Iron chaperones PCBP1 and PCBP2 mediate the metallation of the dinuclear iron enzyme deoxyhypusine hydroxylase

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Although cells express hundreds of metalloenzymes, the mechanisms by which apoenzymes receive their metal cofactors are largely unknown. Poly(rC)-binding proteins PCBP1 and PCBP2 are multifunctional adaptor proteins that bind iron and deliver it to ferritin for storage or to prolyl and asparagyl hydroxylases to metallate the mononuclear iron center. Here, we show that PCBP1 and PCBP2 also deliver iron to deoxyhypusine hydroxylase (DOHH), the dinuclear iron enzyme required for hypusine modification of the translation factor eukaryotic initiation factor 5A. Cells depleted of PCBP1 or PCBP2 exhibited loss of DOHH activity and loss of the holo form of the enzyme in cells, particularly when cells were made mildly iron-deficient. Lysates containing PCBP1 and PCBP2 converted apo-DOHH to holo-DOHH in vitro with greater efficiency than lysates lacking PCBP1 or PCBP2. PCBP1 bound to DOHH in iron-treated cells but not in control or iron-deficient cells. Depletion of PCBP1 or PCBP2 had no effect on the cytosolic Fe-S cluster enzyme xanthine oxidase but led to loss of cytosolic aconitase activity. Loss of aconitase activity was not accompanied by gain of RNA-binding activity, a pattern suggesting the incomplete disassembly of the [4Fe-4S] cluster. PCBP depletions had minimal effects on total cellular iron, mitochondrial iron levels, and heme synthesis. Thus, PCBP1 and PCBP2 may serve as iron chaperones to multiple classes of cytosolic nonheme iron enzymes and may have a particular role in restoring metal cofactors that are spontaneously lost in iron deficient cells.

diiron enzyme | iron regulatory protein

Transition metal ions, especially zinc, iron, copper, and manganese, serve as cofactors for hundreds of cellular proteins and play key roles in most biological processes (1). Coordination of metal centers within proteins typically involves a small number of amino acid side chains, and yet the identity and configuration of these side chains are frequently not sufficient to account for the incorporation of the native metal cofactor and the exclusion of nonnative cofactors. In vitro and in heterologous cells, many metalloproteins will bind nonnative metal cofactors with equal or greater affinity than the native metal. In both instances, misincorporation is likely to inactivate the enzyme. It is clear that cellular factors, particularly the subcellular compartment and the state of the cytosolic metal pools, may determine the species incorporated into a specific metal center. The pool of "free" metal ions in the cytosol is exceedingly low (2), because most metals are either tightly bound to proteins or coordinated by small molecules, such as glutathione (3). For most metalloproteins, the specific cellular factors contributing to metallation are unknown, but some proteins rely on metal chaperones: proteins that specifically bind metal ions and deliver them to target enzymes through direct protein-protein interactions (4).

Iron is one of the most abundant metals in eukaryotic cells and cofactors in the form of heme, Fe-S clusters, and iron ions bind to a large and diverse group of proteins (1). Details of the cellular systems that distribute iron cofactors to recipient proteins are beginning to emerge. Monothiol glutaredoxins function as carriers of Fe-S clusters in eukaryotic cells and are involved in the transfer of Fe-S clusters to apoenzymes (5, 6). In yeast, cytosolic monothiol glutaredoxins are also involved in the delivery of iron to mitochondria and to a cytosolic nonheme iron enzyme, ribonucleotide reductase (7–9). In human cells, the sole cytosolic monothiol glutaredoxin also functions in the maturation of Fe-S cluster enzymes and the synthesis of mitochondrial heme, but whether it functions in the delivery of iron ions to cytosolic nonheme iron enzymes remains to be determined (10, 11).

A pair of multifunctional adaptor proteins, poly(rC)-binding protein (PCBP) 1 and PCBP2 (also called hnRNP E1 and E2, or αCP-1 and -2), function as iron chaperones in human cells (12). PCBP1 and PCBP2 were initially isolated as heterogeneous nuclear ribonucleoproteins, are found in association with C-rich segments of single-stranded RNA, and have a variety of roles in

Significance

One-third of the proteins expressed in eukaryotic and prokaryotic cells may require bound metal ions or metal cofactors for activity. In human cells, the processes by which proteins acquire their native cofactors and avoid mismetallation are largely unknown. The multifunctional adaptor proteins poly (rC)-binding protein (PCBP)1 and PCBP2 are iron chaperones that deliver ferrous iron via a metal-mediated, protein–protein interaction. Three PCBP targets have been identified: ferritin, an iron storage protein; and prolyl and asparagyl hydroxylase, two mononuclear iron enzymes. Here, we show that PCBP1 and PCBP2 deliver iron to deoxyhypusine hydroxylase, a dinuclear iron enzyme representing a major class of nonheme iron enzymes. PCBPs may generally function to maintain nonheme iron cofactors in cytosolic enzymes, especially when iron becomes scarce.

