MECHANISMS OF LIGAND-DEPENDENT TRANSFORMATION OF THE AH RECEPTOR

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Introduction

AhR receptor is a ligand-dependent transcription factor mediating the toxic and biological effects of TCDD and related compounds. AhR, bound to Hsp90 and co-chaperones in an inactive form, is converted by ligand binding into its high affinity DNA binding form following dimerization with a nuclear protein (Arnt) in a process termed transformation. The precise mechanism of transformation is currently unknown and hypotheses are based on following experimental findings: 1. ligand binding results in nuclear localization of the AhR, 2. Hsp90 remains bound to the AhR complex through nuclear localization, and 3. Arnt could be displacing Hsp90 from overlapping binding sites in the AhR bHLH and PASB domains[1-4]. Transformation is thought to be driven by a conformational change in the AhR resulting from the presumed exposure of the nuclear localization sequence (NLS) at the N-terminal end of the AhR (i.e. in the bHLH domain) leading to initiation of AhR dimerization with Arnt through the available bHLH domains[1]. Previous experimental evidence of a conformation change in the AhR included limited proteolysis experiments, in which ligand- and Arnt-dependent protected AhR fragment appeared spanning Arnt dimerization interface in the AhR[5]. Ligand binding and resulting conformational change(s) in the AhR may play a role in interspecies differences in AhR activation through mechanisms resulting in reduced transformation efficiency of mouse AhR compared to other species examined[6]. Limited transformation of the mouse AhR has been suggested to result from slower conformational change and/or more stable interaction with Hsp90[7]. Here we have examined the mechanistic events of ligand-dependent transformation of the mouse AhR in more detail in order to understand its role in modeling AhR responsiveness.

Materials and Methods

Plasmid constructs. The expression plasmids mβAhR/pcDNA3, mArnt/pBK-CMV have been previously described[8,9]. BstEII and AflII sites were introduced at positions –7 and +2397 (relative to the AhR translation start) of mβAhR/pcDNA3 using the QuickChange technique (Stratagene). Using PCR-based cloning techniques, full length Arnt and 1-860 nt AhR fragment were amplified using mArnt/pBK-CMV and mβAhR/pcDNA3 as templates respectively and inserted at BstEII/AflII and BstEII/BamHI sites respectively in the modified mβAhR/pcDNA3. GTS was amplified from pGEX-2T (GE Healthcare) with added 5'-BstEII and 3'AgeI-AflII sites, and GST-AhR and GST-PASA-PASB was generated by inserting amplified full length mouse AhR or its 247-1257 nt fragment at AgeI and AflII sites of this construct. R217 was generated by site mutagenesis using the QuickChange technique.

Glutathione pull-down and limited proteolysis. GST-PASA-PASB, R217 or GST-AhR were synthesized *in vitro* in the presence of ^{35}S-methionine and incubated in the presence of 20 nM TCDD (or 0.2% DMSO) for 1 h, precipitated with glutathione-agarose, washed once with PBS, incubated with 1 μg/ml trypsin for 5 min, incubated with excess trypsin inhibitor for approximately 1 min and washed 3 times with PBS. Precipitated proteins were analyzed with SDS-PAGE[10] and visualized by PhosphorImaging.

Hydroxyapatite (HAP) ligand binding assays. [^{3}H]TCDD specific binding to the *in vitro* synthesized proteins diluted in MEDG buffer to 8 mg/ml protein was conducted in the presence of 2 nM [^{3}H]TCDD for indicated periods of time and measured as described[10]. Equivalent amounts of unprogrammed *in vitro* synthesized reactions were used as the non-specific binding control[11].

Gel retardation assay. An aliquot (1.5 μl) of *in vitro* synthesized wtAhR, AhRΔPASB were combined with 1.5 μl of *in vitro* synthesized Arnt in 7 μl of MEDG buffer (25 mM MOPS, pH 7.5, 10% (v/v) glycerol, 15 mM KCl, 1 mM

EDTA, 1 mM DTT) and incubated in the presence of 20 nM TCDD or 2% (v/v) DMSO for 2 h at room temperature. Annealed double-stranded oligonucleotides containing the AhR:Arnt DNA binding site (DRE3) from the murine *CYP1A1* upstream regulatory sequence were ^{32}P-labeled and gel retardation analysis was conducted[10,12].

