MOLECULAR BIOLOGY OF THE AH RECEPTOR AND AH RECEPTOR-DEPENDENT SIGNALING

THE IMMUNOPHILIN HOMOLOG XAP2 SPECIFICALLY MODULATES THE INTRACELLULAR LOCATION OF THE AH RECEPTOR

John Petrulis and Gary H. Perdew

Center for Molecular Toxicology and the Department of Veterinary Science, The Pennsylvania State University, University Park, Pennsylvania 16802

Introduction

The *Ah* receptor (AhR) exists in the cytoplasm as a tetrameric core complex composed of a ligand binding subunit, a dimer of heat shock protein 90 (hsp90), and a 38 kDa protein (XAP2/ARA9/AIP)[1-3]. Both hsp90 and X-associated protein 2 (XAP2) have been shown to be important in stabilizing the AhR within the cell and both directly interact with the AhR[4]. In addition, exposure of cells to geldanamycin, an inhibitor of hsp90 function, leads to rapid turnover of the existing AhR pool[5]. In contrast XAP2 levels are not altered by exposing cells to geldanamycin[6]. This would suggest that the AhR is particularly susceptable to proteolytic turnover in the absence of functional hsp90. Importantly, in an *in vitro* translation system XAP2 is not expressed yet the AhR is able to bind to hsp90 and fold into a conformation that is capable of binding ligand as well as subsequently heterodimerizing with ARNT[1]. XAP2 overexpression in cells has been shown to enhance AhR levels and transactivation potential, suggesting that the amount of XAP2 available to interact with the AhR may limit the AhR steady state levels[4]. Despite these advances in XAP2 biochemistry the actual function of XAP2 in the tetrameric complex is not fully understood. In this report we examined the ability of XAP2 to modulate AhR subcellular localization.

Methods and Materials

Construction and Sources of Expression Vectors: pcDNA3-βmAhR used for expression of the AhR was provided by O. Hankinson (UCLA). PCI-FKBP52 was obtained from David Smith. pYFP-Nuc (nuclear localized yellow fluorescent protein) was obtained from Clontech (Palo Alto, CA). PCI-XAP2-FLAG was prepared as previously described[4], as was pCI-XAP2-FLAG G272D (Meyer *et al.*, In Press). pAhR-YFP (yellow fluorescent protein cDNA fused to the C-terminus of the mAhR) was constructed by inserting the mAhR (PCR amplified from pcDNA3/βmAhR with Xhol and Xmal sites added and designed to ligate in frame with YFP) into the Xhol and Xmal restriction sites in the MCS of pEYFP-N1 (Clontech, Palo Alto, CA). The ΔNLS mutants (K13A) of the mAhR and AhR-YFP were generated using the Quickchange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) on pcDNA3-βmAhR and pAhR-YFP, respectively. *Cell Culture*: Cells were grown in α-minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin at 37°C in 94% air, 6% CO_2.

Transient Transfection and Preparation of Cell Cytosol: COS-1 cells were transfected at ~70% confluency in 100 mm culture dishes using LipofectAMINE (Gibco BRL, Gaithersburg, MD) following the manufacturers instructions. Transfected cells were harvested the day following transfection by treatment with trypsin-EDTA and washed once with PBS. Cells were suspended in MENG buffer (25 mM MOPS, 2 mM EDTA, 0.02% NaN_3, 10% glycerol, pH 7.5) + 20 mM MoO_4^{2-} + 1X protease inhibitor cocktail (Sigma), and homogenized with 30 strokes in a stainless steel dounce homogenizer. The crude homogenate was centrifuged at 100,000 x g for 30 min at 4°C to obtain the cytosolic fraction.

