Published in final edited form as:

Protein Expr Purif. 2016 June ; 122: 72–81. doi:10.1016/j.pep.2016.02.011.

Binding studies using *Pichia pastoris* expressed human aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator proteins

Yujuan Zheng^{1,†}, Jinghang Xie^{1,†}, Xin Huang², Jin Dong¹, Miki S. Park¹, and William K. Chan^{1,*}

¹Department of Pharmaceutics and Medicinal Chemistry, Thomas J. Long School of Pharmacy and Health Sciences, University of the Pacific, Stockton CA 95211, USA

²Department of Pharmacy, Qianfoshan Hospital of Shandong University, Jinan, Shandong, 250014, China

Abstract

The aryl hydrocarbon receptor (AHR) is a transcription factor which activates gene transcription by binding to its corresponding enhancer as the heterodimer, which is consisted of AHR and the aryl hydrocarbon receptor nuclear translocator (ARNT). Human AHR can be rather difficult to study, when compared among the AHR of other species, since it is relatively unstable and less sensitive to some ligands *in vitro*. Overexpression of human AHR has been limited to the baculovirus expression, which is costly and tedious due to the need of repetitive baculovirus production. Here we explored whether we could generate abundant amounts of human AHR and ARNT in a better overexpression system for functional study. We observed that human AHR and ARNT can be expressed in *Pichia pastoris* with yields that are comparable to the baculovirus system only if their cDNAs are optimized for *Pichia* expression. Fusion with a c-myc tag at their C-termini seems to increase the expression yield. These *Pichia* expressed proteins can effectively heterodimerize and form the ternary AHR/ARNT/enhancer complex in the presence of β -naphthoflavone or kynurenine. Limited proteolysis using thermolysin can be used to study the heterodimerization of these human AHR and ARNT proteins.

Keywords

human AHR; human ARNT; *Pichia* expression; thermolysin

*To whom correspondence should be addressed at Department of Pharmaceutics and Medicinal Chemistry, Thomas J. Long School of Pharmacy and Health Sciences, University of the Pacific, Stockton, California 95211. Fax: (209) 946-2410. wchan@pacific.edu (WKC).

[†]These authors contribute equally to this work.

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1. Introduction

The aryl hydrocarbon receptor (AHR) is a ligand-activated signaling molecule which is involved in many seemingly diverse biological events. This receptor, for example, affects bioactivation of carcinogenic compounds [1], T cell differentiation [2], fatty acid synthesis [3], cell proliferation [4], hematopoietic stem cell differentiation [5], respiratory reactivity [6], and insulin sensitivity [7]. Although the precise mechanism illustrating the endogenous AHR function remains unclear, there has been intense interest in exploring AHR as a potential target for treatment of diseases such as cancer (for review see [8]) and autoimmune diseases (for review see [9]). Currently, AHR is best known for its ligand-dependent signaling events: the ligand-free AHR resides in the cytoplasm as a complex containing a dimer of Hsp90, a molecule of XAP2, and a molecule of p23. Upon ligand binding, AHR reveals the nuclear localization sequence and the whole complex translocates into the nucleus. While in the nucleus, ARNT dissociates the AHR complex and the resulting AHR/ARNT heterodimer binds to the DRE enhancer of the target promoter, activating transcription of genes such as the cytochrome P450 genes – *cyp1a1*, *cyp1a2*, and *cyp1b1* [10].

Species specific differences in the ligand binding environment of AHR explain why ligand binding affinities vary among species. One of the most notable examples is that a conserved change of alanine to valine at the ligand binding domain of the mouse AHR^b allele (amino acid 375) drastically suppresses ligand binding ability [11, 12]. This change may explain why human AHR, which contains a sterically unfavorable valine-381 at the aligned alanine-375 mouse b allele position, has a much lower ligand binding affinity (10-fold less) than the mouse AHR^b allele [13, 14]. Similarly, alignment of the mouse and guinea pig AHR ligand binding domains revealed that one amino acid change from arginine to isoleucine may explain why 3'-methoxy-4'-nitroflavone is a mouse AHR antagonist but a guinea pig AHR agonist [15].

Human AHR is structurally more similar to the “low affinity” mouse AHR (AHR^d, DBA) than the “high affinity” AHR^b allele of C57BL6 (mouse) AHR [14]. However, human AHR does not have low affinity to all ligands. On the contrary, opposite observation holds true in some scenarios. For example, it has been observed that (1) indirubin displaces the ¹²⁵I-labelled azido ligand much more effectively in AHR of human than of mouse [16]; (2) kynurenic acid, which is an endogenous AHR ligand, activates the DRE-driven luciferase activity 100-fold more potently in human than in mouse when hepatoma stable cell lines were used for the assay [17] and (3) StemRegenin 1 antagonizes the TCDD-induced, DRE-driven luciferase activity in human hepatoma cells but has markedly less or no inhibition in mouse and rat hepatoma cells [5]. Surprisingly, TCDD causes only 18% overlap of up-regulated AHR target genes between human and mouse [18], which is likely due to the low sequence identity (< 60%) between the AHR transactivation domains of these two species [13]. Taken together, it is advantageous if we could conveniently generate abundant amounts of the human form of AHR for studies so that results would be more relevant to humans.

