Role of Mediator in Transcriptional Activation by the Aryl Hydrocarbon Receptor

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Running title: TRAP/Mediator and the Aryl Hydrocarbon Receptor
Summary:

The aryl hydrocarbon receptor (AHR) binds many aromatic hydrocarbon compounds and mediates their carcinogenesis. We demonstrate that the endogenous AHR physically associates with the endogenous TRAP/DRIP/ARC/Mediator complex in a ligand-dependent manner. The Med220 subunit, which is known to interact with several nuclear hormone receptors through its LxxLL motifs, potentiates AHR-dependent reporter gene activity in a LxxLL-independent manner. Depletion of Med220 substantially reduces endogenous AHR-mediated CYP1A1 transcription. Both Med220 and CDK8 (another subunit of TRAP/DRIP/ARC/Mediator) are recruited to the CYP1A1 enhancer in a TCDD (2,3,7,8-Tetrachlorodibeno-p-dioxin) -dependent fashion in vivo and Med220’s LxxLL motifs are not required. Med220 rapidly and persistently associates with the enhancer but not the promoter of the CYP1A1 gene after TCDD treatment with similar kinetics as AHR and the coactivators p300 and p/CIP. Our findings demonstrate a novel role for Med220 in AHR-regulated transcription, which differs mechanistically from its role in transcriptional regulation by other previously studied transcription factors.

Keywords: AHR/ARNT/Med220/TRANSCRIPTION/DIOXIN
Introduction:

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) and certain polycyclic aromatic hydrocarbons (PAHs) are widespread environmental pollutants that are designated human carcinogens and have been shown to be potent toxicants and carcinogens in experimental animals (1). The Aryl Hydrocarbon Receptor (AHR), a ligand activated transcription factor, is the intracellular protein that mediates most of the toxic effects of these compounds. Additionally, AHR may also play an important role in normal developmental and physiological setting possibly via an endogenous ligand(s). The evidence in support of this is largely derived from AHR knock-out mice. These mice are viable, but exhibit many physiological changes and developmental abnormalities, including reduced liver weight, a depressed immune system and reproductive defects such as small litter size (2).

Unliganded AHR forms a complex with the chaperone protein hsp90, a co-chaperone protein called p23 (3) and the Hepatitis B Virus X-associated protein 2 (XAP2) (4). This complex resides in the cytoplasm. Upon ligand binding, the AHR undergoes a conformation change and dissociates from the complex. The resulted exposure of its nuclear localization sequence triggers the nuclear translocation of AHR (5). Inside the nucleus, AHR dimerizes with a related nuclear protein called the Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT). This heterodimer can then recognize its cognate DNA binding site called the xenobiotic response element (XRE) and activates genes involved in xenobiotic metabolism and cell proliferation (6, 7).

AHR-regulated transcription is under intense scrutiny since the biological activities of AHR are largely due to its ability to alter gene expression. A model system frequently used to study AHR regulated transcription is the murine cytochrome P4501A1 gene (CYP1A1). Early
studies established that AHR is capable of initiating enhancer-promoter communication during \textit{CYP1A1} transcriptional activation (8,9): occupation of the enhancer region by liganded AHR/ARNT elicits chromatin remodeling over the promoter and assembly of the general transcription machinery. The mechanistic details of these events are however, not fully elucidated.

Direct interactions between transcriptional activators such as AHR/ARNT and general transcription factors are not sufficient for nucleation of the general transcription machinery. For example, activators are unable to render productive and robust transcription \textit{in vitro} with transcription reactions reconstituted with basal factors alone, indicating that additional cofactors are required (10,11). Biochemical isolation and studies of the related or identical multi-subunit complexes, murine Mediator (12), TRAP/DRIP/ARC/human Mediator (13,14,15,16) (hereafter referred to as the Mediator complex) suggest that these complexes can stimulate in vitro transcription mediated by several activators. Certain subunits of the Mediator complex can directly interact with transcription activators, whereas other subunits associate with the general transcription apparatus (17). Thus Mediator may function as an adaptor to convey regulatory signals from enhancers to promoters.

