The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor belonging to the basic helix-loop-helix/Per-Arnt-Sim (bHLH-PAS) family of proteins, which are critical regulators of gene expression networks underlying many essential physiological and developmental processes (1–5). AHR binds a variety of endogenous and xenobiotic compounds, including polycyclic and halogenated aromatic hydrocarbons, and mediates their toxic effects, such as teratogenesis, immunosuppression, and tumor promotion (1, 2, 6). The AHR/ARNT dimer recognizes and binds to xenobiotic-responsive elements of the mouse CYP1A1 gene, an AHR-responsive gene. Upon ligand binding, endogenous GAC63 was recruited to the xenobiotic response element by means of an ATPase activity (12). The TRAP-DRIP-mediator complex also plays a physiological role in AHR-mediated gene transcription by recruiting and activating RNA polymerase II (13).

Other transcription coactivators, such as p160 coactivators, p300/CBP, RIP140, CoCoA, and TRIP230, have also been shown to be involved in transcriptional activation by AHR (14–18). GAC63 (also known as human embryonic lung protein or HUEL) interacts with the bHLH-PAS domain of p160 coactivators as well as the ligand binding domain of some NRs, such as estrogen receptor and androgen receptor. Overexpression of GAC63 enhanced transcriptional activation by NRs in a hormone-dependent manner. Although GAC63 can interact with NR directly, its coactivator function depends on the presence of a p160 coactivator. GAC63 has also been shown to act as a coactivator in NR-mediated gene transcription. Because p160 coactivators and AHR share bHLH-PAS domains, we investigated the possibility that GAC63 is also a coactivator in AHR-mediated transcription. We report here that GAC63 interacts with AHR and functions as a primary coactivator in AHR-mediated gene transcription, i.e. its coactivator function is independent of the presence of p160 coactivators or any other coactivators. Endogenous GAC63 is recruited to the XRE region of an AHR-responsive gene and is important for optimal transcriptional activation by AHR.

EXPERIMENTAL PROCEDURES

Plasmids—The hemagglutinin (HA)-tagged mouse AHR expression plasmid (pACTAG-2.mAHR) was the kind gift of Dr. Oliver Hankinson (University of California, Los Angeles, CA). pGudluc 6.1, encoding a CYP1A1 promoter-driven luciferase reporter gene, was obtained from Dr. Michael Denison (University of California, Los Angeles, CA). A cDNA fragment encoding full-length mouse AHR was inserted into pGEX-5X1 vector (Amersham Biosciences) to express a fusion protein with N-terminal glutathione S-transferase (GST) in Escherichia coli. The following plasmids were described previously: pGEX-5X1-GAC63, pSG5.HA-GAC63, pSG5.HA-GAC63(1–200), pSG5.HA-GAC63(200–370), pSG5.HA-GAC63(370–567) (19), pCMX-GRIP1 (14), pSG5.HA-ARH(1–374), pSG5.HA-ARH(375–805) (17).

GST Pull-down Assay—[35S]Methionine-labeled full-length AHR, GAC63, and their fragments were synthesized in vitro by using TNT-Quick-coupled transcription/translation system (Promega) according to the manufacturer’s protocol. GST pull-down assays were performed as described previously (17, 19).
Endogenous Coimmunoprecipitation and Immunoblotting—Hepa1c1c7 cells, hereafter referred to as Hepa-1 cells, were lysed in radioimmune precipitation assay buffer. Cell lysates were cleared with protein A/G beads (Santa Cruz Biotechnology) for 1 h at 4 °C. 2 μg of rabbit anti-GAC63 antibody (19) or normal rabbit IgG (Santa Cruz Biotechnology) was added to the cell lysates and incubated overnight at 4 °C on a rotator. 30 μl of protein A/G beads were added and incubated for another 3 h. Beads were washed three times with radioimmune precipitation assay buffer and subjected to SDS-PAGE. Blots were probed with anti-AHR antibody (Affinity BioReagents).

