Lipid raft-dependent endocytosis: a new route for hepcidin-mediated regulation of ferroportin in macrophages

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The Online version of this article has a Supplementary Appendix.

ABSTRACT

Background

Expression of the iron exporter ferroportin at the plasma membrane of macrophages is enhanced by iron loading and is decreased by hepcidin. We previously showed that ferroportin is present in specific cell surface domains suggestive of lipid rafts. Herein, we have clarified the localization of ferroportin in macrophage membranes and tested whether raft-mediated endocytosis plays a role in hepcidin activity.

Design and Methods

Raft/detergent-resistant membranes from murine bone marrow-derived macrophages and J774a1 cells were analyzed by Western blotting. The effect of lipid raft- or clathrin-dependent endocytosis inhibitors was studied on hepcidin activity. For this purpose, after treatment, ferroportin expression was analyzed by fluorescence microscopy, Western blotting of total protein extracts or plasma membrane protein samples, and by quantitative immunofluorescence assay (In-Cell-Western).

Results

Macrophage ferroportin was mostly detected in detergent-resistant membranes containing raft markers (caveolin 1, flotillin 1). Interestingly, iron overload strongly increased the presence of ferroportin in the lightest raft fraction. Moreover, lipid raft breakdown by cholesterol sequestration (filipin) or depletion (methyl-beta-cyclodextrin) decreased hepcidin activity on macrophage ferroportin. Cell surface biotinylation and immunofluorescence studies indicated that the process of both hepcidin mediated endocytosis and degradation of ferroportin were affected. By contrast, the inhibition of clathrin dependent endocytosis did not interfere with hepcidin effect.

Conclusions

Macrophage ferroportin is present in lipid rafts which contribute to hepcidin activity. These observations reveal the existence of a new cellular pathway in hepcidin mediated degradation of ferroportin and open a new area of investigation in mammalian iron homeostasis.

Key words: ferroportin, macrophage membranes, hepcidin.

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Introduction

Ferroportin (Fpn), the sole cellular iron exporter known in mammalian cells, is highly expressed in duodenal enterocytes and tissue macrophages,[1-3] playing a role in intestinal iron absorption and iron recycling by macrophages, respectively. Indeed, macrophages are in charge of the critical process of heme iron recycling from senescent red blood cells (RBCs).[4] After a life span of 120 days in humans, RBCs are specifically cleared from the circulation through recognition and phagocytosis by macrophages. Iron is then extracted from heme and is subsequently recycled into the bloodstream via Fpn transport.[5-7] Perturbation of such processes strongly participates in both anemia in chronic inflammatory diseases[8] and iron overload in hemochromatosis.[9-11]

Fpn is strongly down-regulated by hepcidin (Hepc), a peptide produced by the liver in response to iron stores, erythropoiesis, hypoxia and inflammation.[12-14] Through downregulation of Fpn expression, Hepc controls plasma iron concentration and tissue distribution to maintain iron homeostasis. At the cellular level, Hepc binds Fpn at the plasma membrane and induces tyrosine phosphorylation, a signal leading to its subsequent internalization into the cells.[15] In the cytosol, Fpn is then dephosphorylated, ubiquitinylated and targeted for degradation.[15] More recently, Hepc-induced Fpn phosphorylation was shown to involve the Janus Kinase 2 protein.[16] In addition, a clathrin-dependent process was proposed for the Hepc mediated internalization of Fpn in cells over-expressing a GFP-Fpn.[15]

In mouse macrophages, our previous observations indicated that Fpn expression at the cell surface of macrophages is strongly enhanced by iron and reduced by Hepc.[17] In these cells, Hepc treatment induces a rapid endocytosis of the transporter and its degradation mainly in lysosomes.[17] Interestingly, endogenous macrophage Fpn is detected in specific cell surface compartments suggestive of lipid rafts.[17,18] Lipid rafts are detergent-resistant, liquid-ordered membrane domains enriched in cholesterol and sphingolipids. They play a role in a variety of cellular processes including the compartmentalization of cell signaling events and the intracellular trafficking of certain membrane proteins.[19-21] We, therefore, studied the presence of endogenous macrophage Fpn in raft domains and tested if lipid rafts could play a role in the degradation of Fpn by Hepc in macrophages.

Design and Methods

Reagents and antibodies

The following chemicals were purchased from Sigma: chlorpromazine hydrochloride, filipin, methyl-β-cyclodextrin (MβCD). Human hepcidin (Hepc) was purchased from Peptide International. Transferrin (Tf) or cholera-toxin-B (CT-B) conjugated to Alexa Fluor[555], Alexa Fluor[488] goat anti-rabbit, Alexa Fluor 568 or 555 goat anti-mouse antibodies were purchased from Invitrogen. Fpn was detected using either a homemade rabbit polyclonal antibody (Figures 1-4) or a rabbit polyclonal antibody from Alphadiagnostic (Figure 5), both directed against the mouse Fpn. Both antibodies present the same specificity with a higher titer for that commercially available compared to the homemade version. The production, the purification and the specificity of our rabbit polyclonal anti-mouse Fpn antibody have been described previously.[18] The mouse monoclonal anti-caveolin 1 and anti-flotillin 1 were purchased from Abcam and BD Biosciences, respectively. The mouse monoclonal anti-human transferrin receptor 1 (TfR1) was obtained from Zymed laboratories. The mouse monoclonal anti-vinculin was purchased from Sigma-Aldrich.