We demonstrate here a role for Mediator in AHR/ARNT-dependent transcription of \textit{CYP1A1}. The mechanism of Mediator’s action differs for AHR/ARNT from for other previously described transcription factors. Our observations shed light on the enhancer-promoter communication invoked by AHR. Furthermore, potential differences in the structural composition of the Mediator in different tissue and promoter contexts could help explain differential regulation of gene expression mediated by AHR/ARNT, and impact our understanding of receptor mediated chemical toxicity and carcinogenesis.
Experimental procedures:

Antibodies and Cell culture:

Affinity purified rabbit polyclonal antibodies to AHR and ARNT were described previously (18). Antibodies to p/CIP were generated against amino acid 691-851 of mouse p/CIP in this laboratory. Med220 (M-255), CDK8 (C-19), p300 (N-15) and Pol II (N-20) antibodies were purchased from Santa Cruz Biotechnology. The Med130 antibody was purchased from Rockland Inc. Hepa-1 and human hepatocarcinoma cell line HepG2 were maintained in α-MEM medium supplemented with 10% fetal bovine serum (FBS). Med220+/+ and Med220-/- mouse embryonic fibroblasts (19) were grown in DMEM medium High Glucose with 10% FBS. Med220-/- mouse embryonic fibroblasts stably infected with wild type Med220 and LxxLL mutated Med220 (19) were maintained in DMEM medium High Glucose with 10% FBS and 150μg/ml hygromycin.

Plasmid constructs:

Expression constructs of wild type Med220 and LxxLL-mutated Med220 were derived from pWZLhyg-FH220 (19).

Coimmunoprecipitation:

Hepa-1 cells were treated with DMSO or TCDD at a final concentration of 10^{-8} M for 60 minutes and then washed extensively with ice-cold PBS. Nuclear extracts were prepared as described (20). The final KCl concentration in the nuclear extract was 300 mM. Prior to immunoprecipitation, the buffer content of the nuclear extract was adjusted to contain 20 mM HEPES pH7.9, 12.5% glycerol, 250mM KCl, 1.5mM MgCl$_2$, 0.2 mM EDTA, 0.25 mM DTT and 0.1% NP40, plus 1X complete protease inhibitor (Roch...
Biochemical). 400 µg of nuclear extract was pre-cleared with 35 µl of protein A Sepharose at 4 °C for one hour, and immuno-precipitations were performed overnight at 4 °C with AHR antibody, ARNT antibody or pre-immune IgG. Immuno-complexes were collected using 35 µl protein A sepharose containing 1mg/ml BSA. The sepharose beads were washed 4 times with 20 mM HEPES pH 7.9, 12.5% glycerol, 250mM KCl, 1.5mM MgCl₂, 0.2 mM EDTA and 0.1% NP40. Immuno-complexes were eluted with SDS-sample buffer and separated in 6% SDS-PAGE followed by western blot analysis, probing with Med220, Med130, AHR or ARNT antibodies.

**Transient transfections and reporter gene assays:**

Transfections of expression plasmids were performed using the Superfect reagent (Qiagen). For Hepa-1 and HepG2 cells, a dual-luciferase system (Promega) was used. DNA mixtures of the indicated amount of Med220 wild-type or mutant constructs were cotransfected into 12 well plates with 40ng of Renilla luciferase reporter (pRL-TK) driven by the HSV thymidine kinase promoter, and 200ng firefly luciferase reporter (pGL-CYP1A1) driven by 4.2 kb of the 5’ upstream regulatory region of the rat CYP1A1 gene, including its promoter and multiple XREs. The final DNA concentrations were adjusted using empty expression vector to ensure that equal amounts of DNA were used in each plate. After 24 hours, transfected cells were treated with either TCDD or vehicle (DMSO) for 16 hours. Cells were then harvested and lysed in Passive lysis buffer (Promega). Luciferase activities were measured using the luciferase assay reagent (Promega). The Renilla luciferase activity was used to normalize the firefly luciferase activity of each sample. Each transfection was carried out in triplicate.
RNA interference in HepG2 cells:

A Med220 siRNA oligo (siMed220), 5’-GGCUCUCAAAGUAACAUCU(TT)-3’, a scrambled RNA oligo (SC) with similar GC content 5’-GAGUAAGUCAUCACCUAU(TT)-3’ and their respective complementary oligos synthesized by Dharmacon were deprotected and annealed per the manufacturer’s protocol. For ectopic reporter assays, siMed220 or SC were cotransfected into 12 well plates with a mixture of 200 ng pGL-CYP1A1 firefly luciferase reporter and 40 ng pRL-TK renilla luciferase reporter, or a mixture containing 50 ng of GAL4-VP16 expression construct, 200 ng GAL4 firefly luciferase reporter and 40ng pRL-TK renilla luciferase reporter. Transfections were performed at 90% cell confluency with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Transfection mixtures were replaced with fresh medium 5 hours later. 36 hours post transfection, cells transfected with the pGL-CYP1A1 reporter were treated with DMSO or TCDD for an additional 16 hours, whereas the cells transfected with the GAL4 reporter were left undisturbed. Cell lysates were harvested and reporter activities were determined as described above. For the effect of Med220 knock-down on endogenous CYP1A1 induction, siMed220 or SC oligos were transfected into 6 well plates at 30% confluency using oligofectamine (Invitrogen). 24 hours later, cells were transfected again with the same procedures for an additional 24 hours, cells were treated with either DMSO or TCDD for 6 hours. Total RNA was then isolated using Trizol (Invitrogen) and assayed for CYP1A1, AHR, and β-actin mRNA levels by real time PCR, as described below. For detection of Med220 protein levels, cells were lysed in 50mM Tris-HCl pH 7.9, 300mM NaCl, 1mM EDTA, 0.5% NP40, 10% Glycerol, 1mM DTT and 1x complete
protease inhibitor (Roche Biochemical) and 40 µg of whole cell extract was then separated on 6% SDS-PAGE followed by western blotting with either the Med220 antibody or AHR antibody.

