The iron chaperone poly(rC)-binding protein 2 forms a metabolon with the heme oxygenase 1/cytochrome P450 reductase complex for heme catabolism and iron transfer

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Mammals incorporate a major proportion of absorbed iron as heme, which is catabolized by the heme oxygenase 1 (HO1)–NADPH-cytochrome P450 reductase (CPR) complex into biliverdin, carbon monoxide, and ferrous iron. Moreover, intestinal iron is incorporated as ferrous iron, which is transported via the iron importer, divalent metal transporter 1 (DMT1). Recently, we demonstrated that the iron chaperone poly(rC)-binding protein 2 (PCBP2) can directly receive ferrous iron from DMT1 or transfer iron to the iron exporter, ferroportin 1. To promote intracellular iron flux, an iron chaperone may be essential for receiving iron generated by heme catabolism, but this hypothesis is untested so far. Herein, we demonstrate that HO1 binds to PCBP2, but not to other PCBP family members, namely PCBP1, PCBP3, or PCBP4. Interestingly, HO1 formed a complex with either CPR or PCBP2, and it was demonstrated that PCBP2 competes with CPR for HO1 binding. Using PCBP2-deletion mutants, we demonstrated that the PCBP2 K homology 3 domain is important for the HO1/PCBP2 interaction. In heme-loaded cells, heme prompted HO1–CPR complex formation and decreased the HO1/PCBP2 interaction. Furthermore, in vitro reconstitution experiments with purified recombiant proteins indicated that HO1 could bind to PCBP2 in the presence of heme, whereas loading of PCBP2 with ferrous iron caused PCBP2 to lose its affinity for HO1. These results indicate that ferrous iron released from heme can be bound by PCBP2 and suggest a model for an integrated heme catabolism and iron transport metabolon.

Iron is required by all living organisms on earth because it is central to cellular processes, including respiration, DNA synthesis, and oxygen transport (1, 2). In contrast, excess cellular iron is potentially toxic because it catalyzes the generation of reactive oxygen species that damage proteins, DNA, and lipids (3).

Normally, in humans, about 1–2 mg of iron is absorbed daily by the intestine, and at the same time, an approximately equal amount is non-specifically eliminated from the body (4, 5). Dietary iron occurs in two major forms, heme and non-heme (6). The primary sources of heme iron are hemoglobin and myoglobin from meat consumption, whereas non-heme iron is obtained from vegetables, cereals, and fruits (7). Dietary heme can be transported across the apical membrane (8, 9) and be subsequently metabolized by the heme oxygenase (HO)4 enzymes (10). This process is more efficient than the absorption of non-heme iron (11, 12).

The uptake of non-heme iron in the intestine mainly occurs through the function of divalent metal transporter 1 (DMT1) (13, 14). DMT1 has four isoforms and DMT1A-I is localized at the plasma membrane and is involved in non-heme iron acquisition (9, 15). Another isoform with an IRE domain in its 3’ region (DMT1B-I) is localized in the late endosome/lysosome to retrieve iron released during lysosomal catabolism of iron-containing proteins (16, 17). Other isoforms without the IRE domain in their 3’ regions (DMT1A-II and DMT1B-II) are localized at recycling endosomes and are responsible for cellular iron acquisition during the transferrin receptor cycle (9, 18, 19).

The mammalian HO enzymes catabolize cellular heme to biliverdin, carbon monoxide, and ferrous iron at the expense of molecular oxygen using electrons donated by NADPH-cytochrome P450 reductase (CPR) (20). Notably, HO is anchored to the endoplasmic reticulum (ER) by its C-terminal transmembrane region, whereas the N-terminal cytoplasmic region plays a key role in its catalytic activity for heme catabolism (9, 21).

4 The abbreviations used are: HO, heme oxygenase; Ab, antibody; CDTA, 1,2-cyclohexylenedinitrilotriacetic acid; CPR, NADPH-cytochrome P450 reductase; CPRΔTM, CPR lacking the hydrophobic N-terminal transmembrane region (1–57 amino acids); ER, endoplasmic reticulum; FLAG, FLAG-tag octapeptide consisting of the DYKDDDKD motif; KH, heme homology; MBP, maltose-binding protein; pAb, polyclonal antibody; PCBP, poly(rC)-binding protein; pt, pellet; SnMP, tin mesoporphyrin; sup, supernatant; Ni-NTA, nickel-nitrilotriacetic acid; FL, full length; PNS, post-nuclear supernatant.
PCBP2 interacts with heme oxygenase 1

The HO family is represented by two isoforms, HO1 and HO2, encoded by separate genes (21–23). HO1 expression is induced by numerous factors, including oxidative stress (24), inflammation (25), an elevated level of substrate (26), and also iron deficiency (27). Furthermore, sustained expression of HO1 is known to substantially alter cellular iron metabolism (28–30) and to be involved in various disease states (31, 32).

In contrast to HO1, HO2 is not an inducible protein and appears to function as a sensor for O2, carbon monoxide, and nitric oxide (33). Recent evidence suggests that carbon monoxide generated by HO2 may be a physiological signaling molecule (34). In contrast, HO1 is thought to provide an antioxidant defense mechanism, on the basis of its marked up-regulation in stressed cells (35). Both HO isoforms play a role in iron utilization via their ability to liberate this metal ion from heme (20, 23).

It has been demonstrated that one of the mechanisms of intracellular iron transport and metabolism involves chaperone proteins (36). Recently, our laboratory reported that poly(rC)-binding protein 2 (PCBP2) binds to the key transmembrane iron transporters, DMT1 (37, 38) and ferroportin 1 (FPN1) (39). The PCBP group of proteins (i.e. PCBP1–4) was first reported as RNA-binding molecules (40, 41). In fact, each member of the PCBP family is characterized by their affinity to single-stranded poly(C) motifs in their target mRNAs (42). PCBP2 is a multi-functional protein and regulates gene expression at multiple levels, including mRNA metabolism and translation (42). Interestingly, apart from its RNA-binding activity, PCBP2 can function as an iron chaperone (36, 37, 39). All PCBP family members have been characterized that it might play a key function as a “gateway keeper” to distribute iron safely in the cytosol.

In this investigation, we hypothesized that PCBP2 could function in binding ferrous iron produced in the course of the enzymatic degradation of heme. We demonstrate that both HO1 and HO2 specifically interact with PCBP2 but not PCBP1, PCBP3, or PCBP4. In addition, we report that the KH3 domain of PCBP2 is important for the HO1/PCBP2 interaction. This study also shows that PCBP2 competes with CPR for HO1 binding. Using a substrate analog of heme and a mutant of HO1, we show that mutant HO1 could interact with PCBP2 in the presence of heme. However, PCBP2 did not lose binding efficacy to HO1 in the presence of the substrate analog, tin mesoporphyrin (SnMP), but it did lose activity in the presence of heme. Furthermore, iron-loaded PCBP2 lost its binding activity to HO1. Together, these results suggest an integrated model of a metabolon, where PCBP2 is released from HO1 after receiving ferrous iron liberated by heme catabolism. In fact, HO1, CPR, and PCBP2 form a functional unit that integrates the catabolism of heme (via HO1 and CPR) with the binding and transport of iron by PCBP2.

Results

Both HO1 and HO2 can bind to PCBP2 but not PCBP1

Considering the intricate interactions of PCBP2 with DMT1 and FPN1 as an iron chaperone (37, 39), it was hypothesized that PCBP2 could also act to secure the flux of iron from the key enzyme involved in heme catabolism, HO1.

In initial studies, HeP-2 cells were transfected with the following green fluorescent protein (GFP)-containing constructs, namely HO1-GFP, HO2-GFP, GFP alone (control), and DMT1-GFP and then examined by Western blotting and co-immunoprecipitation (Fig. 1 A, panels i–iii). Notably, DMT1 was used in these experiments as a positive control for the interaction with PCBP2 (37, 39). As reported previously (37, 39), Western blotting demonstrated that the PCBP2 antibody resulted in multiple bands between ~34 and ~43 kDa, with a major band at ~43 kDa, whereas a single predominant band for PCBP1 (with evidence of other minor bands) was observed at ~45 kDa (Fig. 1A, panel i). Notably, α-tubulin expression (~55 kDa) was utilized as a protein loading control and confirmed the gel was evenly loaded with total protein (Fig. 1A, panel i).

Co-immunoprecipitation of these cellular lysates using an anti-GFP Ab (Fig. 1A, panel ii, left panel) demonstrated that a band for HO1-GFP was identified at ~60 kDa, whereas HO2-GFP was at ~65 kDa, and GFP alone was at ~28 kDa, as expected (48). DMT1-GFP was detected at ~96 kDa along with a high-molecular-mass band at ~130 kDa, as shown previously (16, 37). The same samples were also analyzed by Western blotting with anti-HO1 pAb (Fig. 1A, panel ii, right panel). HO1-GFP was specifically detected by this anti-HO1 pAb, and a band at ~60 kDa was identified as HO1-GFP. In all lanes, the immunoglobulin heavy chain at ~55 kDa was also detected (as shown by the arrow in Fig. 1A, panel ii).

Immunoprecipitation with anti-GFP Ab following by probing with PCBP1 or PCBP2 antibodies suggested that both HO1-GFP and HO2-GFP could bind to PCBP2 but not PCBP1 in mammalian HeP-2 cells (Fig. 1A, panel iii). In contrast, the common molecular tag, GFP, did not bind to either PCBP1 or PCBP2 (Fig. 1A, panel iii). As demonstrated in our previous investigations (37, 39), DMT1-GFP could bind to PCBP2 but not PCBP1.

HO1 catalyzes the first and rate-limiting enzymatic step of heme degradation (20). Under physiological conditions, HO1 activity is highest in the spleen, where senescent erythrocytes are sequestered and destroyed (20). Because of these crucial metabolic functions of HO1 and its ability to modulate iron metabolism upon sustained expression (9, 18, 19), this study has concentrated on assessing the interaction of the PCBPs with this protein, rather than HO2, which is involved in signaling and oxygen sensing, etc. (33, 34, 49).

It has been reported that there are four members of the PCBP family, PCBP1, PCBP2, PCBP3, and PCBP4 (40, 41),
and all of these exhibit iron chaperone activity (43). Considering this, we analyzed the interaction between HO1 and each member of the PCBP family (Fig. 1B). Expression of the four PCBPs could be clearly detected in HEp-2 cells by Western blotting. Using short and long exposures of the blots in Fig. 1B, we observed multiple bands on Western blottings for PCBP2 and PCBP4, whereas PCBP1 and PCBP3 resulted predominantly in a single major band. As such, it can be speculated that both PCBP2 and PCBP4 have several major isoforms, which may be generated by alternative splicing of the transcripts (50, 51). Co-immunoprecipitation assays were then performed in cells stably expressing HO1-GFP followed by Western blotting with the relevant antibodies against the PCBP family (Fig. 1B). These investigations demonstrated that HO1 could only bind to PCBP2 and not PCBP1, PCBP3, or PCBP4.
PCBP2 interacts with heme oxygenase 1

Subcellular fractionation demonstrates that a proportion of PCBP2 is found in the membrane and also the cytosol

We previously reported that HO1 was localized to the ER and anchored on the membrane of this organelle in HEp-2 cells (9). Therefore, we prepared subcellular fractions of HEp-2 cells by differential centrifugation and analyzed the intracellular localization of the PCBP family members relative to classical markers (Fig. 1C).

It is well known that P-cadherin is located on the plasma membrane (52), whereas calnexin (53) and CPR (54) are localized to the ER, as is HO1 (9, 21). A post-nuclear supernatant was prepared (see under “Experimental procedures”) and then fractionated by differential centrifugation. The proteins P-cadherin, calnexin, CPR, and HO1 were detected in the pellet (pt) after centrifugation at 10,000 × g for 20 min at 4 °C (10Kpt) and also the supernatant (sup) (10Ksup, post-mitochondrial supernatant) (Fig. 1C, panel i). Subsequent fractionation analyses of the 10Ksup showed that the proteins above detected in the 10Ksup were totally accounted for in the pellet after centrifugation at 100,000 × g for 60 min at 4 °C (100Kpt; Fig. 1C, panel i).