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RNA processing, translation, and stability (13–15). PCBP1 and PCBP2 were then found to deliver iron to ferritin, the iron storage protein (16, 17). Metazoan ferritin is a 24-subunit oligomeric complex composed of H and L peptides that assemble into a hollow sphere and store iron in the interior (18). Yeast cells engineered to heterologously express human ferritins store more iron in ferritin when human PCBP1 or PCBP2 is coexpressed. Human Huh7 cells lacking either PCBP1 or PCBP2 load less iron into ferritin. In vitro, both PCBP1 and PCBP2 bind three equivalents of Fe(II) with low micromolar affinity. Fe-bound PCBP1 and PCBP2 bind to ferritin in multiple molar equivalents and Fe-PCBP1 can donate iron to the ferritin mineral core.

In addition to ferritin, PCBPs can deliver iron to the prolyl and asparagyl hydroxylases (PHDs and FIH1, respectively) that modify the α subunit of the transcriptional regulator hypoxia-inducible factor (HIF) (19). PHDs and FIH1 are members of a large family of Fe(II)- and 2-oxoglutarate–dependent dioxygenases that oxidatively modify many types of protein, nucleic acid, and small-molecule substrates (20–22). These enzymes coordinate a single iron ion in the active site using one carboxylate and two imidazole side chains for ligation. Human cells depleted of PCBP1 or PCBP2 exhibit a loss of PHD and FIH1 activities that are attributable to a loss of the iron cofactors. The loss of PHD activity is exacerbated when cells lacking PCBP1 or PCBP2 are made transiently iron-deficient, suggesting that PCBP1 and PCBP2 are especially important for metal delivery when cellular iron pools are lowered. Coimmunoprecipitation studies indicate that PCBP1 physically interacts with PHD2 and FIH1 in cells. These studies indicate that the iron chaperone functions of PCBP1 and PCBP2 are not limited to ferritin, an oligomeric protein that is specialized for iron binding and storage. PCBPs are also involved in delivering iron to at least two members of a large class of mononuclear iron enzymes, which are structurally unrelated to ferritin.

We wished to define the range of iron enzymes that depend on PCBP1 and PCBP2 for the incorporation of their metal centers. Here, we demonstrate that deoxyhypusine (Dhp) hydroxylase (DOHH), a dinuclear iron enzyme required for the hypusine (Hpu) modification of eukaryotic initiation factor (eIF) 5A (23), requires PCBP1 and PCBP2 for the formation of its diiron center in cells. Two cytosolic enzymes that require Fe-S clusters for activity, aconitase and xanthine oxidase, exhibited divergent dependence on PCBPs for the incorporation of Fe-S cofactors. Mitochondrial iron levels and heme biosynthesis were minimally affected by depletion of PCBP1 or PCBP2. Thus, PCBP1 and PCBP2 may specifically function as iron chaperones for ferritin and the classes of enzymes containing mono- and dinuclear iron centers.

Results

Iron-Dependent Synthesis of Hpu on eIF5A. A potential obstacle in the analysis of endogenously expressed, nonheme iron enzymes is the propensity for their Fe(II) cofactors to oxidize and/or dissociate from active site ligands after cells are disrupted and the contents diluted. Thus, we sought to examine a dinuclear iron enzyme activity that could be measured in living cells. DOHH catalyzes the second step of the posttranslational modification of a conserved lysine in eIF5A to Hpu (Fig. 1*A*) (24). Step one of the hypusination of human eIF5A is the transfer of an aminobutyl moiety from cellular spermidine to the ε-amino group of lysine 50 to form Dhp, a reaction that is catalyzed by Dhp synthase. In the second step, DOHH hydroxylates the Dhp to form Hpu. DOHH is a HEAT-repeat enzyme that contains a peroxo-linked, dinuclear iron center in which the iron ions are coordinated by two sets of conserved histidine and glutamate residues (23, 25, 26). Hpu synthesis occurs only on a single lysine residue on a single protein, eIF5A. However, this modification is conserved among all eukaryotes and archaea examined and is essential for the function of eIF5A, which facilitates translation of polyproline sequences at the ribosome (27). Yeast and mice that lack eIF5A or the hypusination machinery are not viable (28, 29) because of this defect in translation of polyproline-containing proteins. Polyproline-containing proteins are encoded by ∼10% of the yeast genome (27) and, in humans, include Huntingtin and SH3 domain- and WW domain-binding proteins.

