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Signaling via the bHLH-PAS proteins AhR and HIF

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For my family, - past, -present and future

ABSTRACT

This thesis concerns some mechanistic properties of the basic helix-loop-helix/PAS (bHLH-PAS) factors aryl hydrocarbon receptor (AhR) and hypoxia-inducible factor 1 alpha (HIF-1α). The bHLH-PAS family of proteins is a family of factors that controls a variety of developmental and physiological events. A common feature for this family of proteins is that they act mainly as intracellular transcription factors. They bind to DNA as a heterodimeric complex, usually together with a bHLH-PAS protein belonging to the aryl hydrocarbon receptor nuclear translocator (ARNT) subfamily. The AhR bind ligands that are environmental pollutants, as well as possibly physiological compounds occurring in the diet. Known functions of the ligand-activated AhR include activation of genes involved in xenobiotic metabolism and an ubiquitin ligase activity targeting nuclear receptors (such as the estrogen receptor) and beta-catenin. HIF-1α mediates signal transduction and gene regulation in cells exposed to deprived oxygen conditions (hypoxia).

In paper **I**, we have shown that the Ah-receptor can be activated by stimulus other than xenobiotics, e.g dioxin. AhR is recruited to target genes in both ligand treated and in suspension culture, suggesting a common mechanism of activation between these two routes of AhR activation. The gene expression profiles critically differ between xenobiotic and suspension activated AhR signaling. The classical xenobiotic metabolizing AhR targets such as *Cyp 1a1*, *Cyp 1b1* and *Nqo* were regulated by both ligand and suspension conditions. Sequence analysis coupled with ChIP assays and reporter gene analysis identified a functional xenobiotic response element (XRE) within the mouse *TIPARP* gene that features a concatamer of 4 XRE cores residing in the first intron.

In paper **II** we have shown that ectopic expression of ARNT, in mammalian cells and yeast cells, was sufficient to promote nuclear accumulation of the Ah-receptor in a ligand-independent manner. We further observed that overexpression of *ARNT* promotes derepression of Ah-receptor function in the absence of ligand, thereby possibly representing an alternative mechanism of activation that is distinct from activation by xenobiotic ligands and thus may be of physiological relevance. We also describe that an excess of *ARNT* in relation to the Ah-receptor and HIF-1α promotes derepression of the receptor and stabilization of HIF-1α *in vivo* and *in vitro*, representing a possible alternative mechanism of activation of bHLH-PAS proteins.

LIST OF PUPLICATIONS

These papers are the basis for this thesis and will be referred to by their Roman numerals.

I Nan Hao, Kian Leong Lee, Sebastian G.B. Furness, **Cecilia Bosdotter**, Lorenz Poellinger and Murray Whitelaw
Xenobiotics and loss of cell adhesion drives distinct transcriptional outcomes by aryl hydrocarbon receptor signaling
In press.

II **Cecilia Bosdotter**, Jaqueline McGuire, Sarah Karttunen, Kensaku Okamoto, Katarina Gradin and Lorenz Poellinger
Role of the Arnt transcription factor in activation of functional activities of the aryl hydrocarbon receptor and the hypoxia-inducible factor 1 alpha.
Manuscript

LIST OF ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
AhRR	Aryl hydrocarbon receptor repressor
ARNT	Ah-receptor nuclear translocator
bHLH	Basic helix-loop-helix
CBP	CREB-binding protein
CTAD	C-terminal transactivating domain
EPO	Erythroietin
HPH	HIF prolyl hydroxylase
HRE	Hypoxia response element
HSP/HSC	Heat shock protein genes
HIF -1α	Hypoxia inducible factor 1α
3MC	3-methylcholantrene
NTAD	N-terminal transactivating domain
PCDDs	Polychlorinated dibenzo-p-dioxins
PAS	Per /Arnt /Sim
PAH	Polycyclic aromatic hydrocarbon
Per	Period
PHD	Prolyl-hydroxylase domain
Sim	Single-minded
TCDD, dioxin	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
VEGF	Vascular endothelial growth factor
pVHL	von Hippel-Lindau protein
XRE	Xenobiotic response element

