

A Novel Heme-Regulatory Motif Mediates Heme-Dependent Degradation of the Circadian Factor Period 2^{∇†}

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Although efforts have been made to identify circadian-controlled genes regulating cell cycle progression and cell death, little is known about the metabolic signals modulating circadian regulation of gene expression. We identify heme, an iron-containing prosthetic group, as a regulatory ligand controlling human *Period-2* (hPer2) stability. Furthermore, we define a novel heme-regulatory motif within the C terminus of hPer2 (SC⁸⁴¹PA) as necessary for heme binding and protein destabilization. Spectroscopy reveals that whereas the PAS domain binds to both the ferric and ferrous forms of heme, SC⁸⁴¹PA binds exclusively to ferric heme, thus acting as a redox sensor. Consequently, binding prevents hPer2 from interacting with its stabilizing counterpart cryptochrome. In vivo, hPer2 downregulation is suppressed by inhibitors of heme synthesis or proteasome activity, while SA⁸⁴¹PA is sufficient to stabilize hPer2 in transfected cells. Moreover, heme binding to the SC⁸⁴¹PA motif directly impacts circadian gene expression, resulting in altered period length. Overall, the data support a model where heme-mediated oxidation triggers hPer2 degradation, thus controlling heterodimerization and ultimately gene transcription.

Cellular homeostasis depends on a delicate balance between metabolic activity and gene expression. Heme is a prosthetic group essential for transport and storage of oxygen that is involved in the generation of cellular energy by respiration and synthesis and degradation of lipids and in oxidative damage. Heme-based sensor proteins detect and respond to variations in oxygen, carbon monoxide, and nitric oxide levels and cellular redox state by acting on transcription, translation, protein translocation, and protein assembly (8, 25).

Heme binding to transcription factors is found in both prokaryotes and lower eukaryotes; however, only three cases have been identified in higher eukaryotes: the basic leucine zipper transcription factor Bach1, the circadian transcription factor NPAS2, and the nuclear orphan receptor Rev-erb α (6, 23, 30, 43). Although structurally unrelated, both NPAS2 and Bach1 regulate the transcription of genes impacting heme synthesis and degradation (15, 36). Heme binds to NPAS2 through a region of homology called PAS (Per-ARNT-Sim) to form a gas-regulated sensor (6). Although DNA binding of NPAS2 depends on its heterodimerization with another basic-helix-loop-helix-PAS protein termed Bmal1, heme is not required for the dimer to bind DNA (6). Heme-bound NPAS2 acts as a gas-sensing protein by binding carbon monoxide, causing inhibition of the DNA-binding capacity of NPAS2/Bmal1 (6). Interestingly, heme binding to Rev-erb is mediated by a histidine residue located in the carboxy tail of the ligand-binding domain, but unlike NPAS2, Rev-erb activity is not responsive to

diatomic gases and is unlikely to sense redox conditions (30, 43).

Along with PAS, a second domain has been identified as a heme-binding site. The heme-regulatory motif (HRM) comprises a stretch of residues where only a Cys-Pro core is absolutely conserved and a preferred hydrophobic residue is located in the fourth position (X-CP- ϕ). This motif has been identified in functionally diverse proteins and is thought to govern the activity of a neighboring transmitter domain in response to heme binding (45). For example, heme binds to the transcriptional repressor of the heme oxygenase-1 (HO-1) gene, Bach1, through multiple HRMs (37). Binding inhibits Bach1/MafK association with the HO-1 promoter, inducing subcellular relocation of Bach1 and degradation (37, 44). In addition to Bach1, various heme-mediated protein functions require HRMs: the yeast transcriptional activator Hap1 that transcribes genes encoding various cytochromes, catalase, and Rox1, which represses anaerobic genes under high heme concentration (see reference 12 and references within); the heme-regulated inhibitor kinase that controls the activity of the translation initiator factor eIF-2 α in stressed erythroid cells (4, 11); the erythroid 5-aminolevulinic acid synthase precursors whose transport to the mitochondria is mediated by heme binding to HRMs (20); the heme lyase found in both *Saccharomyces cerevisiae* and *Neurospora crassa* (35); the mammalian nuclear factor erythroid 2 that plays a critical role in erythroid differentiation (22); the HO-2 that metabolizes heme (21); the iron regulatory protein 2 (IRP2), a regulator of iron metabolism in mammals (13), and the iron response regulator (Irr) in bacteria whose turnover depends on the cellular iron availability (28, 42).

A second PAS-containing circadian molecule, Per2, has also been implicated in heme binding and mediates *per1* and *per2* transcription in vivo by a mechanism involving NPAS2 (15).

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Disruption of either the *per1* or *per2* gene in mice leads to circadian deregulation of heme biosynthesis by altering the expression levels of the rate-limiting enzymes *Alas1* and *Alas2* (15, 46). Unlike NPAS2, Per2 does not contain a basic-helix-loop-helix domain, and it is hypothesized that heme control of Per2-mediated gene transcription takes place indirectly by modulating the expression of Bmal1. Consequently, while we know much about how heme and Per2 signaling molecules operate in cell metabolism and circadian rhythms, we lack a clear understanding of how these two circuitries are integrated and operate to directly modulate gene expression.

Here we report the discovery of a previously uncharacterized heme-regulatory motif in Per2 with a functional link to protein stability. We show that (i) heme binds to two distinct regions of human *Period-2* (hPer2) and the oxidation state of the heme iron determines binding specificity and degradation; (ii) hPer2 stability is compromised when heme binds to the outermost C-terminal domain of the protein, preventing hPer2 from binding its heterodimeric counterpart human cryptochrome 1 (hCry1); (iii) downregulation of hPer2 is suppressed in the presence of inhibitors of heme synthesis or proteasome activity; and (iv) a point mutation in the C-terminal HRM is sufficient to stabilize hPer2 *in vivo*. Together, our data indicate that an uncharacterized HRM functions as a binding site and triggers heme-induced degradation of hPer2, likely regulating cellular signaling by modulating the formation of hPer2/hCry1 complex.

MATERIALS AND METHODS

Plasmid constructs and site-directed mutagenesis. Various hPer2 and hCry1 cDNA fragments were cloned into the *SalI* and *NotI* sites of pGEX-4T-3. Fragments of hPer2 comprising residues 1 to 172, 173 to 355, 356 to 574, 173 to 574, 822 to 872, and 822 to 1255 are referred to as hPer2(I), hPer2(II), hPer2(III), hPer2(II-III), hPer2(V4), and hPer2(V4-VII), respectively. The Cys residue of each putative HRM (Cys⁸⁴¹ and Cys⁹⁶²) and Ser⁶⁶² in hPer2 was mutated to Ala by site-directed mutagenesis using QuikChange (Stratagene). The hPer2, hPer2(SA⁸⁴¹PA), hPer2(II-III), and hPer2(V4-VII) cDNAs were cloned into pCS2+myc-tag vector modified for ligation-independent cloning (Novagen).

Protein pull-down and heme-agarose-binding assays. Glutathione *S*-transferase (GST) fusion proteins were expressed in *Escherichia coli* strain Rosetta (Novagen) and purified by glutathione-Sepharose chromatography following the manufacturer's instructions (GE HealthSciences). Untagged proteins were generated by digestion of fusion proteins with thrombin followed by concentration and buffer exchange (10 mM Tris-HCl [pH 8.0]). For pulldown assays, a total of 5 μ g of GST-hCry1-bound beads or an equivalent amount of glutathione beads was washed in binding buffer A (20 mM Tris-HCl [pH 7.4], 100 mM NaCl, 5 mM EDTA, and 0.1% Triton X-100) and incubated with 2 μ l of *in vitro*-transcribed and -translated ³⁵S-labeled hPer2 or the indicated fragments at 4°C for 1 h. After the beads were washed with low- and high-salt binding buffer A (with 100 mM and 1 M NaCl, respectively), bound proteins were eluted by boiling in Laemmli sample buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. In other experiments, heme [Fe(III)-heme, 10 μ M] was added to either hPer2 or the preformed GST-hCry1/hPer2 complex and incubated at 4°C for 1 h. In the first scenario, hPer2/heme was loaded onto GST-hCry1 beads, and binding proceeded at 4°C for an additional hour. Samples were analyzed by autoradiography.

For heme-agarose binding, 20 μ l of heme-agarose beads (Sigma) was washed, resuspended in binding buffer B (10 mM sodium phosphate buffer [pH 7.5], 500 mM NaCl, 5 mM EDTA, 1% Triton X-100) and incubated with 5 μ g of the indicated recombinant proteins at 4°C for 1 h. Beads were washed with low- and high-salt binding buffer B (with 250 mM and 1 M NaCl, respectively), and proteins were analyzed by SDS-PAGE.

Spectroscopic analysis of heme-protein binding. Ferric heme binding was determined by absorption spectra of 1 μ M heme in the absence or presence of 1 μ M of indicated proteins in 10 mM Tris-HCl, pH 8.0. The protein/heme molar ratio ranged from 0.25 to 8. Results were plotted as absorbance at the peak

versus the molar ratio of protein to heme. To determine ferrous heme-binding properties, 30 mM sodium dithionite was added to reduce heme to ferrous heme. Absorption spectra were recorded between 300 and 700 nm on a Beckman DU-640 UV-visible spectrophotometer.

CD spectroscopy. Far-UV circular dichroism (CD) spectra were measured on a Jasco J-720 spectropolarimeter using a 1-mm-slit-width cuvette. The hPer2(V4-VII) protein (8.3 μ M) was titrated against increasing concentrations of heme (molar protein/heme ratios of 1:1, 1:2, and 1:4) in 10 mM phosphate buffer (pH 7.6) and 150 mM NaCl. Five accumulated scans for each sample were recorded from 190 to 240 nm with an increment of 0.5 nm, a scan rate of 50 nm min⁻¹, a response time of 4 s, and a sensitivity of 50 millidegrees at room temperature. All CD spectra were corrected by subtraction of the background from the spectrum obtained with either buffer alone or buffer containing heme. Raw data were converted to mean residue ellipticity, θ , in degrees cm² dmol⁻¹. A similar procedure was followed for hPer2(II-III) and hPer2(V4-VII-SA⁸⁴¹PA). Data were analyzed for protein secondary structure using DICHROWEB (38) and deconvoluted using CDSSTR (34).

In vitro degradation assays. For protein degradation experiments, Chinese hamster ovary (CHO) cell extracts were prepared in lysis buffer (Promega) containing 25 mM Tris-HCl (pH 7.8), 2 mM EDTA, 2 mM dithiothreitol (DTT), 10% glycerol, and 1% Triton X-100. Alternatively, commercially available HeLa cell extracts (fraction S100 from Biomol) were also used in these experiments. For *in vitro* degradation assays, ³⁵S-labeled fragments of Cry1, Mdm2, hPer2, hPer2(S⁶⁶²A), and hPer2 proteins were incubated with cell extracts at 37°C supplemented with ubiquitin (0.1 mg/ml) and an energy-regenerating system. Heme was added to the mixture to a final concentration of 10, 25, 50, or 100 μ M. Reactions were stopped by the addition of Laemmli sample buffer, resolved by SDS-PAGE, and visualized by autoradiography. Densitometric quantitation was carried out using a FluoChem digital imaging system (Alpha Innotech).

