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Mechanisms of Activation of the Aryl Hydrocarbon Receptor by Novel Inducers of the *CYP1A1* Gene

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To my family

vägen är målet

ABSTRACT

The aryl hydrocarbon receptor (AhR) functions as a ligand-activated transcription factor that is responsible for the activation of several response genes, of which the best characterised is the *CYP1A1* gene. The prototype high affinity ligand for the AhR is the environmental pollutant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The present study was undertaken in an attempt to elucidate the mechanism of activation of the *CYP1A1* gene by several compounds that recently have been reported to be capable of increasing CYP1A1 levels *in vivo* or in different cellular systems, but are not conventional AhR ligands.

The transcriptional activation of the *CYP1A1* gene by benzimidazoles like omeprazole (OME), thiabendazole, carbendazim and 2-mercapto-5-metoxy-benzimidazole (MMB), as well as the quinoline compound primaquine (PRQ) was shown to involve the AhR. However, rat, mouse and human hepatoma cell lines responded differently to CYP1A1 induction, suggesting that the determinants for AhR-activation differ between species or cell types. The magnitude and duration of induction was much less pronounced as compared to TCDD, indicating that these compounds are weak activators of the AhR. Both MMB and PRQ were capable of transforming cytosolic AhR to a DNA-binding form, as well as of displacing AhR-bound [^{3}H]TCDD *in vitro*, indicating that they are low affinity ligands for the AhR. In contrast, OME was neither capable of transforming AhR to a DNA-bound complex, nor to compete for specific [^{3}H]TCDD-binding, although the potency of OME and MMB to e.g. cause Gal4-AhR activation in rat H4IIE cells was similar.

The involvement of protein kinases in AhR signalling was investigated by using various protein kinase inhibitors. In transfected H4IIE or HepG2 cells, inhibition of OME- or TCDD-dependent Gal4-AhR activation was obtained by using c-src kinase inhibitors as well as by co-transfection of a dominant negative c-src-plasmid, indicating that AhR function is regulated by c-src activity. Three tyrosine kinase inhibitors, genistein, tyrphostin AG17 and tyrphostin AG879, selectively blocked OME- but not TCDD-mediated AhR activation in H4IIE or HepG2 cells. These data strongly support the action of a tyrosine kinase dependent pathway in OME-mediated activation of the AhR, which is different from that of TCDD.

When several different residues in the ligand-binding domain of the AhR was subjected to site-directed mutagenesis, a selective and complete loss of Gal4-AhR activation by OME, MMB and PRQ, but not by TCDD, was revealed by mutation of Tyr^{320} to Phe. Competition of AhR-bound [^{3}H]TCDD by PRQ was completely abolished in extracts form HEK293 cells expressing the mutant Gal4-Y320F protein, while TCDD-binding was unaffected. These findings indicate that different residues in the ligand-binding pocket of the AhR are important for recognition of low and high affinity ligands, alternatively, phosphorylation of Tyr^{320} is critical for activation of the receptor by weak AhR activators, without having a great impact on the activation by high affinity ligands.

In summary, two alternative ways of activation of the AhR by novel CYP1A1 inducers have been demonstrated in this study; i) direct low affinity binding and activation of the receptor by MMB and PRQ, and ii) indirect activation of the receptor in a tyrosine kinase dependent pathway by OME, presumably by a ligand-independent mechanism.

LIST OF PUBLICATIONS

This thesis is based on the following papers and will be referred to in the text by their roman numerals:

I. **Backlund M**, Johansson I, Mkrtchian S, Ingelman-Sundberg M (1997) Signal Transduction-mediated Activation of the Aryl Hydrocarbon Receptor in Rat Hepatoma H4IIE Cells. *J Biol Chem* 272:31755-63.

II. **Backlund M**, Weidolf L and Ingelman-Sundberg M (1999) Structural and Mechanistic Aspects of Transcriptional Induction of Cytochrome P450 1A1 by Benzimidazole Derivatives in Rat Hepatoma H4IIE Cells. *Eur. J. Biochem.* 261:66-71.

III. Werlinder V, **Backlund M**, Zhukov A, and Ingelman-Sundberg M (2001) Transcriptional and Post-Translational Regulation of CYP1A1 by Primaquine. *J Pharmacol Exp Ther* 297:206-214.

IV. **Backlund M**, Ingelman-Sundberg M. Different Structural Requirements of the Ligand Binding Domain of the Aryl Hydrocarbon Receptor for High and Low Affinity Ligand Binding and Receptor Activation. *Manuscript.*

V. **Backlund M**, Ingelman-Sundberg M. Ligand-independent Activation of the Aryl Hydrocarbon Receptor Requires Protein Tyrosine Kinase Activity. *Manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

3MC	3-methylcholantrene
AHH	aryl hydrocarbon hydroxylase activity
AhR	aryl hydrocarbon receptor (also called dioxin or aromatic hydrocarbon receptor)
AhRR	aryl hydrocarbon receptor repressor
arnt	aryl hydrocarbon receptor nuclear translocator
B[*a*]P	bezo[*a*]pyrene
bHLH	basic helix loop helix
CYP	cytochrome P450
EPAS-1	endothelial PAS domain protein-1
ER	estrogen receptor
GR	glucocorticoid receptor
HAH	halogenated aromatic hydrocarbons
Hif-1α	hypoxia inducible factor α
hsp90	heat shock protein 90
LBD	ligand-binding domain
MAPK	mitogen-activated protein kinases
MEF	mouse embryonic fibroblasts
MMB	2-mercapto-5-methoxy benzimidazole
NES	nuclear export signal
NLS	nuclear localisation signal
OME	omeprazole
PAH	polycyclic aromatic hydrocarbons
PAS	Per-Arnt-Sim
Per	period
PI-3K	phosphatidyl-inositol-3-kinase
PKB	protein kinase B (also called Akt)
PKC	protein kinase C
PRQ	primaquine
PTK	protein tyrosine kinase
Rb	retinoblastoma protein
Sim	single-minded
TAD	transactivation domain
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
XRE, DRE	xenobiotic or dioxin response element

INTRODUCTION

GENERAL INTRODUCTION – THE ADAPTIVE RESPONSE

Animals, including humans, are constantly exposed to a multitude of chemicals in the environment. Some of these chemicals are giving the animal valuable information about for instance the presence of food, other members of the kindred or threats, while others are toxic and must be avoided or eliminated. Animals possess a variety of mechanisms to sense and respond to these chemicals. Thus, similar to the adaptive immune system, animals have evolved a chemical surveillance system that recognise a wide variety of chemical structures and initiate an appropriate response. One component of this adaptive surveillance system is the sensory proteins e.g. receptors that detect volatile and soluble chemicals in the environment. The second part of the system constitutes signalling molecules that transduce the signal from the receptor and initiate a biological response, often resulting in induction or activation of enzymes. Finally biotransformation, i.e. metabolism and elimination of the insulting compound can take place with the help of the induced enzymes.

The components of the inducible biotransformation system include monooxygenases of the cytochrome P450 (CYP) superfamily and conjugation enzymes such as the glutathione transferase as well as transporter proteins, which act as efflux pumps to remove xenobiotic chemicals from the cell. The sensory and signalling components of this system consist of soluble receptors, including several members of the steroid/nuclear receptor superfamily as well as the aryl hydrocarbon receptor (AhR) that regulate expression of genes involved in biotransformation.

More recently it has been recognised that the components of the surveillance system responding to the environmental stresses are the same as the ones used in developmental signalling. Thus, one can picture the developing embryo as an organism adapting to environmental challenges imposed by multicellularity. Therefore, identification of the components involved in the surveillance system and understanding of the molecular mechanism by which they function will not only give insight into environmental adaptation but also into important developmental processes.

THE EARLY DISCOVERY OF THE AHR AND ARNT

The discovery of the AhR originates from experiments in which it was observed that exposure of rodents to polycyclic aromatic hydrocarbons (PAH) led to induction of a particular aryl hydrocarbon hydroxylase activity (AAH) – today recognised as CYP1A1 activity – that enhanced the metabolism of the PAHs themselves (Nebert and Gelboin, 1969; Nebert et al., 1993). The AAH activity was found to be highly inducible by PAHs such as bezo[*a*]pyrene (B[*a*]P) and 3-methylcholantrene (3MC) in some inbred mouse strains like the C57BL/6 mice but not in other mice such as the DBA/2 strain (Nebert et al., 1972; Thomas et al., 1972). In crosses between the C57BL/6 and DBA/2 mice the lack of induction was inherited as an autosomal recessive trait, termed

the *Ah* locus that encoded a gene accountable for the responsiveness for PAHs. By the use of the more metabolically stable 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) it was found that the extent of induction of AHH activity was the same in both mouse strains, however, the dose-response curve in the Ah-nonresponsive mouse strain was shifted 15- to 20-fold to the right (Poland et al., 1974). It was suggested that the structural gene product (CYP1A1) was the same in both mouse strains, but that for instance altered affinity of PAHs for a regulatory gene product i.e. a receptor accounted for the difference between the two strains. By the use of radiolabelled TCDD, Poland et al (Poland et al., 1976) showed accumulation of [^{3}H]TCDD specifically bound with high affinity to a protein complex in C57BL/6 liver cytosol, but to a much lesser extent in DBA/2 mice liver cytosol. The putative receptor was termed the aryl hydrocarbon receptor (AhR) but has also been called the dioxin or aromatic hydrocarbon receptor. By comparing different PAHs and HAHs for binding affinity to the cytosolic AhR it was recognised that there was in general a good correlation between their affinity for the AhR and their potency to cause CYP1A1 induction as well as to other toxic or biological effects exerted by these compounds. Thus, these findings implied that the toxic effects of e.g. TCDD were mediated through the AhR.

The mouse hepatoma cell line Hepa1c1c7 has been extremely valuable for studying the mechanism of CYP1A1 induction especially since several induction-defective mutants were isolated. One of these cell clones showed normal levels of AhR but were defective in ligand-dependent nuclear translocation, which was suggested to by due to mutation of a protein, termed the Ah receptor nuclear translocator (arnt), important for nuclear translocation of the AhR (Hankinson, 1979; Legraverend et al., 1982). Subsequently, arnt was cloned by complementing arnt-defective variants with genomic DNA from normal cells. The sequence analysis revealed that segments of the protein showed homology to basic helix-loop-helix (bHLH) proteins, but showed no resemblance to the steroid hormone receptor family as originally thought (Hoffman et al., 1991). Although the amino acid sequence of the AhR at that moment was still unknown, this finding suggested that the AhR and arnt function together as a heterodimer, similar to other bHLH transcription factors, during CYP1A1 induction (Hankinson, 1995; Hoffman et al., 1991).

THE AHR IN DIFFERENT SPECIES

The receptors of the C57BL/6 and DBA/2 mouse strains were called the AhR^b and AhR^d alleles and were shown to encode proteins of 95 kDa and 104 kDa, respectively (Poland and Glover, 1987; Poland and Glover, 1990; Schmidt and Bradfield, 1996). Later on, two additional alleles encoding intermediate affinity receptors have been identified in different mouse strains. By using an AhR photoaffinity ligand, the receptor was successfully purified and a partial amino acid sequence was obtained that was utilised in the subsequent cloning of the AhR cDNA (Bradfield et al., 1991). Cloning of the mouse AhR in 1992 by two independent groups (Burbach et al., 1992; Ema et al., 1992) indeed revealed that the receptor belongs to the bHLH-PAS superfamily of transcription factors. Subsequently, the AhR was cloned from several other species including human and rat (Carver et al., 1994a; Dolwick et al., 1993). The human AhR

gene is located on chromosome 7 and the mouse on chromosome 12 (Micka et al., 1997; Poland et al., 1987; Schmidt et al., 1993).

Recently, the AhR has been identified in other vertebrates, including birds, amphibians and both in bony and cartilaginous fish (Hahn, 2002 and references therein). Fish are extremely sensitive to dioxin effects and have been proposed as a model for studying dioxin toxicity. However, identification of the receptor in killifish *Fundulus heteroclitus* as well as in cartilaginous fish such as *Raja erinacea* revealed that fish contains two AhR forms, AhR1 and AhR2 (Hahn et al., 1997). In general, fish AhR1 and AhR2 share properties of the mammalian AhR such as high-affinity dioxin binding, interaction with arnt and transcriptional activation of target genes. However, in a few species the receptors show a poor ability to bind dioxin (Andreasen et al., 2002), which raises the possibility of multiple functions of these ancestral AhRs.

In addition to the vertebrate AhR, homologous of the receptor have also been found recently in invertebrates such as the nematode *Caenorhabditis elegans,* (Powell-Coffman et al., 1998), the fruit fly *Drosophila melanogaster,* (Duncan et al., 1998) and in several molluscs e.g. *Mya arenaria* (Butler et al., 2001). The invertebrate AhR interacts with arnt and shows poor binding ability of prototypical mammalian AhR ligands such as TCDD and β-naphtoflavone, which is in line with a relative insensitivity towards dioxin-induced toxicity in these species (Hahn, 1998). Characterization of mutants of the AhR homolog, *spineless,* in *Drosophila melanogaster* has revealed that this protein plays a role in defining the distal regions of the antenna and leg in the fruit fly and it has been suggested that the function of *spineless* might be related to the chemosensory function of the antenna (Duncan et al., 1998; Emmons et al., 1999). This idea of an ancestral role of the AhR in chemosensory mechanisms is further supported by expression of the AhR homolog, *ahr-1,* in some chemosensory neurons of *Caenorhabditis elegans*. Thus, these findings provide a link of an ancestral role in development of sensory system to the more established role of vertebrate AhR in chemical sensing and adaptation.

PHYSIOLOGICAL ROLE OF THE AHR

Several recent studies point to a role of the AhR in cell cycle control, although the precise mechanism remains elusive. Examination of an AhR-defective variant of the mouse hepatoma Hepa1c1c7 cell line revealed a prolonged doubling time compared to the wild type counterpart that was attributed to a delayed progress through the G_1-phase (Ma and Whitlock, 1996). In mouse embryonic fibroblasts (MEF) derived from AhR-deficient mice, a slower growth rate was seen due to accumulation of cells in the G_2/M-phase (Elizondo et al., 2000). In the same AhR-null MEF cells the AhR was shown to contribute to p300-mediated induction of DNA synthesis and consequent S-phase progression (Tohkin et al., 2000). These observations suggest that AhR promotes the progression of cells through the cell cycle, in the absence of exogenous ligand.

In addition to a proposed function in cell cycle progression, studies using AhR-deficient mice indicate that the AhR plays an important role in normal development. AhR-deficient mice, created independently in tree different research groups, all show

deficient induction of AhR target genes, but some differences in the phenotypes were reported (Fernandez-Salguero et al., 1995; Lahvis and Bradfield, 1998; Mimura et al., 1997; Schmidt et al., 1996). The AhR-/- mice commonly show defects in liver development, decreased body weight and poor reproductive success. According to Fernandez-Salguero et al (Fernandez-Salguero et al., 1995; Gonzalez and Fernandez-Salguero, 1998) the decreased liver weight was suggested to be due to an increased rate of apoptosis and was associated with fibrosis in the portal tract. On the other hand Lahvis and Bradfield (Lahvis et al., 2000) reported that the reduced liver size was a result of a decrease in hepatocyte size due to massive portosytemic shunting in AhR-/- mice. Extensive analysis of these mice provided several examples of fetal vascular systems remaining in the adult mice, for instance, a shunt in the liver, an open ductus venosus in the liver sinusoids, persistent hyaloid arteries in the eyes and altered vascular pattern in the kidney, indicating that the AhR has a function in vascular development (Lahvis et al., 2000). In addition to these effects, female AhR-/- mice demonstrated reduced fecundity probably due to impaired ovarian follicle development (Benedict et al., 2000; Robles et al., 2000). These data, in conjunction with the findings of AhR function in cell cycle control, indicate that the AhR plays an important role in proliferation, differentiation and apoptosis, however, this function appears not to be critical for survival but compromises the responsiveness to environmental signals.

