MOLECULAR BIOLOGY OF THE AH RECEPTOR AND AH RECEPTOR-DEPENDENT SIGNALING

A MODEL FOR FUNCTIONAL ANALYSES OF COADAPTOR INVOLVEMENT IN DIOXIN-INDUCIBLE GENE EXPRESSION

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Introduction

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) is the prototypical ligand for the aryl hydrocarbon receptor (AhR) which accumulates in the nucleus following dioxin exposure and acts as a ligand-activated transcription factor. Within the nucleus, transcriptional activation by the AhR requires its dimerization with the aryl hydrocarbon receptor nuclear translocator (Arnt) protein and binding of this complex to sequence-specific enhancer elements (dioxin responsive elements, DRE). Recent research has sought to determine if coadaptor proteins, known to facilitate/regulate transcription by other ligand activated transcription factors (e.g., steroid hormone receptors), also interact with the AhR to regulate inducible gene transcription. We have used the adenovirus protein E1A243 to examine the role of two coadaptor proteins in AhR-dependent transcription: a 300 kDa protein (p300) and the 105 kDa retinoblastoma gene product (Rb). While wild type E1A243 binds both p300 and Rb, the RG2 mutant carries a point mutation that disrupts p300 binding while the YH47 and 928-YH47 mutants carry point mutations disrupting Rb binding. We have used E1A243 (wild type and mutant) expression plasmid constructs in transient co-transfection assays with a series of luciferase reporter plasmid constructs representing 1) the CYP1A1 promoter and regulatory regions, 2) the CYP1A1 minimal promoter and 3) the CYP1A1 enhancer. The data suggest that E1A243 inhibits CYP1A1 reporter gene expression by targeting both enhancer and promoter activities. These appear to represent two distinct mechanisms in that CYP1A1 promoter inhibition is independent of E1A's ability to bind p300 or Rb while inhibition of enhancer function requires E1A binding to either coadaptor. These results describe a novel system for functional analysis of coadaptor proteins and provide functional evidence for the involvement of coadaptor proteins in AhR-dependent gene transcription.

Materials and Methods

Reporter Plasmid Constructs Reporter plasmid constructs are depicted in Figure 1. The pGudLuc5.0 reporter plasmid contains a fragment of the murine CYP1A1 gene (-1317 to +256 bp) inserted into pGL2 Basic (Promega) immediately upstream of the firefly luciferase reporter[1]. This fragment contains the dioxin responsive domain (containing four functional DREs), the inhibitory and promoter domains, and the first 256 bp of the CYP1A1 structural gene. pGudLuc1.1 contains the -1317 to -818 bp fragment from the upstream region of the CYP1A1 gene inserted directly upstream of the mouse mammary tumor virus (MMTV) promoter conferring TCDD responsiveness upon the MMTV promoter and luciferase gene[2]. pGudLuc4.2 contains the CYP1A1 promoter (-280 to +256 bp) inserted into the vector pGL2 Basic immediately upstream of the luciferase gene resulting in constitutive expression of the luciferase gene [1].

Expression Plasmid Constructs. Wild type and mutant E1A cDNA expression vectors were generated from plasmid constructs provided by Dr. Elizabeth Moran (Temple University, School of Medicine). The pcDNA3.1/E1A$_{243}$ plasmid construct constitutively expresses wild type E1A$_{243}$. A single Arg to Gly substitution at amino acid position 2 disrupts p300 binding in the product of the pcDNA3.1/RG2 plasmid construct. The gene product of pcDNA3.1/YH47 contains a Tyr to His substitution at amino acid position 47 disrupting binding of Rb. The pcDNA3.1/928-YH47 plasmid construct expresses and E1A243 protein which fails to bind Rb as well as a related protein p103 due to Tyr to His and Cys to Gly substitutions at amino acids 47 and 124 respectively.

Transient Cotransfection Assay. Hepa1c1c7 cells, at 40% confluence in 6-well culture dishes, were incubated with 1 ml of polybrene solution (0.03 mg polybrene, 5 μg reporter plasmid construct, 5 μg expression plasmid construct in growth medium) for six hours. Cultures were exposed to DMSO shock (4 min, 25% DMSO (v/v) in complete growth medium) and allowed to recover for 18 hr prior to addition of DMSO (0.1% v/v) or TCDD (1 nM). Following 4 hr incubation, cell lysates assayed for luciferase activity which was calculated as relative light units (RLU) per mg protein (as determined using the fluorescamine protein assay). Results were expressed as a percent of the TCDD response for inducible reporter plasmids or as a percent of control for constitutively expressed reporter plasmids (± SD, n=3, all experiments conducted at least twice).

Results and Discussion

The functional role of coadaptor proteins in nuclear receptor function (e.g., steroid homone receptors) has focused on how these proteins modulate inducible gene expression. One experimental approach evaluates reporter gene expression from inducible plasmid constructs driven by a selected enhancer element similar to our pGudLuc1.1 which selectively responds to ligand activated AhR. The studies presented here, however, suggest that some factors may act directly at the promoter making accurate assessment of enhancer effects problematic. Using a series of reporter plasmid constructs representing the intact CYP1A1 regulatory region as well as two major regulatory components (the enhancer and promoter sequences; Figure 1), we have been able to dissect the phenomenon of E1A inhibition and identify both the CYP1A1 enhancer and promoter as molecular targets of E1A.

