The Ah Receptor Ligand Binding Domain: Homology Modeling And Functional Analysis

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Introduction

The Ah receptor (AhR) is a ligand-dependent basic helix-loop-helix (bHLH), PAS (Per-Arnt-Sim)-containing transcription factor that regulates the toxic and biological effects of a variety of chemicals, including halogenated aromatic hydrocarbons (HAHs) such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) and related dioxin-like chemicals (DLCs), and it is believed that these effects result from AhR-dependent alterations (induction/inhibition) in gene expression in susceptible cells.[1-3] Binding of ligand (agonist) to the cytosolicAhR protein complex (containing AhR, hsp90, XAP2 and p23) is presumed to stimulate a conformation change in the AhR exposing an N-terminal nuclear localization sequence that results in nuclear translocation of the complex. Release of the AhR and its subsequent dimerization with Arnt (AhR nuclear translocator), a related bHLH-PAS protein, converts the AhR into a form that binds to DNA and stimulates gene transcription. Although it's known that the AhR plays the key pivotal role in mediating the response of a cell to AhRligands, it still remains to be established as to how a given ligand actually binds to and activates the AhR and AhR-dependent signal transduction. While the best characterized and highest affinity AhRligands include HAHs and polycyclic aromatic hydrocarbons, recent studies have demonstrated that the AhR can be activated by a wide range of structurally dissimilar chemicals.[4,5] These and other results strongly suggest that the AhR has a promiscuous ligand binding domain (LBD) and raise questions regarding the actual spectrum of chemicals that can bind to and activate the AhR. Knowledge of the structural diversity of AhRligands can provide some insight into the identity of natural and endogenous AhRligands and analysis of the specific binding interactions of these ligands within the AhR LBD pocket would reveal the key molecular events regulating AhRligand binding and the ligand-dependent AhR activation. While this has not been possible due to the lack of a 3-dimensional experimental structure of the AhR LBD, the high degree of structural similarity of PAS domains whose crystal structure has been resolved provided an avenue for development of a theoretical homology model. Our first homology model of the PAS domain of the AhR LBD based on the crystal structure of the bacterial oxygen sensing FixL protein allowed us to generate initial hypotheses regarding the residues and secondary structures important in AhR functional activity.[6] A subsequent set of structures of PAS domains has provided us with additional and more relevant template structures for modeling of the mouse AhR LBD. The first is an X-ray structure of the LOV2 domain from the phototropin domain of the photoreceptor PHY3, which binds a flavin mononucleotide (FMN) molecule that exhibits stereo-electronic properties in the three-ring moiety similar to that of PCDDs.[7] The second is an NMR spectroscopy structure of the PASB domain of the hypoxia inducible factor 2 alpha (HIF2A) that shows the highest sequence identity with the AhR LBD (32%).[8] We have generated new homology models of the PAS domain of the murineAhR LBD using these new structures as templates and have carried out site-directed mutagenesis and functional analysis of the mutant AhR protein in order to both test the homology models and use them to identify key residues and to make predictions on the mechanism of ligand-dependent activation of the AhR. In addition, differences in residues within the PASB domain of the AhR (or AhR homolog) from several species were also modeled.

Material and Methods

Materials. TCDD, 2,3,7,8-TCDF and [^{3}H]TCDD were from Dr. Stephen Safe (Texas A&M University). The TNT® Quick Coupled Transcription/Translation (TnT) rabbit reticulocyte lysate system was from Promega (Madison, WI, USA), the Quick-Change site-direct mutagenesis kit from Stratagene (LaJolla, CA, USA) and Molecular biology reagents from New England Biolabs (Beverly, MA). Translation grade L-^{35}S-methionine (>400 Ci/mmol) was purchased from ICN Biomedicals(Costa Mesa, CA) and ^{32}P-ATP (~6,000 Ci/mmol) from Amersham (Piscataway, NJ).

