Role of the Per/Arnt/Sim Domains in Ligand-dependent Transformation of the Aryl Hydrocarbon Receptor*

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The aryl hydrocarbon receptor (AhR) mediates the toxic and biological effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin and related compounds. In a process termed transformation, ligand binding converts the AhR into its high affinity DNA binding form that represents a dimer of the AhR and Arnt, a closely related nuclear protein. During transformation, protein chaperone Hsp90 is thought to be replaced by Arnt in overlapping binding sites in the basic helix loop helix and PASB domains of the AhR. Here, analysis of AhR variants containing a modified PASB domain and AhR PASA-PASB fragments of various lengths revealed (i) an inhibitory effect on transformation concomitant with Hsp90 binding in the PASB domain, (ii) an ability of the PASA-PASB fragment of the AhR to reproduce key steps in the transformation process, and (iii) a ligand-dependent conformational change in the PASA domain consistent with increased PASA exposure during AhR transformation. Based on these results, we propose a new mechanism of AhR transformation through initiation of Arnt dimerization and Hsp90 displacement in AhR PASA/B domains. This study provides insights into mechanisms of AhR transformation, dimerization of PAS domain proteins, and Hsp90 dissociation in activation of its client proteins.

The AhR is a ligand-dependent transcription factor that mediates the toxic and biological effects of a diverse spectrum of chemicals including the environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (1, 2). The AhR belongs to the family of basic helix loop helix (bHLH) and Per-Arnt-Sim (PAS) domain proteins that also includes mouse HIF1α, SimI, and CLOCK proteins (3). In its native unliganded state, the AhR is cytosolic and exists in a complex with two molecules of Hsp90 and the proteins XAP2 and p23 (4). In the absence of ligand, Hsp90 binding is thought to maintain the AhR in an inactive state, possibly, through association in the regions that overlap AhR Arnt binding sites (bHLH and PASA/B domains) (5, 6). Hsp90 appears to bind to the ligand-binding PASB and N-terminal DNA-binding bHLH domains and following ligand binding, remains associated with the AhR during nuclear translocation (6–8). Once in the nucleus, Hsp90 dissociates or is more likely displaced by Arnt from their overlapping binding sites in the AhR leading to formation of an active transcriptional dimer of AhR/Arnt (9).

The conversion of the AhR into its high affinity DNA binding form is termed transformation and is presumably driven by a ligand binding-mediated conformational change in the AhR. Although the occurrence of conformational change(s) in the AhR has been suggested by limited proteolysis (10, 11), mechanism and functional significance of the presumed conformational change(s) during AhR transformation remain mostly unknown. It has been previously suggested that ligand-dependent nuclear translocation of the AhR complex results from exposure of its nuclear localization sequence (NLS) in the bHLH domain, with the exposed bHLH domain providing a binding surface that allows initiation of Arnt dimerization (12, 13). However, this mechanism still remains to be proven experimentally, and certain evidence such as the disparate effects of the Hsp90 stabilizing agent sodium molybdate on AhR transformation and nuclear translocation do not suggest a mechanistic connection between these steps of AhR activation (8).

The AhR contains two repeats of the PAS motif, the PASA and PASB domains. Although the PASA domain is essential for formation of the stable AhR:Arnt dimer, the PASB domain contains a ligand binding site and one of the Hsp90 binding sites (5, 6, 14, 15). The PASB domain has been suggested to exert an inhibitory effect on AhR transformation and its deletion results in an AhR that is constitutively active with respect to its transformation/DNA binding and transcriptional activation functions (16–18). Although the ability of Hsp90 to bind to the PASB deleted AhR was not determined, the constitutive activity of this AhR suggests deficient Hsp90 binding and/or exposure of the Arnt dimerization interface(s) on the AhR. Either of these events (loss of Hsp90 binding or exposure of the Arnt dimerization interface) could be initiating AhR transformation, and the PASB domain appears to play central role in ligand dependence of such initiation. Nonetheless, the overall mechanism of the AhR transformation and the role of various AhR domains remain to be elucidated. Accordingly, here we describe a series of studies examining the molecular events involved in the initiation of AhR transformation (loss of Hsp90 and Arnt dimerization). Our results are consistent with a mechanism by which
AhR transformation is mediated through ligand-dependent initiation of Arnt dimerization within the AhR PAS domains and further our understanding of the molecular events in AhR signaling transduction.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Monoclonal 3G3p90 anti-Hsp90 antibody (6, 7) was produced at Antibodies Incorporated (Davis, CA) from hydridoma cells kindly provided by Dr. G. Perdew (Penn State University, PA). Monoclonal M2 anti-FLAG antibody and control murine IgG were purchased from Sigma and AffiniPure goat anti-mouse IgM from Jackson Immunoresearch. The anti-AhR N-terminal end SE6 antibody was kindly provided by Dr. G. Clark (Xenobiotic Detection Systems) and its specificity toward in vitro expressed AhR and Arnt variants, C57BL murine cytosolic AhR (19), and in vitro expressed Arnt (no recognition) is demonstrated in supplemental Fig. S1.