Considering these latter results, it can be speculated that some large membrane structures and organelles were not sheared in the preparation of the cell extracts were pelleted in 10Kpt, as commonly demonstrated using similar protocols (16, 55). As such, proteins in the plasma membrane or ER membrane are detected in the 10Kpt fraction (Fig. 1C, panel i). In agreement with this finding, the mitochondrial protein, translocase of outer mitochondrial membrane 20 (TOMM20), was detected solely in the 10Kpt fraction but not the 100Kpt fraction (Fig. 1C, panel i). Thus, the 10Kpt fraction contains mitochondria, plasma membrane, and ER proteins.

The 100Kpt can be used to prepare a microsome-enriched fraction (Fig. 1C, panel i), as it contained calnexin, CPR, and HO1, which are localized in the ER (9, 21, 53, 54), but not TOMM20 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which are soluble cytoplasmic proteins (56). In fact, GAPDH was prominently detected in both the 100Ksup and the 100Kpt fractions and not in the 10Kpt, nor in 100Kpt fraction (Fig. 1C, panel i). Hence, the 10Ksup, and particularly 100Ksup, represented enriched soluble proteins which are largely cytosolic.

The expression of PCBP1 in HEp-2 cells was then analyzed using the same fractions, and it became clear that the molecular distribution varied between the four PCBP family members (Fig. 1C, panel i). Most (~85–90%) of the PCBP1, -2, and -4 was recovered in the 10Ksup relative to the 10Kpt. Subsequent centrifugation of the 10Ksup fraction demonstrated that ~50–80% of PCBP1, -2, and -4 was detected in the 100Ksup fraction, whereas ~20–50% was detected in 100Kpt fractions (Fig. 1C, panel i). These results suggested PCBP1, -2, and -4 were at least partially cytosolic considering the distribution of GAPDH. Notably, a higher proportion of PCBP2 was present in the 100Kpt fraction relative to the 100Ksup than all other PCBP family members. In fact, the distribution of PCBP2 was approximately equal between the 100Ksup and 100Kpt fractions (Fig. 1C, panel i). These results suggested that PCBP1, -2, and -4 were cytosolic proteins, with a proportion also being associated with the 100Kpt microsome-enriched fraction (particularly PCBP2).

In contrast to the other PCBP family members, PCBP3 had a distinct distribution, with ~50% being similarly partitioned between the 10Kpt and 10Ksup fractions (Fig. 1C, panel i). Notably, the 10Kpt fraction contained mitochondria and other large structures (e.g. plasma membrane, etc.). After further fractionation of the 10Ksup, 80% of PCBP3 was identified in the 100Ksup fraction indicating its presence in the cytosol (Fig. 1C, panel i). Collectively, all PCBP proteins were cytosolic, with PCBP2 having a greater association with the microsome-enriched fraction than all other PCBP members, although the distribution of PCBP3 was unique.

Using the 100Kpt-enriched microsomal fraction containing HO1-GFP from Fig. 1C, panel i, a co-immunoprecipitation assay was then performed using anti-GFP Ab. Using short and long exposures of the blots in Fig. 1C, panel ii, this method clearly showed that HO1 could bind to PCBP2 but not PCBP1, PCBP3, or PCBP4. Collectively, these results using the total extract and the 100Kpt demonstrated that only PCBP2 could interact with HO1 among the four PCBP members. Thus, we investigated the HO1/PCBP2 interaction in detail.

HO1, not CPR, can bind to PCBP2

It is well known that HO enzymes form a complex with CPR and receive electrons from CPR to degrade heme (20, 57). We previously reported using HEp-2 cells that HOs were co-localized with CPR at the ER (9), and we have demonstrated in this study using this same cell type that HOs can bind to PCBP2 by using the co-immunoprecipitation assays in Figs. 1A, panel iii, B, and C, panel ii. However, because HO1 and CPR exist as a complex, it is important to determine whether HO1, CPR, or both can bind to PCBP2. To examine this, cells stably expressing N-terminal hemagglutinin (HA)-tagged CPR were transfected simultaneously with CPR siRNA (or control siRNA) and the HO1-GFP expression plasmid (Fig. 2). Significantly, it was confirmed that CPR localization was not affected by HA tagging (data not shown). Approximately 90% of both HA-CPR and endogenous CPR expression were suppressed by its siRNA, and CPR silencing did not significantly (p > 0.05) affect PCBP2 expression (Fig. 2A).

The expression levels of recombinant HA-CPR and endogenous CPR were examined by probing with anti-CPR antibody, and it was demonstrated that HA-CPR expression was almost two times higher than that of endogenous CPR in the recombinant-expressing cells (Fig. 2A). A co-immunoprecipitation assay of HO1-GFP using anti-GFP Ab showed that both HA-CPR and endogenous CPR could bind to HO1-GFP (Fig. 2B). Their binding properties to HO1-GFP must be similar, because both kinds of CPRs were identified in the immunoprecipitates in the same respective ratios relative to their expression levels found in the input (cf. Fig. 2, A and B, left lanes). Furthermore, HA tagging of CPR did not affect the binding of CPR to HO1. The amounts of HA-CPR and endogenous CPR binding to HO1 were decreased after treatment of cells with CPR siRNA (Fig. 2B). Notably, despite marked silencing of CPR, PCBP2 binding to HO1 was not affected (Fig. 2B). Overall, these results indicate that PCBP2 can bind to HO1 but not CPR.
PCBP2 interacts with heme oxygenase 1

It has been reported that each of the three KH domains of PCBP2 have a role in its interaction with RNA, DNA, or proteins (44, 45). We have previously demonstrated that the KH2 domain of PCBP2 was crucial for both the DMT1/PCBP2 and FPN1/PCBP2 interactions (37, 39). Thus, we initially attempted a yeast two-hybrid assay to examine HO1 binding to PCBP2 and to identify the binding domain. However, it was not possible to obtain positive results from the yeast two-hybrid assays assessing this interaction (data not shown). In yeast cells, the interaction between recombinant HO1 and CPR also could not be detected (data not shown). Considering this, it can be speculated that HO1 could not fold properly in yeast cells, and a yeast two-hybrid assay using HO1 as bait or prey was not appropriate for the binding analysis of HO1.

As an alternative, to investigate which PCBP2 domain was important for HO1 binding, we performed co-immunoprecipitation and pulldown assays by using several deletion mutants of PCBP2 (58, 59). The FLAG-tagged full-length form of PCBP2 (FLAG-FL) consists of 39 amino acids (3×FLAG tag) and 366 amino acids (the full-length PCBP2) (Fig. 3A). The FLAG-PCBP2ΔKH1 (ΔKH1) mutant lacks amino acids 12–80 (corresponding to the KH1 domain); the FLAG-PCBP2ΔKH2 mutant (ΔKH2) lacks amino acids 94–192 (corresponding to the KH2 domain); the FLAG-PCBP2ΔKH3 mutant (ΔKH3) lacks amino acids 285–357 (corresponding to the KH3 domain); and the FLAG-PCBP2ΔLinker mutant (ΔLinker) lacks amino acids 193–284 (corresponding to the amino acids between the KH2 and KH3 domains; Fig. 3A).

In initial studies, each deletion mutant was N-terminally FLAG-tagged and was expressed in DMT1-GFP stably expressing HEp-2 cells (Fig. 3B, panel i). The expressions of DMT1-GFP and the FLAG-tagged PCBP2 mutants were examined to ensure their expression at the appropriate molecular weight (Fig. 3B, panels i and ii). The results from co-immunoprecipitation assays showed that the interaction between DMT1-GFP/FLAG-PCBP2 (FLAG-FL) was greatest among the five recombinants. The amounts of co-immunoprecipitated FLAG-ΔKH1 or FLAG-ΔKH3 were significantly (p < 0.001) decreased to ~10–20% of FLAG-FL (Fig. 3B, panels i and ii). Importantly, PCBP2ΔKH2 was no longer able to appreciably bind to DMT1 in mammalian cells (Fig. 3B, panels i and ii), suggesting the critical role of the KH2 domain of PCBP2 in its interaction with DMT1, as described previously in the recombinant yeast cells (37). The binding of FLAG-ΔLinker to DMT1 was significantly (p < 0.05) reduced to ~60% that of FLAG-FL (Fig. 3B). These results are consistent with our previous study in which the KH2 domain of PCBP2 was demonstrated to be important for DMT1/PCBP2 binding (37, 39). Moreover, other groups have indicated that N-terminal FLAG-tagged PCBP2 exhibited equivalent iron delivery activity as authentic PCBP2 (36, 43). Collectively, these results suggest that FLAG-tagged PCBP2 has similar properties to authentic PCBP2.

Next, by using these deletion mutants, the HO1/PCBP2 interaction was analyzed. Each FLAG-tagged deletion mutant was expressed in HO1-GFP stably expressing HEp-2 cells (Fig. 3C, panel i). Initially, the expression of HO1-GFP and the FLAG-tagged PCBP2 mutants was examined to ensure comparable levels of expression at the appropriate molecular weight (Fig. 3C, panels i and ii). The results obtained from co-immunoprecipitation assays showed that the interaction between HO1-GFP/FLAG-FL was greatest among the five recombinants. The amounts of co-immunoprecipitated FLAG-ΔKH1 or FLAG-ΔKH3 were significantly (p < 0.001) decreased to ~10% of FLAG-FL (Fig. 3C, panels i and ii). Importantly, PCBP2ΔKH3 was no longer able to bind appreciably to HO1 in mammalian cells, suggesting the important role of the PCBP2 KH3 domain in its interaction with HO1 (Fig. 3C, panels i and ii). In contrast, the binding of FLAG-ΔLinker to HO1 was significantly (p < 0.001) reduced to less than 60% that of FLAG-FL (Fig. 3C, panels i and ii).

To further assess the interaction between HO1 and PCBP2 deletion mutants, in vitro pulldown assays were performed (Fig. 3D). For these studies, recombinant HO1 was prepared, and it is notable that expression of this gene in bacteria turns the
medium green due to the accumulation of biliverdin, as reported earlier (60–62). After purification of HO1 using affinity chromatography, biliverdin could not be detected using UV-visible spectrophotometry.

The recombinant HO1 was then used to generate maltose-binding protein (MBP)-tagged HO1 (MBP-HO1). MBP-HO1 or MBP was shown to bind avidly to amylose resin and could be eluted using an excess of maltose, and the eluate was assessed by

Figure 3. KH3 domain of PCBP2 was most important for the HO1/PCBP2 interaction. A, schematic of the PCBP2 constructs. The full-length of PCBP2 (FL) consists of 366 amino acids. ΔKH1, ΔKH2, ΔKH3, and Δlinker indicate the deletion mutants that lack 12–80 amino acids, 94–192 amino acids, 285–357 amino acids, and 193–284 amino acids, respectively.

B(i) analysis of the interaction between DMT1 and PCBP2 deletion mutants by using a co-immunoprecipitation assay. (i) DMT1-GFP stably expressing HEp-2 cells were transfected with the N-terminal FLAG-tagged full-length form of PCBP2 and the deletion mutants of PCBP2. Notably, the 3× FLAG tag contains 39 amino acids. After 48 h of incubation, cells were lysed in TNE buffer. Then 5 μg of the total cell lysates were analyzed by Western blotting (WB) using anti-GFP pAb, anti-FLAG mAb, or anti-β-tubulin mAb. A total of 60 μg of these samples were co-immunoprecipitated (IP) with anti-GFP pAb-conjugated protein A beads and analyzed by Western blotting using anti-FLAG mAb. (ii) The results are typical of at least three independent experiments. The results shown in the bar graph are presented as the mean ± S.D. of three independent experiments. *, p < 0.05; ***, p < 0.001.