Macrophage cultures and treatments

Bone marrow derived macrophages (BMDMs) were cultured as described previously.[5] Briefly, bone marrow cells were isolated from femurs of 6-8 week-old mice (DBA2 or SWISS strains), and seeded in 10 cm diameter Petri dishes (2×10^5 cells/mL) for protein extraction, in 96-well cell culture plates (4×10^5 cells/mL) for In-Cell-Western (LI-COR) or onto circle glass coverslips (10 mm; 10^5 cells/mL) placed in 24-well cell culture plates for immunofluorescence studies. The mouse monocyte-macrophage cell line J774a1 was cultured in media and under conditions recommended by

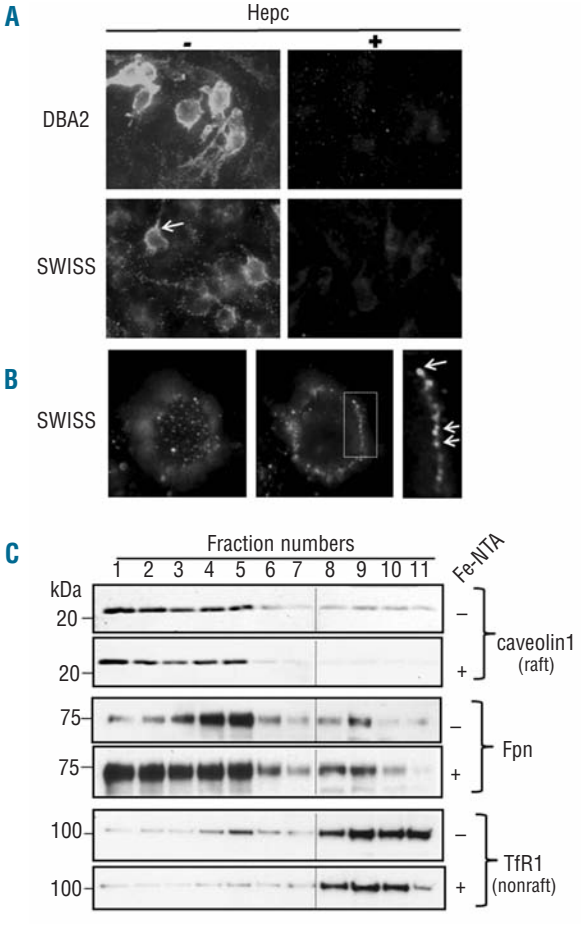

Figure 1. Macrophage ferroportin (Fpn) is present in lipid rafts. (A) and (B) Immunofluorescence studies of Fpn in bone marrow-derived macrophages (BMDM) from DBA2 and SWISS mice. Cells were cultured overnight with Fe-NTA prior to Hepc treatment for 3 h. (B) High magnifications of two different focal sections of a macrophage confirm the presence of Fpn in specific cell surface membrane domains (see white arrows in right panel). (C) Fpn is mostly distributed in rafts/detergent resistant membranes (DRM). Untreated or Fe-NTA treated cells were lysed in buffer containing 1% Triton X-100 before fractionation on a discontinuous iodixanol density gradient by centrifugation. Eleven fractions collected from top (fraction n. 1; low density) to bottom (fraction n. 11; high density) of the gradient were analyzed by immunoblotting for the presence of Fpn, caveolin 1 (rafts marker), and TfR1 (non-rafts marker). Protein species were then revealed using ECL (chemiluminescence). Vertical dashed lines indicate repositioned gel lanes.

ATCC. J774a1 and BMDM were treated with Fe-NTA (200 μM and 100 μM, respectively) during 12h at 37°C in a 5% CO_2 incubator. Cells were incubated with filipin (5 μg/mL), chlorpromazine (14 μM) for 1h or MβCD (10 mM) for 30 min at 37°C in a 5% CO_2 incubator. After drug treatments, cells were treated with Hepc (300 nM) for indicated periods of time at 37°C in a 5% CO_2 incubator.

RIPA cell lysates (total protein extracts)

Cultured BMDMs and J774a1 were washed with cold phosphate buffered saline (PBS), scraped in RIPA buffer (10 mM tris pH8, 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.1% SDS) complemented with protease inhibitors cocktail (Calbiochem). The cells were left on ice for 30 min and then centrifuged at 10,000 x g in a refrigerated microcentrifuge for 15 min. Protein concentrations of supernatants were determined by the Bradford assay (BioRad) and 30 μg for BMDM or 60 μg for J774a1 extracts were then analyzed by Western blotting analysis.

Cell-surface biotinylation

Biotinylation and purification of macrophage cell surface proteins were performed according to the manufacturer's instructions (Pierce Biotechnology). Briefly, intact J774a1 cells were treated with a membrane impermeable biotinylation reagent, EZ-Link® Sulfo-NHS-SS-Biotin (0.25 mg/mL in PBS buffer) for 30 min at 4°C with gentle shaking. The reaction was stopped, cells were lysed and biotinylated proteins from the cell surface were recovered from lysates by incubation with immobilized NeutrAvidin™ Gel (Pierce Biotechnology). A sample of the non-retained proteins (flow-through; intracellular proteins) was also recovered for Western blotting analysis.

Isolation of rafts/detergent resistant membranes

Cells were washed with cold phosphate buffered saline (PBS), scraped into PBS-EDTA (ethylene diamine tetraacetic acid; 2 mM) using a rubber policeman and centrifuged at 500 x g in a refrigerated centrifuge for 5 min. Cell pellets were incubated at 4°C for 30 min in 600 μL lysis buffer (150 mM NaCl, 20 mM MES, pH 6.5, 1% TritonX-100) supplemented with protease inhibitor cocktail. Samples were then homogenized by 20 passages through a 25-gauge needle (5/8-inch) and by sonication. Cell lysates were adjusted to a final concentration of 40% (w/v) iodixanol (Optiprep) and the mixture was then layered under a 20-40% discontinuous iodixanol gradient and centrifuged at 260,000 x g for 13h at 4°C using an SW 41 Ti Rotor (Beckman Coulter). After spinning, eleven fractions of 1 ml were collected from the top to the bottom of the gradient tube. Immunoblotting analysis of these fractions for known raft and non-raft proteins was performed to identify the fractions containing the rafts/DRM.

