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Haifeng Shi
National Institute of Diabetes and Digestive and Kidney Diseases

Krisztina Z. Bencze
Wayne State University

Timothy L. Stemmler
Wayne State University, tstemmle@med.wayne.edu

Caroline C. Philpott
National Institute of Diabetes and Digestive and Kidney Diseases

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A cytosolic iron chaperone that delivers iron to ferritin

Haifeng Shi[1], Krisztina Z. Bencze[2], Timothy L. Stemmler[2], and Caroline C. Philpott[1],†

[1]Liver Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD

[2]Department of Biochemistry and Molecular Biology, Wayne State University School of Medicine, Detroit, MI

†To whom correspondence should be addressed. E-mail: carolinep@intra.niddk.nih.gov

Abstract

Ferritins are the main iron storage proteins found in animals, plants and bacteria. The capacity to store iron in ferritin is essential for life in mammals, but the mechanism by which cytosolic iron is delivered to ferritin is unknown. Human ferritins expressed in yeast contain little iron. The human Poly r(C)-Binding Protein 1 (PCBP1) increased the amount of iron loaded into ferritin when expressed in yeast. PCBP1 bound to ferritin in vivo, and bound iron and facilitated iron loading into ferritin in vitro. Depletion of PCBP1 in human cells inhibited ferritin iron loading and increased cytosolic iron pools. Thus, PCBP1 can function as a cytosolic iron chaperone in the delivery of iron to ferritin.

Ferritins are iron storage proteins that are ubiquitously expressed in animals, plants and bacteria. They serve both to sequester excess iron taken up by the cell and to release stored iron to meet the cell's metabolic needs during iron scarcity (*1*). In animals, ferritin is a cytosolic heteropolymer consisting of 24 subunits of H- and L-isoforms that assemble into a hollow sphere into which iron is deposited. Ferritin H-chains contain the iron-binding and ferroxidase activities that are required for mineralization of the ferritin core. Deletion of the H-ferritin gene is lethal in mice (*2*) and in flies (*3*).

In cells, metallochaperones deliver metals to their cognate enzymes and transporters. Although cytosolic copper and nickel chaperones have been described (*4-7*), no cytosolic iron chaperones have been identified, despite the presence of numerous iron-dependent enzymes in the cytosol. Frataxin, the protein lacking in the neurological disease Friedreich's ataxia, functions as a mitochondrial iron chaperone for iron-sulfur cluster and heme biosynthesis (*8, 9*).

Fungi are anomalous among eukaryotes in that they do not express ferritin. We expressed human H- and L-ferritins in *Saccharomyces cerevisiae*; ferritin assembled into multimeric complexes with properties similar to native human ferritins, but contained only small amounts of iron (fig. S1, A and B). We hypothesized that yeast may also lack the requisite iron chaperones needed for delivery of iron to ferritin and designed a genetic screen to identify human genes that, when expressed in yeast, could increase the amount of iron loaded into ferritin. We introduced an iron-regulated *FeRE/HIS3* reporter construct (*10*) into a yeast strain expressing H- and L-ferritin (Fig. 1A). This construct confers histidine prototrophy to cells when the reporter is bound and transcriptionally activated by Aft1p, the major iron-dependent transcription factor in yeast. Aft1p is activated during periods of cytosolic iron depletion (*11*), which could occur if substantial amounts of cytosolic iron were diverted into ferritin.

Yeast cells containing ferritin and the iron-responsive reporter were transformed with a library synthesized from human liver cDNA engineered into a yeast expression vector. Transformants that exhibited growth on plates lacking histidine were selected for further analysis. We isolated multiple copies of the Poly r(C)-Binding Protein 1 (PCBP1), as well as other unrelated genes including ferritin H-chain. Plasmids containing PCBP1 or the empty vector were retransformed into reporter yeast strains lacking or expressing H- and L-ferritins (Fig. 1B). Expression of PCBP1 did not activate the *FeRE/HIS3* reporter in cells lacking ferritin, as indicated by a lack of growth on medium without histidine. But expression of PCBP1 did activate the *FeRE/HIS3* reporter in the yeast strain expressing ferritins, resulting in growth on medium lacking histidine. Thus, expression of human PCBP1 activated the iron-responsive reporter only in the presence of ferritin. To confirm that reporter activation was due to the delivery of cytosolic iron into ferritin, we directly measured

