Characterising the mechanism of Activation of the bHLH/PAS Dioxin Receptor

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Thesis summary

The mammalian Dioxin Receptor is a ligand activated transcription factor belonging to the bHLH/PAS superfamily of transcriptional regulators. Activation of the Dioxin Receptor occurs by binding dioxin or related aromatic hydrocarbon molecules. In the non ligand exposed state, the Dioxin Receptor remains latent and cytoplasmically localised due to interaction with an hsp90 containing chaperone complex. Upon activation the Receptor translocates from the cytosol to the nucleus whereby it heterodimerises with its bHLH/PAS partner factor ARNT. The Dioxin Receptor/ARNT heterodimer activates target genes which typically encode xenobiotic metabolising enzymes, which chemically modify and subsequently excrete the activating ligand from the body. This general mechanism prevents the possibility of toxic chemicals mutating genomic DNA or having detrimental affects on cell growth or metabolism. In addition to ligand activation, the receptor is also subject to other forms of regulation including nuclear export, degradation and potentially post-translational modification.

This thesis examines the regulation of the receptor in terms of cellular localisation and ligand activation and investigates the contribution that molecular chaperones play in this process. The work herein shows that activation of the Dioxin Receptor is a complex pathway in which redistributing the normally cytosolic receptor to the nucleus is not sufficient for activation, instead ligand is required for concomitant exchange of hsp90 for ARNT in order to process the Dioxin Receptor into a transcriptionally competent form. In addition, upon depletion of the Dioxin Receptor co-chaperone protein Ara9/AIP/XAP2, Dioxin Receptor signalling is abrogated as a result of depleted levels of Dioxin Receptor in the cell. This thesis also proposes a role for the E3 ubiquitin ligase protein CHIP in Receptor degradation, in addition to examining a novel method

of Dioxin Receptor activation, whereby, inhibition of a protein kinase pathway leads to several classical features of receptor activation, implying that Dioxin Receptor regulation can be controlled at the level of post translational modification.

Declaration

This work contains no material which has been accepted for the award of any degree or diploma in any university or other tertiary institution and. Chapter 3 is presented in thesis format and is a reproduction of a publication entitled

"Multiple roles of ligand in transforming the dioxin receptor to an active basic helix-loop-helix/PAS transcription factor complex with the nuclear protein Arnt."

Molecular Cellular Biology. 1999 Aug;19(8):5811-22.

A further publication arising from this work is entitled,

"Mediation of dioxin (aryl hydrocarbon) receptor transcription by Ara9/XAP2/AIP." Toxicology, in press.
By Michael J. Lees and Murray L. Whitelaw

All experiments performed in this thesis were performed by myself.

To the best of my knowledge and belief this thesis contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the university library, being available for loan and photocopying.

..... 10/10/02 .....

Michael Lees Date

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Enough already this is getting ridiculous, lets go, come on lets fire up!

"Ultimately, all our difficulties arise from one basic illusion. We believe in the inherent existence of ourselves and all other phenomena. We project and then cling to, an idea of the intrinsic nature of things, an essence that phenomena do not actually posess."

-The Dalai Lama

"To alcohol! The cause of- and solution to- all of lifes problems"

-Homer (Simpson)

TABLE OF CONTENTS

Abbreviations

AHH Aryl Hydrocarbon Hydroxylase

AIP Aryl hydrocarbon Receptor interacting protein

Ara9 Aryl hydrocarbon associated protein

Arnt Aryl hydrocarbon Receptor Nuclear Translocator

bHLH basic helix loop helix

BMAL Brain Muscle Arnt Like factor

CBP Creb Binding protein

cDNA complementary deoxyribonucleic acid

CHIP C-terminal hsc/hsp70 interacting protein

Cyp4501A1 Cytochrome p450 1A1

DMSO Dimethylsulfoxide

DNA deoxyribonucleic acid

DR Dioxin Receptor

GA Geldanamycin

GST Glutathione-S-transferase

HAH Halogenated aromatic hydrocarbon

HIF Hypoxia-Inducible factor

hsp90 heat shock protein 90

iPAS inhibitory PAS

mRNA messenger ribonucleic acid

ng nanogram

NLS Nuclear Localisation Sequence

NPAS Neuoronal PAS

PAH Polyaromatic hydrocarbon

PAS Per Arnt Sim homology region

PKC Protein Kinase C

PPIase Peptidyl Prolyl isomerase

RNA ribonucleic acid

SIM singleminded

TCDD 2,3,7,8 tetrachlorodibenzo-*p*-dioxin

TPR tetratricopeptide repeat

VHL Von Hippel Lindau

XAP2 Hepatitis B virus X associated protein

XRE Xenobiotic Response Element

µL microlitre

1

Introduction

Chapter 1 Introduction

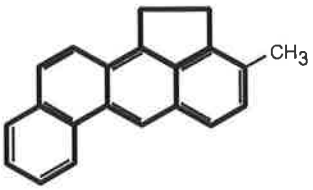
The Dioxin Receptor (DR, also known as the aryl hydrocarbon receptor) is the only member of the bHLH/PAS (basic Helix-Loop-Helix/Per Arnt Sim) family currently known to be activated by ligand. A variety of planar polyaromatic or halogenated hydrocarbons can act as ligands, with the prototypical ligand for the DR being 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD or dioxin). TCDD is formed as a byproduct in the high temperature synthesis of organochlorine pesticides and herbicides, most notably, during the production of Agent Orange, a controversial defoliant used during the Vietnam War. Agent Orange was first used as a herbicide in the 1950's. However, queries began to be raised when it was thought one of its components, 2,4,5-T, lead to birth defects in rodents. It was subsequently found that the causative agent for these abnormalities was TCDD, a contaminant in the preparation. Subsequent studies with TCDD have shown that TCDD produces a plethora of toxic responses in rodent models including thymic involution, chloroacne, palate cleft defects, birth defects and hyperkeratosis, cardiotoxicity in the chick, in addition to TCDD being a potent tumour promoter in rodent models (Poland and Knutson 1982). Currently, the actual danger that TCDD exposure poses to humans is highly controversial (Bradfield *et al* 1994, Kaiser 2000a-c). Several incidents of high-level exposure have occurred that have provided inconclusive results as to the human toxicity profile of TCDD (Koppe *et al* 1991, Bertazzi 1991, Smith and Lopipero 2001, Bertazzi *et al* 2001). One of the major issues concerning TCDD exposure is that the half-life of this chemical in humans is in the order of a decade (Geyer *et al* 2002). It is lipophilic and therefore tends to aggregate in fatty tissue and due to the positioning of the four chlorines on peripheral carbons it cannot be detoxified by standard enzymatic reactions. The classic physiological response to dioxin involves the upregulation of phase I and phase II detoxification

