Dioxin-inducible Transactivation in a Chromosomal Setting

ANALYSIS OF THE ACIDIC DOMAIN OF THE Ah RECEPTOR*

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We analyzed the transactivation function of the acidic segment of the Ah receptor (amino acids 515–583) by reconstituting AhR-defective mouse hepatoma cells with mutants. Our data reveal that both hydrophobic and acidic residues are important for transactivation and that these residues are clustered in two regions of the acidic segment of AhR. Both regions are crucial for function, because disruption of either one substantially impairs transactivation of the chromosomal CYP1A1 target gene. Neither region contains an amino acid motif that resembles those reported for other acidic activation domains. Furthermore, proline substitutions in both regions do not impair transactivation in vivo, a finding that implies that α-helix formation is not required for function.

The halogenated aromatic hydrocarbon 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) is a widespread environmental contaminant that elicits both adverse and adaptive responses in animals and humans (1, 2). An intracellular protein known as the aromatic hydrocarbon receptor (AhR) mediates the biological effects of TCDD (3–6). The most well-characterized action of TCDD is an AhR-dependent adaptive response that leads to increased transcription of the CYP1A1 gene, which encodes the microsomal enzyme cytochrome P4501A1 (7–9). To activate transcription, AhR dimerizes with a second protein, the AhR nuclear translocator (Arnt) (10, 11) thereby generating AhR-Arnt chimeras containing the N-terminal segment of AhR (amino acids 1–494) linked to a 69-amino acid domain from the C-terminal segment of AhR (amino acids 515–583) facilitates CYP1A1 promoter occupancy and restores its responsiveness to TCDD in AhR-defective cells, whereas the N-terminal segment by itself does not (22). The domain spanning amino acids 515–583 is rich in glutamate and aspartate residues (22); by this criterion, it resembles an acidic activation domain (AAD) (23, 24). Here, we have analyzed the function of this acidic domain in more detail. In particular, we have studied its transactivation function in a chromosomal setting by reconstituting AhR-defective cells with chimeric AhR mutants and measuring the response of the native CYP1A1 target gene to TCDD. Our findings reveal similarities, as well as notable differences, between the function of the acidic domain of AhR and that of previously characterized AADs.

EXPERIMENTAL PROCEDURES

Materials—The pGALO vector was provided by Dr. Chi V. Dang (Johns Hopkins University, Baltimore, MD) and contains a G418 DNA-binding domain (amino acids 1 to 147) juxtaposed to a multicloning site (25). The plasmid pFR-Luc, the QuikChange site-directed mutagenesis kit, and Pfu DNA polymerase were purchased from Stratagene (La Jolla, CA). The pRL-cytomegalovirus vector and dual-luciferase reporter assay system were purchased from Promega (Madison, WI). The retroviral vector pMFG was derived from the Moloney murine leukemia virus (26). Transcription of the inserted coding sequences is driven by the retroviral long terminal repeat. The Phoenix-eco retroviral producer cell line was provided by Dr. Garry Nolan (Stanford University, Stanford, CA) (27). Pfu DNA polymerase was from Amersham Pharmacia Biotech. Tissue culture reagents were from Life Technologies, Inc. The AhR antibody was kindly provided by Dr. Gary H. Perdew (Pennsylvania State University, University Park, PA).

Cell Culture—Wild-type Hepa1c1c7 and AhR-defective (TacIIP/+) mouse hepatoma cells were cultured as described previously (28). Phoenix cells were cultured as described previously (27).