Pichia pastoris has been widely used to overexpress functional proteins in the literature. Yeast has been shown to be a useful model system to study the AHR function [19],

suggesting that exogenous AHR expression in yeast could be a viable approach to generate functional AHR protein. Here we explore whether the *Pichia pastoris* expressed human AHR and ARNT proteins can be generated and used for functional study.

2. Material and methods

2.1 Reagents

All restriction enzymes were purchased from New England BioLabs (Ipswich, MA). The AHR ligands (β NF and kyn) and thermolysin were purchased from Sigma (St. Louis, MO). Anti-AHR SA210 rabbit IgG was purchased from Enzo Life Sciences (Farmingdale, NY). Anti-c-myc mouse IgG was purchased from Thermo Fisher Scientific (Grand Island, NY). Anti-ARNT H-172 rabbit IgG was purchased from Santa Cruz Biotechnology (Dallas, TX).

IRDye800 donkey secondary IgG was purchased for LI-COR (Lincoln, NE). IRDye680-conjugated DRE was purchased from Integrated DNA Technologies (San Diego, CA). All oligonucleotides, Dynabeads protein G, zeocin, and chemicals, otherwise specified, were purchased from Thermo Fisher Scientific (Grand Island, NY). The GeneArt codon optimized human AHR and ARNT sequences (GA-hAHR and GA-hARNT) were purchased from Life Technologies (Thermo Fisher Scientific, Grand Island, NY). The pPICZB plasmid and the *Pichia* strains (ySMD1163 and yJC100) were gifts from Dr. Goeff Lin-Cereghino (University of the Pacific). Cobalt agarose beads were purchased from Gold Biotechnology (St. Louis, MO). All ingredients to make bacteria and yeast media (LB, YPD, BMGY, and BMMY) were purchased from either Thermo Fisher Scientific (Grand Island, NY) or Sigma (St. Louis, MO).

2.2 Pichia expression of human AHR and ARNT

Sfu I and Sac II sites were generated at the 5' and 3' ends, respectively, of the GA-hAHR cDNA by PCR. This PCR fragment was then cloned into the Sfu I and Sac II sites of the pPICZB plasmid to generate the pPICZB-GA-hAHR plasmid. This cloned plasmid was used to express human AHR with a c-myc and a 6His tags at the C-terminus. This expression plasmid was linearized with Sac I (which only cut at the plasmid once but not the insert) before being transformed into the freshly prepared ySMD1163 *Pichia pastoris* strain by electroporation using an Eppendorf 2510 electroporator, according to the manufacturer's recommended protocol. The transformed yeast was incubated in 1 mL of 1 M sorbitol at 30°C without shaking for 1 h, followed by the addition of 1 mL of YPD media. The resulting culture was incubated at 30°C for 3 h with rotation at 250 rpm. Cells were then plated onto a YPD plate containing zeocin (100 μ g/mL). After incubating for 2–4 days at 30°C, the transformed yeast colonies were selected and grown on another YPD plate containing zeocin. Colonies were then validated by colony PCR to confirm the presence of hAHR in the genome. For large scale (100 mL) purification, a single colony from a freshly prepared plate was inoculated in BMGY media (50 mL in a 0.5 L baffled flask) and incubated at 29°C with rotation at 250 rpm overnight. After the OD₆₀₀ reached 4 absorbance units, cell suspension (25 mL) was centrifuged at 1,500 g for 5 min at room temperature. The pellet was washed once with BMMY (50 mL) and then resuspended in 100 mL of BMMY containing 0.5% methanol in a 1L baffled flask to induce the GA-hAHR expression

by incubating at 29°C for 12 h with rotation at 250 rpm. To harvest the GA-hAHR protein, cells were centrifuged at 1,500g for 5 min at 4°C. After washing once with 10 mL of cold PBS, the pellet was lysed with the ZrOB05 beads (equal volume as the pellet) in a 5-fold volume of lysis buffer (25 mM HEPES, pH 7.4, 0.3 M KCl, 10% glycerol, 1 mM PMSF, and 2 µg/mL of leupeptin) using a Bullet Blender with the speed setting at 8 for 5 min at 4°C, followed by centrifugation at 14,000g for 30 min at 4°C. The resulting supernatant was collected as the starting material for cobalt-affinity purification as previously described [20]. The enriched GA-hAHR fraction was eluted using the lysis buffer containing 0.5M imidazole, followed by dialysis into the final storage buffer (25 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM DTT, and 50% glycerol).

