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Prion protein functions as a ferrireductase partner for ZIP14 and DMT1

Ajai K. Tripathi^{#1}, Swati Halder^{#1}, Juan Qian¹, Amber Beserra², Srinivas Suda¹, Ajay Singh¹, Ulrich Hopfer³, Shu G. Chen¹, Michael D. Garrick⁴, Jerrold R. Turner⁵, Mitchell D. Knutson⁶, and Neena Singh^{1,*}

¹Department of Pathology, Case Western Reserve University/University Hospitals Case Medical Center, Cleveland, Ohio, USA

²School of Arts and Sciences, Case Western Reserve University/University Hospitals Case Medical Center, Cleveland, Ohio, USA

³Department of physiology and Biophysics, Case Western Reserve University/University Hospitals Case Medical Center, Cleveland, Ohio, USA

⁴Department of Biochemistry, University at Buffalo

⁵Department of Pathology, University of Chicago

⁶Department of Food Science and Human Nutrition, University of Florida, USA.

These authors contributed equally to this work.

Abstract

Excess circulating iron is stored in the liver, and requires reduction of non-Tf-bound-iron (NTBI) and transferrin (Tf)-iron at the plasma membrane and endosomes respectively by ferrireductase (FR) proteins for transport across biological membranes through divalent metal transporters. Here, we report that prion-protein (PrP^C), a ubiquitously expressed glycoprotein most abundant on neuronal cells, functions as a FR partner for divalent-metal transporter-1 (DMT1) and ZIP14. Thus, absence of PrP^C in PrP-knock-out (PrP^{-/-}) mice resulted in markedly reduced liver iron stores, a deficiency that was not corrected by chronic or acute administration of iron by the oral or intra-peritoneal routes. Likewise, preferential radiolabeling of circulating NTBI with ⁵⁹Fe revealed significantly reduced uptake and storage of NTBI by the liver of PrP^{-/-} mice relative to matched PrP^{+/+} controls. However, uptake, storage, and utilization of ferritin-bound iron that does not require reduction for uptake was increased in PrP^{-/-} mice, indicating a compensatory response to

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*To whom correspondence should be addressed: Neena Singh, Department of Pathology, Case Western Reserve University, 2103 Cornell Road, Cleveland, Ohio 44106, USA. Tel: 216-368-2617; neena.singh@case.edu.

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the iron-deficiency. Expression of exogenous PrP^C in HepG2-cells increased uptake and storage of ferric-iron (Fe³⁺), not ferrous-iron (Fe²⁺) from the medium, supporting the function of PrP^C as a plasma membrane FR. Co-expression of PrP^C with ZIP14 and DMT1 in HepG2 cells increased uptake of Fe³⁺ significantly, and surprisingly, increased the ratio of N-terminally truncated PrP^C forms lacking the FR domain relative to full-length PrP^C. Together, these observations indicate that PrP^C promotes, and possibly regulates the uptake of NTBI through DMT1 and Zip14 via its FR activity. Implications of these observations for neuronal iron homeostasis under physiological and pathological conditions are discussed.

Keywords

prion protein; ferrireductase; iron; liver; DMT1; Zip proteins

Introduction

Cells require iron for vital metabolic processes, but excess iron is cytotoxic due to its ability to participate in Fenton chemistry [1]. Iron homeostasis is therefore regulated at the systemic, organelle, and cellular levels by the coordinated action of iron uptake, transport, and storage proteins, which are themselves governed by iron regulatory proteins 1 and 2 as well as additional mechanisms [2-4]. Systemic iron homeostasis is maintained mainly by regulating uptake from the duodenum by the peptide hormone hepcidin released by hepatocytes in response to circulating iron [5, 6]. Most of the absorbed iron binds tightly to plasma transferrin (Tf), and the rest circulates as non-Tf-bound iron (NTBI) in association with small molecular weight compounds. Normally, 30-40% of plasma Tf is saturated with iron, leaving significant buffering capacity for excess iron that may accumulate in pathological conditions. Additional protection is provided by hepatocytes that can internalize and store large quantities of iron before signs of hepatotoxicity appear [7, 8].

Tf-iron and NTBI exist in the relatively inert ferric form (Fe³⁺), and require reduction to the ferrous form (Fe²⁺) for transport across biological membranes [2, 8, 9]. Iron-loaded Tf is endocytosed by the transferrin receptor (TfR), and Fe³⁺ iron released in the acidic environment of late endosomes is reduced to Fe²⁺ by membrane-bound ferrireductase (FR) proteins before transport through divalent metal transporters to the cytosol. NTBI, on the other hand, is reduced at the plasma membrane before transport, though recent reports indicate uptake and transport from the endosomes as well [7, 8, 10, 11]. Known FR proteins that function optimally at low pH of the endosomes include the Steap family of proteins, in particular Steap3 that plays a vital role in iron uptake by hematopoietic cells [12].