Plasmid Construction—Deletion mutants (∆1–5) were generated by cloning PCR-amplified fragments of AhR cDNA into the multicloning site of plasmid pGALO at BamHI and Clal sites. The plasmid pGALO-∆5 (22) was used as a template in PCR reactions, and amplifications were performed using Pfu DNA polymerase according to the manufacturer’s instructions. A linker sequence containing a BamHI site was attached to forward primers, whereas reverse primers contained a Clal site. The following primers were used for amplification: Primer A, 5′-CGGCGATCTTCTCTGGCGGCCTCAGAG-3′; Primer

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§ The abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; AAD(s), acidic activation domain(s); AhR, aromatic hydrocarbon receptor; Arnt, AhR nuclear translocator; bHLH, basic helix-loop-helix; PAS, Per-Arnt-Sim; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; CREB, cAMP-responsive element-binding protein.
Acidic Activation Domain of AhR

B, 5′-TTCATGATTGGTATGAGGCCGAATCTCATCCATCCATTTTAT-3′; Primer C, 5′-TTCATGATTGGTATGAGGCCGCGAGATGTCGACGACC-3′; Primer D, 5′-GCCGGTACATACGTCGAGAAGAACCTTCG-3′; Primer E, 5′-GCCGGTACATACGTCGAGAAGAACCTTCG-3′; and Primer F, 5′-TTCATGATTGGTATGAGGCCGCGAGATGTCGACGACC-3′. Blots were hybridized with 32P-labeled P4501A1 or actin (Stratagene) according to the manufacturer’s instructions. Plasmid pGAR515–583 was used as a template for the appropriate sense and antisense primer pairs. The specific amino acid changes introduced by mutagenic primers are indicated in each figure. All mutations were confirmed by nucleotide sequencing.

Table 1 Relative transactivation activities of AhR mutants

| Mutant | Activity |
|--------|----------|
| E521A | 85 ± 2 |
| D530A | 80 ± 3 |
| D530A | 53 ± 3 |
| D541A | 74 ± 3 |
| E543A/D544A | 42 ± 3 |
| E551A/E552A | 72 ± 3 |
| D557A | 75 ± 3 |
| E563A | 92 ± 7 |
| D565A | 101 ± 2 |
| D568A | 104 ± 3 |
| D570A | 60 ± 2 |
| D573A/E574A | 40 ± 3 |
| D581A | 96 ± 5 |

*Acidic amino acids (D, aspartic acid and E, glutamic acid) were mutated to alanine (A) at the indicated positions in the acidic segment, 515–583, of AhR.*

†Mutants were assayed for their ability to transactivate a luciferase reporter gene, as described under “Experimental Procedures.” Activity is expressed as a percentage of wild-type, which is 100%. Means and standard errors reflect three experiments.

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| D570A | 60 ± 2 |
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Acidic Amino Acids and Transactivation—Previously, we have shown that a 69-amino acid region (amino acids 515–583) of the C-terminal segment of AhR can transactivate a dioxin-responsive target gene when linked to the N-terminal segment of AhR (22). The region containing amino acids 515–583 is rich in glutamate and aspartate residues (24%); thus, it resembles an AAD. We have analyzed the acidic domain in more detail to identify structural features important for transactivation. The specific role(s) of acidic amino acids in AAD function is uncertain (32–35). Therefore, we first asked whether acidic residues contribute to transactivation. For these studies, we constructed single or double mutants in which we substituted alanine for aspartate or glutamate. We fused the mutants to the DNA-binding domain of Gal4 and measured their ability to transactivate a Gal4-dependent luciferase reporter gene in transient transfection experiments. Table I shows the results of these studies. Five mutants (E521A, E563A, D565A, D568A, and D581A) exhibit little change in function. Four mutants (D525A, D541A, E551A/E552A, and D557A) reveal modest reductions (20–30%) in transactivation activity. Four other mutants (D530A, E543A/D544A, D570A, and D573A/E574A) show more substantial (40–60%) losses of function. Our results imply that the acidic side chains at these four sites are particularly important for transactivation capability. However, individual acidic residues in these four regions apparently do not contribute equally to function. For example, the decreased activity of single mutant D530A resembles that of double mutant E543A/D544A. Therefore, on an individual basis, Asp540 may be more important for transactivation than either Glu543 or Asp544. In general, our results indicate that acidic residues vary substantially in the contribution that each makes to transactivation. This finding imposes an important constraint on the possible mechanism by which the acidic residues influence function.

