A Cytosolic Iron Chaperone That Delivers Iron to Ferritin

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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. Human poly (rC)–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 heavy (H) and light (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 ferritins. We expressed human H and L ferritins in the yeast Saccharomyces cerevisiae. The peptides assembled into multimeric complexes, with properties similar to those of native human ferritins, but contained only small amounts of iron (fig. S1, A and B). We hypothesized that yeast might 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 protrophism 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. Transforms that exhibited growth on plates lacking histidine were selected for further analysis. We isolated multiple copies of PCBP1, as well as proteins encoded by other unrelated genes, including H ferritin. 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 media without histidine. But expression of PCBP1 did activate the FeRE/HIS3 reporter in the yeast strain expressing ferritins, resulting in growth on media 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 delivery of cytosolic iron into ferritin, we directly measured the incorporation of iron into ferritin by growing yeast in the presence of [55Fe]Cl3, isolating ferritin on nondenaturing gels, and measuring the amount of 55Fe in the ferritin heteropolymer (Fig. 1, C and D). Substantial amounts of iron-containing protein were detected only in cells expressing ferritin, and iron was detected in a single species that comigrated with the ferritin heteropolymer. Coexpression 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 in the relative ratio of H and L chains (fig. S1A). Similarly, the total amount of 55Fe 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 encoded by other unrelated genes, including H ferritin.
pressed 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 nonferritin strain when transformed with vector alone and four times the 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 or control small interfering RNAs (siRNAs), and partial depletion of PCBP1 mRNA and protein was confirmed (Fig. S2). Transfected cells were loaded with $[^{55}\text{Fe}]\text{Cl}_3$, and ferritin was examined (Fig. 2, A to C). PCBP1-depleted cells exhibited a 63% reduction in the amount of $^{55}$Fe incorporated into ferritin when compared with the amount in control cells at 6, 12, and 24 hours. This reduction in ferritin iron loading was not due to lowered levels of ferritin protein, because 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, because uptake of both $[^{55}\text{Fe}]\text{Cl}_3$ and $[^{55}\text{Fe}]\text{transferrin}$ 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 cotransfected 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, paralogs 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 human embryonic kidney–293 (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, which is 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 with the use of 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 coimmunoprecipitation in yeast cells (Fig. 3). PCBP1 coimmunoprecipitated 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 antiferritin immunoprecipitates (Fig. 3, A and B). The addition of bathophenanthroline disulfonate

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**Fig. 1.** PCBP1 delivers iron to ferritin in *S. cerevisiae*. (A) Activation of an iron-regulated, Aft1-responsive promoter (FeRE) fused to the HIS3 coding sequence when cytosolic iron is transferred into ferritin (Ftn). (B) PCBP1-dependent activation of the FeRE/HIS3 reporter in yeast expressing ferritin, but not in yeast without ferritin. Transformed strains were plated in serial dilutions on media with and without histidine. (C and D) PCBP1 increases ferritin mineralization. Strains transformed as in (B) were grown with $[^{55}\text{Fe}]\text{Cl}_3$, ferritin was isolated by native gel electrophoresis, and iron within ferritin was detected by autoradiography. In (D), replicates were normalized to the vector-transformed strain ($n=5$ independent replicates). *P* < 0.002. (E) PCBP1 did not affect ferritin protein levels ($n=10$ samples). (F) PCBP1-dependent increase in surface ferric reductase activity in yeast expressing ferritin, but not in yeast without ferritin ($n=5$ samples). *P* < 0.002. This and subsequent *P* values were determined via a two-tailed *t* test. Error bars in (D) to (F) indicate SEM.

**Fig. 2.** Depletion of PCBP1 impairs ferritin iron loading in Huh7 cells. Huh7 cells transfected with PCBP1 or control siRNAs were labeled with $[^{55}\text{Fe}]\text{Cl}_3$ for the indicated times. Ferritin was detected by immunoblotting (A) or immunodetection of [55Fe]Cl3 (B). (C) Relative quantitation of ferritin protein, iron, and iron/protein ratio ($n=5$ independent replicates). *P* < 0.0002. (D) Degradation of IRP2 in cells lacking PCBP1. HEK293 cells expressing IRP2 were transfected with PCBP1 and control siRNAs. IRP2, PCBP1, and actin were detected by immunoblotting. (E) Increased labile iron pool in cells lacking PCBP1. PCBP1 was depleted in Huh7 cells, and the relative amounts of the chelatable intracellular iron pool were measured ($n=6$ samples). *P* = 0.003. Error bars in (C) and (E) indicate SEM.
Fig. 3. PCBP1 binds ferritin in vivo. (A and B) Yeast cells expressing ferritin H and L chains, PCBP1, or both were lysed and subjected to immunoprecipitation (IP) with antibodies to PCBP1 or ferritin in buffers containing ferrous iron [both (A) and (B)] or bovine serum albumin (BSA). Ferritin iron was detected by autoradiography. (D) Relative 55Fe(IP) with antibodies to PCBP1 or ferritin in buffers containing ferrous iron [both Atox1 and PCBP1, or both were lysed and subjected to immunoprecipitation].

