Differential Recruitment of Coactivator RIP140 by Ah and Estrogen Receptors

ABSENCE OF A ROLE FOR LXXLL MOTIFS*

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The Ah receptor (AhR), a soluble cytosolic protein, mediates most of the toxic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related environmental contaminants. The mechanism of ligand-mediated AhR activation has been, in part, elucidated. The sequence of events following the binding of the AhR/AhR nuclear translocator protein (ARNT) heterodimer to dioxin response elements has yet to be completely understood. The role of coactivator, RIP140, in the modulation of transcriptional activity of AhR/ARNT heterodimer was examined. RIP140 enhanced TCDD-mediated, dioxin response element-driven reporter gene activity in three cell lines. Co-immunoprecipitation and co-localization assays revealed that RIP140 interacted with AhR, but not with ARNT, both in vitro and in cells. Mapping of the interaction sites revealed that RIP140 was recruited by the AhR transactivation domain via the Q-rich subdomain. The RIP140 domain that interacts with the AhR was mapped to a location between amino acid residues 154 and 350, which is distinct from those involved in estrogen receptor binding. The signature motif, LXXLL, which is responsible for binding of several coactivators to nuclear receptors, is not required for RIP140 binding to AhR. These results demonstrate that the AhR recruits coactivators that are capable of enhancing transcription and, thus, the AhR may compete with steroid receptors for a common coactivator pool. In addition, the data suggest that there are distinct motifs for the recruitment of RIP140 to AhR and possibly other non-steroid receptors/transcription factors.

The Ah receptor (AhR)3 is a ligand-activated transcription factor that mediates most, if not all, of the biological effects of TCDD, an environmental contaminant (1). The AhR is also apparently involved in hepatic growth and development, based on the phenotype of AhR−/− mice (2), and may play a role in the Hepa 1c1c7 cell cycle (3). In addition, the AhR may also be involved in the development of the immune system, as indicated by decreased accumulation of lymphocytes in the spleen and lymph nodes in AhR−/− mice (4). The AhR exists in the cytosol of cells as a heterotetrameric 9 S complex (5–7) with two Hsp90 molecules and the X-associated protein 2 (8–10). Upon ligand binding, the AhR undergoes a conformational change and translocates to the nucleus as a 9 S complex. ARNT (11), which localizes in the nucleus, heterodimerizes with the AhR after Hsp90 dissociation to yield a 6 S complex (12, 13). This heterodimeric AhR/ARNT complex binds to DRE located upstream of the cyp1A1, cyp1A2, glutathione S-transferase Ya, and cyp1B1 genes, and modulates their transcription (14–16). TCDD induces a myriad of species-specific tissue damage, including hyperkeratosis and chloracne of the skin in rabbits and humans, liver damage in rodents, and lymphoid involution and embryotoxicity in many species (for a review, see Ref. 1). In most cases the affected tissues include the epithelium, where TCDD acts to alter proliferation and differentiation. The apparent specificity of these TCDD-induced toxicities suggests that there may be tissue-specific factors that alter the regulation of the AhR-mediated response (17).

Several lines of evidence point to the role of the AhR/ARNT complex in the disruption of chromatin (18, 19) and the direct recruitment of basal transcription factors (20, 21). Mapping of the transactivation domain of ARNT revealed a 34-amino acid transactivation domain consisting of mostly hydrophobic and acidic amino acid residues. In contrast, the AhR has a complex C-terminal activation domain that has been subdivided into acidic, Q-rich, and P/S/T-rich subdomains (17, 22–25). The subdomains are capable of activating transcription when expressed independently, but can act synergistically when expressed together as a chimeric protein (21). Sp1 binds to GC boxes in the promoter region of cyp1A1 and has been shown to interact with AhR and ARNT via the zinc finger domain of Sp1. Sp1, AhR, and ARNT were found to synergistically enhance both transcription in in vitro transcription assays and reporter gene activity in transient transfections (26).

Several proteins in the basal transcription machinery have been shown to interact with the ER, and may contribute to enhancement of transcriptional activity. However, these interactions are unaffected by ER agonists or antagonists (27), suggesting that other factors may be necessary for ligand-dependent transactivation as well as cell and tissue specificity. Several nuclear receptor accessory factors, also referred to as coactivators, may act as bridging factors between the enhancer-binding activator proteins and the basal transcription complex or chromatin remodeling factors. These include SRC-1 (28, 29), CBP

