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The *Drosophila* Nuclear Receptor E75 Contains Heme and Is Gas Responsive

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Summary

Nuclear receptors are a family of transcription factors with structurally conserved ligand binding domains that regulate their activity. Despite intensive efforts to identify ligands, most nuclear receptors are still "orphans." Here, we demonstrate that the ligand binding pocket of the *Drosophila* nuclear receptor E75 contains a heme prosthetic group. E75 absorption spectra, resistance to denaturants, and effects of site-directed mutagenesis indicate a single, coordinately bound heme molecule. A correlation between the levels of E75 expression and the levels of available heme suggest a possible role as a heme sensor. The oxidation state of the heme iron also determines whether E75 can interact with its heterodimer partner DHR3, suggesting an additional role as a redox sensor. Further, the E75-DHR3 interaction is also regulated by the binding of NO or CO to the heme center, suggesting that E75 may also function as a diatomic gas sensor. Possible mechanisms and roles for these interactions are discussed.

Introduction

Nuclear receptors belong to a superfamily of transcription factors that share a common domain architecture, including a highly conserved DNA binding domain (DBD) and a structurally conserved ligand binding domain (LBD). The LBD acts as a molecular switch, undergoing conformational changes upon ligand binding that cast off associated chaperone and co-repressor proteins. These changes, in turn, affect subcellular localization, dimerization, DNA binding, coactivator binding, and/or transcriptional activity (reviewed by Glass and Rosenfeld, 2000). All known nuclear receptor ligands are able to move readily between and within cells, making them ideal inter- and intracellular messengers or hormones.

Three classes of nuclear receptors have been defined based on the type of ligand that they bind (Chawla et al., 2001; Moreau et al., 1988). The endocrine receptors bind ligands such as steroid hormones and fat-soluble vitamins with high affinity. Lipid receptors typically bind dietary, bioactive, or exogenous lipids with lower affinity. Finally, nuclear receptors with no known ligands are classified as orphan receptors. The identification of ligands for nuclear receptors, particularly for the latter class of orphan receptors, has received a great deal of attention, in large part because nuclear receptors play major roles in development, metabolism, reproduction, and disease and because the nature of their ligands makes them excellent targets for pharmaceutical intervention.

Of the 18 classical nuclear receptors found in *Drosophila*, only one, the ecdysone receptor (EcR), has a known ligand. When heterodimerized with Ultraspiracle (USP), which is the *Drosophila* homolog of Retinoid-X receptor (RXR), EcR can respond to ecdysteroid pulses, activating a cascade of nuclear receptor gene interactions (reviewed in Thummel, 1995). One of these EcR/USP target genes, *Eip75B*, encodes the nuclear receptor E75 (Segraves and Hogness, 1990). As an early ecdysone-responsive gene product, E75 is thought to regulate a subset of the developmental processes initiated by ecdysone signals. E75 also appears to function upstream of the ecdysone-mediated nuclear receptor cascade, as it is required for ecdysone synthesis (Bialecki et al., 2002). Germline clones of E75 null mutants arrest during mid-oogenesis (Buszczak et al., 1999), while zygotic mutations are embryonic lethal, with gut and metamorphosis defects. Although not exhaustively researched, later roles include eye and limb development (D'Avino and Thummel, 1998) and the coordination of juvenile hormone and ecdysone signals during larval molts and metamorphosis (Dubrovskaya et al., 2004). Insect orthologs of *Drosophila* E75 are also actively expressed during larval development, pupariation, and oogenesis (Jindra et al., 1994; Palli et al., 1997, 1995; Siaussat et al., 2004; Swevers et al., 2002a; Zhang et al., 2004). A putative ortholog of E75 has been identified outside the insect world in the shrimp *Metapenaus ensis*, where it is also expressed during molting (Chan, 1998). In vertebrates, the closest homologs are members of the orphan Rev-Erb family.

The *Eip75B* gene produces three splice variants, E75A, B, and C, each with a different N-terminal region (Segraves and Hogness, 1990). E75B is unique in that it lacks one of two zinc fingers, both of which are required for DNA binding. Despite their differences, the three protein variants appear to have significant functional redundancy (Bialecki et al., 2002). While zygotic knockout of all three isoforms is embryonic lethal, isoform-specific mutants survive until much later. All three isoforms, including E75 isoforms from other insects, interact with a second nuclear receptor, HR3 (Hiruma and Riddiford, 2004; Swevers et al., 2002b; White et al.,

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1997). These interactions interfere with the ability of HR3 to activate target genes.

Here, we present evidence that the molecule bound within the ligand binding pocket of E75 is protoporphyrin IX coordinated to iron, commonly known as heme. The iron center of E75-heme can be reversibly oxidized and reduced, which affects the ability of E75 to bind the diatomic gas messengers nitric oxide (NO) and carbon monoxide (CO). NO, CO, and redox state also affect the interaction between E75 and its heterodimer partner DHR3. These findings suggest a number of possible developmental and physiological functions known to be regulated by heme, NO, and CO. Although heme and gas binding transcription factors have previously been identified (Aono et al., 1996; Dioum et al., 2002; Ogawa et al., 2001; Shelver et al., 1997), the binding of these molecules to a nuclear receptor is unprecedented.

Results

Does E75 Contain a Ligand?

Nuclear receptor proteins produced in heterologous cell systems often copurify with small molecules that, in many cases, help to stabilize the protein and to provide insights into the properties of the true ligand (Billas et al., 2001; Dhe-Paganon et al., 2002; Potier et al., 2003; Stehlin et al., 2001; Wisely et al., 2002). Upon expression of the E75 ligand binding domain (E75 LBD) in *E. coli* and subsequent purification, the purified protein possesses a deep red color (Figure 1A), suggesting that it copurifies with a small molecule chromophore.

A commonly bound molecule in proteins that display a red color is heme. Heme-containing proteins display characteristic electronic absorption spectra that include a major peak, typically at 400–450 nm (the γ or "Soret" peak), and two minor peaks (β and α peaks) in the range of 500–650 nm. The spectrum of aerobically isolated E75 also produces the characteristic peaks of heme-containing proteins (Figure 1B): specifically, an α peak at 575 nm, a β peak at 544 nm, and a γ peak at 425 nm. The positions of the peaks suggest that the heme iron is oxidized (Fe(III) form), as might be expected given that E75 was purified under aerobic conditions. Consistent with this assumption, the addition of oxidizing agents has no affect on the spectrum of the aerobically purified protein (data not shown). In contrast, addition of the reducing agent dithionite shifts the α, β, and γ absorbance peaks to 559, 531, and 426 nm, respectively, consistent with conversion of the heme iron to the ferrous form (Fe(II)).

To see if the heme group of E75 can be reversibly oxidized and reduced, the reduced form was treated with the oxidizing agent ferricyanide. Ferricyanide absorbs very strongly below 500 nm, masking any effects on the E75 γ absorption peak. However, reversion of the α and β peaks to that seen in the Fe(III) form is clear (data not shown).

We also expressed the E75 LBD and full-length protein in SF9 cells, S2 cells, and developing flies. In all cases, the affinity-purified proteins are red and show the same absorption spectra as shown in Figure 1B. Figure 1C shows the absorption spectrum of a multiply tagged E75 LBD protein affinity purified from pupae.

