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Role of GAC63 in transcriptional activation mediated by the aryl hydrocarbon receptor*

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Abstract

The aryl hydrocarbon receptor (AHR), a member of the basic helix-loop-helix/Per-Arnt-Sim (bHLH-PAS) gene family, binds a variety of polycyclic aromatic hydrocarbons and mediates their toxic effects. GAC63 has been shown to act as a coactivator in nuclear receptor (NR) mediated gene transcription. In this report, we demonstrate that GAC63 interacts with AHR through its bHLH-PAS domain. Overexpression of GAC63 greatly enhanced AHR-regulated reporter gene activity in a ligand-dependent manner in transient transfection assays. Upon ligand treatment, endogenous GAC63 was recruited to the xenobiotic response element (XRE) of the mouse CYP1A1 gene, an AHR-responsive gene. Reduction of the endogenous GAC63 level by small interfering RNA (siRNA) inhibited transcriptional activation by AHR. These findings reveal a new function of GAC63 in AHR-mediated gene transcription.

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 wide 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 best studied AHR ligand is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Unliganded AHR is found in the cytoplasm as part of a multimeric complex containing two molecules of HSP90, the HSP90 cochaperone p23, and one hepatitis B virus X-associated protein 2 (XAP2) (6–8). Upon ligand binding, AHR translocates into the nucleus, and associates with the aryl hydrocarbon receptor nuclear translocator (ARNT) to form a heterodimer (1–3,6). The AHR/ARNT dimer then recognizes and binds to xenobiotic responsive elements (XREs) located in the regulatory domains of AHR-responsive genes, many of which are involved in xenobiotic metabolism, such as CYP1A1, CYP1A2, CYP1B1, CYP2S1 and aldehyde oxydase 1 (AOX1) (1,9–11).

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Upon DNA binding, AHR/ARNT dimer recruits multiple coactivator complexes to the promoter of AHR-responsive genes (1,6). Each coactivator complex constitutes a signal transduction pathway which transmits the activating signal from the AHR to specific downstream targets in the transcription machinery. For example, a member of the Swi/Snf complex, the Brahma/SWI2-related gene 1 protein (Brg-1), has been reported to be involved in transcriptional activation by AHR, and participates in the remodeling of chromatin conformation around the promoter 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, GRIP1 associated coactivator 63, is a newly identified nuclear receptor (NR) coactivator (19). 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 (ER) and androgen receptor (AR). 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 with an intact bHLH-PAS domain. Thus, it functions as a secondary coactivator in NR-mediated gene transcription. Since 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) is 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, Davis). A cDNA fragment encoding full length mouse AHR was inserted into pGEX-5X1 vector (Amersham Pharmacia) to express a fusion protein with N-terminal glutathione S-transferase (GST) in E. 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-AHR(1–374), pSG5.HA-AHR(375–805) (17).

GST Pull-down Assay

[^{35}S]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 (RIPA) buffer. Cell lysates were cleared with protein A/G beads (Santa Cruz Biotechnology) for 1 hour at 4 °C. 2 μg 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 protein A/G beads were added and incubated for another 3 hours. Beads were

washed three times with RIPA buffer and subjected to SDS-PAGE. Blots were probed with anti-AHR antibody (Affinity BioReagents).

Transient Transfection Assay

Hepa-1 cells were maintained in alpha minimal essential medium supplemented with 10% fetal bovine serum and 100 U of streptomycin and penicillin per ml. Typically, 500 ng of the CYP1A1 promoter-driven luciferase vector, pGudluc 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 co-transfected 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 PBS and media was replaced. Cells were treated with either 5 nM TCDD or 0.1% dimethyl sulfoxide (DMSO) and incubated for an additional 20 hours at 37°C. Cells were washed once with PBS, and harvested in 300 μl of 1x Cell Lysis Buffer [25 mM Tris-phosphate (pH 7.8), 2 mM DTT, 2 mM 1, 2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid, 10% (v/v) glycerol, 1% (v/v) Triton X-100]. Lysates were vortexed and cellular debris was pelleted by high-speed centrifugation in a table-top micro-centrifuge. 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 experiments were repeated 2 additional times.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed with Hepa-1 cells from one 15 cm dish treated with or without 10 nM TCDD for 60 minutes as described previously (17,19), using 5 μl 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′-CTATCTCTTAAACCCCACCCCAA-3′ (forward) and 5′-CTAAGTATGGTGGAGGAAAGGGTG-3′ (reverse); β-actin (−527 to −205), 5′-ATTGCTAGCAATTGCTAGCAAGGGGGAGT-3′ (forward) and 5′-GAGAGAAAGCGAGATTGAGGAAGAGG ATGA-3′ (reverse). Quantitative real-time PCR (QPCR) reactions 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′-GCUCUUGCCAGAGAGAAAAdTdT-3′ (sense) and 5′-UUUUCUCUCUGGCAAGAGCdTdT-3′ (antisense). Mismatch siRNA, 5′-GGUCUUGUCAGACAGAAAAdTdT-3′ (sense) and 5′-UUUUCUGUCUGACAAGACCdTdT-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 (RT) by using iScript cDNA Synthesis Kit (Bio-Rad). 2 μl of RT product was subjected to quantitative real-time PCR (QPCR) analysis. The primers used were as follows: mouse GAC63, 5′-TGGTATCATGGAATCATGGG-3′ (forward), 5′-TGCTCCTTCAGACACCAAAG-3′ (reverse); mouse CYP1A1, 5′-