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‡ The abbreviations used are: AhR, aryl hydrocarbon receptor; AR470, AR-associated protein 70; ARNT, AhR nuclear translocator protein; CBP, CREB-binding protein; DRE, dioxin response elements; ER, estrogen receptor; GFP, green fluorescent protein; GRIP1, glucocorticoid receptor-interacting protein 1; GST, glutathione S-transferase; Hsp90, heat shock protein 90; RAR, retinoic acid receptor; RIP140, receptor interacting protein 140; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TAD, transactivation domain; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; TR, thyroid hormone receptor; ERE, estrogen response element; Tricine, N-tris(hydroxymethyl)methylglycine; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)l-propanesulfonic acid; LBD, ligand binding domain.
RIP140 is recruited by the ER and modulates the receptor's transcriptional activity in the presence of estrogen. This coactivator has also been shown to interact directly with the ER and, upon overexpression in cells, caused a 2-fold enhancement of transcriptional activity (27). In addition, RIP140 also enhanced transcription of ER and RAR in vitro in yeast (35). RIP140 was also found to interact with other nuclear receptors, including RAR, TR, and retinoid X receptor, albeit less stably than with ER. It has also been observed that, while the ER, TRβ, and RARβ interact with two distinct sites located in the N and C termini, the retinoid X receptor predominantly interacts through the N-terminal site of RIP140, suggesting a possible mechanism for differential regulation of transcription of cognate genes (36). A short motif (LXXL), also referred to as a NR box or LXD, has been identified in the nuclear receptor-interacting domains of several coactivators, nine copies of which are found in RIP140 (37, 38). Mutation of this motif completely abolished binding of SRC-1 to the ER-AF2 transcription domain, but not to CBP. Mutant SRC-1a also failed to enhance ER-dependent reporter gene activity in transiently transfected HELa cells. Subsequent studies have demonstrated that the NR boxes are necessary for several other coactivator-nuclear receptor interactions (37, 39–48). This has been further characterized by crystallographic analyses of co-crystals of TR ligand binding domain (LBD)/GRIP1 and peroxisome proliferator-activated receptor LBD/SRC-1 (39, 44), clearly demonstrating the role of this motif.

The AhR has been shown to interact with various factors in the basal transcription complex, including TATA-binding protein and transcription factor III (49), but no coactivators have been shown to interact with and enhance AhR-mediated transcriptional activity. Although CBP has been shown to interact with ARNT, there is no direct evidence indicating its role in AhR-mediated transcriptional activity in vivo (50). In this paper, the role of RIP140 in modulating transcriptional activity of the AhR/ARNT heterodimer in a minimal DRE-driven reporter context was examined in co-transfection experiments. RIP140 overexpression resulted in enhanced reporter gene activity in HELa, Hepa 1c1c7, and COS-1 cells. Interaction of RIP140 with the AhR was demonstrated in vitro and in cells using co-immunoprecipitation assays and GFP-tagged proteins. Functional domains necessary for RIP140/AhR interaction in both RIP140 and AhR were mapped and mutations in the LXXL motifs did not significantly destabilize the AhR-RIP140 interaction. Thus, AhR may employ differential mechanisms of coactivator recruitment in the process of transcriptional regulation relative to steroid receptors.

MATERIALS AND METHODS

Plasmids—For the construction of mAhR/DAT/Flag, mAhR sequences (from the T7-HindIII site to 1272 base pairs) from the pcdNA3/mAhR/FLAG (10) were amplified by standard PCR techniques and cloned into the HindIII-Xhol site of pcdNA3 (Invitrogen, San Diego, CA). The Nα315 mAhR/FLAG/pGEM-Zf1 construct was obtained from Christopher Bradfield (University of Wisconsin, Madison, WI) and was used as a template for PCR amplification. The PCR product was subcloned into pcdNA3, resulting in pcdNA3/Nα315/mAhR/FLAG/Flag (mAhREN3315). For the construction of pBOS-GST-AhR 1–418, the cDNA sequences of residues 1–418 of rip140 were amplified and cloned into the ECoRI/KpnI sites in pBK-CMV (Stratagene, La Jolla, CA). The cDNA sequences corresponding to residues 713–848 of hAhR were PCR-amplified and cloned into the BglII/BamHI sites of pGFP-C1 (CLONTECH) to generate pGFP-AhR/TAD. The cDNA sequences corresponding to residues 474–790 of hARNT were PCR-amplified and cloned into the BglII/BamHI sites of pGFP-C1 to generate pGFP-ARNT/TAD.

Encherichia coli strains DH5α (Life Technologies, Inc.) was used for all plasmid preparations, and E. coli BL21 (DE3) (Novagen, Madison, WI) was used in the expression of GST fusion proteins.

Mutagenesis—The two LXXL motifs in RIP140 354–350 were mutated to LXXLA using the Quick Change site-directed mutagenesis kit (Stratagene) following manufacturer's instructions. RIP145–350 lxxl mut was digested with BamHI to cut out the 3.5-kilobase pair fragment containing the transactivation domain. The cDNA sequences corresponding to residues 474–790 of hARNT were PCR-amplified and cloned into the BglII/BamHI sites of pGFP-C1 to generate pGFP-ARNT/TAD.

