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Agonist but Not Antagonist Ligands Induce Conformational Change in the Mouse Aryl Hydrocarbon Receptor as Detected by Partial Proteolysis

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ABSTRACT

The cytosolic transcription factor known as the aryl hydrocarbon receptor (AhR) undergoes transformation to a DNA-binding form by a series of processes initiated by binding of ligand. Subsequent steps include dissociation of several proteins that are complexed with the inactive receptor, nuclear translocation, and dimerization with Arnt. We have used limited proteolysis of the in vitro-translated mouse AhR to determine whether this technique can detect conformational change(s) associated with AhR transformation and whether the effect of agonist and antagonist ligands can be distinguished by this assay. Limited digestion of [^{35}S]AhR/AhR nuclear translocator (Arnt) by trypsin produced a peptide of approximately 40 kDa that was more resistant to proteolysis in the presence of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) than vehicle and was also Arnt-dependent. This trypsin-resistant peptide was also elicited in the presence of other agonist ligands, but not with antagonist ligands that do not form the DNA-binding AhR/Arnt complex. Immunoblot of trypsin-treated AhR/Arnt ± TCDD indicated that the trypsin-resistant peptide did not include the N-terminal portion of the AhR against which the antibody was made. Truncated AhRs were also subjected to limited trypsinization. From AhR(1–399), a TCDD-dependent peptide of approximately 35 kDa was observed; from the constitutively active AhR(1–348), a band of approximately 30 kDa was produced from vehicle- and TCDD-treated protein. From these observations, we hypothesize that the trypsin-resistant peptide from full-length AhR spans approximately from amino acid 80 to 440. We conclude that agonist ligands initiate structural alteration in AhR that is Arnt-dependent and at least partially involves the ligand-binding/Per-Arnt-Sim domain.

Functioning as a transcription factor, the aryl hydrocarbon receptor (AhR) mediates the biological responses, including toxicity, to dioxins and related chemical contaminants. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the highest affinity ligand of the AhR and the most toxic member of this family of halogenated aromatic hydrocarbon chemicals. In the absence of ligand, this cytosolic receptor is complexed with the chaperone hsp90, cochaperone p23, and the immunophilin-like XAP2 (or ARA9 or AIP) (Carver et al., 1998; Meyer and Perdew, 1999). TCDD binding to AhR initiates its transformation to a form that is able to bind to specific dioxin-responsive sites (DREs) in DNA to enhance transcription of the associated gene(s). This process of transformation includes dissociation of one or several of the proteins associated with the unoccupied AhR in the cytoplasm, movement of the receptor-ligand complex into the nucleus, and dimerization with another protein, Arnt, to form the transcriptionally active TCDD-AhR-Arnt complex (Rowlands and Gustafsson, 1997; Denison and Phelan, 1998; Whitlock, 1999). Even before it was discovered that AhR dimerizes with another protein, it was thought that transformation was likely to involve conformational change(s) in the protein, because TCDD binding increases AhR thermostability and alters its sedimentation coefficient and molecular size (Okey et al., 1979; Denison et al., 1986; Kester and Gasiewicz, 1987).

Besides TCDD and related halogenated aromatic hydrocarbons, a diversity of other ligands bind to the AhR, albeit with lower affinity than TCDD. Some of these (e.g., oxidized tryptophan, curcumin, lipoxin, indirubin, indigo) can also transform the AhR to a transcriptionally active form (Sindhu et al., 1996; Ciolino et al., 1998; Heath-Pagliuso et al., 1998; Schaldach et al., 1999; Adachi et al., 2001). Other classes of ligands, notably some flavone derivatives, as well as 9-hydroxyellipticine, phenanthrolines, and 7-ketocholesterol, seem to bind to AhR but are unable (or less able) to elicit the

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ABBREVIATIONS: AhR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; hsp90, 90-kDa heat shock protein; DRE, dioxin response element; Arnt, aryl hydrocarbon nuclear translocator; EMSA, electrophoretic mobility shift assay; HEDG, HEPES/EDTA/glycerol/dithiothreitol; Me_2SO, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis; B[*a*]P, benzo[*a*]pyrene; BNF, β-naphthoflavone; wt, wild type; PAS, Per/Arnt/Sim (periodicity/aryl hydrocarbon receptor nuclear translocator/simple-minded).

DRE-binding conformation of the receptor (Mahon and Gasiewicz, 1992; Kurl et al., 1993; Gasiewicz et al., 1996; Lu et al., 1996; Savouret et al., 2001). Data from our laboratory suggest that 3′-methoxy-4′-nitroflavone antagonizes TCDD activity by binding competitively to the same site on the receptor but not eliciting the subsequent steps of AhR transformation. Instead, the 3′-methoxy-4′-nitroflavone-bound AhR in Hepa cells, unlike the TCDD-bound AhR, remains largely in the cytosol, still complexed with hsp90 (Henry et al., 1999). These observations suggest that ligand binding alone is insufficient to initiate the necessary steps leading to receptor transformation. However, the crucial difference between the interaction of agonist versus antagonist ligands that accounts for their divergent activity remains unknown. Structure-activity analysis of a series of substituted flavones (Henry et al., 1999) showed that the most potent antagonist compounds have high electron charge density external to the ring structure (4′-azido or 4′-nitro). We hypothesized that hydrogen bonding or electrostatic interaction between such flavones and particular but as-yet-unidentified amino acid(s) of the AhR may cause the stabilization of the AhR-ligand complex in a conformation that inhibits the agonist-induced structural change mediating subsequent steps in AhR transformation to the DRE-binding form. The steroid receptor literature has several reports of mutation at a single amino acid causing a change in response to a ligand from antagonism to agonism (or vice versa) (Benhamou et al., 1992; McDonnell et al., 1994; Brzozowski et al., 1997; Ekena et al., 1998). Our preliminary structure-activity analysis of substituted flavones led us to hypothesize that a similar situation might be the case for the AhR and that there could be one or a few amino acids in the ligand-binding pocket that interact with the potent antagonist flavones but not with agonist ligands. However, further investigation of this possibility would be assisted by the use of in vitro-translated protein so that specific amino acid changes could be introduced.