Cell culture and analysis of endogenous Per2 protein. CHO cells were maintained in F-12K medium (Invitrogen) supplemented with 10% fetal bovine serum and gentamicin (50 μ g/ml). To detect endogenous levels of Per2, cells were cultured in serum-free medium containing 5 mM succinylacetone for 24 h prior to heme addition (10 μ M). Cells were harvested at the indicated times after treatment, and pellets were resuspended in lysis buffer (50 mM Tris-HCl [pH 7.5], 10 mM MgCl₂, 200 mM NaCl, 1% NP-40, 5% glycerol). For detection of hCry1 levels, the procedure was essentially the same as the one described above except that cells were first transfected with pCS2+myc-hCry1 using Lipofectamine (Invitrogen) and the protein was allowed to express for 12 h before the addition of succinylacetone. Endogenous Per2 and myc-hCry1 levels were detected by immunoblotting using specific antibodies (Santa Cruz).

Serum shock procedures and sample collection. Low-density CHO cells were plated 4 days before the experiment, transfected with 0.5 μ g of pCS2+myc-hPer2 or -hPer2(SA⁸⁴¹PA) using Lipofectamine and cultured for 12 h before synchronization (1). Briefly, at time zero, the medium was exchanged with 50% F-12K medium supplemented with 50% horse serum and gentamicin (50 μ g/ml). After 2 h of incubation, cells were washed twice with phosphate-buffered saline (PBS), and the medium was replaced with serum-free F-12K medium containing 5 mM succinylacetone. Heme (10 μ M) was added 24 h after serum shock, and the cells were maintained for 6 h before the medium was replaced with serum-free F-12K medium containing 5 mM succinylacetone. At the indicated times, cells were washed with PBS, frozen, and kept at -80°C until the extraction of whole-cell RNA. Reverse transcription-PCRs were performed using specific primers for *Rev-erba* and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase gene) (see supplemental material for details).

Cell transfection and immunofluorescence assays. CHO cells were cultured on coverslips for 24 h. Cells were then transfected with 0.5 μ g of pCS2+myc-hPer2 or -hPer2(SA⁸⁴¹PA) using Lipofectamine (Invitrogen) and cultured for an additional 12 h. The effects of heme on myc-hPer2 and -hPer2(SA⁸⁴¹PA) levels were determined using transfected cells treated with either 10 μ M heme or solvent for 2 h. After incubation, cells were maintained in serum-free medium for an additional 6 h and fixed in 3.7% formaldehyde-PBS-0.5% Triton X-100 at room temperature. Fixed cells were washed with PBS containing 0.5% Triton X-100 and then 0.1% Triton X-100 and blocked with goat serum at room temperature for 30 min. Subcellular localization of myc fusion proteins was detected using a Cy3-conjugated anti-myc antibody (Sigma). Nuclei were detected by incubating fixed cells with 4',6'-diamidino-2-phenylindole (DAPI) (Molecular Probes). Fluorescence was visualized using a DeltaVision Core microscope equipped with a CoolSnap HQ2 camera (Applied Precision) at 457 nm, 528 nm, and 617 nm. Signal intensities were measured using the profile plot analysis.

RESULTS

Heme regulates hPer2 stability. Like other cellular pathways, the circadian clock relies on mechanisms of synthesis and degradation of some of its components to sustain oscillations. Heme stimulates the expression of transcription factors that regulate circadian rhythms by modulating the activity of the Bmal1/NPAS2 complex, which transcriptionally controls the expression of the mammalian *period* genes and of the *alas1* gene (6, 15). Because there is little evidence regarding the mode by which heme acts on eukaryotic circadian transcription factors, we aimed to elucidate the molecular basis by which heme binding influences hPer2 function. First, we monitored the degradation of radiolabeled hPer2 in a cell-free system in response to hemin [Fe(III)-heme] treatment. ^{35}S -labeled hPer2 was incubated with a cell extract as the source for ubiquitination enzymes and proteasome in the presence of various concentrations of hemin. Results show hPer2, but not a non-specific control protein (Mdm2 [see Fig. S1 in the supplemental material]), is degraded shortly after the addition of hemin in a dose-dependent manner (Fig. 1A). Incubation with 1, 10, and 100 μM of ligand resulted in a rapid reduction (~ 20 , 60, and 90%, respectively) of hPer2 levels (Fig. 1A and data not shown). Importantly, this effect was inhibited when cell extracts were preincubated with the proteasome inhibitor MG-132, suggesting that heme-dependent degradation of hPer2 is mediated by the ubiquitin-proteasome pathway (Fig. 1A). Next, we investigated whether heme binding to the hPer2 PAS domain mediates hPer2 turnover. Interestingly, ^{35}S -labeled hPer2(II-III) (residues 173 to 574, comprises the PAS domain) remained stable in a cell-free assay even at high hemin concentrations (Fig. 1B), suggesting that regions other than PAS contain heme-regulated instability elements mediating hPer2 degradation.

Casein kinase I epsilon (CKI ϵ), a central component of the mammalian circadian clock, is the prime kinase involved in hPer2 downregulation by direct targeting of Ser⁶⁶² for phosphorylation (3, 7). To rule out any contribution of CKI ϵ to heme-mediated degradation of hPer2, we analyzed ^{35}S -labeled hPer2(S⁶⁶²A) in a cell-free system for its stability in the presence of hemin (Fig. 1C). hPer2 levels remained stable in the absence of hemin in cell extracts, ruling out the contribution of other phosphorylation events in hPer2 stability. Results indicate that hPer2(S⁶⁶²A) levels remain sensitive to hemin addition, supporting the existence of a novel mechanism for hPer2 degradation that is independent of CKI ϵ phosphorylation but dependent on the presence of heme.

Because hPer2 can be efficiently degraded *in vitro*, we were prompted to look for evidence of heme-mediated degradation *in vivo*. First, endogenous Per2 levels were monitored in CHO cells after hemin addition. Time course experiments showed reduced levels of Per2 protein but not its mRNA upon incubation with hemin (Fig. 1D). This result excludes the possibility of heme-mediated transcriptional effects on the *per2* gene and points toward heme-mediated control of protein stability, since untreated cells showed steady levels of Per2 (Fig. 1D). To further explore the dependence of heme on Per2 stability, CHO cells were pretreated with succinylacetone, an inhibitor of δ -aminolevulinic acid dehydratase, to prevent *de novo* synthesis of endogenous heme (Fig. 1E). Consistent with our *in*

vitro data, downregulation of Per2 in CHO cells was inhibited by succinylacetone but induced by further addition of exogenous hemin, indicating that heme synthesis is essential for Per2 degradation.

Heme binds within the C-terminal domain of hPer2. To identify the region on hPer2 involved in heme targeting, purified GST-hPer2 fragments [GST-hPer2(I), GST-hPer2(II), GST-hPer2(III), GST-hPer2(II-III), and GST-hPer2(V4-VII) (Fig. 2A)] were analyzed for heme-binding activity using hemin-agarose affinity chromatography (Fig. 2B). Direct interactions between hemin and PAS domain-containing fragments GST-hPer2(II), GST-hPer2(III), and GST-hPer2(II-III) were detected, confirming both the role of the PAS domain in heme binding and the reliability of the method to define heme-interacting domains (Fig. 2B). Based on this result, it seems two regions within the PAS might be involved in heme binding. This can be addressed based on the functional homology among the PAS domains of the circadian NPAS2 and Per2 proteins. The PAS domain in NPAS2 typically encompasses ~ 150 amino acids and contains two highly degenerate 50-residue subdomains termed A and B repeats, each of which binds one molecule of heme (for a review, see reference 10). Our results show that hemin is able to bind the truncated forms of PAS domain comprising either subdomain (Fig. 2 and see Fig. 4) (see Fig. S2 in the supplemental material) with equimolar stoichiometry suggesting that, like NPAS2, two independent regions within the hPer2-PAS domain are capable of hemin binding. Interestingly, while the N-terminal fragment of hPer2 comprising residues 1 to 172 [GST-hPer2(I)] did not exhibit any association with hemin-agarose beads (Fig. 2B, right panel), a distinct segment of the protein located within the C-terminal region, GST-hPer2(V4-VII), exhibited strong association with hemin, suggesting that a heme-binding motif is located within this region.

A novel heme-regulatory motif mediates hPer2-heme interaction. Heme-protein interaction is alternatively mediated by evolutionary conserved heme-regulatory motifs where Cys-Pro residues are invariant and where there is a tendency for a hydrophobic amino acid to be in the fourth position. Inspection of the hPer2 sequence determined the presence of two putative HRMs (Fig. 3A). Interestingly, both HRMs were located within hPer2(V4-VII), a fragment that exhibits heme-binding capacity (Fig. 2). Comparative analysis of global multiple Per2 sequence alignments exhibits conserved residues clustered in the HRMs and surrounded by sequence elements of high (for SC⁸⁴¹PA) and low (for AC⁹⁶²PA) conservation (Fig. 3A). Phylogenetic analyses indicate that both putative HRMs are highly conserved modules in Per2 proteins among metazoan lineages, especially in mammals, suggesting that the sequences under investigation have a comparatively young most recent common ancestor (Fig. 3A).