To provide stronger evidence for the presence of heme, purified E75 LBD was subjected to nondenaturing electro-spray mass spectrometry (ES-MS) (Loo, 2000). The multiply charged protein ions observed in the mass spectrum (Figure 2A) correspond to a mass of 30817.4 Daltons (Da), which is 616.3 Da heavier than the predicted mass of the E75 LBD protein (30201.1 Da). A significant peak at 616.2 m/z is also observed, consistent with partial removal of the ligand by the ion beam. These masses correspond well with the theoretical mass of heme (616.48 Da).

Subsequent collision-induced dissociation (CID) was used to remove the ligand of the protein-ligand complex peak at 2802.39 m/z seen in Figure 2A. New masses corresponding to unliganded E75 LBD, at m/z 3020.96 (10+) and m/z 2746.27 (11+), and to heme at 616.19 Da were produced (Figure 2B). A small peak at m/z 557, also seen in the CID spectrum, corresponds to a characteristic ion arising from the loss of a CH_2COOH group from heme. E75 LBD purified from insect cells produces the same sets of peaks (data not shown). We infer from these results that the dissociated heme molecule is tightly bound to the E75 LBD at a stoichiometry of 1:1. This stoichiometry was further verified by comparing the molar ratios of protein (amino acid analysis) and iron (atomic absorption) (data not shown).

Does Heme Function as an E75 Ligand?

Most nuclear receptors can be found in both the apo and ligand bound forms. However, under our expression and purification conditions, no apo-E75 was detected. To test whether the heme moiety in E75 can be dissociated, His-tagged E75 was bound to nickel beads and then treated with increasing concentrations of the denaturant guanidine hydrochloride (GdnHCl). Eluates were collected and monitored for heme or E75-heme by electronic absorption (Figure 3A). The eluates, treated with up to 5M GdnHCl, contained no significant amount of heme, protein bound or free. Extensive dialysis of the protein-heme complex against 100 mM EDTA also failed to dissociate or chelate the heme bound iron (data not shown). Dissociation of the heme-E75 complex also failed to occur when the protein was incubated in the presence of cellular or embryonic extracts (data not shown). These results, taken together with the stability of the E75-heme complex during mass spectrometry, are consistent with the heme moiety being tightly bound and well protected within the ligand binding pocket of the LBD. Furthermore, our inability to detect apo-E75 in any of the tested cell types suggests that heme may be essential for protein folding and accumulation.

Is E75 a Heme Sensor?

If E75 protein is dependent on heme for stability and accumulation, then varying the levels of cellular heme should alter the expression levels of E75 protein. This was tested by supplementing SF9 insect cell media with heme. Figure 3B shows that supplemental heme increased the amount of E75 LBD expressed by about 8-fold (1.57 mg –heme versus 11.6 mg +heme). Similar effects were seen with full-length E75A (data not shown). The addition of heme to SF9 cells infected with

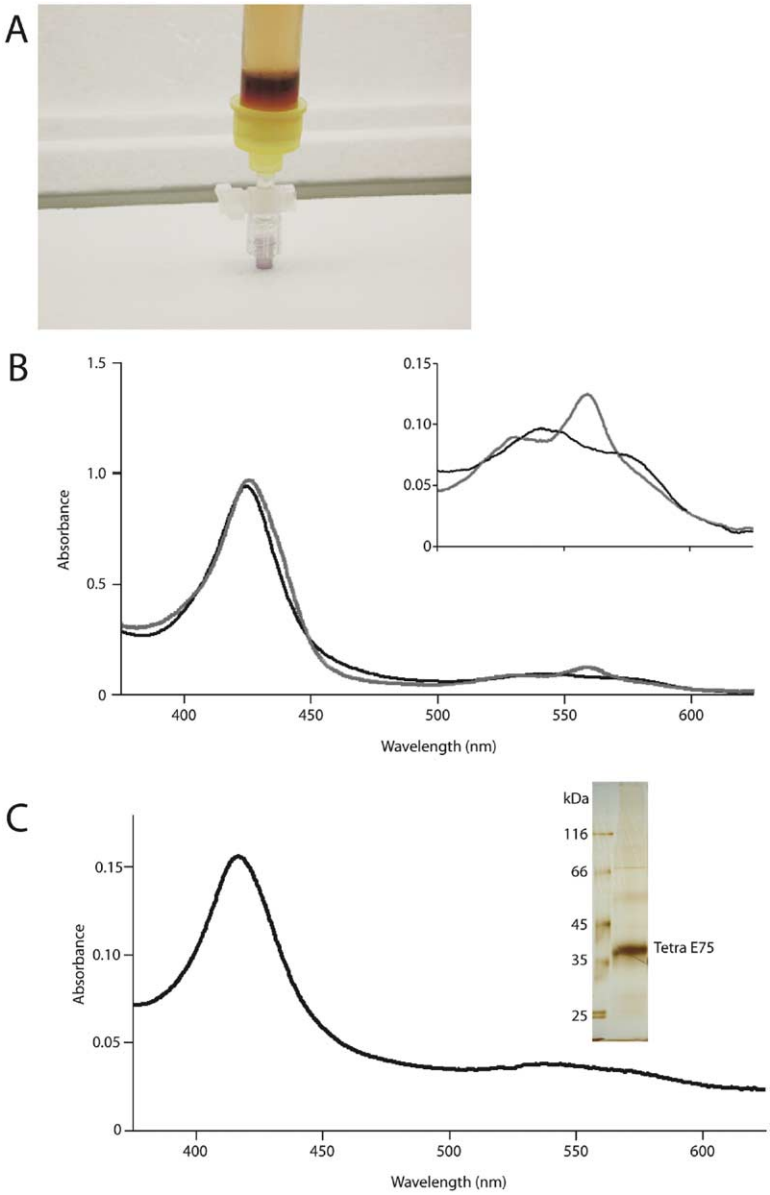

Figure 1. The E75 LBD Copurifies with a Chromophore

(A) Photo of HIS-tagged E75 LBD following expression in *E. coli* and binding to a Ni-NTA column.

(B) Electronic absorption spectra of E75 LBD purified aerobically from *E. coli* (black trace) and after reduction (gray trace).

(C) Electronic absorption spectrum of E75 LBD affinity purified from flies. The inset shows a silver-stained gel of the purified protein.

viruses expressing other nuclear receptor LBDs had no effect on their expression levels (data not shown). We conclude that heme is specifically required for E75 LBD stability, and that levels of E75 expression are proportional to the levels of available heme. Thus, E75 could function as a heme sensor, accumulating and regulating gene expression in accordance with the levels of available heme.

How Is Heme Bound?