Transient Transfection Assay—Hepa-1 cells were maintained in α-minimal essential medium supplemented with 10% fetal bovine serum and 100 units of streptomycin and penicillin/ml. Typically, 500 ng of the CYP1A1 promoter-driven luciferase vector, pGudulc 6.1, was transfected alone or in combination with increasing amounts of pSG5.HA-GAC63 (0.1, 0.5, 1.0, or 2.0 μg) into 6-well plates using 15 μl of Superfect reagent (Qiagen) as per manufacturer’s instructions. For synergy studies with GRIP1, cells were cotransfected with reporter and either 0.5 or 1.0 μg of pCMX-GRIP1 alone, or 0.5 or 1.0 μg each of pCMXGRIP1 and pSG5.HA-GAC63. Final DNA concentration was equalized by the addition of empty plasmid vector. Three hours after transfection, cells were washed once in phosphate-buffered saline, and the medium was replaced. Cells were treated with either 5 nM TCDD or 0.1% dimethyl sulfoxide (Me2SO) and incubated for an additional 20 h at 37 °C. Cells were washed once with phosphate-buffered saline, and the medium was replaced. Cells were treated with either 5 nM TCDD or 0.1% dimethyl sulfoxide (Me2SO) and incubated for an additional 20 h at 37 °C. Cells were washed once with phosphate-buffered saline and harvested in 300 μl of 1× cell lysis buffer (25 mM Tris-phosphate (pH 7.8), 2 mM dithiothreitol, 2 mM 1,2-diamino cyclohexane-N,N,N′,N′-tetraacetic acid, 10% (v/v) glycerol, 1% (v/v) Triton X-100). The lysates were vortexed, and cellular debris was pelleted by high speed centrifugation in a table-top microcentrifuge. Twenty μl of supernatant was added to 100 μl of luciferase assay substrate (Promega, Madison, WI), and luciferase activity was determined using a TD-20e Luminometer (Turner Systems, Sunnyvale, CA). Each transfection was performed in duplicate, and the experiments were repeated two additional times.

Chromatin Immunoprecipitation Assay—Chromatin immunoprecipitation assays were performed with Hepa-1 cells from one 15-cm dish treated with or without 10 nM TCDD for 60 min as described previously (17, 19), using 5 μl of anti-AHR antibody MA1-513 (Affinity Bioreagents), 2 μg of anti-GAC63 antibody 1bg, or 2 μg of normal rabbit IgG. Immunoprecipitated, purified, chromosomal DNA was used for PCR amplification, using the following primers: CYP1A1 (1141/-784), 5′-CTATCTCCTAAACCCCCACCCCA-3′ (forward) and 5′-CTAACTGATGGTGGAAGAAAGGTG-3′ (reverse); β-actin (−522 to −205), 5′-ATGCTAGCAATTTGCTAGCAAGGGGAGT-3′ (forward) and 5′-GAGAAAAGCGAGAGATTTGCGGAGGAGTA-3′ (reverse). Quantitative real time PCRs (QPCR) were performed with 2 μl (from a total of 50 μl) of immunoprecipitated chromosomal DNA with a Stratagene Mx3000P Instrument, using the same primers as for standard PCR.

RNA Interference—RNA interference experiments were performed as described previously (17, 19) using Lipofectamine 2000 (Invitrogen). Small interfering RNA (siRNA) oligonucleotides for GAC63 and mismatch siRNA were synthesized by the USC Norris Comprehensive Cancer Center Microchemical Core Laboratory and annealed to form duplexes. The following siRNA sequences were used: siGAC63, 5′-GCUUCUGGCAGAGAGAAATdTdT-3′ (sense) and 5′-UUUUCUCUCUCGGAAGAG-3′ (antisense). Mismatch siRNA, 5′-GCUUCUGGCAGAGAGAAATdTdT-3′ (sense) and 5′-UUUUCUCUCUCGGAAGAG-3′ (antisense) (mismatched bases are underlined). Two days after siRNA transfection, Hepa-1 cells were treated with or without TCDD. Twenty-four hours after TCDD treatment, total Hepa-1 cell RNA was extracted with TRizol reagent (Invitrogen), and subjected to reverse transcription by using iScript cDNA Synthesis Kit (Bio-Rad). 2 μl of reverse transcription product was subjected to quantitative real-time PCR (QPCR) analysis. The primers used were as follows: mouse GAC63, 5′-TGTTATCATGAACTATGCG-3′ (forward), 5′-TGCTCCTTCAGACACGAAA-3′ (reverse); mouse CYP1A1, 5′-GGCCAGACTCTTACAGCCTTC-3′ (forward), 5′-CTACAGCTGTCCTCCTCC-3′ (reverse); mouse β-actin, 5′-AGTGTGACGGTGAATGAG-3′ (forward), 5′-GTGCTGATCCACACTCTG-3′ (reverse). Cell extracts were also tested by immunoblotting using anti-GAC63 and anti-actin antibodies.