Unlike other heme-binding sites, HRMs establish bonding between the cysteine sulfur and the iron atom of heme (45). Accordingly, we tested whether any of the putative HRMs identified in hPer2(V4-VII) were able to directly bind heme. Untagged hPer2(V4-VII) and its SA⁸⁴¹PA and AA⁹⁶²PA mutant forms were analyzed after hemin addition by absorption spectroscopy (Fig. 3B). The hPer2(V4-VII) protein fragment shifted the peak of the strongest heme absorption band (388 nm), the Soret band, toward a shorter wavelength by ~ 19 nm

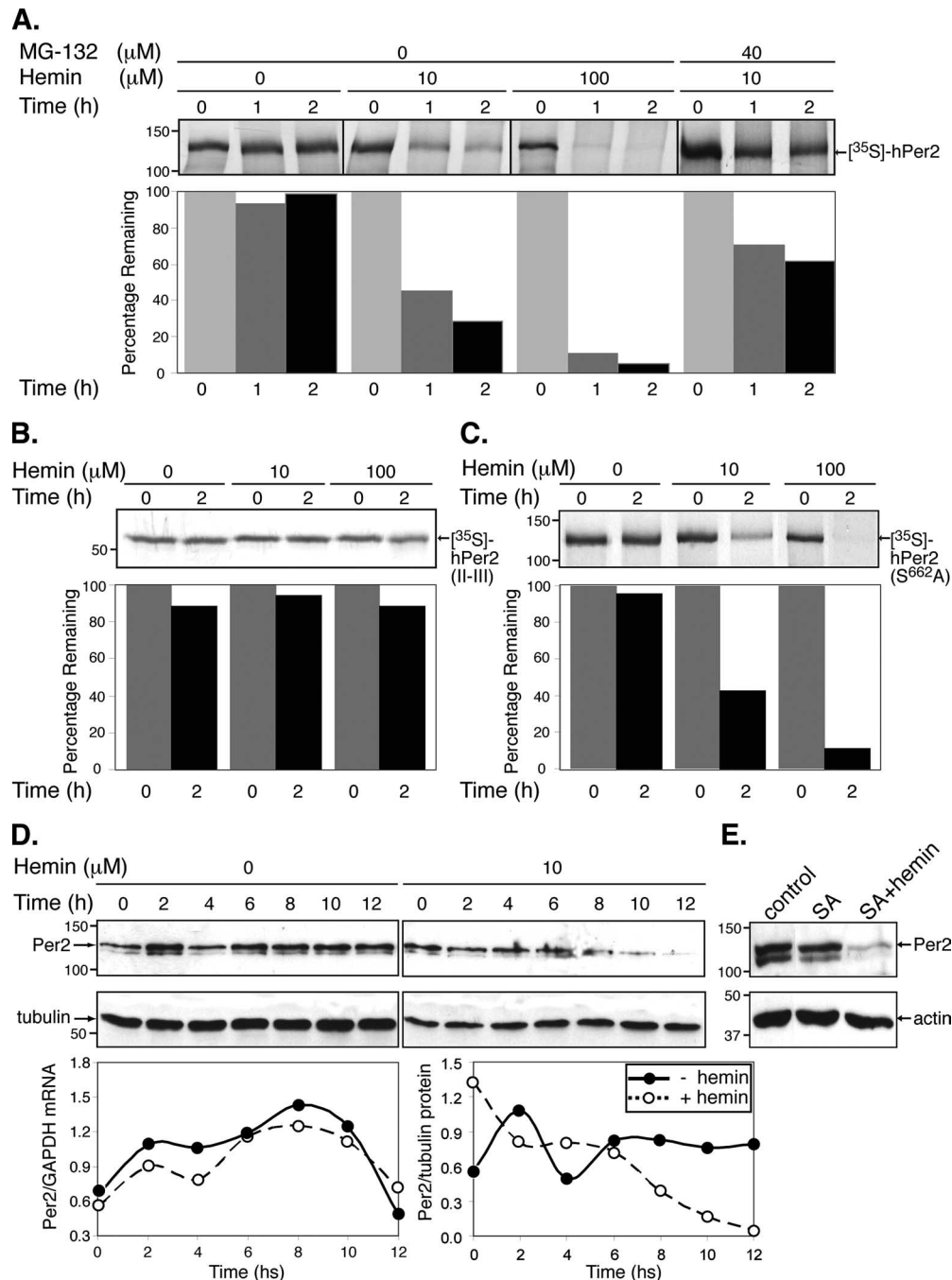


FIG. 1. Heme modulates hPer2 stability in vitro and in vivo. (A, top) 35 S-labeled hPer2 (35 S-hPer2) [35 S-hPer2(II-III) in panel B and 35 S-hPer2(S⁶⁶²A) in panel C] was added to CHO cell extracts in the absence or presence of hemin (10 μ M and 100 μ M) and incubated at 37°C. Aliquots were removed at 0, 1, and 2 h and resolved by SDS-PAGE and autoradiography. In other experiments, CHO extracts were preincubated with MG-132 before the addition of 35 S-hPer2 and hemin (10 μ M). Bands were quantified using an AlphaImager and normalized to the input amount (at time zero in bottom panels). The figure shows data from a single experiment that was repeated three times with similar results. The arrows on the right denote radiolabeled protein. The positions of molecular mass markers (in kilodaltons) are indicated to the left of the gels. (D) CHO cells were incubated with hemin (10 μ M) for 2, 4, 6, 8, 10, or 12 h in serum-free medium. Samples were collected at the indicated times, and endogenous levels of hPer2 were analyzed by immunoblotting (top panel). Bands were quantified using an AlphaImager and normalized to tubulin levels (bottom right panel). Total RNA was isolated from cells harvested at each time point (in hours) and converted to cDNA in reactions that contain equivalent amounts of total RNA. Gene-specific primers (Per2 and GAPDH) were used for PCR amplification. GAPDH was used as internal control (bottom left panel). (E) CHO cells were treated with succinylacetone (SA) to deplete cells of endogenous heme. After removal of the medium, cells were incubated with either serum-free medium (control), SA, or SA plus hemin (10 μ M). Extracts were subjected to SDS-PAGE and immunoblotting. Total protein levels were monitored by either tubulin or actin expression (bottom panels).

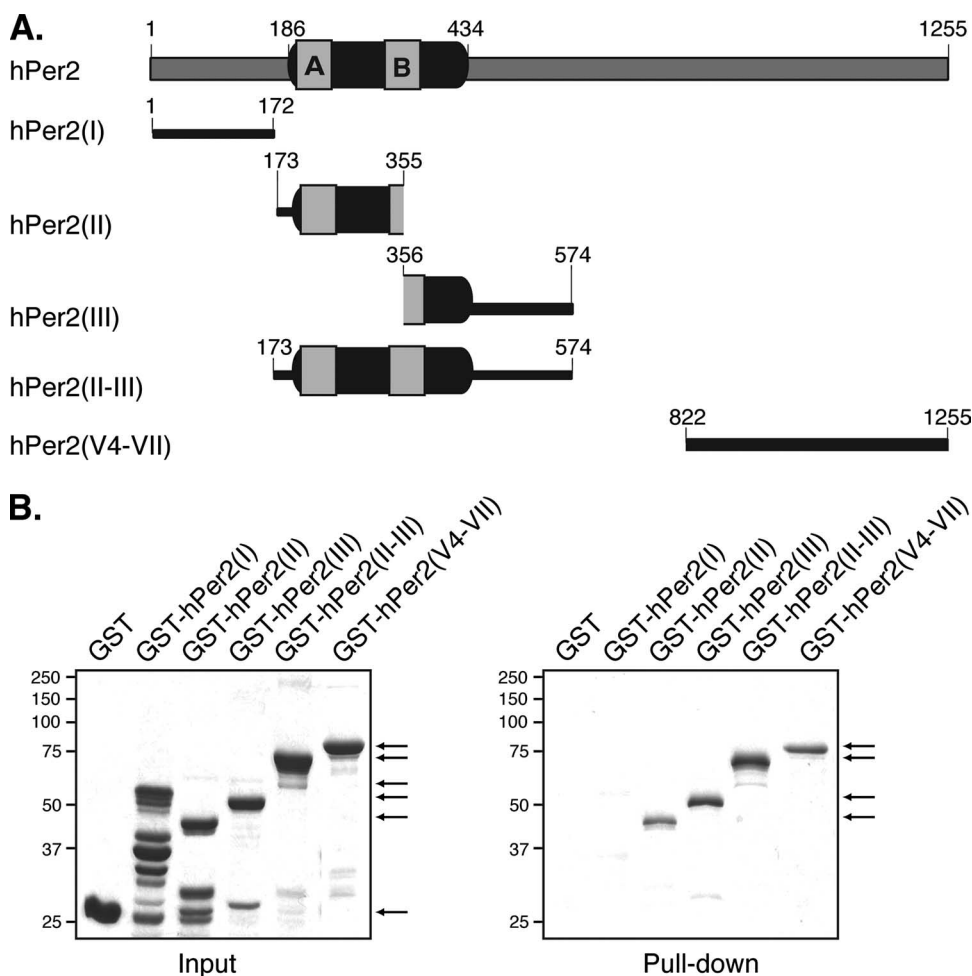


FIG. 2. Heme binds hPer2 in two distinct regions. (A) Schematic representation of hPer2 (1,255 residues) architecture including the PAS domain (residues 186 to 434) and the PAS-A (residues 186 to 235) and PAS-B (residues 322 to 374) subdomains. Fragments hPer2(I) (residues 1 to 172), hPer2(II) (residues 173 to 355), hPer2(III) (residues 356 to 574), hPer2(II-III) (residues 173 to 574), and hPer2(V4-VII) (residues 822 to 1,255) are represented below. (B) GST-tagged hPer2 fragments were purified using affinity chromatography (left panel). Samples were analyzed for heme binding using heme-agarose (right panel).

(369 nm [Fig. 3B]), consistent with heme binding to HRMs (45). The SA⁸⁴¹PA mutant form of hPer2(V4-VII), but not the AA⁹⁶²PA mutant form, abolished the protein's ability to shift the heme absorption spectrum to shorter wavelengths and confirmed the essential role of Cys⁸⁴¹ in heme binding (Fig. 3B). A second slight shift in the Soret peak (421 nm) was detected in the wild-type fragment (Fig. 3B). Whereas an additional residual shoulder was observed at a shorter wavelength for the SA⁸⁴¹PA protein, we believe this shoulder results from excess amounts of free heme in the sample. Our studies indicate that neither Cys⁸⁴¹ nor Cys⁹⁶² is responsible for the peak observed at 421 nm, suggesting the existence of a secondary component involved in heme binding that we later mapped between residues 1,121 and 1,255 of hPer2 (data not shown). Overall, our results pinpoint SC⁸⁴¹PA as a novel heme-binding motif located at the C terminus of hPer2. As a direct test of the role of the SC⁸⁴¹PA motif in heme binding, we examined whether a shorter fragment of hPer2 [GST-hPer2(V4), residues 822 to 872] and its HRM mutant form [GST-hPer2(V4-SA⁸⁴¹PA)] were able to bind heme by affinity

chromatography (Fig. 3C). As expected, GST-hPer2(V4) displayed a strong interaction for heme, and the mutation on Cys⁸⁴¹ completely abrogated binding. Collectively, these data indicate that heme binds to hPer2 directly through Cys⁸⁴¹.