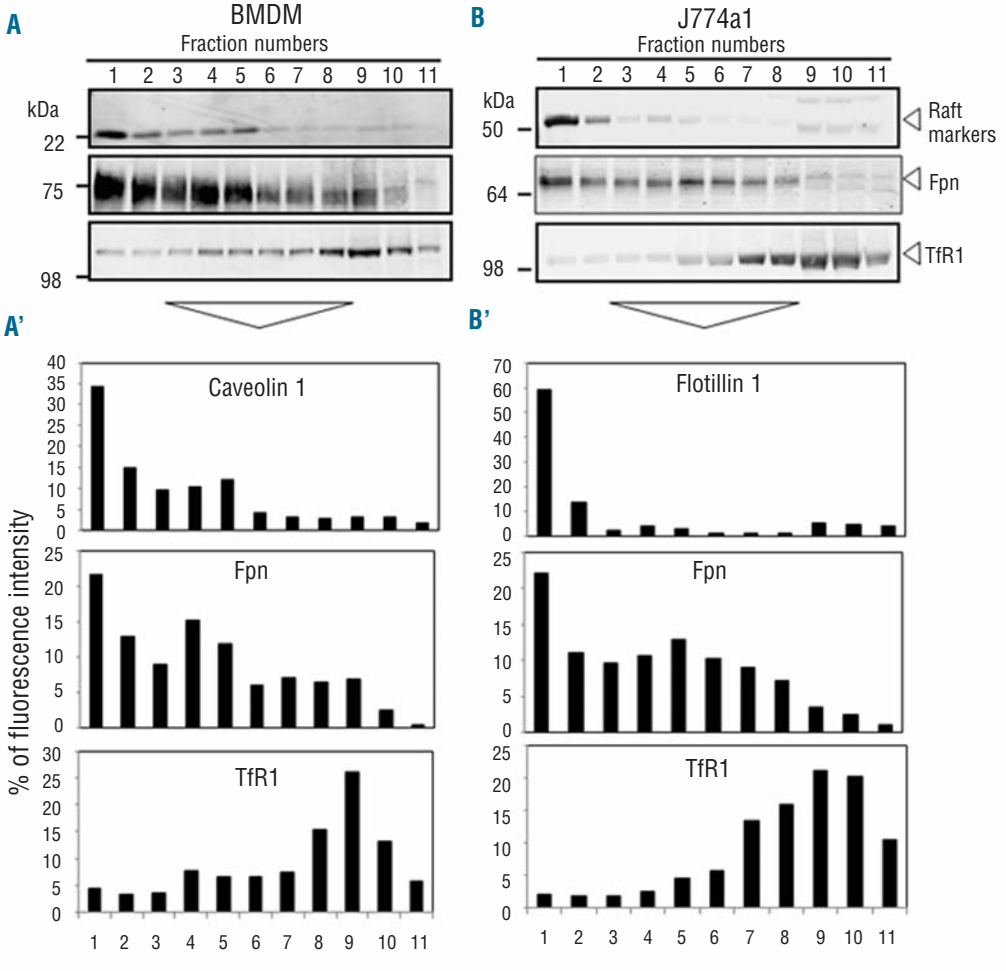

Figure 2. Distribution of ferroportin, rafts and non-rafts markers in Fe-NTA treated BMDMs (A-A') and J774a1 (B-B'). Iodixanol gradient fractions isolated from Fe-NTA treated macrophages were separated by SDS-PAGE, transferred to a PVDF membrane and probed for Fpn, caveolin 1 (A, rafts marker), flotillin 1 (B, rafts marker), and for TfR1 (non-rafts marker). After labeling with specific fluorescent secondary antibodies, protein species were visualized with the Odyssey infrared imaging system (LI-COR Biosciences; A and B). Quantification of specific fluorescent signals in each fraction were represented in A' and B'. The values (% of fluorescence intensity) in histograms are expressed as a percentage of the sum of fluorescence labeling from all fractions.

Western blotting analysis

Collected fractions from iodixanol gradient, RIPA cell lysates or biotinylated samples were solubilized in Laemmli buffer, resolved by a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred on polyvinylidene fluoride (PVDF) membranes. The membranes were incubated in a blocking solution (5% skim milk in TBST [0.15% Tween-20, in Tris-buffered saline]) for 2h at room temperature, and incubated with primary antibodies (anti-Fpn, 1/500; anti-flotillin 1, 1/500; anti-TfR1, 1/1,000; anti-caveolin 1, 1/200 or anti-vinculin, 1/20,000) overnight at 4°C. After washing with TBST, blots were incubated for 1h at room temperature with either peroxidase-labeled secondary antibody (1/50,000) or LI-COR IRDye 800 or 700-labeled secondary antibodies (1/10,000) and revealed either by ECL chemiluminescence (Millipore) or by Infrared Imaging System Odyssey® (LI-COR Bioscience).

Quantitative immunofluorescence assay (In-Cell-Western assay)

After treatments, cells cultured in 96-well cell culture plates were fixed with 100% methanol at -20°C for 15 min, washed with PBS, and then permeabilized with Triton X-100 (0.1% in PBS) for 10 min. Cells were blocked with Odyssey blocking buffer (LI-COR Bioscience) for 90 min at room temperature. Cells were then incubated in a humid chamber overnight at 4°C with anti-Fpn antibody (1/200) in Odyssey blocking buffer. After 5 washes with PBS/0.5% Tween-20, cells were incubated for 1h at room temperature with LI-COR IRDye 800 labeled secondary antibodies (1/400) and with Sapphire700 (1/1000) and DRAQ5 (1/2000) for cell number normalization. Sapphire700 is a non-specific cell stain that accumulates in both the nucleus and cytoplasm of fixed cells whereas DRAQ5 is a cell permeable DNA-interactive agent. Finally, cells were washed 5 times with PBS/0.5% Tween-20 before the scan of the plate by Odyssey® Infrared Imaging System (LI-COR Bioscience).

