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Identification of heme as the ligand for the orphan nuclear receptors REV-ERBα and REV-ERBβ

Srilatha Raghuram[1], **Keith R Stayrook**[2], **Pengxiang Huang**[1], **Pamela M Rogers**[3], **Amanda K Nosie**[2], **Don B McClure**[2], **Lorri L Burris**[2], **Sepideh Khorasanizadeh**[4], **Thomas P Burris**[3,5], and **Fraydoon Rastinejad**[1,5]

[1] Department of Pharmacology and Center for Molecular Design, University of Virginia Health System, 1300 Jefferson Park Avenue, Charlottesville, Virginia 22908-0733, USA

[2] Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, Indiana 46285, USA

[3] Nuclear Receptor Biology Laboratory, Pennington Biomedical Research Center, Louisiana State University System, 6400 Perkins Road, Baton Rouge, Louisiana 70808, USA

[4] Department of Biochemistry & Molecular Genetics, University of Virginia Health System, 1300 Jefferson Park Avenue, Charlottesville, Virginia 22908-0733, USA

Abstract

The nuclear receptors REV-ERBα (encoded by *NR1D1*) and REV-ERBβ (*NR1D2*) have remained orphans owing to the lack of identified physiological ligands. Here we show that heme is a physiological ligand of both receptors. Heme associates with the ligand-binding domains of the REV-ERB receptors with a 1:1 stoichiometry and enhances the thermal stability of the proteins. Results from experiments of heme depletion in mammalian cells indicate that heme binding to REV-ERB causes the recruitment of the co-repressor NCoR, leading to repression of target genes including *BMAL1* (official symbol *ARNTL*), an essential component of the circadian oscillator. Heme extends the known types of ligands used by the human nuclear receptor family beyond the endocrine hormones and dietary lipids described so far. Our results further indicate that heme regulation of REV-ERBs may link the control of metabolism and the mammalian clock.

REV-ERBα was originally identified as an orphan member of the nuclear hormone receptor (NHR) family on the basis of its canonical domain structure and sequence conservation[1,2]. REV-ERBβ was subsequently identified by its homology to other NHRs and its pattern of expression, which overlaps greatly with that of REV-ERBα. Both receptors have particularly high expression in the liver, adipose tissue, skeletal muscle and brain[3–8], where they are transcribed in a circadian manner[9–11]. The REV-ERBs are unique in the NHR superfamily in that they lack the carboxy-terminal tail (helix 12) of the ligand-binding domain (LBD), which is required for coactivator recognition[12] (Supplementary Fig. 1a online). Both receptors act as

Correspondence should be addressed to F.R. (fr9c@virginia.edu) or T.P.B. (Thomas.Burris@pbrc.edu).
[5]These authors contributed equally to this work.

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AUTHOR CONTRIBUTIONS

S.R. produced the wild-type and mutant constructs and, together with P.H., purified the proteins and carried out mass spectrometry and ultraviolet-visible spectroscopy. P.H. and S.K. designed and performed the ITC and circular dichroism studies. K.R.S. performed co-transfection and luminex assays. P.M.R. performed the co-immunoprecipitation and ChIP assays. D.B.M. expressed REVERBs in HEK293 cells. A.K.N and L.L.B. created REV-ERB mutants and performed co-transfection assays. T.P.B. and F.R. conceived and designed the studies and wrote the manuscript.

constitutive repressors of transcription and bind DNA response elements termed 'ROREs'[13–15]. Although considerably more is known about the function of REV-ERBα than that of REV-ERBβ, the similarities in their expression patterns and DNA-binding and transcriptional activities indicate that they are likely to overlap substantially in function. Nevertheless, more complete understanding of their functional properties has been hindered by the lack of known physiological ligands.

REV-ERBα is a principal regulator of the cyclic expression of *BMAL1* (refs. [16–18]), a key component of the mammalian circadian clock. Circadian rhythms play an essential part in aspects of physiology and behavior, including the sleep-wake cycle, body temperature, blood pressure and renal function. The circadian rhythms are generated by feedback loops in gene expression in which heterodimers of BMAL1 and CLOCK activate expression of the *cryptochrome* (*Cry*) and *period* (*Per*) genes (the negative limb)[19,20]. When CRY and PER reach a critical level of expression, they block the stimulatory effect of the CLOCK or NPAS2–BMAL1 complex on their own genes, thereby completing the loop[20,21]. The *BMAL1* promoter contains two ROREs, and *BMAL1* transcription is directly repressed by REV-ERBα[16,22]. Mice deficient in *NR1D1*, the gene encoding REV-ERBα, show loss of the diurnal pattern of expression of *BMAL1* and alterations in the period and phase of their circadian behavior patterns[17].

Clues to a possible physiological ligand for the REV-ERB proteins has come from a study on E75, a member of the NHR family in *Drosophila* that has been shown to contain a heme prosthetic group[23]. The oxidation state of iron-heme in E75 determines whether this NHR can interact with its heterodimer partner, DHR3, and this dimer interaction is further regulated by binding of either nitric oxide or carbon monoxide to heme[23]. The heme in E75 is highly resistant to dissociation, and its forcible removal produces an unstable protein[23,24]. Thus, heme could be involved in the regulation of E75 activity by influencing its stability, or by functioning as a sensor of redox and/or diatomic gases[23]. Alignment of the LBDs of E75 and the REV-ERB receptors shows good conservation overall (Supplementary Fig. 1b). Because heme concentrations change in a circadian manner and can entrain the mammalian clock[25,26] and because heme is used as a cofactor by proteins that control metabolic functions and the circadian clock including NPAS2 (ref. [27]), we have examined whether the human REV-ERB nuclear receptors use heme as their ligand.