**Plasmid Constructs**—The expression plasmids mBAhR/pcDNA3 and mArnt/pBK-CMV have been previously described (20, 21). BstEII and AflIII sites were introduced at positions -7 and +2397 (relative to the AhR translation start) of mBAhR/pcDNA3 using the QuikChange technique (Stratagene) and used to generate the following plasmid constructs by inserting the indicated PCR-amplified fragments at these sites: mBArnt/pcDNA3 amplifying mArnt from the plasmid mArnt/pBK-CMV using primers A1 and A2 (all primers presented in supplemental Fig. S2), C-EYFP/pcDNA3 by amplification of EYFP from pEYFP-N1 (Clontech) using primers Y1 and Y2, and N-GST/pcDNA3 by amplification of GST from the plasmid pGEX-2T (GE Healthcare) using primers Y3 and Y4. Point mutations in the GST-AhR3 were generated using the Stratagene QuikChange technique. All cloned constructs were verified by sequencing.

**In Vitro Protein Expression and Ligand-dependent Transformation**—Wild type mAhR, mArnt, mAhR variants, GST fusion constructs, and GST were synthesized in vitro in the presence of L-[35S]methionine (MP Biomedicals) or unlabeled L-methionine using the TNT Quick-coupled transcription/translation rabbit reticulocyte lysate kit (Promega). The resulting AhR and Arnt translation mixtures were mixed in a 1:1 (v/v) ratio for DNA binding and dimerization experiments and in a 1:3 ratio for Hsp90 dissociation studies, and incubated with 20 nM TCDD or 2% (v/v) DMSO (the solvent control) for 1–2 h at room temperature. For FLAG co-immunoprecipitations, the in vitro synthesized 35S-labeled AhR variants were transformed in the presence of Arnt-FLAG protein instead of Arnt. For gel retardation experiments, 3 μl of AhR-Arnt translation mixture was diluted to 10 μl with MEDG buffer (25 mM MOPS, pH 7.5, 10% (v/v) glycerol, 15 mM KCl, 1 mM EDTA, 1 mM dithiothreitol) and incubated in the presence of 20 nM TCDD or 2% (v/v) DMSO for 2 h at room temperature.

**Co-immunoprecipitation Assays**—Goat anti-mouse IgM was conjugated to Affi-Gel 10 (Bio-Rad) as described by the manufacturer and incubated with 5% (w/v) bovine serum albumin in MEDG buffer for 1 h at 4 °C. Aliquots of the conjugated matrix were incubated with excess 3G3 antibody (2–3 μg/reaction) for 1 h at 4 °C with shaking and washed once with MEDGN (MEDG, 0.1% Nonidet P-40) buffer. Ten μl of transformation mixture (or 35S-labeled protein mixture) was incubated with a total of 10 μl of the prepared 3G3-bound matrix for 1 h at 4 °C with shaking. The beads were washed 3 times with 1 ml of MEDGN (supplemented with 20 mM sodium molybdate where indicated), analyzed by SDS-PAGE as previously described (22), and resolved by Phosphorimaging (GE Healthcare or Fujifilm). In FLAG co-immunoprecipitations, the transformation mixtures were incubated with 1 μg of the anti-FLAG antibody or IgG control for 1 h at 4 °C and then with 10 μl of Protein G Plus-agarose (Santa Cruz) (pre-washed with 2% (w/v) bovine serum albumin in PBS) for 1 h at 4 °C with shaking. The samples were washed 3 times with PBS and proteins were resolved by SDS-PAGE as previously described (22).

**GST Pull-down and Limited Proteolysis Assays**—Following AhR transformation reactions, 35S-labeled GST-AhR1–3 and mutated GST-AhR3 constructs were incubated in the presence of 20 nM TCDD, 2% (v/v) DMSO for 1 h at room temperature, and incubated with 10 μl of prewashed (with PBS) glutathione-agarose (Sigma) for 30 min at room temperature with shaking. Then, the samples were washed once with PBS, incubated with 1 μg/ml trypsin for the indicated periods of time at room temperature, washed 3 times with PBS, and analyzed by SDS-PAGE as previously described (22).

**Hydroxyapatite Ligand Binding Assays**—[3H]TCDD (13 Ci/mmol) was obtained from Dr. Safe (Texas A&M University). [3H]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 [3H]TCDD for the indicated periods of time and measured following the previously published protocol for cytosolic AhR (22). Equivalent amounts of unprogrammed in vitro synthesized reactions were used as a nonspecific binding control as previously described (23).

