Myb-binding Protein 1a Augments AhR-dependent Gene Expression*

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The aromatic hydrocarbon receptor (AhR) is an intracellular protein that mediates transcriptional responses to certain hydrophobic ligands, the most notorious of which is the widespread environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) (1–4). The liganded AhR heterodimerizes with a second protein, known as the AhR nuclear translocator (Arnt), to form a complex that activates transcription by binding to an enhancer in the vicinity of the TCDD-responsive target gene (5, 6). Both AhR and Arnt are prototypical members of the basic helix-loop-helix/Per-Arnt-Sim class of transcription factors, which regulate gene expression in response to a variety of environmental and developmental signals (7–11).

Much of our understanding of AhR/Arnt-dependent transcription stems from analyses of the TCDD-inducible CYP1A1 gene in mouse hepatoma cells; this experimental system benefits from the availability of AhR-defective and Arnt-defective cells. Efficient reconstitution of such cells by retroviral infection permits analyses of AhR and Arnt mutants in a relatively physiological setting (12–14). Studies of CYP1A1 gene regulation in mouse hepatoma cells reveal that exposure to TCDD leads to the binding of AhR/Arnt to an enhancer upstream of the CYP1A1 gene. The C-terminal portion of AhR (which contains several transactivation domains) communicates the induction signal to the neighboring promoter, which then assumes a more accessible chromatin structure and binds general transcription factors (12, 13). Such observations reveal that the transactivation domains of AhR facilitate enhancer-promoter communication by a process that involves changes in the structure of promoter chromatin and occupancy of promoter binding sites by the transcriptional machinery.

Here, we have studied AhR-defective transcription in more detail; we have focused on a relatively small (69 amino acid) domain of AhR that is rich in acidic residues and can transactivate the native chromosomal CYP1A1 gene (13). Our observations indicate that this acidic activation domain (AAD) of AhR associates with a factor previously identified as Myb-binding protein 1a (Mybbp1a) and that Mybbp1a substantially augments the ability of AhR/Arnt to activate transcription. These findings reveal new aspects of AhR and Mybbp1a function.

EXPERIMENTAL PROCEDURES

Materials—The pGudLuc6.1 vector was provided by Dr. Michael S. Denison (University of California, Davis, CA); it contains an AhR/Arnt-defective enhancer and the mouse mammary tumor virus promoter upstream of a firefly luciferase reporter gene (15). The QuikChange Site-Directed Mutagenesis Kit and Pfu DNA polymerase were purchased from Stratagene (La Jolla, CA). The pRL-CMV vector and Dual-Luciferase Report Assay System were purchased from Promega (Madison, WI). The retroviral vector pMFG was derived from the Moloney murine leukemia virus (16). The Phoenix-eco retroviral producer cell line (17) was provided by Dr. Garry Nolan (Stanford University). The pGEX-2T vector and glutathione-Sepharose were purchased from Amersham Biosciences. [α-32P]dCTP (3,000 Ci/mmol) and Renaissance Chemiluminescence Kit were purchased from PerkinElmer Life Sciences. The RNase K kit was from Qiagen (Valencia, CA). Reagents for SDS-PAGE and silver staining were from Bio-Rad. Hyperfilm MP was purchased from Amersham Biosciences. Tissue culture reagents were from Life In Vitrogen.

Cell Culture—Wild-type (Hepa1c1c7) and AhR-defective (Taoc1BPrcl) mouse hepatoma cells were cultured as described previously (18). Phenoxin culture media were used from Amersham Biosciences. Tissue culture reagents were from Life In Vitrogen.

Plasmid Construction—Mutations to alanine were made in AhR's acidic segment at Phe48 and Leu49 using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. Plasmid pGAhR515–583 (13) was used as a template for the sense and antisense mutation primers. Mutations were confirmed by nucleotide sequencing.

Wild-type and mutant pGAhR515–583 plasmids were used as tem-
Expression of GST Fusion Proteins and GST Pull-down Assays—GST fusion proteins were expressed in Escherichia coli by induction with 0.5 mM isopropyl-thiogalactoside. Cells were lysed 3 h after induction by five successive freeze-thaw cycles. After centrifugation, the lysates were incubated with GST-Sepharose beads (500 μl per 500 ml culture) for 30 min at room temperature. The beads were gently pelleted and then washed extensively with phosphate-buffered saline.

Whole-cell extracts were prepared from mouse hepatoma cells as described previously (20) and were incubated with GST fusion proteins (undiluted and then washed extensively with NETN buffer (20 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40) for 1 h at 4 °C with shaking. After the binding reaction, the beads were washed five times with binding buffer and then boiled in SDS sample buffer. The solubilized proteins were fractionated on SDS gels and visualized by silver staining. For protein sequencing, binding reactions were scaled up 10-fold, fractionated by SDS-PAGE, and transferred to Immobilon-P for protein microsequencing. Protein sequence determination was performed by the Protein/DNA Technology Center of the Rockefeller University (21, 22).

Transient Transfections—Wild-type or reconstituted AhR-defective mouse hepatoma cells were plated in 35-mm six-well tissue culture dishes and incubated overnight. Cells were co-transfected using a polybrene (1 μg) in DMEM containing 5% nonfat milk) overnight at 55 °C using ExpressHyb hybridization solution (Promega) according to the manufacturer’s instructions. Light production was measured using a Lumat LB 9507 luminometer. All experiments were performed at least three times, and the data are expressed as mean ± S.E.

Retroviral Expression of AhR—Five micrograms of pMFGAhR, pMFGAhR 494, pMFGAhR 494/mutAAD and the pMFGAhR 494/mutAAD mutants were transfected into the ectotropic packaging cell line, Phoenix, as described previously (17). Recovery of retroviruses and infection of AhR-defective mouse hepatoma cells was carried out as described previously (12).