Expression of human ARNT in *Pichia* is essentially the same as the human AHR expression with following differences: the codon optimized full-length human ARNT (GA-hARNT) cDNA was cloned into Pml I site of the pPICZB plasmid. Sequence-specific primers with a Pml I overhang were used to amplify the full-length ARNT cDNA, followed by the Gibson Assembly method (New England BioLabs, Ipswich, MA) to generate the pPICZB-GA-hARNT plasmid. For large scale (200 mL) preparation, single colony was inoculated in 100 mL of BMGY in a 1 L baffled flask, followed by incubation at 29°C with rotation at 250 rpm until OD₆₀₀ reach 2.5 (about 42 h). Cell suspension (80 mL) was pelleted and washed once with BMMY (20 mL) and then resuspended into 200 mL of BMMY in a 2 L baffled flask to induce the ARNT expression by incubating at 29°C for 6 h with rotation at 250 rpm.

2.3 PCR

Genomic DNA PCR was performed to determine the relative amount of the AHR expression cassettes in the *Pichia* genome after transformation as follows: the wild type ySMD1163 and the protease-deficient ySMD1163 strain transformed with GA-hAHR, WT-hAHR or WT-mAHR were grown in 25 mL of BMGY in a 250 mL flask at 29°C with rotation at 250 rpm until OD₆₀₀ reached 1 (in about 24 h). Aliquots (250 µl) of each culture were centrifuged at 1,000g for 5 min, followed by one time cold PBS wash. Equal pellet volume of ZrOB05 beads was added to the pellet with five times pellet volume of cold buffer (25 mM HEPES, 10% glycerol, 0.3 M KCl, 1 mM PMSF, and 2 µg/mL leupeptin), followed by Bullet Blender treatment at a speed setting of 8 for 5 min at 4°C. The resulting lysed samples were centrifuged at 12,000g for 30 min at 4°C. Each sample was subjected to RNase A treatment for 30 min at 37°C, followed by proteinase K treatment for 2 h at 45°C. Genome DNA was then extracted using Wizard SV gel and PCR clean-up system (Promega, Sunnyvale, CA). The amount of DNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Grand Island, NY). For RT-qPCR, Zymo Direct-zol kit (Irvine, CA) was used to extract RNA, followed by first strand synthesis using Epicentre (Madison, WI) MMLV reverse transcriptase. Bio-Rad (Hercules, CA) SYBR green supermix was used for qPCR. PCR was performed using a CFX Connect Real-Time system (Bio-Rad, Hercules, CA) using the following primer sets: GA-hAHR and WT-hAHR (sense, OL701, GACATCAGACACATGCAGAA and antisense, OL702, AAGTCAATGTCTCTGAAGTCAA; 77 bp, nt 1654–1730 of hAHR open reading frame), WT-mAHR (sense, OL703, TTGGTCTTCTATGCTTCCT and antisense, OL704, TACGCTCTGATGGATGAC; 75 bp, nt 394–468 of mAHR open reading frame), and *Pichia*

actin gene ACT1 (sense, OL705, TTCTTATTCTTCCGATTGATTC and antisense, OL706, AATGAGATGACTGCGTATT). Data was analyzed by the 2^{-CT} method [21].

2.4 LI-COR Western analysis

Protocols for Western analysis were previously described [22]. Anti-c-myc (1:5,000), anti-AHR SA210 (1:5,000), and anti-ARNT H-172 (1:1,000) polyclonal antibodies were used as primary antibodies for overnight incubation at 4°C, followed by IRDye-conjugated donkey secondary antibody (1:10,000) incubation for 2 h at room temperature. LI-COR Odyssey imaging system was used for analysis.

2.5 Gel shift assay

Gel shift assay using the IRDye680-conjugated probe was previously described [22]. In brief, *Pichia* expressed human AHR (GA-hAHR, 2 µL) and human ARNT (GA-hARNT, 0.2 µL) in 50% glycerol buffer were incubated at 30°C for 10 min in the presence or absence of a ligand (10 µM βNF or 30 µM kyn) and 5 µg of proteins (*Pichia* ySMD1163 extract or BSA). *Pichia* extract was the 16,000g supernatant which was prepared by resuspending *Pichia* cell pellet using HEDG buffer, followed by three times freeze/thaw cycle. Gel shift samples were resolved on a 5% native 1XTBE polyacrylamide gel at 4°C for 2 h at 185 V, followed by analysis using a LI-COR Odyssey system.