Immunoprecipitations: FLAG-tagged proteins (mAhR-FLAG and mAhR-ΔNLS-FLAG) were immunoprecipitated using anti-FLAG M2 affinity gel (Sigma). AhR-YFP and AhR-ΔNLS-YFP were immunoprecipitated using anti-AhR mouse monoclonal Ab RPT9[7] pre-bound to protein G-sepharose. Immunoprecipitations were carried out in IP buffer (MENG with 20 mM MoO_4^{2-}, 50 mM NaCl, 2 mg/mL bovine serum albumin, 2 mg/mL ovalbumin). Briefly, aliquots of cytosol containing equal amounts of protein were incubated with the appropriate resin in a total volume of 750 μL for 1 h at 4°C. Each immunoprecipitation was washed 3 times with IP buffer, 3 times with MENG + 20 mM MoO_4^{2-} + 50 mM NaCl, mixed with an equal volume of 2X tricine sample buffer, heated at 95°C for 5 min and resolved by tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (TSDS-PAGE), followed by transfer to PVDF membrane. Bands were visualized by western blot analysis using the appropriate primary antibodies: AhR; RPT1 mouse monoclonal Ab[7], hsp90; anti-hsp84/86 rabbit polyclonal Abs[8], XAP2: anti-XAP2 rabbit polyclonal Ab provide by E. Croze (Berlex Laboratories). Primary antibodies were detected with either peroxidase conjugated goat anti-mouse IgG or peroxidase conjugated donkey anti-rabbit IgG (Jackson Immunoresearch) and visualized using the Vector VIP substrate.

Luciferase Reporter Gene Assay: COS-1 cells grown 6-well culture dishes were transfected using LipofectAMINE (Gibco BRL, Gaithersburg, MD) according to the manufacturers instructions. Each transfection included 200 ng of the DRE-driven luciferase reporter construct pGudLuc 6.1 (a generous gift from M. Denison), 100 ng of an AhR construct, and control vector (pcDNA3) to a total of 2 μg DNA per well. The day following transfection, cells were treated with 10 nM TCDD or DMSO (vehicle) for 6 h, following which luciferase activity was assessed using a Turner TD-20e luminometer. Luciferase activities determined in triplicate were normalized to cytosolic protein levels.

Fluorescence Microscopy: Cells grown on glass cover slips in 6-well culture dishes were transfected with 2 μg DNA using LipofectAMINE (Gibco BRL, Gaithersburg, MD) according to the manufacturers instructions with the following modifications, transfection mixtures were supplemented with 10% fetal bovine serum after 1 h, and cells were incubated in this mixture for an additional 5 h following which the transfection mixture was removed, cells were rinsed with PBS, and replaced in culture medium followed by incubation overnight. Before visualization, cells were rinsed twice with PBS, fixed for 15 min in 4% formaldehyde/PBS at room temperature, rinsed twice with PBS, and the inverted cover slips mounted onto microscope slides with Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA). Fluorescence micrographs were obtained with a SPOT SP100 cooled CCD camera fitted to a Nikon Optiphot-2 upright microscope with EFD-3 episcopic fluorescence attachment using a Nikon Pan Fluor 100X oil immersion objective.

Results and Discussion

AhR-YFP assembles into the AhR core complex with XAP2 and hsp90. In order to confirm that the fusion of YFP onto the C-terminus of the mAhR imparted no loss of function to the AhR, COS-1 cells were co-transfected with pCI-XAP2 and either pcDNA3 (control), pcDNA3-βmAhR, or pAhR-YFP, and cell cytosol isolated after 36 h. Cytosol was then incubated with anti-AhR Ab (RPT9) bound to protein G-sepharose to immunoprecipitate the AhR complex. The immunoprecipitates were then resolved by TSDS-PAGE, electroblotted onto PVDF membrane and visualized by western blot analysis. Control transfected cells showed no immunoprecipitated AhR, AhR transfected cells showed a band at ~97 kDa corresponding to the AhR, and AhR-YFP transfected cells showed a band at ~ 125 kDa corresponding to the AhR-YFP fusion protein (visualized with anti-AhR monoclonal Ab RPT1). This band was also detected using anti-GFP Ab (Clontech, Palo Alto, CA). The AhR and AhR-YFP both co-immunoprecipitated hsp90 and XAP2 indicating that the AhR-YFP fusion is capable of assembling into a core complex that appears analogous to that of the wild-type AhR.