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INTRODUCTION

Aryl hydrocarbon receptor (AhR) signaling

Industrial processes and organic combustion are sources for polycyclic aromatic hydrocarbons. One such polycyclic aromatic hydrocarbon is the deadly poison dioxin, i.e. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, TCDD (Figure 1). Diet, skin contact or inhalation can expose animals and humans to these compounds. Endogenous molecules with structural similarity to these compounds exist, e.g. indolo compunds (flavonoids, indigoids and derivatives of the indole-3-carbinole). Events of occupational and accidental exposure to polycyclic aromatic hydrocarbons (PAHs) are a source of environmental and public health concern. The half-life of dioxin in humans is about seven years, illustrating that it represents a persistent environmental contaminant. One important structural feature is shared among aryl hydrocarbon receptor (AhR) ligands: planarity, or the ability to become co-planar (Gillner et al., 1993). Responses to these molecules, e.g. dioxin, beta-naphtoflavone, and 3-methyl–cholanthrene (3MC), can be both adaptive and toxic (Cespedes et al., 2010). The liver produces xenobiotic metabolizing enzymes in response to many of these compounds. The enzymes include the P450 family, a family of drug metabolizing enzymes. Dioxins are difficult to metabolize and they often provoke an additional battery of toxic responses; these include recalcitrant acne (chloracne), tumor promotion, thymic involution, wasting and death (Mimura and Fujii-Kuriyama, 2003). The recalcitrant chloracne is the result of hyperkeratinization and metaplasia of hair follicles and interfollicular epidermis followed by formation of cysts and abscesses (Suskind, 1985; Zugerman, 1990). The lesions can persist for 30 years after severe exposure to polychlorinated hydrocarbons (Moses and Prioleau, 1985).

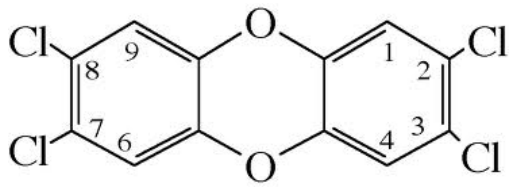

FIGURE 1. Chemical structure of dioxin or 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD).

The AhR binds PAHs and functions as a ligand-activated transcription factor. Ligand binding increases nuclear accumulation of the AhR receptor via a process presumed to involve structural transformation and/or nuclear localization signal presentation (Kazlauskas et al., 2000). Once in the nucleus, the PAS domain of AhR mediates dimerization with the corresponding PAS domain of a constitutively nuclear protein known as ARNT (Figure 2). This complex binds xenobiotic response elements (XREs) of target genes and recruits several coregulatory proteins, including NCOA1 and

NCOA3, p300 (Lee et al., 2000) and the general transcription factor transcription factor IIB (TFIIB) that is required for transcriptional initiation by RNA polymerase II (Beischlag et al., 2002; Hankinson, 2005).