the incorporation of iron into ferritin by growing yeast in the presence of $^{55}FeCl_3$, isolating ferritin on non-denaturing gels, and measuring the amount of ^{55}Fe in the ferritin heteropolymer (Fig. 1C and D). Significant amounts of iron-containing protein were only detected in cells expressing ferritin. Iron was detected in a single species that co-migrated with the ferritin heteropolymer. Co-expression of PCBP1 in these cells resulted in a 2.3-fold increase in the amount of iron in ferritin. This increase was not due to changes in the overall amount of ferritin (Fig. 1E) or due to changes in the relative ratio of H- and L- chains (fig. S1A). Similarly, the total amount of ^{55}Fe taken up by the cells expressing ferritin alone was not different from the amount taken up by cells expressing both PCBP1 and ferritin (fig. S1C).

The delivery of cytosolic iron to ferritin in the presence of PCBP1 activated the *FeRE/HIS3* reporter. We asked whether other proteins expressed during yeast iron deficiency, such as the plasma membrane ferric reductases, were also activated by expression of PCBP1 (Fig. 1F). Ferric reductase activity was low in cells that did not express ferritins, regardless of whether the cells contained pPCBP1 or the empty vector. The ferritin-expressing strain exhibited slightly greater reductase activity than the non-ferritin strain when transformed with vector alone and 4-fold greater reductase activity when the ferritin strain also expressed PCBP1. Thus, when human PCBP1 was expressed in yeast cells containing human ferritins, iron was diverted into ferritin and the cellular iron deficiency response was activated.

PCBP1 is an RNA-binding protein that is ubiquitously expressed in mammalian cells and is located in both the cytosol and the nucleus (*12*). We tested whether PCBP1 was involved in ferritin iron loading in human cells by depleting cellular PCBP1, loading cells with ^{55}Fe, and measuring the amount of ^{55}Fe loaded into endogenous cytosolic ferritin. Huh7 cells were transfected with PCBP1 siRNA or control siRNA, and partial depletion of PCBP1 mRNA and protein was confirmed (fig. S2). Transfected cells were loaded with $^{55}FeCl_3$, and ferritin was examined (Fig. 2, A-C). Cells depleted of PCBP1 exhibited a 63% reduction in the amount of ^{55}Fe incorporated into ferritin when compared to control cells at 6, 12 and 24 hrs. This reduction in ferritin iron loading was not due to lowered levels of ferritin protein, as these levels did not change significantly when PCBP1 was depleted (Fig. 2B). The reduction in ferritin iron loading was also not due to loss of ^{55}Fe uptake in the PCBP1-depleted cells, as uptake of both $^{55}FeCl_3$ and $^{55}Fe_2$-transferrin was equivalent in cells transfected with control or PCBP1 siRNAs (fig. S3). The loss of ferritin iron loading in cells treated with PCBP1 siRNA was not due to off-target effects of the siRNA. Ferritin iron loading was restored in cells co-transfected with a plasmid expressing human PCBP1 containing silent mutations (fig. S4). The ferritin mineralization that remained after PCBP1 depletion might have been due to the activity of residual

PCBP1. Alternatively, paralogues of PCBP1, such as PCBP2, which also activated the *FeRE/HIS3* reporter in yeast (fig. S5), may contribute to ferritin iron loading.

   To determine whether the loss of ferritin iron loading during PCBP1 depletion also resulted in an increase in cytosolic iron, we measured the levels of iron regulatory protein 2 (IRP2). The half-life of IRP2 is inversely related to cytosolic iron levels (*13*), with IRP2 levels increasing when iron is scarce and decreasing when iron is abundant (fig. S6). We transfected HEK293 cells stably overexpressing IRP2 (*14*) with control and PCBP1 siRNAs and measured the levels of IRP2 (Fig. 2D). Loss of PCBP1 was associated with a decrease in IRP2, consistent with the loss of PCBP1 leading to an increase in the cytosolic iron pool. The relative levels of the chelatable cytosolic iron pool can be measured using fluorescent iron chelators (*15*) and depletion of PCBP1 in Huh7 cells led to a 67% increase in the chelatable iron pool (Fig. 2E).