**Reverse Transcription and Real-time quantitative PCR:**

Reverse transcriptions were carried out with Thermoscript RT kit (Invitrogen) using 500 ng of Total RNA in 20 µl volume. 10% of the reaction product was used together with SYBER-GREEN PCR master mix (Applied Biosystem) to assemble a 100 µl reaction that was divided into three 30 µl replicas and run on I-cycler PCR machine (Bio-Rad). Real time PCR reaction parameters were: 94 °C for 10 minutes, then 40 cycles of 15 seconds at 94 °C and 1 minute at 60 °C. First derivative plot of the melting curve obtained following individual PCR reaction reveals a single peak at the melting temperature of the individual amplicon. PCR primers were: 5’-TAAATCGCCAGGCAGTGCA-3’ and 5’-CCCACCATCATGTTCCCTTA-3’ for human Med220, 5’-CCTGAATGCCTGGAC-3’ and 5’-ACGAAGGAAGAGTGCCTG-3’ for human *CYP1A1*, 5’-AGAAGGTCACTCTCTTTGTTTTGC-3’ and 5’-GCAGCAAGATGCGCCAGGAA-3’ for murine *CYP1A1*, 5’-GCTCCTCCTGAGCGCAAG-3’ and 5’-CATCTGCTGGGAGGTGGCA-3’ for human β-actin, 5’-AGAGGGAAATCGTGACGACTG-3’ and 5’-CAATAGTGACCGTCCCTG-3’ for murine β-actin. Med220 and *CYP1A1* mRNA levels were normalized with the amount of β-actin mRNA.

**Chromatin Immuno-precipitation (ChIP) assays:**

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ChIPs were carried out as we described previously (21). Briefly cells were treated with TCDD for 60 minutes except for the time course analysis. The sizes of the majority of chromatin fragments were reduced to below 600 bps by sonication. The primers used to amplify the murine CYP1A1 promoter region were 5’-AATGGAGGCCCATCTTAC-3’ and 5’-AGAACTACCACCTTCAGGGTTAGG-3’. ChIP-reIP utilized a similar ChIP protocol, except that the primary immuno-complex obtained with the p300 antibody was eluted by 10mM DTT with agitation at 37 °C for 30 minutes. The eluate was diluted 50 times with buffer (20 mM Tris-HCl pH8.1, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) and immunoprecipitated with the Med220 antibody.
Results:

**AHR/ARNT interacts with the Mediator Complex in vivo.**

In order to explore the potential involvement of Mediator in AHR/ARNT regulated transcription, we set out to test whether the AHR/ARNT dimer can interact with Mediator. Hepa-1 cells were treated with TCDD or vehicle (DMSO) for 60 minutes. Nuclear extracts from these cells were prepared and immuno-precipitations were performed using an AHR antibody. Two of the Mediator subunits Med220 and Med130, were co-immunoprecipitated with AHR (Figure 1A) from cells treated with TCDD, but not from cells not treated with this AHR ligand. Whereas Med220 may be a loosely attached subunit, since nullification of Med220 does not seem to compromise the overall integrity of the Mediator complex (19), Med130 is an integral part of the Mediator: absence of Med130 destabilizes the associations of Med100 and Med95 with the Mediator complex (22). Hence this co-IP experiment indicates that AHR/ARNT interacts with the Mediator complex. We also immunoprecipitated ARNT from a nuclear extract of Hepa-1 cells untreated with TCDD. We were unable to detect co-immunoprecipitation between Med220, Med130 and ARNT (Figure 1B). These last results indicate ARNT, at least when not associated with AHR, does not interact with the Mediator complex.