C(i) analysis of the interaction between HO1 and PCBP2 deletion mutants by using a co-immunoprecipitation assay. (i) HO1-GFP stably expressing HEp-2 cells were transfected with the N-terminal FLAG-tagged full-length form of PCBP2 and the deletion mutants of PCBP2. After 48 h of incubation, cells were lysed in lysis buffer. Protein (5 μg) from total cell lysates was analyzed by Western blotting using anti-GFP pAb, anti-FLAG mAb, or anti-β-tubulin mAb. Then 60 μg of protein from these samples was co-immunoprecipitated with anti-GFP pAb-conjugated protein A beads and analyzed by Western blotting using anti-FLAG mAb. (ii) The results are typical of at least three independent experiments. Data are shown in the bar graph as the mean ± S.D. of three independent experiments. ***, p < 0.001.

D analysis of the interaction between HO1 and PCBP2 deletion mutants by using a pulldown assay. (i) MBP or MBP-HO1 was mixed with amylose resin for 30 min at room temperature and washed with p-lysis buffer. The resin-bound MBP or MBP-HO1 was eluted with 20 mM maltose/p-lysis buffer and analyzed by SDS-PAGE and stained with Coomassie R-250. (ii) An alternative method was performed with E. coli-expressed GST or GST-PCBP2. Arrowheads indicate the recombinant protein of interest. (iii) The crude extract from ii was mixed with the MBP or MBP-HO1-bound resin and incubated at 4 °C overnight. The resin was washed with p-lysis buffer three times. The binding proteins were eluted with 20 mM maltose/p-lysis buffer. MBP or MBP-HO1, which was bound to amylose resin or the crude extract that contained GST or GST-PCBP2, was analyzed by SDS-PAGE and stained with Coomassie R-250. The eluates were analyzed by Western blotting with anti-GST mAb. (iv) The results are typical of at least three independent experiments. The results shown in the bar graph are presented as the mean ± S.D. of three independent experiments. **, p < 0.01; ***, p < 0.001.
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SDS-PAGE and Coomassie R-250 staining (Fig. 3D, panel i). The amount of MBP (~55 kDa), or MBP-HO1 (~72 kDa) eluted from the resin was highly reproducible in six different experiments for each protein (Fig. 3D, panel i).

Following these successful preliminary studies, glutathione S-transferase (GST)-tagged deletion mutants of PCBP2 were then prepared and expressed in Escherichia coli, and the extracts were examined by SDS-PAGE and Coomassie R-250 staining (Fig. 3D, panel ii). The recombinant proteins were shown to be the appropriate size (see arrowheads; Fig. 3D, panel ii), namely ~28, 65, 57, 54, 57, and 54 kDa corresponding to GST and GST-tagged FL, ΔKH1, ΔKH2, ΔKH3, or ΔLinker, respectively. Some degradation products were detected in the GST-PCBP2ΔKH3 extract. Importantly, it was confirmed that these recombinant products, including GST-PCBP2ΔKH3, could not be further degraded during the reaction time with MBP-HO1 (data not shown).

The cell extracts containing GST-tagged recombinant proteins were reacted with the resin premixed with MBP or MBP-HO1 in vitro. The resin was washed extensively to remove E. coli-derived contaminants and then eluted with maltose. Western blotting with an anti-GST mAb was then performed using these eluates (Fig. 3D, panel iii). It was revealed that MBP alone (negative control) resulted in no binding to any of the recombinant proteins. In contrast, when assessing MBP-HO1, the GST-PCBP2FL recombinant protein demonstrated the greatest interaction in vitro with MBP-HO1 (Fig. 3D, panel iii). The amounts of PCBP2ΔKH1 or PCBP2ΔKH2 pulled down by MBP-HO1 were significantly (p < 0.001) decreased to ~20 or ~10% that of PCBP2FL, respectively (Fig. 3D, panel iv). Importantly, it was observed that the deletion of KH3 domain of PCBP2 resulted in no significant binding to HO1, demonstrating the important role of this domain. There was a significant (p < 0.01) 60% decrease in HO1-PCBP2 binding by the deletion of the linker domain (GST-ΔLinker) relative to GST-FL (Fig. 3D, panel iv). This decrease is comparable with that found for the linker deletion in the co-immunoprecipitation assay (Fig. 3C).

In summary, the KH3 domain was important for the PCBP2/HO1 interaction (Fig. 3, C and D). The KH1 or KH2 deletions of PCBP2 also evoked to a lesser extent the HO1/PCBP2 binding interaction. Hence, it can be suggested that a lack of each KH domain may induce a conformational change in the PCBP2 structure, and/or all three KH domains might be involved in HO1 binding.

Heme, but not the heme analog, tin mesoporphyrin (SnMP), causes HO1/CPR interaction and HO1/PCBP2 dissociation

Considering the interaction of PCBP2 and HO1, studies were then performed to assess how this is regulated by heme binding to HO1 (Fig. 4). To examine this, the association between HO1 and CPR would also be crucial to determine because the latter provides electrons to HO1 to enable heme degradation (63, 64). As an appropriate metabolic probe to elucidate this mechanism, these studies utilized SnMP as a structural analog of heme composed of tin (Sn) and mesoporphyrin, which substitutes for iron and the porphyrin ring, respectively (48). It is well known that SnMP is a potent competitive inhibitor of HO1 and cannot be degraded like heme (65).

To assess the effect of heme relative to SnMP on the interaction between HO1 and CPR and also HO1 and PCBP2, HEp-2 cells were transfected with both HA-CPR and HO1-GFP and incubated with 50 μM heme or 25 μM SnMP for 2–12 h at 37 °C (Fig. 4A, panel i). Preliminary experiments demonstrated that the concentrations were optimal for these studies. In fact, SnMP levels higher than 25 μM caused cytotoxicity (data not shown). Western blotting was then performed using anti-HA, anti-GFP, anti-PCBP2, anti-HO1, anti-ferritin heavy chain, or anti-α-tubulin Abs (Fig. 4A, panel i). In these studies, the incubation with heme or SnMP had no marked or significant (p > 0.05) effect on the expression of HA-CPR, HO1-GFP, PCBP2, or α-tubulin as a function of time.

However, after incubation of cells with heme, there was a pronounced and significant (p < 0.001) time-dependent increase in endogenous HO1 expression at 4, 8, and 12 h relative to the 0-h time point (Fig. 4A, panel i). This is consistent with the well-known cellular response to heme loading, where heme is degraded by the HO1–CPR complex leading to the release of ferrous iron from the enzyme complex (66, 68). The expression of endogenous HO1 in cells treated with heme for 12 h was increased to almost the same level as that observed with HO1-GFP expression. Hence, the levels of HO1 hyper-expression used in our experiments were within the range observed after incubation of cells with heme, which is a physiological inducer of this protein (21, 23).

The liberated iron induced by HO1-mediated heme degradation is known to induce ferritin synthesis (68, 69). Indeed, a significant (p < 0.05) time-dependent increase in ferritin expression was observed from 4 to 12 h relative to the 0-h time point (Fig. 4A, panel i). In contrast to heme, incubation of cells with SnMP did not induce the expression of endogenous HO1, and there was no increase in ferritin expression (Fig. 4A, panel i). This is in agreement with the inability of SnMP to act as a competitive inhibitor of HO1 and the fact it cannot be degraded by this enzyme (65). However, some reports have indicated that SnMP induced HO1 expression in mouse fibroblasts (65) and in rat liver (70). We repeatedly attempted to induce HO1 in HEp-2 cells using SnMP under a range of conditions, but this was unsuccessful. In this study, SnMP was incubated with HEp-2 cells for up to 12 h, and longer incubations could not be used as the agent caused marked cell death. It has been reported that cobalt-protoporphyrin possessed a feature as a strong inducer of HO1 in human peripheral blood mononuclear cells, although tin–protoporphyrin did not (71). These different effects of heme analogs on HO1 induction between studies cannot be currently explained and could be dependent on the various cell types used.

Immunoprecipitation using anti-GFP Ab was then performed on the lysates prepared above to assess the interaction of HO1-GFP with either HA-CPR or PCBP2 (Fig. 4A, panel ii). Hence, anti-GFP Ab-conjugated protein A beads incubated with the lysates were analyzed by Western blotting using anti-HA, anti-GFP, and anti-PCBP2 Abs (Fig. 4A, panel ii). The amount of HA-CPR co-immunoprecipitated with HO1-GFP was markedly and significantly (p < 0.001) increased >100

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Figure 4. Heme causes the HO1/CPR interaction and HO1/PCBP2 dissociation. A, heme binding to HO1 decreases the interaction between HO1 and PCBP2. (i) Both HA-CPR and HO1-GFP were transfected into HEp-2 cells and then incubated for 48 h at 37 °C. Cells were washed with PBS and incubated with serum-free DMEM with 50 μM heme or 25 μM SnMP for 2–12 h at 37 °C. Cells were lysed in lysis buffer, and 10 μg of protein from each sample was analyzed by Western blotting using anti-HA mAb, anti-PCBP2 mAb, anti-HO1 pAb, anti-ferritin heavy chain pAb, or anti-α-tubulin mAb. In contrast, 1 μg of protein was analyzed by Western blotting using anti-GFP pAb. An arrowhead indicates a non-specific band detected by anti-HO1 pAb. The results shown in the bar graphs are presented as the mean ± S.D. of three independent experiments. *, p < 0.05; ***, p < 0.001. (ii) A total of 60 μg of protein from each lysate was co-immunoprecipitated (IP) with anti-GFP pAb conjugated protein A beads and analyzed by Western blotting using anti-HA mAb, anti-GFP pAb, or anti-PCBP2 mAb. Arrow indicates the heavy chain of anti-GFP pAb. The results shown in the bar graphs are presented as the mean ± S.D. of three independent experiments. ***, p < 0.001. B, analysis of the interaction between HO1 and PCBP2 under both heme and SnMP loading. (i) Both HA-CPR and HO1-GFP were transfected into HEp-2 cells and then incubated for 48 h at 37 °C. Cells were washed with PBS and incubated with serum-free DMEM with 10 μM SnMP and 0, 1, 10, or 50 μM heme for 12 h at 37 °C. Cells were lysed in lysis buffer, and 10 μg of protein from each sample was analyzed by Western blotting using anti-HA mAb, anti-PCBP2 mAb, anti-HO1 pAb, or anti-α-tubulin mAb. An arrowhead indicates a non-specific band detected by anti-HO1 pAb. The results shown in the bar graphs are presented as the mean ± S.D. of three independent experiments. *, p < 0.05; **, p < 0.01. (ii) A total of 60 μg of protein from each lysate was co-immunoprecipitated with anti-GFP pAb conjugated protein A beads and analyzed by Western blotting using anti-HA mAb or anti-PCBP2 mAb. The results are typical of at least three independent experiments. The data shown in the bar graphs are presented as the mean ± S.D. of three independent experiments. ***, p < 0.001.

Times by heme loading between 2 and 12 h relative to the 0-h time point (Fig. 4A, panel ii). In contrast, heme loading of cells had no significant (p > 0.05) effect on HO1-GFP levels relative to the control, in good agreement with the Western blotting results of HO1-GFP (Fig. 4A, panel i). These results demonstrated that heme plays no role in regulating HO1-GFP expres-
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Because it is the result of the transfection with the vector encoding HO1. These data are in contrast to the expression of endogenous HO1, which is potently regulated by heme levels (21, 23). Concurrently, co-immunoprecipitated PCBP2 with HO1 was markedly and significantly \((p < 0.001)\) decreased by heme loading between 2 and 12 h relative to the 0-h time point (Fig. 4A, panel ii).

In contrast, when cells were incubated with SnMP, HA-CPR co-immunoprecipitated with HO1-GFP did not increase during the period of SnMP loading (Fig. 4A, panel ii). This observation indicated that the HO1–CPR complex was not formed during the loading of the non-degradable substrate analog of HO1 (Fig. 4A, panel ii). The HO1-GFP band was also not significantly \((p > 0.05)\) affected in terms of its density relative to the control after loading cells with SnMP (Fig. 4A, panel ii).