For in vitro binding assay for interaction of RIP140 with AhR/mAhR/Flag, mARNT/Flag (51) or other cDNA constructs were in vitro transcribed and translated separately using the TNT Coupled Reticulocyte Lysate system (Promega, Madison, WI) and mixed with in vitro translated 35S-methionine-labeled RIP140 or its deletion mutants and incubated at 4 °C for 1.5 h. The complexes were immunoprecipitated by incubation with anti-Flag M2 affinity gel in CSB buffer. The radioactivity was quantitated using a phosphorimager (Bio-Rad).

Ligand Dependence Assays—GST pull-down assays, GST, GST-ARNT/TAD or GST-AhR/TAD fusion proteins immobilized on 30 l of glutathione-agarose were expressed in E. coli (BL21) and purified using glutathione-agarose by standard techniques.

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For GST pull-down assays, GST, GST-ARNT/TAD or GST-AhR/TAD fusion proteins immobilized on 30 μl of glutathione-agarose (Sigma) were incubated with 35S-methionine-labeled in vitro translated RIP140 or its deletion derivatives in CSB buffer. Following a 1.5-h incubation at 4 °C, the agarose was washed four times with CSB buffer without bovine serum albumin. The immobilized proteins were eluted with 2× Tricine sample buffer, separated using SDS-PAGE, and analyzed by fluorography.

Ligand Dependence Assays—GST pull-down assays were performed on 30 μl of glutathione-agarose for 30 min at 4 °C. The agarose was washed three times in CSB buffer with 1 mM 20 μl of 50 μM of CSB buffer containing 150 μM NaCl plus 3 mg/ml soybean trypsin inhibitor. The elution was repeated, and eluted proteins were separated on an 8% polyacrylamide gel and visualized by fluorography.

Cell Culture and Transient Transfection Experiments—Cells were routinely cultured in α-modified Eagle's medium containing 10% fetal bovine serum (v/v). Cells were transfected with a total of 750 μg of DNA using LipofectAMINE reagent (Life Technologies, Inc.) in 24-well plates.
Enhanced AhR Transactivation by RIP140

Modulation of Transcription of AhR/ARNT Activated Reporter Genes by RIP140—In an effort to understand the mechanism of AhR/ARNT-mediated transcriptional regulation of target genes, we analyzed the potential effects of overexpression of a number of steroid receptor corepressors on the activity of a minimal DRE-driven luciferase reporter gene in transient co-transfection experiments. Preliminary data had suggested that RIP140 overexpression resulted in a marked enhancement of DRE-driven reporter gene activity (data not shown). Therefore, the role of RIP140 in this minimal promoter context was examined in three cell lines: HeLa 1c1c7, HeLa, and COS-1 (Fig. 1). COS-1 cells contain negligible amounts of endogenous AhR and were used to clearly delineate the role of RIP140 in AhR-mediated transcription. COS-1 cells, transfected with increasing amounts of RIP140, resulted in a dose-dependent biphasic response only in the presence of TCDD and co-transfected AhR (Fig. 1A). In the absence of co-transfected RIP140, there was a 4.1-fold increase in TCDD-mediated reporter gene activity compared with MeSO4 treatment. An additional 2-fold TCDD-dependent induction of DRE-driven reporter gene activity was seen in the presence of 10 ng of co-transfected RIP140 (Fig. 1A). When the amount of co-transfected RIP140 was increased to 50–100 ng, TCDD-stimulated reporter gene activity was reduced; when RIP140 was overexpressed in the absence of co-transfected AhR, no RIP140-mediated increase in reporter gene activity was seen (Fig. 1A), clearly suggesting that the transactivation process involved and required the AhR. In the absence of TCDD, no increase in reporter gene activity was seen, further supporting the role of AhR in the transactivation process.

In order to determine whether the modulation of DRE-driven reporter activity was cell line-dependent, HeLa and Hepa 1c1c7 cells were transfected with increasing amounts of RIP140. In the absence of exogenous expression of RIP140 in HeLa cells TCDD caused a nearly 6-fold activation of reporter gene activity when compared with controls (Fig. 1B). An additional TCDD-dependent 2.6-fold increase in reporter gene activity was observed when cells were transfected with 100 ng of RIP140 (Fig.
In Vitro Interaction of AhR and RIP140—The in vitro interaction between RIP140 and AhR or ARNT was examined in co-immunoprecipitation assays using in vitro transcribed/translated FLAG-tagged AhR and ARNT and [35S]methionine-labeled in vitro translated RIP140. While RIP140 was found to co-immunoprecipitate with AhR-FLAG (Fig. 2), no RIP140 was found to co-immunoprecipitate with ARNT-FLAG. This is consistent with earlier reports, which indicate that the AhR has a dominant transactivation domain, displayed greatly reduced binding to RIP140 (Fig. 2), suggesting that RIP140 interacts, directly or indirectly, with the transactivation domain.