Hydropathic Amino Acids and Transactivation—Transactivation domains can interact with their target proteins via hydrophobic contacts (35–37). Therefore, we asked whether hydrophobic amino acids in the acidic segment of AhR contribute to transactivation capability. We mutated hydrophobic residues to alanine and tested the resulting mutants (schematically represented in Fig. 1 as ‘Φ’–‘Φ’) for their ability to transactivate the luciferase reporter gene. Our findings reveal that four of the mutants (Φ3, Φ4, Φ5, and Φ7) show reduced activity in transactivation assays. Of these four, mutant Φ4 exhibits the largest decrease in function (25–30% of wild-type), whereas mutants Φ3, Φ5, and Φ7 exhibit somewhat smaller changes (45–75% of wild-type). In contrast, mutants Φ1, Φ2, and Φ5 show no loss of function (Fig. 1). These results imply that the hydrophobic residues targeted in mutants Φ3, Φ4, Φ6,
and $\Phi_7$ are important for function and that hydrophobic amino acids vary considerably in the degree to which they influence transactivation.

To extend these studies further, we analyzed mutants $\Phi_3$, $\Phi_4$, $\Phi_6$, and $\Phi_7$ using a more stringent assay. Reconstitution of AhR-defective cells allows us to measure the transactivation of a known AhR target gene ($CYP1A1$) in its native chromosomal setting (21). For these studies, we linked mutants $\Phi_3$, $\Phi_4$, $\Phi_6$, and $\Phi_7$ to the N-terminal segment of AhR (amino acids 1–494), introduced the resulting constructs into AhR-defective cells by retroviral infection, and assessed the ability of the reconstituted cells to respond to TCDD (as measured by the induction of $CYP1A1$ mRNA). Figure 2A and C schematically depicts the constructs we analyzed. Positive and negative control experiments indicate that the acidic region of AhR, 515–583, restores TCDD responsiveness to AhR-defective cells when linked to the N-terminal segment of AhR (construct AhR$_{494}$AAD) whereas the N-terminal segment of AhR alone (construct AhR$_{494}$) does not (Fig. 2B). Our analyses also reveal that each of the four mutants is defective in its ability to activate $CYP1A1$ transcription (Fig. 2C). The mutations in $\Phi_4$ have the greatest adverse impact on function, substantiating the results in Fig. 1 and revealing the importance of these hydrophobic residues for function in vivo. Immunoblotting studies confirm that the wild-type and mutant proteins are expressed at similar levels in the reconstituted cell lines (Fig. 2D). Therefore, our findings do not represent artifacts because of unequal expression of the mutant proteins.

**Bipartite Organization of Acidic Segment**—Our findings indicate that the hydrophobic residues and the acidic residues that are important for transactivation map to two smaller regions of the acidic segment of AhR (Fig. 3). For example, the mutations in $\Phi_3$, $\Phi_4$, D530A, and E543A/D544A map to the region spanning amino acids 530–545 (which we designate R1), and the mutations in $\Phi_6$, $\Phi_7$, D570A, and D573A/E574A map to the region spanning amino acids 564–579 (which we designate R2). These results imply that regions R1 and R2 are crucial for transactivation. To assess the relative contributions of R1 and R2 to transactivation in vivo, we substituted alanine for all of the hydrophobic residues in R1 or R2 (the mutants are designated mR1 and mR2, respectively). We linked each mutant to the N-terminal segment of AhR, introduced the resulting constructs into AhR-defective cells by retroviral infection, and measured the response of the CYP1A1 gene to TCDD. Our findings (Fig. 4) reveal that mutation of the hydrophobic residues in either R1 (construct AhR$_{494}$/mR1) or R2 (construct AhR$_{494}$/mR2) substantially (>80%) impairs the ability of the acidic segment of AhR to transactivate the CYP1A1 gene in response to TCDD. Furthermore, mutation of the hydrophobic residues in R1 and R2 simultaneously (construct AhR$_{494}$/mR1mR2) abolishes function. These results imply that both R1 and R2 are crucial for transactivation and R1 and R2 function synergetically, because mutation of either one produces >50% loss of function. The mechanism responsible for the synergy remains to be determined.