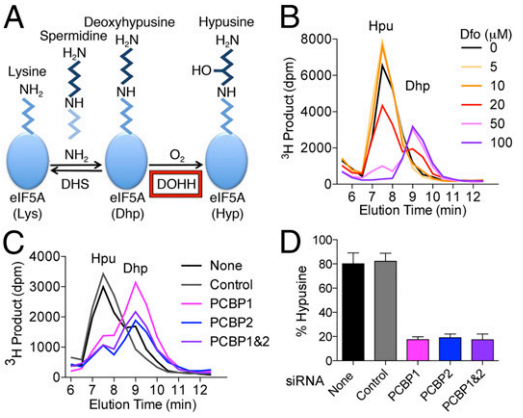

Fig. 1. Inactivation of DOHH in vivo by an iron chelator and by PCBP1 or PCBP2 depletion. (*A*) Two-step modification of eIF5A to form Hpu. Dhp synthase (DHS) catalyzes the formation of deoxyhypusine (Dhp) on eIF5A. DOHH, a diiron enzyme, catalyzes the hydroxylation of Dhp to Hpu. (*B*) Progressive inactivation of DOHH after treatment with Dfo. HEK293T cells were incubated with [^{3}H]spermidine and increasing concentrations of Dfo. Labeled Dhp and Hpu were separated by ion-exchange chromatography. (*C* and *D*) Loss of DOHH activity in cells depleted of PCBPs using siRNA. HEK293T cells were transfected with control, PCBP1, PCBP2, or PCBP1 and -2 siRNA and then treated with 20 μM Dfo and [^{3}H]spermidine. Hpu and Dhp were measured as in *B*, and the percentage of Hpu in the total modified lysine (Hpu + Dhp) is shown. Assays were replicated three times; error bars represent SEM.

Impaired Conversion of Dhp to Hypusine in Cells Lacking PCBP1 or PCBP2. We measured the activity of DOHH in intact HEK293 cells by incubating them with [^{3}H]spermidine and analyzing the labeled amino acids by ion-exchange chromatography. DOHH activity can be inhibited in cells by treatment with desferrioxamine (Dfo), an iron chelator (30). In untreated cells (0 Dfo), nearly all of the labeled amino acid eluted in fractions that comigrated with Hpu (Fig. 1*B*). However, when cells were incubated with increasing concentrations of Dfo, a peak of labeled amino acid corresponding to Dhp appeared. Dhp increased and Hpu decreased with increasing concentrations of Dfo, indicating that cellular iron deficiency could lead to partial or full inactivation of DOHH. We then examined the effects of PCBP depletion on DOHH activity (Fig. 1 *C* and *D*). Because depletion of PCBP1 or PCBP2 in iron-replete cells had little effect on the synthesis of Hpu, we treated all cells with a low concentration of Dfo (20 μM) for 2 h before labeling. Cells treated with no siRNA or a nontargeting control siRNA exhibited a very low level of Dhp accumulation (18–20% Dhp; 80–82% Hpu), indicative of a low level of DOHH inhibition. In contrast, cells treated with siRNA against PCBP1, PCBP2, or both PCBP1 and -2 exhibited a marked accumulation of Dhp (81–83%) and a corresponding reduction in Hpu (17–19%), indicative of a marked inhibition of DOHH activity. By Western blotting, levels of DOHH protein were similar in control and siRNA-treated cells, and PCBPs were efficiently depleted to <10% of control levels (Fig. S1*A*). Thus, the loss of DOHH activity could not be attributed to an absence of the DOHH protein in PCBP-depleted cells. Deletion of both PCBPs simultaneously produced an inhibition of DOHH that was similar to the inhibition produced by deleting either PCBP individually. This suggested that PCBP1 and PCBP2 were not functionally redundant but instead functioned cooperatively to activate DOHH. We confirmed that the observed inhibition of DOHH activity was not attributable to off-target effects by demonstrating inhibition of DOHH in cells depleted of PCBPs using siRNAs of alternative, unrelated sequences (Fig. S1*B*).

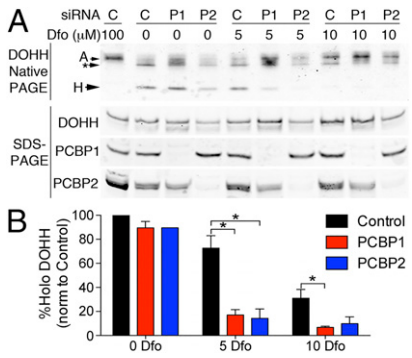

Fig. 2. Impaired iron insertion into DOHH in cells lacking PCBP1 or PCBP2. (*A*) HEK293 cells were transfected with control (C), PCBP1 (P1), or PCBP2 (P2) siRNAs, treated with Dfo for 18 h as indicated, and then analyzed by native PAGE (*Upper*) or SDS/PAGE (*Lower*) and immunoblotting. Small arrow, apo-DOHH (A); large arrow, holo-DOHH (H); *, intermediate form. (*B*) Quantitation of *A*. The ratio of holo-DOHH to total DOHH was expressed as a percentage of untreated control. Assays were replicated three to four times. Error bars indicate SEM. *$P < 0.05$.

