We delineate a mechanism by which dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin or TCDD)-mediated formation of the aryl hydrocarbon receptor (AhR) DNA binding complex is disrupted by a single mutation at the conserved AhR tyrosine 9. Replacement of tyrosine 9 with the structurally conservative phenylalanine (AhRY9F) abolished binding to dioxin response element (DRE) D, E, and A and abrogated DRE-driven gene induction mediated by the AhR with no effect on TCDD binding, TCDD-induced nuclear localization, or ARNT heterodimerization. The speculated role for phosphorylation at tyrosine 9 was also examined. Anti-phosphotyrosine immunoblotting could not detect a major difference between the AhRY9F mutant and wild-type AhR, but a basic isolectric point shift was detected by twodimensional gel electrophoresis of AhRY9F. However, an antibody raised to recognize only phosphorylated tyrosine 9 (anti-AhRPY9) confirmed that AhR tyrosine 9 is not a phosphorylated residue required for DRE binding. Kinase assays using synthetic peptides corresponding to the wild-type and mutant AhR residues 1–23 demonstrated that a tyrosine at position 9 is important for substrate recognition at serine/threonine(s) within this sequence by purified protein kinase C (PKC). Also, compared with AhRY9F, immunopurified full-length wild-type receptor was more rapidly phosphorylated by PKC. Furthermore, co-treatment of AhR-deficient cells that expressed AhRY9F and a DRE-driven luciferase construct with phorbol 12-myristate 13-acetate and TCDD resulted in a 30% increase in luciferase activity compared with AhRY9F treated with TCDD alone. Overall, AhR tyrosine 9, which is not a phosphorylated residue itself but is required for DNA binding, appears to play a crucial role in AhR activity by permitting proper phosphorylation of the AhR.

The aryl hydrocarbon receptor (AhR) is a ligand-activated member of the basic helix-loop-helix/Per-ARNT-Sim (bHLH-PAS) transcription factor family, which includes Per, AhR nuclear translocator (ARNT or hypoxia-inducible factor 1-β), Sim, and HIF 1-α (1–4). This protein is believed to mediate the biological and toxic effects of a class of environmental pollutants, best exemplified by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD or dioxin) (5). TCDD binding initiates the process of transformation, which includes dissociation of one or several of the chaperone-related proteins from the unliganded AhR in the cytoplasm, translocation of the receptor-ligand complex into the nucleus, and dimerization with ARNT, to form the active TCDD-AhR-ARNT transcription factor complex (6, 7). The endogenous ligand for the AhR remains unknown.

The active AhR-ARNT complex specifically recognizes a consensus sequence, termed a dioxin response element (DRE, 5’-CACGCGA-3’), located in the upstream regulatory region of AhR-responsive genes, such as Cyp1A1, leading to transcription initiation. The basic region of the AhR contains multiple essential structural components for direct contact to DNA. It has two basic clusters separated by about 20 amino acids, both of which appear to be engaged in direct protein-DNA contact. Mutation of certain positively charged residues in either cluster (e.g. arginine 14 in the more N-terminal basic region or arginine 39 in the nominal basic region) to alanine or lysine has been found to abolish the formation of the ternary complex between the AhR-ARNT dimer and the DRE, suggesting that it is these basic residues that directly contact the DRE (8–11).

Previous investigations had determined that when N-terminal deletion analyses were performed to map the DNA binding domain of an AhR mutant lacking the C-terminal half and the minimal ligand binding domain (AhRCA516), the first 9 amino acids, including tyrosine 9, were dispensable for constitutive DNA binding activity (11). In contrast, N-terminal deletion analyses of the full-length mouse AhR indicated that the first 9 amino acids are required for TCDD-induced DNA binding of the AhR but not required for heterodimerization with ARNT (10). Also, amino acid scanning mutations to alanine, serine, or tryptophan showed that tyrosine 9 is solely responsible for the loss of DNA binding and transcriptional activity of these N-terminal deletion mutants (9, 10). Besides arginines 14 and 39, AhR tyrosine 9 was the only residue in the N terminus whose mutation resulted in dramatic loss of both DNA binding and transcriptional activity (9, 10). These data clearly demonstrate that AhR tyrosine 9 is a critical residue required for full-length AhR activity. However, the mechanism by which AhR tyrosine

12-myristate 13-acetate; GFP, green fluorescent protein; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; NTA, nickel nitritolactric acid; PVDF, polyvinylidene difluoride; BSA, bovine serum albumin; HRP, horseradish peroxidase; CHAPS, 3-[3-cholamidopropyl(dimethylammonio)-1-propanesulfonic acid; IEF, isoelectric focusing; HA, hemagglutinin; WT, wild type; Me2SO, dimethyl sulfoxide; IPG, immobilized pH gradient.

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‡The abbreviations used are: AhR, aryl hydrocarbon receptor; ARNT, AhR nuclear translocator; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; DRE, dioxin response element; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; GFP, green fluorescent protein; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; NTA, nickel nitritolactric acid; PVDF, polyvinylidene difluoride; BSA, bovine serum albumin; HRP, horseradish peroxidase; CHAPS, 3-[3-cholamidopropyl(dimethylammonio)-1-propanesulfonic acid; IEF, isoelectric focusing; HA, hemagglutinin; WT, wild type; Me2SO, dimethyl sulfoxide; IPG, immobilized pH gradient.

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9 plays a role in AhR activity remains controversial. While this residue is not expected to directly contact DNA, several mechanisms including phosphorylation of this residue have been speculated (9, 12, 13). Here, we examine three known or speculated aspects of AhR activation in which tyrosine 9 may potentially play a role. We analyze the impact of a tyrosine 9 mutation on various known steps of the AhR mechanism of action, including ligand binding, nuclear localization, and ARNT heterodimerization, as well as the ability to bind to various DRE sequences. We also test the hypothesis that tyrosine 9 is required for ligand-dependent DNA binding (i.e., for full-length AhR) (11, 14) and the hypothesis that tyrosine 9 is required for N-terminal cleavage of the AhR prior to DNA binding (15). Finally, we address the hypothesis that phosphorylation of, or mediated by, tyrosine 9 is critical for normal AhR activation (10, 13).