RESULTS

REV-ERB proteins are associated with heme

We purified recombinant histidine-tagged human REV-ERBα and REV-ERBβ LBD proteins after expression in *Escherichia coli*. We also prepared Flag-tagged full-length REV-ERBα and REV-ERBβ by transient transfection of human embryonic kidney HEK293 cells. Both cell systems are known to produce heme that associates with heme-binding proteins[28,29], and recombinant E75 protein expressed in *E. coli* has been shown to associate tightly with heme[23].

Both full-length receptors and LBDs were extensively purified, and the samples were notably red or pink in color (Supplementary Fig. 2 online). Matrix-assisted laser desorption-ionization (MALDI) mass spectra of these samples showed a heme peak, corresponding to iron-protoporphyrin IX, with the expected mass of 616 ± 2 Da (mean ± s.d.; Fig. 1a and Supplementary Fig. 3 online). The distinct features of heme bound to the proteins were observed by ultraviolet-visible spectroscopy: namely α and β peaks in the 500–600 nm range and γ (Soret) peaks in the 390–450 nm range (Fig. 1b and Supplementary Fig. 4 online). In addition, the associated heme was in the oxidized state because the Soret peak was shifted on addition of the reducing agent dithionite (Fig. 1b).

Heme binding thermodynamics

To characterize the thermodynamics of heme binding to REV-ERB proteins, we used isothermal titration calorimetry (ITC) after removing heme from the purified LBDs. In contrast to *Drosophila melanogaster* E75, heme can be removed from the REV-ERB LBDs by extensive washing of Ni-NTA–immobilized proteins or through dialysis (Supplementary Fig. 5 online).

Heme-depleted receptors were appropriately folded and fully competent to bind exogenously added heme, as shown by the ITC measurements; both REV-ERB LBDs bound to heme with a dissociation constant (K_d) of 2–3 μM (Fig. 2a,b). The enthalpy of heme binding was approximately −5 kcal mol^{-1}, and the stoichiometry of heme to receptor binding was roughly 1:1. Thus, heme binding to the REV-ERB LBDs is fully reversible. Although the concentration of heme in different tissues is not known, it has been suggested that they vary in a circadian manner[29], indicating that REV-ERBs might function as heme sensors.

Heme binding increases REV-ERB LBD thermal stability

Nuclear receptor ligands are generally lipophilic and increase protein thermal stability by binding a hydrophobic central cavity in the LBDs. We examined the effect of heme binding on thermal stability of the LBD by using circular dichroism spectroscopy. Heme binding increased the melting temperature (T_m) of each LBD by 4–5 °C (Fig. 2c,d), consistent with ligand effects on other nuclear receptor LBDs, for which it typically increases the T_m by 1–6 °C (refs. [30,31]). These results suggested that a change in protein conformation occurs on heme binding to REV-ERB LBDs, and this idea was supported by peptide interaction ‘conformation sensing’ assays (Supplementary Fig. 6 and Supplementary Methods online).

Taken together, these data are consistent with the hypothesis that REV-ERBs act as physiological sensors of heme concentrations in cells and that heme binding may act as the molecular switch that regulates receptor conformation.

A REV-ERBα LBD mutant deficient in heme binding

Most heme binding proteins use histidine side chains to coordinate the heme molecule. In E75, mutation of particular histidine or cysteine residue eliminates heme binding[23,24], and some of these residues are conserved in REV-ERBα and REV-ERBβ (Supplementary Fig. 1). We individually mutated residues Cys389 (conserved in REV-ERBα and REV-ERBβ), and Cys418 and His602 (conserved in E75 and both REV-ERBs) in the LBD of REV-ERBα. All three mutant LBDs were stable when expressed in *E. coli* and were purified for ITC analysis of their ability to bind heme.

C389A and C418A showed affinities for heme similar to that of the wild-type LBD (data not shown). By contrast, REV-ERBα LBD H602F showed substantially diminished affinity for binding heme (K_d: wild type, 3.5 ± 0.2 μM; H602F, > 120 μM; Fig. 3). In addition, ultraviolet-visible spectrum analysis indicated that the H602F mutant was not associated with heme (Fig. 3), implying that H602 is essential for heme binding to the REV-ERBα LBD.

Heme is required for the repressor activity of REV-ERBα LBD

To examine the role of heme in REV-ERBα function in cells, we assessed the transcriptional repressor activity of REV-ERBα LBD and the H602F mutant, fused to the DNA-binding domain (DBD) of Gal4 in both HuH7 hepatoma cells and HEK293 cells. Use of the Gal4 DBD chimeric receptors facilitated direct assessment of the transcriptional repressor activity of the LBDs in isolation.

Both fusions were expressed in similar amounts, as determined by immunoblotting (Supplementary Fig. 7a online). There was, however, a complete loss in transcriptional repression activity of the H602F LBD, as compared with the wild-type LBD (Fig. 3), suggesting that heme binding is required for repression.

Heme regulates the expression of REV-ERBα target genes

The lack of ability of the Gal4 DBD REV-ERBα LBD H602F mutant to repress transcription suggested that heme might be required for the repressive effect of REV-ERBα on its target genes. To test this idea, we modulated intracellular heme in human HepG2 hepatoblastoma cells and examined the effects on the REV-ERBα target genes *BMAL1* and *ELOVL3*. We inhibited heme biosynthesis in cells with succinylacetone, a highly specific inhibitor of aminolevulinic acid dehydratase that has been used to decrease intracellular heme in several cell lines and *in vivo*[32–37].