**Med220 and also Med220 with mutated LxxLL motifs enhance AHR/ARNT mediated reporter gene activity in Hepa-1 and HepG2 cells.**

To assess the potential contribution of individual subunits of the Mediator complex to AHR/ARNT regulated transcription, cDNA expression constructs for various Mediator subunits, including Med78, Med95, Med100, Med130, Med150, Med220 and Med230 and
Med220 were cotransfected into Hepa-1 cells together with a reporter plasmids containing 4.2 kb of the 5’ upstream regulatory region of the rat CYP1A1 gene, including its promoter and multiple XREs. 24 hours post-transfection, cells were treated with DMSO or TCDD for an additional 16 hours and reporter activities were then measured. Among all the cDNA constructs tested, only Med220 elicited a significant enhancement of AHRC regulated reporter activity (Figure 2B and 2C and data shown). Although the results cannot rule out potential involvements of other Mediator subunits, because relevant cellular protein abundance and the stoichiometric composition of the Mediator complex could also impact the results of the experiments, they do reveal a role for Med220.

Med220 is the interacting subunit within the Mediator complex for several nuclear receptors (NR), including thyroid hormone receptor (TR), vitamin D receptor (VDR), peroxisome proliferator-activated receptor-γ (PPARγ) and estrogen receptor (ER) (23, 14, 19, 16). Mutations of two LxxLL motifs in Med220 abolish its interaction and transcriptional coactivation activity with NRs (24,25). Another important class of NR coactivators is the p160 family including SRC-1, NCoA2 and p/CIP, which can also interact with NRs via their LxxLL motifs (26). The p160 family of coactivators have also been implicated in AHR/ARNT mediated transcription (27, 28). SRC-1 can interact with both AHR and ARNT (27), and the interaction between AHR and SRC-1 depends on the latter’s LxxLL motifs (28). We therefore extended our study using Med220 derivatives with mutated LxxLL motifs. We found that Med220 with either one or both of its LxxLL motifs mutated enhanced AHRC regulated reporter activity with equal potency as wild-type Med220 in Hepa-1 and HepG2 cells (Figure 2B and 2C).
Depletion of endogenous Med220 compromises AHR-regulated CYP1A1 transcription.

We used RNA interference (RNAi) to deplete endogenous Med220 in order to examine the contribution of Med220 to AHR regulated transcription. We were precluded from using Med220 wild type and Med220 knockout mouse embryo fibroblasts (MEF) in this study because CYP1A1 expression is known to be repressed in MEF (29) and also because the receptor appeared to be more abundant in Med220-/- MEF than in Med220+/+ MEF (data not shown). HepG2 cells were transfected with a small interference RNA oligo against human Med220 (siMed220) or a corresponding scrambled control oligo (SC) for 48 hours, followed by DMSO or TCDD treatment for 6 hours. Total RNA was then prepared and analyzed by quantitative real time RT-PCR to determine Med220 mRNA levels. Whole cell extracts of the transfected HepG2 cells were also analyzed for Med220 protein levels. As shown in Figure 3A and 3B, the siMed220 oligo drastically reduced Med220 mRNA and protein levels but did not affect AHR protein levels. For Med220’s effect on AHR/ARNT mediated reporter gene transcription, either siMed220 or SC oligos was cotransfected with the CYP1A1-luciferase reporter plasmid into HepG2 cells. 36 hours after transfection, cells were treated with DMSO or TCDD for an additional 16 hours and reporter activities were then determined. As showed in Figure 3C, siMed220 treatment significantly reduced TCDD induced reporter activity, but SC had no effect. When the same oligos were cotransfected into HepG2 cells along with GAL4-VP16 expression and GAL4 based reporter plasmids, no significant difference in reporter activities were observed between siMed220 treated and SC treated cells (Figure 3D), in accordance with previous studies showing that VP16 targets the TRAP80 subunit in the Mediator complex.
and that GAL4-VP16 mediated reporter activities are unaffected by the absence of Med220 (30). To study the effects of siRNA on the endogenous \( CYP1A1 \) gene, HepG2 cells were transfected with the siMed220 oligo or the control oligo SC for 48 hours, followed by DMSO or TCDD treatment for 6 hours. Total RNA was then prepared and analyzed by quantitative real time RT-PCR. TCDD triggered \( CYP1A1 \) transcription was significantly compromised in siMed220 treated cells (Figure 3E).