Binding of heme to both HRM and PAS follows a precise stoichiometry. Further evidence of direct binding of heme to hPer2(V4) and the PAS domain-containing fragment hPer2(III) was obtained by absorption spectra and titration experiments. Among hPer2 PAS-containing fragments, hPer2(III) was chosen because of its signal intensity. Heme binding to hPer2(V4) shifted the Soret band from 388 to 370 nm, an event that was prevented by the mutation of Cys⁸⁴¹ to Ala, confirming Cys⁸⁴¹ as the axial heme ligand (Fig. 4A, left panel). Since hPer2(V4) does not have any appreciable absorption between 300 and 700 nm, the observed spectral changes on free heme are due to alterations in the electronic structure and coordination state of the heme iron caused by its interaction with hPer2(V4). To examine the specificity of heme binding, heme was titrated with increasing amounts of GST-hPer2(V4). The absorption peak

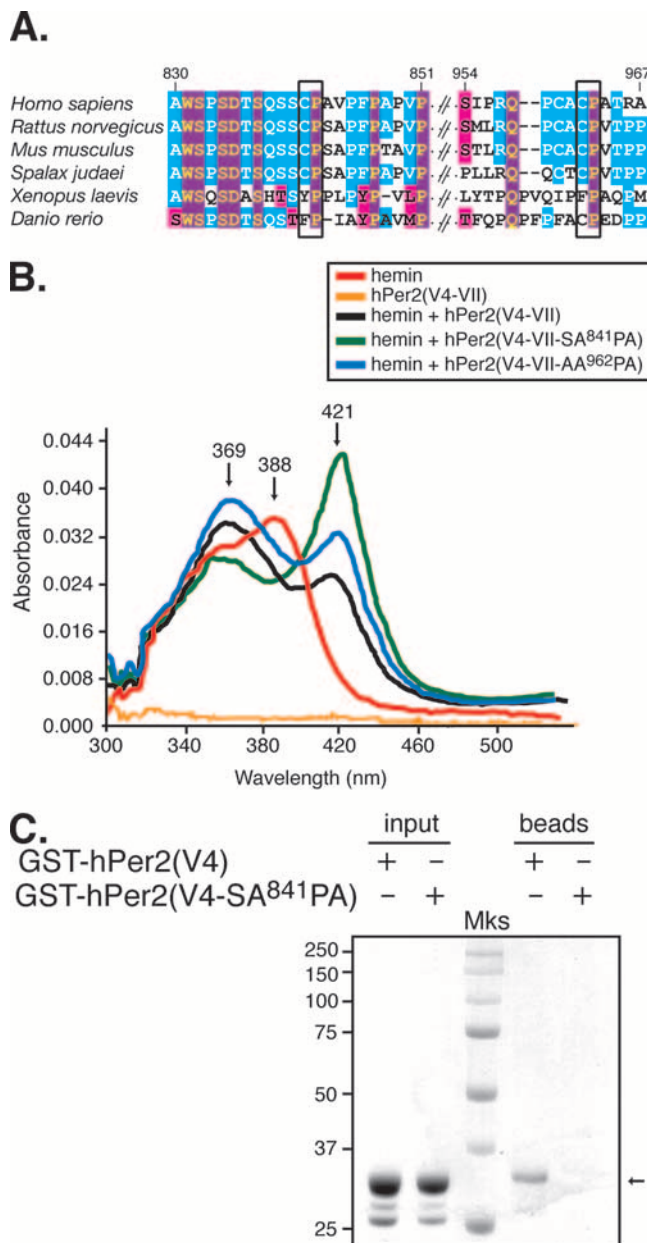


FIG. 3. A novel heme-regulatory motif is present in hPer2. (A) Sequence alignment of the surrounding residues of the putative HRMs (SC⁸⁴¹PA and AC⁹⁶²PA) in hPer2 and its homologs in other species. Multiple alignment was performed with the program BenchWork and refined manually. The numbers at the beginning and end of the top sequence correspond to the positions of the first and the last amino acid residues in hPer2. Conserved residues (blue), similar residues (pink), identical residues (purple), and nonconserved residues (black) are indicated. The CP cores in both HRMs of hPer2 are boxed. (B) Absorption spectra of heme (1 μ M) in the presence of various constructs of hPer2(V4-VII) were recorded between 300 and 700 nm on a Beckman DU640 spectrophotometer. (C) GST-hPer2(V4) (residues 822 to 872) and -hPer2(V4-SA⁸⁴¹PA) were purified and analyzed for heme binding using agarose-hemin beads (+). The arrow on the right denotes the recombinant protein. The positions of molecular mass markers (Mks) (in kilodaltons) are indicated to the left of the gel.

of free heme was blue shifted to 370 nm after the addition of the smallest amount of protein, whereas the amplitude of the peak increased accordingly with protein concentration (Fig. 4B, left panel). Titration curves show a well-defined inflection point corresponding to a molar stoichiometry of heme and GST-hPer2(V4) of 1:1 (Fig. 4C). Specific binding of heme to hPer2(V4-SA⁸⁴¹PA) was not detected by absorption experiments (Fig. 4A, left panel). Titration analysis showed initial diminution of the absorption peak of free heme (388 nm) after hPer2(V4-SA⁸⁴¹PA) addition followed by a continuous shift around the heme peak wavelength, likely caused by nonspecific binding by excess protein (Fig. 4B, middle panel, and C).

Evidence shows that the PAS domain in mouse *Per2* (mPer2) mediates heme binding (15), but there is no spectroscopic data illustrating binding details in this domain. We determined that hPer2 PAS domain heme binding is mediated by either methionine/histidine or bis-histidine coordination, since the free heme absorption spectra (388 nm) shifts to a Soret peak at 412 nm (Fig. 4A, right panel) (see Fig. S2 in the supplemental material). Titration experiments also defined the stoichiometry of the interaction and established that heme binds to PAS-A and -B subdomains, forming an equimolar complex in each case (Fig. 4B and C) (see Fig. S2 in the supplemental material). Altogether, these results demonstrate that (i) Fe(III)-heme binds to hPer2 at two distinct sites (SC⁸⁴¹PA motif and PAS domain), (ii) binding is mediated by different coordination in HRM and PAS, and (iii) both subdomains in PAS are able to bind heme.

Degradation of hPer2 depends exclusively on binding of oxidized heme to HRM. Because heme interacts strongly with both PAS and HRM (Fig. 4), we next examined whether either site was able to bind the reduced form of heme. To study this possibility, heme was reduced by the addition of sodium dithionite and incubated with GST-hPer2(III), GST-hPer2(V4), or GST-hPer2(V4-SA⁸⁴¹PA), and their interactions were monitored by absorption spectra (Fig. 5A). As with ferric heme, ferrous heme has distinct absorption characteristics that shift upon protein binding. Accordingly, a Soret peak at 421 nm was observed exclusively in the presence of GST-hPer2(III) (Fig. 5A) and GST-hPer2(II-III Δ PAS-B) (see Fig. S2C in the supplemental material), suggesting that only this domain is able to bind both forms of heme. Neither GST-hPer2(V4) nor GST-hPer2(V4-SA⁸⁴¹PA) exhibits any apparent peak in the spectra when incubated in the presence of a reducing agent, indicating that ferrous heme is not a suitable ligand for HRM. Therefore, we conclude that both forms of heme are able to bind PAS but that only oxidized heme binds to HRM, which suggests that this interaction takes place exclusively under specific redox conditions.

We next asked whether degradation of hPer2 depends on the redox state of the bound heme iron. To address this question, we used a cell-free system and evaluated hPer2 turnover in the presence of oxidized and reduced forms of heme (Fig. 5B). hPer2 stability was initially monitored in CHO cell extracts in the absence or presence of DTT (control). As expected, hPer2 remained stable in either condition, suggesting that factors other than heme are not required for hPer2 degradation (Fig. 5B, top panels). In agreement with Fig. 1, the sole addition of heme (25 or 50 μ M) showed an increased rate

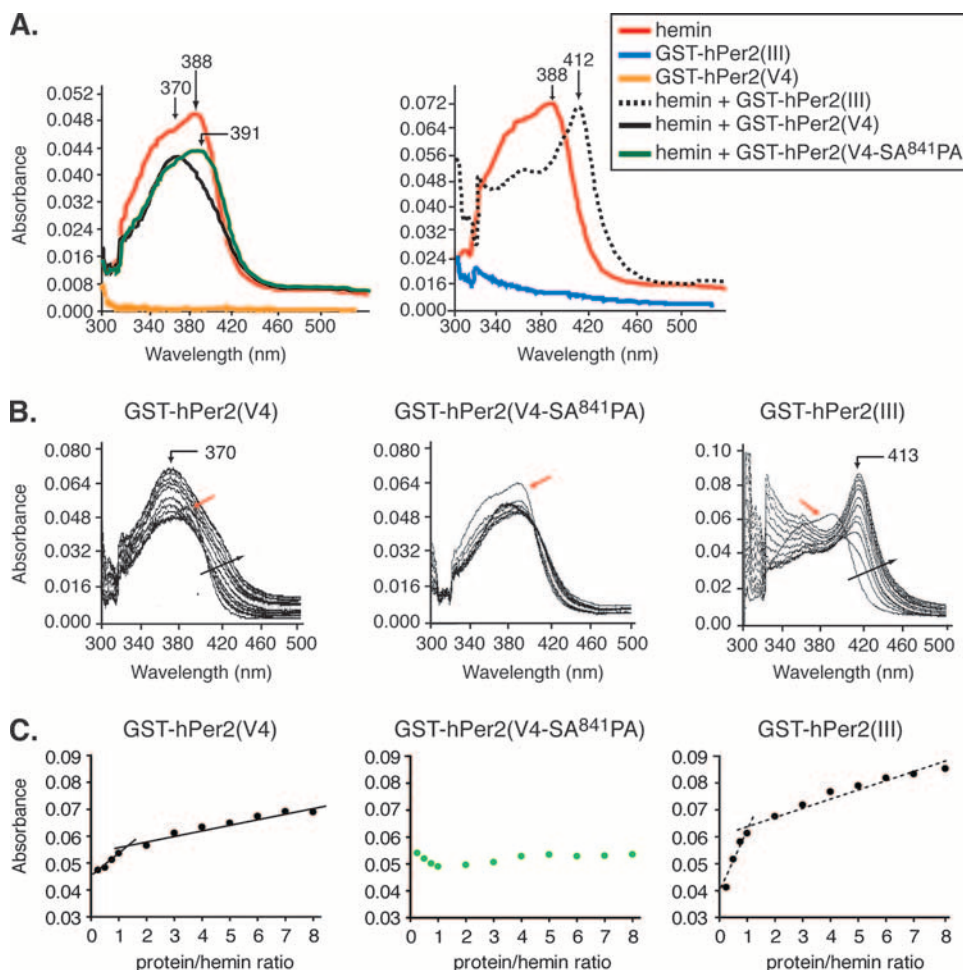


FIG. 4. Heme binds to the PAS domain and the HRM motif. (A) Absorption spectra of ferric heme (hemin) for GST-hPer2(V4), -hPer2(V4-SA⁸⁴¹PA), and -hPer2(III). (B) Absorption spectra of heme after the addition of increasing concentration of GST-hPer2(V4), GST-hPer2(V4-SA⁸⁴¹PA), and GST-hPer2(III) up to 8 mol equivalent of the heme amount (black arrow). Free heme spectrum is indicated with a red arrow. (C) Titration curves of heme with increasing amounts of the indicated protein are represented as absorbance at 370 nm [GST-hPer2(V4)], 382 nm [GST-hPer2(V4-SA⁸⁴¹PA)], and 413 nm [GST-hPer2(III)] as a function of the molar ratios of the protein to heme.

of hPer2 degradation compared to controls (Fig. 5B, left panels). Interestingly, the stability of hPer2 was restored when heme was preincubated with DTT before its addition to the extract, suggesting that the redox state of the heme iron is a determinant of hPer2 stability (Fig. 5B, right panels, and C). To rule out the possibility that DTT reduced proteolysis of hPer2 by a nonspecific toxic effect in the extract, we measured the degradation of a nonrelated protein (a cyclin-dependent inhibitor p27Xic1T²⁰⁴D) in the presence or absence of DTT (Fig. 5D). As a test of the role of HRM in heme-mediated degradation of hPer2, we examined whether the levels of hPer2(II-III) were altered in either redox condition (Fig. 5E and F). Labeled hPer2(II-III) showed steady levels in a cell-free assay throughout the time course analyzed independently of the redox state of the heme iron (Fig. 5E and F). Thus, a heme-binding site other than PAS must be responsible for hPer2 degradation, supporting the role of HRM in mediating hPer2 stability.