Although intracellular heme is usually tightly regulated, we effectively reduced intracellular concentrations by about 40% using succinylacetone (Fig. 4a). This reduction was accompanied by a 2.6-fold increase in the expression of *ALAS1* (Fig. 4b), the gene encoding aminolevulinic acid synthase. Because *ALAS1* expression is known to be under negative transcriptional feedback control by heme[34], the observed upregulation of *ALAS1* on succinylacetone treatment indicates that the decrease in heme is physiologically significant. By contrast, addition of hemin to the cells resulted in a significant ($P < 0.05$) 33% increase in intracellular heme (Fig. 4a), accompanied by a 40% decrease in *ALAS1* expression, again consistent with the physiological response to an excess of intracellular heme. As an alternative to succinylacetone method, the effect of inhibition of *ALAS1* expression by short interfering RNA (siRNA) was also examined. We found, however, that there was no correlation between inhibition of *ALAS1* and a decrease in intracellular heme (potentially owing to compensatory effects of heme oxygenase) and, as a result, we did not pursue further the siRNA approach for modulation of heme.

In heme-depleted cells, we noted a significant ($P < 0.05$) increase in expression of *BMAL1* (3-fold) and *ELOVL3* (5.5-fold; Fig. 4c,d). The increase in expression of REV-ERB target genes that followed heme depletion was consistent with our data on the chimeric H602F LBD, suggesting that an ability to bind heme is required for the repressor activity of REV-ERB LBD. We expected that increasing intracellular heme would result in a decrease in expression of REVERB target genes, owing to an increase in repressive activity. Indeed, raising intracellular heme caused significant ($P < 0.05$) decreases in *BMAL1* and *ELOVL3* expression (Fig. 4c,d). These data are consistent with our hypothesis that heme selectively regulates the transcription of REV-ERB target genes.

Heme regulates NCoR recruitment to REV-ERBα

REV-ERBs repress the transcription of their target genes through recruitment of the co-repressor NCoR[38,39]. The inability of the H602F REV-ERBα LBD mutant to repress transcription, in addition to the observation that intracellular heme regulates the expression of REV-ERBα target genes, suggests that heme may regulate the ability of REV-ERBα to interact with NCoR. Consistent with this hypothesis, depleting intracellular heme resulted in a decreased interaction of REV-ERBα with NCoR, as determined by co-immunoprecipitation. (Fig. 5a). Addition of 30 μM hemin to the heme-depleted cells resulted in partial recovery of the interaction between REV-ERBα and NCoR (Fig. 5b). Depletion of heme also resulted in a large decrease in NCoR occupancy of the RORE in the *BMAL1* promoter, as determined by chromatin immunoprecipitation (ChIP) and quantitative PCR (Fig. 5b). Similar to the co-immunoprecipitation results, addition of hemin to the heme-depleted cells resulted in partial recovery of NCoR occupancy of the *BMAL1* promoter (Fig. 5b). It should be noted that the co-immunoprecipitation and ChIP studies were performed in HepG2 cells expressing endogenous amounts of REV-ERBα and NCoR. These data indicate that heme can regulate the interaction of REV-ERBα with NCoR and are also consistent with the hypothesis that heme regulates REV-ERB–mediated recruitment of NCoR to the *BMAL1* promoter.

REV-ERB LBDs appear not to be redox or gas sensors

In other types of heme–protein complexes, the heme prosthetic group confers the ability to respond either to alterations in redox state or to diatomic gases. We found that reduction of the heme iron through the addition of 10 μM dithionite did not substantially change the binding activity of the CoRNR box peptide, suggesting that the redox state of heme does not alter LBD conformation (Supplementary Fig. 8a,b online). Addition of pharmacological nitric oxide donors did not affect the transcriptional activity of the REV-ERB LBD (Supplementary Fig. 8a,b). Thus, in contrast to E75, REV-ERBs seem to be insensitive to the redox state and diatomic gasses.

DISCUSSION

We have shown that heme meets the requirements of a physiological ligand for the human orphan nuclear receptors REV-ERBα and REVERBβ. Heme binding took place in a 1:1 stoichiometry with the REVERB LBDs, as determined by ITC (Fig. 2a,b), and, consistent with the role of heme as a REV-ERB ligand, the receptor LBDs underwent changes in thermal stability and conformation on heme addition (Fig. 2c,d and Supplementary Fig. 6). Modulation of intracellular heme resulted in a change in the expression of REV-ERB target genes (Fig. 4) and in alteration of the interaction of REV-ERBα with NCoR (Fig. 5a). The effects of heme were specific to the REV-ERB LBDs because we observed no effect on other nuclear receptors such as LXR (Supplementary Fig. 6). These results are consistent with the idea that the REV-ERBs function as physiological sensors of intracellular heme. The identification of a member of the nuclear receptor superfamily as a heme receptor indicates that the diversity of endogenous ligands for this superfamily is more extensive than the steroids, retinoids, thyroid hormone and lipids identified so far. The affinity of heme for the REV-ERB LBDs ($K_d \approx 3$ μM) suggests that these receptors belong in the category of physiological nuclear receptor 'metabolite' sensors, such as FXR (bile acids), LXR (oxysterols) and PPARs (fatty acids), for which K_d values of 1–10 μM have been observed[40].

Although the *Drosophila* E75 protein also associates with heme, there are many important differences between E75 and the two human REV-ERB proteins characterized here. In biochemical assays, the REV-ERB LBDs show reversible binding with heme and stability in both the ligand-bound and apoprotein state (Supplementary Fig. 6). By contrast, binding of heme to E75 seems to be constitutive and is required for protein stability[23]. We found that, in the REV-ERB LBDs, a change in the oxidation state of the iron does seem to alter receptor conformation (Supplementary Fig. 9a,b online). In addition, the transcriptional activity of the REV-ERB does not seem to be responsive to diatomic gases. Thus, it seems that REV-ERBs function as sensors of heme, rather than using heme to sense redox conditions or gases.