A prominent plasma membrane FR is duodenal cytochrome b (Dcytb) that reduces non-heme Fe³⁺ in the duodenum to Fe²⁺ for uptake by duodenal epithelial cells [13, 14]. The FR activity of Steap3 and Dcytb is coupled with the divalent metal transporter-1 (DMT1) that transports reduced iron across the lipid bilayer [15]. Recently, the ZIP family of divalent metal transporters have been described that function at the plasma membrane and in late endosomes [16, 17]. Transported Fe²⁺ joins the cytosolic labile iron pool (LIP), is oxidized

by ferritin and stored in its shell, or exported by the coupled action of a ferroxidase such as ceruloplasmin or hephaestin and the iron export protein ferroportin [3, 7, 18].

Recent reports indicate that prion protein (PrP^C), a mainly neuronal protein implicated in the pathogenesis of prion disorders, promotes cellular iron uptake by functioning as a FR [19-21]. In neuroblastoma cells, PrP^C exhibits FR activity on the plasma membrane, and requires the N-terminal octa-peptide repeat region for this activity [19]. Likewise, PrP^C promotes retrieval of iron from the glomerular filtrate via its FR activity, and increases trans-cellular transport of NTBI from the apical to the basolateral domain of kidney proximal tubule epithelial cells [22]. Deletion of the N-terminal octa-peptide repeat region abolishes this function, implicating the FR activity of PrP^C in kidney iron uptake [22]. However, the metal transporter(s) involved in PrP^C-associated iron uptake and transport are not known. Since liver is the principal organ that stores excess circulating iron, we used a combination of PrP-knock-out (PrP^{-/-}) transgenic mouse models and HepG2 cells expressing exogenous PrP^C to explore the significance of FR activity of PrP^C and associated divalent metal transporters in hepatic iron uptake. Here, we report that PrP^C promotes the uptake of Fe³⁺ iron by hepatocytes through divalent metal transports ZIP14 and DMT1.

Materials and Methods

Animals and Ethics statement

Wild type (PrP^{+/+}) and PrP-knock-out mice (PrP^{-/-}) [23] were originally obtained from George Carlson and bred on the FVB/NJ background for more than 10 years at the Animal Resource Center (ARC) at Case Western Reserve University (CWRU). Animal protocols and procedures were approved by the IACUC committee at CWRU.

Materials and cell culture

HepG2 cells (ATCC) were cultured in high glucose DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. Plasmid encoding DMT1-GFP (-IRE) was from Jerry Kaplan (University of Utah). PrP^C and PrP^C-GFP constructs were prepared by ligating cDNA from previously reported constructs [24] in modified PiggyBac vectors (System Biosciences, Mountainview, CA). HepG2 cells were co-transfected with the PiggyBac vectors and a transposase-expression vector using Lipofectamine® LTX (Invitrogen, Carlsbad, CA), and mixed cultures of ~30 stable clones selected with Blastcidin-S were used for all experiments to avoid clonal artifacts. PrP antibody specific for residues 109-112 (3F4) was from Signet Laboratories (Dedham, MA). The specificity of anti-C antibody against C-terminal residues of human PrP (anti-C) has been described previously [25]. Antibodies specific to ferritin and β -actin were obtained from Sigma (Sigma-Aldrich, St. Louis, MO) and Millipore (Bedford, MA) respectively. Wheat Germ Agglutinin (WGA)-Alexa Fluor-594 was from Molecular Probes (Invitrogen). HRP-conjugated secondary antibodies and ECL Detection Kit were from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Radiolabeled ⁵⁹FeCl₃ was from Perkin-Elmer (Boston, MA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Iron uptake in vivo

Age and sex-matched PrP^{+/+} and PrP^{-/-} mice were provided unrestricted access to food and water, and equal number of mice from each group were subjected to the following experimental paradigms: 1) Carbonyl-iron (200µg) dissolved in PBS was gavage-fed daily for 1 month. Controls received an equal volume of PBS [26]. 2) Equal counts of ⁵⁹Fe-citrate or ⁵⁹Fe-ferritin (purified from mouse liver) [27] containing 11µg of total iron were injected intraperitoneally (IP). 3) Unlabeled ferric-ammonium citrate (FAC, 70µg/22g body-weight) was injected IP followed by ⁵⁹Fe-citrate after 30 min by the same route [28]. A parallel set of animals received ⁵⁹Fe-citrate without prior injection of FAC. At the indicated times, animals were euthanized and processed as described [20, 29]. Liver homogenates and lysed RBCs were resolved by SDS-PAGE and transferred proteins were probed with specific antibodies by Western blotting, or resolved by native PAGE and subjected to autoradiography [20, 29]. Serum iron, tissue non-heme iron, and iron content of purified ferritin was quantified by the iron estimation kit (Stanbio Laboratory, Boerne, TX). Serum ferritin was measured using an ELISA assay (Abcam) [20, 29].