Fluorescence microscopy studies

The effect of inhibitors (filipin, MβCD and chlorpromazine) on rafts or clathrin endocytosis was assessed by fluorescence microscopy analysis of Alexa^{555}-cholera toxin B-subunit (CT-B) or Alexa^{555}-transferrin (Tf) *in vivo* uptake.[22] Briefly, after treatment, cells were incubated with labeled CT-B or Tf for 30 min at 4°C followed by 30 min incubation at 37°C in a 5% CO_2 incubator allowing endocytosis. Cells were fixed in 100% methanol at -20°C for 15 min, washed with PBS and mounted with antifading mounting reagent (VECTASHIELD® Hard Set™ Mounting Medium). For immunofluorescence, cells were fixed with 100% methanol at -20°C for 15 min and permeabilized with Triton X-100 (0.1% in PBS) for 10 min. After PBS washes, cells were incubated in a blocking solution (BSA 1% and 10% heat-inactivated goat serum in

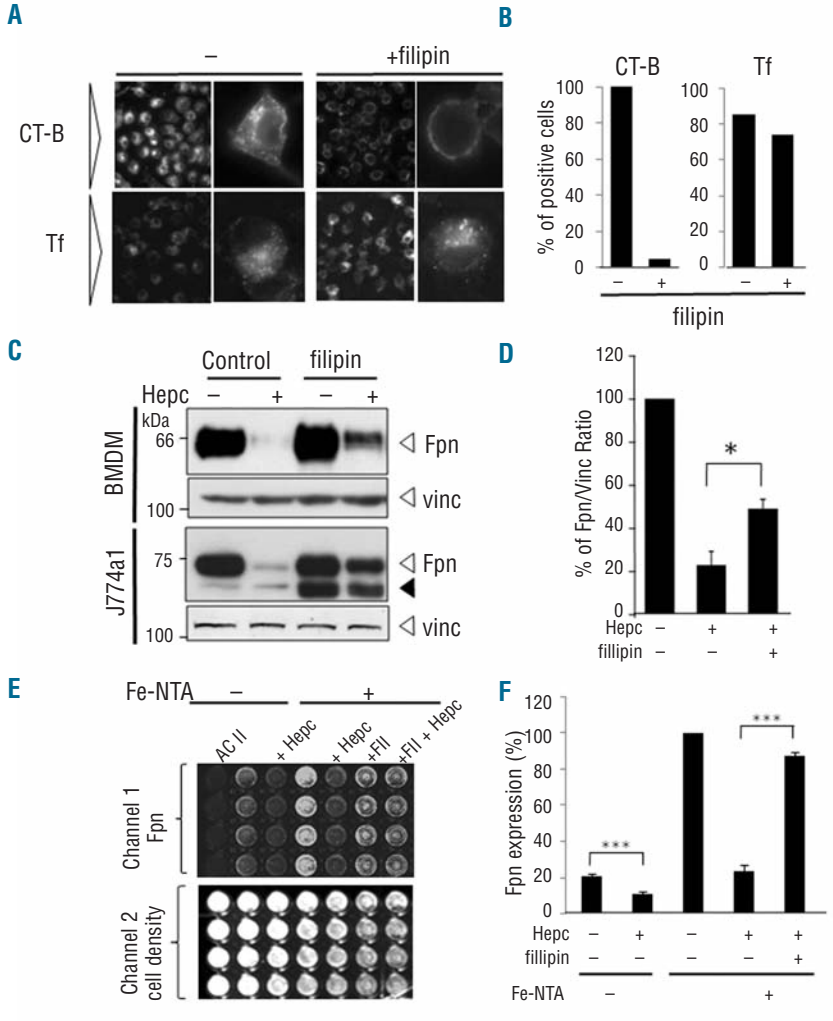

Figure 3. Effects of the lipid rafts disrupter filipin on ferroportin degradation by hepcidin in macrophages. (A) BMDM cultures were incubated overnight with Fe-NTA before treatment with filipin. Endocytosis of Alexa^{555}-cholera toxin-B (CT-B, rafts) or Alexa^{555}-transferrin (Tf, non-rafts) was analyzed by fluorescence microscopy and the percentage of positive cells for intracellular staining of CT-B or Tf was counted (B). (C) Iron-treated macrophages were pre-treated or not with filipin prior to Hepc incubation (3 h). Using RIPA cell lysates, Fpn and vinculin (vinc: loading control) expression were analyzed by Western blotting and ECL. The position and size in kilodaltons (kDa) of molecular mass markers are indicated on the left. Lower Mr protein species were sometimes detected in J774a1 (black arrowhead) and likely correspond to Fpn degradation products. (D) Similar experiments as in (C) were performed in J774a1 and analyzed using the Odyssey infrared imaging system (LI-COR Biosciences). The Fpn signal was normalized to vinculin and was expressed as a percentage of Fpn level in untreated cells (*P<0.05; Student's t-test). In-cell-Western detection (E, see *Design and methods*) and quantification (F) of Fpn expression in BMDM cultured in microplates (96 wells). Cells were treated with Fe-NTA and Hepc (3 h). The effect of Hepc on Fpn expression was studied after pre-treatment with filipin. After cell fixation and permeabilization, Fpn was detected using our high-affinity purified primary antibody and a near infrared-labeled secondary antibody (E, upper panel, channel 1; ACII: secondary antibody alone). (F) To correct for variations in cell number from well to well, quantification and normalization of Fpn fluorescent signal were performed against fluorescent detection (E, lower panel, Channel 2) of Sapphire700 (nucleus and cytosol marker) and DRAQ5 (DNA marker). In the presence or in the absence of filipin, Fpn signals after Hepc treatment were expressed as a percentage of Fpn levels in respective untreated (without Hepc) cells that were fixed at 100%.