We noticed that siMed220 decreases both the reporter gene activity and the endogenous \( CYP1A1 \) mRNA levels in the absence of TCDD. This is probably due to transcription of \( CYP1A1 \) in the absence of TCDD also depending on AHR. Several lines of evidence support this last claim: constitutive levels of AHR-responsive genes \( CYP1A2 \) and \( UGT1{^*}06 \) decrease dramatically in AHR null mice as compared with wild type mice (2) and a substantial decrease of \( CYP1B1 \) basal transcription is also observed in primary AHR-/- MEF when compared with AHR+/+ MEF (31). To provide additional support for this notion, we used real time QPCR to compare \( CYP1A1 \) mRNA levels in Hepa-1 cells with those in c35, a mutant derivative of Hepa-1 which contains a mutant, substantially inactivated form of AHR (32). We found indeed that in the absence of TCDD, c35 had much less \( CYP1A1 \) mRNA than Hepa-1 (Figure 3F). The measurable level of \( CYP1A1 \) transcription in the absence of the exogenous ligand is probably due to the effect of an endogenous ligand in Hepa-1 cells (33) or to the presence of a small amount of AHR inside the nucleus at any given time resulted from trafficking of the receptor between the cytoplasm and nucleus (34).
Mediator associates with the murine $CYP1A1$ enhancer in vivo in a TCDD dependent fashion.

We carried out Chromatin Immunoprecipitation (ChIP) experiments to investigate whether the Mediator complex is recruited to the endogenous the $CYP1A1$ gene upon its transcription activation. Hepa-1 cells were treated with DMSO or TCDD for 60 minutes, followed by formaldehyde crosslinking. Cells were lysed in lysis buffer and sonicated so that the size of the majority of chromatin fragments were reduced below 600 bps. Antibodies against Med220 and CDK8 were then used in order to pull down their associated chromatin fragments. Reversal of crosslinking and deproteinization were then carried out. DNA fragment were purified and amplified by PCR with primers flanking the m$CYP1A1$ enhancer region (Figure 4A). We thereby detected TCDD dependent enhancer association for both Med220 and CDK8 (Figure 4B), indicating that the Mediator was recruited to the $CYP1A1$ enhancer region via AHR/ARNT. Interestingly, studies from several different laboratories have demonstrated that although sharing the majority of subunits with the Mediator complex, some smaller complexes devoid of certain subunits, such as CyclinC and CDK8, do exist (15, 35). The heterogeneity of the Mediator may provide an additional layer of modulation to different transcription activators and promoters. Our results indicate that in the context of $CYP1A1$ transcription, the large complex of Mediator is utilized.

Similar ChIP experiments in Med220+/+ mouse embryonic fibroblasts (MEF) demonstrated TCDD inducible binding of Med220 and AHR to the $CYP1A1$ enhancer in these cells (Figure 4C). However, no Med220 association with the enhancer was observed in Med220-/- MEF, although TCDD-inducible binding of AHR to the enhancer still
occurred (Figure 4C). Moreover, TCDD inducible binding of CDK8 to the enhancer could only be detected in Med220+/+ MEF (Figure 4C). This strongly suggests that Med220 is critical for the recruitment of the Mediator complex by AHR/ARNT. In agreement with the fact that CYP1A1 expression is suppressed in MEFs, ChIP assays in these cells revealed that although receptor binding and Med220 recruitment to the enhancer were readily observed 60 minutes post TCDD treatment, no increase of Pol II occupancy over the promoter could be detected even after 2 hours of TCDD treatment (data not shown), suggesting that AHR is capable of recruiting Mediator even in the absence of a stabilized preinitiation complex over the promoter.

To study the role of the LxxLL motifs of Med220 in the process of recruitment, we performed ChIP experiments in Med220 null MEF stably infected with a mutant Med220 in which both of LxxLL motifs are mutated. As shown in Figure 4D, Med220 with mutated LxxLL motifs can still be recruited to the enhancer in response to TCDD.

Collectively, therefore these results show that Med220 is recruited to the murine CYP1A1 enhancer after TCDD treatment; that the LxxLL motifs in Med220 are not required for recruitment; and that the large complex of the Mediator containing CDK8 is employed.