In the present article, we have optimized the conditions for use of in vitro translated AhR/Arnt and have used the technique of partial proteolysis to determine that a TCDD-dependent conformational change in AhR can be detected using in vitro-translated AhR and Arnt. This alteration was not elicited by antagonist ligands. Partial proteolysis has been used in the study of steroid hormone receptors and has shown that hormone binding elicits a structural alteration in the receptor that results in a peptide fragment becoming resistant to proteolytic digestion, presumably by changing accessibility to enzyme. Furthermore, in some cases, binding of hormone receptor antagonist elicits a slightly different conformation as indicated by altered molecular mass of protease-resistant peptides (Allan et al., 1992; Kuil et al., 1995; Modarress et al., 1997).

Materials and Methods

Chemicals. [^{3}H]TCDD was purchased from Chemsyn Science Laboratories (Lenexa, KS), and unlabeled TCDD was from Cambridge Isotopes (Cambridge, MA). Trypsin was sequencing grade from Sigma (St. Louis, MO). Oligonucleotides used for EMSA were synthesized by Biosynthesis (Lewisville, TX). The flavones used were synthesized in the laboratory of Dr. Andrew Kende (Dept. of Chemistry, University of Rochester) as described previously (Henry et al., 1999).

Cell Culture and Preparation of Cytosolic Extract. Mouse hepatoma cells, Hepa1c1c7, were grown and harvested as described previously (Henry et al., 1999). Cytosol was frozen (−80°C) until used, at which time the protein concentration, measured by the method of Waddell (1956), was adjusted to 2.5 mg/ml.

In Vitro Transcription/Translation. Murine AhR and Arnt cDNA (obtained from J. Whitlock and O. Hankinson, respectively) were inserted into pcDNA3 (Invitrogen, Carlsbad, CA). AhR and Arnt were generated (separately) by coupled transcription/translation in rabbit reticulocyte lysate using the TNT system according to the manufacturer's instructions (Promega, Madison WI). For some experiments, [^{35}S]methionine was included in the transcription/translation mix to generate [^{35}S]AhR. For ligand treatment, AhR and Arnt were mixed (e.g., 25 μl AhR + 25 μl Arnt), and further diluted with 50 μl of 2× HEDG buffer plus 100 μl HEDG. HEDG buffer is 25 mM HEPES, pH 7.6, 1.5 mM Na_2EDTA, 1 mM dithiothreitol, and 10% (v/v) glycerol. Ligands were added in Me_2SO vehicle (less than 0.5% final dilution), and samples were incubated at room temperature for 1.5 to 2 h. Specific binding of [^{3}H]TCDD was determined by the hydroxylapatite assay (Gasiewicz and Neal, 1982).

Electrophoretic Mobility Shift Assay. Aliquots (9–10 μl) of cytosol or TNT-translated proteins treated with vehicle (Me_2SO) or TCDD (or other ligand) were incubated with herring sperm DNA (0.5 μg for TNT samples; 160 ng for cytosols), 0.08 M NaCl, and ^{32}P-end–labeled DRE oligonucleotide (Gasiewicz et al., 1996). Samples were subjected to nondenaturing electrophoresis (4% acrylamide), and the [^{32}P]DRE/AhR retarded bands were visualized/quantified using a PhosphorImager (PSI; Amersham Biosciences, Piscataway, NJ).

Limited Proteolytic Digestion. For dose-range experiments, 0.8 μl of trypsin diluted in water was added at room temperature to 8 μl of ligand or vehicle-treated samples to achieve the desired final concentrations as shown on figures. After 10 min, 8.8 μl of 2× denaturing loading dye was added to each tube, samples were boiled for 4 min, and analyzed by SDS-PAGE (10% acrylamide resolving gel). For time-course studies, a large volume of ligand- or vehicle-treated sample was treated with trypsin (final concentration, 10 μg/ml unless otherwise noted in figures) at room temperature at time = 0. Aliquots were removed periodically, mixed with 2× denaturing loading dye, boiled for 4 min, and separated by SDS-PAGE as above. Gels were fixed (10% acetic acid, 30% methanol) for 45 min, dried, and visualized using the PhosphorImager.

Western Blotting. After electrophoresis, proteins were transferred to Immobilon-P (Millipore, Bedford MA) using a semidry apparatus (Hoefer Scientific, San Francisco CA). The membrane was blocked for 1.5 h at room temperature with 5% bovine lacto transfer optimizer [5% (w/v) nonfat milk powder in 50 mM Tris, pH 7.5, 150 mM NaCl, and 0.2% (v/v) Tween 20], incubated with primary antibody [anti-AhR, Rpt-1 (Perdew et al., 1995) as ascites, in 1% bovine lacto transfer optimizer] for 1.5 h, followed by secondary antibody (horseradish peroxidase-conjugated anti-mouse IgG; Jackson ImmunoResearch, West Grove, PA) for 1 h. Detection was by chemiluminescence using reagents purchased from KPL (Gaithersburg, MD).

Results

Optimization of Conditions for in Vitro Expressed AhR. Initially, it was necessary to compare in vitro-translated AhR/Arnt with AhR/Arnt contained in Hepa cell cytosol to verify that in vitro translated AhR is an appropriate model. Evaluation of a range of incubation conditions indicated that optimal DRE binding and [^{3}H]TCDD-specific binding (and low nonspecific binding) was obtained when AhR and Arnt, expressed separately in reticulocyte lysate, were mixed 1:1 (v/v), then diluted with an equal volume of 2× HEDG buffer, and further with an equal volume of 1× HEDG buffer (final dilution of AhR, 1:8; final dilution of AhR/Arnt

mix, 1:4 relative to original TNT reaction mix). At 3 nM [^{3}H]TCDD, specific binding was equivalent to that determined in the same volume of Hepa cell cytosol (at ~2.5 mg of protein/ml). Furthermore, competition by 3′-methoxy-4′-nitroflavone for [^{3}H]TCDD binding in Hepa cytosol and in vitro translated AhR/Arnt was also equivalent (data not shown). On a Western blot, the amount of immunodetectable AhR per volume of Hepa cytosol or TNT at this standard dilution was comparable (not shown).

DRE binding as quantified using a PhosphorImager was equivalent in terms of -fold induction by TCDD compared with vehicle control (Fig. 1A). In addition, the [^{32}P]DRE-binding affinity of TCDD-AhR-Arnt in reticulocyte lysate and Hepa cytosol was compared by adding a range of concentrations of unlabeled DRE oligonucleotide as competitor. Very similar apparent affinities of DRE interaction were observed under these conditions (Fig. 1B).