Variations in intracellular heme, which occur in a circadian manner in mammals[25], may allow REV-ERB proteins to modulate repression of their target genes and to shape appropriately the amplitude of the circadian rhythm. REV-ERBα regulates the expression of several genes involved in circadian pathways and in lipid metabolism and adipogenesis[16,41–46]. Our findings indicate that the REV-ERBs sense and respond to dynamic changes in intracellular free heme and may use heme to integrate the circadian clock and metabolic programs. Importantly, the REV-ERB target gene *BMAL1*, an essential component of the circadian oscillator, is also essential for adipogenesis[47,48]. Such links between circadian rhythms and metabolic patterns have been recognized for some time[19,48]. More direct evidence has come from mice carrying mutations in *BMAL1* or *Clock*. These mice show abnormalities similar to metabolic syndrome including obesity, dyslipidemia and impaired glucose metabolism[49,50]. Our finding that the

REV-ERBs are in fact ligand-regulated receptors suggests that synthetic modulators of the REV-ERB proteins may be developed in the future with the goal of treating human diseases related to coordination of the cellular clock, control of glucose and energy metabolism.

Synthesis of heme, the ligand for the REV-ERBs, is linked to the nutritional status of mammals through the regulation of *ALAS1* by the nuclear receptor coactivator PGC-1α (ref. [51]). This coactivator is induced by fasting and mediates the transition from glucose to fatty acid use as an energy source and has been shown to integrate the mammalian clock and energy metabolism[52]. We predict that alterations in PGC-1α–induced expression of ALAS1, along with the rate of heme synthesis, may provide a direct pathway for modulating REV-ERB–mediated regulation of the circadian rhythm and energy metabolism, thereby serving as an additional link between these two crucial and intricately regulated biological processes. Finally, REV-ERBα is involved in the induction of adipogenesis, and we predict that heme has a crucial role in this process. Indeed, heme has been shown to be essential in the induction of adipocyte differentiation[53], and our results suggest that this role of heme may be mediated by REV-ERB, which also plays an important part in adipogenesis.

METHODS

Expression and purification of REV-ERBα and REV-ERBβ

For LBDs, residues 281–614 of REV-ERBα (NM_021724) and 247–579 of REV-ERBβ (NM_005126) were expressed as 6×His-tagged proteins from a pET46 Ek/Lic vector in Rosetta (DE3)-pLysS *E. coli* cells (Novagen). We added 150 μM 5-amino levulinic acid to the cultures to enhance expression of the proteins. The cells were induced with 0.5 mM isopropyl-β-D-thiogalactoside (IPTG) at 16 °C overnight, collected and lysed in 20 mM Tris (pH 8.0), 500 mM NaCl, 20 mM imidazole and 10% glycerol. The proteins were purified with His-Bind Resin (Novagen) and eluted with 250 mM imidazole. The purified LBDs were dialyzed against 20 mM Tris, 200 mM NaCl and 5% glycerol for subsequent experiments. LBD concentrations were estimated by using the calculated molar extinction coefficient (ε_{280}) of 0.619 = 1 mg/ml (for REV-ERBα) and 0.393 = 1 mg/ml (for REV-ERBβ). The bound heme concentration was estimated by using the extinction coefficient for the heme Soret peak (ε_{415}) of 101.85 = 1 mM.

For mammalian expression of full-length receptors, pCMV7.1_3X plasmids containing Flag-tagged REV-ERBα and REV-ERBβ were transiently transfected into suspension-adapted 293-EBNA cells (CRL-10852, ATCC; 10 l at 10^6 cells per ml). After 24 h, bovine hemin (H 5533, Sigma-Aldrich) was added at 1 mg/l. After 48 h, cells were collected, frozen at −80 °C, and lysed with Cell Lytic M buffer (Sigma-Aldrich). The proteins were purified by using a Flag M2 affinity (Sigma) column and eluted with 3×Flag peptide (Sigma-Aldrich). The presence of REV-ERBα and REV-ERBβ in the eluted fractions was assessed by immunoblotting using a Flag M2 monoclonal antibody (Sigma-Aldrich). The presence of heme was determined by the Soret band (415 nm).

Ultraviolet-visible spectroscopy

Soret and α,β absorption spectra were measured on a Cary 50 Bio ultraviolet-visible spectrophotometer at room temperature (22 °C). To reduce the heme group, 10 mM sodium hydrosulfite (dithionite) was added to the protein samples. Spectra of reduced complexes were recorded 5 (for REV–ERBα) or 50 (for REV–ERBβ) min after the addition of 10 mM dithionite.

Mass spectrometry

The REV-ERBα and REV-ERBβ LBDs and the Flag-tagged full-length proteins were first dialyzed against water to remove salt. Protein concentrations of 0.5–1 mg/ml were used for

MALDI mass spectrometry analyses. The samples were analyzed at the Biomolecular Facility of the University of Virginia as described in the Supplementary Methods.

ITC

We carried out ITC experiments at 20 °C on a MicroCal VP-ITC instrument. For heme binding studies, LBDs were dialyzed extensively against 20 mM Tris (pH 8.0), 200 mM NaCl and 5% glycerol, and then concentrated in the same buffer to 0.050 mM (REV-ERBα) or 0.045 mM (REV-ERBβ). The concentration of hemin solution in the ITC binding studies was 0.466 mM (for REV-ERBα) or 0.561 mM (for REV-ERBβ).