While AhR tyrosine 9 does not appear to be essential for ligand-elicited nuclear localization or ARNT dimerization, it is necessary for DNA binding of the full-length protein. Although phosphorylation of the AhR tyrosine 9 is not required for DNA binding, mutating tyrosine 9 alters the isoelectric points of the AhR charged forms suggesting that tyrosine 9 plays a role in post-translational modification of other AhR residue(s). We demonstrate that protein kinase C (PKC) phosphorylates the wild-type (WT) AhR, and that mutating AhR tyrosine 9 decreases PKC-elicited phosphorylation of the AhR. Furthermore, we demonstrate that the decreased transcriptional activity of the AhRY9F mutant can be partially overcome upon co-treatment with TCDD and phorbol 12-myristate 13-acetate (PMA). These data demonstrate for the first time that, while tyrosine 9 itself is not phosphorylated, it can play a crucial role in phosphorylation of the AhR and AhR-mediated gene transcription.

EXPERIMENTAL PROCEDURES

Generation of AhR Constructs—Site-directed mutagenesis and construction of pcDNA3/5AH4R-HIS and pcDNA3/5AHRY9F-HIS were performed as described previously (9, 10, 12). For generation of the pHAM6/AhRs that code for N-terminally HA-tagged and C-terminally 6 histidine-tagged AhRs, the wild-type and mutant AhR coding sequences were amplified from pcDNA3/5AhR or pcDNA3/5AHRY9F mutant DNA by Pfu Turbo DNA polymerase using the forward primer, 5′-CCG CCG CTT CTC TAT CCG CCG GGA GGA GGG GAA-GAG ATG CGG CCG CTA AAA TGG TGA GCA AGG GC-3′ and the reverse primer, 5′-GGT ATG GCT GAT TAT GAT AGA ATG CGG CCG CTA AAA TGG TGA GCA AGG GC-3′. The PCR products were digested with XhoI and NotI and annealed with the unlabelled complementary strand; DRE D (AhRE3), 5′-GAT CCG GCT CCT CT CACGCAACT CGA GCT CA-3′; DRE E (AhRE2), 5′-CCC AGT GCT GTC CACGCTAGCT GGG GGA GGG GAA-3′; DRE A (AhRE5), 5′-TGC GCT TCT CACGGAGCT TGG-3′. The duplex oligonucleotide contains a single AhR binding sequence (underlined). Supershift assays were performed as described for EMSAs with the exception of the use of the HA-tagged AhR expression vectors and the inclusion of antibody at the incubation step just prior to DRE addition. The antibodies that recognize the hemagglutinin epitope (HA.11, Covance, Berkeley, CA), the AhR (Rpt-9), a nonspecific rabbit anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories), or the anti-AHRY9F antibody were used as supershift reagents.

In Vitro Protein-Protein Interaction Study—Interaction of AhR with ARNT was investigated by using radiolabeled AhR expressed in the TaT® (Promega) system as described above with the exception of the inclusion of 15 μCi of [35S]methionine in the reaction mixture. 35S-labeled AhR was incubated with the similarly expressed and unlabelled histidine-tagged AhRs in the presence or absence of 10 μM TCDD (in 0.1% MeSO) for 90 min at room temperature. AhR-bound AHRY9F was precipitated with 70 μl of Ni-NTA-agarose for 30 min at 4 °C in the His tag lysis buffer with 0.3 mM NaCl. The pellet was washed twice with His tag lysis buffer containing 40 mM imidazole and boiled with SDS-PAGE buffer. The co-precipitated ARNT was analyzed by SDS-PAGE and visualized by autoradiography.

Co-transfection Assay of AhR-deficient Cells—To determine AhRWT and AhRY9F transactivation activity, 2 × 105 AhR-deficient cells (BPc1TAO or COS-7) were co-transfected with 0.45 μg of AhR expression vectors (pcDNA3/5AH4R or pcDNA3/5AHRY9F) and 0.1 μg of normalization vector (pRSVLacZ) using LipofectAMINE (Invitrogen) or GenePORTER (Gene Therapy Systems, San Diego, CA) in 6-well plates as described previously (12). After the recovery from transfection, the cells were treated with either 0.1% MeSO or 4 nM TCDD (in 0.1% MeSO) for 22 hr and harvested for the reporter gene assay. DRE-driven luciferase activity was measured with the luciferase assay kit (Promega), and β-galactosidase activity was determined with a β-galactosidase substrate kit (Tropix/Lifetech, MA). Marker proteins were detected by Western blotting using a Turner Model TD-20e Lumimeter (Turner Designs, Sunnyvale, CA).

Immunoblot Analysis of Partially Purified Full-length AhRs with Anti-Phosphotyrosine Antibody—The pcDNA3/5AH4R-HIS and pcDNA3/5AHRY9F-HIS vectors were transiently transfected into the AhR-deficient TAO cells using GenePORTER according to the manufacturer and partially purified exactly as previously described using Ni-NTA-agarose (12). After separation by SDS-PAGE, the proteins were transferred to a PVDF membrane, and the membrane was probed with FP-20 monoclonal anti-phosphotyrosine antibodies (Transduction Laboratory, Lexington, KY) in 3% BSA-TBST. The membrane was anti-phosphotyrosine antibody (Becton Dickinson, MA) and revealed using hybridoma cells that were a kind gift from Dr. G. Perdue (Pennsylvania State University). The primary antibody was located with HRP-conjugated goat anti-mouse IgG and visualized with LumiGLO chemiluminescent substrate (KPL, Gaithersburg, MD).

Two-dimensional Gel Electrophoresis of Wild-type AhR and AHRY9F—Two-dimensional gel electrophoresis was performed utilizing radiolabeled AhRs separately expressed in the TaT® system as described above with the exception of inclusion of 15 μCi of [35S]methionine in the reaction mixture, methods similar to those previously described (13). 50 μl of the TaT® lysis containing [35S]labeled AhR was diluted with 150 μl of HEDG buffer (25 mM Hepes, 1.5 mM Na2EDTA, 1 mM DTT, 10% glycerol) and resolved by 2D-PAGE as described for supershift assays. 50 μl of this mixture was added to 30 μl of HEDG containing 15 μCi/mg carrier protein and added to 215 μl of rehydration buffer (6.4 μm urea, 2.6 mM thiourea, 4% CHAPS, 39 mM DTT, 0.2% Bio-Lytes 3/10 amphotoles, 0.001% bromphenol blue). The Bio-Rad Protein II Cell and reagents

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were used for isoelectric focusing (IEF) and the Criterion Precast Gel System was used for SDS-PAGE (Bio-Rad). ReadyStrip IPG 11-cm strips (pH range 3–10) were actively rehydrated (application of 50 V) at 16 °C covered with mineral oil for 26 h. For IEF, a slow ramping protocol was followed: a conditioning step of 250 V for 15 min, followed by voltage ramping up to 8000 V over 2.5 h, and final focusing at 8000 V for 80,000 Vh. Maximum current limit was set at 50 µA per gel, with total Vh not exceeding 100,000 Vh. Following IEF, the IPG strips were equilibrated for 30 min in DTT equilibration buffer (5 mM urea, 0.8 mM thiourea, 4% SDS, 0.375 mM Tris-HCl pH 8.8, 20% glycerol, and 130 mM DTT), followed by 30 min in iodoacetamide equilibration buffer (5 mM urea, 0.8 mM thiourea, 4% SDS, 0.375 mM Tris-HCl pH 8.8, 20% glycerol, and 135 mM iodoacetamide). IPG strips were loaded to the well of a 7.5% Tris-HCl precast gel and run at a constant voltage of 200 V for 1 h. Proteins from the 2nd dimension gels were transferred to Sequi-Blot PVDF membranes (Bio-Rad) and 35S-labeled AhRs were visualized with a PhosphorImager (Amersham Biosciences).