Fig. 4. PCBP1 binds ferrous iron and increases ferritin iron loading in vitro. (A) and (B) PCBP1 binds ferrous iron with micromolar affinity by ITC. (A), raw ITC spectrum; (B), heat evolved per addition of titrant. Metal binding was enthalpically favorable (enthalpy ΔH = −9.9 kcal/mol) with an overall favorable free energy ΔG. Binding was entropically unfavorable [entropy ΔS = −10.7 cal/(K⋅mol)]. (C and D) Increased ferritin mineralization in the presence of PCBP1. Equine apoferritin was incubated with 55Fe(II) and PCBP1 or bovine serum albumin (BSA). Ferritin iron was detected by autoradiography. (D) Relative 55Fe incorporated into ferritin. Samples were normalized to the sample without added PCBP1 (*P < 0.007). Error bars indicate SEM.

References and Notes
3. F. Missirlis et al., Genetics 177, 89 (2007).
10. A. Dauts et al., Cell 76, 393 (1994).
17. J. D. Cook et al., Biochemistry 45, 7767 (2006).
Massive Horizontal Gene Transfer in Bdelloid Rotifers

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Horizontal gene transfer in metazoans has been documented in only a few species and is usually associated with endosymbiosis or parasitism. By contrast, in bdelloid rotifers we found many genes that appear to have originated in bacteria, fungi, and plants, concentrated in telomeric regions along with diverse mobile genetic elements. Bdelloid proximal gene-rich regions, however, appeared to lack foreign genes, thereby resembling those of model metazoan organisms. Some of the foreign genes were defective, whereas others were intact and transcribed; some of the latter appeared to lack foreign genes, thereby resembling those of model metazoan organisms. Some of these genes may represent an important force in bdelloid evolution.

Horizontal gene transfer (HGT), the movement of genes from one organism to another by means other than direct descent (vertical inheritance), has been documented in prokaryotes (1) and in phagocytic and parasitic unicellular eukaryotes (2–4). Despite the large number of sequenced genomes, documented HGT is rare in metazoans, at least in part because of the sequestration of the germ line (5, 6). HGT may be facilitated by long-term association with organelles or with intracellular endosymbionts and parasites (7, 8), or it may involve transposable elements (TEs) (9, 10).

Bdelloid rotifers are small freshwater invertebrates that apparently lack sexual reproduction and can withstand desiccation at any life stage (11, 12). Their genomes contain diverse TEs, including DNA transposons and retrovirus-like env-containing retrotransposons, such as Juno and Vesta, possibly acquired from exogenous sources and concentrated near telomeres (13, 14). We investigated TE distribution in bdelloids by sequencing clones from an Adineta vaga fosmid library hybridizing to Juno1 probes. Unexpectedly, in two Juno1 long terminal repeat (LTR)–containing clones (contigs Av240A and Av212A), we found 10 protein-coding sequences (CDS) yielding strong database hits (BLAST E-values of 8E–102 to 0.0) to bacterial and fungal genes (Fig. 1A, Table 1, fig. S1A, and table S1). Half of these CDS have no metazoan orthologs, and three apparently bacterial CDS are interrupted by canonical spliceosomal introns, which are nonexistent in bacteria.

![Fig. 1. Structural and functional analysis of the Av240 genomic region. (A) The 85-kb Av240B contig. The colinear 50-kb Av240A contig contains the Juno1 LTR (red triangle) and extends from TPR to LRR RI (not shown). CDS (boxes) are colored according to their putative origin: metazoan, gray; bacterial, blue; fungal, purple; plant, green; indeterminate, striped; hypothetical, white. Intron positions are indicated. Pseudogenes are denoted by ψ, and defects in their reading frames appear as vertical lines. Scale bar, 1 kb. (B) RT-PCR analysis of Air, Ddl, and UDP-glycosyltransferase genes. Unspliced and spliced RNA are visible in +RT lanes; −RT, no reverse transcriptase; gDNA, genomic DNA control. (C) 5′ RACE analysis of transcription start sites (arrows) for genes in Fig. 1B. (D) Expression in E. coli of the A. vaga Ddl cloned in pET45b to yield a protein fused to the 6×His tag. Left, SDS–polyacrylamide gel electrophoresis (PAGE) analysis of the Ddl protein after purification by metal-affinity chromatography; right, thin-layer chromatography after incubation of purified AvDdl with d-Ala; co-spot, control for o-Ala and d-Ala; o-Ala, d-Ala migration; pET, vector with no insert. (E) FISH of the Av240A probe, labeled with the red fluorophore, to A. vaga embryo nuclei. The arrow points to a signal in a nucleus with condensed chromosomes.](image-url)