RESULTS

Interaction of GAC63 with AHR—Because GAC63 interacts with the bHLH-PAS motif of GRIP1, and AHR also has the bHLH-PAS motif, GAC63 might also interact with AHR. To test this hypothesis, we performed GST pull-down assays. GST-GAC63 fusion protein was bound to beads and incubated with in vitro synthesized and 35S-labeled AHR, with or without TCDD treatment. Bound proteins were eluted and analyzed by SDS-PAGE and autoradiography. B, full-length GAC63 was synthesized in vitro and tested for binding to bead-bound GST-AHR, with or without TCDD treatment. C, Hepa-1 cells were lysed in radioimmune precipitation assay buffer. The cell extracts were immunoprecipitated with anti-GAC63 antibody or normal rabbit IgG. The immunoprecipitated proteins were detected by immunoblot (IB) with anti-AHR antibody.

Extracted from the text:

FIGURE 1. GAC63 interacts with AHR in vitro and in intact cells in culture. A, in GST pull-down assays, GST-GAC63 fusion protein was bound to beads and incubated with in vitro synthesized and 35S-labeled AHR, with or without TCDD treatment. Bound proteins were eluted and analyzed by SDS-PAGE and autoradiography. B, full-length GAC63 was synthesized in vitro and tested for binding to bead-bound GST-AHR, with or without TCDD treatment. C, Hepa-1 cells were lysed in radioimmune precipitation assay buffer. The cell extracts were immunoprecipitated with anti-GAC63 antibody or normal rabbit IgG. The immunoprecipitated proteins were detected by immunoblot (IB) with anti-AHR antibody.
GAC63 Is a Coactivator for Aryl Hydrocarbon Receptor

A

Zinc finger

GAC63

LXXL

LEU

Acidic region

GAC63

LXXL

LEU

Acidic region

FIGURE 2. Determination of interaction domains of GAC63 and AHR. A, functional domains of GAC63 and AHR. Numbers indicate amino acids. LXXL, leucine-rich motif; LZ, leucine zipper-like motif. B, the indicated GAC63 fragments were synthesized in vitro and tested in GST pull-down assays for binding to GST or GST-AHR. C, the in vitro synthesized AHR fragments were tested in GST pull-down assays for binding to GST or GST-GAC63.

B

GST

1-200

200-370

370-567

GST-AHR

GAC63 fragments

1-200

200-370

370-567

AHR

Determination of Interaction Domains of GAC63 and AHR—To determine the AHR interaction domain within GAC63, we performed GST pull-down assays. Among several GAC63 fragments synthesized in vitro, a fragment of the N-terminal region (amino acids 1–200) efficiently and specifically bound to GST-AHR, but fragments of the central region (amino acids 200–370) and the C-terminal region (amino acids 370–567) only had weak or no binding to GST-AHR (Fig. 2B). Thus, the N-terminal region of GAC63 is the major AHR interaction domain.

C

GST

1-374

375-805

GST-GAC63

GAC63 fragments

1-200

200-370

370-567

AHR

We also tested the GAC63 interaction domain within AHR using GST pull-down assays. The N-terminal fragment of AHR (amino acids 1–374) specifically bound to GST-GAC63, whereas the C-terminal fragment (amino acids 375–805) only showed weak binding (Fig. 2C). These data suggest that the N-terminal bHLH-PAS domain of AHR is the major GAC63 interaction domain.