GGCCAGACCTCTACAGCTTC-3′ (forward), 5′-CTCACGTGCTCCTCCAAGTA-3′ (reverse); mouse β actin, 5′-AGTGTGACGTTGACATCCGT-3′ (forward), 5′-CTTGCTGATCCACATCTGCT-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 attached to glutathione-Sepharose-4B beads efficiently bound full-length AHR in the absence or presence of TCDD treatment, while GST attached to beads did not (Fig. 1A). Conversely, GST-AHR also specifically bound in vitro synthesized GAC63 with or without TCDD treatment (Fig. 1B). The interaction of AHR with GST-GAC63 was enhanced modestly by TCDD treatment (Fig. 1A), while the binding of GAC63 and GST-AHR was not affected (Fig. 1B). These results suggest that GAC63 binds directly to AHR in vitro, in the absence or presence of TCDD treatment.

To test for binding in intact cells in culture, we performed endogenous coimmunoprecipitation assays using the mouse liver cell line Hepa1. Specific binding of GAC63 to AHR was detected without TCDD treatment when anti-GAC63 antibody was used for immunoprecipitation (Fig. 1C). No enhancement of binding was detected in the presence of TCDD (data not shown). These results suggest that GAC63 interacts with AHR in intact cells in culture.

Determination of interaction domains of GAC63 and AHR

In order 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.

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, while 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. Hepa1 cells were transfected with a CYP1A1 promoter-driven luciferase reporter plasmid (pGudluc 6.1) and either empty vector or increasing amounts of GAC63 expression vector alone or in combination with GRIP1 expression vector, with or without TCDD treatment. TCDD treatment alone enhanced CYP1A1 promoter-driven luciferase activity about 8-fold (Fig. 3, assays 1). As expected, overexpression of GRIP1 enhanced the ligand-dependent activity of AHR (Fig. 3, assays 6 &7). Interestingly, unlike in NR-mediated transcription, overexpression of GAC63 enhanced AHR activity up to 7-fold in a ligand-dependent and dose-dependent manner, in the absence of GRIP1 (Fig. 3, assays 2–5).

The combination of GAC63 and GRIP1 did not further enhance AHR activity (Fig. 3, assays 8 & 9). These data suggest that although GAC63 is a secondary coactivator in NR-mediated transcription, it functions as a primary coactivator in AHR-dependent gene activation.

GAC63 is recruited to the XRE region of the CYP1A1 gen

GAC63 interacts with AHR (Fig. 1), and functions as a coactivator in AHR-mediated 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 which are regulated by AHR, we performed ChIP assays to examine the XRE of CYP1A1 gene in mouse Hepa-1 cells. Hepa-1 cells were treated either with 10 nM TCDD in DMSO or with DMSO vehicle for 60 min. The cross-linked, sheared chromatin fractions were immunoprecipitated with various antibodies, and the precipitated DNA was analyzed by PCR amplification of the XRE region of the CYP1A1 gene. As expected, AHR was recruited to 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, since PCR reactions with primers for the mouse β-actin promoter region failed to produce a signal from the same immunoprecipitated chromatin fractions. Real-time 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), while 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, since the results shown are normalized to the level of β-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). Since 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.

Previous study has shown that although GAC63 can interact with NR directly, its coactivator function in NR-mediated transcription depends on the presence of p160 coactivators (19). Interestingly, overexpression of GAC63 alone enhanced AHR activity in a ligand-dependent and dose-dependent manner, in the absence of GRIP1. When GAC63 was co-transfected 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, while 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% (Fig. 5). This partial inhibition could be due to 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 which are regulated by GAC63 are currently under investigation in our lab.