**Kinetics of assembly of AHR, Med220 and other coactivators at the CYP1A1 enhancer after TCDD treatment.**

Different transcription activators recruit different coactivator complexes in distinct kinetic manners to their target promoters, as is evident from a few well-studied nuclear receptor systems. The estrogen receptor (ER) recruits Med220 and the p/CIP member of
the p160 family of coactivators simultaneously to the estrogen responsive regulatory sequences and these proteins appear to associate and disassociate from the regulatory sequences of the pS2 and the cathepsin D genes in a cyclic fashion, the timing coinciding with receptor DNA binding and Pol II occupancy over the regulatory region (36, 37). In the case of thyroid hormone receptor (TR), the receptor mediated recruitment of p160 family of coactivators precedes Med220 recruitment (38). We carried out ChIP kinetic studies on the murine *CYP1A1* regulatory regions using antibodies against AHR, Med220 and various other cofactors in Hepa-1 cells. The use of sonication conditions that reduced the sizes of the majority of chromatin fragments to below 600 bps enabled us to distinguish binding to the promoter from binding to the enhancer element, located 1 kb from the promoter. We detected AHR binding to the enhancer as soon as 10 minutes following the TCDD treatment, and it reached maximal level in approximately 30 minutes (varying somewhat in different experiments: see Figures 5A and 5B). Receptor binding persisted for 105 minutes without significant fluctuation. Interestingly, Med220, p/CIP and p300 were recruited to the enhancer rapidly after TCDD treatment, with similar kinetics as each other and AHR (Figure 5A). As with AHR, there were no consistent fluctuations in binding of these coactivators to the enhancer over a 105 minute time period after TCDD treatment. Neither AHR nor Med220 was detected at the promoter (Figure 5C) suggesting that the Med220 is probably located more proximal to the enhancer than to the promoter in the multimeric protein complex that is associated with the *CYP1A1* gene during transcriptional activation. TCDD treatment stimulated association of Pol II with the promoter (Figure 5C), but as expected, not with the enhancer (Figure 5A). The kinetics of binding of Pol II to the promoter was very similar to the kinetics of AHR, Med220, p300 and p/CIP binding to the
enhancer (Figure 5C). A ChIP reimmunoprecipitation experiment using p300 and Med220 antibodies sequentially was performed (Figure 5D). Briefly, cross-linked chromatins from TCDD treated Hepa-1 cells were first immuno-precipitated with the p300 antibody. The immuno-complex was eluted and subjected to immuno-precipitation with the Med220 antibody. Our results indicate that p300 and Med220 can co-occupy the same enhancer element.

These data suggest that though targeting similar pool of coactivators, AHR recruits them to chromatin with a different kinetic profile from those nuclear receptors that have been studied (36, 37, 38).
Discussion:

Mammalian transcriptional activation is multi-faceted in the sense that inputs from different modulators influence final read-outs. Repression by nucleosomes needs to be overcome in order to allow promoter sequences to be accessible to the general transcription machinery, and in some cases to facilitate binding of transcription factors to their cognate DNA sequences in native chromatin. Complexes containing ATP-dependent chromatin remodeling activities such as mammalian SWI/SNF complexes, as well as histone modifying enzymes such as p300/CBP (39), pCAF (40) and the p160 coactivator family of cofactors (41, 42, 43) have been shown to either independently or cooperatively mediate remodeling of targeted chromatin structures. Although active transcription can be achieved in vitro on a naked DNA template using general transcription factors (GTFs) and core Pol II, additional cofactors are needed to respond to stimulation by activators (10, 11). The Mediator complex purified from yeast (10) and later from mammalian cells contributes towards this role.

Our study demonstrates that AHR can interact with the Mediator complex and that the Med220 subunit is directly involved in AHR-dependent mediated transcriptional activation of the CYP1A1 gene. It is interesting that although AHR belongs to the bHLH-PAS family of transcription factors rather than the nuclear receptor family, these different types of transcription factors target similar pools of transcription coactivators. Previous studies indicate that several known nuclear receptor cofactors including p300/CBP, BRG-1 and three p160 family members are also utilized by AHR. Now we extend this list with Med220. It is important to mention that in contrast to the nuclear receptors, AHR’s ligand binding domain (LBD) has no intrinsic transcription activation capacity and that its rather
complex transcription activation domain is located elsewhere in the molecule. Three subdomains of AHR’s TAD can turn on transcription individually and also synergistically when combined together (44, 45, 46). Coimmunoprecipitation experiments between endogenous AHR and the endogenous Mediator subunits Med220 and Med130 indicate that the interaction of Med220 with AHR probably occurs in the context of an intact Mediator complex. Using the ChIP assay, recruitment of the intact Mediator complex was supported to the demonstration of TCDD-inducible association of Med220 and CDK8 with the \textit{CYP1A1} enhancer in vivo. Consistent with the lack of requirement for the LxxLL motifs for the enhancement of AHR-dependent transcription elicited by Med220, the LxxLL motifs were also not required for TCDD-induced association of Med220 with the endogenous \textit{CYP1A1} enhancer. The above scenario is quite different from the interaction of SRC-1 with AHR, where SRC-1 interacts with the Q-rich subdomain of AHR and there is a dependence on the LxxLL motif of SRC-1 (28). It also contrasts with the observation that the LxxLL motifs of Med220 are required for its coactivator activity with several nuclear receptors.