2.6 Co-immunoprecipitation assay

For *Pichia* human AHR and ARNT interaction study, GA-hAHR (4 µL) was incubated with GA-hARNT (5 µL), and BSA (100µg) in HEDG buffer to a 200 µL final volume at 30°C for 10 min. For *Pichia* AHR and Hep3B ARNT interaction study, GA-hAHR (40 µL) was incubated with Hep3B lysate (150 µg) at 30°C for 15 min. Anti-ARNT H-172 rabbit IgG (0.5 µg) was used to immunoprecipitate ARNT at room temperature for 30 min. Prewashed Dynabeads protein G (5 µL) was then added to each sample, followed by incubation for 1 h (GA-hAHR-Hep3B ARNT) or 4 h (GA-hAHR-GA-hARNT) at 4°C. Beads were washed four times (5 min each) with HEDG buffer containing 0.1% Tween-20. SDS-PAGE electrophoresis sample buffer was then added to each sample, followed by LI-COR Western analysis using anti-AHR antibody (SA210) to determine the amount of AHR co-immunoprecipitated.

2.7 Thermolysin-mediated proteolysis of AHR and ARNT

Thermolysin digestion was used to study the interactions between AHR and ARNT. Affinity enriched GA-hAHR and GA-hARNT were dialyzed into the proteolysis buffer (25 mM HEPES, pH 7.4, 10% glycerol) to a concentration of 2 and 1 mg/mL, respectively. Then, 1 µL of the dialyzed sample was added into 19 µL of the proteolysis buffer, followed by 10 min incubation at room temperature. Samples were placed on ice for 5 min, before the addition of thermolysin (2 µL, 1mg/mL stock solution). After incubation on ice for 1 min, proteolysis was stopped by adding 2 µL of the EDTA solution to make the final concentration of 12.5 mM EDTA. SDS-PAGE electrophoresis sample buffer was immediately added to the sample, followed by vigorous mixing and heat treatment at 95°C for 2 min. Samples were subjected for the LI-COR Western analysis. To study the effect of

AHR on the limited proteolysis of ARNT by thermolysin, GA-hARNT (1 μ L) was incubated with various amounts (0, 1, 5, 10 or 15 μ L) of GA-hAHR (2 mg/mL) or *Pichia* lysate (2 mg/mL) in the presence or absence of β NF (10 μ M) in a final volume of 20 μ L. Limited proteolysis was performed using 1,000-fold less of thermolysin (2 μ L, 0.001 mg/mL stock solution) at 30°C for 10 min.

2.8 Statistical analysis

GraphPad Prism 5 software was utilized for statistical analysis. The significant difference was determined by two-tailed unpaired *t* test to determine the statistical significance with 95% confidence intervals with **p* < 0.05, ***p* < 0.01, ****p* < 0.001, and ns, not significant (*p* > 0.05).

3. Results

3.1 GeneArt cDNA sequences of human AHR and ARNT

In an effort to express human AHR and ARNT proteins in *Pichia pastoris*, we obtained the GeneArt cDNA sequences of human AHR (GA-hAHR) and ARNT (GA-hARNT) which have been optimized for *Pichia* expression. When compared with their wild type counterparts, 23.67% (Fig. S1) and 24.05% (Fig. S2) of nucleotides have been changed in the GA-hAHR and GA-hARNT cDNAs, respectively; however, these changes did not alter the final amino acid sequences.

3.2 Characterization of recombinant human AHR expression in *Pichia pastoris*

We transformed the pPICZ plasmid carrying the GA-hAHR, WT-hAHR, or WT-mAHR cDNA into the protease-deficient *Pichia* strain ySMD1163. After genome integration of the AHR sequence, we observed that the GA-hAHR and WT-hAHR contents were comparable – WT-hAHR was 0.78-fold when compared with GA-hAHR whereas ySMD1163 had negligible background (0.0005-fold) (Fig. 1A). The mAHR content was similarly abundant when compared with the negligible background in wild type ySMD1163 (0.0001-fold) (Fig. 1A). However, only GA-hAHR was noticeably induced after 12 h of methanol treatment at both transcriptional and translational levels (Fig. 1B). Substantial induction of the GA-hAHR protein expression was observed in both the soluble and insoluble (pellet) fractions whereas expression of the WT-hAHR and WT-mAHR proteins was undetectable by Western analysis. Although the crude GA-hAHR protein production was optimal between 6 and 9 h of methanol induction (Fig. 2A), soluble expression of GA-hAHR was actually quite similar between 3 to 12 h (Fig. 2B). The yield of the enriched GA-hAHR by cobalt-affinity purification was modest. The hAHR protein was detectable only by Western but not by Coomassie staining. However, this amount was quite abundant when we compared it to the amount of AHR obtained from human cell lysates (Hep3B, HeLa, and MCF-7) and in vitro transcription/translation using rabbit reticulocyte lysate or wheat germ extract (Fig. 3A). From the Western analysis of the affinity enriched hAHR, we observed that 5 μ L (which is 0.6% of the total yield) of baculovirus AHR from a purification of ten T75 flasks of S₉ cells showed 2.5-fold higher amount than 10 μ L (which is 0.6% of the total yield) of GA-hAHR from a 10 mL *Pichia* culture (Fig. 3B, top). Thus, 25 mL (2.5 X 10 mL) of the *Pichia* culture should generate roughly the amount of recombinant human AHR obtained from ten T75