It has been previously reported that the rabbit reticulocyte lysate contains hsp90 and p23, both of which seem to mediate the activation to fully functional AhR (Allan et al., 1992; Kazlauskas et al., 1999, 2001). Indeed, anti-hsp90 and anti-p23 antibodies coimmunoprecipitate in vitro-translated AhR (Kazlauskas et al., 1999, 2001). These observations are consistent with the functional similarity that we show (Fig. 1) in TCDD binding and DRE binding between Hepa cytosolic and in vitro translated AhR/Arnt. We therefore felt justified in using the expressed proteins for further experiments probing ligand-induced structural alterations.

TCDD-Dependent Change in Trypsin Sensitivity. AhR was expressed in the presence of [^{35}S]methionine and mixed with unlabeled in vitro-expressed Arnt, diluted as described above, then incubated with TCDD or vehicle. Because the mouse AhR contains 73 trypsin cleavage sites (trypsin cleaves C-terminal of Arg and Lys residues), it was not surprising that treatment with trypsin at 2.5 to 20 μg/ml rapidly degraded [^{35}S]AhR. However, we observed that a ^{35}S-labeled peptide of approximately 40 kDa was consistently more resistant to proteolytic degradation when TCDD was bound to the [^{35}S]AhR (Fig. 2A). Although another partially stabilized peptide is seen in Fig. 2, this band at ~30 kDa was not consistently observed to be TCDD-dependent. At the intermediate concentration of 10 μg trypsin/ml, the 40 kDa fragment was more stable in the presence of TCDD at least through 25 min incubation at room temperature with trypsin (Fig. 2B). One explanation for these observations is that TCDD binding initiates a structural change in the AhR such that certain trypsin-sensitive sites become less accessible to the enzyme. Two other proteases, V8-protease and chymotrypsin, which have been used to probe steroid receptor and other protein structures (Allan et al., 1992; Kallio et al., 1997), were also tested, but no consistent differences were observed between vehicle- and TCDD-treated samples under various conditions of concentration and treatment time (data not shown). Trypsin-digested AhR was also analyzed on 12% acrylamide gels to enable detection of smaller fragments, but no other TCDD-dependent peptides were seen (not shown).

Arnt-Dependence of TCDD-Induced Trypsin Resistance. The observed conformational change in AhR might be elicited directly by ligand or be a consequence of dimerization with Arnt. The in vitro translation system enables us to separate these processes to try to distinguish the possible mechanisms. [^{35}S]AhR was incubated with TCDD or vehicle in the absence or presence of Arnt and subsequently treated with trypsin. Aliquots were removed periodically to follow the time course of proteolysis. It was clear from this experiment, shown in Fig. 3A, that TCDD was necessary but insufficient to elicit the increase in trypsin-resistance; the presence of Arnt, and presumably its dimerization with [^{35}S]AhR, was also required. Similarly, both AhR and Arnt are necessary for binding to [^{32}P]DRE (EMSA not shown).

To further examine the role of Arnt in stabilizing this 40-kDa AhR peptide, [^{35}S]AhR was separately incubated with TCDD before addition of Arnt and additional incubation time. This subsequent addition of Arnt to the preformed TCDD-AhR complex restored the trypsin resistance of the 40 kDa peptide (Fig. 3B) and partially restored [^{32}P]DRE binding (data not shown). This finding is consistent with our earlier observation of separation of ligand binding and AhR

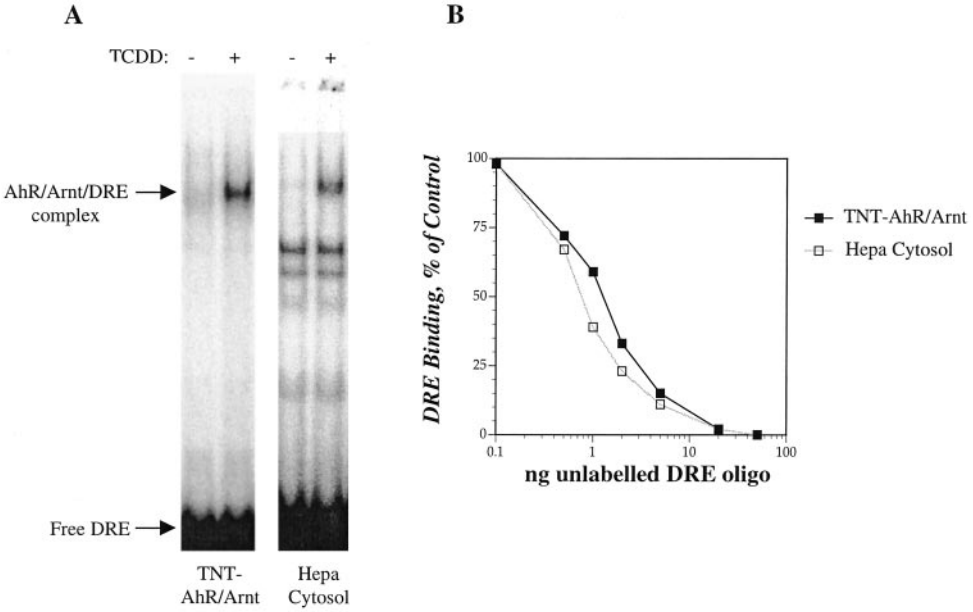

Fig. 1. EMSA of in vitro expressed AhR/Arnt compared with Hepa cell cytosolic AhR transformed in vitro. Separately transcribed/translated AhR and Arnt were mixed, diluted, and treated with Me_2SO or TCDD (5 nM) as described under *Materials and Methods*. Hepa cytosolic extract was adjusted to 2.5 mg protein/ml, and treated with Me_2SO or TCDD (5 nM). A, aliquots (10 μl) of each sample were incubated with [^{32}P]DRE and assayed by nondenaturing electrophoresis as described under *Materials and Methods*. Dried gel was visualized by PhosphorImager. B, aliquots (10 μl) were incubated with [^{32}P]DRE and a range of amounts of unlabeled DRE oligonucleotide (0–50 ng per 25-μl reaction mix) and analyzed by EMSA. DRE-bound AhR was quantified by PhosphorImager, and data are presented as percentage of the value with no added unlabeled DRE oligonucleotide. Each point is the average of two separate experiments.