Analysis of CYP1A1 Gene Expression—Wild-type, AhR-defective, and reconstituted mouse hepatoma cells were grown to ~80% confluence in 100-mm tissue culture dishes and were treated with 1 μM TCDD or 0.1 μM Me2SO for 18 h. Total RNA was isolated using RNeasy spin columns (Qiagen). Total RNA (5 μg) was fractionated on 1.2% agarose-2.2 M formaldehyde gels, transferred to Nytran by capillary blotting in 20 × SSC, and cross-linked to the membrane in a UV Stratalinker 2400 (Stratagene). Blots were hybridized with 32P-labeled CYP1A1 or actin cDNA overnight at 55 °C using ExpressHyb hybridization solution (CLONTECH). Blots were washed as described previously (24) and then autoradiographed with Hyperfilm MP (Amersham Biosciences).

Immunoblotting Analysis—Whole-cell extracts were prepared from wild-type, AhR-defective, and reconstituted cells as described above. Forty micrograms of cellular proteins were dissolved in 2× Laemmli sample buffer (Bio-Rad), fractionated by SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. Blots were incubated in blocking buffer (5% nonfat milk in TBS-T (pH 7.6)) containing 0.1% Tween 20 (TBS-T) containing 5% nonfat milk) overnight at 4 °C. Incubation with primary antibody (anti-AhR, 1:2000) (25) was carried out for 1 h at room temperature. After washing in TBS-T, blots were incubated with secondary antibody (anti-mouse-HRP, 1:2000) for 1 h at room temperature. After washing in TBS-T, blots were developed using the Renaissance Chemiluminescence Kit (PerkinElmer Life Sciences) and visualized on Hyperfilm MP.
abolishes function and provides a useful negative control for GST pull-down experiments.

We constructed GST fusion proteins containing either the wild-type or mutated AAD, attached the fusion proteins to glutathione-Sepharose beads, and allowed the beads to interact with extracts prepared from mouse hepatoma cells. Interacting proteins were purified and analyzed by SDS-PAGE and silver staining. Our findings reveal three proteins, with molecular masses of about 160, 180, and 200 kDa, that interact with the GST-AAD fusion protein but not with the GST-mutAAD fusion protein (Fig. 2A). We isolated enough of the most prominent (160 kDa) AAD-interacting protein to permit its microsequencing; the data revealed that the sequences of two tryptic peptides were identical to sequences within Mybbp1a, a nuclear protein that interacts with c-Myb (19).

We confirmed the identity of the AAD-interacting protein in immunoblotting experiments using an anti-Mybbp1a antibody. Our findings indicate the presence of an immunoreactive 160-kDa band in pull-down eluates from GST-AAD, but not in eluates from GST-mutAAD or GST alone (Fig. 2B). In addition, the immunoreactive band co-localized with the 160 kDa silver-stained band in a gel run in parallel (data not shown). Together, these findings imply that Mybbp1a interacts with the wild-type AAD but not with the mutant AAD. It is notable that the inability of Mybbp1a to interact with the mutant AAD is associated with loss of transactivation capability in the mutant (Fig. 1). This observation tends to implicate Mybbp1a in the transactivation function of the AAD.

The above findings led us to ask whether Mybbp1a affects the transactivation capability of AhR’s AAD. To address this issue, we co-transfected mouse hepatoma cells with increasing amounts of a Mybbp1a expression vector together with a dioxin-responsive AhR/Arnt-dependent firefly luciferase reporter construct (pGudLuc) and measured TCDD-inducible luciferase activity in the transfected cells. Our findings (Fig. 3) reveal that transfections with increased amounts of Mybbp1a expression vector are associated with increased responsiveness of the reporter gene to TCDD. For example, in cells that contain no Mybbp1a expression vector, TCDD induces luciferase activity about 11-fold; in contrast, at the highest level of Mybbp1a expression vector used, TCDD induces luciferase activity about 56-fold. Thus, in this experimental setting, Mybbp1a can increase the responsiveness of an AhR-dependent gene by (at least) a factor of five.

The results of the expression studies (Fig. 3), together with those of the pull-down experiments (Fig. 2), imply that Mybbp1a influences gene expression via AhR’s AAD. To test this idea directly, we used retroviral vectors to reconstitute AhR-defective cells with either AhR1–494/AAD, full-length AhR (as a positive control), or AhR1–494 (as a negative control), and we established clonal strains of each reconstituted cell type. Immunoblotting studies confirmed that expression of the AhR constructs in each of the reconstituted cell strains is similar to that of AhR in wild-type cells (data not shown). We then transiently transfected these strains with the AhR/Arnt-dependent firefly luciferase reporter construct (pGudLuc), to document that the augmentation of AhR-dependent gene expression by Mybbp1a requires the AAD. The positive and negative controls reveal, as expected, that Mybbp1a augments the response of
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In vitro observations imply that Mybbp1a can utilize several different mechanisms AhR-dependent transcription. Taken together, these observations indicate that increased expression of Mybbp1a can substitute functionally for the entire C-terminal portion of AhR (which contains several transactivation domains) suggests that the C-terminal portion exhibits redundancy with respect to its transactivation capability. One possible advantage of such redundancy is that it might improve the ability of AhR/Arnt to communicate with transcriptional promoters that differ in their cognate binding proteins. In this respect, it is notable that a second, glutamine-rich transactivation domain within AhR’s C-terminal portion probably interacts with proteins that are different from those that interact with the AAD (37, 38). Given this situation, we speculate that the AAD and the glutamine-rich domain of AhR preferentially communicate with different sets of transcriptional promoters. This may be an interesting area for future research.

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