Modeling. The structural alignment of template proteins was derived from FSSP database.[9] Multiple alignment of the known sequences of AhRs against the templates was driven by the secondary structure prediction on the LBD (residues 230-421) obtained with the PSIPRED web system.[10] The 3D modelling and loop search was performed with the aid of SWISS-MODEL Comparative Protein Modelling Server and the Swiss-PdbViewer, as well as with the Modeller program.[11,12] Side-chain conformations were optimised with SCWRL 3.[13] Resulting models were submitted to the Biotech Validation Suite for Protein Structures and to the ProsaII.[14,15] 3D visualisation and images were generated using PyMol.[16]

Site-Directed Mutagenesis, In Vitro Protein Synthesis and AhR Functional Analysis. Full length mouse AhR cDNA in pcDNA3.1 was subjected to site directed mutagenesis as described by the manufacturer (Stratagene) using specific primers designed to contain a nucleotide substitution(s) such that the resulting protein product would contain the desired amino acid change. In all cases, initial mutagenesis of the selected amino acids was to Ala with subsequent mutagenesis to other amino acids with respect to questions being asked (i.e. charged, bulky, hydrophobic etc.). Internal deletions of the AhR for use in hsp90 immunoprecipitation experiments were generated by a combination of PCR and restriction digestion. ^{35}S-Labeled mouse AhR and unlabeled ARNT were synthesized in separate reactions in vitro using the Quick Coupled Transcription and Translation kit (Promega) and the resulting protein products mixed and used in [^{3}H]TCDD ligand and [^{32}P]DNA binding experiments as described.[17] To confirm the level of expression of each mutant AhR, ^{35}S-radiolabeled proteins were subjected to SDS-PAGE as described were visualized by autoradiography and quantitated by phosphorimager analysis (Molecular Dynamics, Sunnyvale, CA, USA). Coimmunopreciptitation experiments examining the binding of ^{35}S-labeled in vitro synthesized wild-type and mutant AhRs with hsp90 were conducted as described.[18]

Results and Discussion

On the basis of experimental data on the structure of the LOV2 domain of PHY3 as well as the PAS domain of HIF, we have proposed a revision of our previous mAhR LBD model.[6] The PAS domain of PHY3 and HIF retains the same general structural elements present in PAS domains previously analyzed (FixL, HERG and PYP), namely containing a five-stranded antiparallel b-sheet, a region with three short helices and a long central a-helix, the so-called helical connector. While the overall homology models of the AhR PAS structure derived from these templates are fairly similar (Fig. 1), there are some local differences. Interestingly, there is significantly less separation between the end of the helical connector and the beta sheet in the HIF-based AhR model, therefore the entrance to the LBD pocket is somewhat larger as compared to that of the PHY3 model and this may facilitate entry and binding of the AhRligands of diverse size that have been previously identified. The distinct differences among all PAS structures in the region of the three small helices suggest that these substructures may contribute to the unique functional activities of each PAS protein. The presence of a long loop at the N-term of this region in the HIF-based AhR model suggests a general flexibility at that side of the cavity entrance. This feature could be associated to a high conformational adaptability to ligand binding.

To analyze our hypotheses derived from the initial model, site-directed mutagenesis experiments on the key residues predicted from the model to influence the ligand binding pocket and ligand binding were carried out. Wild-type and mutant AhRs were synthesized in vitro using reticulocyte lysate and the effects of these mutations on AhR ligand binding were compared with ligand-stimulated changes in ligand and DNA binding. These studies revealed the critical nature of helical connector, since insertion of a helix-breaking proline into this helix disrupted AhRligand and DNA binding. Additionally, mutation of Ala375 to Val dramatically reduced AhRligand binding and essentially eliminated AhR DNA transformation and DNA binding. Interestingly, the analogous residue in the human AhR and mouse AhRd allele is not alanine, but valine and it is this residue that is responsible for the significant reduction in ligand binding and ligand binding affinity to both the human AhR and AhRd allele. Mutagenesis of this residue in the human AhR to Ala increases its ligand binding. Given the location of this residue directly within the center of the modeled LBD pocket, decreased binding with increased amino acid side chain length is not surprising and these results are consistent with our model. Since the level of expression of each of these mutants is very comparable (based on [^{35}S]methionine incorporation) the differences in binding are not simply due to differences in the level of protein expression (data not shown). Additionally, the nonligand (TCDD) binding C. elegansAhR homolog contains a Val at an equivalent position and this would certainly contribute, along with other substitutions and structural difference we have observed in the C. elegansAhR LBD model (not shown), to its reduced ligand binding. We have observed only a select number of mutations adversely affecting AhRligand binding and transformation/DNA binding

and based on the model, the majority of the mutations that exert any effect are those that tend to point into the pocket of the HIF-based model. Finally, we also have identified a mutation in the region of the three small helices that negatively affect AhRligand and DNA binding activity and this mutation falls into one of the small helices, suggesting its importance in AhR functionality. The absence of this helix in the C. elegansAhR that fails to bind ligand is also consistent with a role for this helix in AhR functionality. The majority of our preliminary experimental results provide support for our proposed model and confirm that the modeled pocket, delimited by the highly conserved b-scaffold, is involved in ligand binding. Further sited-directed mutagenesis analysis of the AhR LBD is in progress and will further refine and test our model. Future domain swapping experiments will also allow us to define those regions and specific residues present in AhRs both important for AhRligand binding and functional activity, but also to identify residues/regions that confer species-specific ligand binding and lack of TCDD binding (i.e. C. elegans and clam AhRs).