Iron uptake in vitro

HepG2 cells stably expressing vector or PrP^C were exposed to 30µM FAC (Fe³⁺) or iron ascorbate (Fe²⁺) for 1-4 hours or overnight, and expression of PrP^C and ferritin was evaluated by Western blotting. Short-term experiments were conducted in RPMI medium lacking iron and transferrin. To assess the role of ZIP proteins and DMT1 in PrP^C-mediated iron uptake, stable clones of vector and PrP^C-expressing cells were co-transfected with ZIP8-GFP, ZIP14-GFP, or DMT1-GFP, and cultured for 48 hours to allow protein expression. Transfection efficiency was evaluated by flow-cytometry, and lysates were processed for Western blotting as above.

Immunostaining

Monolayers of HepG2 cells expressing PrP^C-GFP were incubated with WGA Alexa Fluor-594 (1:200) on ice for 30 min, washed, fixed with 4% paraformaldehyde, and stained with Hoechst 33342 (0.5ng/ml) to visualize the nuclei. Mild fixation was necessary to preserve the GFP signal. Images were captured by confocal microscopy.

Results

Uptake and storage of iron by the liver is impaired in PrP^{-/-} mice

Previous reports indicate a phenotype of iron deficiency in transgenic mice carrying a deletion in *PRNP*, the gene encoding PrP^C (PrP^{-/-}) [23], and rescue of this phenotype by inserting *PRNP* in the PrP^{-/-} background [20]. To evaluate whether the underlying cause is reduced availability or impaired uptake, storage, and utilization of circulating iron, age and sex-matched PrP^{+/+} and PrP^{-/-} mice were fed regular chow or supplemented with 200µg of carbonyl-iron daily for 1 month [26]. Absorption from the duodenum and storage by the liver was assessed by quantifying levels of serum iron and ferritin, liver non-heme iron, and expression of liver tissue ferritin, the principal iron storage protein of hepatocytes and reticuloendothelial cells (Figure 1).

Serum iron was significantly lower in $\text{PrP}^{-/-}$ mice on normal chow relative to matched controls, but approached control levels when supplemented with excess carbonyl iron (Figure 1 A). However, serum ferritin remained significantly lower in $\text{PrP}^{-/-}$ samples despite normal serum iron, indicating a deficiency in iron storage (Figure 1 B). Liver stores of non-heme iron and ferritin expression were reduced in $\text{PrP}^{-/-}$ mice, and remained significantly lower than matched controls even after administration of excess iron (Figures 1 C-E). It is notable that despite exposure to excess iron, expression of liver ferritin in $\text{PrP}^{-/-}$ mice remained significantly lower than $\text{PrP}^{+/+}$ controls on normal chow (Figure 1 E).

These results suggest that the principal cause of iron deficiency in $\text{PrP}^{-/-}$ mice is impaired hepatic uptake and storage of circulating iron rather than defective duodenal absorption.

PrP^C promotes uptake of Tf-iron and NTBI by the liver

Since orally introduced carbonyl-iron circulates primarily as Tf-iron and NTBI that require reduction of Fe^{3+} to Fe^{2+} before hepatic uptake [3, 8, 9], we considered the possibility that the recently described FR activity of PrP^{C} on cell models was necessary for the uptake of iron from these sources [19]. Thus, equal amounts of ^{59}Fe -citrate were injected intra-peritoneally in age and sex-matched $\text{PrP}^{+/+}$ and $\text{PrP}^{-/-}$ mice. Since plasma Tf is ~30-40% saturated with iron under normal conditions, this procedure radiolabels mainly plasma Tf with ^{59}Fe . Subsequently, uptake of ^{59}Fe by the spleen and femur, organs that utilize mainly Tf-iron for hematopoiesis, was quantified at the indicated time-points. As expected, a spike in the uptake of ^{59}Fe by newly synthesized red-blood-cells was noted after 24h in both mouse lines. However, the counts in $\text{PrP}^{-/-}$ samples were significantly lower relative to matched $\text{PrP}^{+/+}$ controls (Figures 2 A & B).