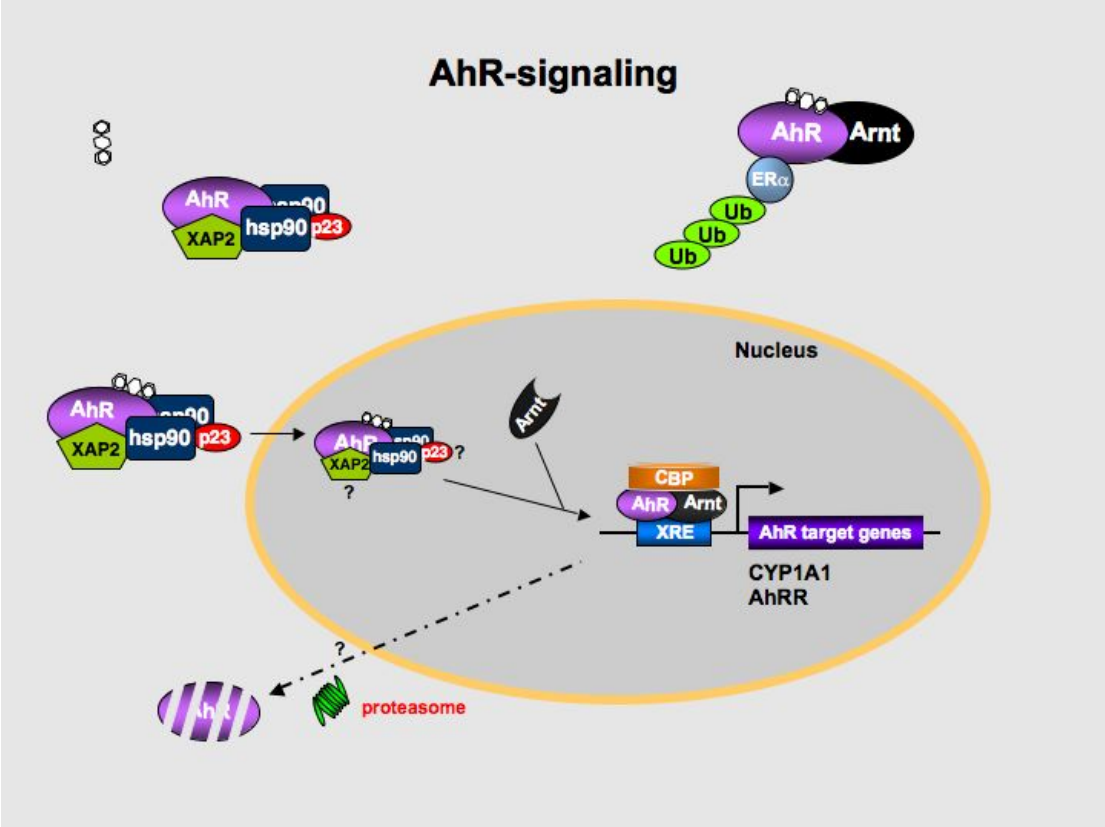

FIGURE 2. A schematic model of the AhR signaling and degradation pathways. The AhR is both a ligand-activated transcription factor and a ligand-dependent ubiquitin E3 ligase targeting itself and substrate proteins such as steroid receptors and β-catenin for proteasomal degradation. Upon ligand activation the receptor translocates from the cytosol to the nucleus, disassociates from hsp90 and dimerizes with the partner factor ARNT. Subsequently, the AhR/ARNT complex binds to xenobiotic response elements (XREs) in the promoter of target genes such as *CYP1A1* and *AhRR*. See text for details.

The AhR belongs to the basic helix-loop-helix/PAS (bHLH-PAS) family of factors. PAS is an acronym based on the founding members of this family of proteins, i.e. in *Drosophila*, period (PER), single-minded protein (SIM), and mammalian ARNT. The functional architecture of the bHLH-PAS proteins is characterized by the N-terminally situated basic region, responsible for DNA binding, followed by the HLH domain, which, together with the PAS domain is important for dimerization specificity and stability (Figure 3).

The AhR seems to have a limited set of target genes (please see below, Paper 1) but these genes include the drug metabolizing enzymes such as CYP1A1 that are induced by dioxin. The AhR also induces expression of an aryl hydrocarbon receptor repressor

(*AhRR*) - an inhibitor of AhR. The AhRR lacks a transactivation domain and works like a repressor: it dimerizes with ARNT and acts as a repressor through steric hindrance at the level of DNA binding and also through its endogenous transrepressor capacity (Evans et al., 2008). The repressor has three SUMOylation sites that are conserved across vertebrate species. All three sites must be SUMOylated for full suppressive activity (Oshima et al., 2009). Prolonged ligand-dependent activation of AhR induces its repressor to displace it and to actively repress its targets. The *AhRR* promoter has functional XRE sequences, and gene expression is enhanced upon ligand activation of AhR, resulting in the inhibition of AhR signaling activity (Baba et al., 2001; Mimura et al., 1999)