enzymes. The enzyme battery that is employed to void the body of planar aromatic hydrocarbons was first characterised in the 1970's, and includes the microsomal Cytochrome P450 family of metabolic enzymes. These enzymes are so named by their literal translation of cyto (cellular) chrome (colour) and 450 relating to their unique absorbance properties having an unusual major optical absorbance peak at 450 nm once the proteins have been reduced and combined with carbon monoxide. This enzyme battery can cope with a broad range of chemicals and the method of detoxification is complex and can vary for a given chemical (Pelkonen and Nebert 1982). Two phases of enzymes are employed to combat xenobiotic assault. Phase I enzymes modify the chemical to increase slightly the water solubility of the ligand, generally by adding a hydroxyl group, ultimately preparing the ligand for a further increase in solubility by the action of the phase II enzymes that conjugate a soluble carrier such as a sugar or glutathione to the hydroxyl group. This greatly increases the overall water solubility to allow excretion (Pelkonen and Nebert 1982). To date, several enzymes (that have a defined role in xenobiotic metabolism) have been demonstrated to be upregulated in response to dioxin and they include CYP1A1, 1B1, 1A2, (the so-called phase 1 enzymes) in addition to the phase 2 enzymes, which include Glutathione-S-transferase Ya subunit, UDP glucuronyltransferase and NAD(P)H:quinone oxidoreductase (Whitlock 1999, Gu *et al* 2001).

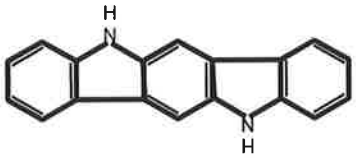
Ligands for the DR

While dioxin is the "best fit" prototypical ligand for the dioxin receptor, many other xenobiotics have been shown to activate the DR, including components from cigarette smoke and exhaust fumes, Benzo-*a*-pyrene (Dertinger *et al* 2001), indolocarbazoles (Kleman *et al* 1994, Chen et al 1995), bilirubin (Sinal and Bend 1997, Phelan *et al* 1998) and plant derived napthoflavones (Ashida *et al* 2000). Furthermore it has been demonstrated that cooking of meats can lead to production of DR ligands (Kleman *et al*

2,3,7,8 Tetrachlorodibenzo-*p*-dioxin

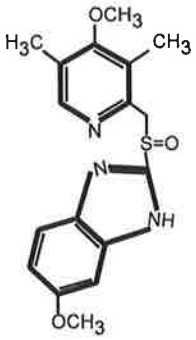


3-methyl cholanthrene

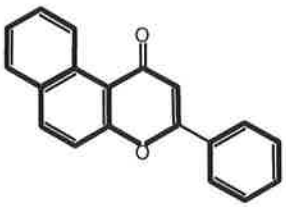


Indolo(3,2-b)carbazole

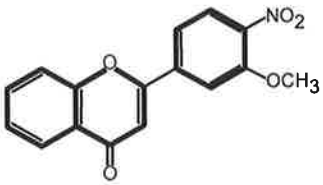
Benzo-a-pyrene



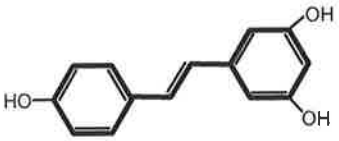
Omeprezole



β-Napthoflavone



3'methoxy4'nitroflavone



Resveratrol

Figure 1.1. *Typical ligands for the DR.* Presented are some of the chemicals known to interact with the DR, the prototypical ligand being 2,3,7,8 Tetrachlorodibenzo-*p*-dioxin (dioxin). The positioning of the peripheral chlorines on dioxin renders it impervious to standard detoxification mechanisms. See text for details.

1992, Kleman *et al* 1994). Generally, ligands for the DR conform to a fairly specific size and shape, that being planar and approximately 10A° x 3A° in the case of dioxin (Figure 1.1). Interestingly, Omeprazole, a drug used in the treatment of stomach ulcers has been shown to be active as a ligand for the DR casting doubt on its suitability for use as a pharmaceutical (Dzeletovic *et al* 1997). Of these ligands, Benzo(a)pyrene, has the ability to be metabolised to chemicals that form DNA adducts (Pelkonen and Nebert 1982). Chemicals also exist which have an antagonistic effect on the DR including 3-methoxy-4-nitroflavone (Lu *et al* 1995, Lu *et al* 1996, Dertinger *et al* 2000) and resveratrol, a component of red wine (Casper *et al* 1999).

Dioxin or dioxin-like chemicals were initially classified as being able to induce Aryl Hydrocarbon Hydroxylase (AHH) activity in rodent livers. However, toxicity initiated by compounds that have the ability to induce AHH activity is complex. Chemicals acting as ligands for the DR can be classified into two classes. The first class, the Polycyclic aromatic hydrocarbons (PAH's), can be said to be capable of undergoing a toxication process (Pelkonen and Nebert 1982). Chemicals that can undergo this process are converted to electrophilically active compounds that can form higher order structures with DNA leading ultimately to carcinogenesis. The second class of chemicals that can lead to DR mediated toxicity are the Halogenated Aromatic Hydrocarbons (HAH's), which are incapable of being modified enzymatically due to their chemical composition (Figure 1.1). Chlorinated dioxins are an example of this second group of compounds by virtue of the chlorines on peripheral carbons rendering them incapable of standard enzymatic detoxification (and hence toxication) and do not directly damage cellular DNA (Pelkonen and Nebert 1982). Dioxins are not tumour initiators but act as tumour promoters via unidentified mechanisms.