Circular dichroism

Purified LBDs were dialyzed against 10 mM NaH_2PO_4 (pH 7.5) and 50 mM NaCl. Measurements were carried out on an AVIV circular dichroism instrument. For temperature melting experiments, a gradient of 20–70 °C was used with 2 °C jumps and a 2-min equilibration time. The change in circular dichroism signal was measured at 223 nm and 224 nm for REV-ERBα and REV-ERBβ, respectively. Protein concentrations were 0.4 mg/ml. Similar measurements were repeated after the addition of 50 μM hemin to the protein samples[54].

Measurement of cellular heme content

Heme in HepG2 cell lysates was measured by using a modified QuantiChrom Heme Assay (BioAssay Systems). The amount of heme in each sample is expressed as μM heme per mg of total protein.

Manipulations of heme biosynthesis

Succinylacetone (Sigma) was added to confluent HepG2 cultures at a final concentration of 0.5 mM (ref. [32]) for 24 h before cell collection. We added 30 μM hemin (Sigma) to confluent cultures for 24 h before cell collection as described[55]. Hemin was added either in the absence of succinylacetone (Fig. 4) or in the presence of succinylacetone (simultaneous treatment; Fig. 5).

Quantitative RT-PCR

Quantitative RT-PCR was performed as described[56]. The *BMAL1* (NM_001178) primers were (5′-GTACCAACATGCAACGCAATG-3′) and (5′-TGTGTATGGATTGGTGGCACC-3′). Cyclophilin B (M60857) was used as a standard with the following primers: (5′-GGAGATGGCACAGGAGGAAA -3′) and (5′-CGTAGTGCTTCAGTTTGAAGTTCTCA-3′). *ELOVL3* primers were obtained from Superarray.

ChIP assay

ChIP assays were performed as described[38,57]. Chromatin-immunoprecipitated DNA was quantified by quantitative PCR with the following primers specific to the human *BMAL1* promoter: *BMAL1*_prom-for (5′-ATTGGTGGCAGGAAAGTAGC-3′) and BMAL1_prom-rev (5′-GTTGTGTGGCGGCTAGAGAG-3′).

Co-immunoprecipitation assay

HepG2 cells were treated with succinylacetone (2 mM) or both succinylacetone (2 mM) and hemin (30 μM) for 24 h before immunoprecipitation. Cells were lysed in Triton lysis buffer (20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulphonyl fluoride, 10 mg/ml of aprotinin and 10 mg/ml of leupeptin). Protein concentrations were measured with BCA Protein Assay Reagent (Pierce) and ~0.5 mg of protein lysate was used for each immunoprecipitation. The antibodies used for immuno-precipitation and immunoblotting were anti–REV-ERBα (Cell Signaling Technologies) and anti–NCoR C-20 (Santa Cruz Biotechnology).

Co-transfection and reporter assays

HuH7 and HEK293 cells were seeded in 96-well plates at 25,000 cells per well as described[58] with the modifications outlined in the Supplementary Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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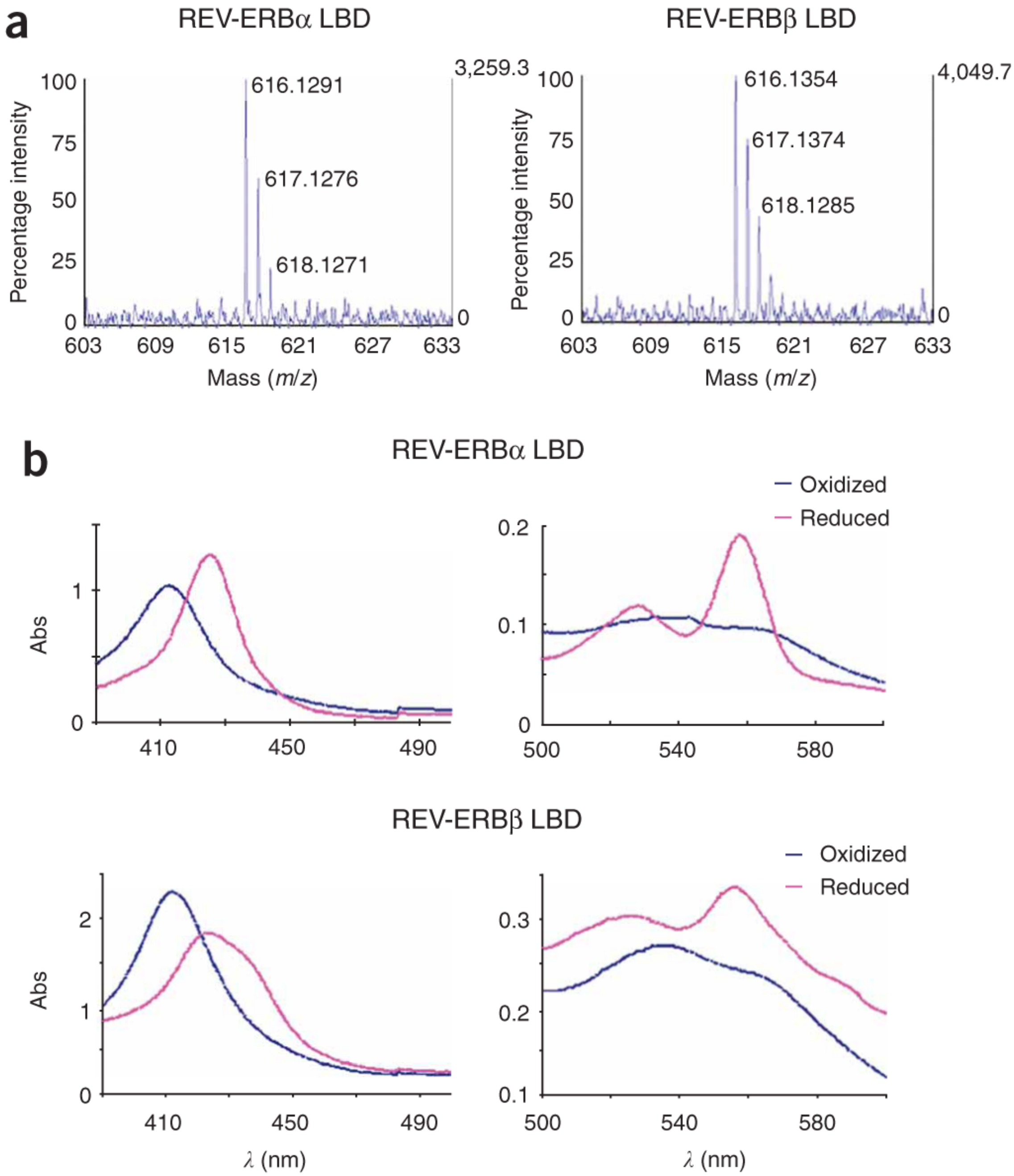