Specific Loss of Holo-DOHH in Cells Lacking PCBP1 or PCBP2. We hypothesized that the loss of DOHH activity in PCBP-depleted cells was attributable to loss of the iron cofactor from the active site, and we examined DOHH from cells depleted of PCBPs for evidence of impaired iron cofactor incorporation. Recombinant DOHH is purified from *Escherichia coli* in two forms, one that migrates slowly in native gels and contains no iron and no enzymatic activity (apo-DOHH) and one that migrates more rapidly and contains both iron and enzymatic activity (holo-DOHH) (23, 31). Furthermore, only Fe(II), and not other divalent metals, can convert apo-DOHH to the more compact holo form, which contains a peroxo-iron bridge, in vitro (23, 26). We confirmed that the faster-migrating, holo form of DOHH corresponds to the iron- and oxygen-bound enzyme by noting its appearance when both iron and air are present but its absence when iron is added in the absence of oxygen (Fig. S2*A*). Endogenous DOHH in HEK293 cell extracts also migrated as multiple species by native-gel electrophoresis and immunoblotting (Fig. 2*A*). Cells made iron deficient by treatment with a high concentration of Dfo (100 μM) contained a single species of DOHH that exhibited the slower migration characteristic of the apo form. This complete conversion to the apoenzyme was consistent with the complete loss of DOHH activity observed under this condition in Fig. 1*B*. DOHH from cells treated with low or no Dfo migrated as three species that corresponded to the apo form, the holo form, and an intermediate form that likely corresponded to partially metallated or incompletely folded DOHH. Addition of iron to cells did not quantitatively convert all DOHH to the holo form, suggesting these forms are interconverted in cells (Fig. S2*B*). Again, no significant difference in the percentage of DOHH migrating as the holoenzyme was observed between cells treated with a control siRNA vs. PCBP1 or PCBP2 siRNAs, when cells were not treated with Dfo (Fig. 2 *A* and *B*). However, addition of very low concentrations of Dfo (5 and 10 μM) was accompanied by a marked decrease in the percentage of holo-DOHH in cells lacking PCBP1 or PCBP2. Control cells treated with 5 μM Dfo exhibited a 27% decrease in holo-DOHH, whereas PCBP1- and PCBP2-depleted cells exhibited 83% and 86% decreases in holo-DOHH, respectively. Cells treated with 10 μM Dfo exhibited proportionally smaller amounts of holo-DOHH and PCBP1-depleted cells lost both holo and intermediate forms of DOHH. The presence of DOHH protein and efficient depletion of PCBP1 and PCBP2 were confirmed by denaturing gel electrophoresis and immunoblotting (Fig. 2*A*, lower gels). These data indicated that cells lacking PCBP1 or PCBP2 exhibited a specific loss of the iron-bound form of DOHH.

In Vitro Metallation of DOHH with Iron or PCBP-Containing Cell Lysates. We examined the conversion of apo-DOHH to holo-DOHH using in vitro synthesis and iron-center metallation. We found that DOHH synthesized in wheat germ extracts, which contain low amounts of iron and no PCBP1, migrated exclusively as apo-DOHH by native-gel electrophoresis and could be converted to holo-DOHH in vitro in the presence of increasing concentrations of Fe(II) (Fig. 3*A*). Using the conversion of apo- to holo-DOHH as an indicator of iron binding, we determined that, at an iron concentration of 1.3 ± 0.2 μM, approximately half of the DOHH bound iron and converted to the holo form. Because iron binding is coupled to oxygen binding and the formation of a very stable complex, this value does not represent an equilibrium dissociation constant. Nevertheless, this concentration is similar to the affinities of PCBP1 and PCBP2 for iron (17).

We then determined whether cell lysates containing PCBP1 and PCBP2 could convert apo-DOHH to holo-DOHH. Labeled apo-DOHH was synthesized by in vitro translation, HEK293 cell lysates were added, and conversion to holo-DOHH was monitored by native gels. Addition of lysate (25 or 50 μg) from cells treated with control siRNA resulted in the dose-dependent conversion of apo- to holo-DOHH (Fig. 3 *B* and *C*). In contrast, lysates from cells treated with siRNAs against PCBP1 or PCBP2 were much less efficient in converting apo- to holo-DOHH. We considered that the reduced capacity of these lysates to metallate DOHH might be attributable to reduced levels of iron in these cells. In previous studies, uptake of iron in Huh7 cells depleted of PCBP1 was not different from control cells (17). Here, we used inductively coupled plasma mass spectrometry (ICP-MS) to measure the iron content of cells and found no difference between control cells and cells depleted of PCBP1 or PCBP2 (Fig. S3*A*). These studies indicated that lysates containing both PCBP1 and PCBP2 could more efficiently donate iron to the active site of DOHH than lysates lacking PCBP1 or PCBP2.

In Vivo Interactions Between PCBP1 and DOHH. Our studies indicated that cellular PCBP1 and PCBP2 were involved in the incorporation of iron into the active site of DOHH. Both PCBP1 and PCBP2 have been found to exhibit iron-mediated binding to ferritin in vivo, when cells are treated with iron, and in vitro, when the PCBPs are loaded with iron (17, 32). Both PHD2 and FIH1 were found in a complex with PCBP1 in cells, but only the FIH1 interaction exhibited a requirement for iron (19). We subjected HEK293 cells to iron, Dfo, or no treatment, then isolated endogenous PCBP1 by immunoprecipitation, and examined immune complexes for the presence of DOHH. DOHH was undetectable in complexes from Dfo or untreated cells but was present in complexes from iron-treated cells (Fig. 4*A*). No DOHH was detected in immunoprecipitates using bulk IgY as