Identification of the AhR locus and protein

The AhR locus was identified by a combination of genetic and pharmacological means when it was discovered that two different strains of inbred mice had differing susceptibilities to dioxin exposure. The C57Bl/6 mouse strain was found to be responsive whilst the DBA/2 mice were classified as non-responsive and exhibited a 10 fold higher tolerance to TCDD exposure. This responsiveness was shown through various backcrosses to segregate to a single autosomal dominant locus termed the AhR (Aryl Hydrocarbon hydroxylase Responsive) locus. Subsequent affinity labelling experiments identified a soluble cytosolic receptor in the liver of the C57Bl/6 "responsive" mouse which exhibited high affinity for ^{125}I radiolabelled dioxin (Poland *et al* 1976, Gasiewicz *et al* 1984). It was later demonstrated the DR from C57Bl/6 mice has an approximate 10 fold higher affinity for TCDD and related congeners than the non responsive mice, correlating with the susceptibilities of these mice to TCDD exposure (Okey *et al* 1989). Biochemical analysis of this protein lead to a model whereby a cytosolic ligand binding receptor was converted from a 9S fraction on sucrose density gradients to a nuclear/DNA binding 6S form. The 6S form was predicted to be the form to upregulate expression of the microsomal enzyme cytochrome P4501A1 (P4501A1) by binding to XRE's or xenobiotic response elements located in the enhancer regions of the Cyp1A1 gene. Large scale purification of this protein enabled N-terminal protein sequencing and subsequent cloning of the murine cDNA for the dioxin receptor (Ema *et al* 1992, Burbach *et al* 1992).

The Dioxin Receptor as a member of the bHLH/PAS family of proteins

Homology based on primary sequence analysis lead to the classification of the DR into a newly classified family of proteins which at the time consisted of 3 members, Per,

Arnt and Sim (or PAS family of transcriptional regulators) (Nambu *et al* 1991, Hoffman *et al* 1991, Huang *et al* 1993). The PAS domain appears to have evolved from early photoreceptor systems still present in bacteria, fungi and plants (Ponting and Aravind 1997, Taylor 1999) and has been utilized by higher organisms to control such diverse processes as circadian rhythm (Dunlap 1996), response to environmental stresses (Gu *et al* 2001), nitrogen fixation in plants (Tuckerman *et al* 2001), ion regulation (Chen *et al* 1999) and kinase cascades (Rutter *et al* 2001).

Adaptive and Toxic response and the mechanism of Dioxin toxicity

The DR pathway presents somewhat of a conundrum in that while it is a pathway suited to deal with assault by xenobiotics, and possible carcinogenic compounds or other toxic chemicals, it is this same pathway which is responsible for many if not all of the deleterious effects caused by exposure to dioxins. In this context the DR system has been classified as both an adaptive response and toxic response pathway (Schmidt and Bradfield 1996). The DR can be thought of as part of an adaptive response in that the assaulting chemical binds to the specific receptor, which leads to the upregulation of a battery of enzymes designed to metabolise and excrete the offending ligand, thus completing a classical negative feedback response. This same pathway is also responsible for a toxic response, such that when it is affronted with man-made chemicals such as halogenated aromatic hydrocarbons, the xenobiotic metabolising enzyme battery is unable to excrete these chemicals from the cell and hence the pathway is continually activated.

Mechanism of dioxin toxicity

One of the conundrums of dioxin toxicity is the nature of the toxicity and the actual molecular mechanisms underlying this process. Several possibilities exist as to how

dioxin can lead to toxicity, however it must be said that these possibilities are largely speculative. The first of these is that dioxin itself is toxic, although dioxin is not genotoxic and does not covalently bind to cellular proteins or DNA (Pelkonen and Nebert 1982). Several observations have been made which provide circumstantial support for this model, including the fact that cellular exposure to dioxin leads to immature thymocyte apoptosis (McConkey *et al* 1998), in addition to upregulation of Protein Kinase C activity (Bagchi *et al* 1997) and activation of src tyrosine kinase activity (Enan and Matsumura 1993, Enan and Matsumura 1994), the implication being that dioxin treatment can disrupt normal cellular kinase regulatory pathways. These processes appear to be mediated independently of the DR as they induce this activation in a timescale of minutes and are thus considered to act far too rapidly to be a product of target gene upregulation induced by the DR. However, these processes were shown to occur either *in vitro,* or using high concentrations of ligand. An alternative to this hypothesis focuses on the inherent stability of TCDD itself. As TCDD has a half life in the order of a decade in humans (Geyer *et al* 2002), it is able to continually activate the DR pathway, perhaps diverting components of the pathway (i.e. the DR, its partner factor Arnt or other transcriptional co-regulators) away from intended house keeping functions. Another potential mechanism of dioxin induced toxicity is that the signature xenobiotic metabolising enzymes are continually present within cells where they would normally only be transiently upregulated and otherwise kept silent. Potentially these enzymes could act on substrates that they would not normally act on and perhaps change the hormone profile of a cell leading to aberrant signalling from a local tissue, leading to more global effects on the whole organism. It is currently unclear as to whether all of the toxic effects of dioxin are mediated through the DR (Okey *et al* 1995, Weber and Stahl 1995), however KO mice studies in combination with structure activity relationship studies comparing ligand affinity for the DR to toxicity *in vivo* suggest that

the majority, if not all, of the toxic effects of dioxin are mediated by the DR (Mimura *et al* 1997, Shimuzu *et al* 2000).

Non-Xenobiotic metabolising DR Target genes

Alternatively, the real clues behind dioxin toxicity could lie behind the target gene profile which is regulated by continual activation of this pathway (again a function of TCDD stability). Indeed, several different assay systems have shown dioxin to regulate a wide variety of genes whose products are non-xenobiotic metabolising in nature (Sutter *et al* 1991, Dong *et al* 1997, Gao *et al* 1998, Puga *et al* 2000, Thomas *et al* 2001). Some of these gene products can be theoretically assigned a role in dioxin toxicity, such as IL-1β and PAI-2 which act as growth regulatory genes as either direct cellular proliferation agents, or proteins concerned with degradation of the extracellular matrix, respectively (Sutter *et al* 1991). In a separate study that also used human keratinocytes, TGFα, a ligand for EGFR, was highly induced in response to TCDD (Choi *et al* 1991). These genes have relevance to dioxin toxicity as the major symptom of TCDD exposure in humans is chloroacne, a hyperplasia of the skin (Poland and Knutson 1982). Other genes, which have no obvious role in xenobiotic metabolism have been identified as dioxin target genes with a requirement for both the DR and its heterodimeric partner factor Arnt. EctoATPase is one such gene (Gao *et al* 1998), which potentially plays a role in purinergic signalling in addition to cell-cell adhesion. Another gene identified by differential display RT-PCR is the Major Histocompatibility Complex Q1b isoform, which is downregulated in a cell culture system following exposure to TCDD, via a complex mechanism involving both transcriptional and post-transcriptional means (Dong *et al* 1997). It is easier to relate the TCDD downregulation of this gene to TCDD toxicity than other dioxin responsive genes as one of the classical responses to TCDD is a suppression of T-cell mediated immunity. The authors

speculate that down regulation of this gene results in a decreased ability of cells to present antigen to Cytotoxic T-cells and hence prolong viral persistence.