To evaluate whether PrP^{C} influences the uptake of NTBI, circulating pool of NTBI in $\text{PrP}^{+/+}$ and $\text{PrP}^{-/-}$ mice was radiolabeled by saturating plasma Tf with excess unlabeled ferric-ammonium citrate (FAC), followed 30 minutes later by intra-peritoneal injection of ^{59}Fe -citrate essentially as described [28]. The mice were euthanized after 6 hours, and uptake of ^{59}Fe -NTBI by the liver and pancreas, the principal organs that store and utilize NTBI respectively, was quantified.

Fractionation of equal aliquots of plasma from untreated (-FAC) and +FAC treated mice on native PAGE followed by autoradiography confirmed ~90% inhibition of ^{59}Fe -labeling of plasma Tf in the latter (Figure 2 C, lanes 1-4 vs. 5-8), ensuring radiolabeling of mainly NTBI in +FAC mice [28]. A comparison of ^{59}Fe uptake by the liver and pancreas in the two mouse strains revealed significantly lower levels in $\text{PrP}^{-/-}$ samples relative to $\text{PrP}^{+/+}$ controls (Figure 2 D). Fractionation of liver homogenates on native PAGE followed by autoradiography confirmed significantly less ^{59}Fe -ferritin in $\text{PrP}^{-/-}$ relative to $\text{PrP}^{+/+}$ controls (Figure 2 E, lanes 1,2 vs. 3,4; arrow-head; Figure 2 F), indicating impaired uptake and/or storage of ^{59}Fe -NTBI in the absence of PrP^{C} .

These results suggest that PrP^{C} promotes the uptake of both Tf-iron and NTBI, and other iron modulating proteins are unable to compensate fully for its absence.

Uptake, storage, and utilization of iron from ferritin is increased in PrP^{-/-} mice

To evaluate whether PrP^C influences the uptake and storage of an alternative source of iron that does not require the activity of FR proteins, uptake of ⁵⁹Fe from intra-peritoneally introduced ⁵⁹Fe-ferritin was evaluated since ferritin is endocytosed by the Tim-2 receptor before the release of associated ⁵⁹Fe in the cytosol [30].

Accordingly, ⁵⁹Fe-citrate and purified, soluble ⁵⁹Fe-ferritin associated with equal amounts of total iron and ⁵⁹Fe-counts were introduced intra-peritoneally in PrP^{+/+} and PrP^{-/-} mice, and uptake of ⁵⁹Fe by the liver was quantified at the indicated times (Figure 3). ⁵⁹Fe counts in the blood and remaining carcass were quantified to eliminate error due to non-specific phagocytosis of ⁵⁹Fe-ferritin in the peritoneal cavity.

Surprisingly, uptake of ⁵⁹Fe from ferritin was significantly higher in PrP^{-/-} samples relative to PrP^{+/+} controls at all the time-points tested (Figure 3 A). However, uptake of ⁵⁹Fe from citrate that is likely to associate with plasma Tf and NTBI was significantly lower in PrP^{-/-} mice (Figure 3 B).

Fractionation of 11 day liver homogenates by native PAGE followed by autoradiography showed significantly higher incorporation of ⁵⁹Fe in liver tissue ferritin in samples injected with purified ⁵⁹Fe-ferritin, and much lower levels in both mouse lines exposed to ⁵⁹Fe-citrate (Figure 3 C, lanes 2-5). Western blotting of the same samples for actin confirmed that the differences were not an artifact of sample loading.

To evaluate whether the stored ⁵⁹Fe in liver ferritin was used for hemoglobin synthesis, the predominant consumer of systemic iron, lysates prepared from 11 day washed RBCs were fractionated by native PAGE followed by autoradiography. The signal from ⁵⁹Fe-Hb was much stronger in samples from PrP^{-/-} mice exposed to ⁵⁹Fe-ferritin relative to matched PrP^{+/+} controls, and from mice exposed to ⁵⁹Fe-citrate (Figure 3 D, lanes 2-5, arrow-head).

Together, the above observations demonstrate that absence of PrP^C limits hepatic uptake of circulating Tf-iron and NTBI that require reduction to Fe²⁺. Uptake of iron from ferritin that does not require reduction and metabolic processes subsequent to iron uptake, i.e., storage in liver ferritin and utilization for hemoglobin synthesis are not affected by the absence of PrP^C. Surprisingly, mRNA levels of liver hepcidin, the master regulator of systemic iron homeostasis, did not show a significant difference between PrP^{-/-} samples and matched PrP^{+/+} controls. However, analysis of the same samples for additional iron modulating proteins revealed significant up-regulation of mRNA for divalent metal transporters ZIP8 and ZIP14, probably as a response to the iron deficiency in these mice (supplemental Figure 1).