Interaction of RIP140 with AhR in Cells—In order to test for an interaction between AhR and RIP140 in cells, COS-1 cells were transiently transfected with pBOS-GST/AhR or pBOS-GST/ARNT, and transfected with only GST-AhR were used. In this case, no detectable RIP140 was found to interact with AhR (lane 6). Protein blot analysis revealed that equal amounts of RIP140 were expressed and loaded in each of these lanes.

To further show interaction of RIP140 in cells, GFP-tagged AhR/TAD (AhR/TAD-GFP), GFP-tagged ARNT/TAD (ARNT/TAD-GFP) fusions, or GFP were expressed in COS-1 cells along with full-length RIP140. Since neither AhR/TAD or ARNT/TAD are known to possess nuclear localization signals, they would be expected to be localized mostly in the cytoplasm. Interaction with RIP140 would lead to the translocation of GFP-AhR/TAD.

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440 hours after transfection, 440–1–418 in the presence or absence of co-transfected RIP140. Thirty-six COS-1 cells were transfected with pBOS/GST-AhR or pBOS/GST-AhR

dentification of AhR/RIP140 interaction in cells using GST pull-down assays. RIP140

eluted and subjected to SDS-PAGE and Western blotting. After extensive washing, the proteins were

with glutathione-agarose. Western blot were detected by anti-RIP140 antibodies.

B

Interaction of RIP140 with AhR in cells. A and B, evidence of AhR/RIP140 interaction in cells using GST pull-down assays. COS-1 cells were transfected with pBOS/GST-AhR or pBOS/GST-AhR 1–418 in the presence or absence of co-transfected RIP140. Thirty-six hours after transfection, 440 μg of whole cell extracts were incubated with glutathione-agarose. After extensive washing, the proteins were eluted and subjected to SDS-PAGE and Western blotting. A, proteins on Western blot were detected by anti-RIP140 antibodies. B, one-tenth input of proteins were used in GST pull-down assays followed by elu-

tion, SDS-PAGE, and Western blotting. Proteins were detected by anti-GST antibodies. C–I, interaction in cells using GFP-tagged proteins. COS-1 cells were transfected with GFP or GFP fusion constructs and RIP140 or control expression vectors and visualized by microscopy (magnification, ×100) 24 h after transfection. C, GFP; D, GFP plus RIP140; E, GFP-AhR; F, GFP-AhR plus RIP140; G, single cell; H, GFP-ARNT; I, GFP-ARNT plus RIP140.

D). These results provide additional evidence for specific interaction of RIP140 with AhR and not with ARNT. Taken together, these results indicate that AhR interacts with RIP140 via the transactivation domain in cells.

Effect of Ligand on AhR-RIP140 Interaction—Preliminary results indicated that the binding of AhR to RIP140, in vitro, occurred even in the absence of apparent ligand. The full-length AhR requires Hsp90 for proper folding, and hence a full-length AhR-GST fusion protein cannot be generated in bacteria. Instead, a GST fusion protein consisting 1–350 amino acid residues of RIP140 was generated and mixed with in vitro translated 35S-labeled AhR and assayed for interaction in the presence or absence of TCDD. Significantly, considerable binding of AhR to RIP140 was observed even in the absence of an apparent ligand (Fig. 4A). GST-RIP 1–350 was able to bind AhR both in the presence and absence of TCDD, although an increase was observed in the presence of TCDD (Fig. 4A). However, some variation in the level of enhanced binding was observed from experiment to experiment. Overall, there appeared to be a rather marginal increase in ligand-dependent binding. Our results are consistent with the fact that in vitro reticulocyte lysate translated AhR and ARNT can successfully form a functional heterodimer capable of binding to DRE in the absence of an exogenous ligand in gel shift assays (26). 2 This is also consistent with the fact that RIP140 can bind efficiently to AhR without its LBD (Fig. 2). This is in contrast to the absolute ligand dependence of ER binding to RIP140 and that of several other nuclear/steroid receptors to other coactivators presenting possible differences in the mechanism of coactivator recruitment.

The relative binding affinities of AhR and ER to RIP140 were compared under increasing salt concentrations. The interaction of RIP140 and ER (in the presence of 1 μM E2) was found to be stronger, with considerable binding at salt concentrations as high as 1.0 M NaCl (Fig. 4B). In contrast, the interaction of RIP140 with AhR was found to be less stable at concentrations higher than 0.5 M NaCl, which appears to be comparable to results observed with the retinoid acid receptor (Fig. 4B (36). RIP140 bound AhR 30% and 35% more weakly compared with the ER, even at concentrations of 250 and 500 mM NaCl, respectively (Fig. 4B). These combined results are consistent with the differential RIP140 recruitment mechanisms between the Ah and estrogen receptors.