The majority of known heme-protein interactions are mediated by coordinate bonds between the heme center and histidine side chains. E75 has four well-conserved His residues in the LBD (Figure 4A). To test if these might be important for heme binding, each residue was mutated to Ala and Phe, residues that are structurally similar to His but do not have the ability to form coordinate bonds. The mutant proteins were expressed in bacteria, purified, and examined for color and absorption. Figure 4B shows spectra of each mutant following identical purifications with a single affinity column. Mutation H585F has no affect on E75 expression or heme binding and, although H416F and H585A result in a substantial reduction of soluble protein recovered, both exhibit characteristic heme absorption peaks. Conversely, His364F/A and His574F/A result in no obvious protein band or heme absorption peak. Since the H416 and H585 mutants still retain the ability to bind heme, they are poor candidates for residues that bind directly to the heme iron. H364 and H574, however, are good candidates, although the effects observed could also be due to disrupted protein folding. Interestingly, these two critical residues are the most highly conserved in E75 orthologs and homologs,

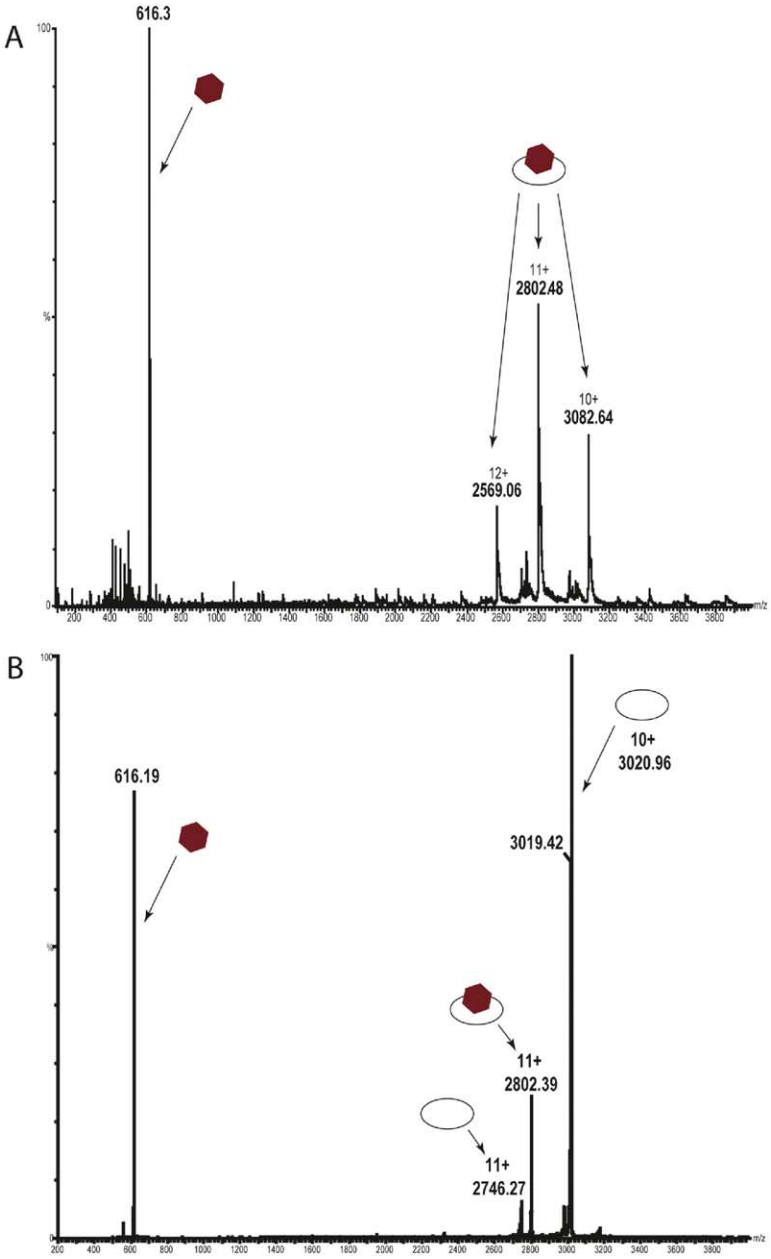

Figure 2. ES-MS Spectrum of the E75 LBD-Ligand Complex

(A) ES-MS under nondenaturing conditions. Deconvolution of the differentially charged peaks (10^+– 12^+) in the higher m/z range reveal a protein-heme complex.

(B) Q-TOF CID Spectrum of the E75 LBD-ligand complex ion (11^+) at 2802.39 m/z (from [A] above).

ranging as far away evolutionarily as the mammalian Rev-Erb proteins (Figure 4A).

Is E75-Heme a Redox Sensor?

To identify possible cofactors for further studies on the role of heme in E75, we designed and obtained peptides corresponding to 40 putative interaction binding motifs. The peptides were then tested in a high-throughput "Stargazer" screen (see Experimental Procedures). Typically, proteins in this screen show increased stability in the presence of ligand, which results in an increase in their denaturation transition temperature. Nonspecific interactions either have no effect or destabilize the target protein, the latter resulting in a decrease in the transition temperature. Figure 5A shows results for the 30 of 40 peptides that generated usable data. Binding of the FTZ-F1 LBD interacting with the LXXLL motif of its Fushi tarazu protein cofactor (Schwartz et al., 2001) is shown as a positive control (Figures 5A and 5B). Addition of the FTZ peptide increases FTZ-F1 thermostability by approximately 4°C (Figure 5B).

Of the 30 peptides tested, one produces statistically significant increases in the E75 LBD transition temperature. This peptide, which corresponds to the AF2 region of the E75 heterodimer partner DHR3 (NVVFPALY KELFSIDSQQD), increases E75 thermostability by 2.9°C (Figure 5C). This result suggests that the E75-DHR3 interaction may be mediated, in part, by contacts between the DHR3 AF2 motif and the E75 LBD. Interest-

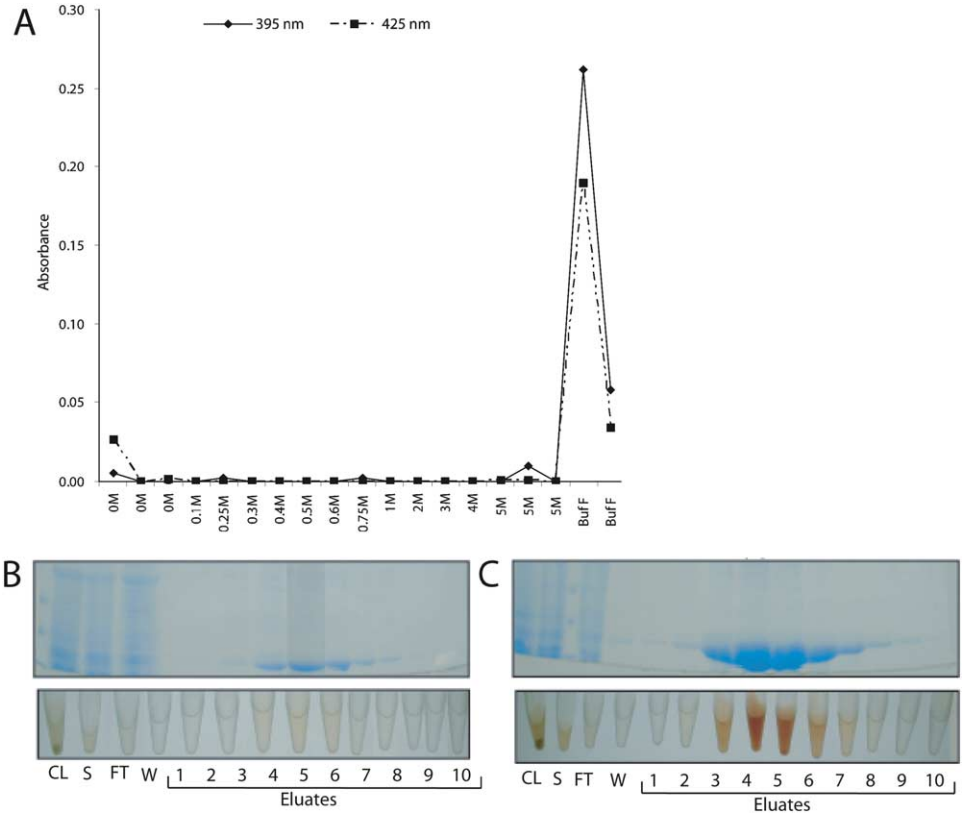

Figure 3. Stability of E75-Heme Interaction

(A) Absorbance readings are shown for Ni^{++} purified His-tagged E75 treated with increasing concentrations of GdnHCl at 395 nm (solid line) and 425 nm (dashed line). Buf F represent the low pH elution.