**Gel Retardation Assays**—Annealed double-stranded oligonucleotides containing the AhR:Arnt DNA binding site (DRE3) from the murine CYP1A1 upstream regulatory sequence were 32P-labeled and gel retardation analysis was conducted as detailed previously (22, 24).

**Cell Culture, Transfection, and Fluorescence Measurement**—COS-1 cells (ATCC) were grown and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum albumin.
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Deletion of the PASB domain was sufficient to destabilize Hsp90 binding to the bHLH domain of the AhRΔPASB protein, although it has been previously reported that C-terminal deletion of the AhR containing the bHLH but not the PASB domains could bind Hsp90 (6, 7). This observation suggested that a change in the overall structure of the AhR resulting from the internal deletion may destabilize Hsp90 binding in the bHLH domain of AhRΔPASB. To minimize destabilizing structural effects of internal deletion, we generated a chimeric AhR, in which the AhR PASB domain (amino acids 263–409) was substituted with the comparable PASB domain of Arnt (amino acids 326–470) (Fig. 1a). The exchanged region in this AhR/PASB-Arnt chimera was slightly different from that of the original deletion in the AhRΔPASB to allow swapping of structurally homologous regions between the two proteins. Interestingly, whereas Arnt does not bind Hsp90 (7) and the AhRΔPASB is deficient in this binding, insertion of the Arnt PASB domain partially restored Hsp90 binding to the chimeric AhR as compared with the AhRΔPASB (compare 29% of input for AhR/PASB-Arnt to 5.6% of input for AhRΔPASB and 54% of input for wtAhR, Fig. 1b). These results suggest that some loss in Hsp90 binding in AhRΔPASB is likely due to a change in the overall structure of the AhR and the remainder due to loss of the Hsp90 binding within PASB. We assume that the restored Hsp90 binding occurs within the AhR bHLH domain binding site of AhR/PASB-Arnt because Arnt (and presumably the Arnt PASB) does not bind Hsp90 (7).

Although removal/reduction of Hsp90 inhibitory association may explain previously reported ligand-independent transformation and nuclear translocation by the AhRΔPASB (17), it was of interest to analyze these activation steps for AhR/PASB-Arnt because this AhR variant retained some Hsp90 binding. AhR transformation of the wtAhR, AhRΔPASB, and the AhR/PASB-Arnt chimeras was examined indirectly by measuring AhR DNA binding using gel retardation analysis (22). As expected, a ligand-dependent increase in complex formation was only observed with the wtAhR, whereas AhRΔPASB exhibited constitutive DNA binding activity (Fig. 2a). The significantly higher amount of DNA binding by the AhRΔPASB (Fig. 2, a and b) is consistent with decreased association with Hsp90 in this AhR variant. Despite retaining some Hsp90 binding, the AhR/PASB-Arnt construct also demonstrated ligand-independent dimerization and DNA binding (Fig. 2a) suggesting that the presumed lack of Hsp90 binding to the PASB domain of this chimera is sufficient to allow constitutive Arnt dimerization, AhR transformation, and DNA binding. Interestingly, the levels of constitutive DNA binding by AhR/PASB-Arnt were similar to that of wtAhR in the presence of TCDD (Fig. 2a). Moreover, the similarity in kinetics of the time course of transformation/DNA binding of these two proteins (Fig. 2b) suggests similarities in the mechanism of dimerization/DNA binding between...
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![Diagram](attachment:figure2.png)

**FIGURE 2.** Constitutive transformation and DNA binding by AhRΔPASB and AhR/PASB-Arnt. *a*, wtAhR, AhRΔPASB (ΔPASB), and AhR/PASB-Arnt (PASB-Arnt) proteins were expressed *in vitro* and incubated with Arnt in the presence of 20 nM TCDD (+) or 2% (v/v) DMSO (−) for 2 h. AhR-Arnt-DNA complex formation was resolved by gel retardation assay and visualized by Phosphorimager analysis. A representative of three independent experiments is shown. *b*, the time course of transformation/DNA binding at room temperature was analyzed as in panel a for *in vitro* expressed AhR (in the presence of DMSO (●) or TCDD (○)), AhRΔPASB (in the presence of DMSO (▲)) and AhR/PASB-Arnt in the presence of DMSO (▼). Values represent the mean ± S.D. of triplicate analyses. Data shown are representative of three independent experiments.

The ligand-bound wtAhR and AhR/PASB-Arnt. These findings are consistent with the previously suggested inhibitory role of the AhR PASB domain in AhR transformation (16, 18) and suggest that although the AhR PASB is important in ligand-dependent control of transformation, it contributes little to maximum levels and kinetics of AhR:Arnt dimerization and DNA binding.