However, it is notable that the HO1-GFP band after SnMP loading was slightly super-shifted to a higher molecular weight (Fig. 4A, panels i and ii). Previous studies have also reported that the HO1 band can be slightly super-shifted after SnMP loading of cells (65, 72). The detailed mechanism to explain this observation is unclear. However, it has been speculated that SnMP enters cells and binds to HO1, which may result in the retardation of migration (65, 72). It is well known that SnMP is a highly lipophilic heme analog that effectively permeates cells and induces migration (65, 72). It is well known that SnMP is a highly lipophilic heme analog that effectively permeates cells and induces its biological effects (65, 72). The evidence that SnMP is penetrating into cells in this investigation is demonstrated by the following facts: 1) there is retardation of migration of HO1 after cells are incubated with this agent (Fig. 4A, panels i and ii); and 2) briefly incubating cells with SnMP immediately prior to lysis did not alter HO1 migration (data not shown), probably because SnMP did not have chance to efficiently enter the cell. In contrast to the treatment with heme, when cells were incubated with SnMP, the co-immunoprecipitation of PCBP2 with HO1-GFP did not decrease during the period of SnMP loading (Fig. 4A, panel ii).

In summary, incubation with heme eliminated the HO1/PCBP2 interaction, whereas it induced HO1–CPR complex formation. In contrast, incubation with SnMP did not affect the HO1/PCBP2 interaction, whereas it prevented the HO1/CPR interaction.

To further investigate the heme or SnMP effects on HO1/PCBP2 or the HO1–CPR complex formation, we performed co-immunoprecipitation using HEp-2 cells incubated with increasing concentrations of heme \((1–50 \mu M)\) in the presence of a constant concentration of SnMP \((10 \mu M)\) for 12 h at 37 °C. The expression of HA-CPR, HO1-GFP, PCBP2, or \(\alpha\)-tubulin were then assessed by Western blotting and showed no change after incubation with heme or SnMP (Fig. 5A). These lysates were then co-immunoprecipitated with anti-GFP and analyzed by Western blotting using anti-HA, anti-GFP, or anti-PCBP2. The amount of HA-CPR co-immunoprecipitated with HO1WT significantly \((p < 0.05)\) increased after incubation of cells with heme, whereas there was no significant \((p > 0.05)\) change in HO1H25A-expressing cells (Fig. 5B).

Upon incubation of cells with SnMP, a slight super-shift of the HO1WT band on the Western blottings was observed, although there were no alteration of the HO1H25A band (Fig. 5B). Hence, this super-shift of the HO1WT band is probably due to SnMP binding, although the mutation of His-25 in HO1H25A prevented ligation to SnMP.

There was a pronounced and significant \((p < 0.001)\) decrease in the interaction between HO1WT and PCBP2 after incubation with heme, whereas the interaction between HO1H25A and PCBP2 did not significantly \((p > 0.05)\) alter, being maintained at control levels. Co-immunoprecipitation of PCBP2 with either HO1WT or HO1H25A did not alter after incubation with SnMP and was maintained at control levels.

Collectively, these studies in Fig. 5 suggest that the binding of heme via His-25 in the binding pocket of HO1WT induced the first step in the interaction between HO1WT and CPR, whereas PCBP2 binding to HO1WT decreased. However, although SnMP binds tightly to HO1WT (65), it cannot be catabolized (65, 76) and does not induce the interaction with CPR nor decrease the interaction with PCBP2. Hence, these results suggest a concerted mechanism of heme degradation consisting of heme binding to HO1, the association of CPR with HO1, and the release of PCBP2 from HO1.
Heme alters the distribution pattern of HO1 in cells and decreases the co-localization of PCBP2 and HO1-GFP

As shown in Fig. 4A, panel i, upon incubation of HO1-GFP stably expressing HEp-2 cells for 0–12 h with heme, ferritin and endogenous HO1 were gradually induced over time, and immunoprecipitable PCBP2 with HO1 was readily decreased (Fig. 4A, panel ii). Considering this, we then investigated the possible alteration in subcellular localization of HO1 and PCBP2 using immunofluorescence analysis via confocal microscopy (Fig. 6).

Under control conditions using HEp-2 cells, HO1-GFP was localized to cytosolic structures consistent with the ER and nuclear membranes, but not the nucleus (Fig. 6), as described previously (9). In contrast, endogenous PCBP2 was widely, but not homogeneously, expressed in the nuclei and cytosol of the control cells (Fig. 6) (77). This observation was in good agreement with the cellular fractionation studies using post-nuclear supernatants, which demonstrated PCBP2 was a cytosolic protein (Fig. 1C, panel i).

Once heme was incubated with cells, the punctate HO1-GFP fluorescence slightly aggregated in cytoplasmic structures that are consistent with the ER. SnMP did not cause any marked alteration in HO1-GFP distribution pattern (Fig. 6). Incubation of cells with heme also led to an alteration in PCBP2 distribution with it becoming slightly aggregated in small puncta (Fig. 6). Examining co-localization (Fig. 6, yellow) between PCBP2 (red) and HO1-GFP (green) under control conditions, Pearson’s correlation coefficient analysis indicated appreciable overlap. Moreover, upon incubation with heme, but not SnMP, there was a significant ($p < 0.001$) decrease in the co-localization of HO1-GFP and PCBP2 (Fig. 6). We previously reported that HO1 was well co-localized with CPR (9). Similar results were observed in this study with the Pearson’s correlation coefficient analysis between HO1-GFP/CPR being 0.9 under control conditions or heme or SnMP treatment (data not shown). Collectively, these results suggest that heme, but not SnMP, alters the distribution pattern of HO1 in cells and decreases the co-localization of PCBP2 and HO1-GFP.

Subcellular distributions of HO1-GFP, CPR, and PCBP2 are altered after incubation of cells with heme

To further analyze the effects of incubation with heme on the cellular localization of HO1-GFP, CPR, and PCBP2, subcellular fractionation was performed, and protein expression in each fraction was analyzed by Western blotting. The HEp-2 cells stably expressing HO1-GFP were treated with 50 μM heme for 4 h, and the 10Ksup was fractionated by OptiPrep discontinuous gradient centrifugation, as described in our previous study (15).

Under control conditions, CPR and HO1-GFP were strongly detected in fractions 2–6 and 11–17 (Fig. 7A). PCBP2 was markedly detected in fractions 1–5 and 13–17 (Fig. 7A). After heme treatment, HO1-GFP and PCBP2 distribution were markedly altered. The expression of CPR in fractions 2–6 decreased, whereas its levels in fractions 11–15 increased (Fig. 7A). A similar change was observed in the distribution of HO1-GFP expression: HO1-GFP levels in fractions 2–6 decreased.
investigated by Western blotting (GAPDH, endogenous HO1, PCBP1, PCBP3, and PCBP4) were an additional seven proteins (namely P-cadherin, calnexin, and expression of other proteins, subcellular distributions of those in fractions 11–17 was markedly reduced (Fig. 7). After heme treatment, PCBP2 expression in fractions 2–4 increased and that in fractions 11–17 was markedly reduced (Fig. 7A).

To thoroughly analyze the effects of heme on the distribution and expression of other proteins, subcellular distributions of an additional seven proteins (namely P-cadherin, calnexin, GAPDH, endogenous HO1, PCBP1, PCBP3, and PCBP4) were investigated by Western blotting (supplemental Fig. 1). The relative expression ratio of these proteins in each fraction is demonstrated in Fig. 7B relative to HO1-GFP, CPR, and PCBP2. In these studies, P-cadherin, which is mainly localized at the plasma membrane (52), was strongly detected in fractions 6–14, with its distribution being almost unchanged after incubation of cells with heme (Fig. 7B). Calnexin is known to be localized in the ER (53), like HO1 (9, 21) or CPR (54), with its expression being marked in fractions 10–17 in control and heme-incubated cells (Fig. 7B). Under control conditions, the distribution of HO1-GFP and CPR were slightly different from calnexin (Fig. 7B). The former two molecules were detected in fractions 2–6, but the latter one was not. This altered distribution might be caused by the effect of their different distribution in the ER or potentially their interaction with other proteins.

GAPDH is a soluble cytoplasmic protein (56), which was mainly detected in fractions 1–7, with its distribution not changing after incubation of cells with heme (Fig. 7B). By examining the expression and distribution of endogenous HO1, it is important to note that before heme induction it was not possible to detect endogenous HO1 by Western blotting. However, after heme induction, endogenous HO1 was markedly expressed and mainly detected in fractions 12–18. The distribution of endogenous HO1 after incubation with heme was very similar to HO1-GFP, and thus, it can be suggested that HO1-GFP demonstrates a physiological distribution (Fig. 7B).

Figure 6. Localization of PCBP2 and HO1-GFP under heme or SnMP treatment. HO1-GFP stably expressing HEp-2 cells were incubated with 50 μM heme or 20 μM SnMP for 4 h at 37 °C. Cells were then fixed with 4% paraformaldehyde/PBS and permeabilized using 0.1% Triton X-100/PBS. Cells were incubated with anti-PCBP2 mAb and with a secondary antibody coupled to Alexa 594. Images were obtained using a Zeiss LSM 700 confocal laser-scanning microscope system. Six images were randomly selected, and Pearson’s correlation coefficient was measured by ImageJ Color2 software. The white box indicates the region shown as an enlargement. Arrowheads indicate the co-localization puncta of HO1-GFP and PCBP2. The results shown for the Pearson’s correlation coefficient are the mean ± S.D. of six independent images. ***, p < 0.001.

All PCBP family members were mainly detected in fractions 1–5. However, PCBP2 could also be detected in fractions 12–17 without heme treatment (Fig. 7B), which are the same fractions where other ER-localized proteins (i.e., HO1, CPR, and calnexin) are also markedly expressed. Notably, after incubation with heme, PCBP2 expression significantly (p < 0.05) decreased in the ER fractions 12–17 and significantly (p < 0.05) increased in fractions 3 and 4 where the cytoplasmic protein, GAPDH, was detected (Fig. 7B). This indicated PCBP2 altered its distribution from the ER to the cytoplasm after heme treatment. Hence, this is consistent with PCBP2 associating with HO1 in the ER in the absence of heme, but dissociating after incubation of cells with heme and moving into the cytosol. These data concur with those in Fig. 6 using confocal microscopy. Significantly, in contrast to PCBP2, PCBP1, PCBP3, and PCBP4 were mainly detected in fractions 3–6 and did not alter their distribution after incubation with heme (Fig. 7B). These studies are consistent with the fractionation experiments in Fig. 1C, panel i, where the PCBPs were shown to be cytosolic proteins, with PCBP2 demonstrating greater microsomal enrichment relative to the other PCBP family members.

PCBP2 binding to HO1 is rapid and saturable indicating specific binding sites

To further investigate the specific interaction between PCBP2 and HO1, time (Fig. 8A) and concentration curve analyses (Fig. 8B) were performed. Purified MBP-HO1 and GST-PCBP2 were used for these assays. The MBP-HO1 was initially mixed with amylose resin, washed, and then incubated with GST-PCBP2 for 0 to 300 s at 37 °C (the molar ratio of MBP-HO1/GST-PCBP2 was 1:1, with the concentrations being 10 μM each). The binding products were retrieved from the amylose resin using maltose, and the eluates were examined by
SDS-PAGE and analyzed by anti-GST mAb (Fig. 8A, panels i and ii). The GST-PCBP2 bound to HO1 was detectable after only 15 s of incubation and increased rapidly from 15 to 120 s and then reached a plateau after 120 s, suggesting completion of the reaction (Fig. 8A, panel ii).