(B) Expression and purification of E75 LBD from SF9 cells without additives to the growth media. The upper panel shows a Coomassie-stained gel of the purified fractions. The lower panel shows photos of the tubes containing the corresponding fractions loaded on the gel above.

(C) Expression and purification of E75 in the presence of 1.5 mM supplemental hemin.

ingly, this thermostabilization only occurrs when binding is carried out with the reduced Fe(II) form of E75 (Figure 5D). As with the previous absorption spectra data, this result is consistent with the possibility that E75 is a cellular redox sensor.

Is E75-Heme a Gas Sensor?

For many heme-containing proteins, the heme moiety facilitates the binding of diatomic gases (for review see Gilles-Gonzalez and Gonzalez, 2005). In these studies, interactions were demonstrated using diagnostic changes in protein electronic absorption peaks in the presence of O_2, NO, or CO.

Addition of O_2 to Fe(II) E75 has no immediate effect on the spectrum. Only after several hours does the spectrum shift gradually to that of the oxidized form (data not shown), suggesting that E75-heme does not act as a direct O_2 sensor. On the other hand, addition of either CO or NO to Fe(II) E75 causes immediate and substantial shifts in the spectra (Figure 6A). CO coordination causes a narrowing and blue shift of the Soret peak (421 nm) as well as broadening of the α/β region. Addition of NO results in a significant broadening and blue shift of the Soret peak (391 nm) and complete loss of peak resolution in the α/β region. Although NO also acts as an oxidizing agent, the rapid shift of the spectrum and the presence of a large excess of reducing agent suggests that this spectrum represents NO coordinately bound to Fe(II) E75. These spectra are highly similar to those of other heme-containing gas sensors such as CooA and sGC (Aono et al., 1996; Reynolds et al., 2000; Shelver et al., 1997; Stone and Marletta, 1994).

The interaction of CO with Fe(II) E75 was also tested using Stargazer (Figure 6B). The short half-life of NO in solution prevented its use in this assay (Stargazer takes 1–2 hr). CO, which is relatively stable in solution, increased the Fe(II) E75 transition temperature by 6.4°C. No effect of CO was observed on oxidized Fe(III) E75. Interestingly, addition of the DHR3 AF2 peptide to Fe(II) E75 had no further effect on E75 stability beyond the effect of CO alone. This lack of an effect beyond that of CO suggests that the DHR3 AF2 is unable to bind E75 in the presence of CO.

To summarize, both NO and CO interact with the reduced form of E75 (NO shown by UV absorption only), and CO, while stabilizing the protein, appears to prevent binding of the DHR3 peptide.

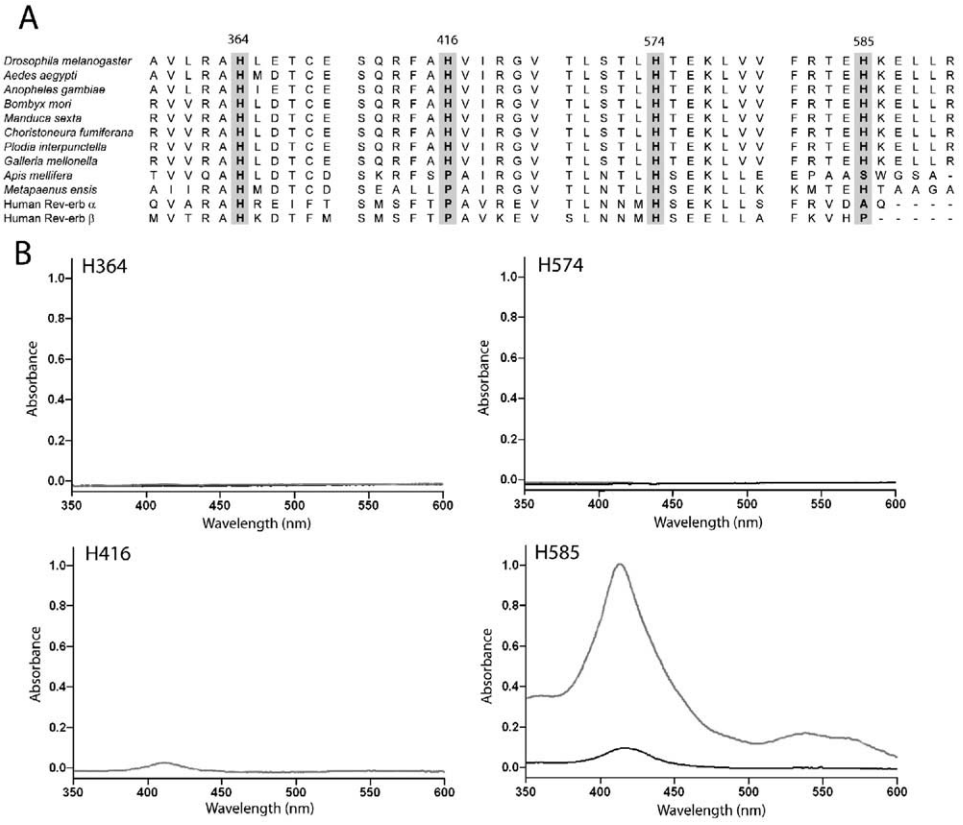

Figure 4. Histidine Residues in the E75 LBD Are Essential for Heme Binding and LBD Stability

(A) Amino acid alignments of conserved histidine residues of *Drosophila* E75 and homologs from other insects, shrimp, and the closest human nuclear receptors. Numbering is based on the *Drosophila* E75A sequence.

(B) Sample spectra from histidine mutants following single-step nickel purification. Histidine to alanine is shown in black, histidine to phenylalanine is shown in gray. No mutant for H416A was obtained.

Effects of Heme and NO on E75 Transcriptional Activity

E75 acts as a repressor in transient transfection assays (White et al., 1997). Both the DNA binding E75A isoform and the non-DNA binding E75B isoform can heterodimerize with DHR3, blocking its ability to activate transcription (Sullivan and Thummel, 2003; White et al., 1997). To determine the effects of heme and NO on these activities, we established a luciferase reporter assay using *Drosophila* Kc cells and tested the effects of supplemental heme and NO donor molecules on E75 activity. The reporter gene constructed contains consensus binding sites for E75A and DHR3 (EAR: sites; White et al., 1997).

Figure 7 shows that, consistent with previous results (White et al., 1997), DHR3 on its own acts as a transcriptional activator (~10-fold over basal) and, when cotransfected with either E75A or E75B, is reduced in activity by 3- to 4-fold (using equal amounts of transfected DHR3 and E75 vector DNA). Adding supplemental heme further reduces the levels of reporter gene expression to near background levels (data not shown). Based on our earlier results with E75 expression in bacteria and cultured cells, we assume the latter is most likely due to an increase in E75 stability and accumulation.