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).

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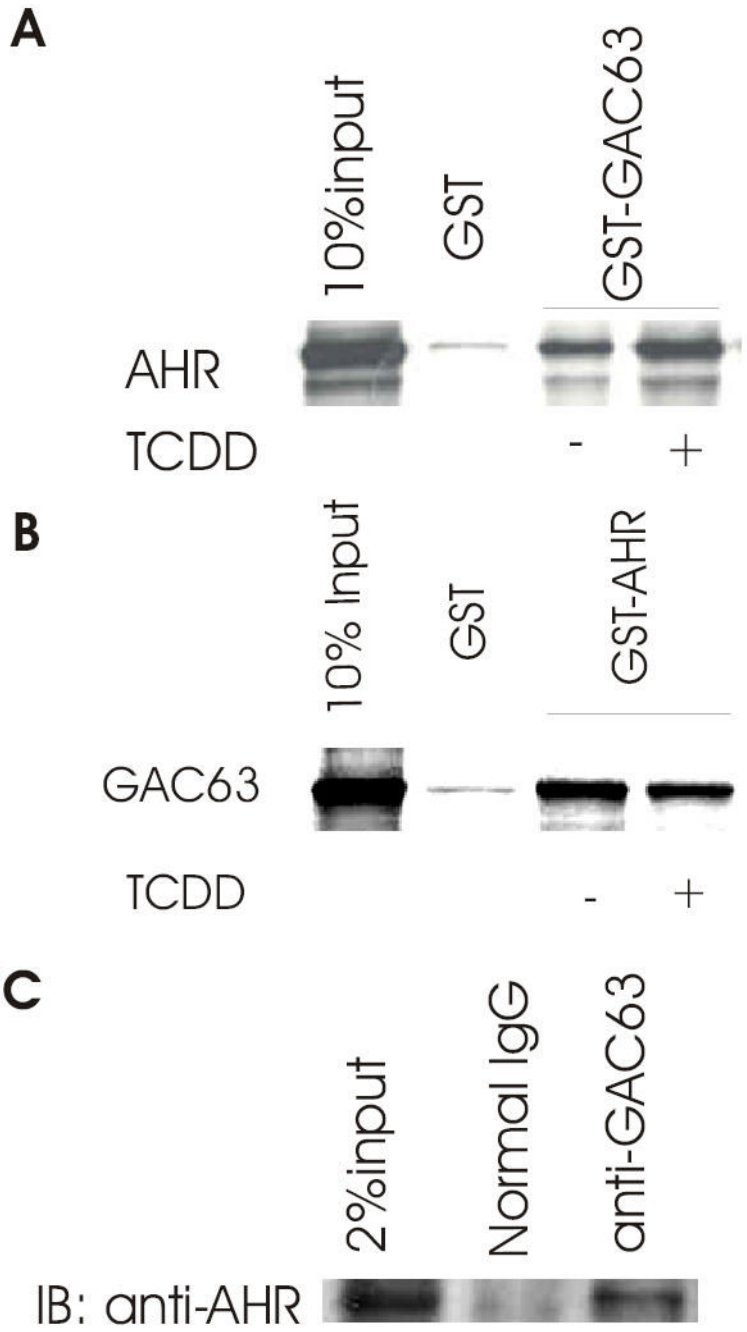

FIG. 1.

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 ^{35}S-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 RIPA buffer. The cell extracts were immunoprecipitated with anti-GAC63 antibody or normal rabbit IgG. The immunoprecipitated proteins were detected by immunoblot with anti-AHR antibody.

