The 90-kDa Heat Shock Protein Is Essential for Ah Receptor Signaling in a Yeast Expression System*

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In an effort to provide a more powerful system to study the Ah receptor (AHR) signaling pathway, we expressed the AHR, its dimerization partner ARNT, and a β-galactosidase (lacZ) reporter gene, driven by two dioxin-responsive enhancers, in the yeast Saccharomyces cerevisiae. In this system, the agonists β-naphthoflavone and α-naphthoflavone induced transcription of the lacZ gene, with EC50 values of 7.9 x 10-8 and 3.0 x 10-7 M, respectively, while the nonagonist dexamethasone was without effect. As a first application of this system, we examined the relationship between the 90-kDa heat shock protein (hsp90) and AHR function. To accomplish this in a manner that was independent of the ARNT protein, we constructed a chimeric receptor in which the DNA binding and primary dimerization domains of the AHR were swapped with analogous domains from the LexA protein. Coexpression of this AHR-LexA chimera and a lacZ reporter gene driven by eight LexA operator sites in a yeast strain with regulatable levels of hsp90, yielded pharmacology that closely mirrored that of the AHR/ARNT/dioxin-responsive enhancer system described above, but only when hsp90 levels were held near their wild type levels. When hsp90 levels were reduced to approximately 5% of normal, AHR signaling in response to agonist was completely blocked despite normal cell growth. These results provide the first genetic evidence for the role of hsp90 in AHR signaling and provide the basis for a powerful new system in which to study this pathway.

The AHR is a ligand-activated transcription factor that mediates a number of biological responses to planar aromatic hydrocarbons (1, 2). One of the classical responses to AHR agonists is the up-regulation of the CYP1A1 gene (3, 4). In the CYP1A1 system, it has been demonstrated that a second protein, known as ARNT, is also required for transduction of the agonist signal (5, 6). Recent cloning studies have demonstrated that the AHR and ARNT harbor N-terminal motifs that conform to the basic tenets of the bHLH structure found in proteins that form heterodimeric DNA binding species (e.g. Myc, Max, Mucy2, E2A) (5, 7–11). It has been shown recently that the bHLH domains of the AHR and ARNT are required for heterodimer formation and the capacity of that complex to recognize cognate enhancer elements, known as DREs (12–15). The AHR and ARNT also have a high degree of sequence similarity in a region now referred to as a PAS domain. In the AHR, this domain has been shown to harbor motifs required for ligand binding, interaction with hsp90, and possibly dimerization with ARNT (7, 16–18). Finally, GAL4 fusion studies with AHR and ARNT suggest that the C termini of these proteins play an important role in the transcriptional activation of target genes by the AHR/ARNT heterodimer (19).

Subcellular fractionation and immunohistochemistry studies suggest that the AHR resides primarily in the cytosol associated with a dimer of hsp90 (20–22). Upon binding ligand the AHR translocates to the nucleus, where it dimerizes with the ARNT protein and the complex attains specificity for DREs (2, 22, 23). Biochemical evidence indicates that AHR-hsp90 association is correlated with an increased ligand binding capacity and that hsp90 dissociation correlates with an increase in receptor affinity for the DRE (24, 25). Interpretations of these results have included the ideas that 1) hsp90 holds the AHR in a conformation capable of binding ligand, 2) ligand binding induces a conformational change that stimulates the dissociation of hsp90, and 3) this dissociation allows nuclear translocation and/or AHR-ARNT dimerization. Despite these provocative biochemical studies suggesting the importance of AHR-hsp90 association, these data are primarily correlative and do not rule out the possibility that hsp90 associations arise in vitro, as an artifact of cell disruption, nor do they establish that hsp90 expression is absolutely required for AHR signaling.

In an effort to address these questions and provide a genetic proof for hsp90’s role in AHR signaling in vivo, we turned our attention to the development of a yeast model system. Since the basic machinery of transcriptional regulation has been shown to be conserved between mammals and yeast, we assumed that a yeast system would serve as a powerful model for AHR signaling (26). Moreover, a mutant yeast strain has been characterized in which the levels of hsp90 can be manipulated (27). This strain has been used to demonstrate a requirement for hsp90 in the signaling pathway of steroid receptors, a family of ligand-activated transcription factors that are structurally unrelated to the AHR and ARNT but whose signaling pathways appear to be mechanistically similar (27). In this communication we demonstrate that the yeast Saccharomyces cerevisiae can provide a useful model system to study AHR-ARNT function and use this system to demonstrate that hsp90 is an essential cellular factor required for the proper AHR signaling in vivo.

