BINDING OF ARYL HYDROCARBON RECEPTOR (AHR) TO AHR-INTERACTING PROTEIN (AIP); THE ROLE OF HSP90

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Introduction

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most potent of a wide range of agonists for the Ah Receptor (AhR)[1]. Activation of the AhR by TCDD leads to a wide variety of responses, including acute toxicity, teratogenicity and cancer [1, 2]. The AhR mediates and is required for all the known responses to TCDD [3, 4], and so it is important to understand how the AhR functions. The AhR is constitutively cytoplasmic, bound to hsp90. Upon binding TCDD, it translocates to the nucleus, where hsp90 is removed and a transcriptionally active AhR-arnt complex is formed. The activated, nuclear AhR is subject to controlled degradation [5-7]; the mechanisms controlling production of the functional, ligand-binding AhR, and its subsequent activation, are much less clearly defined.

It has recently been shown that the AhR associates with a novel protein, known as AIP [8], XAP2[9, 10] or Ara9[11]- this protein was shown to interact with AhR in a yeast two-hybrid assay[8, 11], and also in mammalian cells [9]. AIP appears to enhance the sensitivity and magnitude of ligand-induced signalling through the AhR in yeast [11, 12] and in mammalian cells [8, 13]. However, it is unclear how AIP enhances AhR function.

AIP possesses similarity to the immunophilins, notably FKBP52. Several of the immunophilins, including FKBP52, bind to hsp90 and are co-chaperones associated with nuclear hormone receptors [14]. Characteristic features of the hsp90-immunophilin assembly system include: dependence on cation [15], temperature and ATP[16], and sensitivity to the specific inhibitor of hsp90, geldanamycin [17-19]. Thus one might expect AIP to possess similar properties by analogy to the immunophilins. However, unlike FKBP52, AIP does not bind to FK-506 or rapamycin [11], and there is conflicting evidence about the ability of AIP to bind to hsp90. AIP has been shown to associate with hsp90 in mammalian cells [8], but to bind poorly to hsp90 [12] or not at all [9] in reticulocyte lysates in the absence of the AhR. It is difficult to reconcile these observations, since variation in temperature [16], salt conditions [15] or other conditions used in these papers are critical for the function of hsp90.

Methods and Materials

Plasmids. The murine AIP plasmids pAIP/GEX4T3 and pAIP/ET28a were described in [8]. For construction of pGEX.AIP1-325, the insert of pAIP/ET28a was a template for PCR to introduce BclI and XhoI sites for subcloning into pGEX-4T3. Murine AhR b-1 allele cDNAs were previously decribed [20]. The construct pRSET.LBD containing a minimal AhR ligand binding domain (LBD), amino acids L229-P416, was prepared by PCR subcloning the AhR b-1 cDNA into the vector pRSET (Invitrogen). Mutagenesis was performed with the Promega Gene Editor kit, as described in the manual. Subclones were confirmed by sequencing on both strands. The human hsp90β construct, pET-28a(+) 530-724β, was a kind gift of Professor T. Ratajczak [21]. *Protein expression and purification.* pET and pGEX plasmids were transformed into E.coli BL21 RIL (DE3) and E. coli BL21 (Stratagene), respectively, for expression studies (Invitrogen technical manual, Amersham GST technical manual); samples were dialysed against 100 mM KCl, 10 mM Tris, pH 7.4, 1 mM DTT, and stored at 4C. *PCR of AIP.* AIP was prepared by PCR using pAIP/ET28a as the template, and incorporating a T7 site on the PCR product. PCR products were purified, and used directly for transcription/translation. *Transcription/ translation.* Coupled transcription/ translation used the reticulocyte lysate system from Promega, according to the manufacturer's instructions. For binding assays in reticulocyte lysate, samples containing AIP, AhR or variants thereof, were mixed on ice, then incubated at 30°C for one hour. Alternatively, 100 µl samples of translation product were diafiltered against 50 mM KCl, 10 mM Tris pH 7.5, 5mM $MgCl_2$, 0.2mM DTT, in a Centricon exclusion column with a 10 kDa cut off. The samples were supplemented with as shown; incubation was for one hour at 30 C. Samples were placed on ice, and 0.8µl of α-T7•tag antibody (Novagen) was added in 200 µl of ice-cold 20mM Tris pH 7.5, 150 mM NaCl, 0.5% Ipegal CA-630, 5mM EDTA (wash buffer); the samples were incubated with agitation for one hour at 4C. Sepharose CL4B-protein A conjugate was added, and incubation continued for one hour. Each sample was washed four times, and the protein was eluted into load buffer (Novex), and run on a polyacrylamide gel. The gel was fixed, dried and analysed with a Phosphor Imager. *Protein-protein interactions.* GST, GST-AIP and hsp90Δ530-724 proteins were expressed and purified as described in materials and methods. Protein was incubated in 200 µl of 10 mM KCl, 10 mM Tris, pH 7.4, 1mM DTT for one hour at 4C. GST-Sepharose CL-4B beads were added, and incubation continued at 4C for one hour. The samples were washed 4x with buffer. Proteins were eluted from the beads, followed by electrophoresis on NuPAGE. Gels were stained with Coomassie blue, dried and scanned. *UV-CD spectroscopy.* Far UV-CD spectra were acquired on an AVIV 62DS spectrometer in water or aqueous methanol mixtures at pH 7.0, 60 µM at 20C.