PBS) for 45 min at room temperature. Incubation with primary antibodies was then performed in a humid chamber at room temperature for 1h using the following dilution in blocking solution (rabbit anti-Fpn, 1/100; mouse anti-caveolin 1, 1/50). After 3 washes with PBS/0.5% BSA, cells were incubated for 1h at room temperature with Alexa^{488} goat anti-rabbit or Alexa^{568} or ^{555} goat anti-mouse secondary antibodies (1/200) in blocking solution. Slide coverslips were then washed 3 times in PBS/0.5% BSA, once in PBS, and mounted with antifading mounting reagent. Labeled cells were visualized using a 60X oil objective and a Nikon TE2000E fluorescent microscope (Nikon, Champigny-sur-Marne, France) equipped with a Nikon DXM1200F digital camera.

Results

Ferroportin is present in cell surface domains in primary mouse macrophages isolated from DBA2 and SWISS mice

The localization of the iron exporter Fpn at the cell surface has been reported to be different among macrophages isolated from distinct strains of mice.[23] We previously showed that Fpn is expressed at the cell surface of bone marrow derived macrophages (BMDM) isolated from DBA2 mice.[17] Here, we first studied the presence of Fpn at the cell surface of BMDM isolated from DBA2 and SWISS mice. BMDM were treated overnight with iron (Fe-NTA) to enhance Fpn expression and were analyzed for Fpn expression by fluorescence microscopy (Figure 1A and B). Under these conditions, Fpn was strongly expressed at the plasma membrane of both DBA2 and SWISS mouse macrophages. In addition, treatment with Hepc for 3h induced a strong decrease of Fpn staining in both macrophage populations (Figure 1A). When observed at higher magnification (Figure 1B), Fpn defined a punctuate staining at the cell surface (white arrows) of SWISS BMDM. As previously observed for DBA2 BMDM,[17] these results indicate the presence of Fpn at the cell surface in microdomaine-like structures in SWISS BMDM. Most of our experiments were then designed using SWISS mice.

Macrophage ferroportin is present in lipid rafts

To determine the subcellular compartments of Fpn in macrophages, lipid rafts from BMDM populations were isolated as detergent-resistant (Triton X-100) membranes (DRM).[24,25] Rafts/DRM from BMDM were then analyzed by Western blotting and enhanced chemiluminescence (Figure 1C). In this experiment, the raft/DRM fractions were defined by the presence of the lipid rafts markers, caveolin 1 (Figure 1C). Caveolin forms oligomers and associates with cholesterol and sphingolipids in lipid rafts, leading to the formation of caveolae, a specific subset of lipid rafts.[26] Transferrin receptor 1 (TfR1) that is constitutively internalized through clathrin-coated pits[27] was used to determine the non-lipid raft fractions. In both untreated and iron treated BMDM, the distribution of caveolin 1 defined the top five low-density fractions as the rafts/DRM, whereas non-raft membranes containing TfR1 were defined as the more dense materials in fractions 8 to 11. Interestingly, Fpn was mostly detected in DRM. In addition, the detection of Fpn in the lightest fractions 1-2 enriched in caveolin 1 was increased after iron treatment. In this condition, partial co-localization of Fpn with caveolin1 was also observed by confocal analysis (*Online Supplementary Figure S1*). For accurate quantification on Western blot, detections of the proteins in membrane fractions were performed by fluorescence and analyzed with the LI-COR Odyssey® Infrared Imaging System (Figure 2A). The fluorescent signal quantification showed that in Fe-NTA treated BMDM, more than 70% of total Fpn signal was present in rafts/DRM (fractions 1-5) containing 80% of caveolin 1 (Figure 2A'). In contrast, 70% of TfR1 was found in the last fractions (8 to 11) of the gradient. Similar experiments were performed in the mouse macrophage cell line J774a1 treated with Fe-NTA (Figure 2B). Because of the absence of caveolin 1 in this cell line,[28] flotillin 1 was used as lipid raft marker. As for BMDM, TfR1 was mostly present in the last fractions (around 80% of the staining in fractions 7 to 11). The quantification of the specific signal detected showed the presence of 65% of Fpn in the first five fractions of the gradient (Figure 2B'). In these cells, Fpn was highly present in fraction 1 and 2 colocalizing with 80% of flotillin 1 (Figure 2B'). However, when the fractions 1 and 2 from J774a1 were separated onto a second iodixanol gradient (*Online Supplementary Figure S2*), we observed that flotillin 1 was still enriched in the lightest density fraction 1 whereas Fpn was distributed in other more dense fractions (fractions 1 to 6). This suggests that Fpn could be present in different lipid rafts (different density), with only a few showing overlap and co-