We further evaluated the importance of R1 and R2 by measuring the ability of deletion mutants to transactivate a luciferase reporter gene in response to TCDD. Our findings (Fig. 5) indicate that mutant $\Delta_1$ (which is missing R2) and mutant $\Delta_2$ (which is missing R1) exhibit little transactivation capability (15–25% of wild-type). These results are consistent with those in Fig. 4 and imply that both R1 and R2 are necessary for maximal transactivation. Mutants $\Delta_3$ and $\Delta_4$, which are missing portions of R1 and R2, respectively, but which otherwise contain most of the acidic segment, display substantial losses in function. In addition, mutant $\Delta_5$, which spans amino acids 539–577 and contains partial deletions in both R1 and R2, exhibits virtually no transactivation capability. Taken together, the findings in Fig. 5 are internally consistent with those in Fig. 4 and imply that both R1 and R2 must be intact for complete transactivation activity. Our results indicate that the acidic segment of AhR is composed of two subdomains; this bipartite organization suggests that the segment makes multiple contacts with its target protein(s).

**α-Helices and Transactivation**—Computer analyses of the primary sequence of AhR using the Chou and Fasman (38) and Garnier et al. (39) algorithms predict that the amino acid sequences in the vicinity of R1 and R2 form α-helices. Furthermore, interspecies comparisons indicate that the predicted α-helical regions are >90% conserved among mouse, rat, and human. Therefore, we asked whether the ability to form an α-helix is required for transactivation. We constructed mutants that contain a helix-incompatible proline residue within each of the two predicted α-helices, thereby generating mutants F542P and F566P (Fig. 6). Assays for function reveal that both mutants transactivate a luciferase reporter gene to about the same extent as wild-type (89 and 96%, respectively). Furthermore, a double mutant containing proline substitutions at both positions displays ~75% of wild-type activity (Fig. 6). These observations imply that the capacity for α-helix formation is not a major factor in the transactivation capability of the acidic segment of AhR.

The results in Fig. 6 were unexpected, because studies on other AADs have stressed the importance of α-helix formation for transactivation (32, 36, 40, 41). Therefore, we confirmed our findings using the more stringent reconstitution type of experiment. Our results again demonstrate that reconstitution with the double mutant AhR$_{494}$/2XPro restores TCDD responsiveness to the CYP1A1 gene in AhR-defective cells (Fig. 7). Thus, substitution of proline at positions 542 and 566 does not impair TCDD-inducible transactivation of a chromosomal gene in intact cells. Immunoblotting experiments (Fig. 7C) reveal similar levels of AhR protein in the reconstituted cell lines; therefore, the activity of the double mutant (AhR$_{494}$/2XPro) is not because
of its overexpression relative to wild-type (AhR<sub>494/AAD</sub>). Our findings indicate that the ability to form an α-helix is not required in order for the acidic segment of AhR to transactivate a chromosomal target gene. These observations reveal an interesting contrast between AhR and transactivators that form α-helices as part of their function.

**DISCUSSION**

The mechanism by which AADs increase transcription in vivo is poorly understood. Here, we have analyzed an AhR segment that exhibits transactivation capability and resembles an AAD in its primary amino acid sequence. In addition to using standard reporter gene assays to measure transactivation, we have studied the ability of AhR to transactivate a native chromosomal target gene. Previous studies in other systems imply that transcriptional activators interact with the general transcriptional machinery, either directly or via multivalent coactivators (31, 35, 37, 42, 43). Structural analyses of the prototypical activator VP16, as well as other AADs, suggest that the protein-protein interactions involve a transition from random coil to α-helix with hydrophobic residues along one face of the AAD helix contacting the target protein (35, 37, 43). Our
studies of the AAD of AhR reveal notable differences between it and AADs in other systems.