To analyze cellular localization of the constructs of interest, wtAhR, AhRΔPASB, and AhR/PASB-Arnt were tagged at the C-terminal end with yellow fluorescent protein (YFP). The resulting constructs demonstrated unchanged levels of protein expression, Hsp90 binding, and DNA binding compared with their untagged counterparts (supplemental Fig. S3). COS-1 cells were transiently transfected with YFP-tagged AhR constructs, incubated in the presence of TCDD (or solvent control DMSO) for 1–2 h, and AhR localization determined by fluorescence microscopy. Cells transfected with the control vector pcDNA3 did not produce a fluorescent signal (data not shown). Although incubation of the transiently transfected COS-1 cells with TCDD resulted in a shift of fluorescent signal of the wtAhR-YFP from cytosolic and cytosolic/nuclear localization to predominantly nuclear localization (indicating nuclear import of the AhR complex), AhRΔPASB-YFP fluorescence was primarily localized within the nuclei independent of ligand, as expected (Fig. 3a). In contrast, (AhR/PASB-Arnt)-YFP exhibited predominantly cytosolic and cytosolic/nuclear localization (Fig. 3a) and it was expressed at levels comparable with that of AhRΔPASB as determined by Western blotting assay (Fig. 3b). It is unclear why AhR/PASB-Arnt was less efficient in nuclear translocation than AhRΔPASB or wtAhR in the presence of TCDD, although presumed Hsp90 binding to the bHLH domain of this AhR chimera may mask the NLS. Nonetheless, it is significant that the same AhR protein was fully active in transformation *in vitro* (as evidenced by DNA binding, Fig. 2) and yet inactive in nuclear translocation. Although the previously proposed mechanism of initiation of AhR transformation through the bHLH domain relied on presumed ligand-dependent exposure of this domain (which contains the NLS), the example of the AhR/PASB-Arnt demonstrates that the AhR protein may not need to be able to translocate into the nucleus to be active in AhR transformation (conversion into its DNA binding form).

**Role of the PASA and PASB Domains in the AhR Transformation**—Constitutive transformation of the AhR/PASB-Arnt *in vitro* suggests that this process may be initiated through AhR PAS domains because exposure of the bHLH domain of this chimeric AhR (where the NLS is located) appears to be restricted due to its lack of constitutive nuclear localization in transfected cells (Fig. 3a). Because in the inactive state, Hsp90 is associated with the Arnt dimerization interface in the AhR, the hypothetical mechanism of initiation of AhR transformation through the PASB may involve ligand-dependent dissociation of Hsp90 from the binding site in this domain. To test this possibility, we examined various steps of AhR transformation (ligand binding, Hsp90 displacement and dimerization with Arnt) using AhR-GST fusions containing various lengths of AhR PASA and PASB domains as well as GST full-length AhR. A GST tag was added to stabilize and rapidly isolate the expressed protein fragments for analysis. GST-AhR 1, 2, and 3 span amino acids 230–421, 167–421, and 83–421 of the wtAhR, respectively (Fig. 4a), and were each expressed *in vitro* at levels comparable with that of wtAhR and free GST (Fig. 4b and Table 1), although protein expression levels of GST-AhR-FL were significantly lower. Co-immunoprecipitation experiments revealed that all constructs exhibited high levels of Hsp90 binding (37–45% of input), comparable with that of wtAhR (46% of input, Fig. 4b), whereas the GST negative control protein alone did not bind Hsp90 (Fig. 4b). Thus, whereas GST-AhR-FL was expressed at much lower levels, it had similar Hsp90 binding suggesting that its protein stability was not due to deficient association with Hsp90. However, we chose to not perform functional studies on this construct due to the low levels of expression and functional activity in transformation studies (data not shown). In contrast, we observed that the GST-AhR 1–3 constructs were fully active in AhR transforma-
tion (as detected by dimerization with Arnt and Hsp90 displacement, Fig. 5). Thus, whereas these protein fragments differ from the full-length AhR and have an added tag, their ability to undergo transformation suggested that activities of the PASA and PASB domains in the AhR transformation are retained in isolation from the other AhR domains.

\[ \text{[H]TCDD specific binding to GST-AhR} \]

The small difference in Hsp90 binding to the wtAhR in experiments demonstrated in Figs. 1b and 4b (54 versus 46% of input) could be attributed to the presence of 20 mM sodium molybdate in the washing buffer. We did not include this AhR: Hsp90 binding stabilizing agent in the washing buffer in the latter experiment because at the next step, the process of ligand-dependent Hsp90 dissociation was examined using this co-immunoprecipitation technique.