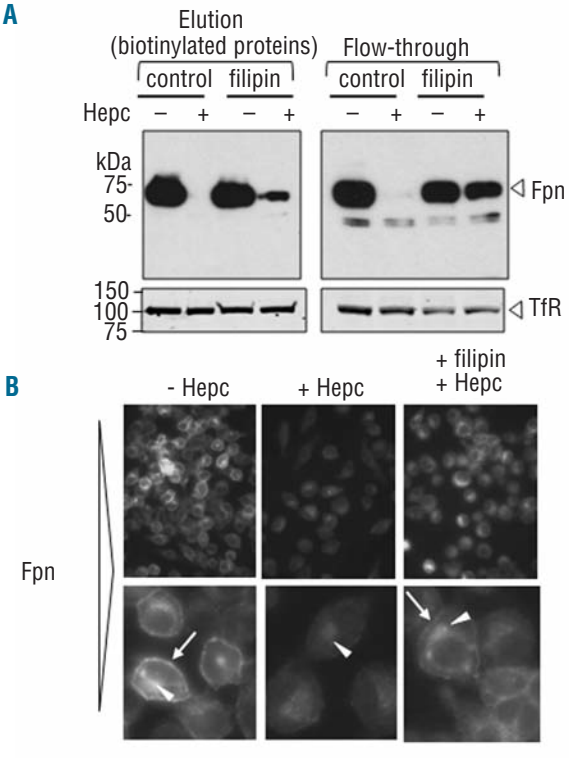

Figure 4. Filipin affects both hepcidin–mediated endocytosis and degradation of ferroportin. Fe-NTA treated J774a1 were incubated or not with filipin prior to treatment with Hepc (300 nM; 3 h). (A) After biotinylation labeling, cell surface (biotinylated proteins) and intracellular (flow-through) extracts were then analyzed by Western blotting using anti-Fpn or anti-TfR1 (control) antibodies. The position and size in kilodaltons (kDa) of molecular mass markers are indicated on the left. (B) Immunofluorescence of Fpn indicates that after filipin treatment and in the presence of Hepc, Fpn is still detectable at the cell surface (arrows) and inside the cells (arrowheads).

localization with raft markers such as caveolin 1 or flotillin 1.

Altogether, these observations clearly indicate that Fpn is present in lipid rafts/DRM in both primary mouse macrophages (BMDM) and in a mouse macrophage cell line (J774a1).

Lipid raft-dependent endocytosis is involved in hepcidin activity in macrophages

The presence of Fpn in lipid rafts at the cell surface of macrophages suggests that such membrane compartments could be involved in Hepc activity. In order to test this hypothesis, filipin, a drug inhibiting lipid raft-dependent endocytosis through the sequestration of cholesterol, was used and the effect of Hepc on Fpn was analyzed (Figure 3). First, the efficiency of the drug was controlled by microscopy (Figure 3A) following labeling with Alexa^{555} conjugated cholera toxin-B (CT-B) or transferrin (Tf) which are endocytosed by cells through lipid rafts or clathrin-dependent mechanisms, respectively.[22] BMDM were treated or not with filipin, incubated with labeled CT-B or Tf and then visualized by immunofluorescence (Figure 3A). In BMDM treated with filipin, the uptake of CT-B into the cells was strongly inhibited, with cells showing only binding of fluorescent CT-B at the cell surface. After filipin treatment, less than 10% of the cells were positive for intracellular CT-B staining (Figure 3B). Importantly, at the concentration used, filipin did not significantly affect the internalization of Tf (Figure 3A and B).

Iron-treated macrophages (BMDM and J774a1) were then incubated with filipin prior to Hepc treatment. Total extracts were performed and Fpn expression was analyzed by classical Western blotting (Figure 3C). After an iron treatment, in both macrophage populations and in both untreated and treated cells with filipin, Fpn is highly detected as a broad protein molecular species with an apparent MW around 70 kDa. In addition, Hepc dramatically reduced the level of Fpn expression in both macrophage populations. However, when Hepc was added to the cells pre-treated with filipin, the amount of Fpn decreased but to a lesser extent than in the control cells. In some experiments but not all, a lower molecular species was detected with our anti-Fpn antibody after filipin treatment in J774a1 (Figure 3C). The relevance of this protein species is not clear but likely corresponds to partial degradation of Fpn during protein extraction. Fpn protein expression in J774a1 cells was then quantified by Western blotting using the LI-COR Odyssey Infrared Imaging System (Figure 3D). Histograms in Figure 3D show that inhibition of lipid raft endocytosis by filipin partially prevents the degradation of Fpn by Hepc. Similar observations were made using methyl-β-cyclodextrin (MβCD) known to inhibit raft-dependent endocytosis through cholesterol depletion from cells (*Online Supplementary Figure S3*).

In-Cell-Western (ICW) experiments were also performed to demonstrate the effect of filipin on Hepc activity (Figure 3E and F). For these assays, Fpn expression was directly detected by infrared immunofluorescence in BMDM cultured in multi-wells (Figure 3E, channel 1). As a control of specificity of the Fpn staining, the fluorescent conjugated secondary antibody alone did not give any specific signal (Figure 3E, AC II). In addition, and as expected, the intensity of Fpn staining strongly increased with iron treatment and was markedly reduced by Hepc. These observations were confirmed by quantification analyses (Figure 3F) obtained through direct measurement