transformation using hepatic cytosols (Henry and Gasiewicz, 1991). In that study, the monomeric AhR-[^{3}H]TCDD complex isolated chromatographically could be subsequently transformed to the DNA-binding form by addition of cytosolic preparations lacking AhR but containing Arnt. Interestingly, in vitro-translated AhRY9F mutant (tyrosine-9 changed to phenylalanine) that binds [^{3}H]TCDD and Arnt to the same extent as does wtAhR (S. Park, E. C. Henry, T. A. Gasiewicz, unpublished observations), was found to undergo the same trypsin-cleavage pattern as wtAhR (Fig. 3C). AhRY9F, however (similar to other Y9 mutants), is unable to bind [^{32}P]DRE or initiate transcription (Bacsi and Hankinson, 1996; Fukunaga and Hankinson, 1996; S. Park, E. C. Henry, T. A. Gasiewicz, unpublished observations). Thus, although TCDD binding and Arnt dimerization are necessary for both DRE binding and the structural change mediating trypsin resistance, there may be other steps and/or other proteins that are involved in producing a fully functional DRE-binding Ah receptor complex and that may be absent or inhibited in the case of AhRY9F. It is also possible that tyrosine-9 has a critical role, such as making contact with DNA, and is therefore intolerant of amino acid substitution, even though normal (wild-type) ligand- and Arnt-induced conformational changes, such as that detected by trypsin cleavage, may occur. This trypsin cleavage protocol is apparently unable to distinguish all these steps leading to a transcriptionally active complex. Nonetheless, formation of the trypsin-resistant

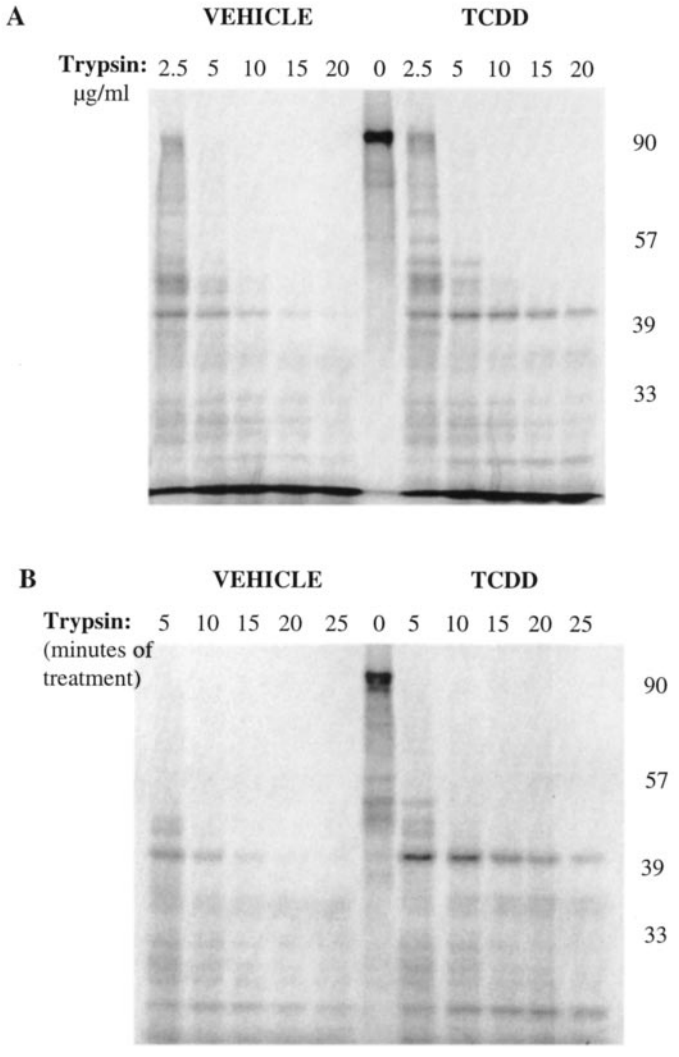

Fig. 2. Partial proteolysis profile of AhR by trypsin is TCDD-dependent. AhR was transcribed/translated in the presence of [^{35}S]Met, mixed with unlabeled Arnt, diluted, and treated with Me_2SO or 10 nM TCDD as described under *Materials and Methods*. A, aliquots of vehicle- and TCDD-treated samples were treated with trypsin at the indicated concentrations for 10 min at room temperature. B, TCDD- and vehicle-treated samples were treated with trypsin at 10 μg/ml, and aliquots were removed after the indicated times of incubation at room temperature. In both cases, all samples were separated by SDS-PAGE, and fixed/dried gels were visualized by PhosphorImager. Figures shown are representative of three (A) or four (B) experiments.