Our observations and those of other investigators on the various coactivators, including the Mediator, suggest that transcriptional cross-talk between AHR and nuclear receptors could occur at multiple levels. Endocrine disruption is a well-known adverse effect of TCDD, and is mediated through AHR. TCDD has been shown to antagonize functions of the estrogen receptor (ER) (47), androgen receptor (AR) (48), progesterone receptor (PR) (49), PPAR\(\gamma\) (31) and possibly VDR (50). Med220 mediates interaction between the Mediator complex and NRs to help assemble the preinitiation complex. Interference between AHR/ARNT and NRs at this crucial step of transcription activation
could contribute to endocrine disruption by TCDD, in addition to other proposed mechanisms, such as enhanced ligand metabolism (51), down regulation of nuclear receptor levels (52) and altered hormone synthesis (53).

To examine the contribution of Med220 to AHR regulated transcription, we monitored CYP1A1 induction after selective reduction of endogenous Med220 using RNAi. Transient knock-down of Med220 using RNAi not only allowed us to study the effect in cell lines highly responsive to TCDD, but also potentially minimized possible perturbation or compensatory effects occurring after elimination of Med220 in long term experiments. Our data show that RNAi reduction of Med220 in HepG2 cells selectively compromised AHR regulated CYP1A1 transcription. However, CYP1A1 induction was not completely abolished. There could be several mechanisms responsible for this remaining activity, including the effects of residual amounts of Med220, or the possibility of weak association between Mediator and AHR via additional subunits other than Med220. A parallel observation was made with TR, showing that it retains a small yet significant amount of transactivation in Med220-/- MEF when compared to Med220 wild type MEF (30).

Transcription of CYP1A1 in the uninduced state is silent. Positioned nucleosomes at the promoter block transcription. TCDD treatment induces chromatin remodeling over the promoter and rapid activation of transcription. The TAD of AHR is indispensable for this process. The remodeled state of chromatin at the promoter is stable after induction rather than being transient (9). Our kinetic ChIP studies on cofactor occupancy provide a means by which AHR mediated coactivator complex assembly can be examined during induction. The kinetics of recruitment of Med220 to the enhancer could not be distinguished from that of p/CIP, p300 or even AHR. Association of all these proteins with the enhancer was
detected as early as 15 minutes after TCDD treatment, reached a maximum after about 30 minutes, and stabilized thereafter. Pol II occupancy over the promoter mirrored the recruitment of these coactivators. Our observations are consistent with previous nuclear run-on assays indicating that a maximal transcription rate is achieved within 30-40 minutes after TCDD treatment of Hepa-1 cells and is maintained for several hours (54, 55) and a previous restriction enzyme accessibility/micrococcus nuclease sensitivity study (8, 9) indicating a persistent open chromatin configuration and sustained maximum transcription rate after TCDD treatment of Hepa-1 cells. Our results indicate that the dynamics of cofactors association with the \textit{CYP1A1} enhancer is different from that of ER and TR. Med220 and a p160 coactivator associate and dissociate in unison on estrogen responsive promoters/enhancers in a cyclic manner similar to the estrogen receptor, whereas the recruitment of p300 is transient. In the case of TR, the p160 coactivator and p300 occupancies are rapid but not sustained, returning to the basal level after 2 hours of ligand treatment: Med220 recruitment follows a much slower kinetics. The unique kinetic profile of p300 in the case of AHR may be a reflection of different roles of p300 in AHR and NR mediated transcription. Our study reveals an integral role for Med220 in AHR driven transcription. Given the multimeric composition of the Mediator complex and potential tissue-specific variation and modification of its subunits, this complex may play a significant role in determining tissue and developmental variations in response to ligands of AHR.
References:


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Figure Legends:

Figure 1. Endogenous AHR interacts with components of the Mediator complex.

A. Nuclear extracts from DMSO- or TCDD-treated Hepa-1 cells were immuno-precipitated with AHR antibodies. Immuno-complexes were fractioned by SDS-PAGE followed by western blotting with AHR, Med220 and Med130 antibodies. B. Nuclear extract from DMSO-treated Hepa1 cells were immuno-precipitated with ARNT antibody and western blotting was performed using ARNT, Med220 and Med130 antibodies.

Figure 2. Med220 wild type and Med220 LxxLL mutants can enhance AHR-dependent reporter gene transcription.
A. Schematics of Med220 and mutant forms in which the two LxxLL motifs in Med220 were replaced with LxxAA either individually (mutant A and B) or together (mutant D). Hepa-1 cells (B) and HepG2 cells(C) were cotransfected with 2 µg of each Med220, 200 ng of rat CYP1A1 regulatory sequence driven firefly luciferase reporter (pGL-CYP1A1) and 40ng of the HSV thymidine kinase promoter driven Renilla luciferase reporter (pRL-TK). 24 hours after transfection, cells were treated with DMSO or TCDD (10⁻⁸M for Hepa-1 cells and 5× 10⁻⁸M for HepG2 cells). Firefly luciferase activities normalized with renilla luciferase activities were determined 16 hours later.