Mapping of AhR Interacting Sites on RIP140—We used a series of N- and C-terminal deletion derivatives of RIP140 to determine the AhR-interacting site(s) using co-immunoprecipitations with AhR/FLAG. Analysis of N-terminal deletion derivatives of RIP140 clearly indicated that AhR, unlike ER, interacted exclusively with the N-terminal region (Fig. 5A). While RIP 393–1158 clearly showed binding, RIP 301–1158 displayed weak binding to AhR. Analysis of the C-terminal deletion derivatives indicated that residues residues 1–439 were necessary and sufficient for AhR binding (Fig. 5A). This is in contrast to results from ER binding analysis, which indicated that ER bound to regions both in the N and C termini, suggesting a different mechanism of recruitment of RIP140 to AhR. Interestingly, these amino acid sequences include one of the acidic regions that were found in RIP140 (28, 37). Further N- and C-terminal deletion constructs were generated and tested for binding to AhR using GST-mAhR/TAD in GST pull-down assays. Among the N-terminal deletion derivatives RIP 53–350, RIP 103–350 and RIP 154–350 were able to bind GST-mAhR/TAD (Fig. 5B), clearly indicating that the N-terminal 154 residues are not required for AhR binding. However, RIP 201–350 failed to bind to AhR (Fig. 5B). Binding of C-terminal derivative, RIP 154–325 was reduced when compared with RIP 154–350 (Fig. 5C), suggesting that, while amino acids 154–325 may contain some of the residues involved in binding function, optimal binding requires amino acid residues 154–350. As ex-

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expected, RIP 154–300 failed to bind to AhR (Fig. 5C). Control assays with GST alone showed no or minimal binding to any of the RIP140 derivatives. These results suggest that amino acid residues 154–350 are involved in binding of RIP140 to AhR. These sequences are clearly distinct from those involved in the binding of ER to RIP 140. However, this region contains two LXXLL motifs, which may or may not play a role in binding of AhR to RIP140 (Fig. 5D). Analysis of this AhR-binding RIP140 sequence indicated that the secondary structure is predicted to be mostly composed of α-helices.

Role of LXXLL Motifs in AhR-RIP140 Interaction—RIP140 and some known coactivators possess several copies of the
short sequence motif, LXXLL This motif has been found to be necessary and sufficient for binding of SRC-1a (37, 44, 48), RIP140 (37, 55), GRIP1 (40, 46), and CBP (37), among others, to the liganded nuclear receptors. The RIP1–439 fragment contains three LXXLL motifs and is capable of binding to the ER transactivation domain (36, 37). Two peptides, P-1, with amino acid sequence, PQAQQKSLLQQLLT, corresponding to the motif at the C terminus of SRC-1a, and P-2 with amino acid sequence, PQAQQKSLQQLAA, containing mutations in the LXXLL motif, were tested for their ability to block the binding of RIP1–439 to the AhR and ER in GST pull-down assays. Increasing concentrations of wild type (wt) peptide, P-1, were able to block the ability of liganded GST-ER-HBD to bind to RIP1–439 (Fig. 6A, upper right panel). In contrast, mutant peptide, P-2, was unable to compete with RIP1–439 for binding to GST-ER-HBD (lower right panel). However, P-1 failed to compete with RIP1–439 for binding to GST-mAhR/TAD (upper left panel), suggesting that the interaction of RIP140 and AhR may be mediated by residues distinct from those involved in the ER-RIP140 interaction. This also suggests the absence of a role for the signature motif, LXXLL in AhR-RIP140 interaction and the presence of other interaction motif(s). However, the possible involvement of the LXXLL motif in other interactions within a transcriptionally active AhR complex cannot be ruled out. The mutant peptide P-2, as expected, was unable to compete with the binding of RIP1–439 to GST-mAhR/TAD (lower right panel).

In order to further determine the lack of requirement for LXXLL motifs, the two LXXLL motifs in the RIP 154–350 truncated protein were mutated at residues 184/186 to LXXAA, translated in vitro in the presence of [35S]methionine, and analyzed for interaction with GST-mAhR/TAD or GST immobilized on glutathione-agarose. C, GST fusions of RIP154–350 wt or RIP154–350 mut 184/186 were generated and assayed for interaction with full-length, [35S]methionine-labeled, in vitro translated AhR using GST pull-down experiments.
RIP154–350mut 184/266 were generated and tested for interaction with full-length in vitro translated AhR in the presence or absence of TCDD. AhR, as noted earlier, bound strongly to GST-RIP 154–350 in both the presence and absence of TCDD, demonstrating again the lack of a direct role of TCDD in the binding of RIP140 to AhR (Fig. 6C). AhR also bound to a mutant derivative of GST-RIP 154–350mut in which the two LXXLL motifs were replaced by LXXAA (GST-RIP 154–350mut 184/266), although to a lesser extent. A marginal decrease in binding in the absence of TCDD was observed. However, clearly the AhR was capable of interacting with the mutant RIP 154–350. In addition, when the interaction was assayed in the presence of TCDD, the binding of mutant RIP 154–350 to AhR was slightly greater with only an 18% decrease compared with that of RIP 154–350mut. The marginal decrease in binding of the mutant proteins suggests that the replacement of the four leucine residues may not be completely innocuous. The mutations may induce changes in tertiary structure, which may affect binding even though they may not be directly involved in binding. Nevertheless, the analysis of the mutants and the peptide competition assays together indicate a lack of a required role of the LXXLL motifs for AhR-RIP140 interaction.