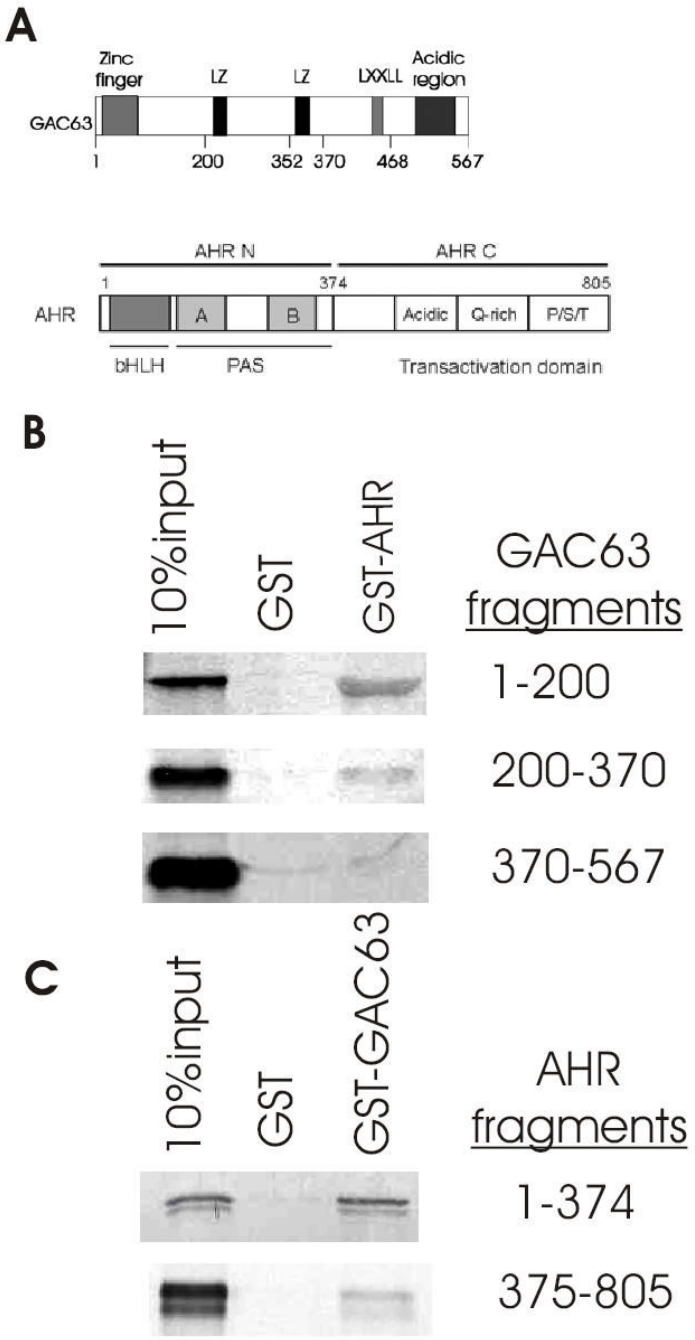

FIG. 2.

Determination of interaction domains of GAC63 and AHR. (A) Functional domains of GAC63 and AHR. Numbers indicate amino acids. LXXLL, 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.

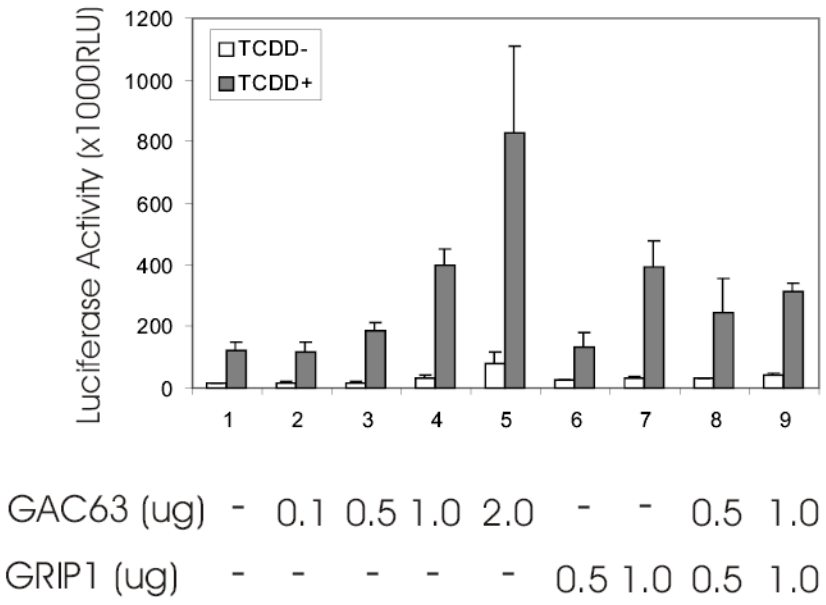

FIG. 3.

GAC63 functions as a transcriptional coactivator of AHR-dependent gene activation. Hepa-1 cells were transfected with a CYP1A1 promoter-driven luciferase construct (pGudluc 6.1) and either 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 hours and lysates were assayed for luciferase activity.