**Anti-AhR Phosphorysine 9 Production and Immunodetection of Phosphorylated AhR Tyrosine 9**—Synthesis of peptides, BSA conjugation, and generation of the phosphopeptide antibody was carried out by Alpha Diagnostic International (San Antonio, TX) as previously described (18–20). A BLAST search was performed to optimize selection of sequence that would be most likely antigenic and exhibit minimal cross-reactivity with nonspecific proteins. The hydrophilic region, residues 4–16 (GANITpY/ASKRRkK) with a cysteine on the C terminus for covalent coupling to keyhole limpet hemocyanin conjugation, was used as the phospho-tyrosine 9 peptide antigen purified to >90% for presentation to rabbits. For affinity purification, a non-phosphopeptide control of the same sequence without a phosphorylated tyrosine 9 was used as an additional affinity support to remove nonspecific antibodies prior to use of the phosphorysine 9 peptide affinity column for capture of the antibodies of interest. Both the control and phosphopeptide were used to test antibody cross-reactivity with non-phosphorylated peptides by ELISA. BSA was conjugated to the phosphopeptide (BSA-<wbr/>pY9) using the glutaraldehyde conjugation method to allow optimization for immunodetection of phosphorylated sone phosphorysine 9. To determine the specificity of the anti-AhRpY9 antibody, the BSA-pY9 conjugate was incubated with a peptide sequence that corresponded to the phosphorylated phospho-tyrosine 9 peptide antigen purified to >90% for presentation to rabbits. For affinity purification, a non-phosphopeptide control of the same sequence without a phosphorylated tyrosine 9 was used as an additional affinity support to remove nonspecific antibodies prior to use of the phosphorysine 9 peptide affinity column for capture of the antibodies of interest. The AhR-deficient cells were transfected with 8 µg of an AhR expression vector (pHM6/4AHR) using GenePORTER in 100-mm plates 24 h after plating 2 × 106 cells in each plate. AhR-deficient cells from confluent plates were not transfected as a negative control. After 13 h of recovery, the cells were treated for 1 h with either 0.1% Me2SO or 10 nM TCDD. Cells were washed with phosphate-buffered saline and lysed in 100 µl of lysis buffer as described by the manufacturer (Promega). The activities of firefly luciferase and Renilla luciferase were determined using the Dual-Luciferase reporter assay system (Promega) as described by the manufacturer. Pelleted protein was solubilized in PBS containing 1% Triton X-100, pH 7.4), and then either incubated for 15 min at 30 °C with alkaline phosphatase or buffer and washed extensively in IP wash buffer. The washed immunocomplexes were then incubated for the indicated times at 30 °C with an active mixture of PKC and [32P]ATP, as described by the manufacturer. Pelleted protein was solubilized in SDS-loading buffer at the time point indicated and loaded to a 7.5% denaturing gel for SDS-PAGE. Protein was then transferred to PVDF membrane and detected using a PhosphorImager. The amount of AhR was analyzed by immunoblot analysis using anti-AhR antibodies or anti-HA antibodies as indicated. The primary antibodies were located with HRP-conjugated IgG and visualized with LumiGLO chemiluminescent substrate.

**PMA Effect on Transcriptional Activity—**AhR-deficient cells (2.0 × 104) were seeded into each well of 24-well plates and incubated at 37 °C. Cells were treated with serum-free medium and transfected with 250 µl of serum-free medium containing 400 ng of the designated pHM6/4AHR-HIS construct, 100 ng of the DRE-driven firefly luciferase reporter construct, pD2Luc, and 15 ng of Renilla luciferase vector pRL-TK (Promega) that was preincubated with 2.6 µl of GenePORTER transfection reagents at room temperature for 45 min. Four hours later, another 250 µl of growth media containing serum-free medium was added to each well to achieve the final serum concentration of 10%. Fresh media was added 24 h later. After 24 h, the cells were treated with either vehicle (0.15% Me2SO), 10 nM TCDD, 100 nM PMA, or a combination of 10 nM TCDD and 100 nM PMA, or a combination of 10 nM TCDD, 100 nM PMA, 1 µM GF 19203X, and 1 µM RO 31–8220. PMA, GF 19203X, and RO 31–8220 were purchased from BioMol. In a separate experiment, similarly transfected cells were pretreated for 15 min with 4 µM chelerythrine chloride (not shown) (Sigma) before the treatment performed above, as described previously (30). All treatment groups were incubated for 4 h before the cells were washed with phosphate-buffered saline and lysed in 100 µl of lysis buffer as described by the manufacturer (Promega). The activities of firefly and Renilla luciferase were determined using the Dual-Luciferase reporter assay system (Promega) as described by the manufacturer. Pelleted protein was solubilized in PBS containing 1% Triton X-100, pH 7.4), and then either incubated for 15 min at 30 °C with alkaline phosphatase or buffer and washed extensively in IP wash buffer. The washed immunocomplexes were then incubated for the indicated times at 30 °C with an active mixture of PKC and [32P]ATP, as described by the manufacturer. Pelleted protein was solubilized in SDS-loading buffer at the time point indicated and loaded to a 7.5% denaturing gel for SDS-PAGE. Protein was then transferred to PVDF membrane and detected using a PhosphorImager. The amount of AhR was analyzed by immunoblot analysis using anti-AhR antibodies or anti-HA antibodies as indicated. The primary antibodies were located with HRP-conjugated IgG and visualized with LumiGLO chemiluminescent substrate.