Figure 1.

Association of heme with the LBDs from REV-ERBα and REVERBβ. (**a**) MALDI mass spectra showing iron protoporphyrin IX in purified REV-ERBα and REV-ERBβ LBDs prepared from *E. coli*. Signals at 616 ± 2 Da correspond to heme iron-protoporphyrin IX. (**b**) Visible absorption spectra of REV-ERB LBD proteins. Peaks characteristic of heme–protein complexes are shown in the 390–450 nm range for the Soret band and in the 500–580 nm range for the α and β bands. Red spectra correspond to the heme–protein complexes after dithionite reduction of the iron moiety.

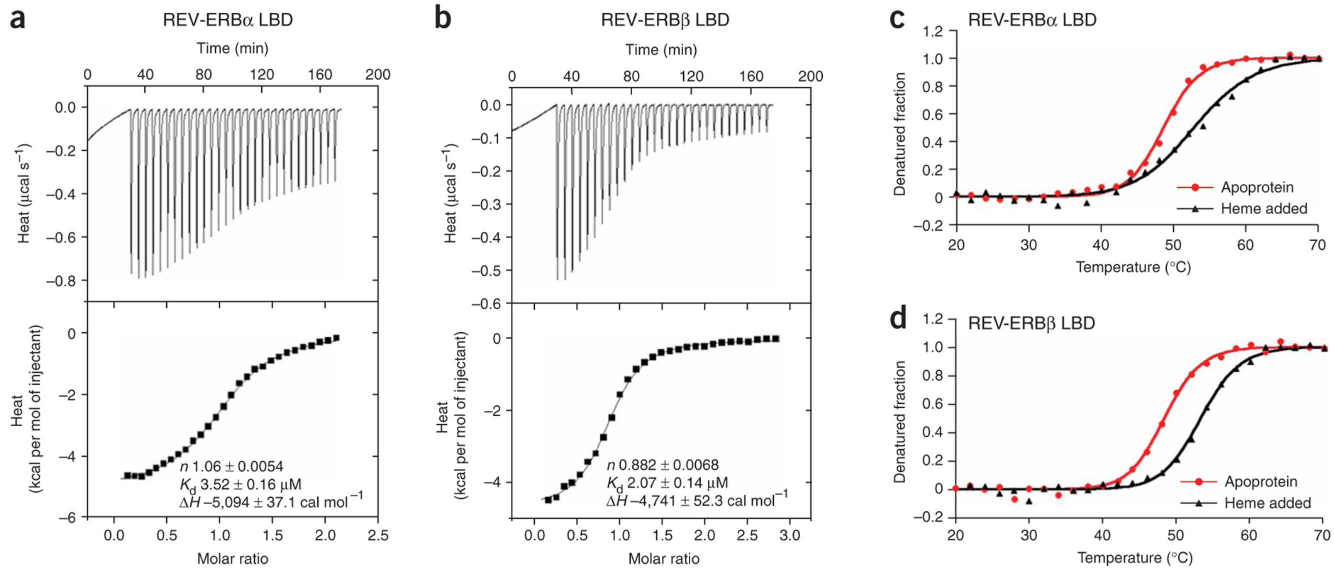

Figure 2.

Thermodynamics of heme association with the REV-ERB LBDs measured by ITC and circular dichroism spectroscopy. (**a**,**b**) ITC data corresponding to the REV-ERBα (**a**) and REV-ERBβ (**b**) LBDs binding hemin. (**c**,**d**) Far-ultraviolet circular dichroism thermal melts corresponding to the REV-ERBα (**c**) and REV-ERBβ (**d**) LBDs in the heme-bound (+heme) and apoprotein (−heme) forms. The T_m values are 48.6 °C (−heme) and 52.9 °C (+heme) for REV-ERBα LBD and 48.4 °C (−heme) and 53.3 °C (+heme) for REV-ERBβ LBD.