OTHER bHLH-PAS PROTEINS

The bHLH-PAS superfamily of transcription factors has been shown to play key roles in development, adaptation to hypoxia, control of circadian rhythmicity as well as metabolism of xenobiotic compounds (reviewed by Gu et al., 2000). The bHLH-PAS proteins contain two conserved domains, the N-terminal bHLH domain that is followed by the PAS domain. The PAS domain constitutes a region of homology encompassing 250-300 amino acids and contains two degenerate 50 amino acid subdomains termed PAS-A and PAS-B. The term PAS is an acronym from the names of the three founding members of the family: the *Drosophila melanogaster* protein Period (Per) protein, the mammalian arnt, and the Single-minded protein (Sim) in *Drosophila melanogaster* (Figure 1). Per was the first PAS factor ever to be characterised, and was identified as a regulator of circadian rhythmicity in *Drosophila melanogaster*. Cloning of the cDNA revealed that Per was a unique member of the bHLH-PAS superfamily in that it did not harbour a bHLH domain, indicating that it does not bind to DNA, however it was shown to dimerize with other PAS containing proteins, implying that it works as a repressor (Hogenesch et al., 1998; Reddy et al., 1986). Sim appears to function as a master developmental regulator of the central nervous system midline development (Nambu et al., 1991).

Arnt

Arnt was shown to be necessary for binding of the AhR to DNA and initially it was thought that arnt is required for translocation of the AhR to the nucleus (Elferink et al., 1990; Hankinson, 1979; Legraverend et al., 1982; Reyes et al., 1992). However, analysis of arnt localisation by immunofluorescence microscopy revealed that arnt is

a nuclear protein (Hord and Perdew, 1994; Pollenz et al., 1994). In addition to the AhR, arnt has been shown to be able to form heterodimers with the mouse Sim and Clock proteins (Probst et al., 1997; Swanson et al., 1995) as well as the hypoxia inducible factor 1α (Hif-1α) and the endothelial PAS domain protein-1 (EPAS-1) (Tian et al., 1997; Wang et al., 1995). Furthermore, it has been shown that in addition to forming heterodimer complexes, arnt can form homodimers and bind the E-box sequence, CACGTG (Antonsson et al., 1995; Sogawa et al., 1995). An important physiological role of arnt was established as arnt deficient mice fail to develop past embryonic day 10.5 and exhibit defects in yolk sac vascularization (Maltepe et al., 1997). The embryonic lethality of the arnt knockout mice probably reflects the participation of arnt in multiple bHLH-PAS signalling pathways. In support of this, the critical role of arnt in both AhR- and hypoxia-signalling was recently established by demonstrating loss of induction of target response genes in a conditional arnt gene knockout mouse (Tomita et al., 2000).

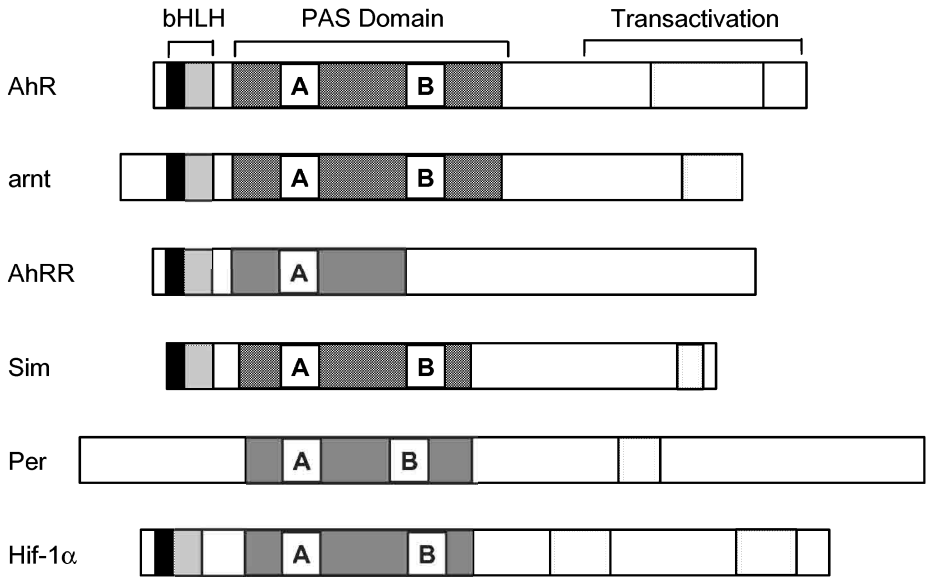

Figure 1. Schematic representation of examples of bHLH-PAS proteins, indicating the basic-helix-loop-helix (bHLH) domain, the Per-Arnt-Sim (PAS) homology domain and the transactivation domains.

Hypoxia inducible factors

The hypoxia inducible factor is a transcription factor, consisted of an inducibly-expressed Hif-1α subunit and arnt, also called Hif-1β (Semenza, 2002; Wang et al., 1995). In addition to Hif-1α, two other α-class homologues, EPAS-1 and Hif-3α, have the capacity to sense low oxygen tension and to dimerize with arnt (Gu et al., 2000; Tian et al., 1997; Wang et al., 1995). Hif-1α functions as a regulator of oxygen homeostasis and has been shown to regulate the expression of more that 40 target genes encoding proteins that play key role in critical developmental and physiological

processes, including angiogenesis, erythropoiesis and glycolysis (Semenza, 2002). Under normal physiological levels of oxygen (normoxia) Hif-1α is rapidly degraded by the ubiquitin-26S proteasomal pathway, however, during hypoxia this process is inhibited resulting in increased levels of Hif-1α. The molecular basis for this regulation is the oxygen-dependent hydroxylation of proline residues in HIF-1α by a prolyl hydroxylase, which is a key component in this unique oxygen sensing mechanism within cells (Ivan et al., 2001; Jaakkola et al., 2001). Prolyl hydroxylation of HIF-1α is required for binding of the von Hippel-Lindau tumour suppressor protein that is the recognition component of an E3 ubiquitin-protein ligase that targets HIF-1α for proteasomal degradation. Intratumoral hypoxia and genetic alterations that dysregulate signal transduction pathways result in overexpression of Hif-1α in the majority of human cancers analysed (Talks et al., 2000; Zhong et al., 1999). Disruption of the HIF-1α gene in mice revealed an essential role in embryonic vascularization and solid tumour formation (Carmeliet et al., 1998; Ryan et al., 1998), and show a similar phenotype as to the arnt knockout mice.

AhR repressor

Recently, a novel PAS containing protein was identified with the capacity to suppress AhR gene regulation, hence the name AhR repressor (AhRR) (Mimura et al., 1999). The protein shows high sequence homology and domain structure to the AhR, however the ligand binding PAS-B region is missing and the C-terminal sequence differs considerably from that of the AhR (see Figure 1). The expression of the AhRR was shown to be regulated by relatively weak binding of AhR-arnt complexes to three XRE elements in the AhRR regulatory region (Baba et al., 2001). When expressed the AhRR is a constitutively active protein that competes with AhR for arnt dimerization and DNA binding (Gu et al., 2000; Mimura et al., 1999). Thus, according to the proposed model the AhRR functions as a negative-feedback regulator of AhR function and may determine tissue-specific responses to AhR signalling (see section “Regulation of the AhR”).

STRUCTURAL AND FUNCTIONAL DOMAINS OF THE AHR

The bHLH domain

The bHLH domain has been found in a variety of transcription factors that function as sequence-specific transcriptional regulators by binding to DNA either as homo- or heterodimers. Although many bHLH proteins form homodimers, the AhR is unable to interact with DNA individually but requires arnt for recognition and binding to the cognate response element on DNA (Dolwick et al., 1993; Swanson, 2002). Thus, the bHLH domains of both AhR and arnt are required for obtaining specific DNA-binding, by means of the two basic regions, whereas dimerization of the HLH domains forms a four-helix bundle structure that stabilises the dimmer (Figure 2). The bHLH-mediated dimerization is further stabilised by interactions between the PAS domains of the AhR and arnt proteins (Reisz-Porszasz et al., 1994). The HLH domain has also been shown

to interact with hsp90 and it appears that this interaction is important for DNA-binding activity (Antonsson et al., 1995).

The N-terminal region of the AhR has been shown to contain a bipartite type of nuclear localisation signal (NLS) between amino acids 13 to 39, thus completely overlapping the DNA-binding domain, and a nuclear export signal (NES) region localised in helix 2 of the bHLH domain (Ikuta et al., 1998). An additional NES was recently identified in the N-terminal part of the PAS domain (Berg and Pongratz, 2001). Proteins that carry both an NLS and NES often dynamically shuttle between the cytoplasm and the nucleus in a process termed nucleocytoplasmic shuttling, which the AhR has been demonstrated to undergo (Ikuta et al., 2000). Treatment of cells with leptomycin B, a specific inhibitor of the nuclear export protein CRM-1 (chromosomal region maintenance protein 1), causes nuclear accumulation of the AhR (Ikuta et al., 2000; Pollenz and Barbour, 2000). Moreover it has been shown that the AhR NES interacts with CRM-1. It has been suggested that the NLS and NES are masked by interaction with the hsp90- and AhR-arnt-XRE-complex, respectively, thereby maintaining the appropriate localisation of the inactive/active receptor (see below).

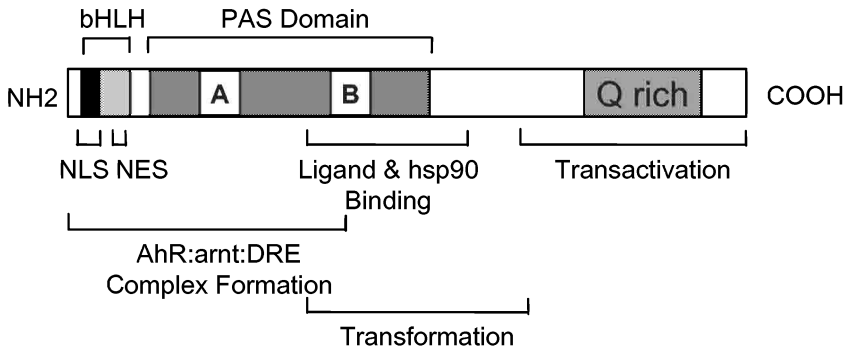

Figure 2. Structural and functional domains of the AhR.

The PAS domain

The PAS domain structure is found in proteins of all three kingdoms of life and is an important signalling molecule that, among others monitors changes of light, oxygen, small ligands and overall energy level of the cell (Gu et al., 2000; Taylor and Zhulin, 1999). Although the amino acid sequence is not very well conserved between different PAS domains, analysis of the crystal structures of the bacterial oxygen sensing FixL, the bacterial photoactive yellow protein PYP, the human potassium channel HERG as well as the LOV2 domain of *Andiantum* phy3, has revealed a highly conserved structural fold characterised by a five-stranded antiparallel β-sheet flanked by one long central α-helix, called the helical connector, and a few shorter α-helixes (Crosson and Moffat, 2001; Gong et al., 1998; Morais Cabral et al., 1998; Pellequer et al., 1998). The hydrophobic core of the PAS domain harbours co-factors as varied as heme (FixL), 4-hydroxycinnamyl chromophore (PYP) and flavin (LOV), however, the actual

positioning of the ligand and binding site interactions differ considerably. Even so, the conservation of the structural fold in rather distant PAS domains suggests some similarity in the mechanism of signal transduction among the PAS-containing proteins.

The PAS domain is thought to function as a surface for both homotypic interactions with other PAS-containing proteins and heterotyptic interactions with various other proteins. The PAS domain of the AhR indeed constitutes a protein-protein interaction surface, since it has been shown to be important for interacting with several proteins, including arnt and hsp90 (Fukunaga et al., 1995; Lindebro et al., 1995; Reisz-Porszasz et al., 1994). The N-terminal part of the PAS domain, spanning the PAS-B region, has been shown to be the most important for maintaining the PAS-PAS interaction between AhR and arnt (Lindebro et al., 1995). In addition, this region is involved in hsp90 binding as well as constitutes the ligand-binding domain (LBD) of the AhR (Burbach et al., 1992; Coumailleau et al., 1995; Whitelaw et al., 1993a). It has been suggested that the folding of the LBD of the AhR occurs via an hsp90-dependent pathway similar to that of steroid hormone receptors (for review, see Pratt, 1997). The role of hsp90 in AhR stabilisation is supported by the findings that treatment of cells with geldanamycin, which binds to the ATP binding site of hsp90 and disrupts hsp90 heterocomplexes, leads to rapid proteolytic turnover of the AhR (Chen et al., 1997). However, hsp90 is probably not only required for proper folding of the AhR but also for maintenance of the receptor in a stable high affinity ligand-binding configuration.

The ligand binding domain

The core ligand binding activity of the mouse AhR has been identified to be located between amino acids 230 and 421, and studies using this minimal ligand-binding domain, translated in reticulocyte extracts *in vitro*, showed similar affinity of TCDD to this fragment as to the full length receptor (Coumailleau et al., 1995; Whitelaw et al., 1993b). The minimal LBD traverse the PAS-B domain, which, as previously mentioned has been shown to be important for association with hsp90 and for heterodimerization with arnt (Coumailleau et al., 1995; Fukunaga et al., 1995; Lindebro et al., 1995). Albeit the known important role of the PAS-containing LBD of the AhR, amazingly few studies have dealt with detailed structural-functional aspects of this region. Analysis of the polymorphic forms of the AhR of the inbred responsive C57BL/6 and nonresponsive DBA/2J mouse strains revealed that whereas the former contain an Ala at position 375 the latter have a Val at this position of the LBD (Ema et al., 1994). This amino acid exchange causes an approximately one order of magnitude decrease in TCDD-binding capacity. Importantly, the corresponding amino acid in the human AhR (position 381) is a Val, thus correlating with the lower affinity of ligands for the human receptor. Since replacement of Ala with Asp at this position completely abolishes ligand binding, it has been suggested that the size and hydrophobicity of the side chain at this position is important for the ligand binding (Dzeletovic et al., 1997; Ema et al., 1994; Procopio et al., 2002).

Recently, a three-dimensional model for the LDB of the mouse AhR was published, using the known three-dimensional crystal structure of the O_2 sensing PAS domain of the bacterial FixL protein as a template (Procopio et al., 2002). According to this model

the ligand-binding pocket is situated in between the β-sheet on the one side and the F_α-helical connector that is preceded by an E_α-helix on the other side. The main difference between all known PAS structures is attributed to the positioning of the F_α-helical connector, which in the PAS proteins that do not bind a cofactor, is placed closer to the β-sheet as compared to in FixL that binds a heme (Gong et al., 1998; Morais Cabral et al., 1998; Pellequer et al., 1998). Hence, in the model of the AhR ligand-binding domain the F_α-helix is placed closer to the β-sheet than in FixL, reducing the size of the hydrophobic core constituting the ligand-binding cavity. In addition to the position of the helical connector closer to the β-sheet also the side-chain volume of some residues within the cavity shows good complementarity with the size of the ligand. For instance, in the position corresponding to Ala^{375} in mouse AhR, a glycine is found in FixL, and importantly, the human AhR and the DBA/2J mouse strain that shows low binding affinity for dioxins has a bigger side chain (Val) at this position. Another residue within the AhR ligand-binding pocket probably important for interaction with dioxins is Cys^{327} that is suggested to interact with the electrophilic central region of TCDD, in analogy to His^{200}, which is coordinating the ferric heme ion in FixL (Procopio et al., 2002). The hydrophobic ligand-binding pocket of the various PAS proteins bind co-factors of rather diverse chemical structures (compare heme of FixL to the hydroxycinnamyl chromophore of PYP). Interestingly, the co-factors are localised in the same general region, but the actual positioning of the ligand and binding site interactions differ considerably (Crosson and Moffat, 2001; Gong et al., 1998; Pellequer et al., 1998). This observation suggests that structurally diverse ligands of the AhR might be positioned differently in the ligand-binding pocket and key residues critical for ligand-protein interactions might be different.

The transactivation domains

The C-terminal regions of both the AhR and arnt contain domains with transactivation capability (Jain et al., 1994; Ma et al., 1995; Sogawa et al., 1995; Whitelaw et al., 1994). The AhR contains three distinct transactivation subdomains i.e. an acidic domain, a glutamine-rich domain, and a proline-serine-threonine rich domain (Ma et al., 1995). The transactivation subdomains of the AhR have been shown to function independently of each other and display different levels of activity, however, when added together they act synergistically. The C-terminal part of arnt contains one single glutamine-rich TAD, which is constitutively active (Whitelaw et al., 1994). The TAD of the AhR, on the other hand, seems to be regulated through an inhibitory domain adjacent to the amino terminal end of the TAD (Ma, 2001; Ma et al., 1995). Experiments involving expression of AhR and arnt mutants in AhR- or arnt-defective cells have revealed that the transactivation function of the AhR is both necessary and sufficient for the induction of the *CYP1A1* gene, however, remarkably the TAD of arnt is dispensable (Ko et al., 1996).