**RESULTS**

AhRpY9F Exhibits Normal Ligand Binding, Subcellular Localization, and ARNT Interaction—We examined the possibility that the AhR tyrosine 9 may be required for ligand binding, nuclear localization, and/or ARNT interaction. The inhibition of DNA binding by the AhR-Y9F mutant was not due to decreased ligand binding, because specific binding of [3H]TCDD to **in vitro** expressed WT and Y9F AhR-ARNT was equivalent as measured by the hydroxylapatite assay (21) (not shown).Tyrosine 9 is located adjacent to the basic cluster in the N terminus that contains the nuclear localization signal (see Fig. 1, NLS); thus, mutation of this residue may have the potential to disturb nuclear localization processes. The N-terminal 495 amino acid fragment of AhR is known to contain all domains for ligand binding, proper nuclear translocation, heterodimerization with ARNT, and association with the DRE sequence in the enhancer region of the CYP1A1 gene upon TCDD treatment (22). To monitor the intracellular location of the AhR, the
N-terminal 495 residues of WT and the Y9F mutant AhRs was fused to the N terminus of green fluorescent protein cDNA to produce AhR495-GFP. Fluorescence microscopic analyses of transiently transfected Hepa1c1c7 cells with these AhR-GFP fusion proteins revealed that GFP, AhR495WT-GFP, and AhR495Y9F-GFP were present both in the cytosol and the nucleus in vehicle-treated cells. After TCDD treatment, however, both AhR495WT-GFP and AhR495Y9F-GFP were compartmentalized mainly in the nucleus, whereas the unconjugated GFP still remained distributed in both the cytosol and the nucleus (Fig. 2A). These data indicate that the nuclear localization signal in AhR495F is still functional and recognized by the nuclear translocation machinery.

Studies were also conducted to determine whether DNA binding of the AhR495F is interrupted by a defect in heterodimerization between the ligand-activated AhR and ARNT. In a co-precipitation assay with His6-tagged AhRs and [35S]methionine-labeled full-length ARNT, the AhR495F mutant was able to heterodimerize with ARNT as efficiently as wild-type AhR (Fig. 2B). Similarly, extracts were also prepared from TAO cells that had been transfected with AhR expression vectors. AhR was immunoprecipitated, and the level of AhR-associated ARNT was determined by Western blotting. The amount of ARNT co-immunoprecipitated with both WT and Y9F AhR was similar (not shown). Therefore, the interaction of the AhR with ARNT was not affected by the mutation of tyrosine 9 to phenylalanine, which is consistent with mutations to alanine, tryptophan, or serine (9, 10). Together, these data suggest that tyrosine 9 is not involved in ligand binding, nuclear localization, or ARNT interaction.

Decreased DNA Binding Activity of AhR495F for DRE D, E, and A—Six distinct DREs (designated DRE A–F), containing a common core sequence but different flanking sequences, are in the upstream DNA region of the TCDD-responsive CYP1A1 gene (17). Southwestern analysis of nuclear extracts from wild-type, AhR-deficient, and ARNT-deficient mouse hepatoma cell lines showed that DRE D is associated with two proteins that are neither AhR nor ARNT (23). One of them is C/EBPα, which interacts with the CAACT sequence present in the DRE D but not in DRE A, C, or E. Compared with other DRE sequences, DRE D is the most active (17). Interaction of C/EBPα with the DRE D sequence was also verified in the glutathione S-transferase Ya subunit gene enhancer region, where C/EBPα further stimulates TCDD-induced AhR transcriptional activation most likely through further stabilization of the AhR-ARNT-DRE complex (24). The other molecule had an approximate mass of 95 kDa and was found to bind both DRE D and A (23). Dissociation of this protein from DNA may be necessary for the access of the AhR-ARNT complex to the same DNA binding sites. Thus, competition with this 95 kDa protein for these sites might determine the DNA binding potential of the AhR-ARNT complex (23). It is possible that the pattern of complex formation of the AhR-ARNT dimer with DRE D may be different from that with DRE E or A. Therefore, it was of interest to test whether the defect in DNA binding by AhR495F is restricted specifically to association with DRE D. However, EMSAs demonstrated that the AhR495F is not capable of binding to any DRE tested as compared with wild-type, suggesting that tyrosine 9 is required for intrinsic AhR DNA binding ability (Fig. 3A).

Disruption of AhR Transcriptional Activity by Mutation of Tyrosine 9 to Phenylalanine Is Not Cell-type Specific—To recapitulate the effect of tyrosine 9 mutation to phenylalanine in intact cells, both wild-type and AhR495F were transiently expressed along with a DRE reporter gene in either AhR-deficient mouse hepatoma (TAO) or monkey kidney cells (COS-7). As expected, the AhR495F exhibited significantly decreased transcriptional activity in both cell types (Fig. 3, B and C, respectively). These data demonstrate that the decrease in transcriptional activity because of a tyrosine 9 mutation is not restricted to a specific cell line.

AhR495F Mutation Disrupts the Ligand-independent DNA Binding Activity of AhR Truncation Mutants—Next, we asked whether tyrosine 9 is required for constitutive DNA binding activity of a PAS-B domain-deleted AhR, because it might utilize a different tertiary structure for DNA binding that no longer requires the first nine amino acids (11). To address this question, a constitutively activated AhR, AhR346, was generated by eliminating only a minimal amount of the ligand binding domain. Truncation mutants with the wild-type sequence and the Y9F mutation were expressed in vitro and incubated with the similarly expressed full-length mouse ARNT in the presence or absence of TCDD. The AhR346 truncation mutant that has the wild-type sequence exhibited strong DNA binding activity in the vehicle-treated samples (Fig. 4A). The level of this constitutive DNA binding activity could not be further elevated by incubation with 10 nM TCDD. Mutation of tyrosine 9 to phenylalanine in the AhR346 PAS-B deleted receptor abolished the constitutive DNA binding activity and TCDD treatment failed to overcome the inhibition of DNA binding activity of AhR346Y9F mutant (Fig. 4A, lanes 7–10). Similar results were obtained with AhR331, even in the presence of the AhR antagonist, 3’-methoxy-4’-nitro-flavone (Fig. 4A, MNF; lanes 5 and 6 and Ref. 25). These data suggest that the DNA binding form of this truncated AhR shares a similar three-dimensional conformation as that of the full-length AhR induced by ligands in which tyrosine 9 plays an essential role.