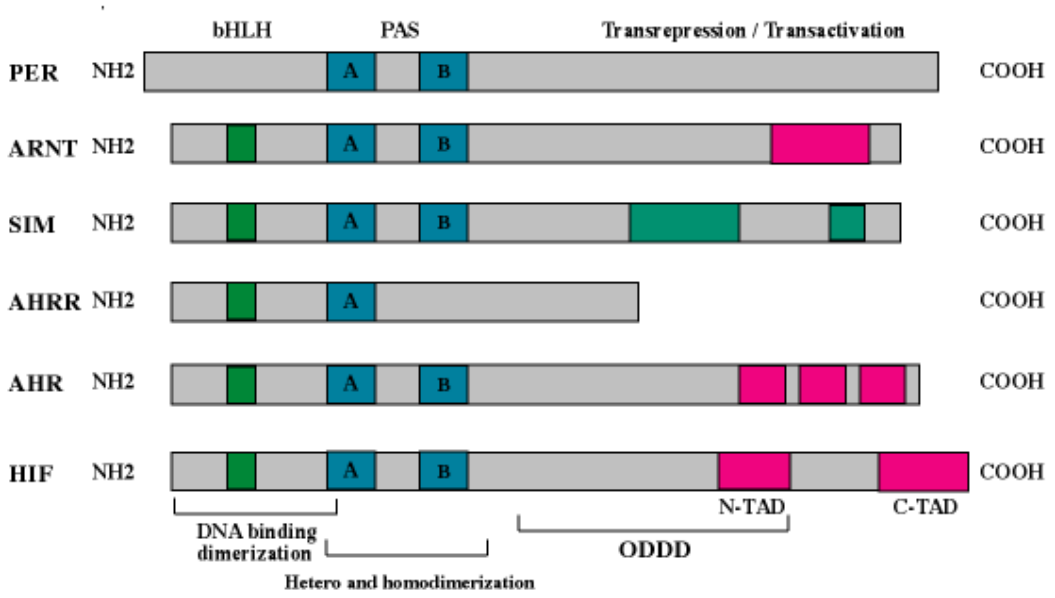

FIGURE 3. Different members of the bHLH-PAS protein family.
The basic-helix-loop-helix domain (bHLH) is depicted in green, with the PAS repeats in blue. The C-terminal region of these proteins is variable, often containing domains for transactivation or repression. ODDD is short for "oxygen dependent degradation domain" that can be found in the HIFs.

In the absence of ligand the AhR is a cytoplasmic protein associated with chaperons and chaperonins: a dimer of hsp90 and p23 and the AhR associated protein, XAP2 (also known as AIP or ARA9). These chaperonins help the receptor fold correctly and maintain its location in the cytoplasm. The PAS B domain partly mediates interactions between the AhR and these proteins (Figure 2). (Dolwick et al., 1993; Reyes et al., 1992; Swanson and Bradfield, 1993)(Reyes, Reisz-Porszasz et al. 1992; Dolwick, Swanson et al. 1993; Swanson and Bradfield 1993)

Possible physiological functions of the AhR

There is strong evidence that AhR plays significant roles in the immune system (Kerkvliet, 1995). Exposure to TCDD leads to severe thymic involution and profound suppression of both the humoral and cellular immune response, resulting in increased susceptibility to infection. In addition, AhR-mutant mice are defective in T-cell differentiation and are more susceptible to bacterial infection (Kerkvliet, 2009; Stevens et al., 2009). AhR null mice are also protected from the toxic effects of dioxins; treatment with these compounds does not cause birth defects (Peters et al., 1999)

In the presence of ligand the AhR can form an E3 ubitquitin ligase complex with Cullin-4B (CUL4B), damaged DNA binding-1 (DDB1), transducin beta-like 3 (TBL3) and ringbox protein 1 (RBX1)/ROC and catalyze the ubiquitylation of the sex steroid hormone receptors, estrogen receptor-α (ER-α), estrogen receptor-β (ER-β), and the androgen receptor (AR), as well as AhR itself (Ohtake et al., 2007). It was recently found that this complex also ubiquitylates β-catenin and functions as a tumor suppressor in the colon (Kawajiri et al., 2009).

Hypoxia Signaling

Our understanding of the molecular physiology of oxygen homeostasis and how it is regulated and dysregulated has increased dramatically during the last two decades.
In the middle of the 1990's the hypoxia inducible factor-1α (HIF-1α) was cloned as the protein that bound to the EPO enhancer together with its partner factor ARNT (Jelkmann and Hellwig-Burgel, 2001; Jiang et al., 1996). Additional components of the intracellular signaling mechanisms were subsequently characterized, and today we have a good understanding of the core signaling machinery that converts the hypoxic stimulus to gene regulatory events in the nucleus.

Cellular functions of HIF

HIF-1α is rapidly degraded under normoxic conditions and is stabilized in response to hypoxia. It was for long unknown what is the oxygen sensor. However, the observations that proline residues within the degradation domain of HIF-1α (termed ODDD, Figure 3) and the cloning of intracellular proline hydroxylases provided a stringent model of intracellular oxygen sensing (Bruick and McKnight, 2001; Epstein et al., 2001; Jaakkola et al., 2001; Mole et al., 2001). Under normoxic conditions the hydroxylated proline residues of HIF-1α are recognized by the VHL protein which functions as an E3 ubiquitin ligase (Figure 4). Cells deficient in functional VHL protein show constitutively high HIF-1α protein levels and constitutive expression of many oxygen-regulated genes. In VHL disease this leads to highly vascularized renal cancer and hemangioblastoma formation (Maxwell et al., 1999; Ohh et al., 2000; Tanimoto et al., 2000).