The bHLH/PAS family of Transcriptional Regulators.

The bHLH motif has been described in detail in a wide variety of proteins and typically these proteins play crucial roles in development by functioning as sequence specific transcriptional regulators. The nature of the bHLH motif has generated elaborate transcriptional networks, utilising spatial and temporal expression and both positive and negative acting factors to control both cellular proliferation and differentiation.

Myogenic bHLH proteins

The best characterised of the bHLH factors are the myogenic regulatory factors (MRFs) which control proliferation and the appropriate differentiation of muscle cells. The key players in the myogenic pathway include MyoD (myogenic determining factor/Myf3), myogenin (Myf-1), myf-5 and MRF4 (Myf-6/Herculin), the so called class B proteins within this family, which preferentially form heterodimers with a class A family member such as E12/E47, E2-2 and E2-5 to bind the canonical E-box sequence CANNTG located in the enhancer regions of muscle specific genes (Molkentin and Olson 1996, Sabourin and Rudnicki 2000). Inhibitor of differentiation proteins (Id 1-4) are HLH proteins lacking the basic DNA binding domain and preferentially heterodimerise with class A family members, inhibiting the action of the myogenic factors (Benezra *et al* 2001, Engel and Murre 2001).

The myc/mad/max family Network

The myc/mad/max family of transcriptional regulators acts as a good model for the bHLH/PAS family of proteins, as they share several overlapping features. Increased

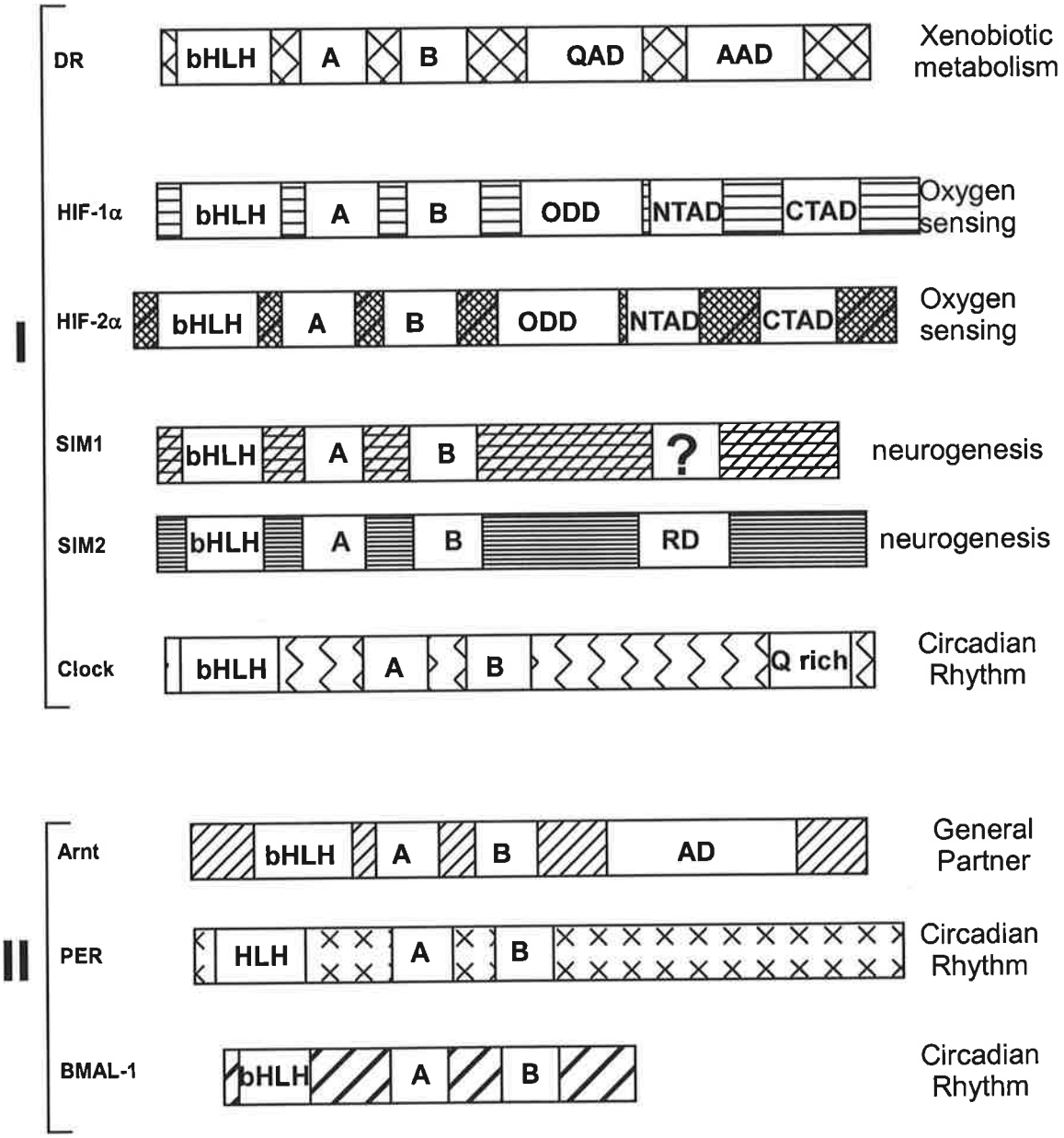


Figure 1.2. *The bHLH/PAS superfamily of proteins*. Presented is a schematic representation of some of the known members of the PAS family. PAS family members typically contain an N-terminal bHLH DNA binding/dimerisation region (with the exception of PER which lacks the Basic DNA binding domain but contains the HLH domain). The PAS domain is a region enriched for hydrophobic amino acids and mammalian PAS proteins typically two of these regions PASA and PASB. PAS family members also typically contain a C-terminal transcriptional regulatory region. Also indicated is the classification of these members as a Class I or Class II family member. Class I members are the signal sensing members and are obligated to form a heterodimer with a Class II member, whilst the Class II members can either heterodimerise with a Class I member or homo- or heterodimerise with a member from Class II. AD, Activation Domain; RD, repression Domain; ODD, oxygen dependent degradation domain.