The N Terminus of the AhR Is Not Cleaved Prior to DNA Binding—It was previously suggested that the first 9 amino acids, including tyrosine 9, are not present in the active AhR.
due to proteolytic cleavage (15). This hypothesis was based on the N-terminal sequencing of the purified AhR from mouse liver that suggested the first residue to be what is now known as alanine 10. However, it is not clear whether the loss of these residues in the purified AhR was due to experimental artifacts or a reflection of a true physiological event (10). An AhR expression vector was constructed to express an N-terminally HA-tagged AhR to examine the hypothesis that the N-terminal of the AhR is proteolytically cleaved to allow normal DNA binding and that tyrosine 9 is required for this speculated cleavage. A supershift EMSA was performed to determine if the N terminus is absent from the AhR in the DNA-bound AhR-ARNT complex. In vitro translated AhRs were treated with 10 nM TCDD, and an EMSA was performed in the presence or absence of an HA antibody. Wild-type AhR tagged on either the N or C terminus (as a control) were supershifted by an HA antibody. Similarly, the AhRY9F, which only binds up to 20% of the level of wild-type AhR (13), was also completely supershifted with the HA antibody (Fig. 4B). These data demonstrate that the N terminus is not required to be cleaved from wild type or AhRY9F prior to/during DNA binding in vitro. Therefore, tyrosine 9 is not required for this speculated N-terminal cleavage to occur prior to DNA binding.

AhRY9F Reacts Similarly to Wild-type AhR with Anti-phosphotyrosine Antibodies, but Exhibits Altered Charged Forms—Previous reports indicated that the AhR requires tyrosine phosphorylation for its DNA binding activity (12, 26). Furthermore, mutational analysis of mouse AhR tyrosine residues suggested that only a mutation at tyrosine 9, a computationally predicted site of phosphorylation, significantly decreases DNA binding activity and DRE-driven luciferase expression (13). These data raise the possibility that phosphorylation at tyrosine 9 may play a role in AhR DNA binding. Phosphorylation of the tyrosine 9 residue was investigated by immunoblot analysis using an anti-phosphotyrosine antibody, mutation to the phosphomimetic residue glutamate and two-dimensional gel electrophoresis. Both wild-type AhR and AhRY9F were conjugated with six consecutive histidines at their C-terminal ends to facilitate purification, and were transiently expressed in the TAO cell line. AhRs fractionated on Ni-NTA-agarose were sep-
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Figure 3. Decreased DNA binding activity for different DREs and disruption of AhR transcriptional activity by mutation of tyrosine 9 to phenylalanine is not cell-type specific. A, both wild-type and mutant AhRs were expressed in vitro in rabbit reticulocyte lysate and incubated with similarly translated ARNT in the presence of vehicle or 10 nM TCDD for 90 min. The DNA binding activity of the AhR-ARNT complexes was measured with different 32P-labeled DRE probes described under “Experimental Procedures” in a non-denaturing gel. Expression level of AhR was monitored by immunoblot analysis of in vitro translated proteins (not shown). Lanes 1–4, DRE D; lanes 5–8, DRE E; lanes 9–12, DRE A. The data shown are representative of three independent experiments. Transcriptional activity of AhRWT and mutant AhRY9F in TAO (B) and COS-7 (C) cells are shown. The cells were transiently transfected with AhR expression vectors, DRE-driven luciferase reporter gene, and β-galactosidase normalization vector using LipofectAMINE. After recovery, the cells were treated with 4 nM TCDD for 22 h before cell lysis. Relative luciferase activity, normalized to β-galactosidase activity, is shown as the percent of vehicle (Me2SO) treated wild-type AhR (±S.D. of triplicate analyses).

Figure 4. Substitution of tyrosine 9 with phenylalanine abolishes DNA binding activity of a ligand-independent AhR truncation mutant, and N-terminally tagged HA-AhR is supershifted with an HA antibody. A, wild-type and mutant AhRs were translated in vitro in rabbit reticulocyte lysate and incubated with similarly translated ARNT in the absence/presence of 10 nM TCDD for 90 min, with or without antagonist 3′-methoxy-4′-nitroflavone (MNF). The DNA binding activity of the AhR-ARNT complexes was measured with 32P-labeled DRE D probe in a non-denaturing gel. Expression level of AhR was monitored by immunoblot analysis of in vitro translated proteins (not shown). Lanes 1 and 2, full-length AhR; lanes 3–6, AhR331; lanes 7 and 8, AhR346WT; lanes 9 and 10, AhR346Y9F. The data shown are representative of three independent experiments. B, non-tagged AhRs and HA-tagged AhRs were translated separately in vitro in rabbit reticulocyte lysate and incubated with similarly translated ARNT in the presence of 10 nM TCDD for 90 min. The DNA binding activities of the AhR-ARNT complexes were measured with 32P-labeled DRE D probe in a non-denaturing gel in the absence/presence of nonspecific (NS) mouse IgG, specific (S) anti-AhR antibody Rpt-9, or anti-HA tag (HA) antibody. Expression level of AhR was monitored by immunoblot analysis of in vitro translated proteins (not shown). Lanes 1–3, wild-type AhR with no tag (WT); lanes 4 and 5, C-terminally tagged wild-type AhR (WT-HA); lanes 6 and 7, N-terminally tagged wild-type AhR (HA-WT); lanes 8 and 9, AhRY9F with no tag (Y9F); lanes 10 and 11, N-terminally tagged AhRY9F (HA-Y9F). The data are representative of three independent experiments.
We tested several different anti-phosphotyrosine antibodies, some of which are known to have differential affinity and specificity for varying substrates. However, we were unable to detect a significant difference in the phosphotyrosine signals between wild-type AhR and AhRY9F (not shown). These data are consistent with the results from anti-phosphotyrosine immunoblotting of wild-type AhR and AhRY9F when they are synthesized in rabbit reticulocyte lysate (13). In addition, phosphotyrosine signals in both WT and AhRY9F remained similar before and after TCDD treatment (Fig. 5A). This indicates that the majority of tyrosine phosphorylation occurs constitutively, and/or tyrosine phosphorylation at several specific sites may change upon TCDD treatment allowing total tyrosine phosphorylation to remain quantitatively unchanged. These data suggest that, although the AhR is tyrosine-phosphorylated, tyrosine 9 may not be phosphorylated at all or, if it is, may not be a major phosphotyrosine residue in the AhR.

On the other hand, the fact that tyrosine 9 cannot be replaced with phenylalanine, which is unable to be phosphorylated but is the structurally most similar amino acid to tyrosine, suggests that phosphorylation of this position may affect the interaction of the AhR-ARNT complex with DNA. In some cases, phosphorylated amino acid residues can be mimicked by phosphomimetic amino acids, such as aspartate or glutamate, whose side chains possess negatively charged carboxyl groups. Therefore, site-directed mutagenesis was performed to replace tyrosine 9 with glutamate to determine whether DNA binding of the AhR involves the negatively charged phosphate group at tyrosine 9. EMSAs showed that in vitro transcribed and translated AhRY9E was unable to bind the DRE sequence when incubated with similarly expressed ARNT in the presence of TCDD (not shown). It also lacked transcriptional activity when it was transiently expressed in CV-1 cells and TAO cells with a DRE-driven reporter gene (not shown). These data are consistent with the suggestion that it is not just the negative charge of a putatively phosphorylated tyrosine 9 that is critical for DNA binding.

