been shown to enhance degradation of the EGF receptor (Sigismund et al., 2005). Such alternative endocytic pathways can have important regulatory functions. For example, the insulin-regulated glucose transporter GLUT4 is internalized via a nystatin-sensitive pathway that is blocked by insulin (Blot and McGraw, 2006). The transforming growth factor β superfamily also utilizes two distinct endocytic pathways for different receptor functions. Internalization through the clathrin-dependent pathway promotes cell signaling whereas receptor uptake via lipid rafts regulates receptor turnover (Di Guglielmo

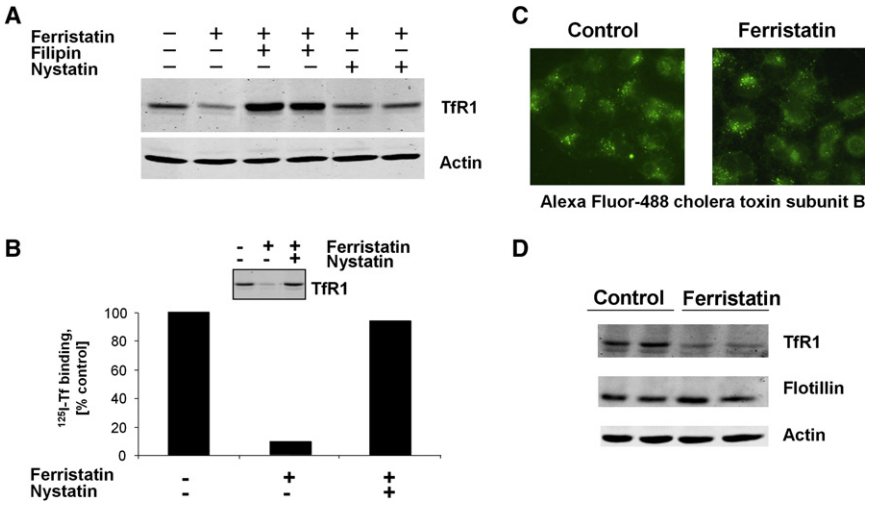

Figure 5. Internalization of Tf Receptors is Sensitive to Cholesterol Depletion

(A) HeLa cells were pretreated for 30 min with 5 μg/ml filipin or 25 μg/ml nystatin or left untreated. After incubation with 50 μM ferristatin for 4 hr, cell lysates were prepared and electrophoresed for immunoblotting to detect Tf receptor (upper) or actin (lower).

(B) HeLa cells were pretreated for 30 min with 25 μg/ml nystatin and then further treated with or without 50 μM ferristatin for 4 hr as indicated. Cells were placed on ice and incubated for 2 hr with 500 nM [^{125}I]Tf in the presence or absence of 5 μM unlabeled Tf. After washing, cell-associated radioactivity was determined, and the difference measured in the presence and absence of unlabeled Tf was calculated. The average of duplicate values is shown; similar results were obtained on two different occasions. Cell lysates were also immunoblotted to determine Tf receptor levels (inset).

(C) HeLa cells were incubated for 4 hr at 37°C with or without 50 μM ferristatin, and then Alexa Fluor 488 cholera toxin subunit B was added to the cells for an additional 30 min incubation. The cells were then placed on ice, rinsed, and fixed with 4% paraformaldehyde and mounted. Cells were examined by fluorescence microscopy using a Zeiss Axiotome microscope equipped with Axiovision software at a nominal magnification of 60×.

(D) HeLa cells were treated with or without 50 μM ferristatin for 4 hr at 37°C and cell lysates were prepared and electrophoresed on a 7.5% SDS-polyacrylamide gel. After transfer to nitrocellulose, immunoblotting was used to detect Tf receptor (top) or flotillin (middle) levels as described in the Experimental Procedures. Actin was also immunoblotted as a loading control (bottom).

et al., 2003). Through the use of the small-molecule inhibitor ferristatin, we have shown that lipid rafts can also mediate internalization of Tf receptors, and that this pathway is associated with receptor degradation.