Interaction of RIP140 with Subdomains of AhR Transactivation Domain—The AhR has an unusually complex transactivation domain comprising acidic, Q-rich, and P/S/T-rich subdomains. In order to determine the subdomain(s) of the human AhR transactivation domain required for RIP140 interaction, several AhR/TAD deletions were made and fused to glutathione S-transferase. The human and murine AhR sequences are 70% homologous and, thus, GST fusion proteins of the transactivation domains of the two proteins were assayed for comparison of their ability to bind full-length RIP140 (Fig. 7). In addition, GST-Sp1, which has a Q-rich transactivation domain, and GST-VP16, which has an acidic transactivation domain, were assayed for interaction with RIP140. The binding of RIP140 to full-length murine and human transactivation domains were comparable, clearly demonstrating the conservancy of specific functions (lanes 2 and 3). The acidic subdomain comprising residues 500–600 was unable to bind RIP140 (lane 7). However, the Q-rich subdomain alone was sufficient for RIP140 binding (lane 6). The Q-rich and the P/S/T-rich subdomains together showed slightly increased binding to RIP140 (lane 4). When the P/S/T-region was deleted the fusion protein showed decreased binding to RIP140 (lane 5). However, P/S/T-rich subdomain alone failed to recruit any RIP140 (lane 8). The deletion of the acidic subdomain made no substantial difference to RIP140 binding function (lane 4), which is consistent with the fact that the acidic subdomain alone was not able to bind RIP140 (lane 7). Interestingly, the Sp1 transactivation domain, which is predominantly Q-rich, failed to bind any RIP140 (lane 9), clearly suggesting specificity in interaction among different transactivating proteins with apparently similar transactivation domains (lane 10). GST-VP16 also failed to interact with RIP140 (lane 10).

An alignment of the transactivation domains of human AhR and Sp1 showed conservation of several glutamine residues and several flanking hydrophobic residues, which have been shown to be important for binding downstream factors. However, these residues do not seem to be highly conserved in AhR from other species, e.g., mice and rat. Interestingly, the human and murine AhRs share extensive homology in other regions of the Q-rich subdomains in certain hydrophobic and glutamine residues, which may be involved in binding coactivators like RIP140.

**DISCUSSION**

In order to determine whether there are tissue-specific coactivator recruitment events that are key determinants in the overall level of gene transcription mediated by the AhR, two important aspects of the assembly of a functional AhR transcriptional complex need to be addressed. The first is the examination of the type of coactivators that are recruited to the complex AhR transactivation domain. The second aspect of this process is a detailed delineation of the subdomain(s) of the AhR TAD, as well as coactivator domains, that are key to formation of the AhR/coactivator complex. This information can then serve as a basis for further studies examining the role of each TAD subdomain in mediating AhR activity through recruitment of specific classes of coactivators in a cell type-specific manner. An additional aspect of coactivator recruitment is the emergence of motifs required for coactivator binding to steroid receptors and whether these motifs also play a role in coactivator binding to other transcription factors, such as the AhR.