It is established that reactive oxygen species encompass a variety of diverse chemical species, including superoxide an-

ions, hydroxyl radicals, and hydrogen peroxide. These various radical species can either be generated exogenously from several different sources (i.e., radiation, hyperthermia, and growth factors) or produced intracellularly as a consequence of metabolic activities, thus perturbing the normal redox balance and shifting cells into a state of oxidative stress. Therefore, we explored the consequences of inducing oxidative stress by diverse sources from different origins on hPer2 stability. Our data show that neither the addition of hydrogen peroxide, high metal concentration, and lipopolysaccharides, nor treatment with ionizing radiation and heat shock resulted in altered levels of hPer2, suggesting that hPer2 degradation is not a general response to oxidative stress conditions (see Fig. S5 in the supplemental material). Moreover, our data point directly toward a heme-mediated response, since the addition of Fe(III), per se, did not cause hPer2 degradation in vitro, but Fe(III)-heme (hemin) addition does (see Fig. S5 in the supplemental material), suggesting that iron must be converted to heme, before it can trigger hPer2 degradation.

It has long been recognized that the conformational stability

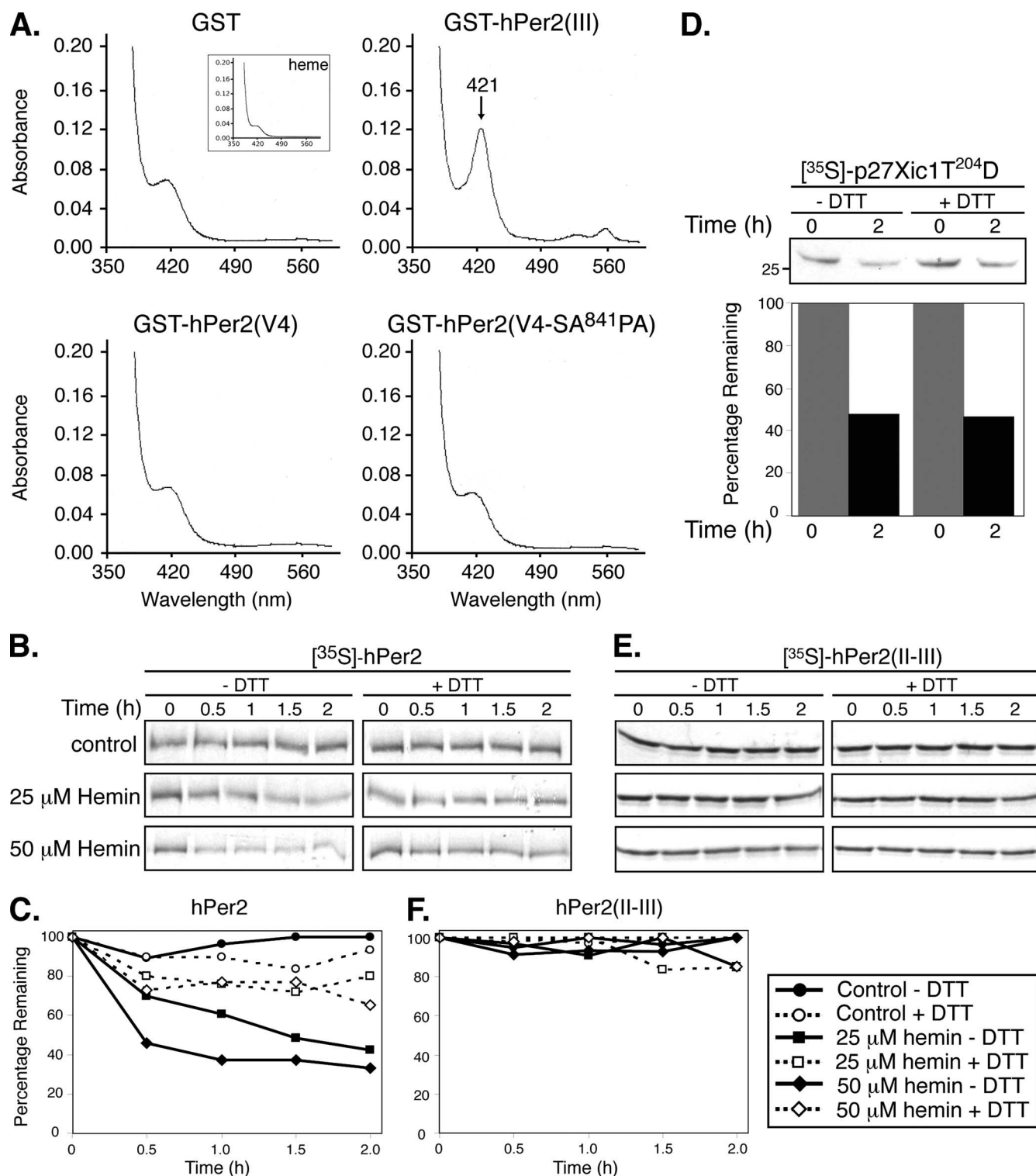


FIG. 5. Degradation of hPer2 depends exclusively on the redox state of the heme bound to HRM. (A) Absorption spectra of ferrous heme (inset) in the presence of GST, GST-hPer2(III), GST-hPer2(V4), and GST-hPer2(V4-SA⁸⁴¹PA). (B) CHO cell extracts were incubated with ³⁵S-labeled hPer2 ([³⁵S]-hPer2) in the absence (control) or presence of hemin (25 or 50 μ M). In a duplicate set of reactions, hemin was preincubated with DTT (+) before adding radiolabeled protein. Aliquots were taken at different times and analyzed by SDS-PAGE and autoradiography. (C) Bands in panel B were quantified using an AlphaImager system, and values were normalized to the input protein (time zero). The figure shows data from a single experiment that was repeated three times with similar results. (D) ³⁵S-labeled p27Xic1T²⁰⁴D ([³⁵S]-p27Xic1T²⁰⁴D) was used as control of extract quality upon DTT addition. Samples were analyzed and later quantified as described above for panels B and C, respectively. (E) Cell-free assays of ³⁵S-labeled hPer2(II-III) ([³⁵S]-hPer2(II-III)) in the absence or presence of hemin with (+) or without (–) DTT addition were performed essentially as described above for panel B. (F) Bands in panel E were quantified as indicated above.

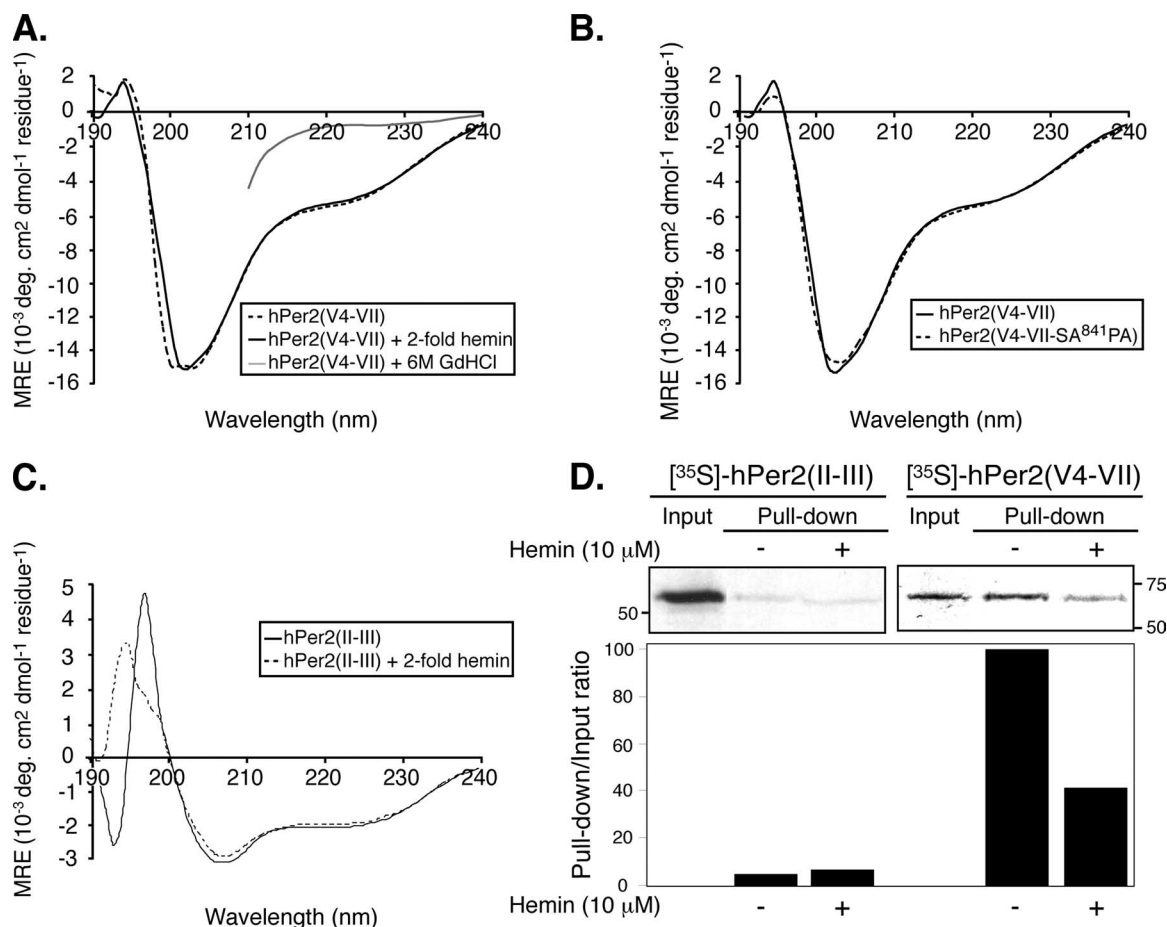


FIG. 6. CD analysis of the heme-binding regions in hPer2. (A) Far-UV CD spectra of untagged hPer2(V4-VII) were collected in the absence (dashed line) or presence (solid line) of heme (twofold) are shown for simplicity and are similar to the spectra for one- and fourfold excess heme. Protein folding was confirmed by preincubating the protein with 6 M guanidinium chloride (GdHCl) prior to data recording (gray line). Mean residue ellipticity (MRE) (in degrees $\text{cm}^2 \text{dmol}^{-1} \text{residue}^{-1}$) is shown on the y axis (B) Comparison of the CD spectra of hPer2(V4-VII) (solid line) and hPer2(V4-VII-SA⁸⁴¹PA) (dashed line) recorded under the same experimental conditions for panel A. (C) CD spectra of free hPer2(II-III) (solid line) and after twofold excess of heme (dashed line). (D) GST-hCry1-bound beads were incubated with either ³⁵S-labeled hPer2(II-III) [³⁵S]-hPer2(II-III) or ³⁵S-labeled hPer2(V4-VII) in the absence (–) or presence (+) of heme (10 μM). Complexes were monitored by SDS-PAGE and autoradiography. Bands were quantified using an AlphaImager. Results similar to those presented here were observed in two independent experiments. The positions of molecular mass markers (in kilodaltons) are shown to the left or right of the gel.