ACTIVATION OF THE AHR

The inactive AhR resides in the cytoplasm in a complex with a dimer of the chaperone protein hsp90 (Perdew, 1988), the co-chaperone p23 (Nair et al., 1996) and the

immulophilin-like protein XAP2 (HBV X-associated protein 2) also known as AIP (AhR interaction protein) and ARA9 (AhR associated protein 9) (Carver et al., 1998; Ma and Whitlock, 1997; Meyer et al., 1998; Rowlands and Gustafsson, 1997). Originally the latent AhR-complex was identified as a 270 to 300 kDa protein complex that sedimented approximately in the 9S fraction by means of sucrose density gradient centrifugation (Gasiewicz and Rucci, 1984; Poellinger et al., 1983). The hsp90-bound form of the AhR exhibits minimal DNA-binding affinity, however, interaction with hsp90 seem to be a prerequisite for ligand-binding and activation of the receptor (Pongratz et al., 1992; Wilhelmsson et al., 1990). The importance of hsp90 in maintaining the AhR in a ligand-binding conformation was clearly shown as block of AhR signalling was revealed when hsp90 levels were reduced (Carver et al., 1994b). Since hsp90 represses AhR DNA-binding activity and both the hsp90- and ligand-binding domain resides in the same region of the AhR, it has been suggested that binding of ligand causes a derepression of the receptor by conformational changes leading to its activation.

The exact functional roles of XAP2 and p23 are not fully established, however, it has been shown that XAP2 stabilises the AhR, probably by protection from ubiquitination, and enlarges the pool of the receptors localised in the cytoplasm (Kazlauskas et al., 2000; LaPres et al., 2000; Meyer and Perdew, 1999). Recently, it was described that XAP2 alters importin β binding (a component of the nuclear pore complex) to the AhR NLS thereby blocking the nucleocytoplasic shuttling of the AhR-complex (Petrulis et al., 2003). The hsp90 accessory protein p23 has been implicated in modulating ligand responsiveness and enhancement of AhR signalling (Cox and Miller, 2002; Kazlauskas et al., 1999).

After ligand binding to the AhR, the receptor undergoes a poorly understood transformation process whereby the receptor translocates to the nucleus, sheds hsp90 and other interacting proteins, and forms a dimer with the general cofactor arnt (Figure 3). Incubation of cytosol *in vitro* with ligand, with high salt concentration or at high temperature causes a shift of the AhR from the 9S form to a 4-6S complex with a estimated molecular weight of 180 to 210 kDa (Denison et al., 1986; Wilhelmsson et al., 1986). This 4-6S form of the receptor consists of an hsp90-free receptor complex that is associated with its partner protein arnt and exhibits a relatively high DNA-binding affinity (Hoffman et al., 1991; Reyes et al., 1992; Wilhelmsson et al., 1990). Originally it was thought that arnt is required for translocation of the AhR to the nucleus, however, TCDD-mediated nuclear accumulation of the AhR has been shown in arnt-deficient Hepa-1 cells (Pollenz et al., 1994). In contrast, attachment between AhR and hsp90 appears to remain throughout the transportation process (Heid et al., 2000; Perdew, 1991; Wilhelmsson et al., 1990), although for the formation of the trancriptionally active AhR-arnt dimer release of hsp90 is required, a process which arnt seems to take active part in (Hord and Perdew, 1994; McGuire et al., 1994). By treatment of cells with geldanamycin, thereby releasing the AhR, it has been shown that dissociation from hsp90 alone is sufficient for obtaining an AhR complex with DNA-binding activity but this complex is incapable of stimulating transcription (Heid et al., 2000). Thus, these findings indicate that the ligand plays multiple mechanistic roles during AhR activation, being important

for nuclear translocation, transformation to an arnt heterodimer and maintenance of the structural integrity required for transcriptional activation.

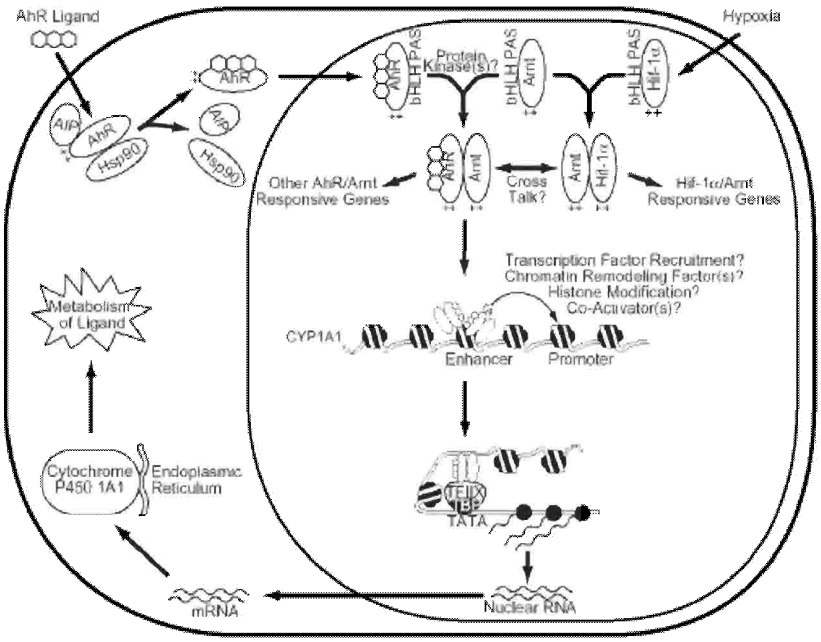

Figure 3. Working model for ligand-dependent AhR activation and transcriptional activation of the *CYP1A1* gene. Adapted from Whitlock (1999).

XRE BINDING AND TRANSCRIPTIONAL ACTIVATION

The regulatory region of AhR target genes, such as the *CYP1A1* gene, contains short segments, called xenobiotic-, dioxin- or AhR-response elements (XRE, DRE or AhRE) that confer TCDD inducibility when linked to a reporter gene. The functional consensus sequence binding AhR-arnt dimers has been identified as TnGCGTG, and mutational analysis has revealed that substitutions in the central core CGTG eliminates binding of the AhR-complex (Lusska et al., 1993; Shen and Whitlock, 1992; Yao and Denison, 1992). However, conserved nucleotides that flank the core XRE has been demonstrated to be important for functionality of the AhR transactivation (Matikainen et al., 2001; Shen and Whitlock, 1989; Swanson et al., 1995). The ligand-activated AhR has been shown to contact both DNA strands at four guanine residues within the recognition motif, indicating that the active AhR complex interacts with the major groove of the DNA double helix (Shen and Whitlock, 1989). In contrast to the majority of bHLH-containing transcription factors, which bind to the E-box sequences CANNTG, the XRE sequence is not symmetrical. Protein-DNA crosslinking studies have shown that the AhR and arnt recognise the 5′-TnGC and the GTG-3′ XRE half sites, respectively (Bacsi et al., 1995; Swanson et al., 1995).

Elucidation of the molecular mechanism of transcriptional activation by the AhR-arnt heterodimers is primarily derived from studies on *CYP1A1* gene induction by TCDD. By using *in vivo* foot printing it has been established that both the enhancer and the promoter region of *CYP1A1* are in a nucleosomal configuration that are not accessible to transcription factors in the absence of AhR agonist (see Whitlock (1999) and references therein). Activation of the AhR leads to the disruption of the chromatin in the enhancer and promoter region of the *CYP1A1* gene, without disrupting the intervening chromatin (see Figure 3). Binding of the AhR-arnt heterodimer to XREs is associated with loss of the nucleosomal structure in the enhancer region, a process that does not require the TADs of the AhR or arnt (Ko et al., 1996; Ko et al., 1997). On the other hand, protein binding in the promoter region is dependent upon the presence of functional TADs of AhR, indicating that the TAD mediates enhancer-promoter communication (Ko et al., 1996; Okino and Whitlock, 1995; Wu and Whitlock, 1992; Wu and Whitlock, 1993). The specific mechanism by which the AhR-arnt dimer mediates *CYP1A1* transcription is not clear. However, in analogy to nuclear hormone receptors it is conceivable that the AhR TAD recruits coactivators and other factors that sequentially initiate the assembly of the general transcription machinery, including the polymerase II complex, at the promoter region. In support of this, the AhR-arnt heterodimer has been shown to interact with members of the p160 and CBP/p300 families, including SRC-1, NCoA-2, p/CIP and p300 (Beischlag et al., 2002; Kobayashi et al., 1997; Kumar and Perdew, 1999; Tohkin et al., 2000). Furthermore, the AhR complex has been shown to be able to directly interact with components of the basal transcription machinery such as the TFIIB and TFIIH factors (Rowlands et al., 1996; Swanson and Yang, 1998).

LIGAND-DEPENDENT ACTIVATION OF THE AHR

A huge number of ligands originating from the environment, diet as well as endogenously formed compounds have been shown to bind to the AhR. However, none of the ligands identified to date has been proven to be a true physiological ligand relevant for all AhR biology. The best-characterised high affinity ligands for the AhR include a wide variety of environmental contaminants such as halogenated aromatic hydrocarbons (HAHs) and PAHs, which all are planar, aromatic and hydrophobic molecules. However, recent studies have demonstrated *CYP1A1* gene induction by a range of structurally diverse chemicals suggesting that the AhR can be activated by multiple endogenous and exogenous compounds. Consequently, the AhR is much more promiscuous than previously thought and can obviously harbour chemicals with great structural differences in its hydrophobic ligand-binding pocket. AhR ligands can be classified in two major groups: i) synthetic ligands that are formed from anthropogenic activities, and ii) natural compounds originating from biological systems by natural processes (reviewed by Denison and Nagy, 2003).

Synthetic AhR ligands

The synthetic compounds are generally formed as by-products in the manufacturing of polychlorinated bifhenyls and chlorinated pesticides, but are also produced by

inefficient cumbustion of organic carbon. Until now it has been assumed that the ubiquitous presence of these compounds in the environment stem from the introduction and expansion of industrialization since the 1950s. However, it was recently reported that burning of coastal peats could represent a pre-industrial source of dioxins (Meharg and Killham, 2003). Since most HAHs are chlorinated in the lateral positions they are generally resistant to biological or chemical degradation and are easily accumulated in the food chain. HAHs like dioxins, dibenzofurans and biphenyls have relative high binding affinity for the AhR i.e. in the pM to nM range. PAHs, on the other hand, are metabolised by e.g. CYP1A1 and can be eliminated from the human body. PAHs like B[*a*]P, 3MC and aromatic amines show significantly lower AhR affinity (in the high nM to μM range) and generally produce a transient AhR-signalling response (Kleman et al., 1992; Poland and Knutson, 1982; Safe, 1990).

Structure-activity relationship studies with a large number of PAH and HAH AhR ligands indicate that the binding pocket can accept planar ligands with the maximal dimension of 14 Å x 12 Å x 5 Å, and that key electronic and thermodynamic properties of the ligand are critical for favourable interactions between the ligand and the receptor (Gillner et al., 1993; Kafafi et al., 1993; Waller and McKinney, 1995). However, the recent findings of various structurally diverse compounds capable of activating the AhR and/or inducing the *CYP1A1* gene, indicate that the current characteristics of AhR ligands and the model of the ligand-binding pocket is too restrictive.

Dietary AhR ligands

The diet is the main source of many naturally occurring AhR agonists or antagonists such as flavonoids, carotinoids and phenolics that can either activate or inhibit the AhR signalling pathway (Bjeldanes et al., 1991; Ciolino et al., 1998a; Ciolino et al., 1998b; Gillner et al., 1993; Gillner et al., 1985; Lu et al., 1995; Lu et al., 1996). Importantly, it has been shown that conversion of dietary indoles, i.e. indole-3-carbinol to more potent AhR ligands occur in the mammalian digestive tract (Bjeldanes et al., 1991; Perdew and Babbs, 1991). In fact, indolo-[3,2-*b*]-carbazole, and acidic condensation product of indole-3-carbinol displays one of the highest AhR ligand binding affinities (~0.2-3.6 nM) of naturally occurring compounds and is a potent inducer of CYP1A1 in cell cultures (Gillner et al., 1993). Considering that most dietary ligands are themselves relatively weak AhR ligands, the formation of relatively potent AhR ligands from these precursor molecules is significant, and as such, they are perhaps the largest class of natural ligands to which humans and animals are exposed. On the other hand, these compounds are readily metabolised and excreted and are therefore generally of less harm than HAHs.

Endogenous AhR ligands

The existence of an endogenous physiological ligand for the AhR has been suggested and supported by numerous studies in which the AhR signalling pathway is activated in the absence of exogenous ligands (Denison and Nagy (2003) and references therein). However, the identification of such a ligand remains elusive, moreover the observation that numerous endogenous compounds, including indoles, tetrapyroles, arachidonic

acid metabolites, carotinoids and oxysterols, can bind to the AhR and/or activate AhR-dependent gene expression support a concept of multiple endogenous ligands. The characterization of endogenous ligands is the subject of several extensive reviews (Denison and Nagy, 2003; Denison et al., 2002) and only a few examples will be given here.

The observed persistent hepatic *CYP1A1* gene expression in congenitally-jaundiced Gunn rats, which serve as a model for the Crigler-Naijar syndrome type I, suggested the existence of an endogenous AhR ligand in the blood of these animals (Iyanagi et al., 1989; Kapitulnik and Gonzalez, 1993; Kapitulnik et al., 1987). Subsequent studies demonstrated that bilirubin, a heme-degradation product, was present in high levels in these rats. Moreover, it was shown that bilirubin as well as biliverdin, a metabolic precursor of bilirubin, induce CYP1A1 expression and an AhR-dependent reporter gene in cell cultures (Sinal and Bend, 1997). Both bilirubin and biliverdin were subsequently shown to be capable of displacing [^{3}H]TCDD in a competitive ligand binding assay, confirming that they indeed were AhR ligands, albeit relatively weak ones (Phelan et al., 1998). Circulating plasma bilirubin possesses antioxidant properties at low concentration, whereas cytotoxic effects have been reported at higher concentrations especially in the central nervous system (Cashore, 1990). Recently it was also shown that bilirubin causes apoptosis in murine Hepa1c1c7 cells (Seubert et al., 2002). Gunn rats and human infants with Crigler-Naijar syndrome type-I lack functional UGT*01, the primary bilirubin detoxification enzyme. The finding that bilirubin is an AhR ligand and can stimulate its own metabolism by increasing the expression of CYP1A1/CYP1A2, offers an alternative mechanism of reducing the circulating levels of bilirubin. Thus, the AhR-dependent increase in CYP1A1/1A2 activity provides a physiologically relevant detoxification system, protecting the body from endogenously produced cytotoxic compounds.

In addition to the indole-containing compounds of dietary origin that have been shown to activate the AhR, endogenously derived indoles have been identified as AhR ligands. Recently, an AhR ligand was isolated form porcine lung and identified as 2-(1´H-inhole-3´carbonyl)thiazole-4-carboxylic acid methyl ester, ITE, (Song et al., 2002), moreover, indigo and indirubin were identified as potent AhR ligands in human urine (Adachi et al., 2001). Although indigo and indirubin predominantly would be derived from plants in the diet, these compounds can be formed by CYP-dependent (2A6, 2C19 and 2E1) metabolism of indole and can be produced in humans *in vivo* (Blanz et al., 1989; Gillam et al., 1999; Gillam et al., 2000). However, the concentration of these compounds in human serum and the function in AhR signalling is at present unknown. The ITE compound was suggested to be produced by a condensation reaction from tryptophan (Song et al., 2002), which probably constitutes one of the main sources of endogenously derived AhR ligands. One of the earliest studies from late 1970s reported that UV illumination of tissue culture media induced AHH (i.e. CYP1A1) activity, and this effect appears to arise from UV photoproducts of tryptophan and histidine that can bind to and activate the AhR (Paine, 1976; Paine and Francis, 1980). Furthermore, it was shown that UV irradiation could induce CYP1A1 in the skin and liver of rat and mice, thus suggesting that photoproducts could be formed *in vivo* (Goerz et al., 1996).