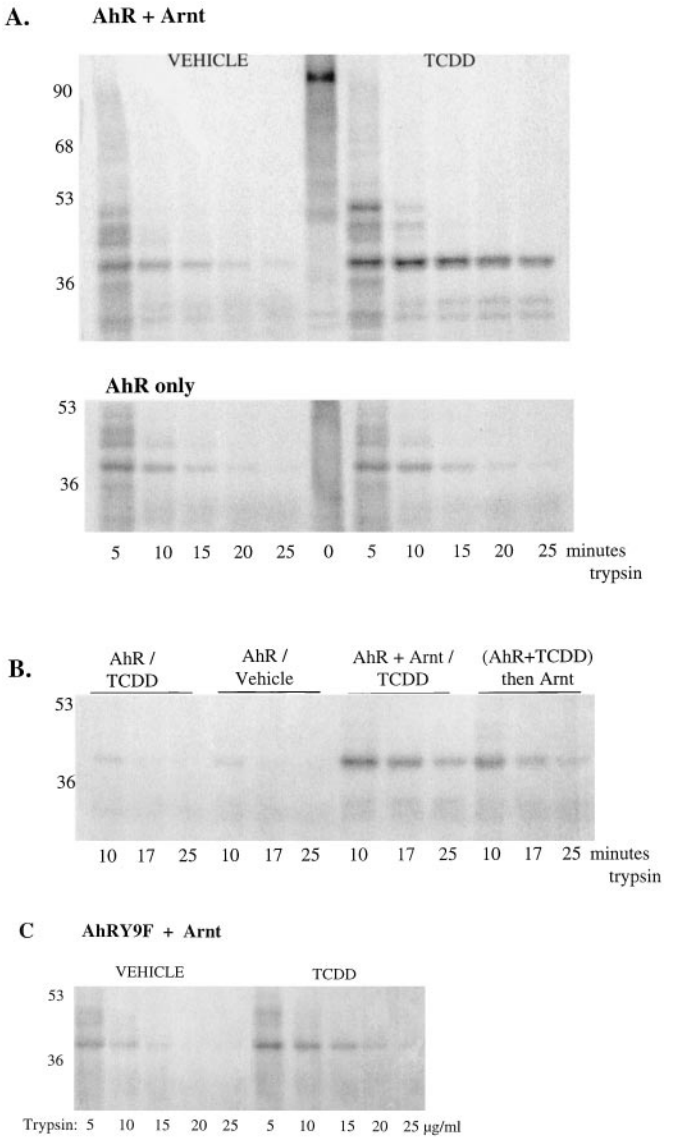

Fig. 3. Arnt-dependence of conformational change. [^{35}S]AhR and unlabeled Arnt were separately transcribed and translated. A, [^{35}S]AhR was mixed with Arnt or an equal volume of blank TNT mix, diluted as usual, and incubated with Me_2SO or 10 nM TCDD. Trypsin (final concentration, 10 μg/ml) was added to each sample and aliquots were removed at the indicated times for SDS-PAGE analysis. B, [^{35}S]AhR was mixed with Arnt or an equal volume of blank TNT mix and treated with either Me_2SO or TCDD (as indicated above lanes 1–9). In lanes 10 to 12, TCDD-treated [^{35}S]AhR was then mixed with unlabeled Arnt for an additional 30-min incubation at 30°C before SDS-PAGE. C, [^{35}S]AhRY9F mutant was mixed with Arnt and incubated with Me_2SO or TCDD. Trypsin was added to aliquots at the indicated concentrations for 10 min before analysis by SDS-PAGE. Representative results of two experiments are shown.

peptide does depend upon TCDD and Arnt, although its formation apparently is not necessarily indicative of a DRE-binding conformation.

Effect of Other Ligands. Several other AhR ligands were tested to determine whether the structural alteration elicited by TCDD is ligand-specific and particularly whether agonist and antagonist ligands produce a distinct or similar change in trypsin cleavage pattern. As expected, gel shift assay of the DRE-binding ability of in vitro-expressed AhR/Arnt in the presence of B[*a*]P and BNF at 16 μM indicated that these compounds were good AhR agonists (Fig. 4A). Furthermore, the same trypsin-resistant peptide was produced in the presence of B[*a*]P (Fig. 4B) and BNF (not shown) as with TCDD.

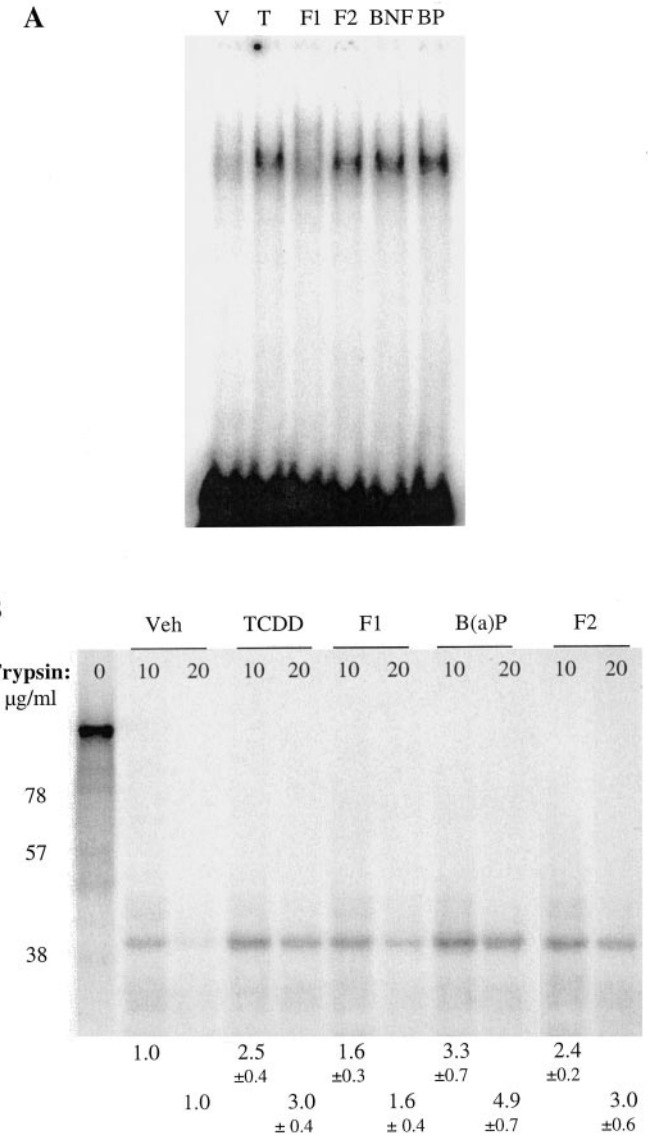

Fig. 4. Interaction of various ligands with in vitro translated AhR. [^{35}S]AhR or unlabeled AhR and Arnt were transcribed and translated, mixed together, diluted, and incubated with Me_2SO (veh), 10 nM TCDD (T), 1 μM 3′-dimethylaminoflavone (F1), 1 μM 3′-methoxy-7,8-benzoflavone (F2), 16 μM β-naphthoflavone (BNF), or 16 μM benzo[*a*]pyrene (BP). A, aliquots of unlabeled AhR/Arnt were assayed by EMSA. B, [^{35}S]AhR/Arnt samples were subjected to partial proteolysis by trypsin at 10 or 20 μg/ml for 10 min before SDS-PAGE analysis. Representative results from three experiments are shown. The 40-kDa bands in B (and the other 2 equivalent experiments) were quantified using a PhosphorImager; the numbers below each lane indicate the mean ± S.D. of the ratio of the band intensity for ligand-treated relative to the vehicle-treated samples treated with the same concentration of trypsin.

A number of substituted flavones have been found to be effective competitors of [^{3}H]TCDD binding in Hepa cell cytosol and to antagonize TCDD-elicited DRE binding and DRE-driven reporter gene transcription (Henry et al., 1999; E. C. Henry and T. A. Gasiewicz, unpublished observations). We chose several flavones to test for their ability to alter AhR proteolysis susceptibility and pattern. When tested alone at 1 μM (without TCDD), 3′-dimethylaminoflavone (F1) was a poor inducer of DRE binding whereas 3′-methoxy-7,8-benzoflavone (F2) had substantial agonist activity (Fig. 4A), an analogous pattern to that seen using Hepa cell cytosol (not shown). [^{35}S]AhR/Arnt treated with F1 was more sensitive to degradation by trypsin than was the TCDD-treated complex, whereas F2-treated [^{35}S]AhR/Arnt resembled TCDD- and B[*a*]P-bound receptors in its trypsin-resistance (Fig. 4B). Quantitation of the 40-kDa band and normalization to the appropriate vehicle control clearly shows this difference (Fig. 4B, numbers below each lane). Several other substituted flavones with antagonist activity (including 3′-methoxy-4′-nitroflavone, 3′-methoxy-4′-CN-flavone, 3′-methoxy-4′-nitro-7,8-benzoflavone, and 3′-methoxyflavone) (Lu et al., 1995; Henry et al., 1999; E. C. Henry and T. A. Gasiewicz, unpublished observations) showed a similar inability to elicit the trypsin-resistant conformation of the [^{35}S]AhR/Arnt (data not shown). These data indicate that the ability of a ligand to induce AhR transformation to a DRE-binding form in a cell-free system and in whole cells correlates with its ability to induce the conformational change detected as a decrease in susceptibility to trypsin cleavage.