Results and Discussion

AIP and the murine AhR b-1 allele were translated separately in a coupled reticulocyte lysate transcription/ translation system in the presence of [^{35}S]-methionine. The AhR and AIP translation reactions were mixed, and incubated at 30 C for an hour. The anti-T7•tag antibody failed to immunoprecipitate the AhR protein by itself but when T7•tagged AIP was mixed with AhR, both proteins were immunoprecipitated, demonstrating the presence of a complex containing both AhR and AIP. We examined the interaction between AIP and AhR orthologues from *C.elegans* [22], *Drosophila* [23] and Fundulus *heteroclitus* AhR2 [24]. The Fundulus AhR2 was co-immunoprecipitated with AIP, but the C. *elegans* and *Drosophila* orthologues, which do not bind dioxin, failed to show any co-immunoprecipitation with AIP. These data show a correlation between the dioxin-binding

functionality of the AhR and association with AIP, suggesting that the ligand-binding domain may have a crucial role in AIP-AhR interaction. However, a minimal fragment of the AhR LBD containing amino acids L229-P416 was not co-immunoprecipitated by the T7•tag antibody; this minimal AhR LBD containing amino acids L229-P416 retains ligand binding activity (D.R. Bell and A. Poland, unpublished results)[25].

We examined the effect of nucleotides on AIP-AhR interactions to determine if AIP-AhR interactions are an ATP-dependent process. After translation of AIP and AhR cDNAs, the reticulocyte lysates were extensively dialysed by centrifugation using a Centricon column with a molecular weight cut-off of 10 kDa [19]. Nucleotides were then added to the mixture, and AhR-AIP interactions were assayed by co-immunoprecipitation with AIP. The process of diafiltration reduced AhR-AIP interactions compared with the non-diafiltered controls, showing that AhR-AIP interactions require a small molecular weight molecule, or molecules. 2mM ATP, the non-hydrolysable ATP analogue, ATPγS, or ATP and 10 mM sodium molybdate restored AIP-AhR interactions. To characterise the requirement for ATP in AIP-AhR interactions, we used a specific inhibitor of hsp90, geldanamycin, which is a ligand for the hsp90 ATPase domain. Reticulocyte lysates containing AIP or AhR translation products were mixed and incubated in the presence of geldanamycin in DMSO, or the vehicle, followed by immunoprecipitation. Geldanamycin showed almost total inhibition of AIP-AHR interactions at 1 μg/ml. AhR was stable in the presence of geldanamyin in reticulocyte lysates, demonstrating that the reduced co-immunoprecipitation of AhR with AIP is not due to degradation of the AhR. The known specificity of geldanamycin as a ligand of the ATP-binding site of hsp90 provides evidence that the active step in AIP-AhR interactions is ATP hydrolysis catalysed by hsp90 during the process of assembly of an AhR-AIP chaperone complex. To test whether AIP binds directly to hsp90, AIP was expressed as a glutathione S-transferase fusion protein [8] and bound to the purified C-terminal hsp90 fragment hsp90β530-724 [21], although GST alone failed to bind, demonstrating that the interaction is between AIP and hsp90β530-724. To test whether the capacity to bind hsp90 is required for AIP function, we made use of the known structure of the related tetratricopeptide repeat protein, protein phosphatase 5 [26], and two conserved basic residues (K97 and R101) bind hsp90 [27]. The analogous Lysine 270 in AIP was mutated to alanine, and expressed as a GST-fusion protein. The purified protein failed to bind the hsp90 C-terminal fragment.

An AIP mutant truncated at amino acid 325, lacking only the last 5 amino acids, failed to co-immunoprecipitate AhR, even when present at higher levels than the full-length AIP. Alanine-scanning mutagenesis of the last 5 amino acids (325-GIFSH) was undertaken. Replacement of amino acids 327-330 with an alanine resulted in almost complete loss of binding to AhR; however, replacement of Gly 326 with alanine yielded a mutant which retained partial immunoprecipitation of AhR. It was of interest to determine whether a C-terminal mutant of AIP, which failed to co-immunoprecipitate AhR in reticulocyte lysate, was also deficient in binding hsp90. The C-terminally truncated AIP, AIP1-325, binds to the hsp90β530-724 protein, demonstrating that a failure of AIP C-terminal mutants to bind hsp90 is not responsible for the failure of these mutants to bind to the AhR. The C-terminus of AIP is scored as an α-helix by secondary structure prediction programmes, and a peptide containing the C-terminal 28 amino acids of AIP with an N-terminal cysteine readily assumed an α-helical formation. The data support a model where (1) AIP binds to both

hsp90 and AhR (2) hsp90 is required for AhR-AIP binding (3) the binding of AhR to AIP stabilises the AIP-hsp90-AhR complex.

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