The fact that tryptophan is a strong near-UV absorbing amino acid, led to the search of photoproducts with the ability to induce CYP1A1 and CYP1A2 (Helferich and Denison, 1991; Rannug et al., 1987; Sindhu et al., 2000). Subsequently, Rannug et al identified two tryptophan photoproducts with the capacity to induce CYP1A1 and determined their structures as 6,12-diformylindolo[3,2-*b*]carbazole (dFICZ) and 6-formylindolo[3,2-*b*]carbazole (FICZ) (Rannug et al., 1987; Rannug et al., 1995). In a competitive ligand binding assay, the K_d value for dFICZ and FICZ was determined to 0.44 nM and 0.07 nM, respectively, whereas a K_d value of 0.48 nM for TCDD was obtained under the same experimental conditions. These findings indicate that both FICZ and dFICZ bind to the AhR with a higher affinity than TCDD, however, the high affinity binding is not reflected in as high CYP1A1 induction response, most likely due to rapid metabolism of these compounds (Wei et al., 2000; Wei et al., 1998). Although the formation of FICZ in biological systems remains to be elucidated, the relationship between light exposure and tryptophan and/or tryptophan metabolites is well documented and Rannug and co-workers have proposed that FICZ and other tryptophan photooxidation products might be novel messengers of light (Wei et al., 2000). Given that other members of the PAS domain superfamily include a variety of light-activated photoreceptors and proteins involved in circadian rhythm give further emphasis to this hypothesis. In support of this, a diurnal expression of CYP1A1 and Per in the posterior pituitary and the liver, as well as the expression of AhR and arnt in the liver of rats, was shown to be highly coordinated, indicating a possible interaction between AhR signalling and circadian response pathways (Huang et al., 2002b).

LIGAND-INDEPENDENT ACTIVATION OF THE AHR

Activation of the AhR signalling pathway and/or transcriptional activation of the *CYP1A1* gene have been reported to occur in several instances without the presence of an exogenous ligand. These situations include stress conditions such as hydrodymanic shear (Mufti et al., 1995; Mufti and Shuler, 1996), growth in methylcellulose suspension (Monk et al., 2001; Sadek and Allen-Hoffmann, 1994) and during hyperoxia (Couroucli et al., 2002; Hazinski et al., 1995; Okamoto et al., 1993). Another situation where AhR signalling is activated is during differentiation, which has been shown in the case of monocytes or keratinocytes, whereas in adipose 3T3-L1 cells AhR overexpression suppressed differentiation (Crawford et al., 1997; Hayashi et al., 1995; Sadek and Allen-Hoffmann, 1994; Shimba et al., 2001; Wanner et al., 1995). Moreover, nuclear accumulation of the AhR has been reported in cell culture or tissue slices in the absence of ligands (Abbott et al., 1994; Chang and Puga, 1998; Singh et al., 1996). In the case of hydrodynamic shear it has been suggested that a metabolite of arachidonic acid (Mufti and Shuler, 1996), which is released by the shear stress, constitutes a ligand for the AhR thereby triggering the AhR signalling response. Similarly it was suggested that the constitutive activation of the AhR observed in CYP1A1-deficient mouse hepatoma c37 cells is due to accumulation of a CYP1A1 substrate, which is a ligand for the AhR (Chang and Puga, 1998). However, this ligand or ligands has yet not been identified. In addition to these observations several investigations, including the previously mentioned studies with AhR knockout mice and the studies pointing to a role of the AhR in cell cycle control, raise the question whether an alternative ligand-independent pathway might be responsible for activation

of the AhR under certain conditions. Since the AhR is a phosphoprotein and protein kinase inhibitors has been shown to inhibit AhR mediated signalling, one possible molecular mechanism of ligand-independent activation could involve protein kinases.

Although steroid receptors represent the textbook example of ligand-dependent transcription factors, more and more evidence indicate that several steroid receptors can be activated in the absence of cognate hormone. Thus, steroid receptors, including estrogen, progesterone and androgen receptors has been shown to be activated by agents such as epidermal growth factor, insulin, insulin-like growth factor I as well as dopamine and other agents that elevate the intracellular levels of cAMP (see Cenni and Picard, 1999; Weigel and Zhang, 1998). Although the precise mechanism of ligand-independent activation of these receptors has not been elucidated, changes in phosphorylation status of the receptor or interacting proteins has been described (Bai et al., 1997; Kato et al., 1995). In the case of estrogen receptor (ER) activation by epidermal growth factor, phosphorylation of Ser^{118} by mitogen-acitvated protein kinases (MAPK) was shown to be required, although additional processes also seem to be necessary (Bunone et al., 1996; Kato et al., 1995). Until recently the glucocorticoid receptor (GR) seemed to be refractory to ligand-independent activation. However, recently Eickelberg et al (Eickelberg et al., 1999) reported on activation of the GR upon stimulation of human lung fibroblasts or vascular smooth muscle cells with β_2-adrenergic receptor agonists, salbutamol and salmeterol. The effect of these drugs was dependent on binding to β_2-adrenergic receptors and was suggested to involve cAMP. Ligand-independent activation of the GR by β_2-adrenergic receptor agonists explains, at least in part, the potent anti-inflammatory properties of β_2-agonsist observed *in vivo* and *in vitro* (Pauwels et al., 1997).

An additional example of alternative mechanisms of activation of transcriptional regulators can be illustrated by Hif-1α signalling. In addition to the oxygen sensing mechanism described above, Hif-1α has been demonstrated to be activated by receptor-mediated factors such as growth factors and cytokines as well as by oncogenes ($HER2^{NEU}$, Ras) and tumor suppressor PTEN (reviewed by Bilton and Booker, 2003). The amplitude of induction by these agents is generally lower compared to stimulation by hypoxia, on the other hand, these stimuli often continue over extended periods of time. Although, both the MAPK and the PI-3K/PKB pathways have been shown to be involved in hypoxia-independent Hif-1 activation, the molecular mechanism is not fully elucidated, besides, several mechanisms appear to exist and may differ depending on cell type and stimuli (Sheta et al., 2001; Treins et al., 2002; Zelzer et al., 1998). Since Hif-1α is a phosphoprotein and can be phosphorylated by p42/p44 and p38 MAPK *in vitro* (Richard et al., 1999; Sodhi et al., 2000), it has been suggested that phosphorylation of the inhibitory domain, which lies between the two transactivation domains of the Hif-1α subunit, leads to derepression and increased transcriptional activity of Hif-1α. However, augmented Hif-1α activity has also been described owing to increased synthesis of Hif-1α protein.

Several compounds have recently been shown to increase *CYP1A1* gene expression through AhR-dependent processes without apparent binding to or any direct interaction with the AhR. These compounds include several drugs e.g. omeprazole, and other benzimidazoles, primaquine, quinine, mevinolin and nicardipine, naturally occurring

food components (isosafrole, myristicin), as well as stimulants like caffeine and nicotine (Table 1). Some of these chemicals probably are weak ligands of the AhR, as for carbaryl that recently was shown to be capable of displacing AhR bound [^{3}H]TCDD, although it was initially reported not to bind to the AhR (Denison et al., 1998; Ledirac et al., 1997; Sandoz et al., 2000). However, it is likely that some of these compounds could activate the AhR by a receptor transactivation mechanism, in analogy with activation of the GR by β_2-adrenergic receptor agonists, salbutamol and salmeterol. Alternatively, it is possible that binding of certain chemicals to other components of the AhR-complex could result in AhR activation.

Table 1 Compounds that mediate AhR-dependent responses but have not been shown to competitively bind to the AhR.

Compound	Reference
Vitamin E, vitamin K	Chen and Ding, 1987; Chun et al., 2001
Methylenedioxybenzenes (isosafrole, piperonyl butoxide)	Adams et al., 1993; Marcus et al., 1990; Owens and Nebert, 1975
Pyridine	Kim et al., 1995; Kim et al., 1991
Mevinolin	Puga et al., 1992b
Albendazole	Souhaili-el Amri et al., 1988
Omeprazole	Daujat et al., 1992; Diaz et al., 1990
Lanzoprazole	Curi-Pedrosa et al., 1994; Daujat et al., 1996; Kikuchi et al., 1996
Oxfendazole	Gleizes et al., 1991; Gleizes-Escala et al., 1996
Thiabendazole	Aix et al., 1994
Carbendazim	Rey-Grobellet et al., 1996
Canthaxanthin	Astorg et al., 1994; Gradelet et al., 1997; Gradelet et al., 1996
β-apo-8´-carotenal	Astorg et al., 1994; Gradelet et al., 1997; Gradelet et al., 1996
11-Ethoxy-cyclopenta[a]phenanthren-17-ones	Boyd et al., 1995
Caffeine	Ayalogu et al., 1995; Goasduff et al., 1996
Metyrapone	Aubrecht et al., 1996; Harvey et al., 1998
Myristicin	Jeong et al., 1997
Primaquine	Fontaine et al., 1999
Quinine	Bapiro et al., 2002
Nicotine	Iba et al., 1998
Cocaine	Wang et al., 2002a
Oltipraz	Langouet et al., 1997; Le Ferrec et al., 2002
Nicardipine	Konno et al., 2003

REGULATION OF THE AHR

Phosphorylation

Several studies have been published concerning the ability of protein kinases to regulate the activity of the AhR-complex and subsequently modulate expression of AhR response genes. Both the AhR and arnt are phosphoproteins and analysis of chemical cleavage patterns of ^{32}P-labeled AhR suggests that phosphorylation occurs predominantly on its carboxyl-terminal half (Mahon and Gasiewicz, 1995). However, the significance of phosphorylation of the AhR-complex on e.g. DNA-binding or transcriptional activity remains unclear. Initially, the XRE-binding activity of the AhR-complex was shown to be lost upon phosphatase treatment *in vitro* (Pongratz et al., 1991) and further investigations indicated that at least one phosphorylation event of the AhR *per se,* positively regulates DNA binding activity (Berghard et al., 1993). Moreover, phosphorylation of arnt appears to be required for dimerization with the AhR.

A number of protein kinase activities, particularly protein kinase C (PKC), have been implicated in modulating AhR-arnt activity and in fact, mutual cross-talk between PKC and AhR-signalling pathways has been reported (Carrier et al., 1992; Chen and Tukey, 1996; Long et al., 1998; Puga et al., 1992a). Inhibition of PKC activity was shown to cause suppression of Cyp1a1 mRNA induction by TCDD in mouse Hepa-1 cells, while simultaneous treatment with TCDD and phorbol ester of human cell lines stably transfected with an AhR-driven reporter gene led to enhanced expression of the reporter. By domain swapping and deletion analysis it was found that the TADs of neither AhR or arnt were absolutely required for PKC-mediated enhancement of AhR-dependent transactivation (Long et al., 1998). Moreover, the DNA-binding activity of the AhR-complex was not influenced by PKC activation or inhibition. Therefore, it was proposed that PKC stimulates AhR-dependent gene expression via the PAS region of the AhR or arnt by facilitating recruitment of a putative PAS-specific coactivator. This is supported by the finding that TFIIB can interact with the PAS domain of the AhR (Swanson and Yang, 1998).

Similar to the cross-talk between PKC and AhR-signalling pathways, there is also compelling evidence for modulation by protein tyrosine kinases (PTK) on the AhR-mediated cellular responses and vice versa (Blankenship and Matsumura, 1997; Enan and Matsumura, 1996; Gradin et al., 1994). TCDD exposure of several tissues and cell culture systems caused activation of PTKs, and at least some of this PTK activity was shown to be related to src-family kinases. Blankenship et al (Blankenship and Matsumura, 1997) have shown that immunoprecipitation of either c-src or AhR from murine hepatic cytosol treated with TCDD exhibited higher PTK activity. Since both c-src and AhR interact with the hsp90, it was suggested that these proteins coexist as part of a multimeric protein complex and that binding of TCDD to AhR activates c-src kinase. Conversely, c-src has been suggested to regulate AhR activity, however, this question has not been adequately addressed thus far. However, studies using PTK inhibitors have revealed an inhibition of dioxin-dependent expression of CYP1A1 in

human keratinocytes by genistein, thus indicating that PTKs could be a possible modulator of AhR signalling (Carrier et al., 1992; Gradin et al., 1994).

Degradation

The proteolytic degradation of transcription factors is a well-established mechanism of regulation of signal transduction pathways and generally serves as a process to switch off or reduce the transcriptional response. The AhR has been shown to be downregulated following ligand exposure in several cell lines derived from different species and tissues, as well as in rat and mice *in vivo* (reviewed by Pollenz, 2002). Pulse-chase experiments in mouse hepatoma cells have shown that TCDD treatment shortens the half-life of AhR from 28 to 3 hours (Ma and Baldwin, 2000). In contrast, the level of arnt protein is unaffected by stimulation with TCDD (Pollenz, 1996). The proteolytic pathway responsible for the degradation of the AhR has been identified as the 26S proteasome, since specific inhibitors such as MG-132 and lactacystin blocks the process (Davarinos and Pollenz, 1999; Roberts and Whitelaw, 1999). Furthermore, studies using a cell line deficient in E1 ubiquitin ligase support the involvement of ubiquitin-mediated degradation through the 26S proteasomal pathway (Ma and Baldwin, 2000). Although the amino acids that are ubiquitinated, generally lysine residues, are still unknown, truncation of AhR indicates that the C-terminal TADs would be required for degradation. Moreover, DNA binding seems to be important for initiating the proteolytic process. The consequence of blocking AhR degradation appears to be an increase both in the magnitude and the duration of gene expression by the AhR-arnt complex, thus, the function of AhR downregulation is to modulate target gene expression (Davarinos and Pollenz, 1999; Ma and Baldwin, 2000; Ma et al., 2000; Pollenz, 1996; Reick et al., 1994).

Regulation by AhRR

The recently identified AhRR protein adds another aspect to the mechanism of AhR regulation. AhRR may constitute a negative-feedback regulator of the AhR, since activated AhR induces expression of the AhRR, which in complex with arnt competes with AhR-arnt dimers for binding to XRE sequences in target genes (Mimura et al., 1999). The expression of AhRR in various tissues of untreated mice is generally low or absent, however, constitutive expression of AhRR mRNA has been shown in the stomach and pituitary of untreated mice (Andersson et al., 2002; Huang et al., 2002a). Upon stimulation with 3-MC a considerable increase in mRNA expression of AhRR was observed in heart and lung, whereas liver, thymus, kidney and intestine expressed relatively small amounts of AhRR mRNA (Mimura et al., 1999). Interestingly, tissues like liver and thymus, which are most susceptible to toxic effects of dioxin, appear to respond poorly to AhRR induction, while these tissues show a substantial induction of CYP1A1 by 3-MC. The AhRR may also be the long-sought-after cycloheximide-sensitive repressor accountable for the CYP1A1 mRNA superinduction phenomenon observed in Hepa1c1c7 cells co-treated with TCDD and protein synthesis inhibitors (Israel et al., 1985; Lusska et al., 1992).

THE ROLE OF AHR IN TOXICITY AND CANCER

The toxic effects caused by TCDD in exposed animals were identified more than 50 years ago, however, despite extensive research in the field, the mechanism whereby TCDD exerts its toxic effects remains an enigma. A plethora of tissue-specific toxic and biological responses, including immunotoxicity, thymic atrophy, wasting syndrome, teratogenesis, tumor promotion and induction of gene expression, have been described in animals exposed to TCDD or other HAHs (Pohjanvirta and Tuomisto, 1994; Poland and Knutson, 1982). One reason for TCDD being the most toxic compound ever produced is probably due to its long biological half-life (up to 10 years in humans) and to its persistence to either enzymatic or chemical degradation. However, there is a marked difference in sensitivity of various mammalian species and strains to TCDD toxicity, for instance, guinea pig is extremely sensitive whereas adult humans are relatively resistant to acute lethality of TCDD exposure. The most characteristic effect of TCDD exposure in humans is chloroacne, a skin disorder with hyperkeratosis and metaplasia of the hair follicles and epidermis (Suskind, 1985).