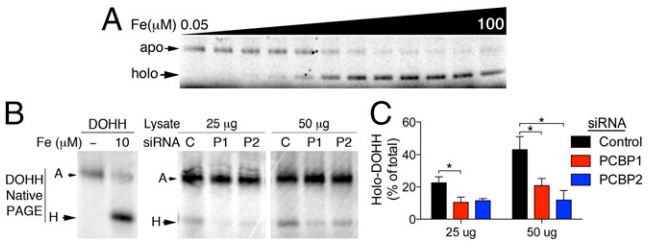

Fig. 3. Conversion of apo-DOHH to holo-DOHH with iron or lysates containing PCBP1 and PCBP2. (*A*) In vitro conversion of apo- to holo-DOHH with Fe(II). ^{35}S-labeled apo-DOHH was synthesized in vitro, incubated with Fe(II), and separated by native PAGE. Labeled DOHH was detected by phosphorimaging. (*B*) Metallation of apo-DOHH with lysates containing PCBP1 and PCBP2. ^{35}S-labeled apo-DOHH was incubated with Fe(II) (*Left*) or lysates from HEK293T cells transfected with control, PCBP1, or PCBP2 siRNAs (*Right*). Apo- (A) and holo-DOHH (H) were detected by native PAGE and phosphorimaging. (*C*) Quantitation of *B*. Assays were replicated three to five times; error bars indicate SEM. *$P < 0.05$.

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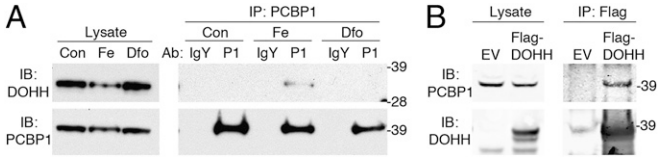

Fig. 4. Iron-dependent interaction between PCBP1 and DOHH in cells. (*A*) Coimmunoprecipitation of DOHH with PCBP1. HEK293T cells were untreated (Con) or treated with $FeCl_3$ (Fe) (20 μM) or Dfo (100 μM) overnight before immunoprecipitation using anti-PCBP1 (P1) or nonspecific IgY. Whole-cell lysates (*Left*) and immune complexes (*Right*) were examined by immunoblotting. (*B*) Coimmunoprecipitation of PCBP1 with Flag-DOHH. HEK293T cells inducibly expressing Flag-DOHH or the empty vector (EV) were treated with doxycycline and $FeCl_3$ for 18 h. Flag-DOHH was isolated using anti-Flag antibodies. Whole-cell lysates (*Left*) and immune complexes (*Right*) were analyzed by immunoblotting. Both endogenous and inducible DOHH were detected in whole-cell lysates. Assays were replicated three times; a representative experiment is shown.

a negative control, demonstrating the specificity of the interaction. Similar amounts of PCBP1 and DOHH were present in each lysate and similar amounts of PCBP1 were isolated in each condition. We then tested whether PCBP1 was detectable in immunoprecipitates of DOHH. We constructed stable cell lines that contained either the empty vector or inducibly expressed, Flag-epitope-tagged DOHH. In this cell line, Flag-DOHH is expressed at levels three- to fourfold higher than the endogenous DOHH (Fig. 4*B*, *Lower Left*). PCBP1 was detected in immunoprecipitates of Flag-DOHH from iron-treated cells but was not detected in immunoprecipitates from the control cell line that did not express Flag-DOHH (Fig. 4*B*, *Upper Right*), although both cell lines contained similar amounts of PCBP1. These data suggested that PCBP1 and DOHH physically interacted in cells and that this interaction was more stable in the presence of elevated intracellular iron.

Disparate Effects of PCBP Depletion on Cytosolic Fe-S Enzymes. We previously demonstrated the roles of PCBP1 and PCBP2 in delivering iron to ferritin and the mononuclear iron enzymes of the 2-oxoglutarate dioxygenase family. We have presented evidence here of the role of PCBP1 and PCBP2 in the metallation of the dinuclear iron enzyme DOHH. In addition to its incorporation into nonheme iron enzymes, cellular iron can be used for the assembly of Fe-S clusters or the synthesis of heme. Because the subcellular localization of PCBPs is restricted to the cytosol and the nucleus (13), they cannot be directly involved in delivering iron to the mitochondrial Fe-S cluster or heme biosynthetic machinery. They could, however, be involved in the delivery of iron to mitochondria or be involved in the assembly of Fe-S clusters in the cytosol. We examined the activity of two cytosolic Fe-S cluster-containing enzymes, aconitase and xanthine oxidase, in cells that lacked PCBP1 and PCBP2. We depleted PCBPs and then treated cells with Dfo for 0, 4, or 18 h to produce progressive degrees of iron deficiency. Cells were lysed, cytosol was separated from mitochondria, and cytosolic aconitase activity was measured. As expected, Dfo treatment led to progressive loss of aconitase activity (Fig. 5*A*). Depletion of PCBP1 or PCBP2 also led to a loss of aconitase activity compared with control siRNA-treated cells with no (0 Dfo) or mild (4-h Dfo) iron deficiency, with only the 4 h Dfo treatment reaching statistical significance. Loss of aconitase activity was not attributable to loss of the aconitase protein, however, because immunoblotting revealed that the protein was present at similar levels (Fig. 5*A*, *Lower*). In contrast, depletion of PCBP1 or PCBP2 in Huh7 cells had no effect on xanthine oxidase activity, although activity was diminished by 18 h Dfo treatment (Fig. 5*B*). We confirmed the separation of cytosol and mitochondria by immunoblotting with antibodies against tubulin and voltage-dependent anion channel (VDAC), respectively (Fig. S4 *A* and *B*). We also confirmed the efficient depletion of PCBP1 and PCBP2 (Fig. S4*C*).