Figure 3. Reduction of Med220 by siRNA in HepG2 cells diminishes CYP1A1 induction by TCDD.

A. HepG2 cells were transfected twice with siMed220 or the scrambled RNA duplex at 24-hour interval during a 48-hour time span. Following 48-hour siRNA-transfections, cells were treated with either DMSO or TCDD for 6 hours. Total RNA was isolated with Trizol and subjected to reverse transcription. Med220 mRNA level normalized to that of β-actin was measured by real time PCR using primers specific for human Med220 and human β-actin. Whole cell extracts were also prepared from HepG2 cells not treated with TCDD and equal amounts of proteins were subjected to western blotting with Med220 or AHR antibodies (panel B). C HepG2 cell were cotransfected with siMed220 or the scrambled RNA duplex SC, 200ng pGL-CYP1A1 firefly luciferase reporter and 40 ng pRL-TK renilla luciferase reporter. 36 hours post transfection, cells were treated with DMSO or 5 x 10⁻⁸M TCDD for 16 hours and reporter activities were determined. D. Similar procedures were performed as in panel C except that the DNA plasmid cocktail contains 50 ng of GAL4-
VP16 expression construct, 200 ng GAL4 firefly luciferase reporter and 40 ng pRL-TK renilla luciferase reporter.  

E. Similar procedures were carried out as in panel A except that the mRNA level of CYP1A1 normalized to that of β-actin was measured by real time PCR using primers specific for human Med220 and human β-actin.  

F. c35 and Hepa-1 were treated with DMSO or TCDD for 10 hours, total RNA were isolated using Trizol and subjected to reverse transcription. CYP1A1 mRNA level normalized to that of β-actin was measured by Real time PCR using primers specific for murine CYP1A1 and β-actin.

Figure 4. Components of the Mediator complex are recruited to the murine CYP1A1 enhancer in a TCDD-dependent fashion.  

A. Schematic of the murine CYP1A1 enhancer located 1kb upstream of the TATA box and locations of the primers used to amplify the enhancer region.  

B. Chromatin preparations from DMSO- or TCDD-treated Hepa-1 cells were crosslinked and subjected to sonication and immunoprecipitation with the indicated antibodies. PCR analysis was performed on the immunoprecipitates using primers flanking the enhancer region of the murine CYP1A1 gene.  

C. Similar procedures were performed on Med220+/+ and -/- MEF.  

D. Similar procedures were carried out on Med220-/- MEF stably expressing Med220 LxxLL mutant D and Med220+/+ MEF.

Figure 5. Kinetics of cofactor recruitments at the murine CYP1A1 enhancer and promoter.  

A. Hepa-1 cells treated with TCDD for various amount of time were subjected to ChIP assays as in Fig. 3, PCR reactions were performed using primers flanking the CYP1A1
enhancer. Antibodies used in the ChIP analysis are indicted on the right of each panel. B. Similar experiments as in A were carried out except that shorter time points were used.

C. ChIP products from A were subjected to PCR reactions with primers flanking the TATA box of murine *CYP1A1*. D. ChIP was first carried out using the p300 antibody (first lane), the immuno-complex was eluted by 10mM DTT. The elution was diluted and immuno-precipitated with the Med220 antibody (second lane). ChIP products were subjected to PCR reactions using primers flanking the enhancer of murine *CYP1A1*. 
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**Fig. 1A**

![Image of protein bands for AHR, Med220, and Med130](image)

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**Fig. 1B**

![Image of protein bands for Med220, Med130, and ARNT](image)
Fig. 2A

Fig. 2B

Fig. 2C

Hepa1

HepG2

Med220 wt
Med mutA
Med220 mutB
Med220 mutD
Fig. 3A

Fig. 3B
Fig. 3C

Fig. 3D
Fig. 3E
Fig. 3F
The enhancer contains functional XREs

mCYP1A1

Fig. 4A

TCDD - +

αMed220

αCDK8

Input

Fig. 4B
Fig4C
Fig. 4D

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Med220-/- MEF stably expressing LxxLL mutated Med220

wt MEF
mCYP1A1 Functional XREs Promoter

-1 kb

CYP1A1 enhancer

0’ 15’ 30’ 45’ 60’ 75’ 90’ 105’ TCDD

AHR

Med220

Pol II

p300

P/CIP

Input

Fig. 5A
Fig. 5B
CYP1A1 promoter

0’ 15’ 30’ 45’ 60’ 75’ 90’ 105’ TCDD

- AHR
- TRAP220
- Pol II
- Input

Fig. 5C
Fig. 5D