Since the early discovery of the AhR, supported by the good correlation between HAH binding affinity and their potency of toxicity, it was believed that the AhR mediates most, if not all, of the toxic and biological effects exerted by these compounds. Recently, the dependence of a functional AhR in the toxicity of HAHs was confirmed by demonstration of resistance to very high doses of TCDD, as well as lack of teratogenic responses in AhR-deficient mice (Fernandez-Salguero et al., 1996; Mimura et al., 1997). Since the AhR is a transcription factor, dysregulation of genes important for proliferation, differentiation and apoptosis are presumably involved, however, the toxic effects mediated via the AhR cannot be fully explained by any of the AhR target genes identified to date. In the past few years, evidence have emerged that an extensive cross-talk occurs between the AhR and other transcription factors, including NF-κB (Tian et al., 1999; Tian et al., 2002), retinoblastoma protein (Rb) (Elferink et al., 2001; Ge and Elferink, 1998; Puga et al., 2000a), ER (Kharat and Saatcioglu, 1996; Safe, 2001) as well as other arnt-dependent factors such as Hif-1α. It was recently shown that AhR and the p65 (RelA) subunit of NF-κB, which is a key component in regulation of the immune system and inflammatory responses, physically associate and that the two pathways can modulate each other's activities (Tian et al., 1999; Tian et al., 2002). The immune system is particularly sensitive to TCDD toxicity and it is possible that these AhR-NF-κB interactions could partially explain the toxic outcome, like immunosuppression and thymic atrophy, elicited by TCDD exposure. In addition to cross-talk with other transcription factors, stimulation of cells with TCDD results in immediate activation of intracellular signalling pathways, including activation of protein kinases and elevation of Ca^{2+} levels, indicating that AhR signalling may interfere with signal transduction pathways without requirement of transcriptional activation (Nebert et al., 1993; Puga et al., 1992a).

As previously mentioned, the AhR has been shown to play a role in cell cycle control without the presence of exogenous ligand. However, AhR ligands such as TCDD, B[*a*]P and indole-3-carbinole have been shown to inhibit cell proliferation in several cell types studied (Cover et al., 1998; Gierthy and Crane, 1984; Hushka and Greenlee,

1995; Vaziri and Faller, 1997). Examination of the rat 5L hepatoma cell line indicated that TCDD-induced G_1-arrest was dependent on the AhR and involved inhibition of CDK2 activity due to $p27^{Kip1}$ induction (Kolluri et al., 1999; Weiss et al., 1996; Wiebel et al., 1991). One major target for phosphorylation by CDK2 is the Rb, which is critical for S-phase entry; hence, inhibition of CDK2 activity by $p27^{Kip1}$ will certainly halt the cell cycle in G1-phase. Interestingly, it has been shown that the AhR can interact with the hypophosphorylated form of Rb, which is restricted to the G_0- and G_1-phases of the cell cycle (Elferink et al., 2001; Ge and Elferink, 1998; Puga et al., 2000a). In sharp contrast to these studies, TCDD can promote liver and skin tumor growth, through involvement of the AhR, in rodents, and is furthermore classified as carcinogenic to humans (IARC, 1997). Numerous studies have shown that TCDD has a blocking effect on some types of cancer, whereas it acts as a tumor promoter in others (Bertazzi et al., 1993; Kayajanian, 1997). In an attempt to elucidate the mechanism of AhR physiology, transgenic mice expressing a constitutively active AhR (CA-AhR mice) was created by Poellinger and co-workers (Andersson et al., 2002; Andersson et al., 2003). These mice showed typical symptoms of TCDD exposure, such as thymic atrophy and increased liver weight. Unexpectedly, however, the CA-AhR mice showed increased mortality that correlated with the development of cystic tumors in the glandular part of the stomach as well as a reduced population of peritoneal B1 cells. Collectively, these results undoubtedly show that the AhR participates in cell cycle regulation; paradoxically, however, AhR seems to act as a tumor suppressor in some cases and as an oncoprotein in other.

AHR TARGET GENES

A battery of genes involved in biotransformation of xenobiotic and endobiotic compounds has been known for a long time to be upregulated via the AhR-mediated response to ligands such as TCDD. These genes include phase I enzymes, like CYP1A1, CYP1A2 and CYP1B1, as well as phase II conjugation enzymes, such as NAD(P)H:quinone oxidoreductase, glutathione S-transferase Ya and UDP glucuronosyltransferase. Furthermore, several genes involved in control of cell growth have also been shown to be upregulated by TCDD-dependent AhR activation, for instance plasminogen activator inhibitor-2, transforming growth factor-α and -β_2, *c-fos* and *c-jun*, (see Hankinson (1995) and references therein). Yet, regulation of these genes cannot explain many of the biological and toxicological effects mediated by the AhR. Several attempts have been made to systematically identify changes in gene expression following TCDD exposure by using different techniques, such as microarrays, differential display, and suppression subtractive hybridisation etc. In a study by Puga et al (Puga et al., 2000b) analysis by high density microarray hybridisation (>8000 genes) of the human HepG2 cell line treated for 8 hours with 10 nM TCDD revealed altered expression of 310 known genes (114 were induced and 196 were repressed), involved in cell cycle regulation, apoptosis, cell adhesion, protein trafficing, among others. Interestingly, genes with related role were in many cases induced or repressed with no apparent underlying rationale. Furthermore, in a recent study it was shown that exposure to B[*a*]P, but not TCDD, resulted in AhR-dependent induction of the proapoptotic Bax gene in oocytes, due to to a single basepair flanking the core XRE-element (Matikainen et al., 2001). These findings indicate that AhR-mediated gene

transcription can occur in a ligand-specific manner, in addition to a cell/tissue specific manner, making the evaluation of target genes even more complicated. These examples indicate that the biological outcome of AhR-dependent gene regulation is far more complex than previously imagined.

THE CYTOCHROME P450 SUPERFAMILY

The cytochrome P450s (CYP) are a superfamily of constitutive and inducible haem-containing oxidative enzymes with an important role in metabolism of endogenous and exogenous compounds. Currently about 100 human CYP genes are identified and classified into families and subfamilies based on amino acid sequence homology (http://drnelson.utmem.edu/CytochromeP450.html). The CYPs can be divided into two main groups: i) those that are mainly involved in metabolism of xenobiotics like drugs, environmental pollutants and carcinogens (CYP1, CYP2, CYP3 and to a lesser extent CYP4), and ii) those that are specifically involved in metabolism of endogenous compounds like fatty acids and steroids (Gonzalez, 1988). Induction of the CYP isoforms that participate in the metabolism of xenobiotics, such as CYP1A1, is usually advantageous in that it enhances the metabolism of lipophilic compounds, a process that help eliminating the compound from the body. Since the inducers often are also substrates of the induced CYP, the induction of the enzyme is generally limited in duration and amplitude as the inducing agents are metabolised by the enzyme. However, induction can be detrimental since CYPs often have broad substrate specificity, induction of one enzyme may lead to increased metabolism by a second, thereby altering the kinetic properties or toxic effect of the other enzyme (Hollenberg, 2002; Rendic, 2002). In addition, induction of CYPs can produce an imbalance between detoxification and activation, leading to formation of reactive species that can bind covalently to cellular components and cause mutagenicity and carcinogenicity.

CYP1 family

Upon exposure to PAHs or HAHs, mainly three CYPs, CYP1A1, CYP1A2 and CYP1B1, are transcriptional upregulated through the AhR signalling pathway. The CYP1A1 and CYP1B1 enzymes are largely responsible for metabolism of PAHs, whereas CYP1A2 preferentially metabolises polycyclic amines and amides (Whitlock, 1999; Whitlock et al., 1996). In addition to PAH metabolism and bioactivation, CYP1 enzymes have been shown to be involved in estrogen metabolism. CYP1A2 is constitutively expressed in human and rodent liver, where upon exposure induction mainly is taking place as well. CYP1A1, on the other hand, is not constitutively expressed in the liver, but is highly inducible in liver, as well as in other tissues including kidney, intestine, lung, skin and the brain. CYP1B1 is primarily expressed in extrahepatic tissues with the highest levels being found in the kidney, prostate, uterus and breast.

The CYP1 family enzymes are accountable for activation of many toxic, mutagenic and carcinogenic compounds. As an example, CYP1A1 metabolises B[*a*]P, found in cigarette smoke, to epoxides and arene epoxides, especially the *trans*-7,8-diol-8,10-

epoxide, which has been shown to readily form DNA-adducts and are associated with mutagenesis. Paradoxically, *Cyp1a1*(-/-) knockout mice exposed to a large daily dose of B[*a*]P were shown to be protected by surviving longer than *Cyp1a1*(+/-) heterozygotes (Uno et al., 2001). However, *Cyp1a1*(-/-) mice displayed higher B[*a*]P-DNA adduct levels probably due to slower clearance of B[*a*]P. It was suggested that the absence of CYP1A1 enzyme protected the animal from B[*a*]P-mediated liver toxicity and death, by decreasing the formation of toxic metabolites. CYPs are considered to have a central role in tumor development and progression, since they can activate or deactivate most carcinogens. Interestingly, CYP1B1 has been shown to be expressed at high levels in several types of human tumors, including cancers of the lung, breast, liver and the gastrointestinal tract (Murray, 2000). The tumor-specific expression of CYPs could provide a basis for the development of novel therapeutical strategies.

The regulatory region of CYP1A1

The human, rat and mouse *CYP1A1* genes have similar structure in their regulatory region and all contain multiple copies of the XRE element in the enhancer region approximately between –800 to –1300 basepair upstream of the transcriptional start site (Denison et al., 1988; Hines et al., 1988; Sogawa et al., 1986). The XREs are spaced at irregular interval, and this organisation will increase the probability that at least one site will face outwards from the histones and be accessible to the AhR-arnt-dimer when the enhancer is in a nucleosomal configuration. The promoter region immediately upstream of the transcriptional start site has binding sites for several transcription factors including the TATA-binding protein, Sp1 and nuclear factor 1, however, except for the TATA site, these binding sites are probably less important (Jones and Whitlock, 1990). The function of the promoter seems to be under enhancer control, thus the promoter region is silenced by nucleosomal binding in the absence of AhR-arnt binding to the enhancer region. Upon binding of AhR-arnt to the enhancer region, communication to the promoter region has been shown to be mediated through the TADs of AhR (Ko et al., 1996; Whitlock, 1999).

Binding of AhR-arnt upregulates *CYP1A1* transcription, however, suppression of the *CYP1A1* gene has been described to occur both in a species- and tissue specific fashion. Exposure of cells with TCDD plus protein synthesis inhibitors, like cycloheximide, activates *CYP1A1* transcription to a greater extent than TCDD alone, a phenomenon termed superinduction (Israel et al., 1985; Lusska et al., 1992). This phenomenon has been attributed to binding of a labile, inhibitory factor to the XRE, however, the identity of this factor remains ambiguous. Fibroblasts were shown to be nonresponsive to CYP1A1 induction by e.g. TCDD due to XRE-binding of arnt and a fibroblast-specific factor that appears to be regulated by histone deacetylase activity (Gradin et al., 1999). Similarly, adult, but not neonatal rabbit liver, is nonresponsive to CYP1A1 induction and it has been shown that rabbit XREs were blocked for AhR-arnt-binding by constitutive binding of the upstream stimulatory factor 1 (Takahashi et al., 1997). In addition to the enhancer elements, negative regulatory regions have also been described in the 5′-upstream flanking region of the *CYP1A1* gene, however, the binding factors and the function of these elements are poorly understood.

Induction of CYP1A1 by omeprazole

Omeprazole (OME), a substituted benzimidazole, is a potent suppressor of gastric acid secretion and has attained an important position in therapy of individuals suffering from gastroesophageal reflux disease. In 1990 Diaz and co-workers (Diaz et al., 1990) reported for the first time that OME produced a time- and dose-dependent induction of CYP1A levels in primary human hepatocytes, a finding that was later confirmed by using different human and rodent cell lines (Daujat et al., 1992; Quattrochi and Tukey, 1993). The *in vivo* induction of CYP1A1 and 1A2 by OME was shown by using biopsies from liver and alimentary tract of patents treated with OME (Diaz et al., 1990; McDonnell et al., 1992). However, by investigating CYP1A2 activity using the ^{13}C-[*N*-3-methyl]-caffeine breath test, therapeutic doses of OME seemed to induce CYP1A2 activity only in poor, but not in extensive metabolisers, of CYP2C19, the main enzyme responsible for OME metabolism (Andersson et al., 1994; Chiba et al., 1993; Rost et al., 1992). Moreover, there is a large interindividual variation in CYP1A2 activity and the extent of induction by OME shown in this study never exceeded the range of variability in healthy volunteers.

The mechanism of induction of CYP1A by OME and other benzimidazoles like lanzoprazole, albendazole, and thiabendazole, has been under debate. Originally, it was found that these compounds were capable of causing an increased expression of CYP1A mRNA in primary hepatocytes as well as in different cell lines, indicating a transcriptional activation mediated via the AhR. (Aix et al., 1994; Curi-Pedrosa et al., 1994; Daujat et al., 1992; Diaz et al., 1990; Kikuchi et al., 1996; Quattrochi and Tukey, 1993). In contrast, no specific binding of OME or lanzoprazole to the human hepatic cytosolic AhR was revealed by competition experiments using [^{3}H]TCDD as an AhR ligand (Curi-Pedrosa et al., 1994; Daujat et al., 1992). However, OME was shown to trigger translocation of the AhR to the nuclei, with subsequent binding to the XRE in the regulatory region of the human *CYP1A1* gene, indeed indicating involvement of the AhR (Kikuchi et al., 1996; Quattrochi and Tukey, 1993). Moreover, whereas induction of CYP1A1 was observed in human cell lines, primary mouse hepatocytes or hepatoma cell lines were refractory to induction. In an attempt to re-establish CYP1A1 inducibility in AhR-deficient mouse Hepa1c1c7 cells, human AhR was expressed, however, these cells did not respond to OME treatment, indicating that other cellular components other than the AhR were important for proper activation of the AhR signalling pathway (Kikuchi et al., 1996). This was supported by experiments using a reconstituted yeast model system showing that both mouse and human AhR could be activated by OME, although a direct interaction by OME to the mouse AhR could not be detected (Dzeletovic et al., 1997). Based on these findings, two models for the mechanism of induction by OME has been proposed, i) a metabolite of OME, but not the parent compound itself, constitutes the true ligand for the AhR, and ii) OME causes ligand-independent activation of the AhR by triggering of a signal transduction system. These two possibilities will be outlined more in detail in the "Discussion" section of this thesis.

AIMS OF THE PRESENT STUDY

Ligand-dependent activation of the AhR has been described to be essential for transcriptional activation of the *CYP1A1* gene. However, numerous compounds, structurally diverse from typical AhR ligands, have been shown to be capable of inducing CYP1A1 *in vivo* or in different cellular systems investigated. These novel CYP1A1 inducers include the inhibitor of gastric acid secretion, omeprazole (OME), the antimalarial drug primaquine (PRQ), as well as the bezimidazole component of omeprazole, 2-mercapto-5-methoxybenzimidazole (MMB). The overall aim of this thesis was to delineate the molecular mechanisms whereby these compounds activate *CYP1A1* gene transcription. The more specific objectives were:

- To elucidate the involvement of the AhR in OME-, MMB- and PRQ-mediated *CYP1A1* induction
- To investigate the possible binding or interaction of OME, MMB and PRQ with the AhR
- To examine the involvement of intracellular signal transduction pathways, mainly protein kinase signalling, on OME-dependent AhR activation
- To investigate the role of phosphorylation of the AhR in AhR-mediated signalling

COMMENTS ON METHODOLOGY

CELL LINES

The cell system most extensively used for studying CYP1A1 induction is the mouse hepatoma Hepa1c1c7 cell line, which has proven very useful especially since cell variants that are defective in AhR, arnt or CYP1A1 exist in addition to the wild type. However, OME does not confer CYP1A1 inducibility in this cell type, as first reported by Kikuchi et al (Kikuchi et al., 1995). Initial experiments carried out in our laboratory indicated on the other hand that the rat hepatoma H4IIE cell line showed a robust response of CYP1A1 induction by OME and was therefore chosen as a model system. However this cell line has some disadvantages; the level of AhR is extremely low, approximately 65-fold lower than in Hepa1c1c7 cells (Holmes and Pollenz, 1997), and cannot be detected by Western blotting, secondly, the transfection efficiency is quite poor. Therefore, the human hepatoma HepG2 cell line was used in some experiments. Interestingly, a large interspecies or cell line difference in response exerted by atypical CYP1A1 inducers has been reported, which is not observed for PAHs or HAHs.