In this report, the effect of RIP140 co-expression on DRE-driven reporter gene activity was examined in transient transfections. We tested the effect of RIP140 overexpression in a variety of cell lines: COS-1, which have negligible amounts of endogenous AhR (Fig. 1A), HeLa (Fig. 1B), and Hepa 1c1c7 (Fig. 1C) cells, which have significant endogenous levels of AhR and ARNT. Upon RIP140 ectopic expression, a TCDD-dependent 2-fold increase in DRE-driven reporter gene activity was seen in COS-1 cells. When AhR was not co-transfected, RIP140 did not enhance reporter gene activity, clearly suggesting that the effects of RIP140 are mediated through the AhR and that RIP140 cannot act alone in transactivating the reporter gene. A 4.4- and 2.6-fold increase in DRE-driven reporter gene activity were observed in Hepa 1c1c7 and HeLa cells, respectively. Although the co-transfection experiments indicate that RIP140 can increase minimal DRE-driven reporter activity to different levels in a cell line-dependent manner, the level of enhanced coactivation could potentially depend on the existing pool of RIP140 and other coactivators in each cell line. In addition, other factors such as cell-specific repressors and promoter contexts may have a significant influence on the level of induction observed. Nevertheless, determining the influence of RIP140 in different cell lines further strengthens the hypothesis that RIP140 is a potential coactivator of AhR-mediated transcrip-
enhanced reporter activity in a RIP140 dose-dependent manner in the presence of estrogen. Additionally, a similar biphasic response to increasing RIP140 doses in reporter co-transfection assays was observed. This is consistent with other reports of coactivators, including SRC-1 (56) and CBP (57). When an increased amount of RIP140 is co-transfected, it could potentially sequence key transcription factors, leading to a decrease in transcription (53). RIP140 itself does not bind to TATA-binding protein or TFIIIB (52), but it could potentially interact with any of the other factors involved in assembling a transcription initiation complex. RIP140 may behave like a repressor when overexpressed by sequestering SRC-1 from transcription complexes (58), as suggested in the case of peroxisome proliferator-activated receptor. RIP140 has also been suggested to have an auto-repressor function (55).

RIP140 and other coactivators have been shown to interact with the transactivation domain of several steroid/nuclear receptors (36). Our results suggest that the AhR also recruits RIP140 via its transactivation domain (Fig. 2). It has been seen in the case of several steroid receptors that the binding of ligand to the LBD induces a conformational change such that the AF-2 amphipathic α-helix (helix 12) is aligned over the LBD, unlike the case of an unliganded receptor, where helix 12 protrudes away from the LBD. This realignment may lead to the formation of a new interaction surface for coactivators and this sequence of events may explain the need for ligands for effective binding of coactivators (59). Unlike the ER and other steroid receptors, where the LBD is in close proximity to the transactivation domain, these two domains are well separated in the AhR, which may explain the lack of enhancement of RIP140 binding in the presence of ligand in vivo. In contrast to several steroid receptors, which require the presence of a ligand to bind RIP140 (36), the AhR ligand, TCDD, had marginal effect on RIP140 binding in vitro. However, in cells, enhanced interaction with RIP140 was observed in the presence of TCDD. In the case of AhR, which possesses a separate and potentially unmasked transactivation domain that can readily interact with coactivators, the need for a ligand for physical interaction with coactivators, at least in part, may be unnecessary. Although the possible role of endogenous ligands or factors in the reticulocyte lysate cannot be completely ruled out, pull-down experiments with GST fusion proteins, containing only the transactivation domain of the AhR without its LBD, clearly indicate that RIP140 can bind AhR in the absence of ligand. It should be noted that, although RIP140 seems to interact with the AhR in the absence of TCDD, in vivo transcriptional enhancement by RIP140 requires TCDD, as seen with the lack of enhanced reporter gene activity in the absence of TCDD (Fig. 1, A–C). There is considerable documented evidence for the role of ligands triggering the nuclear translocation of cytosolic AhR (5, 10). AhR in a tetrameric complex (5, 10) is thought to bind ligand, translocate to the nucleus, and subsequently transform into a heterodimeric complex (5). The modular, well-separated nature of AhR LBD and its transactivation domain suggest that, although TCDD may be required for translocation of AhR to the nucleus, TCDD may not be required for physical interaction between the AhR and certain coactivators. This may preclude the necessity of a ligand to induce a conformational change for protein-protein interaction to occur with the transactivation domain. In contrast, estradiol is required for interaction of RIP140 with the ER, even in the context of the GST fusion protein with AF2 alone, since the AF2 domain includes both the transactivation domain and the ligand binding domain of ER (36). The in vitro interaction of RIP140 and AhR/ARNT heterodimer was also examined using co-immunoprecipitation experiments. There was no observed increase in the co-immunoprecipitation of RIP140 with AhR/ARNT heterodimer as compared with AhR alone (data not shown), further pointing to the dominant nature of the AhR transactivation domain. This points to the existence of a discrete class of receptors, which can apparently physically interact with specific coactivators or other transcription factors in the absence of an exogenous ligand, although a ligand is clearly required for other functions, like transactivation.