Our experiments reveal that the acidic segment of AhR contains two regions that contribute to transactivation. Both regions contain important acidic and hydrophobic residues, and the two regions function synergistically. From a mechanistic standpoint, synergy could reflect either of two scenarios. First, the two regions may contact different sites within a single target protein (such as a coactivator or general transcription factor). For example, the interaction of the transactivation domain of p53 with MDM2 displays this type of synergy (44). Alternatively, the two regions may interact with separate target proteins to produce synergy; for example, two consecutive LXXLL motifs in SRC-1 contact distinct subunits of a PPAR-γ homodimer (45). Although its mechanism remains to be elucidated, the synergy we observe for transactivation by AhR resembles that in other systems where hydrophobic regions mediate interactions with target proteins.

Our studies also implicate acidic side chains in transactivation by AhR. We envision that electrostatic interactions complement hydrophobic protein-protein interactions, because the acidic and hydrophobic residues that strongly affect function are located adjacent to each other. The finding that only certain acidic side chains are important for function suggests that these residues confer local charge to specific regions of the acidic segment of AhR. In this respect, AhR is distinctly different from transactivators whose function depends more on overall negative charge than on the positions of individual acidic residues (33, 35, 46, 47).

Structural analyses in other systems (for example, p53-MDM2 (36), VP16-TAF31 (35), and cAMP-responsive element-binding protein (CREB)-CREB-binding protein (37, 48)) suggest that transcriptional activation domains are largely unstructured in solution but adopt an α-helical conformation upon interaction with their target proteins. Mutational analyses of the AADs of Pho4 (49), peroxisome proliferator-activated receptor α (32), and the ETS family member ERM (41) support this concept, because their capacity to form an α-helix correlates with their transactivation potential. In contrast, our studies of proline substitution mutants imply that the capacity for α-helix formation is not a major factor in transactivation by the acidic domain of AhR even though the potential for α-helix formation has been conserved across species. Furthermore, we note that although they prevent α-helix formation, proline substitutions maintain hydrophobicity, reinforcing our impression that hydrophobicity is more important than α-helicity for transactivation by the acidic segment of AhR. Thus, our studies reveal an interesting example of an AAD whose action at a mammalian chromosomal target gene does not require that it form an
α-helix. This property distinguishes the AAD of AhR from those described previously.

Another noteworthy difference between the acidic segment of AhR and other transcriptional activation domains is the absence of an obvious signature motif. For example, the acidic activators p53 (43) and VP16 (35) contain FXXΦΦ motifs (Φ, hydrophobic residues), which are essential for function. Furthermore, Uesugi et al. (43) have suggested that the FXXΦΦ motif is a general recognition sequence for the coactivator hTAFII31. Similarly, the activation domain of CREB binds to CREB-binding protein via a related motif, YXXIL (37). In contrast, the AAD of AhR contains no comparable amino acid sequence. Furthermore, there is no obvious similarity between the amino acid sequences of R1 and R2 within the AAD of AhR. The absence of known signature motifs in the AAD of AhR implies that its interactions with target proteins are novel and differ from those described for other AADs. A more complete understanding of the interactions between the acidic segment of AhR and target proteins awaits structural studies. Because AhR is a prototypical bHLH/PAS transcription factor, its mechanism of transactivation might be representative of other members of this class of regulatory proteins. If so, future studies of the AhR system may generate insights into transactivation of genes involved in responses to hypoxia, circadian rhythms, development, and other pathways (17).

Finally, our studies indicate that different mutations in the acidic segment of AhR produce quantitatively different effects on transactivation capability. If analogous mutations were to occur in human populations, we envision that they could account, in part, for genetic polymorphisms among individuals in their responsiveness to TCDD. Whether such polymorphisms would be associated with different susceptibilities to dioxin-induced disease is an interesting issue for future research.

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