COMPOUNDS USED IN THIS STUDY

Figure 4 shows the chemical structures for the three main compounds investigated in this study, as well as the structure of TCDD, the prototype AhR ligand. In addition to OME, numerous metabolites and breakdown products of OME have been evaluated in this study for their capacity to induce CYP1A1, wherein MMB was found to be a significant inducer and was therefore chosen for more detailed examination.

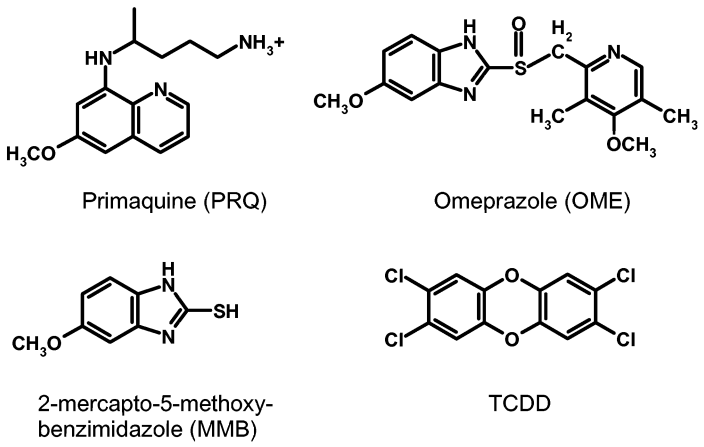

Figure 4. Structures of the compounds used in this study.

ANALYSIS OF hnRNA

Transcriptional activation of the *CYP1A1* gene was analysed by measuring the levels of hnRNA, i.e. nascent, unspliced transcripts, which mirror the rate of transcription of the gene (Elferink and Reiners, 1996). Total RNA was isolated from H4IIE cells by standard techniques and was subjected to reverse transcription using a reverse transcription-primer specific for intron 1 of the *CYP1A1* gene. Following reverse transcription, the amount of hnRNA was quantified by performing a competitive PCR using different concentrations of a heterologous standard. The PCR products were separated by agarose gel electrophoresis and negative Polaroid photographs were taken for analysis of the band intensity with a laser densitometer.

LUCIFERASE REPORTER AND GAL4-AHR HYBRID CONSTUCTS

For analysis of transcriptional activation of the *CYP1A1* gene and to elucidate the involvement of the AhR-complex, several luciferase reporter constructs has been utilised (Figure 5). The XRE-pGL3 reporter plasmid contained a fragment of the rat *CYP1A1* 5'-flanking region extending from nucleotides -1139 to -848 relative to the transcription start site in front of the SV40 promoter and the firefly luciferase reporter gene (pGL3-promoter vector). The 1195pGL3 and 917pGL3 reporter constructs contained fragments of rat CYP1A1 5'-flanking region spanning from nucleotides -1195 to +61 and -917 to +61, respectively.

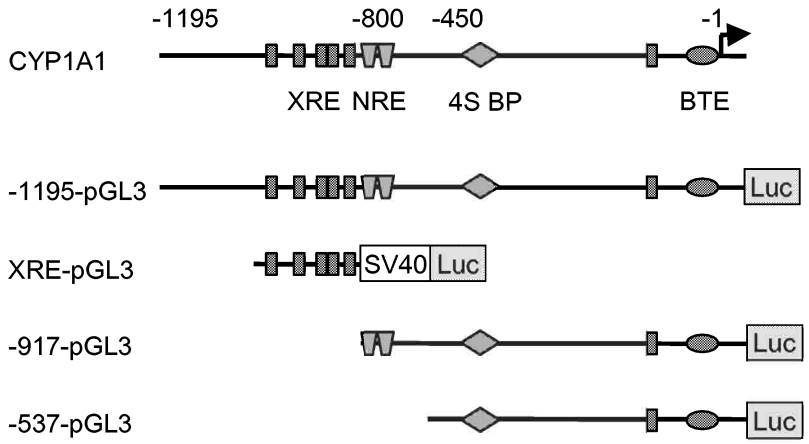

Figure 5. Regulatory region of the rat *CYP1A1* gene and schematic representation of luciferase reporter gene constructs. XRE, xenobiotic response element; NRE, negative regulatory region; 4S BP, region for binding of 4S PAH-binding protein; BTE, basal transcription element, including TATA sequence; Luc, firefly luciferase; SV40, simian virus 40 promoter.

The Gal4-AhR hybrid protein (Figure 6) was created by cloning of the rat AhR into the mammalian expression vector pFA-CMV containing the cDNA for the yeast Gal4 DNA-bindning domain (amino acids 1-147). Several constructs were created starting with amino acid 2, 37 or 83 of rat AhR, respectively, however after initial experiments the Gal4-AhR37 hybrid construct was chosen for all subsequent experiments. The Gal4-AhR expression is driven by the CMV promoter, however, activity of the co-transfected Gal4-dependent luciferase reporter gene is only detected after stimulation of the cells with AhR activators.

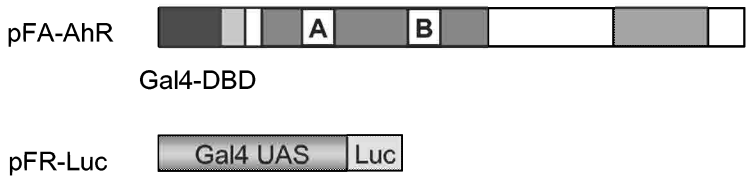

Figure 6. Schematic representation of the Gal4-AhR expression vector, pFA-AhR, and the pFR-Luc reporter gene. The yeast Gal4 DNA-binding domain was fused in front of rat AhR, lacking the first 37 amino acids.

Site directed mutagenesis of amino acids in the AhR ligand-binding domain was carried out by PCR amplification using mutation specific primers and subsequent subcloning of the mutation-containing fragment into the properly cut pFA-AhR vector. To generate some amino acid mutations, a method described by Bi and Stambrook (Bi and Stambrook, 1997), combining a ligase chain reaction and PCR was applied. This method uses two external primers, that amplify the region of interest and span two unique restriction enzyme sites, and one internal mutagenic primer with a 5'-phosphorylation that introduce the wanted mutation. In addition to the DNA polymerase, a thermostable ligase is utilised in the PCR reaction, to ligate the 5´-end of the internal primer to the 3´-end of the fragment extending from the external primer. The PCR products obtained were cut with the unique restriction enzymes and subcloned into the appropriate cloning sites in the pFA-AhR vector.

ANALYSIS OF AHR LIGAND BINDING AND ACTIVATION

Sucrose density gradient experiments have frequently been used to study ligand binding, however, the extremely high AhR-binding affinity of [^{3}H]TCDD makes it technically difficult to demonstrate competitive binding by low affinity ligands. Therefore a low concentration of [^{3}H]TCDD (1 nM/mg protein) was utilised, so that saturation of AhR binding sites is hardly obtained, whereas as high concentration of the competitor as possible was used while retaining its solubility. Both rat liver cytosol and cytosol prepared from HEK293 cells expressing wild type or mutant Gal4-AhR hybrid proteins has been successfully used in this assay. Following centrifugation on a linear 5-30% sucrose density gradient, the [^{3}H]TCDD-labelled AhR was recovered approximately in the 9S region of the gradient, consistent with the sedimentation properties of the AhR-hsp90 complex. Approximately 250 µl fractions were collected

from the bottom of the gradient and radioactivity was measured by scintillation counting.

THE USE OF PROTEIN KINASE INHIBITORS

In recent years, several small, cell-permeable inhibitors of protein kinases have been developed that exhibit relatively high degree of specificity for particular protein kinases. These inhibitors are very useful tools in elucidating the contribution of specific signalling pathways in cellular functions and finding physiological substrates of these enzymes. Nearly all protein kinase inhibitors are competitive towards the enzymes recognition site of ATP, whereas some compete with the substrate-binding site. Although these agents are produced with the intention of being specific for only one particular protein kinase, they are often having a broader specificity. Nevertheless relatively non-specific protein kinase inhibitors can still be useful for excluding the involvement of particular protein kinase(s) in a cellular process.

In this study several PTK inhibitors have been used, mainly genistein and a few tyrphostins. Genistein is a relatively specific inhibitor of PTKs including those of the platelet-derived growth factor, insulin receptor as well as $pp60^{src}$, $pp110^{gag\text{-}fes}$ and $pp56^{lck}$ but does not inhibit serine or threonine kinases such as PKC (Akiyama and Ogawara, 1991). Tyrphostins are a series of synthetic compounds that structurally resemble the tyrosine and erbstatin moieties and therefore in general inhibit PTKs by binding to the substrate binding site (Gazit et al., 1989; Levitzki and Gazit, 1995). Tyrphostins inhibit a smaller group of RTKs than genistein, and was utilised in an attempt to find the signalling pathway involved in OME-mediated CYP1A1 induction. Unfortunately, rather limited information is available in the literature about the exact enzyme inhibition and IC_{50} concentration for each particular tyrphostin.

RESULTS

INDUCTION OF *CYP1A1* GENE EXPRESSION BY NOVEL INDUCERS IN DIFFERENT HEPATOMA CELL LINES (PAPER I, II, III AND IV)

In the rat hepatoma H4IIE cell line, several benzimidazoles, e.g. omeprazole (OME), thiabendazole (TBZ), carbendazim (BCM) and 2-mercapto-5-metoxy-benzimidazole (MBB) were shown to cause an induction of *CYP1A1* gene expression. Northern blot analysis revealed that H4IIE cells treated for 12 h with 200 μM of OME, MBB, TBZ and BCM caused an increase of CYP1A1 mRNA in the range of 20% to 45% of the magnitude seen after maximal induction by 10 nM TCDD. In comparison to TCDD the response of CYP1A1 induction by OME was delayed by 6-9 hours. Examination of the structure–activity relationship of different substituents at the 2- and 5-positions of the benzimidazole moiety indicated that compounds containing a nucleophilic group in the 2-position favoured CYP1A1 induction.

The quinoline compound primaquine (PRQ) was also shown to cause an increase in *CYP1A1* gene expression in H4IIE cells. Maximal induction was seen at a concentration of 45 μM and reached approximately 70% of the effect caused by 10 nM TCDD. In contrast to OME, PRQ caused a rapid increase in CYP1A1 mRNA levels comparable to TCDD, however, the increase was transient with a peak at 6 hours.

The induction of CYP1A1 by OME, MMB and PRQ was further examined in the human HepG2 and mouse Hepa1c1c7 hepatoma cell lines. Western blot analysis revealed that both OME and PRQ, but not MMB, caused induction of CYP1A1 in HepG2 cells. In contrast, MMB was capable of causing CYP1A1 induction in Hepa1c1c7 cells, whereas OME and PRQ were not.

POST-TRANSLATIONAL REGULATION OF CYP1A1 BY PRIMAQUINE (PAPER III)

Although PRQ caused a transient increase in CYP1A1 mRNA, a sustained elevation for at least 24 hours of the CYP1A1 protein was seen, indicating that PRQ could influence the stability of CYP1A1. Indeed, PRQ caused a dose-dependent inhibition of CYP1A1 protein degradation in V79 cells, stably transfected with a human CYP1A1 cDNA. PRQ formed a type II binding spectrum with CYP1A1 and inhibited CYP1A1-dependent 7-ethoxyresorufin *O*-deethylase activity *in vitro*, indicating that the drug protects CYP1A1 from degradation by binding to the active site.

TRANSCRIPTIONAL ACTIVATION OF THE *CYP1A1* GENE BY NOVEL INDUCERS (PAPER I, II AND III)

The transcriptional activation of the *CYP1A1* gene by the compounds examined in this study was investigated either by monitoring hnRNA levels by RT-PCR or by transient transfection assays using reporter constructs derived from the rat *CYP1A1* 5´-flanking region. Increased hnRNA levels were observed after treatment of H4IIE cells with OME or TCDD for 12 hours. Transient transfection of H4IIE cells with an XRE-driven luciferase reporter gene (XRE-pGL3), revealed an increase in luciferase activity caused by OME, MMB, TBZ, BCM as well as PRQ, comparable to the magnitude obtained by Northern blotting. Transfection of pGL3-constructs lacking the XRE element did not display increased luciferase activity by any of these compounds. Taken together these results showed that OME, MMB, TBZ, BCM and PRQ were weak inducers of the *CYP1A1* gene, and indicated that the transcriptional activation was mediated via the AhR.

INVOLVEMENT OF THE AHR (PAPER I, III AND IV)

The involvement of the AhR in the transcriptional activation of the *CYP1A1* gene caused by the compounds investigated in this study was examined by both cellular and *in vitro* assays by i.e. i) XRE-binding of nuclear proteins by EMSA, ii) activation of the Gal4-AhR construct in transfected cells, iii) activation of cytosolic AhR to a DNA-binding from (transformation) and, iv) sucrose density gradient ligand-binding assay. All compounds used in this study were capable of activating the Gal4-AhR construct in transiently transfected H4IIE cells. Furthermore, MMB and PRQ were capable of transforming cytosolic AhR to a DNA-binding form and to displace AhR-bound [^{3}H]TCDD *in vitro*. In contrast, OME failed in both these assays. Thus, based on these four assays one can distinguish two groups of compounds: 1) agents that succeeded both in the cellular and the *in vitro* examination and probably are weak AhR ligands, and 2) agents like OME that activates the cellular AhR but were ineffective in the *in vitro* tests.

PRQ and MMB are low affinity ligands for the AhR (Paper III and IV)

Incubation of cytosols prepared from H4IIE cells or Hepa1c1c7 cells with 200 µM MMB or 30 µM PRQ for 2 hours at 4°C and subsequent incubation with a ^{32}P-labeled XRE-probe revealed formation of an XRE-bound protein complex as analysed by EMSA. The nature of the XRE-bound proteins was identified as AhR and arnt, since addition of antibodies against these proteins produced supershifts in the EMSA.

To evaluate whether MMB and PRQ could compete with [^{3}H]TCDD for AhR binding in rat liver cytosols, sucrose density gradient ligand-binding assay was carried out. Since these compounds most likely were weak receptor ligands a non-saturating concentration of [^{3}H]TCDD and high concentration of the competitor was used in the incubation reaction. Analysis of the AhR containing 9S region of the gradient showed that approximately 50% and 100% of the radiolabelled TCDD was displaced by using

40,000- and 200,000-fold molar excess of PRQ, respectively. At a 200,000-fold molar excess of MMB approximately 65% of the AhR-bound [^{3}H]TCDD was displaced.

Ligand-independent activation of the AhR by OME (Paper I and IV)

Preparation of nuclear extracts from H4IIE cells stimulated with OME for different time points, and subsequent EMSA analysis revealed that formation of an XRE-bound AhR-complex was obtained after 2 hours of treatment. However, maximal formation of a TCDD-induced AhR-arnt-XRE-complex was reached already at 1 hour. Transfection of H4IIE cells with the pFA-AhR vector and stimulation with OME or TCDD revealed a delayed AhR activation response of OME as compared to TCDD. The increase in luciferase activity measured after 20 hours of stimulation with OME and TCDD was 12.5- and 34-fold, respectively.

In contrast to PRQ and MMB, OME (at up to 500 μM) was not capable of transformation of the cytosolic AhR to a DNA-binding form, irrespective of whether the cytosol was prepared from rat liver, H4IIE or Hepa1c1c7 cells. Evaluation of OME in the ligand-binding assay showed that OME was incapable of competition with [^{3}H]TCDD for AhR-binding even at a 600,000-fold molar excess. Given that the potencies of OME and MMB in activation of the Gal4-AhR construct in H4IIE cells were approximately the same and MMB competed with AhR-bound [^{3}H]TCDD at a 200,000-fold excess whereas OME apparently failed to do so at higher concentration, indicates that OME does not directly bind to the AhR.

MECHANISM OF LIGAND-INDEPENDENT AHR SIGNALLING (PAPER I AND V)

To investigate whether OME caused AhR activation by a mechanism distinct from that of high affinity ligands biologically active substances, such as protein kinase inhibitors but also growth factors, were used to try to identify components specifically involved in OME-dependent AhR signalling. Several agents were found that inhibited OME-mediated AhR function exclusively without having a significant effect on TCDD induced AhR activity. Interestingly, the interference occurred at different points of AhR signal transduction as detected by i) Gal4-AhR activation, ii) formation of an XRE-bound AhR-complex, iii) activation of the XRE-pGL3 reporter gene and iv) an increase in hnRNA levels.