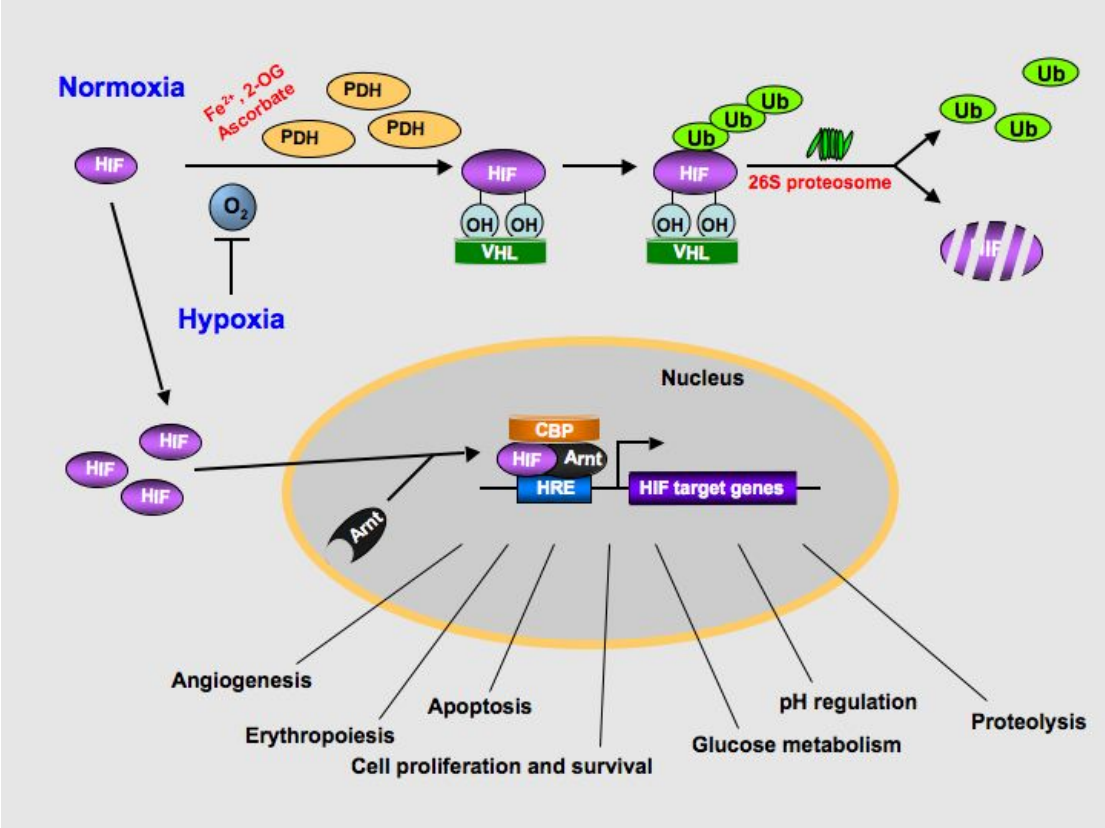

FIGURE 4. The HIF signaling pathway.

The HIF target genes all mediate adaptive responses to hypoxia e.g. EPO (erythropoietin), several glycolytic enzymes, VEGF (vascular endothelial growth factor). There are two proline residues in the oxygen dependent degradation domain that are hydroxylated by proline hydroxylases (PDHs) under normal oxygen conditions. The hydroxylated prolyl residues bind the von Hippel-Lindau protein (pVHL), leading to ubiquitination and subsequent proteasomal degradation. The stabilized HIF-α protein accumulates in the nucleus, interacts with ARNT, binds to hypoxia responsive elements (HREs) of target genes, and recruits coactivator proteins such as p300/CBP to increase gene transcription and mediate adaptive responses to hypoxia.

In analogy to the AhR, HIF-1α interacts with the molecular chaperone hsp90 which helps HIF-1α to adapt and maintain the correct structure (Katschinski et al., 2004; Liu et al., 2007). Both the AhR and HIF-1α interact with hsp90 via the C-terminus of their bHLH-PAS domains (termed the PAS B domains, Figure 3).

