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

SMRT MEDIATED TRANSCRIPTIONAL SILENCING OF THE Ah RECEPTOR SIGNALING PATHWAY NOT OBSERVED IN HUMAN MCF-7 OR BG-1 CELL LINES

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Introduction

Exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or related chemicals such as polycyclic and/or halogenated aromatic compounds leads to a variety of tissue and species specific toxic and biological effects, including a wasting syndrome, thymic involution, chloracne, teratogenesis, and others. Despite this wide range of effects seen at the organismal level, the majority of these effects are mediated by a single protein, the Aryl Hydrocarbon Receptor (AhR). In its inactive, ligand-binding form, the AhR resides in the cytosol complexed with two molecules of hsp90 and one of XAP2 (also known as ARA9 or AIP). Upon binding its target ligand, the AhR is thought to change conformation and expose a nuclear localization signal. After entering the nucleus, the AhR dissociates from its associated protein subunits, dimerizes with its partner, the Ah Receptor Nuclear Translocator (ARNT), and binds its specific DNA recognition site, the Dioxin Responsive Element (DRE). Binding of the ligand:AhR:ARNT complex to the DRE stimulates expression of the adjacent gene. Since it is this basic pathway mediating the various biological responses to exposure to toxins such as TCDD, we have postulated that the xenobiotic response pathway is modulated by other, tissue specific, factors which act on AhR and/or ARNT to regulate their activity. In order to identify some of these factors, we have examined a group of cofactors known to regulate transcriptional activity in other pathways, most notably the steroid hormone receptor pathways. One such cofactor is the Silencing Mediator for Retinoic acid and Thyroid hormone receptors (SMRT). As part of a repressor complex containing mSin3A and histone deacetylase 1 (HDAC-1) (1), SMRT interacts with nuclear receptors, allowing them to remain in a transcriptionally silent state even though they are bound to their DNA response elements. SMRT is also implicated in the silencing of non-receptor transcription factors.

Materials and Methods

Cell Culture and Transfection. Human breast carcinoma MCF-7 cells, obtained from the American Type Culture Collection (Rockville, MD), and human ovarian carcinoma BG-1 cells, kindly provided by Dr. George Clark (Xenobiotic Detection Systems Inc., Durham, NC), were grown and maintained as previously described (3). For transient transfections, cells were seeded into twelve-well plates and transfected at 60% confluence with 1.5 μg of pGUDLUC6.1 and 100 ng of pCMX, pCMX-SMRT, or equal volume of TE buffer, using the previously described *polybrene method (3)*. Forty-eight hours after transfection, cells were treated with 10 nM TCDD or DMSO solvent control and harvested 24 hours later for measurement of luciferase activity as described (3). Luciferase activity was normalized to sample protein concentration as determined

by the fluorescamine protein assay method (4), using bovine serum albumin as the standard.

GST Pull Down Assays. Pull down assays were performed as previously described (5,6). Briefly, Glutathione S-transferase (GST)-SMRT fusion proteins expressed in the *E. coli* strain DH5α were purified and immobilized on glutathione-agarose. Full length ^{35}S-radiolabeled mouse AhR was synthesized *in vitro* using the Quick Coupled Transcription and Translation kit (Promega), treated with 20 nM TCDD or equal volume of DMSO, and incubated with shaking for 2 hours at 30°C with the immobilized proteins. The agarose matrix was washed extensively and bound proteins were eluted with 10 mM reduced glutathione and separated by SDS-PAGE. Dried gels were quantitated by PhosphorImager analysis (Molecular Dynamics SI system, Sunnyvale, CA).

Plasmids. The pGUDLUC6.1 reporter contains the coding region of the luciferase gene (from pGL3 basic (Promega)) under control of the mouse mammary tumor virus basal promoter and four DREs (7). The pCMX-SMRT plasmid (8) was a gift from Dr. Ronald Evans (Salk Institute, La Jolla, CA). The pCMX control was regenerated by excising the SMRT cDNA and religating the empty vector. All GST-SMRT fusions were constructed in pGEX-KG vectors (6,9).

Results and Discussion

Transient Cotransfections. Previous work by Nguyen et al (2) reported that SMRT mediated inhibition of AhR activity in MCF-7 cells cotransfected with up to 1μg of pCMX-SMRT and the pRNH11c reporter (containing the *CYP1A1* promoter fused to the CAT gene) in 60mm^2 culture plates. We repeated these experiments using pGUDLUC6.1 and 100 ng of pCMX-SMRT, an equivalent amount of DNA/well for a twelve well plate. Contrary to their results, we saw no repression of AhR signaling in MCF-7 cells (figure 1, left side). In fact, we saw no difference between the SMRT transfected cells and either the pCMX or the no vector control. Interestingly, when we performed the same transfections in BG-1 cells (figure 1, right side), the activity of the SMRT transfected cells was midway between that of the two controls. Although the difference between SMRT and the no vector control was not statistically significant, SMRT significantly ($p<0.05$) increased the activity as compared to the pCMX controls. This may be due to an inhibitory effect of the pCMX promoter on luciferase transcription (perhaps due to titration, or "squelching", of general transcription factors), which is partially relieved by SMRT.

The fact that we failed to see repression of gene expression where Nguyen and coworkers (2) observed repression may be a result of a number of factors. Although we used the same cell line (MCF-7) and an equivalent amount of expression vector, we are using a different reporter system (our luciferase vs. their CAT), with the transcription of each reporter driven by a different promoter. This raises the possibility that some factor(s), other than the AhR/ARNT complex, may be acting on the promoter to affect reporter gene transcription. One other possibility is that the length of induction has an effect on silencing. While we induced cells with TCDD for 24 hours, Nguyen et al induced for 48 hours, potentially allowing longer term effects to occur. Further studies are planned to examine this discrepancy.