PrP^C promotes NTBI uptake via ZIP14 and DMT1

To determine whether the FR activity of PrP^C is coupled with iron uptake through specific metal transporters, HepG2 cells, a well-characterized hepatoma cell-line, was used. Expression of a fusion construct of PrP^C and green-fluorescent-protein (PrP^C-GFP) [24] revealed expression on the plasma membrane, co-localizing with wheat-germ agglutinin

(WGA) that binds to apical and basolateral surface glycoproteins on cultured HepG2 cells (Figure 4, panels 1-3) [31].

Before proceeding with the identification of PrP^C-associated metal transporters, FR activity of transfected PrP^C in HepG2 cells was confirmed. PrP^C (HepG2-PrP^C) or vector (HepG2-vec)-containing HepG2 cells were exposed to 30μM FAC (Fe³⁺) or iron ascorbate (Fe²⁺), and uptake of iron was measured as a function of ferritin up-regulation (Figure 5 A). Expression of PrP^C induced 2.7-fold up-regulation of intracellular ferritin, indicating increased uptake of iron from the medium (Figure 5 A, lanes 1 vs. 4). Notably, exposure to Fe³⁺ (FAC) induced 3.1-fold up-regulation of ferritin in HepG2-PrP^C-cells relative to vector controls (Figure 5 A, lane 2 vs. 5; Figure 5 B), while exposure to Fe²⁺ from iron ascorbate (Fe²⁺) caused a similar change in ferritin levels in the two cell-lines (Figure 5 A, lane 3 vs. 6; Figure 5 B). Since the amount of added Fe³⁺ and Fe²⁺ far exceeded the iron-binding capacity of Tf in the culture medium, these results suggest a facilitative role of PrP^C in the uptake of both Tf-iron and NTBI from the medium due to its FR activity.

Subsequently, stable HepG2-vec and HepG2-PrP^C cells were co-transfected with GFP-tagged ZIP8, ZIP14, or DMT1, and uptake of iron from complete medium (with no added iron) was quantified after 24 hours as a function of ferritin up-regulation. Expression of ZIP8, ZIP14, or DMT1 in HepG2-vec cells caused ~3.2-fold up-regulation of ferritin relative to controls, consistent with the iron transport function of these proteins (Figure 5 C, lane 1 vs. 2-4, Figure 5 D). However, co-expression of PrP^C with ZIP8, ZIP14, or DMT1 induced a further up-regulation of ferritin by 0.8, 2.8, and 2.6-fold respectively, indicating that PrP^C enhances iron uptake via these transporters, mainly ZIP14 and DMT1 (Figure 5 C, lanes 2-4 vs. 6-8; Figure 5 D).

Notably, a significant decrease in PrP^C expression accompanied co-expression of the iron transporter, particularly ZIP14 and DMT1. The decrease was specific to 3F4-reactive full-length PrP^C, including the un-glycosylated (27kDa) and various glycoforms migrating between 30-37kDa (Figure 5 C, upper panel; Figure 5 E). On the other hand, anti-C reactive PrP^C forms that include N-terminally truncated un-glycosylated (18kDa) and glycosylated forms (18-30kDa) in addition to full-length PrP^C showed a corresponding increase (Figure 5 C, lower panel, lanes 7,8). A darker exposure revealed up-regulation and cleavage of endogenous PrP^C in HepG2-vec cells in the presence of ZIP14 and DMT1 (lower panel, darker exposure, lanes 3 & 4). Since PrP^C undergoes physiological recycling from the plasma membrane and is cleaved at residues 111/112 during this process that eliminates the epitope for 3F4 (Figure 5 E) [25], it is likely that expression of ZIP14 and DMT1 up-regulates recycling, or triggers cleavage of PrP^C by an independent process.

Discussion

This report demonstrates that PrP^C promotes uptake of NTBI, and to a lesser extent, Tf-iron by providing FR activity necessary for the transport of ferric iron through divalent metal transporters ZIP14 and DMT1. This explains reduced hepatic iron stores of PrP^{-/-} mice despite chronic iron supplementation, and enhanced uptake of ferric-iron by HepG2 cells expressing exogenous PrP^C. Co-expression of PrP^C with ZIP14 and DMT1 augments ferric

iron uptake further, suggesting that PrP^C functions as a FR partner for these transporters. In contrast, PrP^C does not influence hepatic uptake and storage or utilization of ferritin-bound iron *in vivo*, or of iron ascorbate (Fe²⁺) by HepG2 cells *in vitro*, sources that do not require reduction of associated iron for uptake. These observations underscore the functional significance of PrP^C as a FR, and the inability of other FR proteins to compensate fully for its absence.