of a protein and its proteolytic susceptibility are linked. The reason for this linkage lies in the assumption that certain protein conformational states are better substrates for proteases, with highly ordered conformations being relatively poor substrates owing to the lack of conformational freedom of the polypeptide chain (24). Therefore, we explored whether binding of heme influences the conformation of the protein using CD spectroscopy. Experiments were carried out in the presence of increasing concentrations of heme and the apo forms of hPer2(II-III), hPer2(V4-VII), and hPer2(V4-VII-SA⁸⁴¹PA). Prediction of the secondary structure of hPer2(V4-VII) revealed the presence of a 24% α -helical, 18% β -strand, and 19% β -turn content (Fig. 6A). The hPer2(V4-VII) secondary structure elements were disrupted after treatment of the protein with guanidinium hydrochloride (Fig. 6A). The hPer2(V4-VII-SA⁸⁴¹PA) fragment showed essentially the same overall fold as the wild-type construct, suggesting that the Cys⁸⁴¹ mutation does not alter the overall structure of the C-terminal

domain (Fig. 6B), supporting a direct role for HRM in heme binding. The N terminus of hPer2(II-III) revealed a 5% α -helical, 29% β -strand, and 21% β -turn content, in agreement with the structural data from other PAS domain-containing proteins (Fig. 6C) (5). Neither hPer2(V4-VII) nor hPer2(II-III) showed a significant rearrangement of their secondary structure even at twofold excess of ligand (Fig. 6A and C), suggesting that hPer2 degradation might result from its inability to form a stable heterodimer with hCry1 rather than a ligand-induced unfolding state. To test this possibility, pull-down experiments were performed using GST-hCry1-bound beads in the presence or absence of heme and radiolabeled hPer2 fragments (Fig. 6D). In agreement with its role in hCry1 binding, the C-terminal fragment of hPer2 showed a reduced interaction to GST-hCry1 in the presence of heme, suggesting that competition prevents this association. The labeled PAS domain-containing protein was unable to interact with hCry1 independently of heme addition and does restrict the heme

effect on hPer2/hCry1 formation to the C-terminal portion of hPer2.

HRM is required for degradation of hPer2 in vivo. To gain further insight into the role of HRM in the degradation of Per2 in vivo, we transiently transfected CHO cells with either *myc*-hPer2 or *myc*-hPer2-SA⁸⁴¹PA and evaluated their subcellular localization and intracellular levels in response to hemin addition by immunofluorescence microscopy (Fig. 7A). In agreement with previous observations (40), immunofluorescence staining showed that *myc*-hPer2 was distributed in both nuclear and cytosolic compartments and that its accumulation was remarkably higher in the former (Fig. 7A). The cellular distribution of *myc*-hPer2-SA⁸⁴¹PA mutant was similar to that of the wild-type protein, and thus, we conclude the Cys⁸⁴¹Ala mutation does not alter hPer2 localization (Fig. 7A). Hemin addition to *myc*-hPer2-transfected cells resulted in decreased levels of the nuclear protein without increasing hPer2 levels in the cytosolic compartment, suggesting that degradation, rather than translocation, was triggered by hemin (Fig. 7A and B). Supporting the role of HRM in heme-mediated hPer2 degradation in vivo, the addition of hemin to *myc*-hPer2-SA⁸⁴¹PA-transfected cells did not result in apparent changes in mutant protein levels (Fig. 7A). Profile plotting of signal intensity along cross sections of cells transfected with wild-type and hPer2 mutants confirmed hemin-induced nuclear degradation of hPer2 and unambiguously confirmed that this phenomenon is mediated by HRM (Fig. 7B).

To further support the concept that HRM is sufficient to promote heme-mediated hPer2 degradation and that heme binding to PAS domain plays a distinct role (15), we transfected cells with *myc*-hPer2, *myc*-hPer2-SA⁸⁴¹PA, or *myc*-hPer2(II-III) and evaluated their total protein levels in response to hemin addition (Fig. 7C). The remarkable stability of *myc*-hPer2(II-III) observed in the presence of hemin contrasted greatly with the levels of *myc*-hPer2 detected under the same condition, suggesting that binding of heme to the PAS domain does not alter its stability in vivo (Fig. 7C). The presence of equivalent levels of *myc*-hPer2-SA⁸⁴¹PA in the absence or presence of hemin further supports our model.

Binding of heme to HRM prevents the formation of the hPer2/hCry1 complex. The C terminus of Per2 physically associates with Cry proteins (9, 19), and the complex translocates to the nucleus where it acts as a negative regulator by directly interacting with Clock/Bmal1 (31). Thus, we first asked whether heme treatment of cells alters the intracellular levels of the hPer2/hCry1 complex (Fig. 8). Immunoprecipitation assays of hemin-treated *myc*-hPer2/FLAG-hCry1 cells were analyzed for the presence of heterodimers by immunoblotting (Fig. 8A). Results show reduced levels of bound hCry1 in hemin-treated samples, indicating that heme alters hPer2/hCry1 levels in cells (Fig. 8A). To rule out the possibility that heme can cause hCry1 degradation and disrupt hPer2/hCry1 interaction, transfected CHO cells were incubated with hemin, and hCry1 levels were monitored at different times. As shown in Fig. S3A in the supplemental material, hCry1 levels remained invariable throughout the time course analyzed, suggesting that hCry1 stability is independent of the presence of heme. A similar result was obtained when hCry1 stability was tested in the presence of hemin in a cell-free assay (see Fig. S3B in the supplemental material).

We then examined whether heme binding to hPer2 prevents

the formation of hPer2/hCry1 or disrupts an already preformed complex instead. To evaluate either model, we first recapitulated the cellular events leading to heme-dependent reduction of hPer2/hCry1 levels in vitro (Fig. 8B). Recombinant GST-hCry1, ³⁵S-labeled hPer2, and hemin were simultaneously incubated, and the amount of ³⁵S-labeled hPer2 present in the complex was analyzed by pull-down experiments (Fig. 8B and 6D). As was the case with transfected cell extracts, our in vitro assay showed lower levels of ³⁵S-labeled hPer2 associated with GST-hCry1 in the presence of hemin, supporting a model where ligand binding compromises hPer2/hCry1 complex formation. Because of the nature of our in vitro assay, only two proteins and hemin were present, which also suggests that heme binding to the C terminus of hPer2 prevents or disrupts its association with hCry1 and that heme-mediated degradation of hPer2 might be a consequence of lack of association.

Next, we established which event of the complex formation is inhibited by heme binding. In the first scenario, hPer2/GST-hCry1 complex was allowed to form and later incubated with hemin (Fig. 8C). In a parallel experiment, hPer2 was preincubated with hemin, added to GST-hCry1, and analyzed by pull-down experiments (Fig. 8C). Results demonstrate that more hPer2 is bound to GST-hCry1 when the complex is preformed, suggesting that hemin is unable to disrupt a stable heterodimer.

Expression of a non-heme-responsive HRM form of hPer2 alters the pattern of circadian gene expression. The observation that circadian gene expression can persist for several days in serum-free medium after an initial serum shock (1, 2) prompted us to test the effects of hPer2 and hPer2(SA⁸⁴¹PA) mutant on the mRNA accumulation profile of circadian genes. We investigated one of the known downstream effectors of Per2 signaling, *Rev-erba*, a transcript that is lowest at times when Per2 expression peaks in the nucleus (26). Circadian *Rev-erba* expression is controlled by components of the general feedback loop, thus influencing the period length and phase-shifting properties of the clock (26). In agreement with our model, cells transfected with hPer2 exhibited reduced levels of *Rev-erba* compared with nontransfected cells (Fig. 9A, right panel), an effect that is reversed when cells were pretreated with hemin (Fig. 9A, bottom left panel). Moreover, transfection with hPer2(SA⁸⁴¹PA) resulted in sustained downregulation of *Rev-erba* transcription throughout the analyzed time course (Fig. 9A, bottom right panel). As predicted, the addition of hemin to hPer2(SA⁸⁴¹PA)-transfected cells did not result in altered levels of *Rev-erba*, since the ligand can no longer bind the mutant protein and is therefore unable to act on its stability.