The ready availability of NO donors with half-lives compatible with the time course of incubation (24–48 hr) allowed us to examine the effects of NO in situ. Addition of 200 μM DETA-NO ($T_{1/2}$ ~20 hr) to the culture media significantly affects the ability of both E75A and E75B to interfere with DHR3-mediated transcriptional activation, returning the levels of DHR3 activity close to the levels obtained in the absence of E75 isoforms. This effect was E75 specific, as no effects were seen on DHR3 on its own (Figure 7B) or on other nuclear receptors (data not shown). To summarize, NO appears to function as an antagonist of E75 repressor activity. Further testing will be required to determine if and how CO affects E75 transcriptional activity.

Discussion

We have shown, using electronic absorption and mass spectrometry, that the *Drosophila* nuclear receptor E75 contains a single tightly associated heme prosthetic group. Thus, nuclear receptor LBDs can now be added to the limited repertoire of known heme binding motifs. All results here are consistent with a conventional heme-protein interaction mediated by a pair of coordinate bonds. Two highly conserved histidine residues are good candidates for the interacting residues.

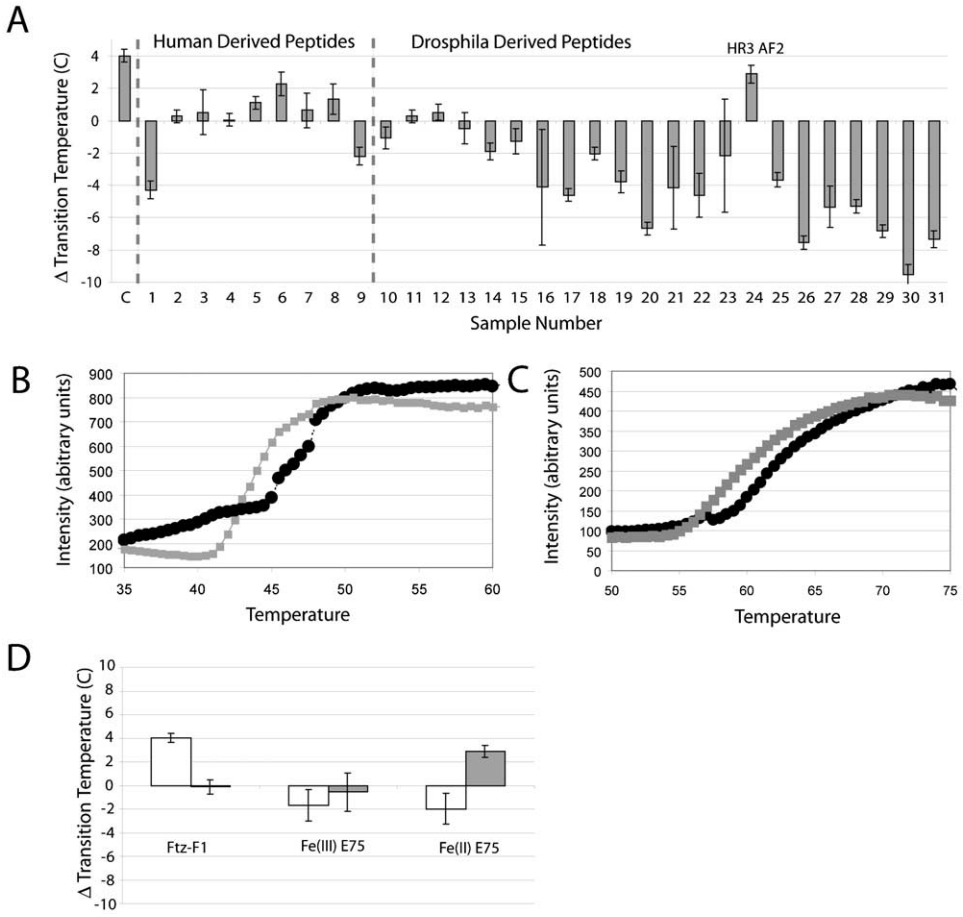

Figure 5. Peptide Binding

(A) Summary of changes in transition temperatures upon addition of peptides to E75 Fe(II). Change is relative to E75 Fe(II) without peptide. The letter C on the left denotes the control reaction: FTZ-F1 binding to the FTZ-derived LXXLL peptide relative to FTZ-F1 alone. The peptide derived from the putative AF2 helix of HR3 is indicated. Results are shown as the average of duplicate data sets ± standard deviation (SD).

(B) Sample transition curves of FTZ-F1 in the presence (black circles) or absence (gray squares) of FTZ LXXLL peptide.

(C) Sample transition curves of Fe(II) E75 in the presence (black circles) or absence of (gray squares) the DHR3 AF2 peptide.

(D) Effect of E75 oxidation state on Stargazer-monitored interactions in the presence of FTZ (white) or DHR3 AF2 (gray) peptides. Changes are relative to FTZ-F1 (shown as a control), E75 Fe(III), or E75 Fe(II) in the absence of peptide. Results are shown as the average of duplicate data sets ± SD.

Our results suggest three general ways in which E75-heme may function. First, the necessity of heme for E75 LBD stability and the changes in expression levels brought about by supplemental heme suggest that E75 may function as a nuclear monitor of cellular heme levels. Second, the ability of E75-heme to switch between oxidized and reduced states and the effects of these states on CO, NO, and cofactor peptide binding suggest a possible role as a cellular redox sensor. Third, the ability of E75-heme to bind NO and CO, and for these gases to modulate cofactor binding and transcriptional activity, suggests a role in mediating NO and/or CO intercellular signaling. This is the first example of a nuclear receptor with a bipartite ligand binding system.

Heme as a Nuclear Receptor Ligand

Nuclear receptors were first characterized based on their ability to bind steroid hormones but are now known to bind a fairly diverse set of lipophilic molecules including fatty acids, phospholipids, retinoids, bile acids, farnesoids, and a range of xenobiotics. Like most other nuclear receptor ligands, heme is also a lipophilic molecule with polarized negative charges. Its molecular weight and solvent-excluded volume, although larger than most, are within the range of other known ligands. For example, one of the closest vertebrate homologs to E75, Peroxisome proliferator activator receptor γ (PPARγ), has a pocket that can easily accommodate a molecule of this size (Nolte et al., 1998; Xu et al., 1999). Although heme is not dissimilar to other ligands in most physical attributes, an iron atom within a protoporphyrin ring brings an exciting new dimension to the potential molecular and physiological roles of nuclear receptor proteins.