Inhibition of AhR activation by PTK inhibitors (Paper I and V)

Several serine/threonine and tyrosine kinase inhibitors were investigated for their capacity to block OME-dependent AhR activation by using the Gal4-AhR system. Interestingly, inhibitors selective for the MAPK or PI-3K/PKB pathways, such as PD98056, SB203580, wortmannin and rapamycin did not considerably affect OME-mediated Gal4-AhR activation. However, our results revealed that OME-dependent Gal4-AhR activation in H4IIE cells was selectively and completely blocked by a few tyrosine kinase inhibitors i.e. genistein, tyrphostin AG17 and tyrphostin AG879.

Northern blot analysis revealed an almost complete lack of CYP1A1 mRNA expression in H4IIE cells co-treated with OME and genistein, tyrphostin AG17 or tyrphostin AG879, as compared to cell treated with OME alone, indicating that these agents were capable of blocking endogenous AhR responses. Moreover, genistein was shown to inhibit OME-induced binding of the AhR-complex to XRE as well as transcriptional activation of the XRE-pGL3 luciferase reporter in H4IIE cells. In contrast, genistein caused an augmentation of TCDD-mediated XRE-binding and XRE-pGL3 reporter transactivation.

In addition to AG17 and AG879 several other structurally related tyrphostins, i.e. AG82, AG494, AG555 and AG1478, were also tested, however, none of these had any major effect on OME-mediated Gal4-AhR activation. The strong effect of tyrphostin AG17 and tyrphostin AG879 prompted us to examine the effective dose of these two tyrphostins, and a 50% inhibitory response of Gal4-AhR activation by OME was revealed at only 85 nM and 400 nM of tyrphostin AG17 and tyrphostin AG879, respectively. The AhR levels were determined in total cell extracts prepared from H4IIE cell treated with OME in the presence or absence of genistein, tyrphostin AG17 or tyrphostin AG879 and found to be similar, indicating that the inhibitors did not cause degradation of the AhR protein.

Inhibition of XRE-dependent AhR transactivation (Paper I and V)

Initially we found that the increase in CYP1A1 mRNA or hnRNA caused by OME was blocked by genistein and daidzein. Examination of an effect of these compounds on the transcriptional activation of the XRE-dependent pGL3-reporter gene revealed that both genistein and daidzein caused an inhibition of OME-mediated reporter activation. However, in contrast to genistein, daidzein was not capable of inhibition of OME-induced XRE-binding of the AhR-complex. Furthermore, activation of the Gal4-AhR by OME was not blocked by daidzein. Thus, these data indicate that the interference of daidzein occurs downstream of the point of genistein action.

Inhibition of transcriptional activation of the *CYP1A1* gene (Paper I)

A third point of inhibition of AhR-dependent transcriptional activation was revealed by treatment of H4IIE cells with insulin in combination with OME or TCDD. Northern blot analysis showed that OME-induced CYP1A1 mRNA expression was completely blocked by insulin while the TCDD-mediated induction of CYP1A1 mRNA was partly inhibited. Measurement of hnRNA levels by quantitative RT-PCR indicated that the OME-mediated transcriptional activation of the *CYP1A1* gene was blocked by insulin. However, no effect of insulin on the transcriptional activation of the XRE-pGL3 reporter gene by OME was observed. Furthermore, a luciferase reporter construct containing the rat *CYP1A1* gene extending from –4244 to + 2557 relative to the transcription start site, did not confer this insulin response (V. Dolžan, unpublished observations).

REGULATION OF AHR BY C-SRC (PAPER V)

The involvement of c-src kinase in AhR signalling was investigated, since genistein has been reported to inhibit c-src activity. An attenuation of the responses caused by OME or TCDD on Gal4-AhR activation was observed in H4IIE cells treated with the specific c-src inhibitors PP1 and PP2. The inhibition obtained by 10 μM PP1 and 10 μM PP2 on OME-mediated Gal4-AhR activation was 69% and 58%, respectively, and on TCDD-mediated activation 60% and 57%, respectively. Northern blot analysis revealed that both PP1 and PP2 caused an attenuation of the OME-dependent increase in CYP1A1 mRNA levels indicating that the endogenous AhR response was blocked as well.

To confirm that the effect of PP1 or PP2 was due to c-src but not other closely related src family kinases, a dominant negative c-src (DN-src) expression vector was co-transfected with the Gal4-AhR in H4IIE cells and the cells were subsequently stimulated by OME or TCDD. The Gal4-AhR-dependent luciferase activity decreased inversely correlated to the amount of DN-src expression vector added and at the highest amount of vector added the inhibition reached 65% and 32% by OME and TCDD treatment, respectively.

Comparable results were also obtained in human hepatoma HepG2 cells, either with PP1 and PP2 stimulation or expression of the dominant negative c-src, indicating no cell specific action of c-src inhibition. Examination of Gal4-AhR levels revealed that expression of DN-src did not have any significant influence on the levels of Gal4-AhR protein expression in transfected HepG2 cells, suggesting that c-src inhibition did not influence on Gal4-AhR stability. Taken together these data indicate that AhR signalling in general is modulated by c-src kinase activity.

MUTATIONAL ANALYSIS OF THE AHR LBD (PAPER IV AND V)

A site-directed mutagenesis approach was used to try to identify potential phosphorylation sites of the LBD that could be important for OME-dependent AhR signalling. A computer-assisted analysis of the 192-amino acid fragment (amino acids 234 to 425) constituting the LBD of rat AhR predicted several potential phosphorylation sites and seven of these amino acids were selected for site-directed mutagenesis in the context of the Gal4-AhR construct. Transfection of the wild type and mutant Gal4-AhR constructs in H4IIE cells and stimulation with TCDD revealed no major difference in the capacity of reporter gene activation. Five of the seven mutants, Gal4-S274A, Gal4-Y376F, Gal4-T380, Gal4-Y412F and Gal4-S419A, were also activated to a similar extent by OME, MMB and PRQ as the wild type Gal4-AhR. In contrast, a selective and total abrogation of activation of the Gal4-Y320F and Gal4-T385V hybrid protein by OME was observed. Analysis of the expression level of all Gal4-hybrid proteins showed that all mutants were expressed to the same level, indicating that the lack of activation of Gal4-Y320F or Gal4-T385V was not due to lower expression levels. Furthermore, the Gal4-hybrid proteins were shown to maintain their interaction with the chaperone hsp90.

Activation of the different mutant Gal4-AhR constructs by MMB and PRQ revealed a lack of activation of the Gal4-Y320F construct, similarly to OME, and a reduced response of the Gal4-T385F mutant. In order to examine whether the Gal4-Y320F-construct showed an impaired ligand-binding capacity compared to the wild type Gal4-AhR, the mutant and wild type Gal4-proteins were expressed in HEK393 cells and [^{3}H]TCDD ligand-binding assay was carried out. Saturation binding analysis was carried out with increasing concentrations of [^{3}H]TCDD and maximal binding was obtained already at approximately 10 nM of [^{3}H]TCDD. Regression analysis verified a similar binding capacity of [^{3}H]TCDD for Gal4-AhR and Gal4-Y320F proteins with similar B_{max} and K_d values. Incubation of Gal4-AhR extracts with 5nM [^{3}H]TCDD in absence or presence of 1 µM TCDD or 1mM PRQ revealed a complete competition by TCDD and approximately 60% competition by PRQ. Similarly a 200-fold molar excess of TCDD was sufficient to completely displace the binding of [^{3}H]TCDD to Gal4-Y320F protein. In contrast, incubation of the cell extracts containing the Gal4-Y320F protein with a 200,000-fold molar excess of PRQ showed no displacement at all of the bound radiolabelled TCDD. In summary, these results identified Tyr^{320} in the LBD of the rat AhR as critical amino acid for binding of MMB and PRQ, as well as for activation by OME, but not for the high affinity ligand TCDD.

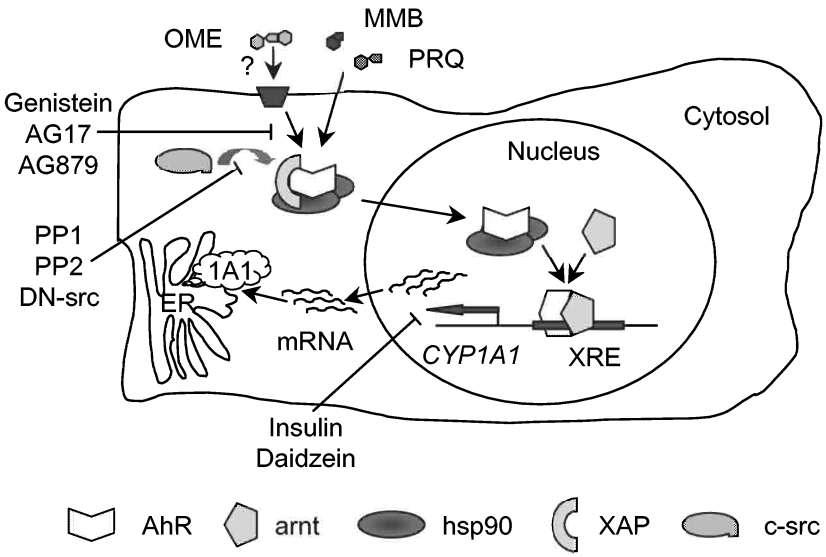

Figure 7. Illustration summarising the main results of this thesis.

DISCUSSION

The mechanism of transcriptional activation of the *CYP1A1* gene has been extensively studied throughout the past 30 years, and the concept of ligand-dependent activation of the AhR, which functions as a transcription factor, is very well established. Although numerous chemicals, originating from the environment, diet or endogenously formed compounds have been shown to bind to or activate the AhR, no true physiological ligand relevant for AhR biology has been identified so far. In fact, it is possible that several biologically active substances are responsible for ligand-dependent AhR activation. Furthermore, a ligand-independent mechanism of AhR activation is plausible. Since the prototype AhR ligand, TCDD, is known to cause a number of serious toxic effects, induction of CYP1A1 traditionally has been associated with deleterious effects, and as such used as a marker in toxicity screening.

This thesis was initiated by the findings that OME, a frequently used drug in the industrialized countries, could cause induction of CYP1A1/CYP1A2 in the liver and the alimentary tract (Diaz et al., 1990; McDonnell et al., 1992). The chemical structure of this drug does not resemble that of typical AhR ligands. Therefore, the finding that OME did not seem to interact with the AhR may not have come as a surprise, and it was even questioned whether the AhR was involved in the process of CYP1A1 induction whatsoever. Thus, the participation of the AhR in OME-mediated CYP1A1 induction needed to be clarified, and if the AhR was involved – how would it be activated by OME?

MECHANISMS OF ACTIVATION OF THE AHR BY OME

By using the rat hepatoma H4IIE cell line, we first confirmed that OME was capable of inducing CYP1A1 (Paper I), as well as CYP1A2 and CYP1B1 (unpublished results), mRNA levels. The increase in CYP1A1 mRNA expression caused by OME was approximately 35% of the maximal induction caused by 10 nM TCDD (Paper I). In addition to OME, several other benzimidazoles like lansoprazole, thiabendazole and carbendazim were also shown to cause an induction of *CYP1A1* gene expression (Paper II). These findings were in agreement with several other studies showing that structurally related benzimidazoles are weak inducers of *CYP1A1* gene expression (Aix et al., 1994; Curi-Pedrosa et al., 1994; Daujat et al., 1996; Daujat et al., 1992; Diaz et al., 1990; Dzeletovic et al., 1997; Kikuchi et al., 1995; Quattrochi and Tukey, 1993; Rey-Grobellet et al., 1996).

The involvement of the AhR was demonstrated by OME's capability of forming a nuclear XRE-binding protein complex, consisting of the AhR and arnt, as well as by activation of a Gal4-AhR construct in transfected H4IIE cells (Paper I and IV). On the other hand, OME was not capable of transforming cytosolic AhR, prepared form H4IIE or Hepa1c1c7 cells, to a DNA-binding form *in vitro*. Furthermore, OME did not displace AhR-bound [^{3}H]TCDD in rat liver cytosols, not even when added at a 600,000-fold molar excess. These results indicate that OME can activate the cellular

AhR, but does not directly interact with the AhR. Similarly to these findings, no interaction of OME was registered by using human or mouse AhR (Daujat et al., 1996; Daujat et al., 1992; Dzeletovic et al., 1997). The activation of the AhR by OME without any apparent binding to the receptor has been explained by two different models, e.g. i) a metabolite of OME is the true ligand, or ii) ligand-independent activation of the AhR is triggered by OME.

The three major metabolites found in plasma of patients treated with OME are hydroxyomeprazole, omeprazole sulfone and 5-*O*-desmethylomeprazole (Andersson et al., 1994). A modest induction of CYP1A1 was recorded by hydroxyomeprazole, whereas 5-*O*-desmethylomeprazole was not capable of inducing CYP1A1 levels in H4IIE cells (Paper II). No displacement of [^{3}H]TCDD from the 9S enriched cytosolic fraction, prepared from human liver, by OME and omeprazole sulfone was observed by Daujat et al (Daujat et al., 1992). The four small 2- and 5-substituted benzimidazole compounds studied in Paper II, have been found in urine of OME-treated rats and could theoretically also be formed in humans. Of these compounds only MMB was capable of causing a significant CYP1A1 induction, comparable to OME, in H4IIE cells. In contrast to OME, MMB was shown to be capable of displacing AhR-bound [^{3}H]TCDD by using a 200,000-fold molar excess (Paper IV). Furthermore, by incubation of rat or mouse cytosol with 200 μM MMB, AhR was transformed to the DNA-binding form. However, based on the dose-dependency of CYP1A1 induction by MMB (Paper II) it is clear that this compound could not constitute the OME-metabolite responsible for AhR activation.

In another attempt to identify a hypothetical OME-metabolite, growth media from H4IIE cells was analysing the by HPLC, without finding any compound capable of inducing CYP1A1 (unpublished observations). The mouse hepatoma Hepa1c1c7 cells do not respond to CYP1A1 induction upon OME treatment, which could be due to lack of production of this hypothetical AhR ligand. However, conditioned media transferred from OME-treated H4IIE cells did not confer inducibility of Hepa1c1c7 cells. Similar experiments were performed by Kikuchi et al using conditioned media from HepG2 cells (Kikuchi et al., 1996).

A planar sulfenamide structure, generated by acidic decomposition of OME, has been proposed by Dzeletovic et al (Dzeletovic et al., 1997) to be a true AhR ligand. In fact, OME as well as lansoprazole and pantoprazole, have a unique chemical feature that contributes to the specificity of these drugs and makes them therapeutically inactive at neutral pH (Lindberg et al., 1986). However, upon entering an acidic compartment of a pH of 3 or lower, the inactive parent compound is transformed to an active cyclic sulfenamide principle that binds covalently to sufhydryl groups of cysteins in the H^+/K^+-ATPase. Such low pH values are encountered in the body exclusively within the canaliculus of the activated, secreting parietal cell, in the close proximity to the enzyme. The sulfenamide structure is very labile and transient in nature and has been difficult to synthesise or trap in conditioned media. Therefore, Dzeletovic and co-workers tested two analogues of OME, timoprazole and deoxytimoprazole, the former capable of forming the sulfenamide ring structure whereas the latter is not. At a concentration of 5 μM timoprazole, but not deoxytimoprazole, was capable of activating the AhR by using a reconstituted yeast model system. However, the

magnitude of induction was similar as to that caused by 5 μM OME in this system, furthermore, deoxytimoprazole was also capable of inducing AhR activity at a concentration of 50 μM.