Results and Discussion

Previous studies utilizing limited proteolysis have detected formation of protected AhR fragment in the presence of Arnt[5]. While these results are consistent with formation of the AhR:Arnt dimer, they do not indicate whether prior to dimerization with Arnt, a conformational change takes place in the AhR complex which could account for NLS exposure or initiation of dimerization with Arnt. We changed the limited proteolysis strategy using a smaller and tagged AhR fragment, specifically GST-PASA-PASB, to be able to detect conformational change(s) in the PASA-PASB region of the AhR and use the GST tag to be able to map the location of the change following glutathione precipitation of the resulting fragments. Consistent with these expectations, we observed a ligand-dependent increase in formation of an approximately 43 kDa fragment in the absence of Arnt (Figure 1, lanes 5 and 6). Since this fragment was glutathione-precipitated, its size was indicative of the cleavage site which mapped approximately to amino acid 225 of the mouse AhR. Tentative trypsin cleavage sites within AhR region of amino acids 165-248 were individually mutated and while none resulted in disappearance of the fragment (data not shown), one mutation, i.e. R217A, resulted in consistently shorter fragment suggesting use of an alternative trypsin cleavage site in this construct (Figure 1, lane 7). These results suggested that following ligand binding, a region around amino acid 217 of the mouse AhR (in the mAhR PASA domain[13]) becomes more exposed to trypsin, and this is the first real experimental evidence of a ligand-dependent conformational change in the mouse AhR. Moreover, increased exposure of the mouse AhR PASA domain could be instrumental in initiation dimerization with Arnt during AhR transformation. Increased formation of fragments in the PASA region was also observed for full length AhR (Figure 1, lanes 8, 9).

We next addressed the phenomenon of limited transformation efficiency of the mouse AhR. Using *in vitro* synthesized AhR, Arnt and AhRΔPASB[14], we were able to demonstrate limited transformation/DNA binding efficiency *in vitro* with levels of [^{3}H]TCDD ligand binding at ~50 fmol/mg of protein (Figure 2A) and those of DNA binding (saturated in the presence of excess [^{32}P]DRE) – at ~15 fmol/mg of protein (Figure 2B). Thus, transformation/DNA binding efficiency was about 30%. In these experiments, two observations suggested a lack of effect of ligand binding and conformational changes on the efficiency of AhR transformation/DNA binding. First, ligand binding decreased significantly over time in the absence of Arnt at room temperature (Figure 2A), indicating early saturation of ligand binding and the importance of Arnt in stabilizing AhR:TCDD complex. The latter observation was consistent with a previous study demonstrating an apparent increase in TCDD binding affinity of the AhR following dimerization with Arnt[2]. Thus, ligand binding could not be responsible for slower increase in DNA binding (Figure 2B) or the limited transformation/DNA binding efficiency since it remained stable at much higher levels than that observed for DNA-bound AhR. Secondly, the kinetic profile of the AhRΔPASB was similar to that of the wild type AhR reaching maximum at around 3-4 hours, although at much higher absolute levels of maximum activation (Figure 2B). Since the AhRΔPASB lacks a ligand-binding domain, ligand-dependent conformational changes could not be responsible for the slow transformation rate indicating that neither ligand binding nor conformational changes control mouse AhR transformation/DNA binding. Moreover, the significantly higher levels of transformation efficiency of the AhRΔPASB compared to those of the wild type AhR provide us with an *in vitro* experimental system to further characterize the underlying mechanism(s).

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Figure 1
^{35}S-labeled GST-PASA-PASB, R217A mutation or GST-AhR were analyzed by glutathione pull-down and limited proteolysis (with 1 µg/ml trypsin for 5 min)

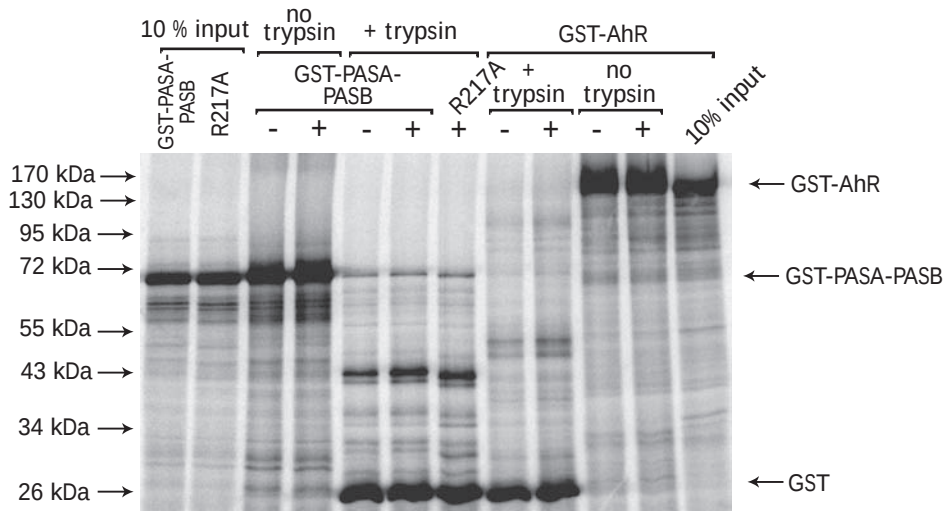

Figure 2
(A) Arnt stabilizes [^{3}H]TCDD binding to the in vitro expressed AhR. *, statistically different in t-test at P<0.005 (N=3). (B) Reduced transformation/DNA binding by in vitro expressed AhR.