partner dimerisation and specificity is achieved via the leucine zipper secondary dimerisation interface (Kadesch 1993, Baxevanis and Vinson 1993), and positive or negative regulation of target genes occurs depending on tissue specific expression of a partner for ubiquitous max (Luscher 2001). For example, myc/max heterodimers bind to E-box regulatory elements and myc then recruits several positive transcriptional regulatory co-factors including Swi/SNF and TRAPP via the myc box motif to regulate the target genes (Luscher 2001). Conversely, mad/max heterodimers upon binding E-box elements have been shown via an N-terminal localized interacting region to recruit the SID co-repressor complex containing HDAC activity (Luscher 2001). Deletion of this region leads to an impaired ability of Mad proteins to induce growth arrest, differentiation and survival (Luscher 2001).

The bHLH/PAS family members

Members of this family are identified by the presence of an N-terminal basic DNA binding domain contiguous with a Helix-Loop-Helix motif, which acts as a primary dimerisation domain between members of the family, and the PAS domain, a region of 200-300 amino acids which is enriched for hydrophobic amino acids in two regions of approximately 50 amino acids (termed PAS A and PAS B, Figure 1.2). The PAS domain is analogous to the leucine zipper by acting as a secondary dimerisation interface (Huang *et al* 1993, Lindebro *et al* 1995). The presence of this secondary dimerisation interface acts two-fold, firstly it enhances the strength of interactions between family members (Sogawa *et al* 1995, Antonsson *et al* 1995b) in addition to providing specificity between family members (Pongratz *et al* 1998), such that members can be classified into two groups. Class 1 members are the signal sensing/responsive members of the family and do not homodimerise but instead are obligated to form heterodimers with a Class 2 member. Class 2 members have the ability to both

heterodimerise with Class 1 and other members of Class 2 in addition to the ability to homodimerise (Antonsson *et al* 1995b, Sogawa *et al* 1995). This enables hierarchical networks to be established to enable priority pathways to function during competing cellular stress events (Gradin *et al* 1996, Chan *et al* 1999). The following, paragraphs contain a brief description of several of the bHLH/PAS superfamily members, which often display a high level of regulation to control gene output. This regulation includes novel forms of post translational modification, regulated nuclear import mechanisms, regulation of the ability of these factors to interact with transcriptional machinery and upregulation of target genes which act in negative feedback loops. Such a high level of control underpins the importance of the factors and the roles they have been assigned in physiological processes.

Negative regulation of bHLH/PAS proteins

The bHLH/PAS system is widely recognised as requiring a transcriptional regulatory unit comprising a Class 1 member eg DR or HIF-1α which senses stimulatory cues and is responsible for transmitting a signal from the cytoplasm to the nucleus, and a constitutively nuclear factor of the Class2 bHLH/PAS group which is absolutely required to achieve a DNA binding form and hence transcriptional competency. By placing a requirement on the transcriptional complex to be devised of two parts the system has the capacity to become highly intricate, which is exemplified by the PER factors which lack the DNA binding domain (in analogy to the Id factors of the myc/mad/max family) and abolish gene upregulation. Further forms of negative regulation are typified by IPAS (inhibitory PAS protein, a splice variant of HIF-3α) which lacks the C-terminal transactivation domain and acts as a dominant negative partner factor for the HIF's (Makino *et al* 2001, Makino *et al* 2002). Alternatively a third option exists to control gene regulation via active repression of target genes by

recruitment of a complex containing Histone Deacetylase Activity. Candidate proteins for the bHLH/PAS family to utilise this mechanism are the AhR Repressor (AhRR) and mSIM2 proteins, however this is yet to be demonstrated experimentally.

Hypoxia Inducible Factors

To date the Hypoxia inducible factors (HIF's) provide the most advanced level of regulation of bHLH/PAS family members. Oxygen delivery to cells is an essential component of multicellular organisms, and as such an exquisite system has evolved to sense changes in both global and local changes in cellular oxygen concentration. In mammals, cells must sense decreases in oxygen levels and be able to respond rapidly in an attempt to switch from aerobic to anaerobic respiration by switching the respiratory enzyme profile. In addition to this the body must respond to more global decreases in oxygen concentrations by increasing erythropoiesis (Red Blood Cell formation), and in the case of ischaemia or other disease whereby a tissue has prolonged oxygen deprivation, that tissue must be able to induce local angiogenesis to promote new blood vessels to increase the supply of oxygen. This procedure is not only applicable in disease states but is also an essential part of development, as both HIF-1α and HIF-2α knockout mice die of blood vessel malformation (Iyer *et al* 1998, Ryan *et al* 1998) and for HIF-2α, potentially disrupted neurotransmitter signalling (Tian *et al* 1997). In response to low oxygen HIF-1α has been shown to upregulate EPO, VEGF and a number of glycolytic enzymes (Fedele *et al* 2002). In normoxic conditions HIF-1α and HIF-2α (also known as HIF Like Factor/HLF or Endothelial PAS factor/EPAS) protein levels are rapidly turned over via the ubiquitin proteasome pathway (Salceda and Caro 1997, Kallio *et al* 1999). Hydroxylation at a critical proline residue (Ivan *et al* 2001, Jaakola *et al* 2001) by a family of prolyl hydroxylases (PHD 1,2 and 3, Epstein *et al* 2001, Bruick and McKnight 2001) enables recruitment of the Von Hippel Lindau/E3