The AhR isolated from cells or synthesized in vitro has been shown to exhibit charge heterogeneity that is consistent with phosphorylation as determined by two-dimensional gel electrophoresis (13, 27, 28). Cytosolic 35S-dioxin-labeled AhR exhibited an apparent isoelectric point (pI) range of 5.2–5.7, while the apparent pI nuclear AhR ranged from 5.5 to 6.2 (28). This differential charge heterogeneity is consistent with a means of dynamic post-translational modification occurring at some point between the cytosol and the nucleus, though no evidence has been generated to directly implicate phosphorylation at specific residues. The 35S-labeled wild-type AhR and AhRY9F were separately synthesized in vitro and analyzed by two-dimensional gel electrophoresis. Both AhRs showed charge heterogeneity similar to previous reports, but AhRY9F isoforms were more basic than the wild-type AhR (Fig. 5B). These results suggest that either tyrosine 9 is a phosphorylated site and/or that mutating tyrosine 9 results in a change in charge (for example, by post-translational modification) at other unidentified residues.

The Unoccupied, TCDD-bound, and DNA-binding Forms of the AhR Are Not Phosphorylated on Tyrosine 9—To determine whether or not phosphorylation of AhR tyrosine 9 is required for DNA binding, we produced an antibody (anti-AhRty9) for immunodetection of the putatively phosphorylated AhR tyrosine 9. The polyclonal antibody recognized specifically a phosphorylated tyrosine 9 that is critical for DNA binding (13). The anti-AhRty9 antibody did not recognize a non-phosphorylated negative control peptide corresponding to AhR residues 4–16, containing AhR tyrosine 9, and did not recognize a non-phosphorylated negative control peptide using both ELISA and immunoblot methodologies (not shown).

To verify the specificity of the anti-AhRty9 antibody for whole protein containing a phosphorylated tyrosine 9, BSA was conjugated to the phosphopeptide antigen (BSA-pY9). The anti-AhRty9 antibody did react specifically with the BSA-pY9 (Fig. 6A, top panel, lane 1) but did not recognize the BSA-pY9 conjugate when it was dephosphorylated (Fig. 6A, top panel, lane 2). Interestingly, another polyclonal AhR antibody (immunogen, recombinant AhR N-terminal fragment (residues 1–402) obtained from BioMol) only recognized the dephosphorylated BSA conjugate (Fig. 6A, bottom panel, lane 2). These results suggest that the anti-AhRty9 antibody is both specific and sensitive enough to detect an AhR-phosphotyrosine 9 sequence. However, the anti-AhRty9 antibody did not recognize vehicle (not shown) or TCDD-treated AhRs synthesized in vitro, even when incubated with TCDD and ARNT (Fig. 6A, lanes 3 and 4). Also, the anti-AhRty9 antibody did not recognize AhRs from...
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Fig. 6. The anti-AhR phosphotyrosine 9 antibody (anti-AhRpY9) does not recognize denatured AhRs. A, immunoblot control and detection of denatured AhR with the anti-AhRpY9 antibody. BSA was conjugated to a peptide corresponding to AhR residues 4–16, containing a phosphorylated tyrosine at the position representing tyrosine 9 to produce BSA-pY9. An aliquot of BSA-pY9 lane 1 was dephosphorylated with a mixture of alkaline phosphatase (AP) and potato acid phosphatase (PAP) as a control lane 2 for the specificity of the anti-AhRpY9 antibody. AhRWT lane 4 and AhRY9F lane 3 were translated separately in vitro in rabbit reticulocyte lysate and incubated with similarly translated ARNT in the presence of 10 nM TCDD for 90 min. The BSA conjugates and the AhR samples were loaded on a 7.5% denaturing gel for SDS-PAGE, and the protein was transferred to a PVDF membrane. The anti-AhRpY9 antibody was used for the immunoblot in the top panel, while polyclonal anti-AhR antibody (BicMo) was used for immunoblot detection in the bottom panel after stripping the membrane. B, immunodetection of AhR isolated from transiently transfected AhR-deficient cells with the anti-AhRpY9 antibody. TAO cells were transfected with AhR expression vectors (pH6/AHRWT, pH6/AHRY9, lanes 4 and 5 and pH6/AHRWT, lanes 6 and 7). After 48 h of transfection, the cells were treated with either vehicle (0.1% MeSO4) (lanes 4 and 6) or 10 nM TCDD (lanes 5 and 7) for 1 h prior to cell lysis. The HA-tagged AhR protein was purified by immunoprecipitation using the anti-HA antibody. The purified AhR proteins were separated by SDS-PAGE, transferred to PVDF membrane, and probed with anti-AhRpY9 antibody (top panel). The same membrane was stripped and reprobed with the anti-AhR antibody (bottom panel). These data are representative of three independent experiments.

It is possible that phosphorylation at AhRY9 may occur in only a small population of the AhR pool, specifically in the DNA binding form. We addressed this issue in two different ways. First, we labeled AhR with [35S]methionine and attempted to concentrate any AhR phosphorylated on tyrosine 9 by immunoprecipitating with an excess amount of anti-AhRpY9. The HA-tagged AhRs that had been incubated with ARNT and TCDD to produce the DNA binding form of the AhR could be immunoprecipitated by an anti-HA antibody (Fig. 7A, lanes 1–4) but not by the anti-AhRpY9 antibody (Fig. 7A, lanes 5–8). Also, we hypothesized that the anti-AhRpY9 antibody would result in either a supershift or an attenuation of the TCDD-mediated AhR-ARNT-DRE complex if the DNA binding form of the AhR is phosphorylated on tyrosine 9. However, the anti-AhRpY9 antibody failed to alter the normal mobility of the wild-type AhR-ARNT-DRE ternary complex when AhR and ARNT were synthesized in rabbit reticulocyte lysate (Fig. 7B, pY9, lane 7) or prepared from Hepa1c1c7 cells (not shown). The anti-HA positive control antibody supershifted this complex (Fig. 7B, HA, lane 8) whereas the anti-AhR antibody, which recognizes residues within the DNA binding region of AhR, attenuated DNA binding as expected (Fig. 7B, Rpt9, lane 6).