AhRΔNLS-FLAG assembles into the AhR core complex with XAP2 and hsp90. In order to confirm that the introduction of the K13A mutation into the AhR did not disrupt the formation of the AhR core complex, COS-1 cells were co-transfected with pCI-XAP2 and either pcDNA3 (control), pcDNA3-βmAhR-FLAG, or pcDNA3-βmAhR ΔNLS-FLAG, and cell cytosol isolated. The K13A mutation in the AhR was chosen to disable the nuclear localization signal (NLS) as previously reported[9]. Cytosol was then incubated with anti-FLAG M2 affinity gel to immunoprecipitate the AhR-FLAG complex. The immunoprecipitates were then resolved by TSDS-PAGE, electroblotted onto PVDF membrane and visualized by western blot analysis. Control transfected cells showed no immunoprecipitated AhR, while AhR-FLAG and AhRΔNLS-FLAG transfected cells showed a band at ~97 kDa that was visualized with the anti-AhR monoclonal Ab RPT1. The AhR-FLAG, and AhRΔNLS-FLAG both co-immunoprecipitated hsp90 and XAP2 indicating that the AhRΔNLS-FLAG is capable of assembling into a core complex that appears analogous to that of the wild-type AhR-FLAG.

AhR-YFP retains functionality of the wild-type AhR in a DRE-driven luciferase reporter assay. To further confirm the functionality of the AhR-YFP fusion protein, its ability to activate a DRE-driven luciferase reporter plasmid was compared with the activity of the wild-type mAhR. COS-1 cells grown in 6-well culture dishes were transfected with 200 ng pGudLuc 6.1 (DRE-driven luciferase reporter construct) and either pcDNA3 (control), 100 ng pcDNA3-βmAhR, or 100 ng pAhR-YFP, made up to a total of 2 μg DNA/well with pcDNA3. Cells were treated with 10 nM TCDD or DMSO (vehicle) for 6 h, followed by an assay for luciferase activity.The results revealed that the AhR-YFP fusion protein demonstrated the ability to activate the luciferase reporter construct similar to the reporter activity obtained with AhR, suggesting that the fusion protein retains the functional characteristics of the wild-type AhR and thus would serve as an accurate model for studying the intracellular localization of the AhR.

YFP localizes to cytoplasm and nuclei and YFP-Nuc localizes to nuclei in COS-1 cells. COS-1 cells were grown on glass cover slips and transfected with either pYFP or pYFP-Nuc (containing

three tandem repeats of the nuclear localization signal of the simian virus 40 large T-antigen fused to its C-terminus) (Clontech, Palo Alto, CA). Cells were fixed, mounted onto microscope slides, and visualized by fluorescence microscopy as described in Materials and Methods. YFP alone was distributed throughout cells with an apparent concentration in nuclei, while YFP-Nuc was visible only in the nuclei of transfected cells. The localization of YFP and YFP-Nuc were unaffected by treatment with 10 nM TCDD for 1 h. These control experiments demonstrated that NLS-mediated import was functional, that nuclear localized YFP was clearly distinguishable from unlocalized YFP and that YFP did not display distinct localization that could affect localization of the fusion protein. In addition, the results demonstrate that TCDD does not effect localization of either construct and therefore that TCDD does not appear to influence nuclear import machinery.