flasks of S9 cells via baculovirus expression. This amount is roughly translated into about 40 microgram of human AHR per 1 L of the *Pichia* culture. Since the *Pichia* AHR has a c-myc tag which is absent in baculovirus AHR, we investigated whether the c-myc tag would alter the expression of AHR. To do that, we cloned the human AHR cDNA containing a C-terminal 6His tag into the pPICZ plasmid off frame to the c-myc tag. This *Pichia* AHR would be equivalent to baculovirus AHR which also has a 6His tag at its C-terminus. Interestingly, the presence of a c-myc tag upstream to the 6His tag increased the yield of human AHR production in *Pichia* (Fig. 3B, b).

3.3 Characterization of recombinant human ARNT expression in *Pichia pastoris*

Human ARNT was expressed more abundantly than human AHR in *Pichia*: the GA-hARNT protein was the major protein observed in Coomassie staining after it was affinity enriched (Fig. 4, top). A strong band (~70 KDa), which was smaller than the full-length ARNT protein, was observed by Western analysis, suggesting that there might be a truncated ARNT protein expressed in *Pichia*. Nevertheless, the yield of the *Pichia* ARNT protein was substantial. It is known that human ARNT can be expressed abundantly in a baculovirus system [20]. Based on Western analysis of the affinity enriched ARNT, we observed that 1 μ L of GA-hARNT from a 200 mL of *Pichia* culture showed similar amounts as 1.5 μ L of baculovirus ARNT from ten T75 flasks of S9 cells (Fig. 4, c). We estimated that 130 mL of the *Pichia* culture should generate roughly the same amount of ARNT obtained from a routine baculovirus expression. This amount can be roughly translated into about 2 milligram of human ARNT per 1 L of the *Pichia* culture. In addition, wild type ARNT was undetectable in the crude *Pichia* lysate by Western analysis even when five times the amount of GA-hARNT crude lysate (which was the loading sample) was analyzed (Fig. 4, d), suggesting that codon optimization is essential for the expression of human ARNT in *Pichia*.

3.4 *Pichia* expressed human AHR and ARNT can form heterodimeric complex

After we expressed the human AHR and ARNT proteins in *Pichia*, we investigated whether these proteins are functional. First, we addressed whether these proteins can heterodimerize with each other in a co-immunoprecipitation experiment. We observed that GA-hAHR could be co-immunoprecipitated only in the presence of GA-hARNT (Fig. 5, a), suggesting that the *Pichia* expressed AHR and ARNT are capable of forming the heterodimer in the absence of ligand, similar to what we observed with baculovirus AHR and ARNT. Next, we addressed whether GA-hAHR could interact with cellular ARNT protein. We performed similar co-immunoprecipitation experiment using Hep3B lysate and GA-hAHR and observed that GA-hAHR was co-immunoprecipitated only in the presence of Hep3B lysate, suggesting that ARNT in Hep3B cells interacted with GA-hAHR, allowing the co-immunoprecipitation of GA-hAHR by the anti-ARNT antibody (Fig. 5, b).

3.5 *Pichia* expressed human AHR and ARNT can form the ligand-dependent gel shift complex

Gel shift assay was performed to address whether the *Pichia* AHR-ARNT heterodimer can bind DRE. We conducted similar gel shift experiment which we routinely performed to observe the ligand-dependent formation of the AHR gel shift complex from nuclear extracts and the baculovirus expressed AHR and ARNT proteins [22, 23]. Results from the gel shift

assay showed that GA-hAHR can effectively form a gel shift complex with GA-hARNT in a ligand-dependent manner (Fig. 6, lanes 1–2, 7–8). Both β NF and kyn were able to cause the formation of the gel shift complex. The affinity enriched GA-hAHR and GA-hARNT could not form the gel shift complex unless *Pichia* extract, which is the 16,000g supernatant in the gel shift buffer after *Pichia* whole cell lysis, was reconstituted (lanes 1, 5, 7, 9, 11). This observation is similar to what we observed with the baculovirus expressed human AHR and ARNT proteins that formation of this gel shift complex requires exogenous protein factors [20].