E75 as a Heme Sensor

The tight interaction generally formed between heme molecules and their protein partners, also observed

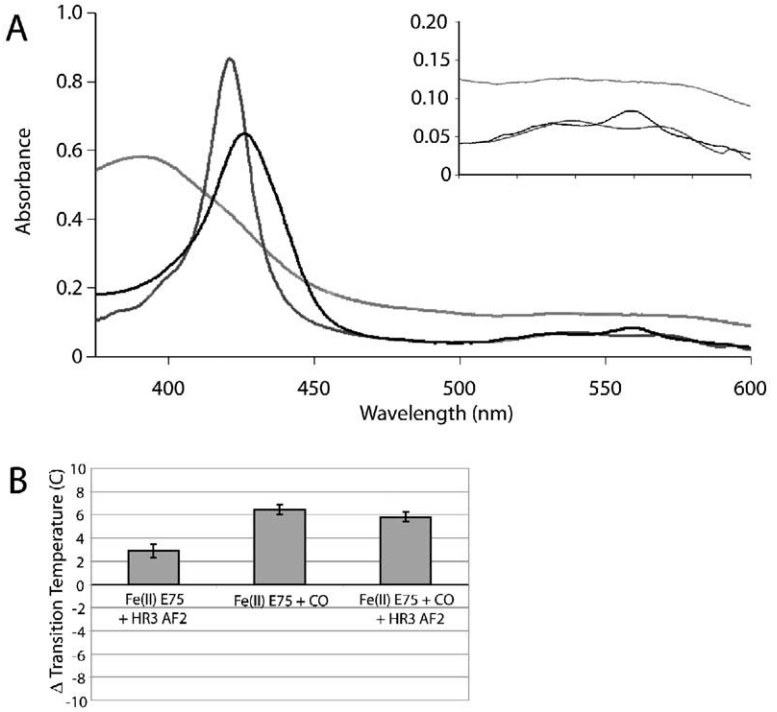

Figure 6. Fe(II) E75 Binds Diatomic Gases

(A) Electronic absorption spectra of Fe(II) E75 without gas (black), plus CO (dark gray), and plus NO (light gray).

(B) Effect of CO binding as observed by Stargazer. Changes are relative to E75 Fe(II) in the absence of peptide. Results are shown as the average of duplicate data sets ± SD.

with E75, and our inability to detect apo-E75 suggest that E75 and heme are unlikely to associate and dissociate readily. For this to occur, dedicated cofactors would be required to shuttle the heme in and out of the LBD and to stabilize the LBD in the absence of heme. Although we have not found evidence for such cofactors, it is possible that endogenous E75 is expressed in certain tissues or stages in which such cofactors exist. If, on the other hand, these cofactors are not present, or do not exist, heme would be required as a dedicated structural component. In the latter case, the levels of free heme in the cell would determine the levels of E75

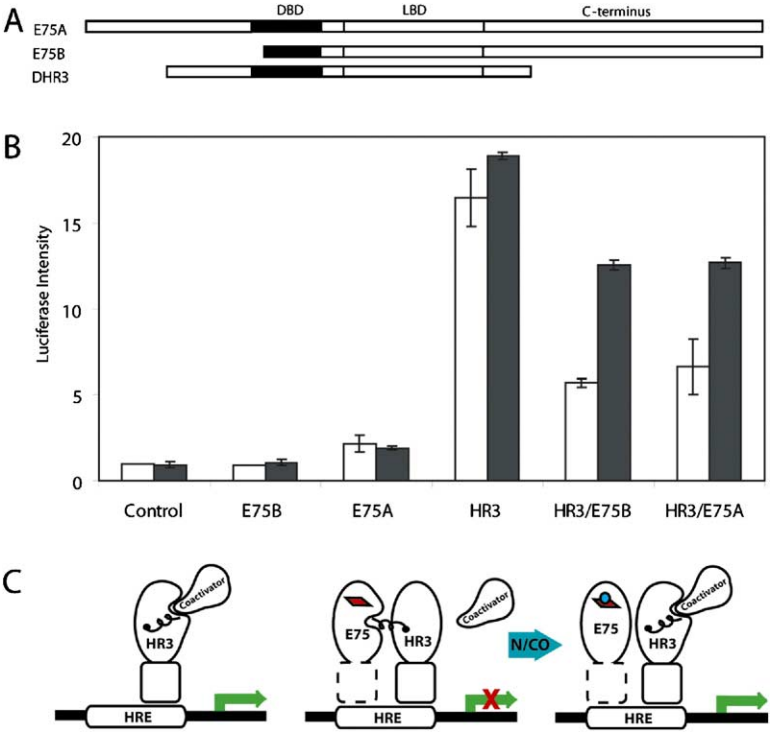

Figure 7. Effects of NO on E75 Transcriptional Activity

(A) Schematic representation of the constructs used for the full-length nuclear receptor reporter assay.

(B) Effects of exogenous NO on transcriptional activity. Empty bars represent untreated cells and black bars represent cells treated with NO donor. Labels below each pair of bars indicate the combination of expression constructs transfected. "Control" represents reporter only. Results are shown as the average of triplicate data sets ± SD.

(C) Proposed model of E75-gas regulation of HR3 transcriptional activation.

that can accumulate. Either way, heme availability would determine the levels of active E75 in the cell. If E75 target genes include regulators of heme metabolism, these properties would make E75 an ideal regulator of heme homeostasis. Processes potentially affected by this are discussed below.

Heme is required by proteins that control an enormous range of cellular and biological processes. Examples of these processes (and proteins) include energy transfer (mitochondrial cytochromes), lipid and drug metabolism (cytochrome P450s), heme metabolism (heme oxygenase [HO]), oxygen radical detection and removal (superoxide dismutases, catalase, NADPH hydroxylase), gas transport (hemoglobin, myoglobin), iron transport (HO-FE-ATP pump), neuronal differentiation, and behavior (guanylate cyclase, nitric-oxide synthase [NOS]) and circadian rhythm (HO, NPAS2) (see Pardee et al., 2004; Ryter and Otterbein, 2004; Wagener et al., 2003 and references therein). Interestingly, NOS and HO, the enzymes that produce NO and CO in the cell, are also heme-containing proteins (discussed further below).

Of the physiological processes listed above, particularly intriguing and relevant ones are lipid and xenobiotic metabolism. In vertebrates, most of the enzymes that regulate these reactions are cytochrome P450s, which typically use heme to transfer oxygen or hydroxyl groups to or from their substrates (Poulos, 1988). Homeostasis between lipid absorption/production and secretion/breakdown is controlled by nuclear receptors, largely through transcriptional regulation of cytochrome P450 (*cyp*) genes (reviewed in Chawla et al., 2001). In turn, the substrates or products of the P450-regulated reactions are often ligands for the nuclear receptors that regulate expression of the respective P450 genes. This triangular relationship between transcriptional regulators, enzymes, and reaction products provides the necessary feedback for homeostasis. Given that a close link between heme and lipid metabolism is also well documented (Bauer et al., 2004; Chawla et al., 2001; Lorenz and Parks, 1991; Pardee et al., 2004), the ability of a nuclear receptor to monitor levels of heme could provide a general mechanism for coordinating these processes.

E75 as a Redox Sensor

The iron center of E75 can reversibly switch between Fe(III) and Fe(II) oxidation states. Furthermore, the interaction with an HR3-derived peptide is selective for the Fe(II) oxidation state of E75-heme. This set of characteristics sets up the intriguing possibility of E75 acting as a direct redox sensor. An interesting example of this behavior is the reported heme-based redox sensor activity of Ec-DOS (Kurokawa et al., 2004; Sasakura et al., 2002), a phosphodiesterase in *E. coli*. Enzymatic activity is modulated by an allosteric change in the heme-containing PAS domain. The heterodimerization and transcriptional activities of the NPAS2 and Bmal1 transcription factors, which regulate circadian rhythm, have also been shown to depend on redox potential in vitro (Rutter et al., 2001). NPAS2 has two heme-containing PAS domains (Dioum et al., 2002), which could provide the mechanism for redox detection. Ec-DOS and NPAS2 also function as gas sensors (see below).