Hypoxia in physiology and pathology

HIF plays a major role in normal development of an organism. HIF-deficient mice (knockout of HIF-1α or ARNT protein) show early lethal phenotypes (around day E8.5-10.5) with a collapsed vascular development (Carmeliet et al., 1998; Maltepe et al., 1997; Tian et al., 1998). HIF signaling plays a significant role in a number of pathophysiological settings, such as inflammation, cardiovascular diseases, tumor metabolism, stroke and tumor metastasis (Acker and Acker, 2004; Anagnostopoulos et al., 2008; Zinkernagel et al., 2007)

HIFs come in many different flavors

There are a number of HIF paralogues: HIF-1α, -2α, -3α. HIF-3α is alternatively spliced to give rise to inhibitory PAS protein (IPAS) (Makino et al., 2001). IPAS lacks a trans-activating domain and functions as a negative regulator of HIF-1α. It is expressed in the cornea epithelium and plays a role in maintaining an avascular phenotype of this tissue.

The physiological role of HIF-3α still remains to be elucidated, however it has been shown that expression of HIF-3α during embryonic and neonatal stages is important for negative regulation of endothelin-1 expression since there is massive pulmonary hypertension and cardiac hypertrophy in HIF-3α-deficient mice (Yamashita et al., 2008). At least five different splice variants may be expressed from the human HIF-3α locus that are suggested to exert primarily negative regulatory effects on hypoxic gene regulation (Tanaka et al., 2009).

Hsp90 is known to bind HIF-1α , and hsp90 inhibitors have been shown to inhibit tumor growth and to induce proteasomal degradation of HIF-1α, even in cells lacking pVHL (Isaacs et al., 2002). RACK1 was shown to compete with hsp90 for binding to the PAS A – domain of HIF-1α. Treatment with an hsp90 inhibitor such as geldanamycin results in unopposed RACK1 binding leading to increased ubiquitination and degradation of HIF-1α. The ability of hsp90 inhibitors to induce HIF-1α degradation is dependent on *RACK1* expression (Liu et al., 2007).

ARNT - a master regulator of bHLH PAS proteins

As outlined above, ARNT interacts with both HIF-1α and the. The ARNT protein is also capable of forming a homodimer with binding preference for the palindromic E-box sequence, CACGTG (Swanson and Yang, 1999). It was recently shown, both *in vitro* and *in vivo*, that ARNT as a homodimer can regulate the *CYP2a5* promoter (Arpiainen et al., 2007). The CYP2a5 was found to be important in murine primary hepatocytes, and is involved in degradation of bilirubin, a breakdown product of heme. The CYP2a5 is speculated to be involved in nicotine-degradation as well as nitrosamines and aflatoxins (Nakajima et al., 1996; Pelkonen et al., 1997).

The tissue distribution of ARNT is ubiquitous. In contrast to HIF-1α and AhR, ARNT is not regulated by ligands or by hypoxia. There are several paralogues of ARNT: ARNT (= ARNT1), ARNT2, and ARNT3. Only ARNT and ARNT2 can dimerize with HIF-1α. The role, if any, of ARNT3 (MOP3) in hypoxia signaling is unclear. It has been shown that ARNT3 interacts with CLOCK to regulate circadian rhythms. Mice lacking ARNT3 show severe deficits in circadian rhythmicity (Cowden and Simon, 2002).

AIM OF THE STUDY

The aim of the study was to investigate if ligand-dependent activation of the AhR results in activation of different target genes in comparison to ligand-independent activation of AhR function upon loss of cell-cell contacts (suspension culture; Paper I). Possibly, this information could shed light on the physiological function of the AhR. Secondly, based on preliminary observations that an excess of ARNT induces nuclear accumulation of both the AhR and HIF-1α, we were interested in investigating whether ARNT can provide a mode of regulation of these proteins under conditions of no environmental pollution or hypoxic stress (Paper II).