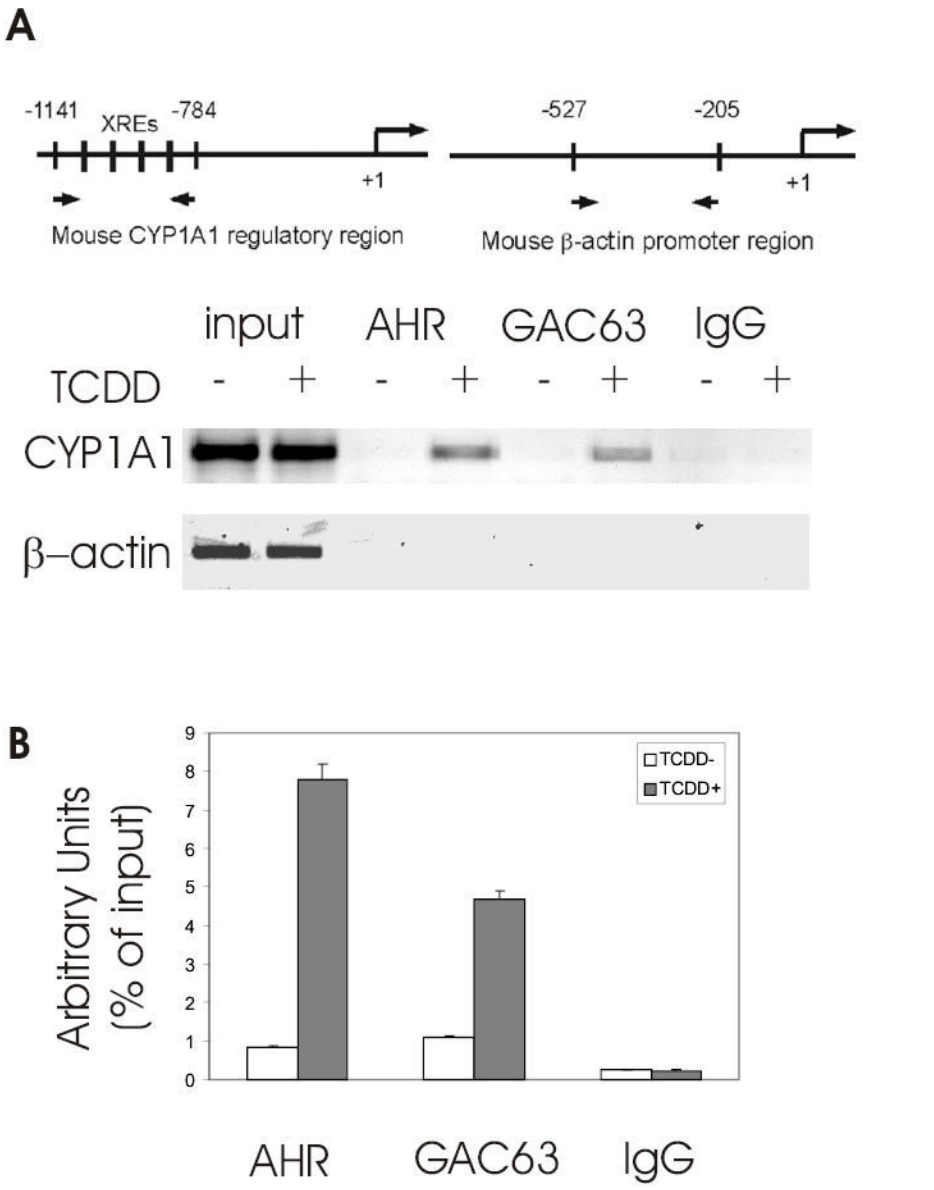

FIG. 4.

GAC63 is recruited to the XRE region of CYP1A1 gene. (A) Hepa-1 cells were grown with or without TCDD for 60 minutes and analyzed by ChIP assays, using the indicated antibodies for immunoprecipitation. The immunoprecipitated DNA was amplified by PCR using primers to amplify the XRE region of CYP1A1 gene or the β-actin promoter. The results shown are representative of three independent experiments. (B) Real-time QPCR analysis of immunoprecipitated Hepa-1 chromosomal DNA using primers for the XRE region of CYP1A1 gene was performed with 1ul samples. The results are shown as percentage of input, are the mean and standard deviation from triplicate reactions, and are representative of two independent experiments.