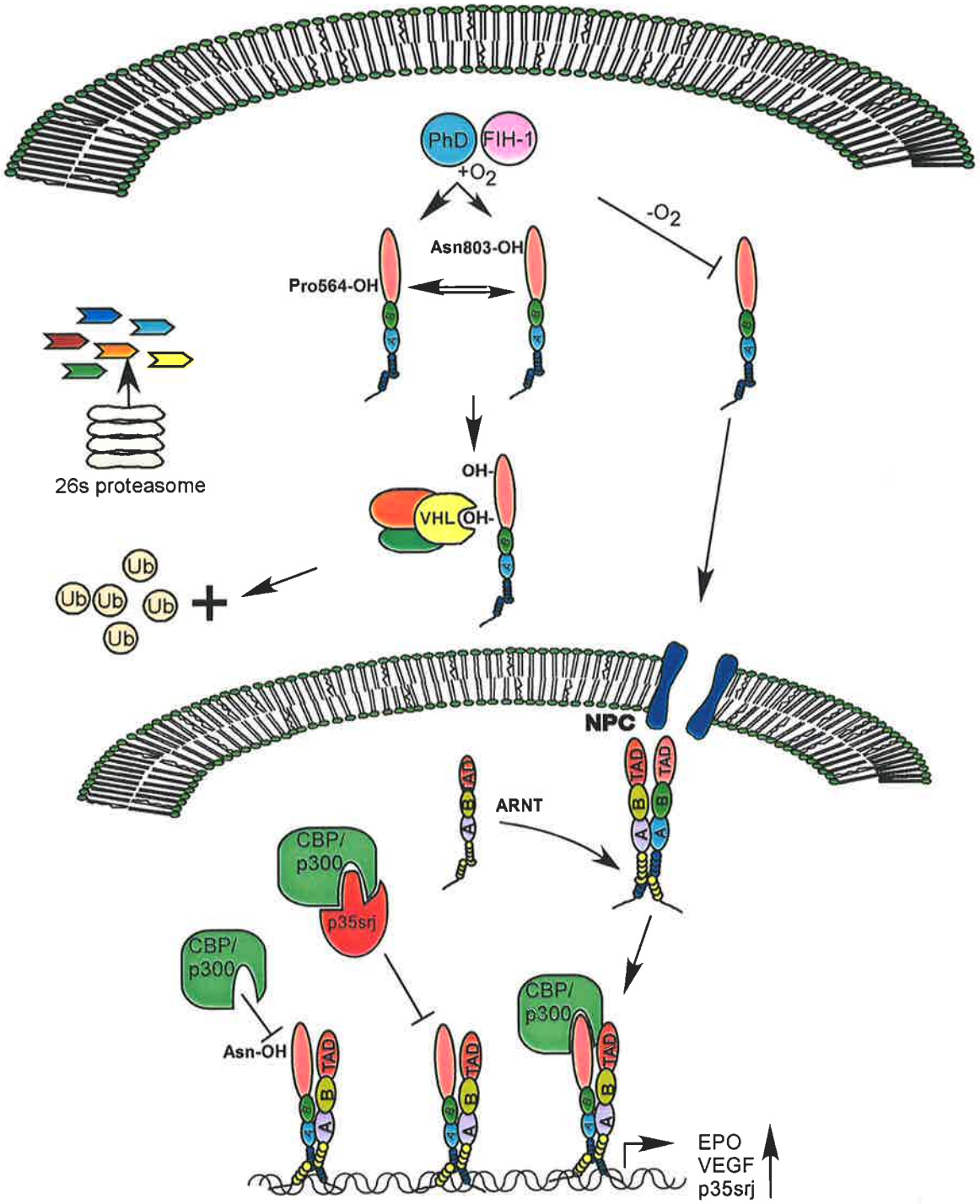


Figure 1.3. *The HIF-1α pathway*. Under normoxic conditions, HIF-1α is rapidly degraded by hydroxylation of a critical proline residue within an Oxygen Dependent Degradation Domain. Hydroxylation of Pro564 enables recognition by the VHL/E3 ubiquitin ligase complex and degradation by the 26S proteasome. A second hydroxylation event, Asn 803 prevents association of the HIF-1α with the CBP/p300 transcriptional machinery. Oxygen depletion, prevents these hydroxylation events, enabling stabilisation of HIF-1a heterodimerisation with ARNT and transcriptional activation of genes including erythropoietin and VEGF. HIF-1α also upregulates p35srj/CITED2 which acts to negatively regulate HIF-1 mediated transcription by blocking the HIF-1 recognition site of CBP/p300.

ubiquitin ligase complex (Ivan *et al* 2001, Jaakola *et al* 2001, Yu *et al* 2001, Min *et al* 2002, Hon *et al* 2002) to affect degradation by the proteasome. During periods of low oxygen stress, the PHD enzymes are unable to function, the HIF-1α subunit becomes stabilised and translocates to the nucleus whereby it forms a DNA binding complex with the Arnt partner factor and is able to interact with transcriptional co-activators such as CBP/p300 to activate target genes (Arany *et al* 1996, Kallio *et al* 1998, Freedman *et al* 2002). In normoxia a second hydroxylation event occurs on an asparagine residue within the C-terminal transactivation domain by a separate enzyme (FIH-1), which abolishes the interaction of the activation domain with co-activator proteins (Mahon *et al* 2001, Lando *et al* 2002a, Lando *et al* 2002b, McNeil *et al* 2002). The HIF pathway is summarised if Figure 1.3. An additional level of regulation for the HIF system has recently been proposed (Makino *et al* 2001, Makino *et al* 2002) whereby a novel bHLH/PAS factor termed IPAS (inhibitory PAS) is upregulated in specific tissues in response to transient hypoxic conditions (i.e. during sleep), whereby the cornea is exposed to hypoxia by means of the eyelid closing. It is proposed that during sleep, hypoxia triggers HIF-1 activation and upregulation of target genes including IPAS in the cornea, IPAS lacks a C-terminal regulatory domain common to all bHLH/PAS factors so far identified, and thus acts as a dominant negative PAS factor. In this context IPAS acts as a dominant negative class 2 factor, sequestering activated HIFs, preventing the upregulation of HIF-1 target genes during periods of sleep and hence preventing aberrant processes such as angiogenesis occurring in the eye (Makino *et al* 2001).

Circadian Rhythm.