As reported previously [20] and expanded here, absence of PrP^C results in systemic iron deficiency in PrP^{-/-} mice as evidenced by significantly lower levels of serum iron, serum ferritin, liver non-heme iron, and liver ferritin relative to PrP^{+/+} controls. Although chronic exposure to excess oral iron restored serum iron in PrP^{-/-} mice to control levels, serum ferritin, liver nonheme-iron, and liver ferritin remained significantly lower than iron-supplemented PrP^{+/+} mice, suggesting impaired hepatic uptake and storage of iron as the principal cause of iron deficiency, not insufficient absorption from the duodenum.

It is notable that impairment of hepatic iron uptake in PrP^{-/-} mice is specific to NTBI and Tf-iron that require reduction to the ferrous form for transport through divalent metal transporters [7, 9]. Uptake of ⁵⁹Fe-Tf by the spleen and bone marrow, main hematopoietic organs that utilize Tf-bound iron, and ⁵⁹Fe-NTBI by the liver and pancreas, principal organs that store and utilize NTBI, was significantly lower in PrP^{-/-} mice relative to PrP^{+/+} controls despite systemic iron over load [28]. On the other hand, uptake, storage, and utilization of ⁵⁹Fe from iron-loaded ferritin that utilizes receptor-mediated uptake pathway [30] was increased in PrP^{-/-} mice, highlighting the iron-deficiency of these mice, and implicating PrP^C in the uptake of ferric iron, not ferritin iron that is stored and used effectively in its absence. It is likely that the relatively mild iron deficiency in PrP^{-/-} mice is due to the participation of PrP^C in the uptake of mainly NTBI from the plasma membrane, not Tf-iron that can utilize alternative FR proteins such as Steap3 in late endosomes. Consistent with this assumption, transcription of liver hepcidin, the master regulator of systemic iron homeostasis, was not altered in PrP^{-/-} mice, though ZIP8 and ZIP14 showed significant up-regulation (Figure 6).

In vitro observations on HepG2 expressing exogenous PrP^C support the *in vivo* observations in PrP^{-/-} mice. Thus, expression of PrP^C in HepG2 cells increased the uptake of Fe³⁺ from FAC, and had minimal influence on Fe²⁺ from iron ascorbate, leaving little doubt that PrP^C is a functional FR on the plasma membrane of these cells. However, this is an unusual function for a glycosylphosphatidylinositol-linked protein that is unlikely to utilize reducing equivalents from the cytosol because of linkage to the outer leaflet of the plasma membrane. It is likely that PrP^C utilizes extracellular reducing agents for its FR activity such as conversion of ascorbate released from cells to dehydroascorbate through the intermediate ascorbyl free radical [32]. We believe that PrP^C itself functions as a ferrireductase based on the following observations: 1) uptake of Fe³⁺ is reduced in the liver and kidney of PrP^{-/-} mice, organs involved in storage and reuptake of iron from the glomerular filtrate respectively and likely to express distinct set of proteins on the plasma membrane [this study; 22], and 2) exogenous expression of PrP^C on HepG2 cells, proximal kidney tubule epithelial cells [this study; 22], neuroblastoma cells [19], and K562 cells, a hematopoietic cell line [unpublished observations] increases FR activity on the plasma membrane and in

cell lysates, indicating a primary role of PrP^C in iron uptake. The increase in FR activity due to PrP^C is sensitive to heat, pH, and is saturated at a specific concentration of PrP^C and Fe³⁺ [19], suggesting an enzymatic nature. However, the possibility that PrP^C interacts with a ubiquitously expressed FR via its octa-peptide repeat region cannot be ruled out from our data. Further studies are necessary to characterize the FR activity of PrP^C fully.

It is notable that co-expression of PrP^C with ZIP14 or DMT1 increased uptake of Fe³⁺ from the medium, indicating a close functional interaction. Interestingly, expression of ZIP14 and DMT1 up-regulated endoproteolytic cleavage of PrP^C, a physiological process that increases the proportion of N-terminally cleaved, C-terminal fragment of PrP^C relative to the full-length form [25]. Since PrP^C is expressed abundantly on neurons, it possibly functions as a FR partner for ZIP14 and DMT1 on the neuronal plasma membrane and endosomes, facilitating the uptake of NTBI and Tf-iron by these cells. Concomitant cleavage of the N-terminus necessary for its FR activity is likely to regulate iron uptake, a phenomenon unique to PrP^C and absent in other FR proteins such as Dcytb and Steap3 abundant on the plasma membrane and endosomes respectively of specific cell types (Figure 5 F). A detailed understanding of the relationship between iron transport and endoproteolytic cleavage of PrP^C is required for defining the contribution of PrP^C to cellular iron metabolism.