Hsp90 is the name given to a family of highly conserved, heat-inducible proteins that have an average apparent molecular mass of 90 kDa. In this manuscript “hsp90” is used generically while “hsp82” and “hsc82” refer to the two members of this family expressed in yeast.
Strains and Plasmids—S. cerevisiae strain A303 (Mata, ura3-52, trplΔ1, his3Δ200, leu2Δ1) was used as a host to transform the AHR, ARNT, and the lac2 reporter gene driven by two DREs. Strain GRS4 (Mato, can1-100, ade2-1, his3-11, 15, leu2-3, 12, trp1-1, ura3-1, hsc82-Leu2+, hsp82-Leu2+), deleted for the hsp82 and hsc82 alleles, contains the low copy number plasmid pMT8 carrying hsp82 under the control of the galactose-inducible GAL1 promoter (27). When grown in media containing 2% galactose, hsp82 is expressed at levels comparable to the combined wild type levels of hsc82 and hsp82. In glucose media, hsp82 is expressed at only 5% of the wild type levels (27). The low level expression of hsp82 appears to be the result of an uncharacterized mutation allowing limited expression from the GAL1 promoter in glucose media (27). pCW10 is a CEN6/ARS4, HIS3-marked expression plasmid containing a phosphoglycerate kinase promoter to drive expression (28). To construct the pCWuAHR, the full-length human AHR was excised from plasmid phuAHR (8) with XmaI and cloned into the corresponding site of pCW10. pYARP1 was constructed by digesting plasmid pBM5/NEO-M1-1 with BamHI and subcloning the ARNT fragment into the corresponding site of the expression vector pYFGE2, a 2p, TRP1-marked plasmid containing a phosphoglycerate kinase promoter to drive expression (5, 29). The reporter plasmid pDRE23-Z was constructed by first subcloning the HindIII/EcoRI fragment of pGEMLS3.2 (30) containing DRE2-DRE3 into pBluescript SK (Stratagene) to generate pSKDRE23. The DRE2-DRE3 fragment was subsequently amplified by polymerase chain reaction from pSKDRE23 using 50 pmol of primers OL146 (5′-GAATTGAATACGACTAATAGGG-3′) and OL290 (5′-CCCTCGAGAGCATCTGATGGCC-3′) in a 50-μl reaction containing 200 μM each dNTP, 2 μM MgCl2, 10 μM Tris, 50 μM KCl, 0.001% gelatin (w/v). The reaction was incubated at 95 °C 5 min, then 72 °C for 5 min, during which 2.5 units of Taq polymerase were added and the reaction continued at 95 °C (1 min), 50 °C (1 min), and 72 °C (1 min) for 25 cycles. The resulting polymerase chain reaction product was digested with XhoI and cloned into the XhoI site of p2UGZ (31) in the same orientation as originally found in pGEMLS3.2. pEG202 is a 2p, HIS3-selectable plasmid containing the coding sequence for amino acids 1-202 of the bacterial repressor LexA under the control of the alcohol dehydrogenase-1 promoter. The plasmid pEGAHRRN1166, containing the AHR with amino acids 1-186 replaced by residues 1-202 of LexA, was constructed by cloning the EcoRI fragment of pSGNA166 (16) into the EcoRI site of pEG202. The reporter plasmid pSH18-34 is a 2p, URA3-selectable vector containing the GAL1 promoter fused to the bacterial lac2 gene in which the UAS, has been replaced with eight LexA binding sites.

Transformations—Plasmids were sequentially transformed into the appropriate yeast strain by electroporation (32) or by a modified LiAc method (33). For electroporation, 100–500 ng of plasmid DNA was added to 50 μl of competent cells and incubated on ice for 5 min. The mixture was then transferred to a 2-mm cuvette and electroporated using a BTX electrophorator manipulator 600 (BTX, San Diego, CA) at 2.5 kV per cm and then immediately placed on selection plates containing 1 μl sorbitol and incubated at 30 °C until colonies appeared. For the LiAc protocol, a single colony of A303 cells was picked and added to 10 μl of crude plasmid DNA in a sterile microcentrifuge tube. After mixing, 500 μl of PLATE solution (40% polyethylene glycol 4000, 100 μM LiAc, 10 μM Tris, pH 7.5, 1 μM EDTA) was added and the mixture incubated at room temperature overnight. Following incubation, the cell/DNA mixture was heated at 42 °C for 20 min and plated on selection media.