Together, these data are consistent with the interpretation that the AhRY9 is not a phosphorylated residue required for DNA binding.

Immunopurified AhR Is Phosphorylated by PKC, and AhRY9F Inhibits PKC-elicited Phosphorylation. The inability of the anti-AhRpY9 antibody to recognize the AhR together with the two-dimensional gel electrophoresis data suggesting that a mutation of tyrosine 9 still results in a less negatively charged receptor support a hypothesis that tyrosine 9 is required for normal post-translational modification of other AhR residues. There is much published data implicating the importance of phosphorylation for AhR activity and, in particular, PKC has been observed to be important in the regulation of AhR activity in whole cells (for example, Refs. 26 and 29–32). In an attempt to determine if PKC was capable of phosphorylating the full-length AhR, wild-type and mutant AhRs were immunoprecipitated from rabbit reticulocyte lysate and incubated with purified PKC and [32P]ATP. Notably, wild-type AhR was phosphorylated by the purified PKC, demonstrating for the first time that PKC can directly phosphorylate the AhR (Fig. 8A). AhR from Hepa1c1c7 cells similarly immunopurified (using the anti-AhR antibody, Rpt-9) also demonstrated PKC-elicited phosphate incorporation (not shown). Furthermore, AhRY9F incorporated less phosphate relative to wild-type AhR (Fig. 8A) even when immunopurified AhRs were treated with alkaline phosphatase prior to PKC incubation (not shown) indicating a difference in PKC substrate recognition and/or kinetics. The AhRY9F did incorporate a similar amount of labeled phosphate compared with AhRWT after a 16-h incubation with PKC. This demonstrates that the mutant is capable of incorporating phosphate but at a slower rate than the wild-type AhR (not shown). The control lane (Fig. 8A, Cntrl, lane 7) represents an immunopurification of lysate containing no AhR and subsequent incubation with PKC. The lower band that incorporates phosphate represents PKC autophosphorylation. These data suggest that tyrosine 9 is required for proper kinase substrate recognition of serine(s/threonine(s) within the full-length AhR.

Synthetic AhRY9F-mutated Peptides Are Phosphorylated Less Efficiently by PKC. To test the hypothesis that tyrosine 9 may be required for optimal phosphorylation of serine(s) and/or threonine(s) in the N terminus of AhR, we utilized peptides corresponding to the wild-type AhR residues 1–23 (AhR(1–23)WT), the mutant (AhR(1–23)Y9F) sequence, and a phosphorylated tyrosine 9 (AhR(1–23)pY9) sequence. Interestingly, if we consider the phosphate incorporated into the AhR(1–23)WT peptide as 100%, then significantly less phosphate was incorporated into the peptides that had the tyrosine mutated to a phenylalanine (69%) or to a phosphorylated tyrosine (36%) (Fig. 8B). These data, together, suggest that tyrosine 9 itself is not phosphorylated, but that a tyrosine at position 9 is required for normal substrate recognition by serine/threonine kinase(s), such as PKC.

PMA Augments AhRY9F Transactivation Activity. To test the hypothesis that the decreased activity of the AhR9F mutant is, at least in part, due to less efficient PKC-elicited phosphorylation, we treated AhR-deficient cells that were transfected with the AhRs and a DRE-driven luciferase construct transiently transfected and vehicle or TCDD-treated TAO cells (Fig. 6B).
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AhR tyrosine 9 is conserved across species, suggesting a crucial role for this residue in AhR function. Previously, we have indicated that the DNA binding activity of a purified AhR-ARNT complex is controlled by tyrosine phosphorylation apparently directly on the AhR (12). We have demonstrated that the AhR is tyrosine-phosphorylated when synthesized in both rabbit reticulocyte lysate (13) and cells (Fig. 5A). In an attempt to identify the putative phosphotyrosine(s) in the AhR, we carried out investigations using site-directed mutagenesis. Of all the tyrosines located in the N-terminal region of the AhR (amino acids 1–399), only mutation of tyrosine 9 leads to significant loss in AhR DNA binding and transcriptional activity, suggesting that tyrosine 9 may be the phosphotyrosine required for AhR DNA binding (13). Additionally, two-dimensional separation based on size and charge of the AhR showed that there is a basic shift in charge distribution due to a single mutation at tyrosine 9, which suggests that this residue is phosphorylated itself and/or that the AhR-Y9F mutation alters normal covalent modification(s) at other sites (Fig. 5B). To determine conclusively if tyrosine 9 is itself phosphorylated or if it is otherwise involved in maintaining charge distribution on the AhR, an antibody was raised using a peptide antigen (AhR residues 4–16 with a phosphotyrosine corresponding to tyrosine 9). This anti-AhR9 antibody did not immunoreact with any AhR examined, while all the controls indicate it is capable of specific immunodetection of a phosphorylated tyrosine 9 (Figs. 6 and 7). We conclude that tyrosine 9 is not a phosphorylated residue required for DNA binding but that it is required to maintain normal post-translational modification of other AhR residues.

Following our determination that a mutation of tyrosine 9 to phenylalanine did not interfere with ligand-activated nuclear localization or ARNT heterodimerization, we sought to determine a mechanism by which tyrosine 9 may be responsible for maintaining post-translational modification of the AhR. Treatment of AhRs synthesized in rabbit reticulocyte lysate with tyrosine or serine/threonine phosphatases resulted in a basic shift of the receptor as determined by two-dimensional gel electrophoresis (13). While continuing to analyze the hypothesis that tyrosine 9 is a phosphoshope, peptides corresponding to AhR residues 1–23 (MSSGANITYASRKRKKPVQKTVK) were synthesized in order to evaluate whether or not tyrosine...
9, the only tyrosine in this sequence, could serve as a substrate for the unknown tyrosine kinase(s) present in the lysate. Under in vitro kinase assay conditions suitable for supporting tyrosine kinase activity, we detected no significant levels of incorporation into these peptides (not shown). However, in a similar experiment using assay conditions suitable for maximal serine/threonine kinase activity, we observed high \( \gamma^{32}\text{P} \) incorporation (not shown). In an attempt to identify the serine/threonine kinase(s) activity in the reticulocyte lysate responsible for this peptide and full-length AhR phosphorylation, kinase assays were performed utilizing synthetic peptide substrates for various serine/threonine kinases. We observed moderate PKC-like activity in reticulocyte lysate, and subsequently demonstrated the presence of PKC in rabbit reticulocyte lysate by immunoblotting. To determine if PKC was capable of phosphorylating the AhR peptides, we incubated purified PKC with the peptides and \( \gamma^{32}\text{P} \)ATP. Peptides with a mutated (Y9F) or modified (phosphorylated) tyrosine 9 incorporated significantly less phosphate than the wild-type sequence (Fig. 8). These data suggest that tyrosine at position 9 may be critical for serine/threonine kinase(s) recognition and that it may be this ability that accounts for some of the altered charge forms of AhRY9F consistent with a less phosphorylated AhR.