Underpinning both the importance and diverse function of bHLH/PAS factors is the observation that a high proportion of the proteins involved in circadian rhythm are PAS superfamily members. Indeed one of the founding members of the PAS family (in terms

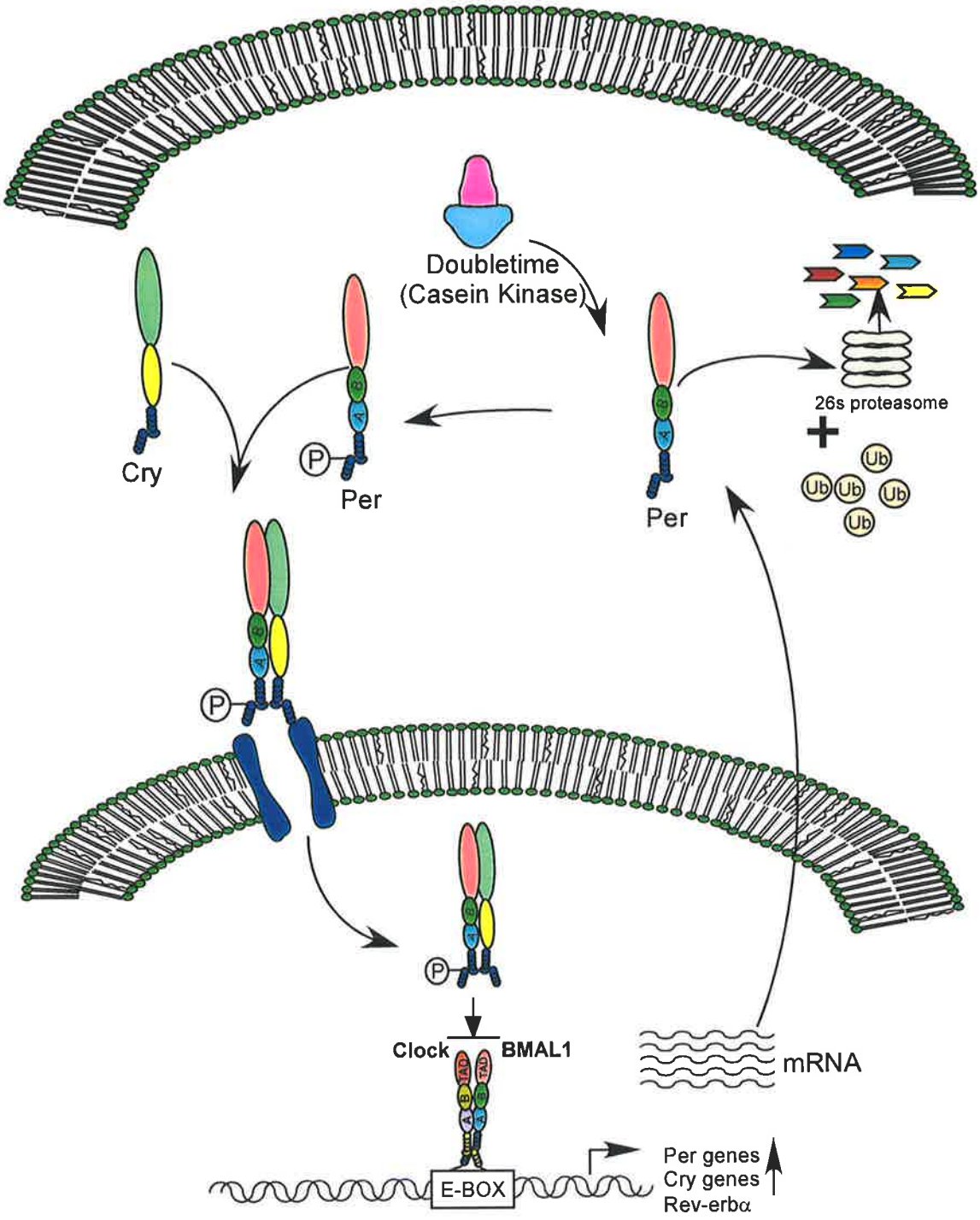


Figure 1.4. *The Circadian Feedback Loop.* Presented is a simplified version of the mammalian circadian rhythm system, which displays multiple levels of regulation including phosphorylation regulated nuclear entry and degradation, and postive and negative acting target genes. The loop begins by the transcripional upregulation of Per, Cry and Rev-erbα genes by the bHLH/PAS factors Clock and BMAL through binding to E-box recognition sequences of target genes. Upon translation the PAS containing Per proteins can be degraded via the 26s proteasome or undergo phosphorylation dependent nuclear entry in concert with heterodimerisation with a CRY(cryptochrome) factor to shut down transcription of their own genes. The Rev-erbα protein has been proposed to shut down transcription of the BMAL1 gene to further regulate the loop.

of discovery) is the PER or Period protein, which was isolated on the basis that it altered the light/ dark cycle of flies which carried a mutation within this locus. Biological rhythms are crucial elements in vertebrate biology and they include the menstrual cycle, hormone levels and the day/night cycle (circadian rhythm). Since the initial cloning of Per (Citri *et al* 1987), a multitude of PAS containing circadian factors have also been cloned which combine in a complex system of positive and negative regulatory factors to co-ordinate a molecular response to external cues, a process known as entrainment (Reppert and Weaver 2002). There exists a remarkable conservation between the components of the systems of simple eukaryotes (Crosthwaite *et al* 1997), flies and mammals (Dunlap 1999). Central to the process are the Per proteins (Per 1-3). As Per proteins lack a DNA binding domain it was proposed that these factors act as repressive proteins (Hardin *et al* 1990). The circadian rhythm proteins perform gene regulation via a combination of degradation of the signal sensing units, regulated nuclear/cytosolic shuttling and a complex interplay with negative acting factors. (Price *et al* 1998, Kloss *et al* 1998, Jin *et al* 1999, Yagita *et al* 2000, Bae *et al* 2001, Vielhaber *et al* 2001, Akashi *et al* 2001, Reppert and Weaver 2002). The pathway is summarised in Figure 1.4. Furthermore, two recent studies using High Density Array Analysis and oligonucleotide arrays has shown that there are approximately 600 genes regulated in a circadian manner in the circadian pacemaker centre the Suprachiasmatic Nucleus. In addition, an equally high number of genes appear to be regulated in peripheral tissue such as the heart and liver (Panda *et al* 2002, Storch *et al* 2002).