GST Pull Down Assays. In order to determine whether SMRT could interact directly with the AhR, we performed GST pull down assays. These studies utilized GST fusions of three domains of SMRT shown to be involved in its silencing activity, Silencing Domain 1 (SD-1), Silencing

Domain 2 (SD-2), and the Receptor Interacting Domains (RIDs 1 and 2). Non-recombinant GST was included as a background control. Our results reveal that SMRT interacts strongly with AhR within SD-1, with a preference for TCDD treated AhR (figure 2, 96-566 results). Within the SD-2 domain, SMRT interacts weakly with AhR, with no preference for TCDD or non-TCDD treated AhR (figure 2, 566-680). The RID domain also shows no preference for treated or non-treated AhR, and the strength of its interaction with AhR is intermediate between those of the other domains (figure 2, 751-1495).

The ability of the AhR to interact strongly with the SD-1 region of SMRT suggests that this interaction could potentially disrupt the SMRT/mSin3A/HDAC-1 silencing complex, since it has been shown that this is the same domain that interacts with mSin3A (10). Thus, although SMRT interacts moderately with AhR within the RIDs--comparable to its interactions with DNA-bound nuclear receptors--AhR may affect silencing of transcription factors negatively regulated by SMRT. The interaction of the AhR with a region of SMRT distinct from the hormone receptor interacting domains is consistent with our results showing that SMRT does not appear to have a repressive effect. In addition, the other levels of regulation of AhR response (its cytosolic location, ligand dependent activation, and relatively rapid ligand dependent degradation) argue against a need for a nuclear repressor protein for the AhR.

Acknowledgements

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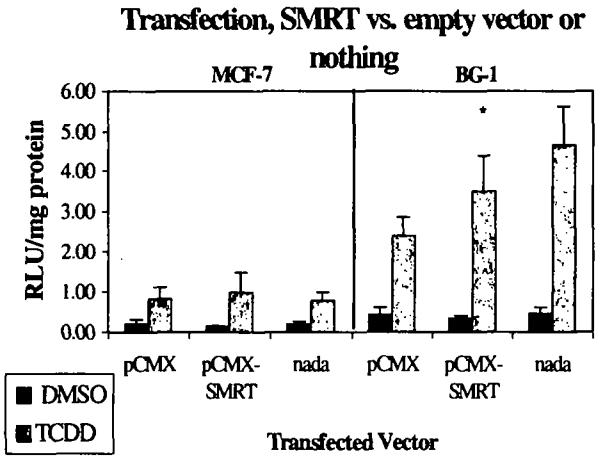
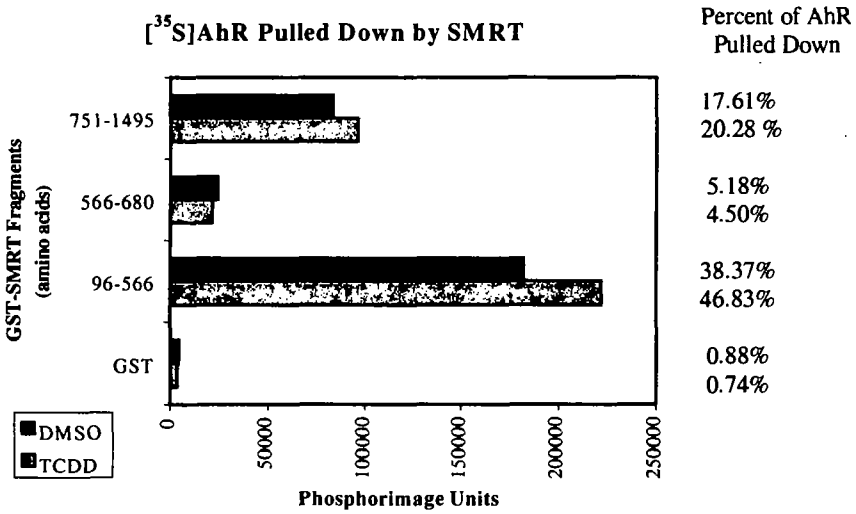


Figure 1. BG-1 and MCF-7 cells were transfected with pGUDLUC6.1 and SMRT, empty vector (pCMX), or no vector (nada). The asterisk indicates a significant ($p < 0.05$) increase in luciferase activity over the empty pCMX vector. Each bar represents the mean and standard deviation of 6 independent measurements.



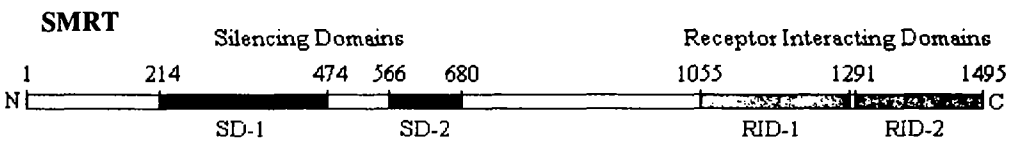


Figure 2. Top - PhosphorImager analysis and quantitation of [^{35}S]-AhR "pulled down" with the indicated GST-SMRT fragments. Bottom - Domain map of SMRT. The GST-SMRT fusions used are SD-1 (96-566), SD-2 (566-680), and the RIDs (751-1495).