It was of particular interest to determine if PKC could directly phosphorylate the full-length AhR and whether or not a mutation at tyrosine 9 in the sequence interfered with this phosphorylation. We demonstrate for the first time that the AhR can be phosphorylated by PKC (Fig. 8). Additionally, as hypothesized from the peptide data, AhRY9F was phosphorylated efficiently by PKC.
PKC activity has been linked to TCDD-mediated AhR-dependent processes (26, 29–32). The AhR and PKC signaling pathways apparently converge as TCDD increases PKC activity that then enhances TCDD-elicted AhR transcriptional activity. Furthermore, compounds structurally related to TCDD also increase PKC-elicited phosphorylation activity in vitro and in vivo, in various cell lines (34). Several studies have suggested that PKC-elicited phosphorylation causes a synergistic increase in TCDD-induced, AhR-mediated CYP1A1 gene induction (29, 30, 31). Co-treatment of cells with TCDD and PMA induces AhR-mediated gene transduction above levels induced by saturating concentrations of TCDD (30, 31). This PMA effect was not caused by message/gene product stabilization or stimulation of basal transcription machinery. Also, this PKC stimulation did not alter the protein levels of AhR or ARNT or the TCDD-induced down-regulation of the AhR, nor did PKC stimulation result in changed nuclear accumulation levels of the AhR-ARNT heterodimer (30, 31). Furthermore, domain-swapping analyses revealed that neither the transactivation domain (TAD) of AhR (see Fig. 1) or ARNT was required for the PMA effect (31). These results suggest that the PKC activity is required either for activation of co-activators that interact with the N terminus of the AhR and/or ARNT and/or that PKC can directly phosphorylate the N terminus of the AhR and/or ARNT. Together, the results presented in this work demonstrate that PKC can directly phosphorylate the AhR (Fig. 8A). Furthermore, the AhR Y9F “DNA binding mutant” can be made to induce transcription when PKC is stimulated in cells while mutation of a residue that is thought to directly contact DNA, AhR S39A, cannot be made to induce gene transcription (Fig. 9). These results suggest that enhanced PKC activity may partially overcome the inhibitory effect of a mutation of tyrosine 9, and PKC may be directly phosphorylating the AhR to mediate this effect.

By what mechanism may PKC-elicited phosphorylation or similar serine/threonine kinase-elicited phosphorylation be responsible for AhR activity? It has been shown that a basic leucine zipper transcription factor, Nrf2, could be phosphorylated on serine 40 by PKC. Lack of phosphorylation on a Nrf2-S40A mutant resulted in enhanced interaction with a cytosolic dimerization partner, Keap1, resulting in decreased antioxidant response element-mediated transcription (36). It may be that altered phosphorylation status of the AhR results in changed affinity for one or multiple associating proteins, such as XAP2 and/or the importins, in the cytoplasm and/or the nucleus resulting in altered DNA binding and transcriptional activity. Also, although this distinct composition of the AhR DNA binding domain has been proposed to be essential for binding to a unique sequence (5′-GCNA-3′) of the DRE rather than a conventional E-box sequence, it is not known how two basic clusters separated by about 20 amino acids can make contact with a short DNA sequence of about four nucleotides. In order for these two separate domains to recognize the same four-nucleotide-long sequence (5′-GCNA-3′), there must be a mechanism that brings the two clusters into the small region of the DNA double helix. Current findings suggest that the tyrosine 9 in the vicinity of the first basic region may fulfill this role. It is conceivable that tyrosine 9 participates in a conformational change, possibly induced by phosphorylation at adjacent serine/threonine(s) that are necessary to pull the first basic cluster, containing arginine 14, into the proximity of the second basic region, containing arginine 39, perhaps requiring another associated protein, and thus maintains an optimal three-dimensional structure of the unique AhR DNA binding domain.

We conclude that AhR tyrosine 9, a conserved residue, is not a phosphorylated residue but that it is required for appropriate AhR phosphorylation by serine/threonine kinase(s). These data allow us to reexamine the hypothesis that tyrosine phosphorylation of the AhR is required for DNA binding, because the two most likely candidates, tyrosine 372 in the major region of phosphorylation (12) and now tyrosine 9, can be ruled out. The major evidence in support of this hypothesis was the data demonstrating that AhR not ARNT phosphorylation is required for DNA binding (26) and the ability of tyrosine- but not serine/threonine-specific phosphatase treatment of purified rat AhR-ARNT to disrupt DRE binding (12). A possible explanation for these results is that multiple tyrosines, not just a single tyrosine, were dephosphorylated leading to a significant conformational change and inability to bind DRE. The data presented here also focus on the potential functional role of AhR serine/threonine(s) phosphorylation sites. There are five sites of the AhR that correspond to a PKC motif: serine 11, threonine 21, serine 35, threonine 376, and threonine 429. Data already exist suggesting that DNA binding can be altered by mutation of serine 11, threonine 21, or serine 35 to Alanines (8–10). It is important to note that although these residues are predicted PKC target sites, each has a context sequence suitable for several serine/threonine kinases, including multiple PKC iso-types. For example, serine 11 also has a context sequence that may serve as a substrate for cyclin-dependent protein kinase 5 (Cdk5) and casein kinase 1 (CK1), while the sequence context of serine 35 is suitable for phosphorylation by Cdk1 and CK1.2 It is becoming clear that many transcription factors are phosphorylated and dephosphorylated within and/or surrounding their nuclear import signals and nuclear export signals to regulate protein-protein interactions and compartmentalization (37). While this manuscript was in preparation, for example, it was reported that phosphorylation of human serum 68 within the AhR nuclear export signals by p38 mitogen-activated protein kinase plays a role in subcellular localization of the AhR (38). Studies are under way to address the hypothesis that altered phosphorylation at possibly more than one residue by kinases in addition to PKC, because of a mutation of tyrosine 9 near the AhR nuclear import signals, can significantly impair the ability of the AhR to interact with certain proteins, bind to DNA, and induce transcription.

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The Aryl Hydrocarbon Receptor (AhR) Tyrosine 9, a Residue That Is Essential for AhR DNA Binding Activity, Is Not a Phosphoresidue but Augments AhR Phosphorylation

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