Mapping of the AhR-interacting sites on RIP140 revealed significant differences between the binding of AhR and ER to RIP140 (Fig. 5, A–C). While ER interacts with RIP140 via two sites on the N and C termini of RIP140 (36), it was found that AhR interacts predominantly with the N terminus, involving residues 154–350 in RIP140. No interaction was seen with any of the C-terminal residues of RIP140, which indicates a significant difference in binding mechanism of RIP140 to the ER versus the AhR. The AhR interacting RIP140 fragment, RIP 154–350, contains two LXXL motifs, which are necessary for binding of several coactivators to ER and other nuclear receptors (36). However, peptide competition assays showed that a peptide, containing a wild type LXXL motif, competed with RIP140 for binding to the transactivation domain of the ER, but failed to compete with RIP140 for binding to the AhR (Fig. 6A). The lack of a necessary role for LXXL motifs in RIP140/ AhR interaction was further established when LXXL mutants of RIP 154–350, the minimal AhR interaction domain, were found to bind AhR similar to unt RIP 154–350 (Fig. 6B). This suggests that a different motif(s) may be involved in binding of RIP140 to non-steroid receptors like the AhR, which suggests the existence of diverse mechanisms for recruiting limited coactivator pools to different receptors, or classes of receptors, depending on the signal. There are numerous reports of the requirement of the LXXL motifs for interaction with nuclear receptors (37, 39–48) and of differential requirements for these motifs in cases where more than one motif exists in response to different ligands and to different receptors (39). The AhR presents a unique case where the LXXL motif is not required for interaction with RIP140. However, this may depend on the actual biological situation existing in the cell, in terms of signals, promoter contexts, and cell cycle status. It is possible that other receptors which harbor similar transactivation domains may display differential mechanisms of recruiting common coactivators also. The flanking sequences clearly play a vital role in differential recruitment to nuclear receptors and the affinities of their interaction. The requirement of a relatively long RIP140 sequence for stable AhR binding also is a distinct feature that must be further investigated.

Analysis of AhR transactivation domain deletions indicated that the Q-rich subdomain was necessary and sufficient for binding of RIP140, while the acidic and P/S/T-rich subdomains clearly appeared to be dispensable for RIP140 interaction (Fig. 7). The lack of a clear role for the P/S/T-subdomain in cyp1A1 activation has also been underscored in AhR transactivation in vivo studies in other reports (24, 60). However, reporter gene assays have suggested that the acidic subdomain (which also has some conserved glutamine residues) is required for optimal activation (24). However, the fact that the P/S/T and acidic subdomains do not interact with RIP140 does not rule out the
requirement of these subdomains for interaction with other transcription factors, including basal transcription factors, repressors, and other coactivators. Indeed, while VP16 fused to the N terminus of AhR has been previously shown to induce cyp1A1 expression (60), suggesting that the VP16 activation domain could substitute for the AhR activation domain, we failed to detect any RIP140 binding to VP16. Sp1 fused to the N terminus of AhR was shown to be unable to induce cyp1A1 expression (60). Sp1 in the present study showed no binding to RIP140. It has been observed in reporter gene assays with a fusion protein of truncated AhR transactivation domains that, while individually the subdomains are incapable of substantial induction, a combination of two or more subdomains clearly leads to synergy in induction (21). It is possible that the different subdomains may be involved in recruiting different coactivators and/or basal transcription factors. This differential recruitment may also be dependent on cell, species, and tissue types along with promoter contexts.

Several coactivators have been isolated that modulate the transcriptional activity of steroid hormone receptors (28, 32, 33, 52), but there is as yet no evidence for AhR-specific coactivators. Coactivators like ARA70 have been shown to be involved in regulating androgen receptor-mediated transcription of certain genes in specific tissues and thus, at least in part, help explain tissue- and cell line-specific phenotype(s) (34). AhR may differentially recruit coactivators relative to the ER and other steroid hormone receptors, depending on the signal impinging on the cell. There may also be other classes of coactivators, which may be uniquely recruited by transcription factors with transactivation domains similar to that of the AhR. Nevertheless, this report demonstrates that the AhR and steroid receptors share at least one common coactivator. However, whether these receptor systems compete for the existing coactivator pool will require additional investigation. Finally, determination of the type(s) of common or distinct motifs involved in the recruitment of various coactivators to AhR or AhR-like transcriptional enhancer proteins will aid in understanding possible differential coactivator recruitment mechanism(s) employed by various transcription factors and how these mechanisms may lead to cell-, species-, and tissue-specific activation of certain subsets of genes.

Acknowledgments—We thank Malcolm Parker for RIP140 constructs, P1 and P2 peptides, and helpful advice. We also thank Michael requirement of these subdomains for interaction with other transcription factors, including basal transcription factors, repressors, and other coactivators. Indeed, while VP16 fused to the N terminus of AhR has been previously shown to induce cyp1A1 expression (60), suggesting that the VP16 activation domain could substitute for the AhR activation domain, we failed to detect any RIP140 binding to VP16. Sp1 fused to the N terminus of AhR was shown to be unable to induce cyp1A1 expression (60). Sp1 in the present study showed no binding to RIP140. It has been observed in reporter gene assays with a fusion protein of truncated AhR transactivation domains that, while individually the subdomains are incapable of substantial induction, a combination of two or more subdomains clearly leads to synergy in induction (21). It is possible that the different subdomains may be involved in recruiting different coactivators and/or basal transcription factors. This differential recruitment may also be dependent on cell, species, and tissue types along with promoter contexts.

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