Next, increasing concentrations of GST-PCBP2 were reacted with a fixed amount of MBP-HO1 (Fig. 8B, panels i and ii). In these studies, MBP-HO1 (20 μM) was mixed with amylose resin, washed, and then incubated with increasing concentrations of GST-PCBP2 (0.25 to 20 μM). The resin was again washed, and the bound proteins were eluted with maltose and examined by SDS-PAGE and Western blotting using an anti-GST Ab. Examining the blot using densitometry demonstrated a rapid increase in binding up to 5 μM, which plateaued from this point up to 20 μM (Fig. 8B, panel ii). This suggested a single, saturable class of non-interacting PCBP2-binding sites on...
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PCBP2 competes with CPR for HO1 binding

Both Figs. 4 and 5 showed that CPR and PCBP2 could interact with HO1 and that the interaction of CPR with HO1 appears opposite that of PCBP2. Thus, we investigated the interaction among HO1, CPR, and PCBP2 in vitro and particularly the competition between CPR and PCBP2 for binding to HO1 (Fig. 9).

In Fig. 9A, E. coli extracts were used that expressed the following recombinant proteins: 1) pGEX2T (GST; this vector was used as a negative control); 2) pET32 (His; this vector was also used as a negative control and encodes a His$_6$-tag, thioredoxin tag, and S tag (S tag: N-terminal 15-amino acid sequence of ribonuclease S), and thus appeared at 28 kDa on SDS-polyacrylamide gels, referred to as “His” herein and in Fig. 9); 3) pGEX2T-PCBP2 (GST-PCBP2); or 4) pET32-CPR$_{ΔTM}$ (His-CPR). Notably, the His-tagged full-length form of CPR was insoluble (data not shown). Therefore, the soluble deletion mutant, CPR$_{ΔTM}$, was implemented that lacked the hydrophobic N-terminal trans-membrane region (1–57 amino acids). This CPR mutant was previously confirmed to have appropriate enzymatic activity (57), and it was used in this study.

These recombinant proteins were purified from the cell extracts by glutathione-Sepharose 4B beads or Ni-NTA-agarose, then electrophoresed on an SDS-polyacrylamide gel, and stained with Coomassie R-250 (Fig. 9A). It was confirmed that the recombinant proteins appeared at the appropriate size, namely ~28, 28, 60, and 68 kDa corresponding to GST, His, GST-PCBP2, and His-CPR, respectively (see arrows, Fig. 9A). These purified recombinant proteins were then used in the second and third reactions in Fig. 9, B and C.

By using these purified recombinant proteins, competition experiments were then examined in vitro to assess how the HO1–CPR complex affects HO1–PCBP2 complex formation (Fig. 9B). Before starting the following reactions, heme and iron contents in each purified recombinant protein solution were measured and shown to be below the detection limit. Previous reports indicated that apo-HO1 lacking the 22-amino acid C-terminal hydrophobic segment could not bind to CPR (57), and hence, the C-terminal region is necessary for efficient HO1–CPR complex formation (78). In this study, we prepared MBP-HO1 (full-length) and His-CPR$_{Δ1–57}$ and could detect binding between MBP-HO1 and His-CPR (data not shown).

Thus, we used these recombinant proteins in the following experiments.

GST mAb. The results are typical of at least three independent experiments.

Figure 8. PCBP2 binds rapidly to a single class of non-interacting saturable binding sites on HO1 in vitro. A, time course of the binding of PCBP2 to HO1. (i and ii) The purified MBP-HO1 (10 µM) was mixed with amylase resin for 30 min at room temperature and washed with p-lysis buffer. Then, the purified GST-PCBP2 (10 µM) was loaded to MBP-HO1 (10 µM)/amylose resin and incubated at 37 °C for 0, 15, 30, 45, 60, 90, 120, 150, 180, or 300 s. MBP-HO1/amylase resin was washed with p-lysis buffer four times and eluted with 20 mM maltose/p-lysis buffer. MBP-HO1, bound to amylase resin was analyzed by SDS-PAGE and stained with Coomassie R-250. GST-PCBP2, which was used in this procedure, was analyzed by Western blotting using anti-GST mAb. The eluates were analyzed by Western blotting using anti-GST mAb. The results shown in the line graph are presented as the mean ± S.D. of at least three independent experiments.
In these studies, three reaction steps were utilized to assess whether competition occurred between CPR and PCBP2 when binding to HO1. In the first reaction, MBP or MBP-HO1 expressed in E. coli and lysed in p-lysis buffer by sonication. The recombinant proteins were purified from E. coli cell extracts by GSH 4B beads or Ni-NTA beads and then analyzed by SDS-PAGE. The results shown in the gel are typical of three independent experiments. B, competitive effect of His-CPR on the HO1/PCBP2 interaction. These studies were conducted in three stages. In the first step, the purified MBP or MBP-HO1 (10 μM) was mixed with amylose resin and then washed with p-lysis buffer (first reaction). In the second step, 10 μM purified pET32 alone (His), pGEX2T-PCBP2 (GST-PCBP2), or pET32-CPRΔTM (His-CPR) was mixed with the post-first reaction resin for 8 h at 4 °C and washed with p-lysis buffer (second reaction). In the third step, 10 μM purified GST-PCBP2, His, or His-CPR was mixed with the post-second reaction resin for 8 h at 4 °C and washed with p-lysis buffer (third reaction). The amylose resin-bound proteins were eluted with 20 mM maltose/p-lysis buffer. Eluates were analyzed by SDS-PAGE and stained with Coomassie R-250 staining or Western blotting using anti-GST mAb. MBP and MBP-HO1 were detected at 55 and 70 kDa, respectively. Results shown in the gels or blots are typical of three independent experiments. C, competitive effect of GST-PCBP2 on the HO1/CPR interaction. These experiments were conducted in three stages: (1) MBP or MBP-HO1 (10 μM) was mixed with amylose resin and then washed with p-lysis buffer (first reaction); (2) 10 μM purified pGEX2T alone (GST), pET32-CPRΔTM (His-CPR), or pGEX2T-PCBP2 (GST-PCBP2) were mixed with the post-first reaction resin for 8 h at 4 °C and washed with p-lysis buffer (second reaction); and (3) then 10 μM purified His-CPR, GST, or GST-PCBP2 was mixed with the post-second reaction resin for 8 h at 4 °C and washed with p-lysis buffer (third reaction). The resin-bound proteins were eluted with 20 mM maltose/p-lysis buffer. Eluates were analyzed by SDS-PAGE and stained with Coomassie R-250 staining or Western blotting using anti-His mAb. MBP and MBP-HO1 were detected at 55 and 70 kDa in eluates, respectively. Results shown in the gels or blots are typical of three independent experiments. The results shown in the bar graph are presented as the mean ± S.D. of three independent experiments. *** p < 0.001.

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Figure 9. PCBP2 competes with CPR for binding to HO1 in vitro. A. expression of recombinant proteins. pGEX2T alone (GST, negative control), pET32 alone (His, negative control), pGEX2T-PCBP2, or pET32-CPRΔTM was expressed in E. coli and lysed in p-lysis buffer by sonication. The recombinant proteins were purified from E. coli cell extracts by GSH 4B beads or Ni-NTA beads and then analyzed by SDS-PAGE. The results shown in the gel are typical of three independent experiments. B, competitive effect of His-CPR on the HO1/PCBP2 interaction. These studies were conducted in three stages. In the first step, the purified MBP or MBP-HO1 (10 μM) was mixed with amylose resin and then washed with p-lysis buffer (first reaction). In the second step, 10 μM purified pET32 alone (His), pGEX2T-PCBP2 (GST-PCBP2), or pET32-CPRΔTM (His-CPR) was mixed with the post-first reaction resin for 8 h at 4 °C and washed with p-lysis buffer (second reaction). In the third step, 10 μM purified GST-PCBP2, His, or His-CPR was mixed with the post-second reaction resin for 8 h at 4 °C and washed with p-lysis buffer (third reaction). The amylose resin-bound proteins were eluted with 20 mM maltose/p-lysis buffer. Eluates were analyzed by SDS-PAGE and stained with Coomassie R-250 staining or Western blotting using anti-GST mAb. MBP and MBP-HO1 were detected at 55 and 70 kDa, respectively. Results shown in the gels or blots are typical of three independent experiments. The results shown in the bar graph are presented as the mean ± S.D. of three independent experiments. *** p < 0.001.
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Indeed, in the third reaction step, GST-PCBP2, His (negative control), or His-CPR was added. Hence, in this third step, the addition of His-CPR or GST-PCBP2 might compete with MBP-HO1 on the resin that had already bound GST-PCBP2 or His-CPR from the second step (Fig. 9B). The resin-bound MBP complexes were then eluted using an excess of maltose and analyzed by Coomassie R-250 staining and by Western blotting with an anti-GST antibody to detect PCBP2 (Fig. 9B). Importantly, the amount of MBP (~55 kDa) or MBP-HO1 (~72 kDa) eluted from the resin and stained with Coomassie R-250 was equivalent among the reaction conditions used and highly reproducible in eight different experiments in each pulldown assay (Fig. 9, B and C).

Comparing lanes 1 and 2 of Fig. 9B, these studies demonstrated that MBP did not bind to GST-PCBP2, whereas GST-PCBP2 could bind to MBP-HO1. Furthermore, irrespective of whether the E. coli extract of His was added in the second or third reaction, it did not interfere with the interaction between HO1 and PCBP2 (Fig. 9B; cf. lanes 2 and 4). When the resin/MBP-HO1 was mixed with His-CPR in the second reaction, and subsequently mixed with GST-PCBP2 in the third reaction (Fig. 9B, lane 6), then the amount of GST-PCBP2 bound to MBP-HO1 was markedly and significantly ($p < 0.001$) decreased relative to adding His in the second reaction followed by GST-PCBP2 in the third (Fig. 9B, lane 2). This observation demonstrated that mixing CPR with HO1 first interfered with subsequent PCBP2 binding to HO1. When the sequential order of these reactions was exchanged, namely GST-PCBP2 in the second reaction and His-CPR in the third (Fig. 9B, lane 8), the amount of GST-PCBP2 bound to MBP-HO1 was significantly ($p < 0.001$) increased relative to when His-CPR was added in the second reaction followed by GST-PCBP2 in the third (Fig. 9B, lane 6). This finding indicated that once PCBP2 binds to HO1, the interaction could not be interfered with by the subsequent addition of CPR.

Using the same sequential reaction protocol in Fig. 9B, we next investigated how the HO1/PCBP2 interaction affects HO1–CPR complex formation (Fig. 9C). In these studies, we used anti-His antibody to detect CPR in the eluates from the resin (Fig. 9C). Comparing lanes 1 and 2 of Fig. 9C, His-CPR did not interact with the MBP control but was demonstrated to bind to MBP-HO1. Notably, comparing lanes 2 and 4 of Fig. 9C, an E. coli extract of GST did not significantly ($p > 0.05$) interfere with the interaction between HO1 and CPR, irrespective of whether it was added before or after His-CPR.

When the resin/MBP-HO1 was premixed with GST-PCBP2 in the second reaction and then mixed with His-CPR in the third reaction (Fig. 9C, lane 6), the amount of His-CPR bound to HO1 was significantly ($p < 0.001$) decreased relative to when GST was added in the second reaction followed by His-CPR in the third (Fig. 9C, lane 2). This finding suggests that premixing PCBP2 with HO1 interfered with subsequent CPR binding to HO1. When the sequential order of these reactions was exchanged, namely His-CPR in the second reaction and GST-PCBP2 in the third (Fig. 9C, lane 8), the amount of His-CPR bound to MBP-HO1 was significantly increased ($p < 0.05$) relative to adding GST-PCBP2 in the second reaction and His-CPR in the third (Fig. 9C, lane 6). These studies demonstrate that once CPR binds to HO1, its binding could not be disrupted by the subsequent addition of PCBP2.

In summary, these results demonstrate that PCBP2 could compete with CPR for complex formation with HO1. Indeed, pre-binding of either PCBP2 or CPR to HO1 precludes the binding of the other partner protein. Thus, the interaction of HO1, PCBP2, and CPR may sequentially change during the process of heme catabolism.