Addition of ligand and/or Arnt did not alter levels of Hsp90 binding to GST-AhR1 and GST-AhR2 as determined by co-immunoprecipitation with anti-Hsp90 antibody (Fig. 5a) suggesting that ligand binding to the PASB domain of this AhR fragment (Table 1) does not result in Hsp90 dissociation from its binding site(s) within this domain. However, with GST-AhR3, which includes the PASA and PASB domains, Hsp90 binding was reduced in the presence of both ligand and Arnt to a level comparable with that of wtAhR (Fig. 5b).

\[ \text{TABLE 1} \]

Protein expression and ligand binding activity of GST fusions relative to that of wtAhR

| GST fusion | Number of methionines | Protein expression* | [H]TCDD binding* |
|------------|-----------------------|---------------------|------------------|
| 1          | 15                    | 93 ± 9%             | 34 ± 6%          |
| 2          | 16                    | 97 ± 13%            | 39 ± 4%          |
| 3          | 16                    | 83 ± 9%             | 47 ± 5%          |

*Values presented are relative to the amount of [H]TCDD specific binding to wtAhR and are expressed as the mean ± S.D. of three to four independent experiments.

\[ \text{FIGURE 4. In vitro protein expression and Hsp90 binding of AhR PASA/B fragments.} \]

\[ \text{FIGURE 3. Ahr/PASB-Arnt does not translocate into the nucleus in transient transfections in COS-1 cells.} \]
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FIGURE 5. The AhR PASA domain is required for efficient displacement of Hsp90 and dimerization with Arnt. a, GST-AhR fusion proteins 1, 2, 3, and wtAhR were 35S-labeled, incubated in the presence of 20 nM TCDD (+) or 2% (v/v) DMSO (−) and Arnt or unprogrammed TnT lysate and co-immunoprecipitated with anti-Hsp90 antibody or IgM control. Precipitated proteins were analyzed by SDS-PAGE. b, specific bands in panel a were quantitated by Phosphorimager analysis. Values represent the mean ± S.D. of three replicates. Asterisks (*) indicate the values that are statistically different from the matched (−/−, +/−, and −/+ ) reactions at p < 0.01 as determined by the Student’s t test. The results are representative of five to six independent experiments. c, GST-AhR fusion proteins 1, 2, 3, and AhR were 35S-labeled, incubated in the presence of 20 nM TCDD (+) or 2% (v/v) DMSO (−), and FLAG-tagged Arnt and precipitated with anti-FLAG antibody or IgG control. Proteins were quantitated using Phosphorimager analysis. The results are representative of three independent experiments.

to either wtAhR or GST-AhR3 in the presence of TCDD and Arnt (+/+ ) was statistically lower than that in the presence of any other TCDD/Arnt combination (Fig. 5b). Thus, whereas ligand binding did not result in Hsp90 dissociation from the PASB domain, the presence of the PASA domain was sufficient to initiate Hsp90 dissociation from the PASA-PASB fragment, but only in the presence of TCDD and Arnt.

Co-immunoprecipitation experiments with anti-FLAG antibody revealed that ligand-dependent dimerization of GST-AhR3 with Arnt-FLAG was comparable with that of wtAhR, whereas GST-AhR1 and GST-AhR2 demonstrated significantly lower levels of dimerization, which was, however, ligand-dependent (Fig. 5c). This finding suggests that although the PASB domain by itself is capable of initiating dimerization with Arnt, it cannot support the full measure of dimer formation.

The observed correlation of Arnt dimerization and Arnt-dependent Hsp90 dissociation is consistent with the previous suggestion of Hsp90 displacement by Arnt during AhR transformation (6, 13, 26, 27) and, being contingent on the presence of the PASA, implicates this domain in stabilizing the AhR:Arnt dimer in transformation. Taken together, these data suggest a mechanism of ligand-dependent transformation of the AhR PASA-PASB fragment, in which Hsp90 is displaced by Arnt from the ligand-bound PASB domain binding site due to stabilization of the AhR/Arnt interaction by the AhR PASA domain.

Although GST-AhR1 exhibited ligand-dependent dimerization with Arnt (at approximately half of that observed with GST-AhR3 (Fig. 5c)), there was no detectable Hsp90 dissociation in contrast to that observed with GST-AhR3 or wtAhR (Fig. 5b). It is possible that a lower level of dimerization of this construct with Arnt masks corresponding changes in its association with Hsp90 or that an alternative dimerization interface is used for Arnt dimer formation in this AhR PAS domain fragment.