METHODOLOGICAL CONSIDERATIONS

Reconstitution of AhR function in Yeast

Sacchromyces cerevisiae, or bakers yeast, is recognized as an ideal eukaryotic microorganism for biological studies. Despite the small genomic size, $1{,}4x10^7$ bp, the haploid nuclear DNA content is only 3.5 times that of E. Coli, yeast displays most of the cellular house keeping features of higher eukaryotes. Many cellular processes, like cell cycle regulation and the basic transcriptional machinery are structurally and mechanistically conserved among different species. Some of the properties that make yeast particularly suitable for biological studies include rapid growth, the ease of replica plating and mutant isolation, and a highly versatile DNA transformation system. Plasmids can be introduced into yeast either as replicating molecules or by integration into the genome. We used yeast to study the activation of AhR by an excess of ARNT. Important is also the ability of certain ligands, in particular the dietary indole derivative indolo[3,2-b]carbazole to activate the AhR in *S. cerevisiae*

Western blot

Western blot or immunoblot is the golden standard of molecular biology methods to detect and identify specific proteins with antibodies. The first step at a western blot analysis is to purify the protein content from a cell or a tissue of interest. The proteins are then separated by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), together with a known molecular weight marker according to size. SDS binds and denatures proteins and make them negatively charged. Subsequently the proteins are transferred to a support, e.g. a membrane (most commonly a nylon or a nitrocellulose membrane). To check the quality of the transfer the membrane can be stained with Ponceau S solution staining proteins. To reduce nonspecific binding of antibodies the membrane is blocked with nonfat milk powder or bovine serum albumin. After the blocking the membrane is incubated with a primary antibody that recognizes the protein of interest. In order to detect the primary antibody, a second antibody coupled to an enzyme, directed against the primary antibody, is added. Finally a substrate for the conjugated enzyme is used to detect the protein. Even though the primary antibody is made for detection of a specific protein it is always a risk that the antibody will bind to other proteins. Both monoclonal and polyclonal antibodies can be used for Western blot analysis but monoclonal antibodies react only with one epitope and are considered to be more specific to the protein of interest. On the other hand, a good polyclonal antibody has higher sensitivity. Washing the membrane thoroughly after each antibody application is also important to avoid nonspecific binding of the antibodies. This is usually done with a phosphate or Tris buffer together with the detergent Tween 20.

Quantitaive PCR

Quantitative PCR is a method to measure a certain amount of DNA or RNA in cells or tissue. Most PCR methods amplify the template from minute starting materials, and end with measurable amounts. qPCR detection methods are based on changes in fluorescence, which are proportional to the amount of increased target (quantitative) and the process is measured as time goes, so called real-time PCR. RNA is a very labile molecule and does not survive the high temperature that is necessary for PCR run. Therefore the RNA has to be converted to cDNA by using reverse transcriptase. The

measurement can be done in two different ways, either by absolute measurement or by a ratio in relation to another gene, usually a gene that exists in most cell types (a housekeeping gene). Commonly used housekeeping genes are, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), β-actin, or hypoxanthine-guanine phosphoribosyltransferase (HPRT). To calculate how many copies of RNA or DNA that are in the starting material, a standard curve using material with already known amounts of cDNA or DNA has to be run parallel with the sample.

Chromatin immunoprecipitation (ChIP)

ChIP is a powerful method in connecting transcription factors, co-activators, repressors or DNA-modifications to a specific endogenous DNA sequence as well as for examining protein-DNA interactions at a genome-wide scale. The result is often very useful to postulate the function of a specific transcription factor or histone modification. Despite the excellent value of ChIP assay, it is important to be aware of the limitations. One big issue of the ChIP method is the antibody specificity and quality of different batches. Also blocking of epitopes caused by cross-linking and varying quality of chromatin fragmentation could affect the result of ChIP analysis. In brief, ChIP involves formaldehyde treatment of a cell population to cross-link proteins to their target DNA, cell lysis, sonication of the chromatin to 300-500 bp fragments, and then immunoprecipitation of specific protein/chromatin complexes using an antibody against the protein of interest. After removal of the cross-links, various methods of analysis of bound DNA can be used, e.g. PCR amplification, microarray or sequencing.