Although xanthine oxidase and cytosolic aconitase are both Fe-S cluster enzymes, they differ in important ways. Xanthine oxidase contains two [2Fe-2S] clusters in domains separate from the active site, which contains a molybdopterin cofactor (33). Cytosolic aconitase contains a single, labile [4Fe-4S] cluster in the active site (34). Under specific cellular conditions, this cluster can partially disassemble into a [3Fe-4S] form or it can completely disassemble (35, 36). Partial disassembly is associated with loss of enzyme activity, and complete disassembly is associated with both loss of enzyme activity and acquisition of RNA-binding activity. Cytosolic aconitase, also known as iron regulatory protein (IRP)1, is a bifunctional protein. In its iron-free form, it can bind to specific stem-loop structures [called iron-regulatory elements (IREs)] in mRNAs and alter their stability or translation (37). If the loss of cytosolic aconitase activity in the PCBP-depleted cells were attributable to complete absence of the [4Fe-4S] cluster, these cells would exhibit a reciprocal gain in IRE-binding activity. We measured IRE binding in PCBP-depleted cells by mixing lysates with radiolabeled IRE-containing oligonucleotides and separating IRP1/IRE complexes from free IREs by gel electrophoresis. In cells treated with control siRNA, the increasing iron deficiency produced by Dfo treatment resulted in increasing amounts of IRP1/IRE complexes (Fig. 5*C*, *Upper*). In contrast, cells depleted of PCBP1 or PCBP2 exhibited lower levels of IRE-binding activity for each level of Dfo treatment compared with control siRNA cells. Addition of high concentrations of the sulfhydryl reductant 2-mercaptoethanol (2ME) to lysates can convert non–IRE-binding forms of IRP1 to the IRE-binding form, thereby providing a measure of the total amount of IRP1-mediated, IRE-binding activity in the lysate (38). Addition of 2ME indicated that levels of IRE-binding activity were similar in all cells (Fig. 5*C*, *Lower*). IRP2, which does not contain an Fe-S cluster but does bind IREs, migrates as a separate species under the electrophoretic conditions used in these assays. No IRP2/IRE complexes were detected, likely because of the very low levels of IRP2 protein in these cells. These data indicated that, although depletion of PCBPs impaired aconitase activity, it did not promote

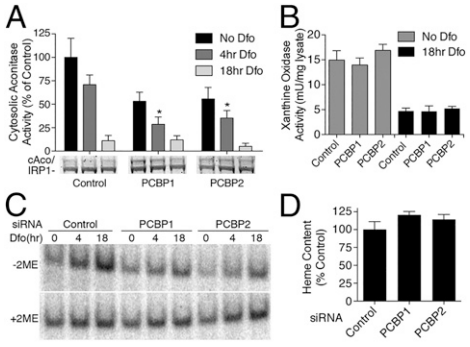

Fig. 5. Disparate effects of PCBP depletion on Fe-S cluster enzymes. (*A*) Loss of c-aconitase activity in cells depleted of PCBPs. PCBP1 or PCBP2 was depleted in HEK293 cells before treatment with Dfo (100 μM). Mitochondria were separated from cytosol and c-aconitase activity measured; c-aconitase protein was detected by immunoblotting (*Lower*). Assays were replicated four times and activities normalized to untreated control. $*P < 0.05$ in unpaired *t* test with corresponding control. (*B*) Lack of change in xanthine oxidase activity in PCBP-depleted cells. Huh7 cells were depleted of PCBPs then treated with no or 100 μM Dfo for 18 h. Xanthine oxidase activity was measured, assays were replicated thrice. (*C*) Loss of IRE-binding activity in cells depleted of PCBP1 or PCBP2. HEK293 cells were treated as in *A* and then analyzed for [^{32}P]IRE binding by electrophoretic mobility shift in the presence or absence of 2% (vol/vol) 2ME. IRP1/IRE complexes are shown. Assays were replicated thrice and representative data are shown. (*D*) Lack of change in heme synthesis in cells lacking PCBPs. HEK293T cells were depleted of PCBPs and labeled overnight with ^{55}Fe(II). Cellular heme was extracted and measured by scintillation counting. Assays were replicated thrice and normalized to control. All error bars indicate SEM.

the acquisition of IRE-binding activity and likely did not produce complete loss of the [4Fe-4S] cluster in cytosolic aconitase/IRP1.