GAC63 Functions as a Coactivator in AHR-mediated Transcription—GAC63 is a secondary coactivator in NR-mediated gene transcription. Although it can bind NR directly, its coactivator function depends on the presence of GRIP1. Moreover, GRIP1 has been reported to be a coactivator in AHR-dependent gene activation. To test whether GAC63 also functions as a coactivator in this pathway, we performed transient transfection assays in AHR/ARNT-positive Hepa-1 cells to study the effect of overexpression of GAC63 on AHR-mediated gene transcription. Hepa-1 cells were transfected with a CYP1A1 promoter-driven luciferase construct (pGudluc 6.1) and empty vector or increasing amounts of GAC63 expression vector, GRIP1 expression vector, or both. Cells were treated with 5 nM TCDD or vehicle for 20 h, and lysates were assayed for luciferase activity.

GAC63 Is Recruited to the XRE Region of the CYP1A1 Gene—GAC63 functions as a transcriptional coactivator of AHR-dependent gene expression when GAC63 is overexpressed in transient transfection assays (Fig. 3). To test whether endogenous GAC63 is recruited to the XREs of known genes that are regulated by AHR, we performed chromatin immunoprecipitation assays to examine the XREs of CYP1A1 gene in mouse Hepa-1 cells. Hepa-1 cells were treated either with 10 nM TCDD in Me2SO or with Me2SO vehicle for 60 min. The recruitment of AHR and GAC63 was specific for the XRE region after TCDD treatment. In addition, anti-GAC63 antibody efficiently immunoprecipitated this region in a ligand-dependent manner (Fig. 4A). Normal IgG served as a negative control, and the input chromatin levels from TCDD-treated or untreated cells were equivalent. The recruitment of AHR and GAC63 was specific for the XRE region of CYP1A1 gene, because PCRs with primers for the mouse β-actin promoter region failed to produce a signal from the same immunoprecipitated chromatin fractions. QPCR analysis confirmed the TCDD-dependent recruitment of AHR and GAC63 to the native, chromosomally integrated XRE region of CYP1A1 gene (Fig. 4B).

GAC63 Is Important for Efficient Transcriptional Activation by AHR—Overexpression of GAC63 enhances AHR-regulated reporter gene expression (Fig. 3). To test for a physiological role of endogenous GAC63 in the
process of transcriptional activation by AHR, we performed siRNA experiments to decrease the expression of endogenous GAC63. The GAC63 siRNA specifically reduced the level of endogenous GAC63 mRNA (Fig. 5A, upper panel) and protein (Fig. 5B), whereas the mismatch siRNA did not. The expression of AHR-regulated CYP1A1 gene was inhibited by 40% in the presence of GAC63-directed siRNA but not affected by an equivalent amount of mismatch siRNA (Fig. 5A, lower panel). A similar result was obtained with another pair of siRNAs, one targeting a different region of GAC63 and the other a scrambled-sequence control (data not shown). The effect on CYP1A1 gene expression was specific, because the results shown are normalized to the level of \(	ext{\beta}-\text{actin}\) transcripts. Thus, although GAC63 is only one of several known coactivators for AHR, endogenous GAC63 makes an important contribution to the efficient transcriptional activation of endogenous genes by AHR.

**DISCUSSION**

Ligand-activated AHR/ARNT dimer binds to the XRE of AHR-responsive genes and transmits the activating signal through a variety of coactivator complexes (1). Each coactivator complex fulfills a specific task to help the transcriptional activation by AHR. For example, the SWI/SNF complex contributes to chromatin remodeling (12), and the TRAP-DRIP-mediator complex helps to recruit and activate RNA polymerase II (13).

In the present study, we showed that GAC63, a newly identified nuclear receptor coactivator, also functions as a coactivator in transcriptional activation by AHR. In our study, GAC63 interacts with AHR in a ligand-independent manner (Fig. 1). Several other transcription coactivators, such as p160 coactivators and CoCoA, have been shown to interact with AHR in a similar manner (14, 17). The human orthologue of GAC63, HUEL, has several potential nuclear localization signals and is primarily cytoplasmic in interphase cells, but it undergoes nuclear translocation during the S-phase of the cell cycle (20). Because AHR undergoes nuclear translocation upon ligand binding, it is possible that GAC63 exists in a complex with unliganded AHR and travels with liganded AHR into nucleus. We also tested the interaction between GAC63 and ARNT in GST pull-down assays, and found that GAC63 also interacts with ARNT directly in vitro (data not shown). However, we failed to detect their interaction in intact cells in culture using coimmunoprecipitation assays. Whether GAC63 binds to ARNT within the context of the AHR/ARNT heterodimer bound to DNA will need to be determined.