Our study further shows that ferristatin-induced Tf receptor downregulation by the lipid raft pathway is associated with degradative loss of the receptor in a step that is sensitive to both proteasomal and lysosomal inhibitors. Neither of these inhibitors blocks receptor internalization, an observation that indicates endocytosis occurs independently of these functions. Our studies implicate a role for the proteosome in membrane protein degradation that is unexpected, particularly as Tf receptors are thought to be degraded by the lysosome (Rutledge et al., 1991). Although it is possible that ubiquitinylation of the Tf receptor itself could be involved in this process, we have not been able to detect this protein modification on the receptor (our unpublished observations). One speculation is that upon lipid raft internalization of Tf receptors, ferristatin's action promotes the proteasomal degradation of a factor that would otherwise prevent trafficking of receptors to the lysosome in the lipid raft endocytic pathway. However, other explanations for the influence of both proteosomal and lysosomal inhibitors cannot be excluded, and it is possible that ferristatin has multiple cellular effects. In fact, we have found that it disrupts non-Tf-bound iron uptake as well as the Tf-mediated delivery of iron; how these observations may be related remains unknown. Future use of the small-molecule inhibitor ferristatin should provide new insights about membrane trafficking of Tf receptors and its regulation, as well as the necessary means to identify new factors involved in iron transport.

SIGNIFICANCE

Iron is an essential nutrient required for DNA synthesis, energy production, and normal cellular growth. Iron circulates in the plasma bound to transferrin (Tf) (Hentze et al., 2004). Most cells express Tf receptors on their surface to bind and internalize Tf by receptor-mediated endocytosis (Karin and Mintz, 1981; Klausner et al., 1983b). Elements of Tf-mediated iron delivery have been exploited to elucidate a mechanistic understanding of the endocytic process (Conner and Schmid, 2003; Kirchhausen, 2000; Ungewickell and Hinrichsen, 2007). Using a chemical genetics approach, we have previously established that the small-molecule inhibitor ferristatin (NSC306711) blocks iron assimilation from Tf (Brown et al., 2004). Here we show that the drug's mechanism of action is to induce internalization and degradation of Tf receptors through an unexpected endocytic pathway. Unlike classical clathrin-mediated Tf receptor endocytosis, internalization promoted by ferristatin is independent of clathrin and dynamin, and is sensitive to the cholesterol-depleting agents filipin and nystatin. After nystatin-sensitive internalization, ferristatin-induced Tf receptor degradation is blocked by inhibitors of both the proteasome and lysosome. One plausible explanation for these observations is that ferristatin promotes degradation of a factor associated with intracellular Tf receptor trafficking by the proteosome, thereby releasing internalized receptors to the lysosomal degradative pathway. Regardless of how intracellular degradation occurs, the nystatin-sensitive degradation of Tf receptors induced by ferristatin ultimately accounts for its ability to block Tf-mediated iron uptake. The discovery of this novel cholesterol-dependent Tf receptor internalization pathway through use of the inhibitor demonstrates the utility of small molecules in the study of complex cellular processes.

EXPERIMENTAL PROCEDURES

Cell-Culture and Transfection Conditions

HeLa cells were grown in Dulbecco's minimal essential medium (DMEM) containing 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum (FBS). Cells were treated with ferristatin in serum-free DMEM. Cells were transiently transfected to express wild-type or K44A mutant dynamin-1 with plasmids kindly provided by Sanja Sever (Massachusetts General Hospital) using LipofectAMINE 2000 (Invitrogen) according to the manufacturers' instructions. Cells were transfected with siRNA duplexes against the clathrin heavy chain (Dharmacon) as described by Huang et al. (2004). Briefly, subconfluent HeLa cells were transfected twice with 20 μM siRNA duplex (day 1 and day 2) and then placed in fresh DMEM supplemented with 10% FBS for 14 hr prior to experiments, which were performed on day 3.