3.6 Thermolysin-mediated degradation of *Pichia* expressed human AHR and ARNT

Since thermolysin remains active in the presence of urea, it can be used to discern protein conformation by degrading substrates in a urea-dependent manner since proteins unfold differently in a urea gradient. Although BSA was (as expected) stable against thermolysin treatment in the absence of urea, both GA-hAHR and GA-hARNT were completely degraded in 0.09 mg/mL of thermolysin (Fig. 7A, a). Next, we investigated whether binding of GA-hAHR to GA-hARNT could sterically interfere with the thermolysin degradation of the ARNT protein. We observed that when we used 1,000-fold less of thermolysin (0.09 μ g/mL), GA-hARNT was sufficiently detectable by Western analysis. Presence of up to 10-fold of GA-hAHR protected GA-hARNT from thermolysin degradation (Fig. 7A, b, lanes 1, 4, 5). This protection appeared to be dependent upon the folding status of GA-hARNT since 90°C heat treatment for 15 min of GA-hARNT effectively eliminated the GA-hAHR protection (lanes 3, 8, 9) whereas 70°C heat treatment for 1 h appeared to retain the protection (lanes 2, 6, 7). Unlike GA-hAHR, equal amounts of the protein content from *Pichia* extract did not protect the GA-hARNT degradation by thermolysin, suggesting that this protection is specific to GA-hAHR and not a general protein effect (Fig. 7B). This protective effect appeared to be ligand-independent since similar protection was observed in the presence or absence of β NF (Fig. 7B and C). This GA-hAHR-mediated protection was statistically significant when compared with 1-fold of GA-hARNT/GA-hAHR (Fig. 7C), further supporting that the interaction surface of AHR and ARNT contains the recognition site for ARNT degradation by thermolysin.

4. Discussion

Human AHR and ARNT were first overexpressed in a baculovirus system for functional study in two laboratories around mid 1990s [20, 24]. Since then, our laboratory has been utilizing the baculovirus expressed AHR and ARNT proteins to study the human AHR binding to its enhancer. Other researchers subsequently generated other baculovirus expressed human and rat AHR for the ligand binding study [25]. Due to the nature of baculovirus expression, cloned baculovirus must be regularly generated via low multiplicity of infection (MOI) of insect cells in order to replenish the high titer viral stock for continual recombinant protein expression. This process of high titer viral stock generation could introduce mutation to the baculoviral genome over time, which might lower the yield and possibly alter the properties of the recombinant protein. In contrast, *Pichia pastoris* expression is relatively reliable since the AHR or ARNT cDNA is integrated into the yeast

genome so that the transformed yeast, which can be stored at -80°C for a long period of time, can be continuously used to generate the human AHR or ARNT protein.

Our initial attempt of expressing human AHR in *Pichia pastoris* resulted in very modest yield, suggesting that *Pichia pastoris* may have problems processing the human AHR cDNA sequence for protein expression. To circumvent this problem, we made two changes: (1) we used a protease-deficient strain of *Pichia pastoris*, ySMD1163, to increase the yield of human AHR expression and (2) we used the GeneArt human AHR (GA-hAHR) cDNA, which has been optimized for *Pichia* protein expression, to allow for the optimal usage of codons and to minimize unstable mRNA sequences. We observed higher levels of GA-hAHR expression in ySMD1163 than the wild type strain yJC100 (data not shown), suggesting that it is beneficial to use the protease-deficient yeast strain for AHR production. Our results showed that optimization of the human AHR cDNA sequence is required for accumulation of the AHR transcript in *Pichia pastoris*. To our surprise, the c-myc tag at the C-terminus of AHR markedly increases the yield of the human AHR expression. Presence of the c-myc sequence in between the AHR and the 6His sequences might provide some structural stability for the AHR protein expression. The amount of the *Pichia* expression of human AHR is comparable to the yield we obtained from a typical baculovirus expression. For baculovirus expression, we observed that S9 cells generate a higher amount of recombinant AHR in monolayer than suspension culture. A typical, reproducible purification would require infection of ten 75cm^2 flasks of monolayer S9 cells for human AHR production. In the case of the human AHR production in *Pichia pastoris*, equivalent amount of this baculovirus expression can be obtained from just 25 mL of the *Pichia* culture. It is rather easy and economical to grow 200 mL of the *Pichia* culture at any given time, thus making the *Pichia* expression a better alternative of obtaining large amounts of functional human AHR for mechanistic study. One might expect that since human ARNT can be expressed rather abundantly in a baculovirus system [20], it should express well in a *Pichia* system. However, optimization of the human ARNT cDNA sequence is also a prerequisite for the *Pichia* expression, similar to what we observed for the human AHR expression. Considering the cost of cell culture plasticware and insect media, expression of the human ARNT protein in *Pichia* is more cost effective than in a baculovirus system, making it more attractive to obtain the human ARNT protein from the *Pichia* expression. Currently homologous modeling coupled with mutagenesis data is the only means to provide structural information regarding AHR [26, 27]. Providing that it would be rather straightforward and economical to scale up the production of human AHR and ARNT in *Pichia pastoris*, we will explore the possibility of purifying large amounts of these full-length human AHR and ARNT and their truncated proteins for structural study.