In contrast to a previous study (6), we did not observe a significant decrease in bound Hsp90 following ligand binding to the wtAhR in the absence of Arnt (Fig. 5b), although a small decrease in bound Hsp90 was observed for the wtAhR and GST-AhR3 (which contains PASA and PASB domains) in the presence of ligand (compare +/− to +/+ reactions in Fig. 5b). Although we hypothesized that following ligand binding Hsp90 association is weakened with the wtAhR (or the PASA-PASB fragment), a higher stringency washing buffer did not result in ligand-dependent differences in binding in the absence of Arnt (data not shown). It is unlikely that Hsp90 fully dissociates from the AhR simply as a result of ligand binding and this is consistent with previous findings that Hsp90 remained bound to the AhR complex through ligand-induced nuclear translocation, and that the ligand-bound nuclear AhR complex contained Hsp90 (8, 28). It is possible that ligand binding results in weakened AhR/Hsp90 interactions, however, this remains to be confirmed.

Ligand Binding Results in a Conformational Change in the AhR PASA Domain—Previously, a conformational change in the AhR was reported using limited proteolysis (10). In these experiments, a protected AhR fragment was only formed in the presence of both Arnt and TCDD, which approximately spanned the AhR Arnt dimerization interface (bHLH through PASB domains) (10). Thus the apparent conformational change(s) could have represented a difference between the AhR-Hsp90 and AhR-Arnt complexes. Seeking to demonstrate a conformational change in the absence of Arnt, we modified this approach and performed limited proteolysis on 35S-labeled GST-AhR3 bound to glutathione beads. The assumption of this strategy was that first, a smaller length of the analyzed fragment would generate fewer proteolytic fragments and second, only proteolytic fragments containing the GST moiety would be isolated (on glutathione beads) following limited proteolysis. Such an approach would allow approximate mapping of the cleavage site(s).

Glutathione precipitation/limited proteolysis of 35S-labeled GST-AhR3 revealed a fragment of ~43 kDa, which was moderately increased in the presence of ligand (Fig. 6a). Assuming the GST moiety at the N-terminal end of this fragment, the cleavage site was tentatively mapped to amino acid 225 of AhR.
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AhR, or within the PASA domain according to the domain alignment of the bHLH-PAS proteins from a recent study (29).

Quantitation of the limited proteolysis results is presented in Fig. 6c and was carried out as follows. The specific bands (of the GST-AhR3 and 43-kDa proteolytic fragment) from the DMSO and TCDD reactions were quantitated using the phosphorimager technique, and normalized to corresponding GST-AhR3 intensities from DMSO and TCDD-treated “no trypsin” control reactions (to correct for differences in the input of [35S]GST-AhR3). In addition, the percentages of the 43-kDa band were adjusted using a factor of 16/10, which reflects the fact that this fragment would contain 10 methionines (when its C terminus is mapped around AhR amino acid 209) compared with 16 methionines in the GST-AhR3 fragment.

Comparison of the digestion time profiles revealed that the amount of the 43-kDa fragment was ~2-fold higher in the presence of TCDD as compared with control (DMSO) reactions at all incubation times (Fig. 6c). The ligand-dependent difference in formation of the 43-kDa fragment can result from either higher accumulation in the presence of TCDD or faster degradation in the presence of DMSO, or a combination of both. At 5 and 10 min the amount of undigested GST-AhR3 is lower than that of the 43-kDa fragment indicating higher stability of the 43-kDa fragment compared with the undigested form. Moreover, the amounts of the 43-kDa fragment in the DMSO and TCDD reactions decreased proportionally (Fig. 6c) suggesting that the 43-kDa fragment has similar degradation rates in the DMSO and TCDD reactions. Hence, the ligand-dependent difference in its formation could be due to differences in accumulation rather than degradation of the fragment.

Given that no higher molecular mass product(s) was present in amounts close to that of the 43-kDa fragment at 2 min (about 25% of bound input, Fig. 6c), the 43-kDa fragment and GST appear to be the primary products of proteolytic digestion suggesting that the 43-kDa fragment is formed directly from GST-AhR3. The increase in ligand-dependent accumulation of the 43-kDa fragment indicates that ligand binding results in increased cleavage of the GST-AhR3 in the PASA domain. Such preferential cleavage would be consistent with increased ligand-dependent exposure of the PASA domain.

Next, we attempted to identify the specific residue(s) responsible for producing the 43-kDa fragment. Predicted trypsin cleavage sites spanning amino acids 106 to 354 were mutated to alanine, and the resulting GST-AhR3 variants were subjected to limited proteolysis (Fig. 7). Although no single mutation resulted in complete disappearance of the 43-kDa band, mutation of arginine 217 to alanine (R217A) resulted in a substantial reduction of the 43-kDa band (Fig. 7). Moreover, this cleavage site was close to the predicted position (amino acids 209–225). For these reasons, further analysis involving this mutation was performed.