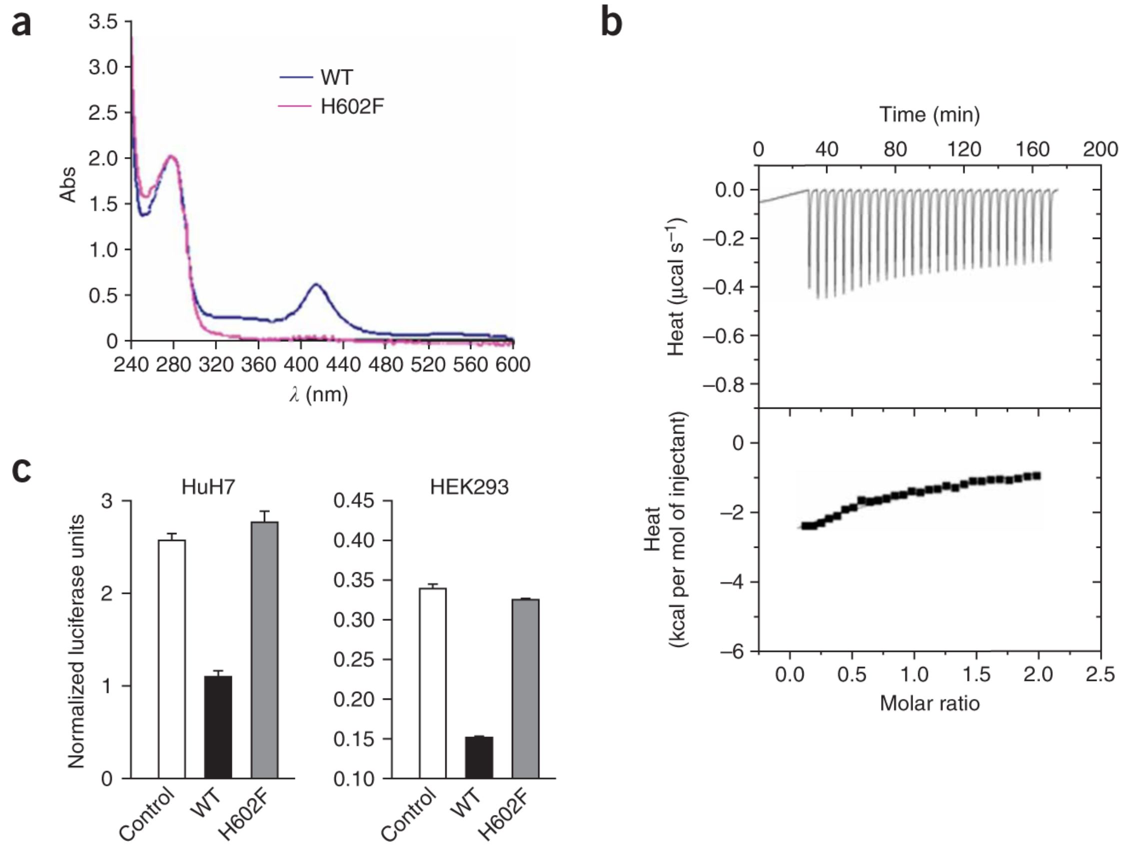

Figure 3.

Role of heme in regulation of REV-ERBα LBD activity. (**a**) Ultraviolet-visible spectrum of wild-type REV-ERBα (blue) and H602F REV-ERBα (red) LDBs. The absorbance peaks at 280 nm indicate that similar amounts of wild-type and H602F protein were examined, whereas the loss of the absorbance peak at ~420 nm for the H602F protein indicates lack of heme binding. (**b**) Lack of heme binding by the H602F REV-ERBα LBD mutant determined by ITC. Minimal binding to heme was not saturable; thus, K_d could not be estimated (**c**) Co-transfection reporter assay, comparing the transcriptional activity of the wild-type and H602F REV-ERBα LBDs fused to the Gal4 DBD in HuH7 hepatoma cells and HEK293 cells. The cells were co-transfected with a vector containing five copies of the Gal4 UAS upstream of luciferase (5×UAS-SV40 pGL3 firefly luciferase reporter vector). The wild-type and H602F chimeric proteins were expressed in comparable amounts, as determined by immunoblotting (Supplementary Fig. 8). Experiments were performed in triplicate a minimum of three times, and the mean ± s.d. of a representative experiment is shown.

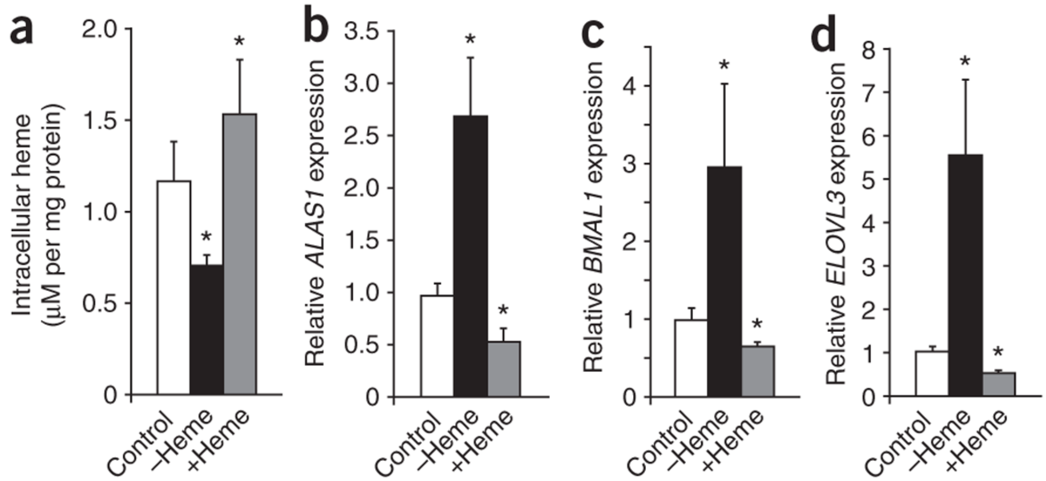

Figure 4.