These human AHR and ARNT proteins from the *Pichia* expression are certainly functional: they interacted with each other in a co-immunoprecipitation experiment and formed a ligand-dependent DRE complex *in vitro*. Formation of this ternary gel shift complex required reconstitution of the yeast extract, consistent with our previous observation that protein factors, such as the co-chaperone p23 [28] and the cyclophilin CyP40 [29], can promote the formation of the AHR/ARNT/DRE complex. This observation suggested that the human AHR and ARNT proteins from *Pichia* behave similarly to those expressed in a baculovirus system, even though the *Pichia* expressed proteins contain a c-myc tag and are

likely glycosylated. In an effort to study protein conformation, we examined whether human AHR and ARNT are subjected to the urea-dependent degradation by thermolysin. This protease cleaves at many hydrophobic residues (I, L, V, F, M, A) which can usually be found in the hydrophobic core of a folded protein. Therefore, thermolysin proteolysis in a urea gradient could reveal interesting protein folding properties [28]. Both *Pichia* expressed human AHR and ARNT, however, are degraded readily by thermolysin in the absence of urea, suggesting that the hydrophobic residues of AHR and ARNT are exposed to the environment. This observation suggests that these proteins might be relatively lipophilic, which may partly explain why the bacterially expressed AHR and ARNT proteins are prone to form inclusion bodies. A careful examination of the PAS domains of AHR and ARNT, which are the water accessible interaction domains, showed many leucine and valine residues which are potential sites for thermolysin degradation. Interestingly, we are able to use AHR to protect ARNT from degradation by thermolysin, suggesting that the interaction surface between AHR and ARNT is important for the ARNT degradation by thermolysin. There is a great potential of developing such proteolysis assay using the *Pichia* expressed AHR and ARNT proteins to study the species-specific AHR/ARNT heterodimerization.

5. Conclusions

Functional full-length human AHR and ARNT proteins can be rather easily generated and affinity enriched in relative abundant amounts using *Pichia pastoris* once the nucleotide sequences are optimized for the *Pichia* expression. Long term generation of these proteins in *Pichia* should be more cost effective and stable than their expression in a baculovirus system. These proteins unveil the possibility of developing new means to study the AHR and ARNT interaction by limited proteolysis using thermolysin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Goeff Lin-Cereghino (University of the Pacific) for providing *Pichia* reagents and for his technical helps in *Pichia* expression. This work is partly supported by the National Institutes of Health (R15ES023104).

Abbreviations

GA-hAHR	GeneArt optimized human aryl hydrocarbon receptor
WT-hAHR	wild type human aryl hydrocarbon receptor
WT-mAHR	wild type mouse aryl hydrocarbon receptor
GA-hARNT	GeneArt optimized human aryl hydrocarbon receptor nuclear translocator
WT-hARNT	wild type human ARNT
βNF	β-naphthoflavone
kyn	L-kynurenine

ySMD1163	protease-deficient <i>Pichia</i> strain
BMGY	buffered glycerol complex medium
BMMY	buffered methanol complex medium
HEDG	25 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM DTT, and 10% glycerol
DRE	dioxin response element

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Highlights

- Human AHR and ARNT proteins can be expressed abundantly in *Pichia*, only when the sequences are optimized for *Pichia* expression.
- *Pichia* expressed human AHR and ARNT behave similarly to baculovirus AHR and ARNT.
- *Pichia* expressed human AHR and ARNT can dimerize and form ligand-dependent gel shift complex.
- *Pichia* expressed human AHR and ARNT can be used for limited proteolysis to study AHR-ARNT interaction.

Fig. 1. Incorporation of the AHR gene into the yMSD1163 *Pichia* strain genome

(A) qPCR of *Pichia* genomic DNA showing relative content of different AHR sequences integrated in the genome. GA-hAHR (a), WT-hAHR (a), and WT-mAHR (b) were cloned into the pPICZ plasmid and then transformed into yMSD1163. (B) Expression $\pm$ methanol at the transcriptional (a and b) and protein (c) levels. P, pellet; S, soluble extract. Two antibodies (anti-c-myc and anti-AHR SA-210) were used to detect the AHR expression. Expression was induced with methanol for 12 h. All qPCR values in A and B were calculated using the *Pichia* actin gene ACT1 as the internal standard using the 2^{-C_T} method with different conditions set as 1 as indicated in the graphs. All error bars represent means $\pm$ SD, $n = 3$.