Unchanged Heme Synthesis and Mitochondrial Iron in PCBP-Depleted Cells. We examined the role of PCBPs in the delivery of iron to mitochondria using two methods. First, we isolated mitochondria from cells depleted of PCBPs and measured the iron content by ICP-MS (Fig. S5*A*). Although cells depleted of PCBP2 exhibited a slightly reduced mitochondrial iron content, the difference was small, and no effect was seen upon PCBP1 depletion. Depletion of PCBPs and separation of intact mitochondria from cytosol was confirmed by immunoblotting (Fig. S5*B*). Second, we measured the biosynthesis of heme in cells depleted of PCBPs and found that the synthesis of heme was not different from control cells (Fig. 5*D*). These measurements indicated that PCBPs did not play a major role in the delivery of iron to mitochondria.

Discussion

The studies presented here indicate that PCBP1 and PCBP2 act as iron chaperones to enhance the incorporation of iron into the diiron enzyme DOHH. Four separate lines of evidence support this conclusion. (*i*) Deletion of either PCBP1 or PCBP2 in a human cell line was associated with a marked decrease in DOHH activity, particularly in cells exposed to mild iron limitation. (*ii*) The loss of activity was attributable to loss of the iron cofactor, because depletion of either PCBP1 or PCBP2 resulted in a specific loss of the iron-bound form of the enzyme. This loss was not attributable to diminished cellular iron pools because both total and mitochondrial iron remained unchanged. (*iii*) Lysates containing both PCBP1 and PCBP2 were more efficient than lysates lacking either protein in restoring iron to the active site of DOHH in vitro. This observation indicated that PCBPs could facilitate iron incorporation into DOHH posttranslationally, when the protein is incompletely folded. (*iv*) PCBP1 and DOHH were detected together in a complex but only in cells that had been treated with iron. This observation suggests a metal-mediated interaction in which direct transfer of iron from PCBP to DOHH is possible.

Several features of the interactions between PCBPs and DOHH are similar to those observed for PHDs, FIH1, and ferritin (16, 17, 19). First, both PCBP1 and PCBP2 play a role in iron delivery, but they cannot functionally substitute for each other. Cells depleted of PCBP1 contain wild-type levels of PCBP2 and vice versa, and yet depletion of either paralog resulted in loss of DOHH metallation. Furthermore, simultaneous depletion of both PCBP1 and PCBP2 produced the same degree of DOHH inhibition as individual depletion of either paralog. A similar situation was observed in the case of PHDs and ferritin in that depletion of either or both PCBP paralogs interfered with iron delivery to the target protein. PCBP1 can be detected in a complex with PCBP2 in human cells, but the pair is not quantitatively isolated as a stable complex, suggesting that the interaction is relatively weak (16, 19). Coimmunoprecipitation studies with ferritin indicate that depletion of one PCBP paralog diminishes the binding of the other paralog to ferritin, suggesting that the heterooligomer of PCBP1 and PCBP2 binds more stably to ferritin than either a homo-oligomer or monomeric PCBPs (16). Together, these observations suggest that PCBP1 and PCBP2 physically interact to bring iron to targets such as ferritin, PHDs, and DOHH. Second, the interactions between PCBP1 and ferritin, FIH1, and DOHH were detectable only when cells were made iron replete, and the binding of PCBP1 or PCBP2 to ferritin in vitro was only measurable in the presence of iron. The observations indicate that the interactions between PCBPs and their target proteins are stabilized by the presence of iron but remain weak and transient.

The roles of PCBP1 and PCBP2 in iron delivery to PHDs and DOHH are most prominent in cells made mildly iron-deficient with low concentrations of Dfo. These conditions of mild iron deficiency had relatively small effects on iron-dependent enzymes (*ca.* 20% reductions in aconitase or DOHH activity). However, PHDs and DOHH exhibited marked sensitivity to PCBP depletion under these conditions. One interpretation of these observations is that PCBPs are necessary to maintain metallation of iron enzymes under conditions of mild iron deficiency. PCBP1 and PCBP2 are very abundant proteins, and expression levels do not change under conditions of iron deficiency or iron excess. Thus, the cell does not appear to regulate iron delivery via changing PCBP levels. Our in vitro measurements indicated that 50% of DOHH was in the iron-bound form at an iron concentration of 1.3 μM. This value would suggest that under iron-replete conditions, where the labile iron pool has been estimated at 1–2 μM, some fraction of DOHH could acquire iron in a PCBP-independent manner, and our data support this prediction. Because the iron centers of DOHH and PHDs are solvent accessible and potentially in equilibrium with the cytosol, spontaneous dissociation of the iron cofactors would be expected in cells, especially under conditions of iron deficiency. PCBPs may have an important role in restoring iron to active sites that spontaneously lose the iron cofactor.