Effect of modulation of intracellular heme on expression of REV-ERB target genes in HepG2 cells. (**a**) Treatment of HepG2 cells with succinylacetone (−heme) results in depletion of intracellular heme, whereas addition of hemin results in an increase in intracellular heme (+heme). (**b**) Expression of *ALAS1* increases in response to a decrease in intracellular heme, and decreases in response to an increase in intracellular heme. (**c**,**d**) Expression of *BMAL1* (**c**) and *ELOVL3* (**d**) increases with heme depletion and decreases with increased intracellular heme; both genes are known to be repressed by REV-ERBα. Data are the mean ± s.d. of triplicate wells. $*P < 0.05$.

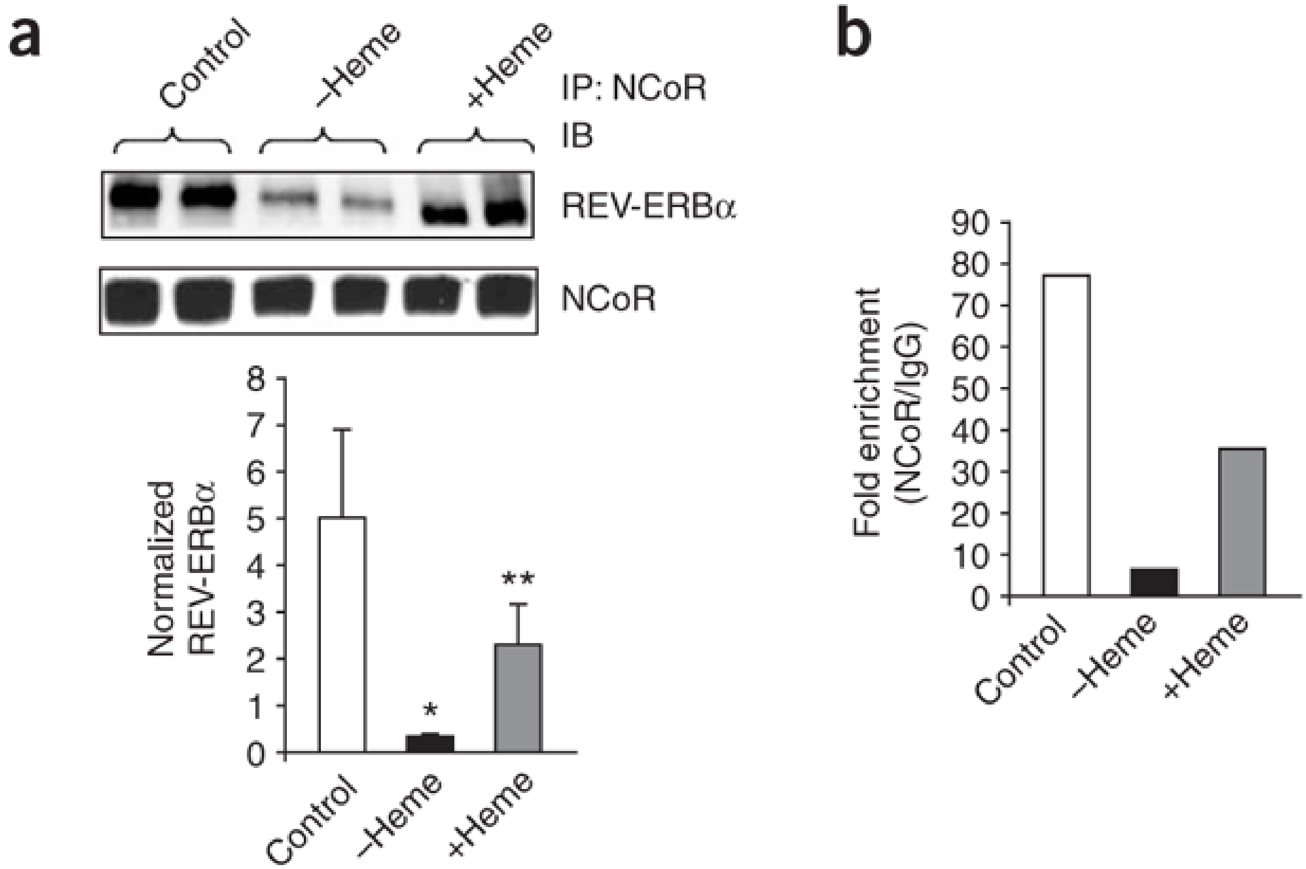

Figure 5.

Effect of intracellular heme on NCoR interaction with REV-ERBα and recruitment to promoters. (**a**) The interaction of REV-ERBα with NCoR in HepG2 cells was determined by coimmunoprecipitation. Heme was depleted by succinylacetone treatment (−heme), and added back (+heme) by supplying hemin to heme-depleted cells. Cellular extracts were immunoprecipitated with antibody to NCoR, and samples were analyzed by immunoblotting with an antibody to REV-ERBα. Experiments were performed a minimum of three times and a representative gel is shown (top). Use of IgG did not result in detectable REV-ERBα (Supplementary Fig. 7b). Histogram shows quantification of the REV-ERBα immunoblots (bottom). Data are the mean ± s.d. from three individual experiments. Student's *t*-test: $*P < 0.05$ versus control, $**P < 0.05$ versus heme depletion. (**b**) Effect of intracellular heme on NCoR occupancy in the *BMAL1* promoter in HepG2 cells. Heme was depleted by succinylacetone treatment (−heme), and added back by hemin supplementation to heme-depleted cells (+heme). Chromatin was immunoprecipitated with an antibody to NCoR, and DNA was quantified by quantitative PCR. A control using rabbit IgG was used for normalization. Histogram shows the results from a representative of three independent experiments.