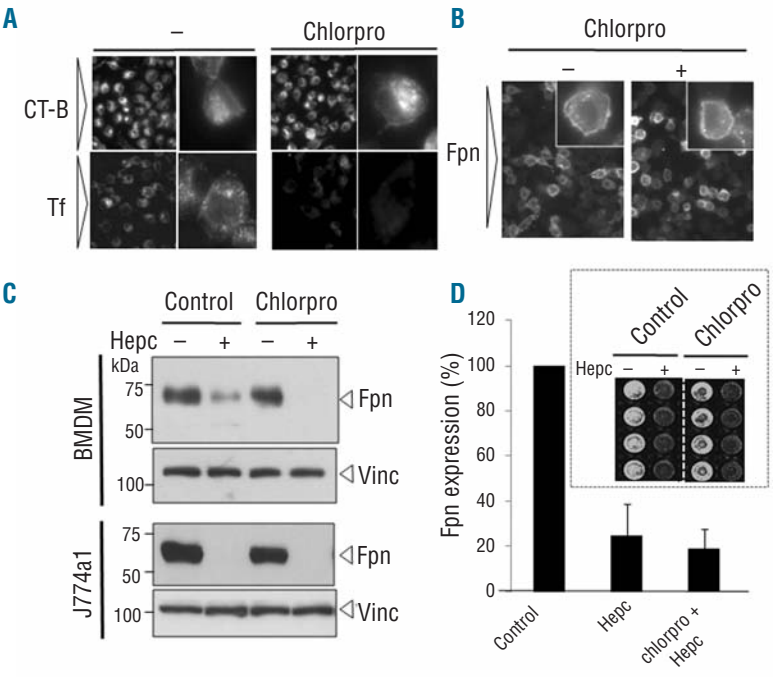

Figure 5. Inhibition of clathrin-dependent endocytosis and effect on ferroportin degradation by hepcidin in macrophages. (A) BMDM cultures were incubated overnight with Fe-NTA before treatment with chlorpromazine (Chlorpro), a clathrin-dependent endocytosis inhibitor. (A) Endocytosis of Alexa^{555}-cholera toxin-B (CT-B, rafts) or Alexa^{555}-transferrin (Tf, non-rafts) was then analyzed by fluorescence microscopy to control the efficiency of the treatment. (B) Localization of ferroportin (Fpn) assessed by immunofluorescence after Chlorpro treatment. (C) Western blotting analysis of Fpn expression. Macrophage cultures were treated overnight with Fe-NTA to enhance Fpn expression. Before Hepc incubation (3 h), cells were pre-treated or not with chlorpromazine. RIPA cell lysates (30 μg for BMDMs to 60 μg for J774a1 extracts) were then analyzed by Western blotting using anti-Fpn or anti-vinculin (vinc: loading control) antibodies. Blots were revealed using enhanced chemiluminescence. The position and size in kilodaltons (kDa) of molecular mass markers are indicated on the left. (D) In-cell-Western analysis of macrophage Fpn expression after chlorpromazine treatment. BMDM treated with Fe-NTA were incubated with chlorpromazine before treatment with Hepc and analyzed for Fpn expression using the Odyssey® infrared imaging system. Inset in D shows visualization of the specific Fpn signal. After normalization, the level of Fpn expression was expressed as a percentage of the Fpn levels in iron treated cells (control).

of the fluorescent staining of Fpn level normalized by the cellular density in each well (Figure 3E, channel 2). Combined use of the two molecules allows an accurate normalization of cell numbers. In Figure 3F, results were expressed as a percentage of the signal detected in cells only treated with Fe-NTA (100%). We observed that Fe-NTA increased the expression of Fpn protein 5-fold. In both untreated and iron treated BMDM, Fpn protein levels were decreased after Hepc treatment by 50% and 80%, respectively. Importantly, filipin significantly prevented the decrease of Fpn expression induced by Hepc. In ICW assay, filipin was shown to impair the degradation of Fpn by Hepc in a dose-dependent manner (*Online Supplementary Figure S4*).

We then tested the hypothesis that filipin could reduce cell surface expression of Fpn thereby impairing action of Hepc. The presence of Fpn at the cell surface of iron-treated J774a1 macrophages was studied by biotinylation experiments (Figure 4A). Fpn was strongly detected in both the biotinylated (cell surface proteins) and the flow-through (intracellular proteins) fractions and disappeared in both cell fractions in the presence of Hepc (3h). As a control, the level of TfR1 expressed both at the plasma membrane and inside the cells (recycling endosomes) was not affected by Hepc. Importantly, the detection of Fpn was not affected by filipin. On the other hand, when cells were treated with filipin prior to Hepc, the disappearance of Fpn was impaired. This effect was more pronounced in the flow-through fraction. Similar observations were made after a shorter Hepc treatment (1h) and the amount of Fpn at the cell surface was estimated to be around four times more important in filipin treated cells than in untreated cells (*Online Supplementary Figure S5*). According to these results, immunofluorescence studies showed that filipin reduced both cell surface (arrow) and intracellular (arrowhead) decrease of Fpn by Hepc (Figure 4B). Similar observations were also made after MβCD treatment (*Online Supplementary Figure S6*).

Taken together, these results indicate that lipid raft perturbation through cholesterol sequestration or depletion interferes with Hepc-mediated endocytosis and degradation of the transporter, and suggest that Hepc-mediated degradation of Fpn in macrophages is, at least in part, raft-dependent.

De Domenico *et al.* found that Fpn-GFP transfected in HEK293T is internalized after Hepc treatment through a clathrin-dependent process.[15] Therefore, the role of clathrin endocytosis was also investigated in Fpn internalization by Hepc in our cells using chlorpromazine previously described as an inhibitor of this pathway.[22] Indeed, chlorpromazine specifically reduced clathrin-dependent endocytosis of Tf (80% decrease by microscopy counts of positive labeled cells; *data not shown*) but had no effect on CT-B uptake (Figure 5A). Furthermore, chlorpromazine did not delocalize Fpn (Figure 5B; about 65% of the cells expressed Fpn on plasma membrane after treatment). After Fe-NTA treatment, BMDM and J774a1 cells were then incubated or not with chlorpromazine prior Hepc (Figure 5 C and D). Fpn expression was analyzed both by Western blotting (Figure 5C) and ICW assay (Figure 5D). In both experiments, Hepc led to the degradation of the same amount of Fpn in cells treated or not with chlorpromazine. According to these results, clathrin endocytosis did not seem to be involved in the internalization of Fpn by Hepc in our macrophage cultures.