The lack of complete disappearance of the 43-kDa protein band by a single mutation could suggest that this protein band actually results from cleavage at multiple trypsin sites that are close to each other. In fact, there are four putative trypsin sites in the RC-rich stretch where Arg217 is located. The apparent slightly shorter size of the band corresponding to the 43-kDa fragment with the R217A mutant (Fig. 8c, compare position of
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DISCUSSION

Ligand-dependent AhR transformation is a complex event and is thought to be triggered by a ligand binding-driven conformational change(s) in the AhR protein. Previous studies have demonstrated that inactive unliganded AhR is associated with Hsp90, and that during transformation ligand-bound AhR dimerizes with Arnt in the nucleus, releasing Hsp90 at some step prior to dimer formation (9, 30). Because Hsp90 and Arnt have overlapping binding sites in the AhR (in the bHLH and PASB domains) and do not appear to bind to the AhR at the same time, it was hypothesized that Hsp90 is displaced by Arnt during transformation and that in the absence of ligand Hsp90 maintains the AhR in a dimerization-incompetent state by masking the Arnt dimerization interface(s) on the receptor (6, 13, 25, 27). In agreement with this hypothesis, we found that AhRΔPASB, which is constitutively active in transformation, was deficient in Hsp90 binding. Moreover, AhR/PASB-Arnt was constitutively active in transformation/DNA binding while retaining ~50% of Hsp90 binding compared with wtAhR indicating that loss of Hsp90 association specifically in the PASB domain of the AhR is likely sufficient for constitutive activation of transformation. Interestingly, loss of some Hsp90 binding to the AhR/PASB-Arnt protein did not appear to affect the kinetics of AhR transformation that was similar to that of wtAhR in the presence of TCDD (Fig. 2b) suggesting similarities in the mechanisms of transformation/DNA binding between these two proteins. Further analysis of transformation events in the PASA-PASB fragments of the AhR revealed that this region is sufficient to reproduce transformation events and that following ligand binding, a conformational change can be detected in the PASA that is consistent with increased exposure of this domain. Such increased exposure of the PASA domain could be coincidental with an increase in dimerization potential of the PASA-PASB fragment following ligand binding and would explain the observed stabilizing role of the PASA on AhR:Arnt dimerization resulting in more efficient Hsp90 displacement. Together, these data are consistent with a mechanism of AhR transformation that is initiated through the PAS domains.

In contrast, the previously proposed mechanism suggested that initiation of AhR transformation occurred at the bHLH domain (12, 13). This mechanism was based on predicted exposure of the NLS contained within the bHLH domain following ligand binding due to the observed nuclear import of the AhR complex (12). However, a more recent study suggested that nuclear import of the AhR complex may depend on a change in the NLS conformation rather than its exposure and that XAP2 could be involved in this process (31). Moreover, the prediction of ligand-dependent dissociation of Hsp90 from the bHLH domain (to account for presumed NLS exposure) was also questioned by the findings that the Hsp90 binding stabilizing agent sodium molybdate did not inhibit nuclear translocation of the AhR complex (8), and by the observation that deletion of the C-terminal transactivation domain of the AhR, which does not affect Hsp90 binding, resulted in constitutive ligand-independent AhR nuclear localization (32, 33).

In this study, we found that the chimeric AhR variant, AhR/PASB-Arnt, was capable of transformation but did not translo-
cate into the nucleus (Fig. 3). These results further support the idea of a certain degree of independence between the effect of ligand binding on the transformation potential of the AhR and its ability to undergo nuclear localization. We suggest that the inability of the AhR/PASB-Arnt to undergo nuclear translocation is due to the loss of the ability of ligand to affect Hsp90 bound in the bHLH region. Although ligand binding should clearly affect conformation or exposure of the wtAhR NLS, the mechanism of this effect remains unclear. However, together with previous studies, our data suggest that the bHLH exposure is not responsible for initiation of dissociation of Hsp90 and dimerization with Arnt in AhR transformation.

Our studies also rule out another possible mechanism of initiation of transformation, namely through ligand-dependent

FIGURE 8. R217A mutation results in an altered proteolytic pattern. a. 35S-labeled GST-AhR3 and its indicated point mutation variants were incubated in the presence of TCDD and analyzed by pull-down limited proteolysis analysis as described in the legend to Fig. 7. The gel was visualized and quantitated by Phosphorimager analysis, and density lanes profiles are presented (b and c). Input controls (10% input) are shown. The 43-kDa fragment is indicated with an arrow. Results are representative of three independent experiments. b, density lane profiles for the wild type GST-AhR3 (blue), R212A (green), and R215A (cyan) variants. PSL, photostimulated luminescence units. c, density lane profiles for the wild type GST-AhR3 (blue), R217A (pink), R212A/R217A (peach), and R215A/ R217A (orange) variants.
dissociation of Hsp90 in the AhR PASB domain. Such a mechanism was suggested by notion of constitutive activation of AhR/PASB-Arnt (which presumably lacks Hsp90 binding in the PASB), however, ligand binding to the GST-PASB (GST-AhR1) did not result in Hsp90 dissociation (Fig. 5, a and b). On the other hand, the PASB domain can dimerize with Arnt although less efficiently than the wtAhR or GST-AhR3 (Fig. 5c). These findings not only suggest that the PASB domain is capable of initiation of dimerization and that the PASA domain is necessary for successful completion of this process (resulting in Hsp90 displacement), but are also consistent with the previously reported inhibitory effect of the PASB domain on transformation (16, 18) and demonstration of PASA as a major contributor to the stability of the AhR:Arnt dimer (14). Moreover, it has been found that a conformational change in the AhR is required for stable dimer formation because geldanamycin- or salt-mediated dissociation of Hsp90 from the AhR resulted in less stable dimer formation (5, 13).