A previous study (19) has shown that although GAC63 can interact with NR directly, its coactivator function in NR-mediated transcription depends on the presence of p160 coactivators. Interestingly, overexpression of GAC63 alone enhanced AHR activity in a ligand-dependent manner in the absence of GRIP1. When GAC63 was cotransfected with GRIP1, no synergy was observed (Fig. 3). However, we cannot rule out the possibility that GAC63 and GRIP1 may function synergistically as coactivators for AHR/ARNT under different conditions. Thus, GAC63 functions as a primary coactivator in AHR-mediated transcription, whereas it serves as a secondary coactivator in transactivation by NR.

Endogenous GAC63 was recruited to the XRE region of the CYP1A1 gene in a ligand-dependent manner (Fig. 4), and reduction of endogenous GAC63 by siRNA inhibited the expression of CYP1A1 gene by 40%
This partial inhibition could be because of residual GAC63, or the compensatory effects by other transcription coactivators, such as p300/CBP, BRG-1, p160 coactivators, Med220, and CoCoA, which are also known to mediate AHR function. Thus, we conclude that GAC63 is important for optimal transcriptional activation by AHR and is a physiological part of AHR-mediated gene transcription.

We also identified the domains within GAC63 and AHR required for their mutual interactions. The N-terminal and central regions of GAC63 have been reported to be responsible for interaction with GRIP1 (19). Similarly, the N-terminal region of GAC63 is the major AHR interaction domain, and the central region also showed weak binding to AHR (Fig. 2B). The N-terminal region has a zinc finger-like motif, and the central region has two leucine zipper-like motifs (21). It remains to be tested whether these motifs might contribute to GAC63 interaction with AHR. We also found that the N-terminal bHLH-PAS domain of AHR is the major GAC63 interaction domain. Similarly, the N-terminal bHLH-PAS domain of GRIP1 has been shown to be the GAC63 interaction domain (19). Thus, the N-terminal and central regions of GAC63, especially the N-terminal region, may generally recognize and bind bHLH-PAS domains.

AHR, ARNT, and p160 coactivators all belong to bHLH-PAS gene family (4, 5). Our findings suggest that GAC63 might also interact with other members of bHLH-PAS gene family, and serve as a general coactivator for all the bHLH-PAS transcription factors. Future studies of the physical and functional interaction between GAC63 and other bHLH-PAS proteins will help us to understand the transactivation mechanisms of the bHLH-PAS gene family and their functions in regulating target gene expression. Furthermore, GAC63 might also function as a coactivator for transcription factors other than the bHLH-PAS transcription factors. The nature of the downstream targets and the specific components of the transcription machinery that are regulated by GAC63 are currently under investigation in our laboratory.

AHR-mediated gene transcription proceeds in a manner similar to NR-mediated transcription (6). Although AHR and NR have different ligands and target genes, they share a variety of transcriptional coactivators. For example, acetyltransferase p300/CBP, SWI/SNF ATPase...
subunit BRG-1, p160 coactivators, mediator subunit Med220, CoCoA, and TRIP230 are all involved in both AHR- and NR-mediated gene transcription (1, 6, 12–18). GAC63 provides another example of a transcriptional coactivator that is involved in both signaling pathways. Furthermore, TCDD has been shown to antagonize functions of several nuclear receptors, including estrogen receptor (22), androgen receptor (23), progesterone receptor (24), and peroxisome proliferator-activated receptor-γ (25). The antagonistic effect by TCDD could be due to competition for coregulators, in addition to other proposed mechanisms, such as enhanced ligand metabolism, down-regulation of nuclear receptor levels, transrepression, and altered hormone synthesis (6, 26–28).

Acknowledgments—We thank Dr. Oliver Hankinson (University of California, Los Angeles, CA) for AHR expression plasmid and Dr. Michael Denison (University of California, Davis) for pGudLUC 6.1 reporter plasmid. We thank Daniel Gerke and Kelly Chang (University of Southern California) for expert technical assistance.

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