Interestingly, PrP and the Zip family of proteins share an evolutionary link [33, 34], and certain members of the Zip family are expressed in close proximity with PrP^C at the plasma membrane [35] and are altered during prion infection [36]. ZIP14 is most abundant in the liver, pancreas, and heart, and like PrP^C, is localized at the plasma membrane where it mediates the uptake of NTBI [16, 17, 37]. Recent reports indicate localization of ZIP14 in late endosomes and lysosomes in addition to the plasma membrane, suggestive of a role in the transport of Tf-iron across the endosomal membrane [38]. ZIP8, on the other hand, is most abundant in the lung, testis, and kidney [39]. Impaired uptake of ⁵⁹Fe-NTBI by the liver and pancreas of PrP^{-/-} mice therefore suggests that PrP^C is the principal FR partner for ZIP14 on the plasma membrane of these organs. Although physical interaction of PrP^C with the metal transporters is not necessary for the coupled reduction and transport of Fe³⁺ iron, limited co-localization of PrP^C with ZIP14 was evident in HepG2 cells by confocal microscopy (unpublished observations).

In conclusion, this report provides convincing evidence that PrP^C functions as a FR partner for ZIP14 and DMT1, and possibly regulates the uptake of NTBI and Tf-iron by shedding its N-terminal FR domain during recycling from the plasma membrane. Since DMT1 and ZIP14 mediate the transport of other cations including copper and zinc, aggregation of PrP^C to the disease-associated PrP-scrapie form during prion disease pathogenesis is likely to alter the homeostasis of other metals in addition to the reported effect on brain iron homeostasis [29, 40]. In addition, these observations have significant implications for Alzheimer's disease where brain metal imbalance is a prominent feature, and PrP is believed to play an important pathogenic role [41-44].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Dcytb	Duodenal cytochrome b
DMT1	Divalent metal transporter-1
FAC	Ferric ammonium citrate
FR	Ferrireductase
GFP	Green-fluorescent protein
IP	Intra peritoneal
LIP	Labile iron pool
NTBI	non-transferrin bound iron
PrP^C	Prion protein
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tf	Transferrin
TfR	Transferrin receptor
ZIP	Zrt Irt-like protein

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Highlights

1. Cellular prion protein (PrP^C) functions as a ferrireductase (FR) partner for divalent metal transporters ZIP14 and DMT1 at the plasma membrane and endosomal compartment.
2. Endoproteolytic cleavage of PrP^C with the release of N-terminal FR domain is increased by co-expression with ZIP14 and DMT1, suggesting a regulatory role of PrP^C in iron uptake.
3. The FR activity of PrP^C is likely to modulate neuronal iron metabolism.

Figure 1. Uptake and storage of iron by the liver is impaired in PrP^{-/-} mice
 (A-C) Serum iron (A) serum ferritin (B), and liver non-heme iron (C) are lower in PrP^{-/-} mice relative to PrP^{+/+} controls. Supplementation with oral iron restores serum iron of PrP^{-/-} mice towards control values, but serum ferritin and liver non-heme iron remain significantly lower than matched controls. (D & E) Ferritin in liver homogenates of PrP^{-/-} mice on regular chow is lower than PrP^{+/+} controls (panel D, lanes 1 & 2 vs. 5 & 6, panel E). Iron supplementation up-regulates ferritin in both mouse lines (panel D, lanes 3, 4, 7, 8; panel E), but the levels in iron supplemented PrP^{-/-} mice remain significantly lower than control and iron-supplemented PrP^{+/+} mice (panel D, lanes 7, 8 vs. 1-4; panel E). (All samples were fractionated on the same gel that was re-arranged using Photoshop). Reaction for β -actin was used to normalize densitometric data in panel E. Values are mean $\pm$ SEM of the indicated n. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2. PrP^{-/-} mice show impaired uptake of Tf-iron and NTBI

(A & B) Uptake of ⁵⁹Fe from intra-peritoneally administered ⁵⁹Fe-citrate by the spleen and femur is maximal after 24h, and significantly lower in PrP^{-/-} samples relative to PrP^{+/+} controls. (C) Intra-peritoneal administration of excess unlabeled FAC prior to ⁵⁹Fe-citrate inhibits radiolabeling of plasma Tf by ~90% (lanes 1-4 vs. 5-8) (D) Uptake of ⁵⁹Fe-NTBI is significantly lower in the liver and pancreas of PrP^{-/-} mice relative to PrP^{+/+} controls. (E & F) Incorporation of ⁵⁹Fe-NTBI by liver ferritin (+FAC) is significantly lower in PrP^{-/-} samples relative to PrP^{+/+} controls (lanes 3, 4 vs. 1, 2). Western blotting of the same samples

and probing for β -actin provides a control for protein loading. Values are mean $\pm$ SEM of the indicated n. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3. Uptake, storage, and utilization of intra-peritoneally administered ferritin-iron is increased in PrP^{-/-} mice