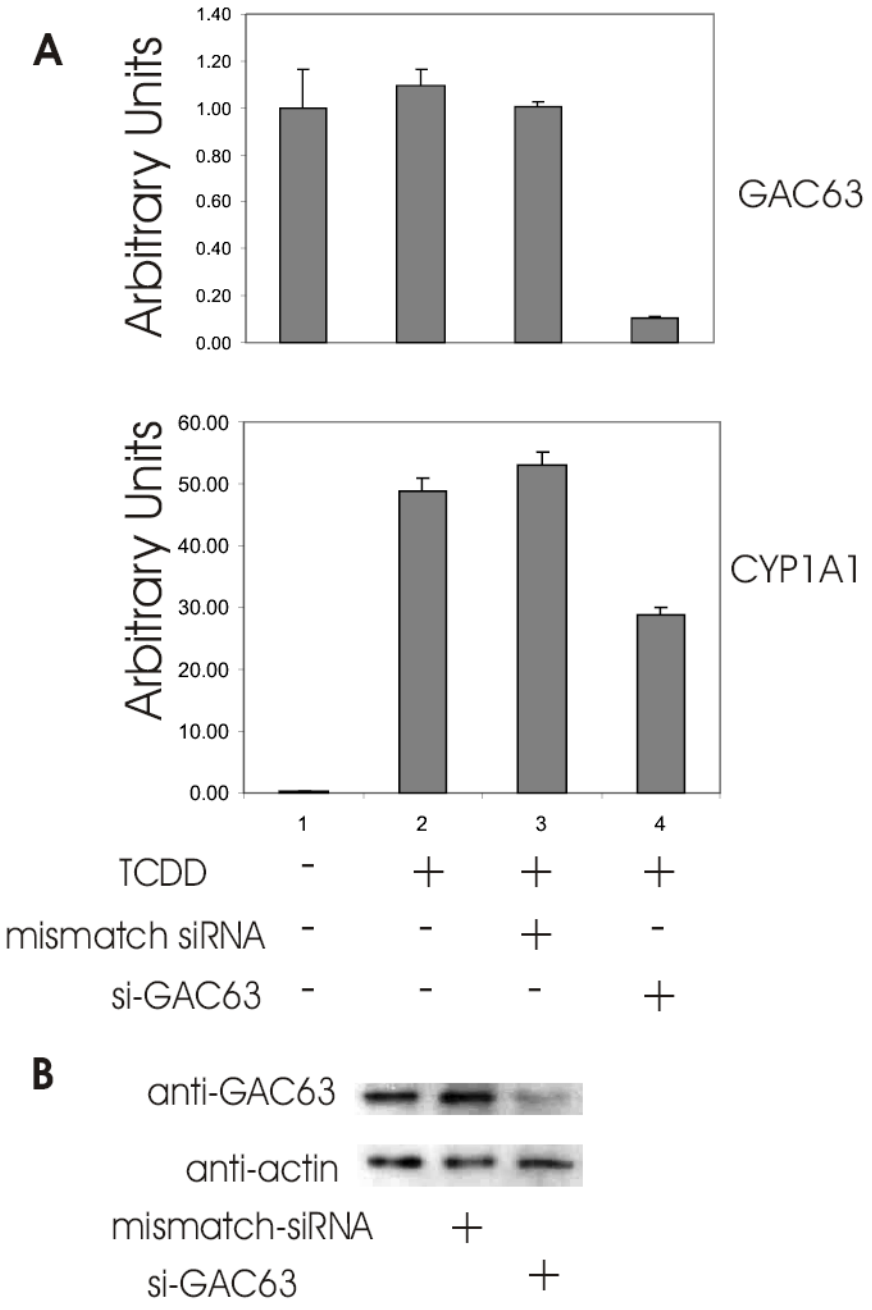

FIG. 5.

Endogenous GAC63 is important for efficient transcriptional activation by AHR. (A) Hepa-1 cells were transfected with 100 pmol of siRNA duplex against GAC63 or mismatch siRNA duplex. After 48 hours, cells were treated with 10 nM TCDD or left untreated for an additional 24 hours before harvest. mRNA was analyzed by RT-QPCR to measure the levels of GAC63, CYP1A1 and β-actin transcripts. Results shown for GAC63 and CYP1A1 transcripts were normalized by the levels of β-actin transcripts and are representative of three independent experiments. A paired, two-tailed t-test performed on the values from the three experiments indicated that the siRNA against GAC63 caused significant decreases in the TCDD-induced levels of CYP1A1 mRNA (p=0.003). For the three experiments, the mean decrease and 95%

confidence interval was 94±2% for GAC63 mRNA, and 42±3% for CYP1A1 mRNA. (B) Cell extracts were also tested by immunoblotting using anti-GAC63 and anti-actin antibodies.