E75 as a Substrate for Gas Ligands

In many heme bound proteins, the heme molecule serves as a cofactor or prosthetic group for the binding of diatomic gases. Our results show that this is also the case for E75-heme, and that NO and CO may serve as E75 ligand(s). Although small, these diatomic gases have been shown to bring about significant changes in protein structure. In heme-containing proteins such as hemoglobin, myoglobin, guanylate cyclase, CooA, and FixL, for example, the binding of O_2, CO, or NO causes allosteric rearrangements that modulate protein multimerization, enzymatic activity, and/or the ability to interact with cofactors or target molecules (reviewed in Gilles-Gonzalez and Gonzalez, 2005).

In the case of E75, our peptide binding studies suggest that CO interferes with the ability of E75 to interact with the DHR3 AF2 motif. Although unable to confirm this by direct binding assays, our results with NO on E75 transcriptional activity suggest that NO may act similarly. This may be analogous to the case of soluble guanylate cyclase, where NO and CO binding both lead to functional activation, but to varying degrees (for review, see Jain and Chan, 2003). As with other nuclear receptors, proper placement of the DHR3 AF2 helix within its ligand binding domain is most likely required for DHR3 to bind transcriptional coactivators. If E75 were to sequester the DHR3 AF2 helix, then DHR3 activity would be compromised. By reversing the AF2-E75 interaction, NO and/or CO could then restore normal DHR3-coactivator interactions (modeled in Figure 7C).

General Roles of NO and CO

NO and CO are coming to be implicated in a rapidly growing list of processes in vertebrates (Ignarro, 2000; Ryter and Otterbein, 2004), plants (Neill et al., 2003), and insects (Bicker, 2001). Their ability to diffuse readily between and within cells, and their short half-lives, make them ideal intercellular signaling molecules. In many cases, they act together or in opposition. Examples of processes regulated by NO and CO include blood pressure, cell division, cell death, inflammation, metabolism, hypoxia, diurnal cycles, behavior, and memory (Ignarro, 2000; Ryter and Otterbein, 2004; Snyder and Ferris, 2000). The primary source of NO in tissues is the enzyme NOS and for CO, HO. In flies, mutation of the *dnos* gene results in embryonic lethality (Regulski et al., 2004). Flies also contain a single HO gene (Zhang et al., 2004), but genetic and functional analyses of dHO are yet to be carried out.

The fact that CO production is dependent upon heme as a substrate, that NOS and HO both have heme centers, and that they have related physiological functions poses an interesting set of coincidences. This and the convergence of heme with lipid synthesis and related processes, NO and CO gas binding and now E75 function (reviewed in Pardee et al., 2004), suggests that E75 may provide a unifying role in the regulation of these processes.

Possible Roles for E75-Heme in Insects

Numerous studies have placed E75 genetically, transcriptionally, and functionally in the ecdysone response pathway, both upstream and downstream of the ecdy-

sone receptor (Bialecki et al., 2002; Segraves and Hogness, 1990; Sullivan and Thummel, 2003). Thus, E75 appears to play roles in both ecdysone production and response. Ecdysone-regulated processes during insect development include cuticle formation, molting, programmed cell death, neurogenesis, imaginal disk development, and oogenesis (Brennan et al., 1998; Buszczak et al., 1999; Ward et al., 2003). The binding of E75 to heme suggests possible connections between these processes and heme metabolism or function. This could occur at a variety of different levels. Some intriguing possibilities include the regulation of hormone-synthesis pathways, oogenesis arrest, metabolism, and the control of circadian rhythm. These possible roles are discussed briefly below.

As discussed earlier, an intimate functional triangle exists between nuclear receptor function, cytochrome P450 expression, and cytochrome P450 substrates. This relationship, taken together with the apparent need for E75 in ecdysone production and response, suggests the possibility that E75 could control ecdysteroid metabolism by regulating the transcription of key *cyp* genes, or more globally by sensing the levels of available heme. The possible ability of E75 to regulate hormone synthesis, and its known role in controlling the progression of molts, metamorphosis, and oogenesis, also brings up other intriguing possible functions. One example might be the ability to monitor energy resources such as lipids and to coordinate these levels with developmental progression. More specifically, the close link between heme, NO, and lipid metabolism could be used to block or postpone molting, pupariation, or oogenesis when energy resources are low, and vice versa.

Interestingly, in mosquitoes, oogenesis is halted prior to chorion deposition until the insect obtains a blood meal. Although the blood-meal components that release the oogenesis arrest have yet to be identified, one of the major components of the blood meal is heme removed from the metabolized hemoglobin. Taken together with the observations that one of the responses of the blood meal is a pulse of ecdysone synthesis and action and that in *Drosophila*, E75 functions in both a feed forward and downstream role in the ecdysone signaling pathway, E75 may play a key role in this response by responding to the ingested heme and inducing ecdysone synthesis.

A number of possible links also exist between E75 function and a role in regulating circadian rhythms. First, NO, CO, and heme appear to be important regulators of circadian oscillators. In vertebrates, "light" and "dark" inputs are thought to be converted to signals of NO and CO, which in turn modulate the phase and period of the circadian cycle (Artinian et al., 2001; Ding et al., 1994). Heme biosynthesis is also reciprocally regulated by the circadian clock (Kaasik and Lee, 2004). Second, the closest homolog to E75 in vertebrates, Rev-Erbα, is a well-established regulator of circadian rhythm in mammals, also acting as a transcriptional repressor and competing with the nuclear receptor RORα, which is a transcriptional activator of the circadian regulator Bmal1 (Akashi and Takumi, 2005; Nakajima et al., 2004; Preitner et al., 2002; Sato et al., 2004). The interaction between Rev-Erbα and RORα is analogous to that of E75 and HR3, their closest insect homologs. Third, each larval instar molt, as well as the onset of metamorphosis and adult eclosure, are coupled to the circadian clock. Molts between first and third instars occur every 24 hr, and metamorphosis and eclosure generally begin on the mornings of the fifth and ninth days. These events are regulated by rhythmic pulses of juvenile hormone and ecdysone (Thummel, 1995), which are under E75 control (Bialecki et al., 2002; Dubrovskaya et al., 2004; Thummel, 1995). In insects that take longer to develop, such as Rodnius (21 days), ecdysone levels have been shown to oscillate daily, with levels highest at night (Vafopoulou and Steel, 1996a; Vafopoulou and Steel, 1996b). E75 may time these oscillating events indirectly by monitoring feeding activity (lipid intake) or through a more direct role in circadian rhythm.

An interesting implication of this study is that the orphan receptor, Rev-Erbα, may also bind heme and respond to diatomic gases. If it does not, compensating evolutionary steps may have been adopted to maintain the circuitry of E75-heme-regulated processes. It is also possible that other nuclear receptors may have adopted or conserved the capacity to bind heme and diatomic gases and to regulate corresponding developmental and physiological functions.

Experimental Procedures

Expression Constructs

For bacterial expression, the E75 LBD, consisting of amino acids G341–S602, was subcloned into the vector pET15B (Invitrogen) between the NdeI and BamHI restriction sites. For baculovirus expression, the vector pFASTBAC DUAL (Invitrogen) was modified to include a His_6 tag and a thrombin cleavage site. These were taken from pET15b (Novagen) and inserted into the BamHI and NotI sites of the modified baculovirus vector. Coding regions for full-length E75A and E75 LBD (residues A345–G604) were inserted into the NotI and XbaI sites of pFASTBAC DUAL (Invitrogen), to generate recombinant baculoviruses. For expression in flies and *Drosophila* cells grown in culture, E75 LBD (A345–G604) was subcloned into a derivative of pCaSpeR-hs-act (C. Thummel) named pTetraCasper, made by adding a 4-tag cassette, consisting of protein A, Calmodulin, 3×FLAG, and His_6 upstream of the multiple cloning site (plasmid construction is described in detail in Yang et al., 2005).