Overall, our observations favor a scenario where heme plays an essential role in controlling hPer2 cellular levels by targeting hPer2 for degradation and preventing hPer2/hCry1 complex accumulation (Fig. 9B). Our tryptophan fluorescence spectroscopy data show that both PAS and HRM bind heme with roughly equal affinity in the nanomolar range [K_d hPer2(V4-VII) [dissociation constant], 9.20 ± 0.94 nM; K_d hPer2(II-III), 12.31 ± 0.43 nM; see Fig. S4 in the supplemental material}. Interestingly, whereas ferrous heme will bind only to the PAS domain (Fig. 5A), its ferric form could, in principle, target either binding site. At this point, we hypothesize that binding of ferric heme to either PAS or HRM might depend on their availability. For

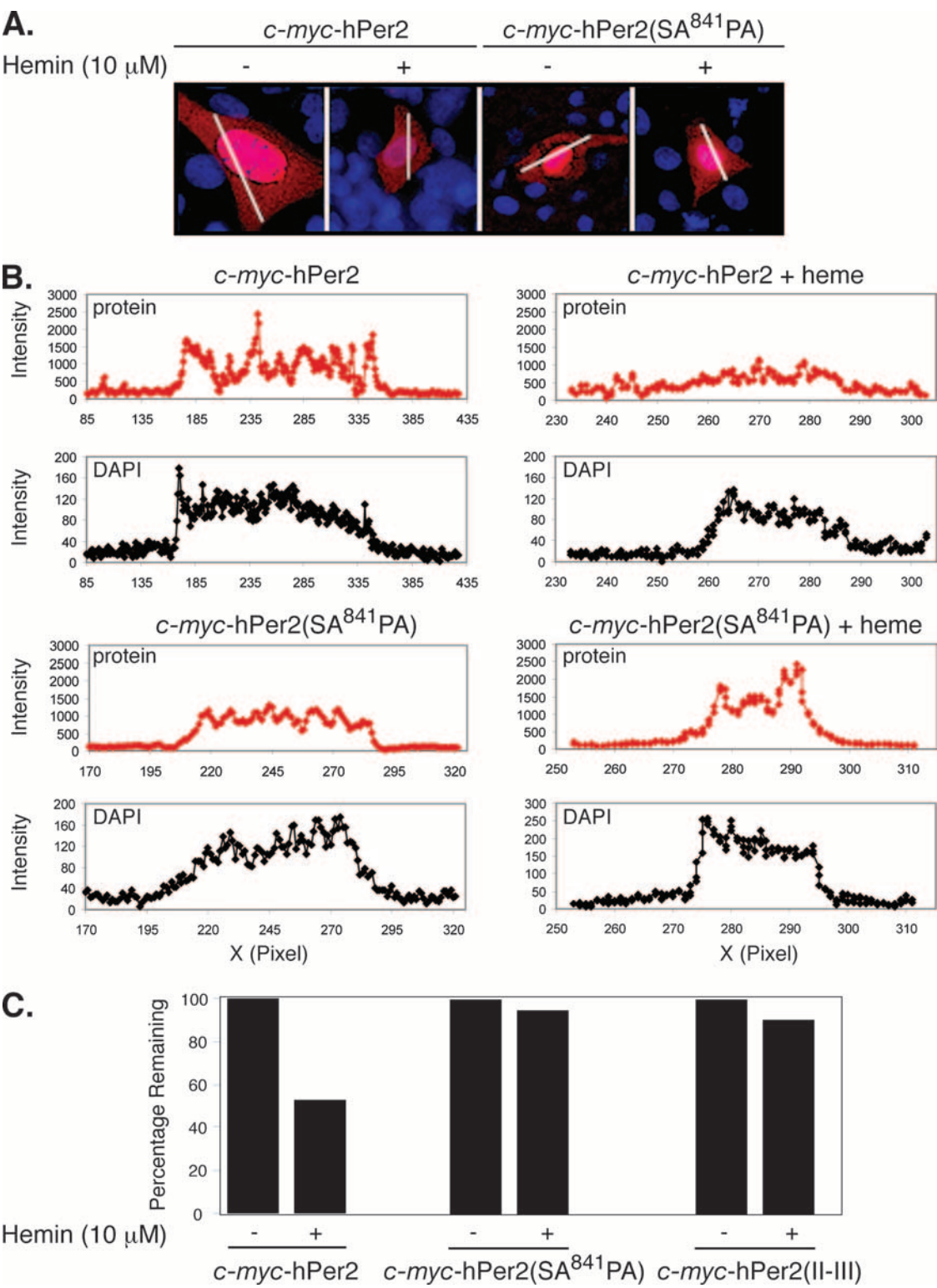


FIG. 7. The HRM modulates hPer2 stability in CHO cells. (A) CHO cells were transfected with either *myc*-tagged hPer2 or -hPer2(SA⁸⁴¹PA) plasmids and incubated with hemin (+). Expressed proteins and DNA were detected using a Cy3-conjugated anti-*myc* antibody and DAPI, respectively. (B) Profile plots of signal intensity across the cell. Recombinant protein and DNA levels were scored along the white lines shown in panel A and represented as intensity values (red for protein; black for DNA). (C) CHO cells were transfected with either *myc*-hPer2, -hPer2(SA⁸⁴¹PA), or -hPer2(II-III) and treated with (+) or without (–) hemin. Cell extracts were analyzed for the presence of recombinant proteins by immunoblotting using an anti-*myc* antibody. Bands were quantified using an AlphaImager system, and values were normalized to β -galactosidase activity. Results similar to those presented here were observed in three independent experiments.

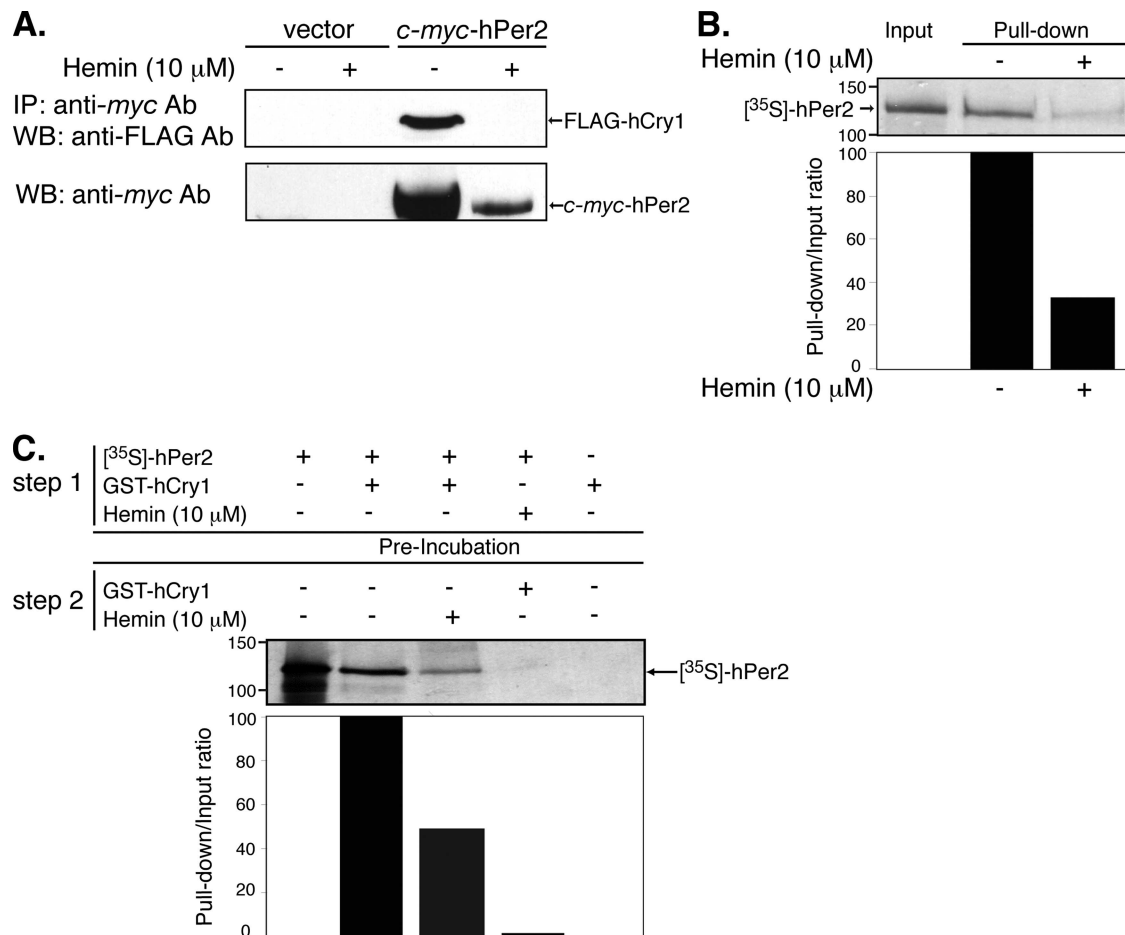


FIG. 8. Binding of heme prevents hPer2/hCry1 heterodimerization. (A) CHO cells were cotransfected with either *myc-hPer2* or pCS2+ (*myc*)₆ empty vector and FLAG-hCry1 and treated with hemin (10 μ M) (+) as shown in Fig. 7. Extracts were incubated with anti-*myc*-tagged beads, and samples were analyzed by SDS-PAGE and immunoblotting using anti-*myc* and anti-FLAG antibodies (Ab). IP, immunoprecipitation; WB, Western blotting. (B) GST-hCry1-bound beads were incubated with [35 S]-hPer2 ([35 S]-hPer2) in the absence (-) and presence (+) of hemin (10 μ M), and binding was monitored by SDS-PAGE and autoradiography. Bands were quantified using an AlphaImager. (C) GST-hCry1-bound beads were incubated with [35 S]-labeled hPer2 (+) before or after hemin (10 μ M) addition (+). In all cases, beads were exhaustively washed, and samples were analyzed by SDS-PAGE and autoradiography. The figure shows data from a single experiment that was repeated three times with similar results.

example, preassociation of hPer2 to hCry1 prevents the access of heme to HRM but not PAS (Fig. 8 and 9B) and thus affects signaling downstream. Accordingly, binding of heme to PAS in the mPer2/hCry1 complex regulates the transcriptional activity of Bmal1/NPAS2 and the expression of the *alas1* gene (15). Conversely, the absence of hCry1 will allow heme to bind HRM (or both HRM and PAS simultaneously) and promote instability of hPer2 (this study), an event that is exclusively mediated by HRM, since heme binding to PAS does not alter hPer2 instability (Fig. 1B). In this scenario, downregulation of hPer2 directly impacts the oscillatory expression of circadian genes. Thus, this novel pathway ensures an alternative mechanism to physiologically controlling the circadian clock by acting on gene expression.