Transferrin Uptake and Binding Assays

Prior to Tf uptake assays, HeLa cells were washed with phosphate-buffered saline containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (PBS++) and then incubated for 4 hr at 37°C with [^{125}I]Tf in PBS++, with or without 50 μM ferristatin. As a control, cells were incubated with vehicle alone (0.5% DMSO). To quench uptake, cells were chilled on ice, washed with ice-cold PBS++, and incubated with 500 nM unlabeled Tf for 1 hr at 4°C to displace any surface-bound radioactivity. After washing with ice-cold PBS++, cells were lysed with 0.1% Triton X-100 containing 0.1% NaOH, and cell-associated radioactivity and protein levels were determined. Uptake, calculated as pmol [^{55}Fe]Tf or [^{125}I]Tf/mg cell protein, was normalized to control (vehicle alone). Cell-surface Tf binding was determined in a similar fashion, except that HeLa cells were treated with or without ferristatin, washed in ice-cold PBS++, and then incubated on ice for 2 hr with 500 nM [^{125}I]Tf in the presence or absence of 5 μM unlabeled Tf. At the end of incubation the cells were washed and solubilized, and surface-bound radioactivity was measured by gamma counting. Specific binding was calculated as the difference in cell-associated radioactivity displaced by unlabeled Tf, normalized to cell protein.

Fluorescence Microscopy

HeLa cells were treated with 50 μM ferristatin or vehicle control (0.5% DMSO) for 4 hr and then incubated with 5 μg/ml Alexa Fluor 594 Tf (Alexa-red Tf; Invitrogen), 25 μg/ml FITC-dextran, or 20 μg/ml DiI-LDL for an additional 20 min, or with 40 nM Alexa Fluor 488 cholera toxin subunit B (Invitrogen) for an additional 30 min. Cells were washed with PBS++ and fixed with 4% paraformaldehyde. For dynamin and clathrin staining, fixed cells were permeabilized with 0.1% Triton X-100 and rinsed with 1% NH_4Cl in PBS. The cells were then blocked with 5% goat serum and immunoreacted with mouse anti-dynamin (1:200; Upstate) or mouse anti-clathrin (1:100; Santa Cruz Biotechnology) in PBS containing 2% goat serum and a secondary goat anti-mouse conjugated to Alexa Fluor 488 (1:400; Invitrogen) for 40 min. Cells were washed with PBS++ and fixed with 4% paraformaldehyde. After copious rinsing with PBS, cells were briefly dried and coverslips were mounted using fluorescent mounting medium (DakoCytomation).

Western Blot Analysis

To determine the effects of ferristatin on Tf receptor levels, HeLa cells were washed three times with PBS++ and lysed in NET lysis buffer (150 mM NaCl, 5 mM EDTA, 10 mM Tris [pH 7.4], 1% Triton X-100). The cells lysates were spun at 14,000 rpm for 10 min and 50 μg of the supernatant was loaded on 7.5% SDS-polyacrylamide gels. After electrophoresis, samples were transferred to nitrocellulose for immunoblotting using a monoclonal anti-human Tf receptor antibody (1:1000; Zymed), a monoclonal anti-human LDL receptor antibody (1:2000; a kind gift of Monty Krieger, Massachusetts Institute of

Technology), or a monoclonal anti-flotillin-1 antibody (1:500; BD Biosciences). As a loading control, blots were also probed with mouse anti-actin (1:12,000; ICN). Secondary IRDye800-conjugated goat anti-mouse (1:10,000; Rockland) was used to detect immunoreactivity using an Odyssey Infrared Imaging System (LI-COR). Relative intensities of protein bands normalized to actin were determined using Odyssey version 1.2 software.

SUPPLEMENTAL DATA

Supplemental Data include one figure and can be found with this article online at http://www.chembiol.com/cgi/content/full/15/7/647/DC1/.

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