(A & B) Hepatic uptake of ^{59}Fe by PrP^{-/-} mice is higher when exposed to intra-peritoneal ^{59}Fe -ferritin, and lower when exposed to equal counts of ^{59}Fe -citrate relative to PrP^{+/+} controls at the indicated time-points. Values are mean $\pm$ SEM of the indicated n. * p <0.05, ** p <0.01, *** p <0.001. (C) Autoradiography of 11 day liver homogenate samples fractionated by native PAGE shows significantly more ^{59}Fe in PrP^{-/-} samples exposed to ^{59}Fe -ferritin relative to PrP^{+/+} controls (lanes 2,3), and minimal incorporation in both mouse lines exposed to ^{59}Fe -citrate (lanes 4,5). A representative fluorogram from three independent experiments is shown. (D) Incorporation of ^{59}Fe in hemoglobin is significantly higher in PrP^{-/-} samples exposed to ^{59}Fe -ferritin relative to PrP^{+/+} controls (lanes 2,3), and equivalent in both mouse lines exposed to ^{59}Fe -citrate (lanes 4,5). A representative fluorogram from three independent experiments is shown. (Samples in panels C and D were fractionated on the same gel that was re-arranged by Photoshop). Reaction for β -actin confirms equivalent protein loading.

Figure 4. Distribution of PrP^C on HepG2 cells

Confocal imaging of HepG2-PrP^C-GFP cells immunostained with WGA shows mainly plasma membrane localization of PrP^C-GFP (panels 1-3). Scale bar: 10 μ M.

Figure 5. PrP^C functions as a FR partner for ZIP14 and DMT1

(A & B) Expression of PrP^C in HepG2 cells up-regulates ferritin by 2.7-fold relative to vector-expressing controls (A, lane 1 vs 4; B). Exposure to FAC (Fe³⁺) causes 3.1-fold up-regulation of ferritin in HepG2-PrP^C cells relative to HepG2-vector controls (A, lane 2 vs. 5; B). Uptake of iron from iron-ascorbate (Fe²⁺) is similar in HepG2-vector and HepG2-PrP^C cells (lane 3 vs. 6; B). A representative gel from three independent experiments and corresponding densitometric analysis is shown. (C & D) Transfection of HepG2-vector cells with ZIP8, ZIP14, or DMT1 up-regulates ferritin by ~3.2 fold (C, lane 1 vs. 2-4, D).

Expression of ZIP14 and DMT1 in HepG2-PrP^C cells causes a further up-regulation of ferritin by 2.8 and 2.6 fold respectively (C, lanes 5,6 vs. 7,8; D). Surprisingly, 3F4-reactive PrP^C is down-regulated in the presence of ZIP8, ZIP14, and DMT1 (C, upper panel, lane 5 vs. 6-8, darker exposure), while the anti-C reactive N-terminally truncated forms of PrP^C increase in abundance especially in the presence of ZIP14 and DMT1 (C, lower panel, lane 5 vs. 6-8, darker exposure; E). Notably, anti-C-reactive truncated forms of endogenous PrP^C are visible even in vector transfected cells co-expressing ZIP14 and DMT1 (lower panel, darker exposure, lanes 3, 4). A representative gel from four independent experiments and corresponding densitometric analysis is shown. **(E)** Schematic representation of epitopes for 3F4 and anti-C. **(F) Proposed function of PrP^C as a FR.** PrP^C at the plasma membrane and endosomal membrane reduces Fe³⁺ to Fe²⁺, which is then transport across the lipid bilayer through ZIP14 and DMT1. This process results in cleavage of PrP^C at residues 111/112 and loss of the N-terminal FR domain. PrP^C lacking the N-terminal domain is transported back to the plasma membrane, precluding further FR activity. Tf-iron is endocytosed by the Tf/TfR complex, and Fe³⁺ released from Tf in acidic endosomes is reduced to Fe²⁺ by PrP^C and other membrane-bound FR proteins such as Dcytb and/or Steap3. Fe²⁺ transported to the cytosol joins the cellular labile iron pool, and excess is oxidized to Fe³⁺ and stored in cytosolic ferritin. PM: plasma membrane; End: endosome; Nuc: nucleus, Tf/TfR: transferrin/transferrin receptor.