The theory of a planar OME sulfenamide structure is especially intriguing, however, a few questions remain unanswered; 1) OME, lansoprazole and pantoprazole would all readily form the active sulfenamide principle, however, in several systems investigated only OME and lansoprazole are capable of activating the AhR (unpublished observations and (Krusekopf et al., 1997; Krusekopf et al., 2003). 2) Although OME can activate the mouse AhR in a reconstituted yeast system, it is not capable of activating the receptor in intact Hepa1c1c7 cells or the human AhR transfected into the AhR-deficient variant (Dzeletovic et al., 1997; Kikuchi et al., 1996). Since the formation of the sulfenamide occurs through a non-enzymatic reaction the non-responsiveness of these cells cannot be explained by lack of the cellular system responsible for generating this principle. 3) OME is a weak base that would accumulate in acidic compartments where the conversion to a sulfenamide could take place. At neutral pH, like in the cytoplasm of cells, this formation would be extremely slow, if existing at all. Moreover, if the sulfenamide would be formed it is more likely that it would attack cysteins and form covalent disulfide-bonds to proteins than bind as a ligand by hydrophobic interactions.

An alternative mechanism of OME-mediated AhR activation includes the participation of cellular signalling systems that probably involves PTKs. This hypothesis is based on the findings that several tyrosine kinase inhibitors, including genistein, herbamycin and several tyrphostins, can selectively block OME-mediated AhR activation and/or CYP1A1 induction, without having any major effect on the TCDD-mediated response (Paper I and V; Kikuchi et al., 1998). In transfected H4IIE cells OME-mediated activation of the Gal4-AhR protein was completely blocked by treatment of 80 μM genistein. Similarly, two tyrphostins, AG17 and AG879 selectively blocked OME-, but not TCDD-dependent activation of the Gal4-AhR construct. In support of a distinct mechanism of AhR activation by OME, as compared to AhR-ligands, Kikuchi and co-workers have reported that two antagonists of the AhR, α-naphtoflavone and 3′-methoxy-4′-aminoflavone, did not inhibit CYP1A1 induction produced by OME in HepG2 cells, whereas both of them inhibited the response elicited by TCDD (Kikuchi et al., 1998).

Receptor transactivation between different types of cell surface receptors is a signalling process obtaining increasing awareness. For instance, receptors for insulin, platelet derived growth factor and epidermal growth factor are optimally activated by their ligands only under appropriate attachment conditions, thus depending on integrins (Giancotti and Ruoslahti, 1999). Cross-talk between G protein-coupled receptors and tyrosine kinase receptors has also been described. Recently, several examples of activation of intracellular receptors by stimulation of plasma membrane receptors have been presented (Cenni and Picard, 1999; Mazure et al., 2003; Weigel and Zhang, 1998). One such example is the finding of ligand-independent activation of the GR upon stimulation of human lung fibroblasts or vascular smooth muscle cells by β_2-adrenergic receptor agonists, salbutamol and salmeterol (Eickelberg et al., 1999).

It is reasonable to think that OME could interact with a cell surface receptor thereby initiating a signalling cascade that ultimately leads to a conformational change of the AhR, which is necessary for obtaining the nuclear translocation and the transcriptionally active complex. The final structural conformation obtained by the ligand-independent and -dependent activation of the AhR might be identical, but the mechanism of transducing the signal would differ. Interestingly, only tyrosine kinase inhibitors were shown to selectively block OME-mediated AhR activation, although several different serine/threonine protein kinase inhibitors were also tested for their capability to induce OME-mediated Gal4-AhR activation. Tyrphostins are generally competitive inhibitors towards the substrate-binding site of the enzyme, thus making them rather specific tyrosine kinase inhibitors. However, limited information about which particular PTK they block and their efficacy in cellular systems is available in the literature. In this study 50% inhibition of Gal4-AhR activation by OME, was obtained at a concentration of 85 nM and 400 nM of tyrphostin AG17 and tyrphostin AG879, respectively, which is a remarkably low dose (Paper V). At this concentration tyrphostin AG17 has been reported to reduce the TNFα induced integrin-dependent respiratory burst in primary human neutrophils, which was attributed to inhibition of Pyk2 (proline-rich tyrosine kinase) a member of the focal adhesion kinase family (Avdi et al., 2001; Fuortes et al., 1999). In another study, tyrphostin AG17 was shown to block c-src- and Pyk2-dependent recruitment of accessory molecules to integrins in osteoclasts during bone resorption (Lakkakorpi et al., 2001). However, in agreement with the results by Lakkakorpi et al showing that tyrphostin AG17 was incapable of blocking c-src or Pyk2 kinase activity *in vitro,* no inhibition of tyrphostin AG17 or tyrphostin AG879 was observed in an *in vitro* kinase assay using recombinant c-src kinase (Paper V). Furthermore, the dose of tyrphostin AG879 shown in our investigation to be capable of blocking OME-mediated AhR activation is, to our best knowledge, the lowest concentration reported to block any intracellular event. Thus, our results suggest that other as yet unidentified protein kinase(s) are the direct targets of tyrphostin AG17 and tyrphostin AG879 and their activity is critical for OME-dependent activation of AhR signalling. The identity of these protein kinases remains to be elucidated.

PRQ IS A LOW AFFINITY LIGAND FOR THE AHR AND PROTECTS CYP1A1 FROM DEGRADATION

The antimalarial drug PRQ was found to be a very potent inducer of *CYP1A1* gene expression, reaching maximal induction, approximately 70% of the effect caused by 10 nM TCDD, at a concentration of 45 μM in H4IIE cells. Northern blot analysis showed a time-dependent, but transient, increase in CYP1A1 mRNA levels by PRQ with a peak at 6 hours (Paper III). Ligand binding assay revealed a complete competition of 5 nM [^{3}H]TCDD by using 1 mM PRQ in rat liver cytosol. Furthermore, PRQ was capable of causing transformation of cytosolic AhR, prepared from either rat H4IIE or mouse Hepa1c1c7 cells, to a DNA-binding form *in vitro* (Paper V). These findings are in sharp contrast to the results obtained by OME, and indicate that PRQ probably is a weak ligand for the AhR. PRQ was initially shown by Fontaine and co-workers (Fontaine et al., 1999) to increase both ethoxyresorufin-O-deethylase activity and

CYP1A1 mRNA in human hepatocytes as well as in HepG2 cells, but no displacement of [^{3}H]TCDD from AhR in competitive binding studies using 9S-enriched fractions of human hepatic cytosol was seen. However, the conditions used by this group, namely 50 nM [^{3}H]TCDD and 400 μM PRQ, would result in a fairly large pool of unbound [^{3}H]TCDD and 8000-fold molar excess of competitor would not be sufficiently high to allow detection of low affinity competition. The apparent discrepancy between their study and ours is therefore not a consequence of species differences, since human and rat hepatic cytosol was used, respectively, but due to the modified ligand-binding assay used in our study.

By serendipity we found that PRQ in addition to the transcriptional activation of the *CYP1A1* gene, also affected CYP1A1 by a mechanism involving post-translational regulation. By using V79 cells, stably transfected with CYP1A1, we showed that PRQ caused a moderate inhibition of CYP1A1 degradation. It has been reported that CYP1A2 can be protected from degradation by its substrates isosafrole and methylcholanthrene (Silver and Krauter, 1988; Steward et al., 1985; Voorman and Aust, 1988), however a similar mechanism has not been reported for CYP1A1. On the other hand, post-translational regulation of CYP2E1 is an important mechanism for enzyme induction, where degradation of the enzyme has been shown to be inhibited by binding of its substrates (Eliasson et al., 1988; Zhukov and Ingelman-Sundberg, 1997). All substrates reported to protect CYP2E1 enzyme from degradation are type II or reverse type II substrates that bind to the heme iron and prevent oxygen activation of the enzyme. In line with this, PRQ was capable of forming a type II binding spectrum with CYP1A1 and inhibited ethoxyresorufin-O-deethylase activity *in vitro*. These findings suggest that PRQ protects CYP1A1 from degradation by binding to its active site, by a similar mechanism as found for CYP2E1, indicating that protection from degradation by substrates could be a general phenomenon for a number of CYPs.

AHR SIGNALLING IS REGULATED BY C-SRC KINASE ACTIVITY

A mutual cross-talk between the AhR and c-src signalling has been suggested, and the ability of TCDD to affect protein kinase activity, especially c-src, has been extensively studied. It has been shown that TCDD treatment *in vivo* increases the overall protein kinase activity in an AhR-dependent manner and that this increase is an important step in the mechanism of action of TCDD induced toxicity (Blankenship and Matsumura, 1997; Dunlap et al., 2002; Enan and Matsumura, 1996; Matsumura, 1994). We have showed that two specific c-src inhibitors, PP1 and PP2, were able to inhibit Gal4-AhR activation by OME or TCDD in H4IIE cells (Paper V). Futhermore, co-transfection of a dominant negative c-src expression vector attenuated the response caused by OME or TCDD on Gal4-AhR activation. These results indicate that the cross-talk between AhR and c-src is bi-directional, since c-src can also act upstream of AhR activation. Several nuclear hormone receptors have been reported to be phosphorylated in a ligand-dependent manner. For example, estrogen-induced MAPK phosphorylates directly the ER on Ser^{118} altering its transcriptional activity (Kato et al., 1995), and, recently it was reported that dexamethasone-dependent phosphorylation on Ser^{211} of the GR is correlated with its nuclear localisation and transcriptional activity (Wang et al., 2002b). Given that both the AhR and c-src have been shown to associate with hsp90, and

TCDD can cause an increase in c-src activity in AhR-immunocomplexes (Blankenship and Matsumura, 1997), it is possible that c-src directly phosphorylates the AhR in a ligand-dependent manner. However, induction of the *CYP1a1* gene has been observed in c-src-deficient mice (Enan et al., 1998). Our attempts to show increased c-src activity in H4IIE cells treated with OME or TCDD have been inconclusive. This might indicate that c-src is rather a constitutive modulator of AhR activity in a ligand-independent manner, than being an absolute requirement for ligand-dependent AhR signalling. Interestingly, the effect of c-src on AhR activation is more pronounced with OME than TCDD as activator, implying that the modulatory function by c-src is more important for transducing the signalling event caused by OME than by high affinity ligands.

THE LBD IS REQUIRED FOR LIGAND-INDEPENDENT ACTIVATION OF THE AHR

The LBD of the mouse AhR has been identified to be located between amino acids 230 and 421, and studies using this minimal LBD or full length AhR, translated in reticulocyte extracts *in vitro*, showed similar affinity of TCDD to this fragment as to the full length receptor (Coumailleau et al., 1995; Whitelaw et al., 1993b). Despite the fact that OME apparently does not directly interact with the AhR, the LBD was shown to be important for OME-dependent activation, since mutation of Val^{375} to Ala abolished activation of the receptor (Dzeletovic et al., 1997). In our hands, similar results were obtained by mutation of the corresponding amino acid (Ala^{379}) of the rat AhR, in the context of the Gal4-AhR construct (unpublished results). Furthermore, we were able to show a complete and selective loss of OME-mediated activation of two variants of the AhR containing single-point mutations at positions Tyr^{320} and Thr^{385} (Paper V). Activation of these mutant Gal4-AhR constructs by MMB and PRQ revealed a lack of activation of the Gal4-Y320F and an attenuation of the response of the Gal4-T385V mutant (Paper IV). In contrast, activation of these Gal4-AhR mutants by TCDD was not affected, moreover, saturation ligand binding analysis indicated that [^{3}H]TCDD bound to Gal4-Y320F equally efficient as to the wild-type Gal4-AhR protein. On the other hand, low affinity binding by PRQ to the Gal4-Y320F protein was shown to be impaired (Paper IV).

Interestingly, the Tyr^{320} is situated in a well-conserved region of the PAS domain, and the amino acid triplet SGY^{320} is conserved in almost all AhR proteins known today from different species. Furthermore, this SGY-triplet or a SXY-triplet is conserved in other α-class sensory proteins, such as Clock and neuronal PAS domain protein 2, but not in the family members belonging to the β-class partners, e.g arnt, or γ-class coactivators (see Gu et al, 2000). Thus, it is possible that the conservation of the Tyr^{320}-region has been important for maintaining some functional property contributed to the sensory role of this group of proteins, for instance, recognition of a ligand. According to the recently published 3-dimensional model of the AhR the SGY^{320}-triplet is located in the E_α-helix, preceding the F_α-helical connector. Based on our findings that activation of the Gal4-Y320F by TCDD and binding of [^{3}H]TCDD to the mutant variant was unaffected, it is unlikely that TCDD would directly be recognised by the Tyr^{320} residue. On the other hand our results could indicate that other molecules, e.g.

PRQ, could be positioned differently in the ligand-binding cavity of the AhR, hence, other amino acid residues of the LBD would become critical for the ligand-receptor interaction. Additionally, Tyr^{320} might constitute a phosphorylation site of the AhR. The specificity of protein kinases is determined, among other things, by residues adjacent to the phosphorylated residues and the accessibility and flexibility of the site. In support of the idea of a phosphorylation site at Tyr^{320}, examination of a Gal4-AhR construct containing a Ser^{318} to Ala mutation also revealed a complete abrogation of activation by OME but not by TCDD, identical to the results with the Gal4-Y320F protein (unpublished data). In contrast to the Tyr^{320} region, the Thr^{385} site is not as well conserved among AhR homologs or other PAS proteins, although, this site was predicted to be a putative phosphorylation site for several serine/threonine protein kinases. However, our results using various protein kinase inhibitors would rather indicate involvement of PTKs than serine/threonine kinses in ligand-independent AhR phosphorylation. Moreover, Park et al (Park et al., 2000) have shown that the DNA-binding activity of the ligand-activated AhR is sensitive to tyrosine-specific but not to serine/threonine-specific protein phosphatases. At present it is unclear whether Tyr^{320} is phosphorylated or not. However, the complete loss of Gal4-Y320F activation by OME, MMB and PRQ, but not by TCDD, certainly indicates that this amino acid is critical for ligand-independent activation of the receptor as well as for activation by low affinity ligands.

FUTURE PERSPECTIVES

The recent identification of a range of structurally diverse endogenous and exogenous compounds that can activate the AhR, challenges the current understanding of ligand-protein interaction and indicates that the AhR ligand-binding pocket is much more promiscuous than previously thought. How does this “loose” AhR ligand-binding pocket fit with the great deal of specificity demonstrated in several structure-activity relationship studies? A question that has not been addressed so far is whether different ligand-binding sites of the AhR could harbour these diverse compounds. Furthermore, is the developmental defects found in AhR-deficient mice or the function of the AhR in cell cycle control dependent on activation of the receptor by a ligand? Or could some of these events occur through a ligand-independent mechanism? Is it possible that OME as well as other atypical AhR activators mimic such a mechanism?

It is apparent that the LBD plays a cruical role in AhR activation. In lack of an X-ray structure of the LBD the theoretical model provides a good basis for carrying out site-directed mutagenesis and ligand-binding experiments to identify key residues involved in low and high affinity binding, as well as in defining species-specific differences in ligand-binding. However, a simple key-lock complementarity does not seem to be sufficient to explain the whole signal transmission mechanism of the AhR. Therefore, a deeper functional analysis of the LBD, containing the PAS signalling module, is warranted for gaining insight into AhR signal transduction.

CONCLUSIONS

The results of the present thesis can be summarised as follows:

- Transcriptional activation of the *CYP1A1* gene by OME, MMB and other benzimidazoles, as well as by PRQ was shown to involve AhR activation. The magnitude and duration of *CYP1A1* expression indicates that these compounds are weak AhR activators.

- Two novel CYP1A1 inducers, MMB and PRQ were recognised as low affinity ligands for the AhR. No apparent direct interaction of OME with the AhR-complex was established.

- CYP1A1 was protected from degradation by PRQ binding to its active site. Protection of CYP enzymes from degradation by their substrates could be a more general mechanism for regulation of CYPs than previously thought.

- Both OME- and TCDD-mediated AhR-signalling was shown to be modulated by c-src kinase activity in hepatoma cell lines.

- OME-dependent activation of the AhR was shown to depend on tyrosine kinase activity, as activation was specifically blocked by genistein, tyrphostin AG17 and typhostin AG879.

- A residue (Tyr^{320}) in the LBD of the AhR was shown to be critical for activation of the receptor by OME, as well as for low affinity binding by PRQ and MMB, but not for TCDD.

In summary, two alternative ways of activation of the AhR by novel CYP1A1 inducers have been demonstrated in this study; i) direct low affinity binding and activation of the receptor by PRQ and MMB, and ii) ligand-independent activation of the AhR by OME, which appears to be dependent on tyrosine kinase activity.

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har ingen ödestro
den tanken ger ingen ro
men vem vänder vindarna
vem får mej att gå dit jag aldrig gått
(E. Dahlgren)

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