The occurrence of a ligand-dependent conformational change in the AhR was previously suggested by the observation of the formation of a transiently protected trypic AhR fragment in limited proteolysis assay (10). This fragment was observed only in the presence of Arnt and an AhR agonist and it overlapped the bHLH/PAS domains (i.e. the Arnt dimerization region of the AhR). Although these results demonstrate the ligand-dependent formation of the AhR:Arnt dimer, they do not directly demonstrate a ligand-dependent conformational change in the AhR because the protected fragment could simply result from differences in exposure of regions of the AhR due to the presence of bound Arnt rather than bound Hsp90. Modification of the limited proteolysis assay reported here revealed a ligand-dependent change in the proteolytical pattern of the AhR and AhR fragments in the absence of Arnt. In addition, these analyses allowed tentative mapping of the cleavage site close to Arg217, which is located in the AhR PASA domain (29). Interestingly, Arg217 is located within PASA in an arginine/cysteine-rich stretch of amino acids (212RCFRC217LRC), previously reported to affect AhR transformation (34). Thus, the PASA domain may play an additional regulatory role in the AhR activation.

At present, it is not clear how ligand-dependent conformational changes in the PAS domains of proteins lead to activation of distinct functional domains in the same protein. α-Helical elements outside the canonical PAS structure have been suggested to mediate PAS-PAS contacts in PAS-containing protein sensors (35). Also, in the PASA-PASB homodimerization of the Drosophila periodicity protein Per, α-helices C-terminal to the PASB domain mediate contacts between subunits (29). One can envision that elements outside the AhR PASB-fold, specifically in the C-terminal region, change their position or orientation following ligand binding, resulting in increased PASA exposure. Such re-orientation of C-terminal domains relative to the rest of the protein could also be relevant for the reported ability of the C-terminal end of the AhR to modulate its nuclear translocation (32, 33). Understanding the mechanism of AhR dimerization with Arnt would provide insights into dimerization mechanisms of other PAS-containing proteins, including HIF1α, SimI, soluble GC, and proteins of circadian cycles.

Our proposed mechanism is also important for understanding involvement of Hsp90 in activation of its protein clients. This chaperone has been found associated with functionally important domains of its clients including DNA binding and/or dimerization domains in the AhR, HIF1α, SimI, soluble GC, and p53, the regulatory polo domain in Polo kinase and kinase domains in multiple protein kinases including Raf, Akt, Cdk2, and Erb2 (6, 36–44). The functional activity of these sites of Hsp90 association imply that Hsp90 dissociation could lead to activation of the client protein, a mechanism suggested for many nuclear hormone receptors (45, 46), although the exact mechanisms of Hsp90 dissociation/displacement from different Hsp90 clients remain to be elucidated. For example, ligand binding does not lead to Hsp90 dissociation from the AhR or glucocorticoid receptor, nor does phosphorylation of the activation segment result in Hsp90 dissociation from the kinase domain of Akt (8, 47, 48). It is possible that during activation of these client proteins, Hsp90 could simply be displaced from functional domains by dimerization partners, allosteric co-activators, or substrate proteins. However, such a mechanism would suggest a regulatory role for this chaperone in the functional activity of the client protein (through competitive displacement). Contrary to this notion, in the mechanism proposed for the AhR transformation, dimerization with Arnt is stabilized through the AhR PASA domain, a non-Hsp90 binding region. In this mechanism, Hsp90 would function as a passive chaperone, whose presence obscures the Arnt dimerization domains in the unliganded AhR, rather than it functioning as a specific regulator of AhR:Arnt dimer formation. Studies of Hsp90 involvement and dissociation in the activation mechanisms of other client proteins will be required to further characterize its functions in signal transduction.

In conclusion, we characterized the role of the PAS domains in the ligand-dependent transformation of the AhR and proposed a novel mechanism of this process with initiation of Arnt dimerization occurring through PAS domains. These results provide valuable insights into mechanisms of ligand-dependent AhR signal transduction as well as of dimerization of PAS-containing proteins and Hsp90 displacement in activation of its client proteins.

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