HSP90 coimmunoprecipitation experiments with AhRs containing a progressive series of deletions and mutations have not revealed of any specific amino acids responsible for hsp90 binding. In fact, our results are more consistent with the hypothesis that multiple regions of the AhR LBD are involved in hsp90 binding interactions, including contacts with the backside and upper loop of the beta-sheet as shown in the AhR LBD models (Fig. 1). Multiple regions of interaction of the AhR with hsp90 makes sense when we consider that ligand binding does not displace hsp90 (since the ligandedAhRtranslocates into the nucleus as a hsp90-bound complex), but results in a structural change that exposes the nuclear localization sequence. The interaction with hsp90 with these regions of the AhR LBD is also consistent with our hypothesized model of the events following AhRligand binding. It should be noted that the PAS homology model we have generated is derived from PAS templates that do not bind hsp90 and as such, the structure is likely to be altered from what we have presented. However, when we consider the structural diversity of AhRligands (many of which could not fit in the pocket of our current LBD model) combined with the fact that this region may be somewhat flexible (at least potentially more than the rest of the model), we hypothesize that the region of the three small helices is partially elevated, resulting in a larger ligand binding cavity and channel for ligand entrance. We propose that ligand binding leads to a conformational change in the pocket resulting in disruption of protein (hsp90?) interactions within the region of the small helices and/or the beta-sheet region at the top of our model. This ligand-dependent disruption could lead to a downward movement of the three helical region that could trap the ligand within the pocket. This "trapping" could explain previous studies demonstrating the essentially irreversible nature of AhRligandbinding.[19] Additional modeling, mutational and functional analysis should provide insights into AhRligand binding and mechanisms of ligand-dependent AhR activation.

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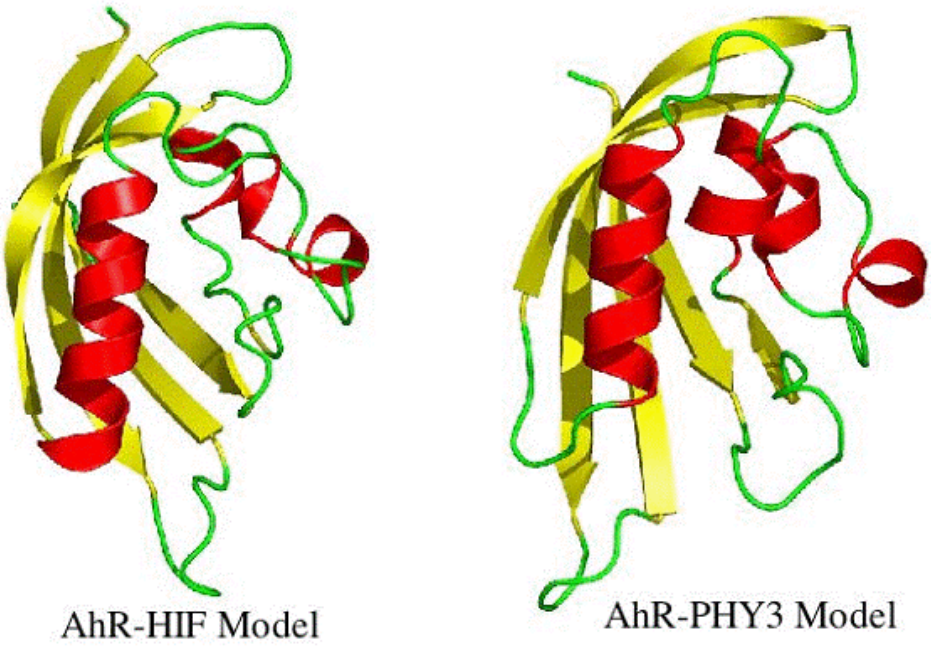

Figure 1. Proposed structures for the mAhR LBD. Representations of the mAhR LBD structures obtained by homology modeling using HIF2A and PHY3 as the template structure and PyMol.[16]