Discussion

In macrophages, we and others have demonstrated that Fpn expression is strongly enhanced by iron loading and down-regulated by Hepc.[4,17,29] In both untreated or iron treated macrophages, Fpn presents a specific punctuate staining at the cellular surface, accumulating in some particular domains, suggestive of lipid rafts as previously observed.[17,18] Using two murine macrophage cell cultures, a mouse macrophage cell line (J774a1) and bone marrow-derived macrophages (BMDM), we demonstrate here that endogenous macrophage Fpn is physically associated with lipid rafts. By subcellular fractionation using iodixanol density gradient and Western blotting analysis, Fpn was mostly detected in rafts/DRM, identified in BMDM and J774a1 by the presence, respectively, of caveolin 1 and flotillin 1, two well-known lipid raft markers. On the other hand, Fpn was poorly detected in the fractions containing the non-raft protein TfR1. Moreover, when Fpn expression was up-regulated by iron, the transporter was enriched in DRM, in particular in the lightest DRM fractions containing high amounts of caveolin 1 (in BMDM) and flotillin 1 (in J774a1). The shift of Fpn from higher density to the lowest density fractions after iron treatment is not completely understood but could reflect some iron-mediated changes in the density of the Fpn containing rafts. Further investigations are needed to clarify this point. In a previous study, we also showed that Fpn colocalizes with CD11b at the cell surface of macrophages.[17] Interestingly, CD11b is also known as a raft-associated antigen in human monocytic cells.[30, 31] Together, these observations indicate that the iron exporter Fpn is preferentially present in lipid rafts at the plasma membrane of macrophages. Different modes by which Fpn could integrate lipid rafts exist. They are called lipidation (either acylation of the protein, GPI-linkage or covalent attachment of cholesterol), protein-protein interactions or presence of a CRAC domain.[32] The CRAC is a common cholesterol recognition and interaction amino acid consensus pattern.[33] Interestingly, analysis of the Fpn sequence reveals the presence of four putative cholesterol recognition/interaction amino acid consensus (CRAC) motifs in transmembrane domains or near the membrane interface according to a recent topology model of Fpn[34] (*Online Supplementary Figure S7*). Further investigations are needed to determine if such CRAC motifs play a role in Fpn integration into lipid rafts.

Lipid rafts control key biological events dependent on the functional organization of the plasma membrane, such as signaling cascades, protein and lipid sorting and trafficking, as well as immune responses.[19-21] The preference of the iron exporter Fpn to partition into raft domains strongly suggests that lipid rafts are important for the function of the transporter and, therefore, for the process of iron recycling in macrophages. A particular lipid and protein environment of Fpn at the cell surface is likely important not only for the transport activity but also for the trafficking of the transporter at the cell surface. One can speculate that a specific membrane compartment such as lipid rafts may stabilize the protein at the cell surface when its role of iron exporter is essential. In addition, lipid raft-mediated endocytosis is a well-described process of protein trafficking from the cell surface to the intracellular space and multiple studies have shown the role of lipid rafts in the endocytosis of various ligands.[19] After binding

to Hepc, cell surface Fpn was shown to be rapidly internalized and degraded.[7,14,15,17] Importantly, using different experimental approaches, we demonstrate here that raft integrity is important for Hepc action on macrophage Fpn. More precisely, we show that two specific drugs disrupting lipid rafts decreased Hepc activity on macrophage Fpn affecting both the process of endocytosis at the cell surface and the intracellular degradation of the transporter.

Disruption of lipid rafts containing Fpn could impair interactions of the transporter with key mediators of the Hepc effect. Indeed, previous studies indicate that the signal that triggers internalization of the transporter after Hepc binding is phosphorylation by the tyrosine kinase Jak2 of two tyrosine residues of Fpn.[15,16] Numerous receptor and non-receptor tyrosine kinases including Jak2 are important signaling molecules in raft domains.[19,21,35] Disruption of lipid rafts may, therefore, affect tyrosine kinase recruitment at the cell surface and limit their action on Fpn phosphorylation after Hepc binding. However, in preliminary co-immunoprecipitation experiments, we did not detect any phosphorylation of Fpn after Hepc treatment (*data not shown*). Experiments are in progress to clarify this point. Dimerization of Fpn was also proposed for Hepc-mediated JAK2 activation.[16] Therefore, lipid rafts could facilitate cooperativity between Fpn monomers for an efficient Hepc effect. On the other hand, using bioluminescence resonance energy transfer analysis, other authors have demonstrated that Fpn behaves preferentially as a monomer.[36] The multimeric nature of Fpn still needs to be clarified, especially in primary cells expressing endogenous Fpn. Finally, another hypothesis is that disruption of lipid rafts could also induce conformational changes in Fpn, affecting accessibility of Hepc to its binding domain.[37]

In our study, the depletion or sequestration of cholesterol that affects lipid raft integrity only partially prevents the effect of Hepc on Fpn. These observations could be explained by the formation of new lipid rafts during the time of the Hepc treatment. This could also suggest the existence of other pathways for Hepc-mediated Fpn degradation. Previous studies have shown that after Hepc treatment, Fpn is internalized in the cells through clathrin-coated pits.[15] However, our observations indicate that clathrin-dependent endocytosis is not involved in the degradation of Fpn induced by Hepc in macrophages. The relevance of such a discrepancy is not clear but may reflect cell type–specific differences and different experimental procedures. Recently, some observations suggested that the action of Hepc is cell- and tissue-specific. Interestingly, macrophages seem to be more sensitive than enterocytes to a Hepc challenge.[38] This highlights the need to investigate endogenous Fpn properties in different physiological cellular contexts, such as macrophages, enterocytes and hepatocytes. In addition, it will be of importance to study in a raft macrophage specific context the disease-related Fpn mutants, as recently and carefully performed by the Bjorkman group.[34]

In conclusion, this work emphasizes the role of lipid rafts in macrophage Fpn regulation revealing the existence of a new cellular pathway in Hepc-mediated endocytosis and degradation of Fpn. In order to gain insights into Hepc regulation and iron transport activity of Fpn, it is now important to study how Fpn partitions into lipid rafts and to determine the proteomic environment of Fpn in cell membranes. Due to the critical role of macrophages in iron homeostasis, the study of lipid rafts containing macrophage Fpn could help to shed some light on the physiopathology of iron-related disorders including hemochromatosis and anemia of chronic disease. Moreover, rafts are of medical importance as they contribute to the pathogenesis of other human diseases, including cancer and atherosclerosis.[26,39] Therefore, the presence of the only known iron exporter Fpn in raft membranes may be of biological significance in the relationship between iron metabolism and human diseases and this needs to be explored.

Authorship and Disclosures

AA designed and performed research, analyzed data and contributed to the writing of the paper. AW performed research and analyzed data. FCH designed and performed research, analyzed data and wrote the paper.

The authors report no conflicts of interest.

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