For cotransfection studies, the reporter pEar1-Luc was obtained by inserting three recognition sites for E75A and DHR3 (Horner et al., 1995) upstream of the basal promoter of the luciferase reporter vector pGL3 (Promega). Expression vectors pMT-E75A, pMT-E75B, and pMT-HR3 were constructed by inserting full-length E75A, E75B, and HR3 sequences into the NotI and XbaI sites of pMTv5HisA (Invitrogen). cDNA templates for the PCR reactions were kindly provided by C. Thummel. All constructs were verified by sequencing.

Protein Purification

Protein expressed in *E. coli* was purified using Ni-NTA affinity chromatography as described elsewhere (Zhang et al., 2001). Following overnight cleavage by thrombin proteinase at 4°C, the sample was diluted to 20 mM salt using buffer Q_0 (10 mM tris•HCl [pH 8.0] at 4°C, 5% v/v 1,2-propanediol, 5 mM DTT) and loaded onto an anion exchange column (Q Fast-Flow [Amersham Biosciences]). Protein was eluted with a linear salt gradient (Q_0 plus 30 mM–2M NaCl) using an AKTA FPLC (Amersham Biosciences). Fractions containing pure E75 were identified by 280/425 nm absorbance and verified by SDS-PAGE. E75 constructs purified from *Spodoptera frugiperda* (SF9) were prepared as described elsewhere (Orlicky et al., 2001). When used, aminolevulinic acid and hemin (50 mg/l, Sigma) were added 2 days postinfection. *Schneider 2* (S2) cells carrying a

multiply tagged E75 LBD construct (pCaSpeR-Tetra-E75LBD) and full-length E75 proteins were grown as suspension cultures and purified using tandem anti-FLAG and Ni-NTA affinity columns. Transgenic fly lines expressing pCaSpeR Tetra E75 LBD were grown to the pupal stage, induced by a 20 min heat shock, harvested, homogenized, clarified, and purified as above (described in detail in Yang et al., 2005). Reduction of the heme center of E75-heme was achieved by adding 1 mM sodium dithionite (Sigma).

His Mutants

The bacterial E75 LBD expression vector, described above, was used as the template to mutagenize the four conserved His residues using the quickchange mutagenesis protocol (Invitrogen). All mutants were verified by sequencing, except for H416A, which was not obtained. Proteins were expressed and purified as above with a single affinity column and analyzed by electronic absorption.

Mass Spectrometry

Mass spectrometry analyses were carried out using a quadrupole time-of-flight mass spectrometer (Q-TOF, Micromass) equipped with a nano electrospray (ES) source. In preparation for MS, an additional gel filtration step, using a Superdex 16/60 column (Amersham), was performed. Samples (5 mg/ml) were then desalted in four dilution/concentration steps using centrifugal concentration into 20 mM ammonium acetate (pH 6.2) until a concentration of 1 mg/ml was reached. Samples were loaded on a gold-coated capillary (Protona) with the tip manually opened to produce an orifice of approximately 10 μm. Positive ES was performed at a capillary voltage of 1.8 to 2 kV and cone voltage of 50V. CID experiments were carried out with a collision energy of 80V using argon as the collision gas. Average molecular masses were calculated using Mass Lynx 4.0 (Micromass).

Guanidine-HCl Denaturation

Nickel beads (1 ml) bound with E75 LBD were washed with 1.5 ml wash buffer (500 mM NaCl and 3% 1,2-propanediol with GdnHCl at concentrations of 0, 0.1, 0.25, 0.3, 0.4, 0.5, 0.6, 0.75, 1, 2, 3, 4, or 5 M. The absorbance of eluted fractions was monitored at two wavelengths; 425 nm is λ_{max} of native E75-heme (see Figure 1), 395 nm represents λ_{max} of free heme as well as E75-heme under denaturing conditions (data not shown). Protein remaining on the column after the last treatment was eluted using buffer F (0.2 M acetic acid, 6 M GdnHCl).

Gas Binding In Vitro

Storage buffer (10 mM Tris [pH 8.0] at 4°C, 500 mM NaCl) was degassed and then saturated with CO (Praxair). Based on the mole fraction solubility of CO in water (Gevantman, 2004), the concentration of this CO stock solution was estimated at 1 mM. CO-saturated solution was added to 1 mg/ml reduced protein samples at 1:10 volume ratio, providing an approximately 3-fold molar excess of CO in solution. Storage buffer supplemented with 200 mM Tris•Cl was degassed and saturated with NO gas (Aldrich), then adjusted to pH 8.0. The estimated concentration of NO saturated solution was 1.9 mM and was added to protein samples at a 1:20 volume ratio. Argon and O_2 gases (Praxair) were added to samples directly by bubbling for 10 min.

Peptide Assays

Forty-six unique fluorescein-labeled peptides based on LXXLL-containing and other suspected nuclear receptor-interacting motifs from fly and human proteins were designed and ordered (Sigma Genosys). Forty of the peptides were successfully synthesized. Crude peptides were screened for binding to E75 using Stargazer (Affinium Parmaceuticals). Briefly, protein samples were heated from 27°C to 80°C at the rate of 1°C per minute in clear bottom 384 well plates (Nunc), and protein aggregation was monitored. Images of scattered light were captured every 30 s and the light intensities were translated to arbitrary numbers. Intensities were plotted against temperature for each sample well, and transition curves were fitted using the Boltzmann equation. The midpoint of each transition was identified and is referred to as the transition temperature. Each well contained 50 μl of 200 μM peptide, 0.4 mg/ml E75 LBD (13 μM), 200 mM Tris (pH 8.0) at 4°C, 150 mM NaCl, and 2.5 mM CaCl covered by 50 μl of mineral oil. Each peptide was tested with and without reducing agent and in duplicate. Samples were also tested with CO present at a 5 molar excess (60 μM).

Transcription Assays

Kc cells (15 × 106) were transfected in duplicate with 8 μg each of one of the following expression plasmids: pMT-E75A, pMT-E75B, or pMT-HR3, together with 8 μg of the reporter pEarl-Luc and 4 μg of the transfection control plasmid pPAC-β-gal using Cellfectin (Invitrogen). Cells were grown overnight at 27°C, washed, and induced 8 hr later with 500 μM $CuSO_4$. After 48 hr, cells were collected, lysed, and assayed for luciferase activity (Promega). β-galactosidase (β-gal) activity was measured by adding equal volumes of lysate and buffer containing *o*-nitrophenyl-β-D-galactopyranoside (OPNG). Hydrolysis of OPNG (colorless) to *o*-nitrophenyl (yellow) by β-gal was measured at 420 nm. Luciferase measurements were normalized to β-gal activities and presented as a ratio of the values obtained with untreated cells. Transfections were conducted in parallel in four separate experiments and the numbers averaged. Where used, hemin (Sigma) dissolved in DMSO, was added to the cells to a final concentration of 1.5 mM 16 hr after induction with $CuSO_4$, and 2,2′-(Hydroxynitrosohydrazino) bis-ethanamine (DETA-NO) (Sigma) was added 24 hr following $CuSO_4$ induction.

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