β-Galactosidase Assays—For the AHR/ARNT/DRE-Z system in A303 cells, a single colony was inoculated into a 2-ml primary culture of glucose media and incubated at 30 °C overnight. Two hundred microliters of this culture was then used to inoculate a secondary 2-ml culture followed by addition of agonist dissolved in Me2SO. Cultures were grown for 16-18 h at 30 °C and then pelleted and resuspended in 700 μl of Z-buffer (60 μM NaHPO4, 40 μM Na2HPO4, 10 mM KCl, 1 mM MgSO4, 35 mM β-mercaptoethanol). The cells were permeabilized by the addition of 50 μl of Z-buffer and 50 μl of the LacZ solution followed by vigorous vortexing for 30 s. Following permeabilization, 160 μl of an ONPG solution (4 mg/ml in Z-buffer) was added to each tube, mixed, and incubated at 30 °C for 20 h. The reaction was stopped by the addition of 400 μl of 1 M Na2CO3, the cell debris removed by centrifugation at 16,000 x g for 10 min, and the A420 of the sample was determined.

β-Galactosidase assays carried out on the LExAHRNA166 chimera system were performed as above except for the following changes. Yeast strains were grown from a single colony overnight at 30 °C in 2 ml of selection medium containing either 2% glucose or 2% galactose as a carbon source. Fifty microliters of this primary culture was used to inoculate a 1-ml culture of the same media containing appropriate concentrations of agonist dissolved in Me2SO. The culture was grown for 16–18 h with vigorous shaking at room temperature. β-Galactosidase activity was determined by adding a 50-μl aliquot of the cultures to 650 μl of Z-buffer, permeabilizing the cells, and incubating with ONPG at 30 °C for 30 min. β-Galactosidase units were determined using the following formula: (A420/A007) of 1/10 dilution of cells x volume of culture x length of incubation) x 1000.

RESULTS AND DISCUSSION

Experimental Design—One of the most powerful systems available to study hsp90 function in vivo is the yeast S. cerevisiae. Yeast harbor two genes encoding hsp90 homologues (34). One, hsc82, is constitutively expressed at high levels and is only moderately heat-inducible. The second, hsp82, is constitutively expressed at relatively low levels and is highly heat-inducible. Yeast deleted for either gene are viable, while a double mutation is lethal (34). The strain GRS4 carries disruptions for these two yeast hsp90 genes but has been rescued by insertion of a plasmid, pTT8, that contains hsp82 driven by the GAL1 promoter (27). The result of this genetic manipulation is that the levels of hsp90 can be regulated by the presence or absence of galactose in the growth media. When grown in media containing 2% galactose, hsp82 is expressed at levels comparable to the combined wild type levels of hsc82 and hsp82 (27). In glucose media, hsp82 is expressed at only 5% of the wild type levels. Although the low hsp82 expression in glucose is sufficient for cell growth at room temperature or 30 °C (Fig. 1 and data not shown), the levels of hsp82 are insufficient to allow cell survival at 37 °C or ligand-induced signaling through a number of steroid receptor pathways (27).

Reconstitution of the AHR/ARNT/DRE Signaling Pathway in Yeast—To characterize the AHR signaling pathway in yeast, strain A303 was transformed with expression plasmids containing the full-length AHR, its dimerization partner ARNT, and a lacZ reporter plasmid driven by two DREs derived from the CYP1A1 promoter (30). The transformed yeast were exposed to various concentrations of βNF and αNF and the activity from the DRE-driven lacZ reporter was measured (Fig. 2A). The results of at least three independent experiments indicated that both βNF and αNF activated the AHR an average of 12-fold over background in a dose-dependent manner, while dexamethasone, which does not bind the AHR, did not
activate signaling (Fig. 2B). For data presentation, the β-galactosidase units were normalized to the maximal response seen for βNF, a sigmoidal curve was constructed, and the EC₅₀ values generated from the curve. The EC₅₀ values were 7.9 ± 3.6 × 10⁻⁸ M and 3.0 ± 0.9 × 10⁻⁷ M for βNF and αNF, respectively. These values are in general agreement with the known rank order potencies and AHR binding constants (Kᵦ values) reported for these compounds (i.e. 1.8 × 10⁻⁸ M for βNF and 2.9 × 10⁻⁹ M for αNF) (35). In control experiments, we found that βNF did not activate the lacZ reporter in cells expressing only the AHR or ARNT (data not shown). Additionally, reporter gene activity was not activated by vehicle alone (data not shown).