Our examination of the role of PCBPs in the maturation of cytosolic Fe-S cluster enzymes produced disparate results, with depletion having significant effects on cytosolic aconitase and no effects on xanthine oxidase. Only one of the [2Fe-2S] clusters of xanthine oxidase is solvent-accessible, and neither is located in the enzymatic active site. These Fe-S clusters are stable and not prone to oxidative damage or disassembly in vivo (33, 39). The lack of effect of PCBP depletion on xanthine oxidase activity, even under conditions of iron deficiency, would suggest that PCBPs play no role in the de novo assembly/delivery of [2Fe-2S] clusters. In contrast, the [4Fe-4S] cluster of c-aconitase directly coordinates substrate in the active site and readily undergoes partial or complete disassembly in living cells (40). This lability is attributable in part to the coordination of the cluster by only three cysteinyl ligands, rather than the usual four. Under conditions of oxidative stress (e.g., treatment with hydrogen peroxide), aconitase may coordinate a catalytically inactive [3Fe-4S] cluster (35). Under conditions of iron deficiency, the cluster may further disassemble, and the enzyme may convert to the RNA-binding form. Because PCBP depletion was accompanied by loss of c-aconitase activity without reciprocal acquisition of IRE-binding activity, the loss of c-aconitase activity is not likely attributable to complete loss of the [4Fe-4S] cluster. These data are therefore consistent with the xanthine oxidase data, suggesting PCBPs are not required for de novo Fe-S cluster assembly. The loss of c-aconitase activity could point to a role for PCBPs in the restoration of iron to partially disassembled [3Fe-4S] clusters. Alternatively, the increase in the labile iron pool associated with PCBP1 depletion could promote the formation of reactive oxygen species through Fe(II)-mediated Fenton chemistry. These species could also attack c-aconitase and result in the formation of [3Fe-4S] clusters or otherwise damage the enzyme. Clarification of the role of PCBPs in Fe-S cluster maintenance awaits further study.

The studies presented here and published previously indicate that PCBP1 and PCBP2 have a broad role in delivering iron to several different types of cytosolic nonheme iron enzymes and may even have a role in restoring the labile fourth iron to the Fe-S cluster of c-aconitase/IRP1. The simplest model for PCBP-mediated iron delivery would include a direct physical interaction between iron-bound PCBP1 and PCBP2 oligomers and the enzymatic target protein. Bridging ligands between the iron and the protein partners could stabilize the complex and facilitate transfer. PCBP1 binds three iron ions at sites that range in affinity from 0.9 to 5.8 μM. Thus, binding of one, two, or three equivalents of iron may occur under different cellular conditions and transfer of iron may preferentially occur from a particular binding site to a specific target or class of target enzyme (mononuclear, dinuclear, ferritin). Given the potentially large numbers of target enzymes and the relatively weak interactions observed, the binding may more closely resemble that of classical protein chaperones such as HSP70, in which targets are recognized nonspecifically by solvent-exposed hydrophobic patches (41). The source of iron that metallates the

PCBPs in cells is unclear. The readily exchangeable pool of iron in the cytosol is thought to be largely coordinated by reduced glutathione (3) but may also be comprised of iron-bound PCBPs as well, because the proteins are very abundant in cells.

Materials and Methods

Cell Culture and Protein Depletion by siRNA. Huh7, HEK293, and HEK293T cell propagation is described in *SI Materials and Methods*. PCBP1 and PCBP2 were depleted by single (Huh7) or two sequential (HEK293 and HEK293T) transfections of RNAi (Invitrogen) (19). A nontargeting, scrambled sequence siRNA pool was used as a control. Cells were harvested 4 d after the first transfection.

Immunoblot Analysis. Immunoblotting was performed as described in *SI Materials and Methods*.

Enzymatic Assays. Detailed protocols appear in *SI Materials and Methods*. For measurement of DOHH activity, cells were labeled with [1,8-^{3}H]spermidine, and radiolabeled Hpu and Dhp were separated by ion-exchange chromatography (30); c-aconitase activity was measured using the coupled aconitase/isocitrate dehydrogenase assay (42, 43). Xanthine oxidase activity was determined using the Amplex Red assay kit (Invitrogen).

Analysis of Apo- and Holo-DOHH. DOHH apo- and holoenzyme were separated using native PAGE (23). For in vitro metallation, ^{35}S-labeled apo-DOHH was incubated with Fe(II) or with HEK293T lysate. Detailed protocols appear in *SI Materials and Methods*.

Immunoprecipitation. Immunoprecipitations of PCBP1 were performed as described (19). A stable cell line expressing Flag-DOHH under the doxycycline-inducible CMV promoter was constructed, and Flag-DOHH was isolated (16). Detailed protocols appear in *SI Materials and Methods*.

Other Assays. To measure IRP1 binding to [^{32}P]IRE, HEK293 cells were depleted of PCBPs and assayed by electrophoretic mobility shift (38, 44). Total cellular and mitochondrial iron content were analyzed by ICP-MS (45, 46). Detailed protocols appear in *SI Materials and Methods*.

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