Iron evokes PCBP2 dissociation from HO1

The process of heme degradation has been well-studied and proceeds via a multistep mechanism (79, 80). The first step is heme binding to the ligating amino acids of HO1, particularly His-25, etc. (80). The second step is the oxidation of heme to α-hydroxy-heme, requiring O$_2$ and reducing equivalents supplied by CPR (81). The third step is the formation of α-verdoheme with the concomitant release of hydroxylated α-meso carbon as carbon monoxide. The fourth step is the conversion of α-verdoheme to a biliverdin-iron chelate, for which O$_2$ and electrons from CPR are also required (20, 57). In the final step, the iron of the biliverdin-iron chelate is reduced by CPR, and finally, ferrous iron and biliverdin are released from HO1 (82).

Recently, our laboratories have demonstrated that iron-loaded PCBP2 and iron-chelated PCBP2 had different binding affinities to DMT1 or FPN1 (37, 39). Thus, we hypothesized that iron-free PCBP2 may associate with HO1 for receiving ferrous iron in the final step of heme degradation. To examine this, an in vitro pulldown assay was performed to investigate the relationship between heme, ferrous iron, PCBP2, and HO1. In these experiments, 10 μM MBP-HO1 was mixed with amylose resin and incubated with 2.5–10 μM heme, washed, and then mixed with 2.5 μM GST-PCBP2 (Fig. 10A, panel i). In this in vitro assay, heme is bound but not catabolized by HO1 in the absence of CPR, and hence, heme degradation could not progress.

To investigate the molecular change in the states of heme-loaded (holo)-HO1 and heme-free (apo)-HO1, UV-visible spectrophotometry was performed (Fig. 10A, panel ii). Previous reports indicated that a change of heme content in the reaction mixture of HO1 and heme could be quantitatively measured by the absorbance at 405 nm (Soret for HO1-heme) and ~395 nm (Soret for free heme) (57, 61, 79). In this study, heme was added to purified MBP-HO1/amylose resin, and then the supernatant was collected and the change in absorbance of free heme measured (Fig. 10A, panel i). The concentration of free heme in the reaction mixture (i.e. Soret ~395 nm) was decreased by the addition of increasing amounts of MBP-HO1 (Fig. 10A, panel ii). Hence, this suggested HO1 bound heme in solution, reducing its concentration (MBP alone had no effect; data not shown).

Furthermore, the amount of PCBP2 pulled down with HO1 was not significantly ($p > 0.05$) affected by incubation with heme (Fig. 10A, panel i). Thus, the interaction of HO1 and PCBP2 was not influenced by heme, because in these in vitro experiments HO1 was unable to degrade heme due to the absence of CPR and reducing equivalents. Thus, it can be con-
included that holo-HO1 still maintains its binding activity to PCBP2 and that heme degradation is required for the release of PCBP2 from HO1, in accordance with the experiments above.

Next, the effect of depleting or loading PCBP2 with iron (i.e., apo- or holo-PCBP2) was assessed in terms of its interaction with HO1 (Fig. 10B, panels i and ii). In these studies, GST-PCBP2 was depleted or loaded with iron using standard methodology in our laboratories (37, 39), namely implementing the chelator 1,2-cyclohexylenedinitrilotetraacetic acid (CDTA; 10 μM) to deplete PCBP2 of iron or 1–8 μM FeCl₂ (freshly prepared to prevent iron polymerization) to load this protein with iron. These proteins were then incubated with MBP-HO1 bound to amylose resin; the resin was washed, and the bound proteins were eluted with maltose. The eluates were subsequently analyzed by Western blotting using anti-GST Ab.

Depletion of iron from PCBP2 using CDTA did not significantly (p > 0.05) affect the interaction of PCBP2 with HO1-HA relative to the control in these pulldown studies (Fig. 10B, panel i and ii). However, relative to the control, there was a marked and significant (p < 0.001) decrease in the interaction of PCBP2 with HO1-HA after loading PCBP2 with FeCl₂ at 2–8 μM. These results suggest that during the final step of HO1-mediated heme catabolism, where biliverdin and ferrous iron are released, apo-PCBP2 binds this iron and then dissociates from HO1. Iron-loaded holo-PCBP2 may then deliver its metal cargo to other recipient proteins.

In vitro reconstruction of the HO1, CPR, and PCBP2 metabolon

Considering the results from Figs. 4, 9, and 10, it was extrapolated that PCBP2 could receive iron generated during heme degradation, and the iron-loaded PCBP2 might dissociate from the HO1–CPR complex. To further elucidate this enzymatic process, a spectroscopic assay of HO1 activity (57, 61, 83, 84) was performed (Fig. 11A) under the same conditions as the binding studies (Fig. 11B). Using this combined method, we reconstituted the heme catabolism metabolon in vitro using purified recombinant proteins to examine HO1 activity (Fig. 11A) and whether PCBP2 or CPR became bound by HO1 during heme degradation (Fig. 11B).

First, examining HO1 activity in the reconstituted metabolon, once NADPH was added into the MBP-HO1/His-CPR/GST-PCBP2/heme mixture, a reduction in absorbance at 405 nm (the main Soret peak of heme) and 340 nm (the peak of NADPH) was observed and was time-dependent, indicating visible absorbance was measured. After mixing heme with the MBP-HO1/amylose resin, the mixture was centrifuged and the supernatant collected. The absorbance was measured by using a 1:10 dilution of the sample. 8, decrease in the interaction of PCBP2 with HO1 after loading PCBP2 with iron. (i) The purified MBP-HO1 (10 μM) was mixed with amylose resin for 30 min at room temperature and incubated with 2.5, 5.0, or 10.0 μM heme for 10 min at 37 °C. The resin was washed with p-lysis buffer and mixed with 2.5 μM purified GST-PCBP2 for 5 min at 37 °C. The resin was washed with p-lysis buffer, and protein was eluted with 20 mM maltose in p-lysis buffer. MBP-HO1 bound to amylose resin was analyzed by SDS-PAGE and stained with Coomassie R-250. The eluates were analyzed by Western blotting (WB) with anti-GST mAb. The results are typical of at least three independent experiments. (ii) To confirm the formation of the holo-HO1 complex, the UV-visible absorbance was measured. After mixing heme with the MBP-HO1/amylose resin, the mixture was centrifuged and the supernatant collected. The absorbance was measured by using a 1:10 dilution of the sample. 8, decrease in the interaction of PCBP2 with HO1 after loading PCBP2 with iron. (i) The purified MBP-HO1 (10 μM) was mixed with amylose resin for 30 min at room temperature. Then the purified GST-PCBP2 (2.5 μM) was preincubated with either the chelator, CDTA (10 μM; to deplete PCBP2 of iron), or freshly prepared solutions of 1, 2, 4, or 8 μM FeCl₂ (to load PCBP2 with iron) for 10 min at 37 °C. MBP-HO1/amylose resin was loaded with iron-loaded GST-PCBP2 and incubated for 5 min at 37 °C. The resin was washed with p-lysis buffer three times. The protein was eluted with 20 mM maltose/p-lysis buffer. MBP-HO1, which was bound to amylose resin, was analyzed by SDS-PAGE and stained with Coomassie R-250. The eluates were analyzed by Western blotting (WB) with anti-GST mAb. The results are typical of at least three independent experiments. (ii) The results shown in the bar graph are presented as the mean ± S.D. of three independent experiments. ***, p < 0.001.
Figure 11. Reconstruction of the HO1/PCBP2/CPR metabolon in vitro. A, recombinant GST-HO1–His-CPR complex can degrade heme into biliverdin. This study was performed in three steps. In the first step, the purified MBP-HO1 was mixed with amylose resin and then washed with p-lysis buffer (first reaction). In the second step, the purified GST-PCBP2 could bind to MBP-HO1 on the resin and might compete with GST-PCBP2, which was added in the second reaction step, either His-CPR alone, His-CPR with heme, His-CPR with heme and NADPH, or GST-PCBP2 was added. Hence, during heme degradation, His-CPR added in this third step might compete with GST-PCBP2, which was added in the second step and had already coupled with MBP-HO1 on the resin (Fig. 11B). The resin-bound MBP complexes were then eluted from the resin using an excess of maltose and analyzed by Western blotting with either an anti-GST antibody to detect CPR (Fig. 11B) or anti-His antibody to detect PCBP2 (Fig. 11B, left panel) or anti-His antibody to detect CPR (Fig. 11B, right panel).

Once GST-PCBP2 was loaded to MBP-HO1 in the second step and His-CPR as a third step, GST-PCBP2 could bind to heme degradation (Fig. 11A). Moreover, after a 30-min incubation, the 680-nm absorbance peak of biliverdin increased (Fig. 11A). Similar spectral profiles leading to the decrease in Soret absorbance of heme and an increase in the absorbance of the product, biliverdin, have been demonstrated in previous studies using HO (57, 61). Hence, this reconstituted metabolon could effectively degrade heme into biliverdin in vitro.

Notably, biliverdin generation appears low (see Fig. 11A, inset) because of the relatively weak extinction coefficient of this metabolite at 680 nm (28 mm$^{-1}$ cm$^{-1}$; (83)), which is much lower than heme at 405 nm (121 mm$^{-1}$ cm$^{-1}$ (62)). Quantitating the decrease in heme at 405 nm using its extinction coefficient (62) demonstrates that at 10 and 30 min, the heme levels are 2.14 and 1.24 μM, respectively, and thus, 0.9 μM heme is consumed. Using the extinction coefficient of biliverdin (83), the concentration of biliverdin at 10 and 30 min is 0.34 and 1.04 μM, respectively, and thus 0.7 μM biliverdin is generated. Thus, the degradation of heme is quantitatively similar to the generation of biliverdin, indicating the competency of the reconstituted metabolon in heme metabolism.

Second, our studies then reconstructed the HO1/CPR/PCBP2 metabolon in vitro and examined its activity via a competition assay (Fig. 11B) similar to the binding protocol used in Fig. 9B. In these experiments, three reaction steps were utilized to assess whether competition occurred between CPR and PCBP2 when one of these molecules binds to HO1 in the presence of heme and NADPH. In the first reaction, MBP-HO1 was mixed with amylose resin (Fig. 11B). In the second reaction, GST-PCBP2 or His-CPR was then mixed with the MBP-HO1-bound resin. Based on our studies described above, it was expected these molecules added in the second reaction step could bind to MBP-HO1 on the resin and might compete with the molecules added in the subsequent third reaction step. In the third reaction step, either His-CPR alone, His-CPR with heme, His-CPR with heme and NADPH, or GST-PCBP2 was added. Hence, during heme degradation, His-CPR added in this third step might compete with GST-PCBP2, which was added in the second step and had already coupled with MBP-HO1 on the resin (Fig. 11B). The resin-bound MBP complexes were then eluted from the resin using an excess of maltose and analyzed by Western blotting with either an anti-GST antibody to detect PCBP2 (Fig. 11B, left panel) or anti-His antibody to detect CPR (Fig. 11B, right panel).

Once GST-PCBP2 was loaded to MBP-HO1 in the second step and His-CPR as a third step, GST-PCBP2 could bind to...
PCBP2 interacts with heme oxygenase 1

MBP-HO1 on the resin (Fig. 11B, left panel, lane 1) and His-CPR could not (Fig. 11B, right panel, lane 1). This MBP-HO1/GST-PCBP2 binding was not significantly affected by heme alone (Fig. 11B, lane 2). However, in the presence of both heme and NADPH (third step), GST-PCBP2 (second step) could be released from MBP-HO1 (Fig. 11B, left panel, lane 3), and His-CPR (third step) was observed bound with MBP-HO1 (Fig. 11B, right panel, lane 3). As described in Fig. 10, once His-CPR was mixed with MBP-HO1 before GST-PCBP2 loading, GST-PCBP2 could not bind to MBP-HO1 (Fig. 11B, left panel, lane 4) but His-CPR could (Fig. 11B, right panel, lane 4). Thus, GST-PCBP2 became dissociated from MBP-HO1, and His-CPR was associated with MBP-HO1 during the process of heme degradation in vitro.