Trypsin-Resistant Band Does Not Include the AhR N-Terminal Peptide. Our observations suggest that the agonist-induced conformational change in AhR is necessary for DRE binding and is probably dependent upon dimerization with Arnt. However, because the AhRY9F-TCDD complex cannot yet bind DRE shows wild-type resistance to trypsin cleavage, we hypothesize that the conformational change detected by our partial proteolysis assay may not be in the DNA binding domain of AhR. To localize the 40-kDa trypsin-generated fragment, we assayed in vitro-expressed AhR/Arnt treated with vehicle or TCDD and then trypsin by Western blot using an antibody that recognizes the N-terminal amino acids 12 to 31 of AhR (Perdew et al., 1995). The major trypsin-resistant fragment from [^{35}S]AhR/Arnt was apparently not immunodetectable; however, at very early time points, a larger fragment (~50 kDa) was detectable and was more prominent in TCDD-treated samples (Fig. 5). ^{35}S-labeled bands in this size range were also present at early time points or lower concentrations of trypsin, but the smaller, ~40-kDa–labeled band rapidly became predominant (Fig. 2). These observations suggest that the immunodetectable band containing the N-terminal portion of AhR is somewhat stabilized by TCDD but is rapidly (within 5 min) further cleaved by trypsin to yield the 40-kDa band, which no longer includes the N-terminal antigenic region. Note that there is only one methionine residue (amino acid 1) within the N-terminal ~200 amino acids; therefore, possible conformational changes in the DNA-binding region would be difficult to detect based on ^{35}S-labeling and proteolysis.

To further probe what portion of the TCDD-AhR becomes trypsin-resistant, the N-terminal [^{35}S]AhR (amino acid

1–399) was translated in vitro using mutant cDNA (prepared by D. Vorojeikina) and subjected to the same trypsin treatment as AhRwt. The AhR399 binds TCDD and Arnt and shows TCDD-dependent DRE binding (G. Minsavage, D. Vorojeikina, and T. A. Gasiewicz, submitted). AhR399 is approximately 45 kDa; partial trypsin digestion yielded a TCDD-dependent, trypsin-resistant band at about 35 kDa (Fig. 6B) that was not recognized by the α-AhR (data not shown). This pattern could arise if both wtAhR and AhR399 were similarly cleaved by trypsin to remove the N terminus (~10 kDa), which includes the portion that is recognized by the α-AhR antibody. An additional AhR truncation mutant, AhR(1–348) (cDNA prepared by S.-K. Park), was translated in vitro and subjected to trypsin digestion. If the above hypothesis regarding trypsin cleavage of AhR399 and AhRwt is correct, then trypsin treatment of [^{35}S]AhR348 should yield a peptide that is shorter than that formed from AhR399 by 51 amino acids (or ~5–6 kDa). AhR348 binds Arnt and DRE constitutively (S.-K. Park and T. A. Gasiewicz, unpublished observations), so it was not unexpected that the effect of trypsin was not different between vehicle- and TCDD-treated samples. However, there was a fragment of approximately 30 kDa observed after trypsin digestion (Fig. 6C), consistent with the above proposal. Together, these data suggest that the peptide fragment generated from full-length AhRwt probably represents amino acids ~80 through ~440, as illustrated in Fig. 7.

Discussion

The ability to transcribe and translate proteins in vitro is a valuable tool for probing protein structure and function, and the role of specific amino acid residues in protein activity. Large amounts of the protein of interest, with or without targeted mutations, can be produced much more quickly and can be radiolabeled to a much higher specific activity than in whole cells. Furthermore, the rabbit reticulocyte lysate system, as used for these studies, provides many of the eucaryotic post-translational modification activities, cofactors, hsp90, and other heat-shock proteins that are lacking in bacterial and yeast systems. Indeed, we found that when murine AhR and Arnt were separately translated in reticulocyte lysate and mixed together, TCDD binding and DRE binding of the resultant complex were very similar to that formed in mouse hepatoma cell cytosol. Coimmunoprecipitation of unliganded [^{35}S]AhR with α-hsp90 antibody further indicates its comparability to the cytosolic AhR complex (Kazlauskas et al., 2001). It has also been reported that glucocorticoid receptor translated in reticulocyte lysate forms a multimeric complex that includes hsp90 and sediments at 9S as does the cytosolic receptor (Denis and Gustafsson, 1989). Similarly, the properties of other steroid receptors translated in vitro have been found to be analogous to receptors isolated from cells or tissues (e.g., Allan et al., 1992). Based on these basic characteristics, we concluded that the in vitro-translated Ah receptor was a suitable model for further analysis of the protein and ligand-induced conformational effects. Nevertheless, conclusions from any studies must be qualified by the consideration that the environment in reticulocyte lysate differs greatly from that in cells, or in cytosol, notably in concentration of total protein and individual proteins, and the fact that cofactors and other components of the lysate are

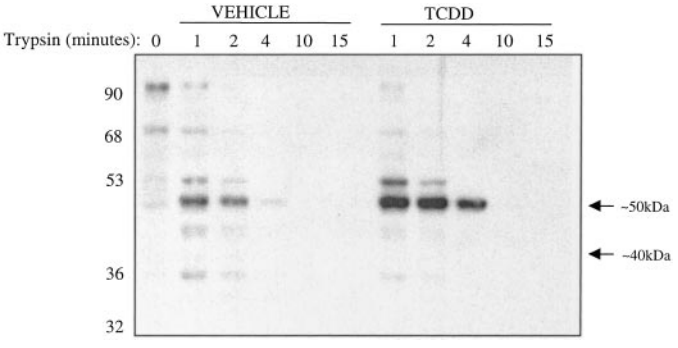

Fig. 5. The trypsin-resistant fragment does not include the N-terminal residues. AhR and Arnt transcription/translation, treatment with vehicle or TCDD, and partial proteolysis with trypsin were as described under *Materials and Methods*. After SDS-PAGE, proteins were transferred to polyvinylidene difluoride membrane and probed with anti-AhR antibody (Rpt-1, which was made against an N-terminal peptide, amino acids 12–31). Note that sampling times are much shorter than in other figures. Blot shown is representative of five experiments.