RESULTS AND DISCUSSION

Paper I

Even though the AhR was activated by different modes, i.e. by ligand treatment of upon loss of cell-cell contacts in suspension culture, a similar battery of AhR-specific target genes was induced. For instance, genes encoding drug metabolizing enzymes such as *Cyp1A1* and *Cyp1B1* and *Nqo1* (NADPH- quinone oxidoreductase1) were induced in a similar fashion by both modes of activation. These genes represent classical already known AhR target genes. Interestingly, the identified AhR-specific battery of target genes remained relatively small even if the mode of AhR activation was switched to a ligand-independent mode. However, there were some subtle differences between the two modes of activation. *Po*r (P450 cytochrome oxidase) and *Cldnd 1* were regulated predominately by ligand treatment, while in contrast, *ApoER2* (Apolipoprotein E receptor 2) and *Ganc* (neutral alpha-glucoside C) were regulated predominately by the suspension condition. In addition to activation of AhR target genes, suspension culture induced (as expected) expression of a large number of target genes in an AhR-independent manner, presumably due to activation of other distinct signaling pathways. *Tiparp* (TCDD-inducible poly[ADP-ribose] polymerase) represents an AhR target gene that is not encoding a drug metabolizing enzyme but is induced in a similar fashion by the two different modes of receptor activation. Sequence analysis coupled with AhR chromatin immunoprecipitation (ChIP) assays and reporter gene analysis identified a functional XRE within the mouse *Tiparp* gene that features a concatamer of 4XRE cores residing in the first intron about 1.2 kb downstream of the *Tiparp* transcription start site. This work gives novel insights into how AhR signaling drives transcriptional programs via the ligand versus suspension modes of activation.

Paper II

In paper II we observed that AhR and HIF-1α function can be induced in the presence of high levels of ARNT. This is a novel finding and suggests a new way for ARNT to activate its partner factors HIF 1α and AhR in the absence of AhR ligand or hypoxia, respectively. Consistent with these observations, in the absence of AhR ligand and hypoxia, an excess of ARNT induced translocation of either AhR or HIF-1α from the cytoplasm to the nucleus, and stabilized HIF-1α from proteasomal degradation. As a negative control we used the delta basic-helix-loop-helix protein ARNT, which has been shown not to dimerize with the AhR,

Derepression of AhR function by high levels of ARNT in yeast

To further explore regulation of AhR function by ARNT we co-transformed yeast cells with high or low copy number vectors encoding mAhR, ARNT, or, as negative controls, the ARNT mutants ARNT delta basic or delta basic helix-loop-helix. We then monitored activation of AhR function in the absence or presence of AhR ligand. Expression levels of the constructs were monitored by Western blot analysis. We could see a clear pattern where full-length ARNT, but not the truncated versions, was activating AhR to a functional transcription factor in the absence ligand.

Derepression of AhR function and stabilization of HIF-1α by high levels of ARNT in mammalian cells

In the presence of an excess of ARNT but in the absence of AhR ligand or hypoxia we observed activation of AhR and HIF-1α function, as assessed by reporter gene assays (please see above). Immunostaining showed nuclear translocation of endogenous HIF 1α. In agreement with these observations, upon ectopic expression of increasing levels of ARNT together with GFP-fused AhR or HIF-1α in COS cells, nuclear fluorescence can be observed. The subcellular localization of HIF-1α and ARNT was also determined by indirect immunofluorescence. The nuclei were stained with 4-6 diamino-2-phenylindone (DAPI), and we show that the two bHLH –PAS proteins co-localize in the nuclear compartment of the cells, consistent with the activation of HIF-1α function under these conditions.

CONCLUSIONS

This work provides novel insights into how AhR signaling drives transcriptional programs via the ligand versus suspension modes of action. Following either route of AhR activation, AhR-specific target genes remained relatively small and included the classic xenobiotic metabolizing AhR targets such as *Cyp1a1*, *Cyp1b1*, and *Nqo1*. There were some differences in gene expression profiles between xenobiotic and suspension activated AhR signaling. *Por*, and *Cldnd1* were regulated predominately by ligand treatments, while in contrast, *ApoER2* and *Ganc* were regulated predominately by the suspension condition. Temporal expression patterns of AhR target genes were also found to vary, with examples of transient activation, transient repression, or sustained alterations in expression.

The present data establish that an excess of ARNT can induce nuclear accumulation of the AhR and activate its function as a transcription factor in the absence of ligand. In a similar fashion, an excess of ARNT stabilized HIF-1α against proteasomal degradation in normoxia, induced its nuclear accumulation and activated its function as a transcription factor. Thus, these results indicate that modulation of the levels of ARNT or the size of the intracellular pool of ARNT available for dimerization with these partner proteins, may provide an alternative route of activation of these signaling pathways in the absence of environmental or hypoxic stress.

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