In summary, these results demonstrate that the formation of both the HO1–CPR complex and HO1–PCBP2 complex are functioning as a metabolon during heme degradation and that PCBP2 serves as an iron chaperone, binding the iron as it is released by heme catabolism.

Discussion

For the first time in this study, interactions between HO1 and the iron chaperone, PCBP2, were examined, with binding between these two molecules being demonstrated by a variety of methods. It is well known that HO1 and CPR form a complex via their cytosolic regions and catabolize heme into biliverdin, carbon monoxide, and ferrous iron (23, 57, 63). The iron released from heme is an important source of this nutrient for subsequent metabolism of this metal ion (85).

This investigation also demonstrated that HO1 can interact with PCBP2 but not PCBP1, PCBP3, or PCBP4 (Fig. 1, B and C). Interestingly, studies by others using Pcbp2−/− mice have demonstrated that PCBP2 possesses a non-redundant role in vivo, especially during hematopoiesis (86), suggesting it plays an essential role in iron metabolism.

The PCBPs have no transmembrane domains and are known as soluble proteins, mainly residing in the cytosol (37, 42, 43). However, fractionation showed that considerable PCBP2 was detected in the 10Kpt, 10Ksp, and 100Kpt fractions that corresponded to membrane (i.e. P-cadherin) and microsomal proteins (i.e. calnexin, HO1, and CPR; Fig. 1C, panel i). This observation suggested that PCBP2 may interact with membrane-associated protein(s) such as HO1 (9, 21), DMT1 (37), and FPN1 (39). Of interest, the amount of PCBP1 in the 100Kpt microsomal fraction was less compared with PCBP2 (Fig. 1C, panel i). Hence, the membrane-associated properties must be somewhat different between these two latter molecules.

Other differences in these two chaperones include that PCBP2 exhibited ~2-fold higher binding affinity for ferritin than iron-loaded PCBP1 (43). Additionally, in contrast to PCBP2, it is notable that PCBP1, PCBP3, or PCBP4 did not bind to the transmembrane transporters DMT1 and FPN1 (37, 39), and it was demonstrated in the current study that HO1 interacts only with PCBP2 during heme catabolism. Further analysis using discontinuous gradient centrifugation revealed differences in subcellular distribution of the PCBP family members (Fig. 7). In fact, in contrast to PCBP1, PCBP3, and PCBP4, PCBP2 changed its subcellular distribution after incubation of cells with heme, leading to a decrease in its levels in the microsomal fraction and an increase in the cytosol. This finding was in accordance with confocal microscopy studies demonstrating a decrease in the association of PCBP2 with the microsomal protein, HO1, after incubation with heme (Fig. 6).

We initially demonstrated the interaction of PCBP2 with both HO1 and HO2 by co-immunoprecipitation assays in mammalian cells. In addition, we prepared several PCBP2 deletion mutants and investigated which domain of PCBP2 was necessary for the HO1/PCBP2 interaction. Previous reports showed that N-terminal FLAG-tagged PCBP2 exhibited equivalent iron delivery activity as authentic PCBP2 (36, 43). Thus, we generated N-terminally FLAG-tagged PCBP2 with various deletions of its KH domains and performed in vivo co-immunoprecipitation assays in mammalian cells (Fig. 3). Binding studies using these FLAG-tagged deletion mutants (Fig. 3) revealed that deletion of the KH2 domain in PCBP2 disrupted its interaction with DMT1, and deletion of the KH3 domain led to the loss of the interaction with HO1. Notably, PCBP2 lacking other KH domains attenuated the binding affinity for HO1 to a lesser extent, suggesting that such deletions may strain the conformation of PCBP2 and weaken the HO1 binding efficacy. Hence, the KH3 domain, as well as the conformation of PCBP2, could be important for the HO1/PCBP2 interaction.

Previous investigations have demonstrated that HO enzymes form dimers/oligomers in vitro in solution and also in cells (87, 88). Considering this, we showed that HO1 and PCBP2 form a complex in a 4:1 molar ratio, and as such PCBP2 may receive multiple ferrous iron atoms from the HO1 oligomer. This could be important to consider especially because PCBP2 has been shown to have three Fe(II)-binding sites (36). Further studies using structural analysis via X-ray crystallography, etc. will be crucial for precise elucidation of the HO1/PCBP2 interaction.

It was previously reported that the authentic protein concentration of PCBP2 in cells was 100 nm (89) and that of HO1 was 0.17 nm (90). In fact, PCBP2 are estimated to occupy ~0.5% of total proteins (91) and are abundantly expressed relative to HO1. Hence, even with transfection of HO1, PCBP2 is still estimated to be present at a large excess, and thus, a complex can form between these proteins. Based on these data, we assess that our current studies are biologically meaningful. As part of assessing their intracellular levels, it is also important to consider the evidence that heme levels alter the cellular distribution of PCBP2 (Fig. 7) and induce the co-localization of PCBP2 and HO1 (Fig. 6). Such changes, as well as the intricate compartmentalized nature of the cellular microenvironment, need to also be considered when examining cellular protein concentrations and protein/protein interactions (discussed further below).

Structural analysis of HO1 and CPR has demonstrated that CPR contains two enzymatic domains (57), each of which catalyzes electron transfer from NADPH to flavin adenine dinucleotide, then to flavin mononucleotide, and finally to heme bound to HO1 (57). Once heme is bound to HO1, heme-bound HO1 is encircled by CPR, and they form a tight complex (57). The studies in Fig. 2 demonstrated that PCBP2 could bind to HO1, but not CPR, and a competition appears to occur between PCBP2 and CPR for HO1 binding (Figs. 4, 5, 9, and 11). Our
experiments show that the HO1/CPR interaction and HO1/PCBP2 dissociation were increased by heme loading. Considering this, it can be suggested that during heme degradation by HO1, each reaction step may cause step-by-step conformational changes in HO1, CPR, and/or PCBP2. Of note, heme-loaded HO1 retains PCBP2-binding affinity, and iron-loaded PCBP2 loses its HO1-binding affinity.

Collectively, the studies in Figs. 1, 2, and 4–11 enable an integrated model of the putative function of PCBP2 in heme catabolism via HO1 to be proposed (Fig. 12). PCBP2 competes with CPR for binding to HO1 (Figs. 4, 5, 9, and 11). Both HO1 (9, 21) and CPR (54) are localized at the ER membrane, and apo-PCBP2 binds to HO1 under steady-state control conditions (Figs. 1, 2, 4, and 8–10). This latter interaction is hypothesized to ensure that PCBP2 remains in the micro-environment of HO1 and CPR. Once heme is transported through the cell (8, 92), it can bind to its binding site on HO1. Heme binding to HO1 is the first trigger, as it alters HO1 structure, which then enables CPR to become tightly bound to HO1 and transfer electrons to commence heme degradation (57). This conformational change of HO1 is suggested to cause HO1/PCBP2 dissociation. Then rapidly, CPR dissociates from HO1 and apo-PCBP2 then binds to HO1 to accept the released ferrous iron. At the final step of the heme degradation process, the ferrous iron–PCBP2 (holo-PCBP2) complex then dissociates from HO1 to transfer its metal to other protein acceptors, e.g. FPN1 (Fig. 12) (39). In fact, iron-loaded (holo-PCBP2) PCBP2 demonstrates markedly lower association with HO1 than apo-PCBP2 (Fig. 10B). After dissociation of holo-PCBP2, another apo-PCBP2 molecule then binds to HO1 (Fig. 12).

Apart from the differential ability of apo-PCBP2 and iron-loaded PCBP2 to bind to HO1 (Fig. 10B), there are probably other mechanisms that ensure that PCBP2 remains in the appropriate micro-environment close to CPR and HO1. In particular, a mechanism must exist that keeps apo-PCBP2 in the vicinity of HO1 when CPR is bound to HO1 and iron is released from degraded heme (Fig. 12). Such a mechanism may involve cytoskeletal or organelle interactions that are known to be involved in the transport of other iron transport proteins, e.g. the uptake of iron via transferrin and its internalization into the cell where iron is released (1, 2, 6). In this study, we demonstrated that there were distinct alterations in the cellular distribution of PBCP2 upon the addition of heme (Figs. 6 and 7). The precise molecular processes involved remain to be investigated in future studies.

In the cytosol, iron-loaded PCBP2 could form a hetero- or homodimer with PCBP1 or PCBP2 (36) and then deliver iron to appropriate target molecules. For instance, we already have demonstrated that iron-loaded PCBP2 can bind to FPN1, and silencing of PCBP2 prevents FPN1-mediated iron efflux (39). Moreover, we and others have demonstrated that HO1 expression results in iron release from cells (28–30). We speculate this may occur through PCBP2 loaded with iron (derived from HO1) subsequently interacting with FPN1.

It has been previously demonstrated that recombinant HO1 possesses enzymatic activity and can utilize electron supply...
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from CPR to degrade heme into iron and its other end products in vitro (60–62). Our results suggest that PCBP2 is not necessary for heme degradation activity. Hence, PCBP2 could play a role as part of an integrated complex of proteins (i.e. HO1-CPR-PCBP2) to form a functional metabolon to prevent the generation of free/labile iron in cytosol, which may cause toxic reactive oxygen species production.

In conclusion, PCBP2 acts as a recipient of iron from heme degradation mediated by HO1. Taken together with our previous studies (37, 39), this investigation supports a model where PCBP2 acts as a common chaperone for cellular iron influx via DMT1, cellular iron efflux via FPN1, and also for iron released by heme catabolism. Considering these interactions, it is conceivable that PCBPs could interact with other iron-containing proteins and transporters, such as the ZIP transporters (94, 95) and mitoferrin (96). Thus, the participation of the PCBP family members in these processes is crucial for further exploration.

Experimental procedures

Antibodies and reagents

A goat anti-human PCBP1 pAb was from Acris (Herford, Germany; catalog no. AP22434PU-N). Mouse anti-human PCBP2 monoclonal Ab (mAb) was purchased from Abnova (Taipei, Taiwan; catalog no. H0005094-M07). Rabbit anti-human PCBP3 pAb was obtained from Atlas Antibodies (Stockholm, Sweden; catalog no. HA030247); rabbit anti-human PCBP4 pAb was from Novus Biologicals (Minneapolis, MN; catalog no. NBP1-76729). Mouse anti-HA mAb was acquired from Cell Signaling Technology (Boston; catalog no. 2367); mouse anti-human pan-cadherin mAb (clone CH-19) was obtained from Sigma (catalog no. C1821-2ML); mouse antihuman α-tubulin mAb was from Sigma (catalog no. T9026-100UL); mouse anti-human TOMM20 mAb (MO1) was procured from Abnova (catalog no. H0009804). Rabbit antihuman cytochrome P450 reductase pAb was from Abcam (Cambridge, UK; catalog no. ab13513); rabbit anti-human calnexin mAb (C5C9) was attained from Cell Signaling Technology (catalog no. 2679P); rabbit anti-human GAPDH pAb was from Merck Millipore (Darmstadt, Germany; catalog no. ABS16); rabbit anti-rat HO1 pAb was acquired from Enzo Life Sciences (Farmington, NY; catalog no. ADI-SPA-895-D); rabbit anti-human ferritin heavy chain was purchased from Santa Cruz Biotechnology (Dallas, TX; catalog no. sc25617). Alexa 594-labeled goat anti-mouse IgG (catalog no. A-11037) was from Thermo Fisher Scientific (Waltham, MA).

We previously raised rabbit polyclonal antibody against GFP (18). Horseradish peroxidase (HRP)-conjugated anti-goat IgG was from Jackson ImmunoResearch (West Grove, PA; catalog no. 705-035-147), and HRP-conjugated anti-mouse (catalog no. 7076) and anti-rabbit IgGs (catalog no. 7074) were from Cell Signaling Technology. All antibodies were utilized in dilutions in the range of 1:100–1:10,000. Most other general reagents were purchased from Wako Chemicals (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), or Sigma.