DISCUSSION

The mammalian circadian system influences most physiological activities, including sleep/wake cycles, cardiovascular activity,

body temperature, blood pressure, glucose and fat metabolism, renal plasma flow, liver metabolism and detoxification, and hormonal secretion (32). Cross talk between the body's circadian rhythm and metabolic systems has been identified within both the gluconeogenic and lipogenic pathways and in organisms as diverse as flies and mammals. Examples include the circadian oscillatory expression of the sterol-regulatory element-binding proteins 1a and 1c, a group of transcription factors that bind to the sterol regulatory element to control the hepatic transcriptome and thus the hepatic physiology. In addition, the orphan nuclear receptor Rev-erb α , a negative regulator of the circadian core gene *bmal1*, is expressed according to a robust circadian pattern and is induced during normal adipogenesis. Conversely, the retinoic acid-related orphan receptors also modulate *bmal1* expression while regulating lipid flux, lipogenesis, and lipid storage in skeletal muscle, providing an additional nodal point interrelating metabolic and circadian physiology. Further studies linked carbohydrate metabolism and circadian rhythms in fruit flies, and strong evidence supports a cross talk mechanism between nuclear

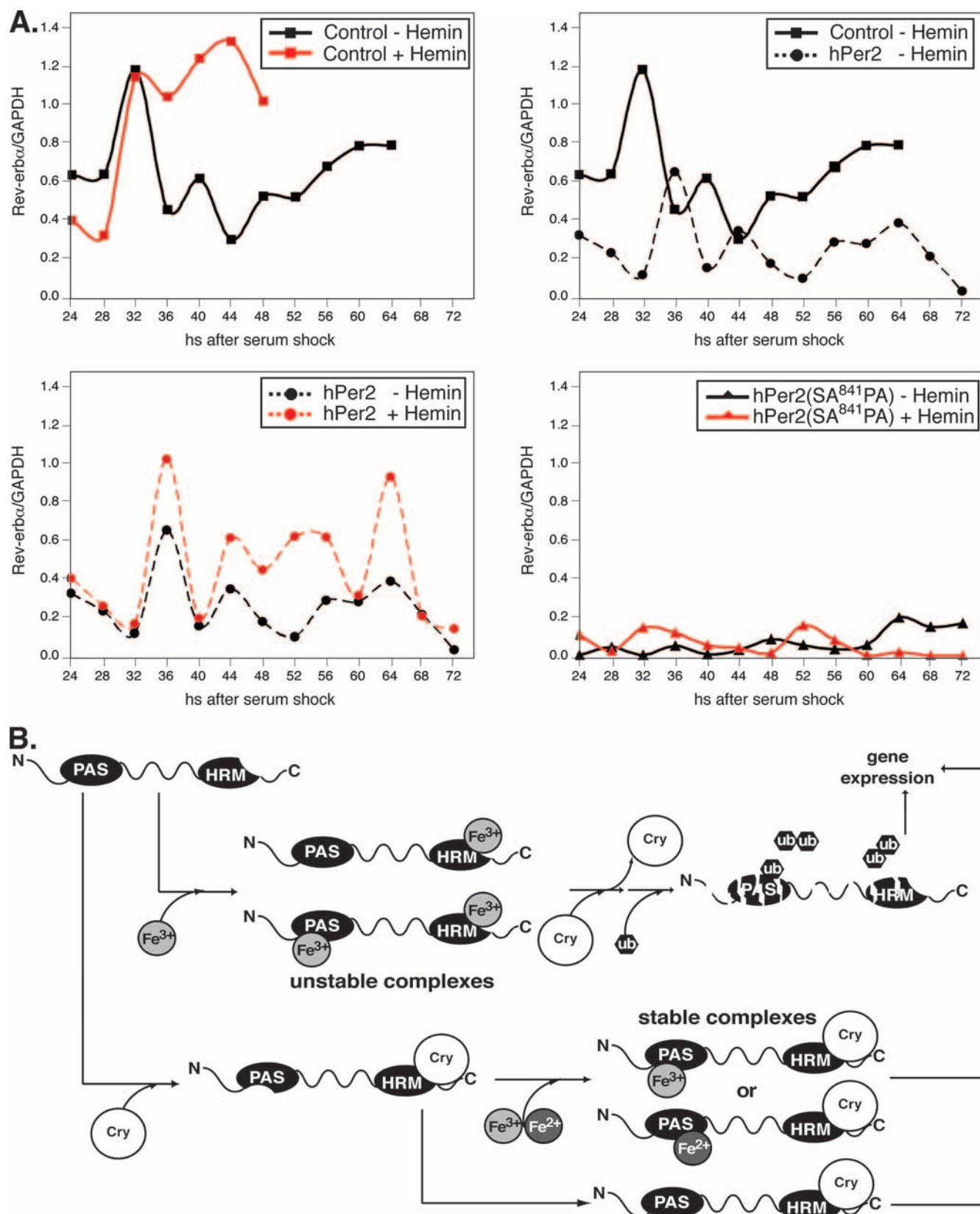


FIG. 9. HRM-heme binding modulates the expression of *Rev-erbα*. (A) CHO cells were transfected with *myc-hPer2*, *myc-hPer2*(SA⁸⁴¹PA), or empty vector (control) before the cells were shifted to a medium containing 50% horse serum and incubated for 2 h (time zero [see Materials and Methods]). Synchronized cells were then maintained in 5 mM succinylacetone followed by hemin (10 μ M) addition (at 24 h). Total RNA was prepared from about 10^7 cells at the times (in hours after serum shock) indicated in the panels, and the relative levels of *Rev-erbα* and *GAPDH* were determined by reverse transcription-PCR. *Rev-erbα* levels were normalized to those of the housekeeping gene. (B) A proposed model for the role of heme binding in hPer2/hCry1 complex formation is depicted. C, C terminus; N, N terminus; ub, ubiquitin; Fe³⁺, ferric heme; Fe²⁺, ferrous heme.

hormone receptors and the core circadian complex Clock/Bmal1 in adipogenesis (for a review, see reference 18).

An additional level of complexity arises from experiments showing that many heme-containing molecules regulate cellular homeostasis which, consistent with the circadian oscillatory nature of heme levels, led us to propose heme as a candidate bridge molecule for the circadian and metabolic mechanisms. We and other groups have reported that heme directly targets circadian clock components modulating both gene transcription and protein stability (6, 15, 43; this article). We established that heme directly binds to a novel regulatory motif in hPer2 in a redox-dependent manner, resulting in hPer2 instability and altered hPer2/hCry1 formation. Therefore, we propose that hPer2 acts as a heme sensor-transducer molecule, coupling metabolic signals to the circadian oscillator.

Control of Per2 stability plays a key role in driving circadian rhythmicity (31). During the transcription-translational feedback loop, Per2 is rapidly degraded as a result of phosphorylation by the double-time kinase in *Drosophila* (27) or CKI ϵ in mammals (3), altering the levels of Per2 available for heterodimerization and nuclear translocation. Although phosphorylation remains the primary mechanism responsible for Per2 degradation, alternative mechanisms to control its stability might exist. We tested the simplest model in which binding of heme to hPer2 induces protein instability in a phosphorylation-independent fashion. Indeed, heme favors hPer2 degradation both in vitro and in vivo. More importantly, this event is independent of both phosphorylation by CKI ϵ and binding of heme to the PAS domain, indicating that degradation of hPer2 can occur by alternative mechanisms. Period protein turnover is mediated by ubiquitination and further degradation by the proteasome pathway (39). Our data agreed, showing that inhibitors of proteasome function restore hPer2 levels, supporting a model where heme-mediated degradation of hPer2 depends on ubiquitination. Similarly, heme-mediated ubiquitination and degradation exist in iron regulatory proteins in other systems (13). Specifically, IRP2 oxidation, which is mediated by heme binding to its regulatory domain, triggers IRP2 ubiquitination-dependent degradation regulating the expression of genes involved in iron metabolism (13, 14, 41). In addition, the DNA-binding activity of the transcriptional repressor Bach1 dramatically decreases upon heme binding through multiple HRMs (23, 36), inducing nuclear export of Bach1 (37), polyubiquitination, and degradation of the repressor (44). Heme also binds to the bacterial iron response regulator through two distinct regions including an HRM, a necessary interaction for normal degradation (28, 29, 42). In this scenario, both redox states are required for rapid turnover of Irr, although its stability is independent of ubiquitination and likely mediated by an unknown specific protease (42). Like IRP2 and Irr, heme-dependent degradation of hPer2 is mediated by a CP core of a HRM. Unlike IRP2, where the Cys and His residues within the HRM participate in coordination and are responsible for axial ligand of ferric and ferrous heme (13), the HRM of hPer2 lacks the His component found in the HRM of IRP2 and exclusively binds ferric heme. More importantly, whereas oxidized heme binds to both HRM and PAS of hPer2, it is only its interaction with the former that is responsible for hPer2 degradation. This is the first demonstration of ligand-induced instability of a clock gene product and is a novel mode of regulation of the circadian feedback loop.

To understand the mechanism underlying heme-hPer2 rec-

ognition, we studied whether conformational changes are associated with ligand binding and heterodimeric complex formation. It is not known whether or to what extent heme binding to hPer2 plays a role in hPer2/hCry1 complex formation. Examples show slight secondary structural changes in helicity in the electron transport protein cytochrome *b*₅₆₂ upon heme binding (16), whereas large changes in secondary structure are revealed when the His-rich protein II is compared to the apoprotein after ferric heme addition (33). Our secondary structural studies of the C-terminal domain of hPer2 show that heme binding does not induce major conformational changes in the protein, suggesting that degradation of hPer2 does not result from unfolding upon ligand binding but is most likely mediated by an unknown, specific ubiquitin ligase enzyme. Much has been done to identify the molecules responsible for selective recognition of oxidized target proteins, including the recent characterization of the heme-oxidized IRP2 ubiquitin ligase-1 responsible for IRP2 turnover (13, 41). Interestingly, mPer2 ubiquitination is reduced by its interaction with Cry and is mediated by the Cry-binding domain residing in the C-terminal portion of mPer2 (9, 19), a mode of regulation closely resembling the organization of the Per/Tim loop in *Drosophila* (17).

All of these findings raise the question of whether heme binding to the C terminus of hPer2 prevents the formation of the hPer2/hCry1 complex or rather perturbs the stability of an already preformed heterodimer. Here, we provide evidence that heme acts by preventing hPer2 from binding to hCry1 when bound to HRM, whereas heme-PAS binding neither promotes hPer2 degradation nor affects hCry1 association. Heme binding to PAS plays a role in mPer2 interaction with the Bmal1/NPAS2 complex and in its transcriptional activity (15). Accordingly, cyanocobalamin, a vitamin B₁₂ analogue with a similar porphyrin ring structure to heme, greatly decreases the binding of NPAS2 and mPer2 to a heme-agarose matrix (15). The overall data are reconciled in a model where heme binding to either HRM or PAS in hPer2 targets different circadian complexes for regulation, likely connecting the cellular response to changes in heme levels. Furthermore, we propose that selectivity of binding is dictated by the redox state of the iron core in the heme molecule. Last, we demonstrate that transcription of the orphan nuclear receptor *Rev-erba*, a major regulator of the circadian oscillator that influences period length and affects the phase-shifting properties of the clock, is responsive to heme binding to the HRM of hPer2. These experiments add a new level of regulation in circadian gene expression by directly coupling metabolic sensing to the transcriptional control of the molecular oscillator.

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J.Y. performed all experiments except those mentioned below. A.L. and D.G.S.C. performed and analyzed CD experiments, C.V.F. performed immunofluorescence experiments, and K.D.K. performed the in vitro hPer2/hCry1 binding and fluorescence spectroscopy experiments. D.G.S.C. processed and analyzed the fluorescence data. K.E.D., C.S.S., and S.P.M. performed the experiments described in the supplemental material and provided technical support at various stages of the project. C.V.F., D.G.S.C., and J.Y. analyzed the overall data. C.V.F. wrote the manuscript.

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