Expression of an AHR-LexA Chimera in Strain GRS4—Once it was established that yeast was an appropriate model system for the AHR/ARNT/DRE pathway, we turned our attention to the development of a model AHR signaling system, where the levels of hsp90 could be regulated. In an effort to apply the yeast GRS4 system, we had to address two issues. First, we had to develop a strategy to study the effects of hsp90 on the AHR in a manner that was independent of ARNT expression. Second, the GRS4 strain has a practical limitation in that its phenotype only allows the use of a limited number of auxotrophic markers and thus transformation with the three independent plasmids required for reconstitution of the entire AHR/ARNT/DRE signaling pathway in GRS4 cells was problematic.

To solve these problems, we constructed a chimera in which the bHLH domain of the murine AHR was replaced with a heterologous dimerization and DNA binding domain from the bacterial LexA protein and used a reporter plasmid containing the lacZ gene driven by eight LexA operator sites upstream of a minimal promoter (Fig. 3). Strain GRS4 was transformed with the LexA-AHRNA166 fusion plasmid pEGAHRNA166 and the LexA operator reporter plasmid pSH18-34. In this system, expression of the reporter gene was dependent on pEGAHRNA166 expression and on the presence of agonist. Incubation with either βNF or αNF caused an average 90-fold induction of lacZ activity over background with EC₅₀ values of 6.1 ± 3.5 × 10⁻⁸ M and 1.9 ± 0.2 × 10⁻⁴ M, respectively. The dose-response curves obtained from this chimeric receptor system closely paralleled those of the complete AHR/ARNT/DRE pathway expressed in A303 cells (Fig. 2C). The EC₅₀ of each agonist used in the chimera system was within 1 order of magnitude of the EC₅₀ values in the AHR/ARNT/DRE-Z system described above, indicating that the LexA-AHR chimera has similar pharmacology to the intact AHR. When transformed into strain A303, ligand responsiveness in this system was unaffected by the carbon source used (data not shown).

Effect of hsp90 on AHR Signal Transduction—Next we used the GRS4 signaling system to test whether the presence of hsp90 is essential for AHR signaling. To confirm that the regulation of hsp82 expression was appropriately controlled in this system, we first examined the heat sensitivity of the strain under conditions of high and low hsp82 expression (galactose or glucose media, respectively). The GRS4 cells transformed with pEGAHRNA166 and pSH18-34 were streaked onto plates containing 2% galactose (high hsp90 expression) or 2% glucose media (low hsp90 expression) and grown at either 23 °C or 37 °C. Cells grown on galactose media grew at either temperature, suggesting normal levels of hsp82 expression, while cells grown on glucose media displayed no growth at 37 °C, consistent with low level expression of hsp82 (Fig. 1).

The dose-response experiments with receptor agonists were repeated with the chimeric receptor system in GRS4 cells under conditions of high and low hsp82 expression (Fig. 4). These experiments revealed that ligand activation of the AHR chimera was highly dependent on hsp82 levels, since low levels of hsp82 completely abolished the response of the receptor to
either βNF or αNF. These results suggest that hsp90 may be required for proper folding of the receptor during or following protein synthesis. If hsp90 is simply an anchoring molecule, which holds the unliganded AHR in the cytosolic compartment, then we might have expected to see some degree of ligand-independent lacZ activation at low levels of hsp82. Since this was not the case, we conclude that the AHR is either improperly folded or destabilized in the absence of hsp82. Data from similar experiments have shown that during low hsp82 expression, the glucocorticoid receptor also signals with reduced efficiency that can be partly overcome with high doses of a potent agonist (27).

In conclusion, this report describes the reconstitution of a functional AHR signal transduction system in yeast and the use of this system to demonstrate that reduction of hsp82 levels abrogates receptor function. This is the first in vivo evidence that hsp90 plays an essential role in AHR activity. The yeast model system reported here should prove to be a useful tool for further investigating the interaction between hsp90 and the AHR. In addition, the ease with which genetic screens can be performed out in yeast will make this a valuable system for performing mutagenesis studies which may advance our knowledge of AHR structure and function. Finally, this system can also be used in conjunction with the recently developed two-hybrid system in yeast to identify possible accessory proteins involved in AHR activity (36) or could be adapted for use in screening for compounds that activate the AHR.

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