Vectors and plasmid constructions

To express human DMT1, HO1, HO2, PCBP2, or CPR, each cDNA was cloned into a pEGFP-N1 vector (TaKaRa; Shiga, Japan), pCMV-FLAG vector (TaKaRa), or pCMV-HA (TaKaRa) vector. The HO1 H25A mutant was made by PCR mutagenesis using KOD plus DNA polymerase (Toyobo, Osaka, Japan) using standard methodology (35, 37). Nucleotide sequences of PCR-oriented constructs were confirmed by the dideoxynucleotide chain-termination method using an ABI 3100 automated DNA sequencer (Thermo Fisher Scientific).

Cell culture and transfection

Human HEp-2 epithelial cells were obtained from ATCC (CCL-23) maintained in Dulbecco’s minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS). HO1-GFP or HA-CPR stably expressing HEp-2 cells were maintained in DMEM supplemented with 10% FBS and 750 µg/ml G418. Cells were transfected with HA-tagged full-length form of CPR, FLAG-tagged PCBP2 using Lipofectamine® 3000 reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions.

siRNA transfection

Silencer Select siRNA targeting a 21-nucleotide sequence of CPR (catalog no. s10838) and Silencer Select Negative Control No. 1 siRNA (catalog no. 4390844) were purchased from Thermo Fisher Scientific. The sequence of CPR Silencer Select siRNA was 5’-CCACCAACCGGAAGCUATT-3’. HEp-2 cells were transfected with 5 nM Silencer Select siRNA using Lipofectamine® RNAiMAX (Thermo Fisher Scientific) and incubated for 48 h at 37 °C. The Lipofectamine® RNAiMAX:siRNA ratio was 1 µl/2.5 pmol.

Subcellular fractionation

HO1-GFP stably expressing HEp-2 cells were grown to confluence in 100-mm dishes. Cells were washed twice with PBS, removed from the plate in homogenization buffer (0.25 M sucrose, 10 mM Tris-HCl (pH 7.4)), and homogenized in a Teflon-coated glass Potter homogenizer rotating at 1000 rpm for 15 strokes at 4 °C. The homogenate was then centrifuged at 1000 × g for 10 min at 4 °C to obtain the post-nuclear supernatant (PNS). The PNS was centrifuged at 10,000 × g for 20 min at 4 °C to obtain the crude mitochondrial fraction (10Ksup). Then, the 10Ksup was centrifuged at 100,000 × g for 60 min at 4 °C to separate the 10,000 × g pellet (10Kpt) and 10,000 × g supernatant (10Ksup, post-mitochondrial supernatant). Then the 10Ksup was centrifuged at 100,000 × g for 60 min at 4 °C to separate the 100,000 × g pellet (100Kpt) and 100,000 × g supernatant (100Ksup, post-mitochondrial supernatant). Then the 100Ksup was centrifuged at 100,000 × g for 60 min at 4 °C to separate the 100,000 × g pellet (100Kpt) and 100,000 × g supernatant (100Ksup) (10, 16, 25). The 10Kpt and 100Kpt were resuspended in homogenization buffer and centrifuged at 10,000 × g for 20 min at 4 °C and 100,000 × g for 60 min at 4 °C, respectively, to wash the pellets. The 10Kpt was subsequently lysed in Laemmli buffer.

To assess the relative amount of the molecule of interest in each fraction, these four samples, namely 10Kpt, 10Ksup, 100Kpt, and 100Ksup, were adjusted to the same volume, and then equal volumes of each fraction were analyzed by Western blotting, as per standard methods (97, 98). The 100Kpt fractions were resolved in lysis buffer (20 mM Tris-HCl (pH 8), 0.1% (w/v) Nonidet P-40, and 60 mM NaCl) and used for the co-immunoprecipitation assay.
For further analysis of subcellular distribution, density gradient fractionation was performed (15). The detailed methods were previously described (15). HEP-2 cells or HO1-GFP stably expressing HEP-2 cells were incubated with 50 μM hemin (Frontier Science, Logan, UT) for 6 h at 37 °C. Cells were collected and homogenized by the method as described above. The 10Ksup was loaded on the top of a step (30, 20, 15, 10, and 5%) OptiPrep (Axis-Shield PoC AS, Oslo, Norway) gradient and centrifuged at 88,000 × g for 18 h at 4 °C. After centrifugation, 20 fractions were collected, and an equal sample volume of each fraction was analyzed by Western blotting.

Co-immunoprecipitation assay and Western blotting
Cells were washed with PBS extensively and lysed in ice-cold buffer to obtain the total cell extract. DMT1-GFP stably expressing cells were lysed in TNE buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 2% (w/v) Nonidet P-40) (35, 37). The total cell extract or 100K pt lysate was co-immunoprecipitated with anti-GFP pAb-conjugated protein A beads and washed extensively with the lysis buffer and then analyzed by Western blotting using the appropriate antibodies via standard methods (99). Protein expression was normalized by the measurement to total protein (i.e. Coomassie R-250-stained). Protein concentrations were determined using Lowry’s method (100), and probing with α-tubulin was implemented as a protein-loading control.

Recombinant protein expression
The full-length of HO1 was cloned into the pMAL-c2x vector (New England Biolabs, Ipswich, MA); the full-length or each of the PCBP2 deletion mutants was cloned into the pGEX2T vector (GE Healthcare), and CPR lacking the predicted transmembrane region (amino acids 1–57) was cloned into the pET32a vector (Merck Millipore). Lemo21 (DE3) E. coli cells (New England Biolabs) was transformed with these plasmids and grown in Luria broth with 100 μg/ml ampicillin and 20 μg/ml chloramphenicol at 37 °C to an *A* 600 of 0.8. Then, recombinant gene expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.5 mM for 6 h at 25 °C. Cells were harvested and disrupted in p-lysis buffer (50 mM sodium phosphate (pH 6.4), 0.1% Nonidet P-40, 60 mM NaCl, and 1 mM DTT) by sonication. Lysates were cleared by centrifugation at 20,000 × g for 10 min at 4 °C.

Purification of recombinant proteins
MBP-tagged proteins were purified using amylose resin (New England Biolabs). After incubation for 2 h at room temperature with amylose resin, the resin was washed four times with p-lysis buffer, and then the recombinant fusion proteins were eluted with 20 mM maltose in p-lysis buffer at room temperature.

GST-tagged proteins were purified using glutathione-Sepharose 4B beads (GE Healthcare). After incubation for 2 h at room temperature with Sepharose beads, the beads were washed four times with p-lysis buffer, and then the recombinant fusion proteins were eluted with 50 mM reduced glutathione in p-lysis buffer at room temperature.

His-tagged proteins were purified using Ni-NTA-agarose (Qiagen, Hilden, Germany). After incubation for 2 h at room temperature with Ni-NTA-agarose, the agarose was washed four times with 20 mM imidazole in p-lysis buffer, and then the recombinant fusion proteins were eluted with 200 mM imidazole in p-lysis buffer at room temperature. These recombinant proteins were dialyzed in p-lysis buffer overnight at 4 °C.

In terms of the biological activity of these recombinant proteins, we have demonstrated that GST-PCBP2 demonstrates saturable binding activity to MBP-HO1 (Fig. 8B, panel ii), illustrating the competency of these proteins. Moreover, recombinant MBP-HO1, His-CPR, and GST-PCBP2 can form a markedly active and functional metabolon in vitro that metabolizes heme and NADPH leading to the generation of biliverdin (Fig. 11A), as well as the association and dissociation of CPR and PCBP2 (Fig. 11B). These studies, using this functional unit of recombinant proteins, confirm the association and dissociation of PCBP2 and HO1 demonstrated in vitro (Figs. 1–5 and 8–10) and also observed in living cells (Fig. 6). Collectively, these properties demonstrate the marked functional activity of the recombinant proteins generated. However, although these proteins were markedly active and functional, we did not determine whether they were fully competent.

Immunofluorescence analysis
HO1-GFP stably expressing cells were grown on the coverslips. Cells were incubated with 50 μM hemin or 25 μM SnMP (Frontier Science, Logan, UT) for 8 h at 37 °C. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. The cells were blocked in 0.1% fish skin gelatin and 0.1% paraformaldehyde and permeabilized with 0.1% Triton

Measurement of heme and iron concentration
Heme concentration was measured by hemin colorimetric assay kit (BioVision, CA) according to the manufacturer’s instruction. Total iron was measured by a ferrozine-based iron assay (67). The detailed method was described previously (39, 67). Briefly, cells were lysed in 50 mM NaOH and then treated with acidic KMnO4 to release iron from proteins. The samples were then incubated with an iron detection reagent (6.5 mM ferrozine, 6.5 mM neocuproine, 2.5 mM ammonium acetate, and 1 mM ascorbic acid dissolved in water). A colorimetric measurement was performed by calculating the absorbance of each sample relative to the absorbance of FeCl3 standards at a range of concentrations.

Holo-HO1 formation assay
MBP-HO1 was mixed with amylose resin and washed with p-lysis buffer. After buffering with the MBP-HO1/amylose resin, the mixture was centrifuged, and the supernatant was collected. The supernatant was diluted 1:10 and then measured using a Beckman DU 800 UV-visible spectrophotometer (Beckman, Brea, CA).
PCBP2 interacts with heme oxygenase 1

**In vitro kinetic assay**

MBP-HO1 was mixed with amylose resin and washed with p-lysis buffer. The purified GST-PCBP2 was pre-incubated at 37 °C and then mixed with MBP-HO1/amylose resin. After an incubation at 37 °C, the mixture was chilled on ice to stop the reaction. The beads were washed with ice-cold p-lysis buffer four times, and then the binding proteins were eluted with 20 mM maltose in p-lysis buffer at room temperature. The eluates were analyzed by Western blotting.

**In vitro heme degradation assays**

In the first step, MBP-HO1 was mixed with amylose resin and then washed with p-lysis buffer (first reaction). In the second step, the purified GST-PCBP2 expressed in *E. coli* harboring pGEX2T-PCBP2 or the purified His-CPR expressed in *E. coli* harboring pET32-CPRΔTM was mixed with the post-first reaction resin for 30 min at room temperature and washed with p-lysis buffer (second reaction). In the third step, the purified GST-PCBP2 or His-CPR was mixed with the post-second reaction resin for 30 min at room temperature and washed with p-lysis buffer (third reaction). During the third reaction, 5 μM heme or 25 μM NADPH (P-L Biochemicals Inc., Milwaukee, WI) was loaded to the mixture. The amylose resin-bound proteins were eluted with 20 mM maltose/p-lysis buffer. Eluates were analyzed by Western blotting using anti-GST mAb or anti-His mAb.

To confirm the heme catabolism in the reaction mixture, we analyzed the consumption of heme and NADPH and the formation of biliverdin during the enzymatic degradation of heme in vitro with the absorbance being measured by the DU 800 UV-visible spectrophotometer described previously (57, 61, 83, 84). In the first step, MBP-HO1 was mixed with amylose resin and then washed with p-lysis buffer (first reaction). In the second step, the purified GST-PCBP2 was mixed with the post-first reaction resin for 30 min at room temperature and washed with p-lysis buffer (second reaction). In the third step, the purified His-CPR was mixed with the post-second reaction resin for 30 min at room temperature and washed with p-lysis buffer (third reaction). During the third reaction, 5 μM heme and 25 μM NADPH were loaded to the mixture. The absorbance was then measured after incubations of 0, 10, and 30 min at room temperature (83).

**Statistics and analysis**

Statistical analysis was performed using Student’s *t* test. All results are typical of at least three experiments. The density of specific bands from Western blotting was measured with a computer-assisted imaging analysis system (ImageJ, version 4.7 software, National Institutes of Health). The images from immunofluorescence analysis were examined using ImageJ.

**Author contributions**—I. Y. conducted most of the experiments, analyzed the results, and wrote and revised the paper. D. R. R. suggested experiments and also was involved in analyzing results and writing and revising the manuscript. S. T. suggested experiments and was involved in revising the manuscript. F. K. conceived the study and wrote the paper in association with I. Y. and D. R. R.

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