AhR-YFP localization in COS-1 and NIH-3T3 cells is specifically modulated by XAP2. NIH 3T3 and COS-1 cells grown on glass cover slips were transfected with pAhR-YFP. Two unrelated cell lines were utilized to ensure that observed localization of the AhR-YFP was not specific to a single cell line. When AhR-YFP alone was transiently expressed in either cell line, it was consistently found to localize primarily to the nuclei of transfected cells in the presence and absence of ligand. To test the hypothesis that the transiently expressed receptor may be overwhelming endogenous levels of other components of the core complex, that might lead to altered function, pAhR-YFP was co-transfected into both cell lines along with pCI-XAP2. The result of co-expression of XAP2 was that the AhR-YFP localized to the cytosolic compartments of the cells. In addition, treatment of these cells with 10 nM TCDD for 1 h resulted in nearly complete nuclear localization. Cells were also co-transfected with pAhR-YFP and hsp90, results revealed that exogenous hsp90 did not have any effect on localization of AhR-YFP. To examine the specificity of the effect of XAP2 on AhR-YFP localization, cells were co-transfected with pAhR-EYFP and pCI-FKBP52. FKBP52 is an hsp90 associated immunophilin that shares significant homology to XAP2 and is present in glucocorticoid and other steroid receptor core complexes. Co-expression of AhR-YFP with FKBP52 had no detectable effect on the localization of AhR-YFP, demonstrating that AhR-YFP localization is specifically modulated by XAP2. To further examine the specificity of the effect of XAP2 on the localization of AhR-YFP, COS-1 cells were co-transfected with pAhR-YFP and pCI-XAP2 G272D which contains a point mutation in the C-terminal tetratricopeptide repeat (TPR) domain[5]. This mutant has previously been shown to be incapable of assembling into the AhR core complex along with hsp90 and was found to have no effect on the cellular localization of AhR-YFP, further suggesting that the altered localization of AhR-YFP is specifically due to the presence of XAP2 in the core AhR complex. These results demonstrate that exogenous XAP2 is able to modulate the cellular localization of the AhR.

AhRΔNLS-YFP localizes to cytoplasm and is unable to undergo ligand dependent nuclear translocation. In order to determine if the localization of AhR-YFP was being mediated by the nuclear localization signal (NLS) of the AhR, a point mutation (K13A) was made in the NLS, to give pAhRΔNLS-YFP, that has been previously shown to abolish nuclear translocation of the AhR[9]. In both NIH 3T3 and COS-1 cells, AhRΔNLS-YFP localized to cytoplasm and did not undergo detectable nuclear translocation following treatment with 10 nM TCDD for 1 h. These observations confirm that AhR-YFP localization in both COS-1 and NIH 3T3 cells is being mediated by the NLS of the AhR, and is unlikely and artifact of transient expression or an alternate means of nuclear entry.

XAP2 does not affect localization of YFP or YFP-Nuc. To determine if XAP2 was specifically affecting the AhR and not influencing general nuclear import processes, COS-1 cells were co-transfected with either pYFP or pYFP-Nuc and pCI-XAP2 or pCI (control). In both cases, XAP2 had no effect on localization. The results further demonstrate that the effect of XAP2 on AhR localization is specific and is not an effect of altered nuclear import.

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References

1. Meyer, B.K., Pray-Grant, M.G., Vanden Heuvel, J.P., and Perdew, G.H. (1998) Mol Cell Biol. 18, 978-988.
2. Ma, Q. and Whitlock, J.P. Jr. (1997) J. Biol. Chem 272, 8878-8884.
3. Carver, L.A. and Bradfield, C.A.(1997) J. Biol. Chem. 272, 11452-11456.
4. Meyer, B.K. and Perdew, G.H. (1999) Biochemistry. 38(28):8907-17.
5. Meyer, B.K., Petrulis, J.R. and Perdew, G.H. (2000) Cell Stress and Chaperones. 5, In Press.
6. Chen, H-S., Singh, S.S., and Perdew, G.H. (1997) Arch. Biochem. Biophys. 348, 190-198.
7. Perdew, G.H., Abbott, B. and Stanker, L.H. (1995) Hybridoma, 14:279-283.
8. Perdew, G.H., Hord, N., Hollenback, C.E., and Welsh, M.J. (1993) Exp. Cell Res. 209, 350-356.
9. Ikuta, T., Eguchi, H., Tachibana, T., Yoneda, Y. and Kawajiri, K. (1998) J. Biol. Chem. 273(5):2895-904.