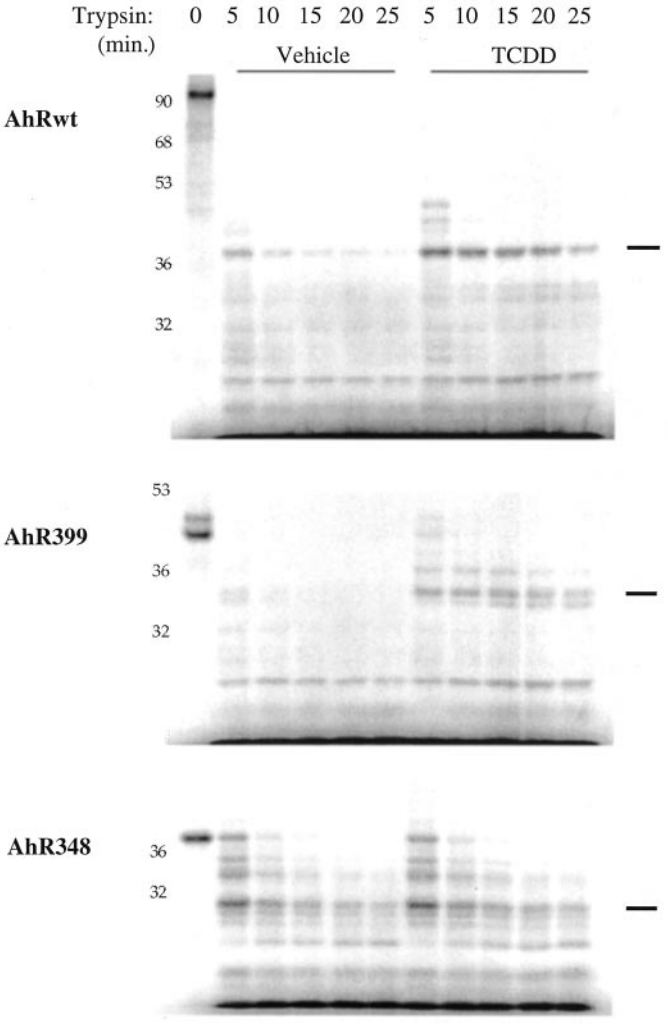

Fig. 6. Trypsin cleavage of truncated AhRs. Indicated [^{35}S]AhRs (full-length or truncation mutants) were translated and mixed with translated Arnt, incubated with vehicle or TCDD, and subjected to partial proteolysis with trypsin (10 μg/ml) as described under *Materials and Methods*, with the exception that for AhR348, trypsin was added at 5 μg/ml and a resolving gel of 11% acrylamide was used. Cleavage pattern of AhR348 was identical in the presence or absence of Arnt. Figure shows representative results from four (AhRwt and 399) or two (AhR348) experiments.

from a different species (rabbit) than are the receptor proteins AhR and Arnt.

Partial proteolysis has become a widely used method of probing protein structure and changes in structure after various treatments, such as ligand binding in the case of receptors. In the present study, limited trypsinization of the ^{35}S-labeled Ah receptor indicated that TCDD induces a structural alteration in the protein that results in a peptide of approximately 40 kDa that is more resistant to proteolysis than in the unliganded receptor. This change occurs only in the presence of both Arnt and TCDD (Figs. 2 and 3), indicating that the process of dimerization, which is TCDD-dependent, probably initiates the structural change that limits accessibility to trypsin. Indeed, the close association of Arnt itself probably restricts the access of trypsin to parts of AhR. It is reasonable to assume that the binding of TCDD to AhR must also elicit some initial conformational change to enable the release of associated proteins (e.g., hsp90, AIP) that are known to comprise the nonligand-occupied AhR complex) and subsequent dimerization with Arnt. Kronenberg et al. (2000) reported detection of a ~35-kDa fragment of [^{35}S]AhR (no Arnt present) treated with trypsin, which they interpreted as a "ligand-induced AhR conformation" but only at TCDD concentrations of 25 nM and higher that are well above AhR saturation levels. We did not detect any change in trypsin-resistance of the AhR alone (in the absence of Arnt) at 10 nM TCDD, even under milder conditions (0°C incubation, lower concentrations of enzyme) (data not shown).

Labeling of Arnt rather than AhR with ^{35}S indicated that Arnt was rapidly degraded under those conditions in which the 40-kDa TCDD-dependent peptide of AhR was observed (data not shown). This suggests that Arnt may initiate a conformational change in AhR distinct from simply a physical block of trypsin sites on AhR; however, it does not preclude the possibility of a small molecular mass (<15 kDa, undetected by our protocol) fragment of Arnt remaining dimerized with AhR and blocking trypsin. Interestingly, Kallio et al. (1997) reported that Arnt initiated a conformational change in another of its dimerization partners, hypoxia-inducible factor-1α, as determined using V8 protease digestion, and that this activity was dependent on the C-terminal portion of Arnt. Thus, Arnt-dependent allosteric regulation of protein activity may be a common mechanism of activation in the basic helix-loop-helix–PAS family. Using the same protocols, Kallio et al. (1997) were unable to detect a change in AhR conformation, consistent with our observation that V8 protease was not effective for probing AhR conformational change.

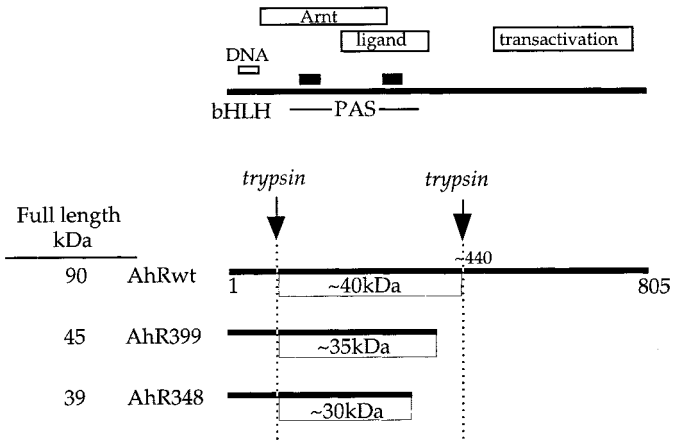

Fig. 7. Hypothesized cleavage of AhRs by trypsin. Full-length and truncated AhRs are aligned with the corresponding domain structure of AhR. Hypothesized ends of trypsin-resistant peptide at approximately amino acids 80 and 440 are shown (arrows in lower portion of figure), based on fragment sizes observed in Fig. 6.