   PCBP1 may facilitate ferritin iron loading by directly interacting with ferritin, or by an indirect mechanism that requires other cellular factors. We tested for a direct, in vivo, interaction between ferritin and PCBP1 by co-immunoprecipitation in yeast cells (Fig. 3). PCBP1 co-immunoprecipitated with ferritin in cells expressing PCBP1 and H- and L-ferritin (Fig. 3, A and B). No PCBP1 was detected in immune complexes from cells lacking either PCBP1 or ferritin. When cells expressed both ferritins and PCBP1, PCBP1 was not detected in immunoprecipitates collected in buffer without iron (Fig.3B), but when ferrous iron was added to the buffer, PCBP1 was detected in anti-ferritin immunoprecipitates (Fig. 3A, 3B). The addition of bathophrenanthroline disulfonate, a ferrous iron chelator, to buffer containing iron, blocked the co-immunoprecipitation of PCBP1 with ferritin (Fig. 3B). Thus, PCBP1 physically interacted with ferritin in the presence of iron and might directly bind iron.

   We used isothermal titration calorimetry (ITC) to directly measure interactions between PCBP1 and iron. PCBP1 overexpressed and purified from *E. coli* was folded with high helical content (fig. S7). Titration of ferrous iron into solutions of PCBP1 under anaerobic conditions produced negative peaks in the raw thermogram, which indicated ferrous iron binds to PCBP1 in an exothermic process (Fig. 4A). An integration of each individual titration peak gave rise to the processed spectrum (Fig. 4B). PCBP1 bound a total of 3 iron atoms with a dissociation constant of 0.9 ± 0.1 mM for the first and an average dissociation constant of 5.8 ± 0.3 mM for the remaining two metal ions.

   Although ferritin mineralization occurs in vitro in the presence of only ferrous iron and oxygen, we tested whether PCBP1 could enhance mineralization of ferritin at low iron concentrations (Fig. 4C and D). Addition of purified PCBP1 to solutions of apoferritin and ^{55}Fe(II) increased the amount of

[55]Fe detected in ferritin in a dose-dependent manner, with mineralization increasing 2-fold at the higher concentrations of PCBP1. Albumin, which binds iron with low affinity, did not significantly alter the amount of iron incorporated into ferritin, indicating that PCBP1 specifically and directly delivered iron for ferritin mineralization in vitro.

Human H-ferritin homopolymers bind ferrous iron with an affinity of 15 mM (*16*), a concentration far above the levels of "free" ferrous iron thought to be present in cytosol, which raises the likelihood that a specific iron carrier is required for delivery of iron to ferritin. PCBP1 bound 3 atoms of ferrous iron with dissociation constants of 0.9-5.8 mM, which is similar to the binding affinity of yeast frataxin, a mitochondrial iron chaperone that binds 2 ferrous iron atoms with dissociation constants of 2-3 mM (*17*). Similarly, the cytosolic copper chaperone Atox1 binds copper with a K_d of ~10 mM (*18*). PCBP1 bound to ferritin only in the presence of Fe(II). Similarly, the interaction between yeast Atx1p and the copper transporter Ccc2p only occurs in the presence of copper (*19*).

PCBP1 is a member of a family of four homologous RNA binding proteins belonging to the KH domain superfamily and is widely expressed and highly conserved among mammals. PCBP1 and 2 bind specifically to sequences within multiple cellular or viral mRNA species with a consequent increase in the stability of the messages or alteration of their translation efficiency (*12*). The bifunctional nature of PCBP1 as both an iron- and a RNA-binding protein is reminiscent of the mammalian iron regulatory protein, IRP1. IRP1 functions as cytosolic aconitase when it contains an iron-sulfur cluster and binds to mRNA transcripts involved in iron homeostasis (such as ferritin) when it does not (*13*). We propose that PCBP1 acts as a cytosolic iron chaperone, directly binding iron and loading ferritin.

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