Fig. 2. Time-dependent expression of GA-hAHR in yMSD1163 *Pichia* strain

(A) a. Time-dependent expression of crude GA-hAHR. GA-hAHR was harvested at various times (0–24 h) after methanol induction. BCA assay was used to determine the protein content of crude lysates. b. The image intensity at zero hour is arbitrarily set as 1 for comparison. (B) a. Time-dependent expression of soluble GA-hAHR. Soluble (S) and insoluble pellet (P) were obtained after various times (3–12 h) of methanol induction. b. The

3 h pellet fraction is arbitrarily set as 1 for comparison. All lanes contained 100 μ g of protein. All error bars represent means $\pm$ SD, n = 3. The Western images on top are the representative results.

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Fig. 3. Analysis of the affinity enriched human AHR

(A) Coomassie (a, 15 μ L per lane) staining and Western (b, 5 μ L per lane) analysis of fractions from metal-affinity purification of a 40 ml culture. L, loading sample; FT, sample after column flow through; W, wash; E1-3, eluted fractions. Arrows indicate GA-hAHR. c, Western analysis comparing the amount of affinity enriched GA-hAHR with human AHR expressed in rabbit reticulocyte lysate (RL), wheat germ extract (WG), and human cell lines (Hep3B, HeLa, and MCF-7). (B) a, comparison of the yield of *Pichia*- and baculovirus-derived affinity enriched human AHR. 5 μ L of baculovirus AHR and 10 μ L of GA-hAHR represents roughly 0.6% yield from ten T75 flasks of S9 cells and 10 mL of *Pichia* culture, respectively. b, Comparison of the yield of GA-hAHR with (+) or without (-) the c-myc tag. Crude GA-hAHR (5 μ L from 10 mL culture) is the loading sample before affinity

purification. Western images include affinity enriched GA-hAHR (7.5 μ L from 10 mL culture) from two separate purifications of GA-hAHR $\pm$ c-myc tag.

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Fig. 4. Analysis of the affinity enriched human ARNT

Coomassie (a, 15 μ L per lane) staining and Western (b, 5 μ L per lane) analysis of fractions from metal-affinity purification of a 25 ml culture. L, loading sample; FT, sample after column flow through; W1-2, wash with purification buffer (W1) or purification buffer with 10mM imidazole (W2); E1-3, eluted fractions. Arrows indicate GA-hARNT. c, comparison of the yield of *Pichia*- and baculovirus-derived affinity enriched human ARNT. Samples were from either 200 mL of *Pichia* culture or ten T75 flasks of *Sf9* cells. d, undetectable amount of wild type *Pichia* ARNT in crude lysate (25 μ L, 5X) which is the same sample as L on top right for GA-hARNT (5 μ L, 1X).

Fig. 5. Co-immunoprecipitation results showing GA-hAHR interacts with GA-hARNT and cellular ARNT

α ARNT, anti-ARNT H-172; IgG, rabbit IgG. % input indicates the expected intensity of the amount of GA-hAHR as 100% equals the amount of GA-hAHR used to start each condition. The ARNT-specific H-172 antibody was used for co-immunoprecipitate AHR and the AHR-specific SA210 antibody was used for Western to detect AHR. Arrows indicate the GA-hAHR and the cellular AHR. Hep3B lysate [22] was the crude cell extract in high salt (0.4 M KCl) buffer.

Fig. 6. Gel shift results showing GA-hAHR and GA-hARNT are capable of forming the ligand-dependent gel shift complex

Upper arrow indicates GA-hAHR/GA-hARNT/DRE gel shift complex whereas the lower arrow indicates the free IRDye680-conjugated DRE. DRE sense, OL74, TCGAGTAGATCACGCAATGGGCCCAGC; DRE antisense, OL74, TCGAGC TGGGCCCATTGCGTGATCTAC, with DRE core sequence underlined.

Fig. 7. Thermolysin proteolysis showing protection of the GA-hARNT degradation by GA-hAHR (A) a, Western and Silver staining images showing the degradation of GA-hAHR, GA-hARNT, and BSA by thermolysin (1 mg/mL stock) in the absence of urea. b, anti-ARNT H-172 Western images showing GA-hARNT degradation by thermolysin (1 μ g/mL stock) in the absence of urea. GA-hARNT (70°C) and GA-hARNT (90°C) are samples heated at 70°C for 1 h or 90°C for 15 min. (B) anti-ARNT H-172 Western images showing GA-hARNT in the presence (+) or absence (–) of thermolysin (1 μ g/mL stock) $\pm$ GA-hAHR (AHR) $\pm$ *Pichia* extract (PE) in the presence (a) or absence (b) of 10 μ M β NF. (C) Graph summarizes the experiment in (B) repeated with the n values indicated on the x-axis. 1-, 5-, 10-, and 15-fold refer to the protein amounts quantified by BCA assay with 1-fold equals the amount of the affinity enriched GA-hAHR added.