Partial proteolysis of steroid hormone receptors (androgen, estrogen, progesterone, glucocorticoid) has shown that distinct conformational changes occur in these receptors upon hormone binding (Allan et al., 1992; Kuil et al., 1995; McDonnell et al., 1995; Modarress et al., 1997). Furthermore, in some cases, binding of some hormone receptor antagonists also elicited conformational change, but distinctly different from that elicited by agonist hormone [molecular mass of protease-resistant peptide(s) differed between agonist- and antagonist-treated receptor]. Such observations are consistent with the hypothesis that one mechanism of antagonism may be induction of an inappropriate conformation of the receptor. Although antagonists of the AhR are less well characterized than steroid receptor antagonists, we and others have identified several substituted flavone compounds that are competitive inhibitors of TCDD binding and also inhibit TCDD-induced DRE binding and transcriptional enhancement in mouse hepatoma cells (Lu et al., 1995; Gasiewicz et al., 1996; Henry et al., 1999). One of the most potent, 3′-methoxy-4′-nitroflavone, was equally effective in competing for TCDD binding to the TNT-expressed AhR/Arnt and in Hepa cytosolic extracts (data not shown), consistent with our conclusion based on data in Fig. 1 that Hepa- and TNT-expressed receptors have comparable ligand-binding behavior.

We tested several flavones for their ability to induce a conformational change detected by trypsin cleavage. No distinctive pattern of cleavage by trypsin was observed. Rather, the presence and trypsin resistance of the 40-kDa peptide correlated with the flavone's agonist activity. Thus, for flavones that were able to inhibit TCDD-elicited DRE binding and that did not themselves induce a DRE-bound complex, the proteolytic pattern resembled vehicle controls, whereas for flavones with agonist activity (DRE binding) the presence of the 40-kDa peptide was similar to TCDD-treated samples. We have shown that in cells treated with the most potent of the flavone antagonists, the AhR primarily remains in the cytosol, still associated with hsp90 rather than moving to the nucleus for dimerization with Arnt (Henry et al., 1999). The lack of increased trypsin resistance when receptor is treated with antagonist is consistent with this lack of receptor activation. Recently, there have been numerous studies characterizing the roles of several AhR-associated proteins, notably hsp90, p23, and ARA9, in regulating receptor stability, ligand binding, intracellular localization, and transcriptional activation (Carver et al., 1998; Kazlauskas et al., 1999, 2000, 2001; Cox and Miller, 2002). It is likely that interactions among and functioning of these associated proteins and the AhR differ between agonist and antagonist-bound receptor. The availability of these proteins for in vitro translation will provide another useful tool, in conjunction with partial proteolysis, for delineating the mechanisms of antagonist versus agonist ligands and the role of AhR structural changes.

Immunoblot analysis (Fig. 5) of the trypsin-treated AhR/Arnt indicated that the 40-kDa peptide did not contain the

N-terminal portion against which the antibody was made (amino acids 12–31). Partial proteolysis of truncated AhRs (amino acids 1–399 and 1–348) yielded peptides of molecular masses that are consistent with the hypothesis that the protease-resistant fragment of full-length AhR may extend from amino acid ~80 to ~440. Furthermore, there are trypsin-specific cutting sites near these estimated residues (e.g., Lys77, 79, Lys432, 442). Within this portion of the AhR are the ligand-binding domain and the PAS domain, which is important in dimerization (Rowlands and Gustafsson, 1997). It seems reasonable that ligand binding would induce a structural alteration centered in this region. Although the trypsin-resistant peptide itself does not include the DRE-binding basic-helix-loop-helix domain, the conformational change observed may in turn enable (or be accompanied by) modification that exposes the N-terminal DRE-binding domain. However, it is evident that formation of the trypsin-resistant peptide, although it is TCDD- and Arnt-dependent, is not definitive for formation of the fully transformed DRE-binding conformation because the peptide was also produced from the AhRY9F-Arnt complex (Fig. 3C), which does not bind the DRE sequence.

Data obtained so far cannot conclusively demonstrate an AhR structural change distinct from the association with Arnt that follows agonist ligand-binding and the consequent physical blocking of protease sites on AhR. The truncation mutant AhR348 binds Arnt and DRE constitutively (not ligand-dependent), indicating that amino acids 348 to 399 include sites that are critical for maintaining a ligand-dependent AhR conformation. Thus, even unliganded AhR348 exists in an altered conformation that partially resembles the DRE-binding full-length AhR. Trypsin digestion of AhR348 yielded a peptide that was equivalent to that produced from TCDD-treated AhRwt (size adjusted for lack of C-terminal amino acids) although it was less stable than the TCDD-AhRwt peptide and was TCDD- and Arnt-independent. This suggests that protection from trypsin reflects more than physical blockage by Arnt, but additional studies are necessary to verify this hypothesis. Our observation that ^{35}S-Arnt seemed to be rapidly degraded by trypsin, whereas the AhR peptide persisted, further suggests that protection of AhR from trypsin depends on a structural change in the AhR protein itself.

In summary, we have used in vitro-transcribed/translated AhR and Arnt under conditions that optimize TCDD binding and DRE binding, to examine possible structural changes during receptor transformation. Partial proteolysis by trypsin yielded a peptide of approximately 40 kDa that was more resistant to further digestion only in the presence of both agonist ligand (TCDD, B[α]P, BNF, or substituted flavone that has agonist activity) and Arnt protein. Substituted flavones that are AhR antagonists did not readily elicit this trypsin-resistant peptide. We tentatively have identified this fragment to span approximately amino acids 80 to 440 of the murine AhR, which includes the ligand-binding and Arnt-binding domains but not the basic region helix-loop-helix motif at the N-terminal end of the receptor. Thus, we conclude that a TCDD- and Arnt-dependent structural change initiated in this PAS region of the AhR probably transmits a structural change to the DNA-binding domain of the molecule that enables DRE-binding.

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