Sogawa and coworkers previously reported E1A-dependent inhibition of TCDD-inducible reporter gene expression suggesting that inhibition by E1A occurred at the enhancer[3]. It was hypothesized that E1A disrupted the interaction between Arnt and the p300 coadaptor[4]. Although inhibition of constitutive gene expression was observed, the significance of the phenomenon was not addressed. These observations, however, led us to consider the possibility that E1A may be directly targeting the promoter for repression rather than the CYP1A1 enhancer. Consistent with this hypothesis, transient cotransfections using the pGudLuc4.2 reporter plasmid construct (Figure 2) demonstrate that E1A directly inhibits CYP1A1 promoter function at high concentrations. These concentrations inhibit both basal and constitutive reporter gene activity in a manner independent of E1A binding to p300 or Rb (Figure 3).

Although our studies demonstrate that the CYP1A1 promoter is a target for E1A inhibition, they do not eliminate the possibility that inhibition also occurs at the enhancer. Reducing wild type E1A levels in cotransfection such that there are minimal effects on the CYP1A1 promoter allowed us to observe select inhibition of inducible reporter gene expression

(Figure 4). Under these conditions, E1A mutants failing to bind either p300 or Rb did not inhibit inducible reporter gene expression. These results provide functional relavence for the physical interactions between AhR or Arnt and p300 and Rb reported by other laboratories suggesting that these coadaptor proteins are integral for AhR-dependent gene transcription[4,5].

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References

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Figure 1: Reporter plasmid constructs .

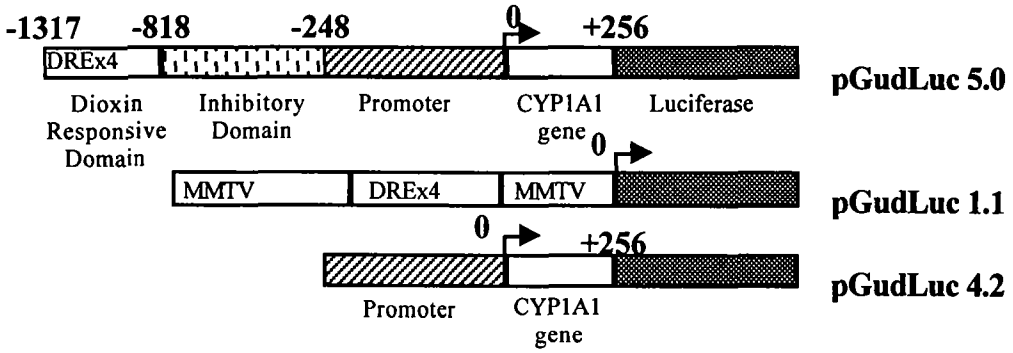

Figure 2: High levels of E1A inhibit CYP1A1 promoter function in transient cotransfection assays.

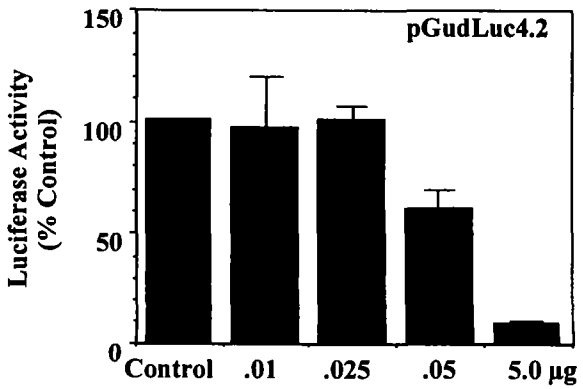

Figure 3: High levels of E1A inhibit both constitutive (DMSO treated) and inducible (TCDD treated) reporter gene expression in a manner independent of E1A binding of p300 or Rb.

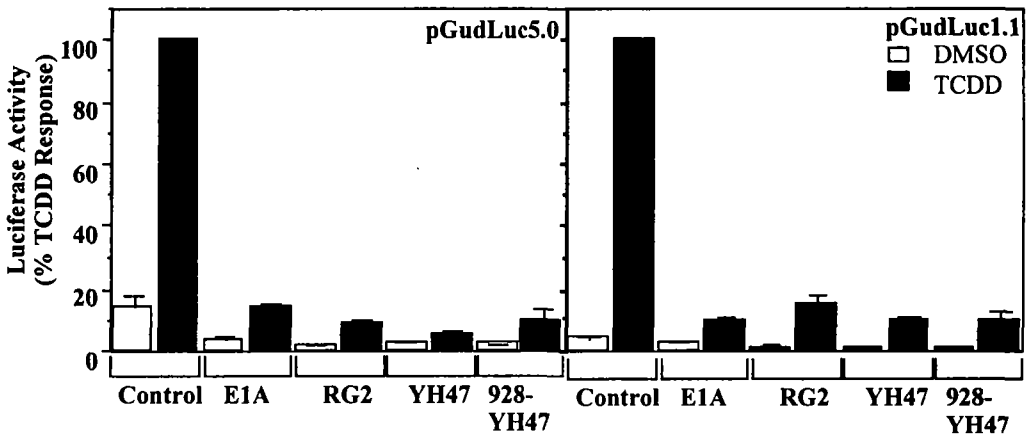

Figure 4: Low concentrations of E1A have minimal promoter effects revealing inhibition AhR-dependent gene expression which is dependent on E1A binding to either p300 or Rb