Single-minded

Single-minded is a master regulator gene of Drosophila neurogenesis (Nambu *et al* 1991). In mice, Sim has been shown to be critical for specifying hypothalamic neurons and involves the upregulation of the Brn2 gene in hypothalamic nuclei to specify their

fate (Michaud *et al* 1998). Using the Drosophila proteins Sim and Trh, new insight into the function of the PAS domain has been provided. That is the PAS domain can function externally to the well characterised role of PAS-PAS interactions and that it may be the PAS domain itself that can potentially specify target gene activation by recruiting non-PAS containing factors to act in a promoter specific context. Evidence for this is provided by PAS domain swapping experiments which lead to altered cellular fate (Zelzer *et al* 1997). The Drosophila proteins Sim and Trh both bind the Drosophila Arnt homolog Tango (Sonnenfeld *et al* 1997), in addition to recognising an identical DNA recognition motif, the CME or central midline element (Wharton *et al* 1994, Zelzer *et al* 1997). In addition to this there is overlapping expression between these two proteins (Zelzer *et al* 1997), confounding the problem of disparate regulation between these two proteins. The domain swapping experiments exchanged the PAS domain of Trh with the PAS domain of Sim as well as the reverse swap and chimeric proteins were expressed from the endogenous promoters. The result being that the chimeric proteins controlled cell fate by means of their PAS domain (Zelzer *et al* 1997), implying that cell fate was dictated by interaction with other cell specific genes. One candidate gene which was identified by its similar expression pattern with Trh was the Pou domain protein fish hook (Zelzer *et al* 2000, Ma *et al* 2000) implying that specific PAS domain interactions are a crucial factor in determining cell fate in Drosophila. In mammals, two SIM proteins (SIM1 and SIM2) exist to date (Ema *et al* 1996, Fan *et al* 1996) both of which are lethal in gene disruption experiments either pre or post-natally (Shamblott *et al* 2002). Interestingly, the SIM2 gene is localised to the Downs syndrome critical region present in trisomic individuals and given the crucial role that Drosophila SIM plays in neurogenesis this makes it an interesting candidate as a potential effector gene in this process. The murine SIM proteins appear to differ from their Drosophila counterparts, in that they appear to be able to function as transcriptional repressors (Ema *et al* 1996, Moffet *et al* 1997, Woods and Whitelaw 2002).

Neuronal PAS proteins

A homology based scan of EST databases lead to the identification of Neuronal PAS proteins 1, 2 (NPAS) (Zhou *et al* 1997) and 3 (Brunskill *et al* 1998). These factors are expressed primarily in neuronal tissue in areas other than the SCN, the traditional circadian pacemaker tissue (Zhou *et al* 1997, Garcia *et al* 2000). Targeted deletion of NPAS2 generates mice that display impaired cued and contextual learning/memory disorders (Garcia *et al* 2000). Fascinatingly, these factors may provide a simple biochemical link between feeding behaviour and consciousness itself. A screen of NPAS2 target genes identified Lactate dehydrogenase A, an enzyme which can regulate NADP/NADPH and NAD/NADH levels within a cell, as a primary target (Reick *et al* 2001). Subsequently it was shown using purified components that the ratios of NADP/NADPH and NAD/NADH, affect the ability of NPAS2/BMAL1 heterodimers to bind E-box elements (Rutter *et al* 2001). Given that NPAS2 is a CLOCK homologue in addition to BMAL1 being a partner factor for the master circadian component CLOCK, this discovery links feeding patterns to Circadian rhythm as NADP/NADPH and NAD/NADH levels fluctuate according to feeding responses.

Arnt

A valuable tool in analysis of DR signalling has been the murine hepatocellular carcinoma cell line Hepa1c1c7. These cells have been widely characterized as having highly inducible levels of DR target genes including 1A1 and 1A2 (Whitlock *et al* 1989) as well as containing high levels of DR (Holmes *et al* 1997). As these cells have readily inducible Aryl Hydrocarbon Hydroxylase (AHH) activity, this feature was utilized to generate a series of chemically mutagenised cell lines which disrupted AHH

inducibility. A range of mutant cell lines which were classified into several complementation groups (Van Gurp and Hankinson 1984). Through these complementation groups it was demonstrated that the DR signalling pathway requires several loci in to function. One of the mutant groups was a cell line exhibiting normal ligand binding activity but failed to retain the DR in the nucleus and subsequent conversion to the 6S/DNA binding fraction following ligand treatment. Using a complementation screen of this mutant cell line and assaying for restored AHH activity, a factor was isolated and termed the Aryl Hydrocarbon Receptor Nuclear Translocator protein (Arnt) by virtue of the fact that it was able to retain the DR in the nucleus (Hoffman *et al* 1991). Additional studies using this cell line have identified a point mutation (Glycine 326) between the PASA and PASB regions which leads to rapid turnover and hence low levels of Arnt protein (Numayama-Tsuruta *et al* 1997). Since the initial cloning of Arnt, several other Arnt like factors have been identified including Arnt2 (Hirose *et al* 1996, Drutel *et al* 1996), BMAL/Arnt3 (Ikeda and Nomura 1997, Takahata *et al* 1998). Arnt or Arnt like factors can be considered as obligate partner factors for other members of the bHLH/PAS family. *In vitro* gel shift experiments and reporter gene assays demonstrate that Arnt has the ability to function as a homodimer by recognising the E-box element CACGTG (Swanson *et al* 1995, Antonsson *et al* 1995b, Sogawa *et al* 1995, Long *et al* 1999, Levine and Perdew 2002). Immunohistochemistry has demonstrated Arnt to be nuclear in both cell culture systems and in tissue sections (Hord and Perdew 1994, Pollenz *et al* 1994). Arnt is ubiquitously expressed throughout development in all tissues (Abbot *et al* 1995, Jain *et al* 1998) and is more ubiquitous in its expression than the other Arnt like proteins which show a more restricted expression pattern, for instance BMAL is expressed primarily in the brain, heart and muscle regions (Ikeda *et al* 1997) whilst Arnt2 is expressed predominantly in the brain and kidney (Hirose *et al* 1996, Drutel *et al* 1996). Mice which have a targeted disruption in the Arnt gene fail to progress past embryonic day 10.5 due to a defect in

vascularisation (Maltepe *et al* 1997) which is coincident with the phenotype of the HIF-1α knockout (Iyer *et al* 1998, Ryan *et al* 1998), demonstrating a crucial role for Arnt in HIF-1α activity and suggesting that there is a lack of functional redundancy between the Arnt proteins at least at this stage of development.

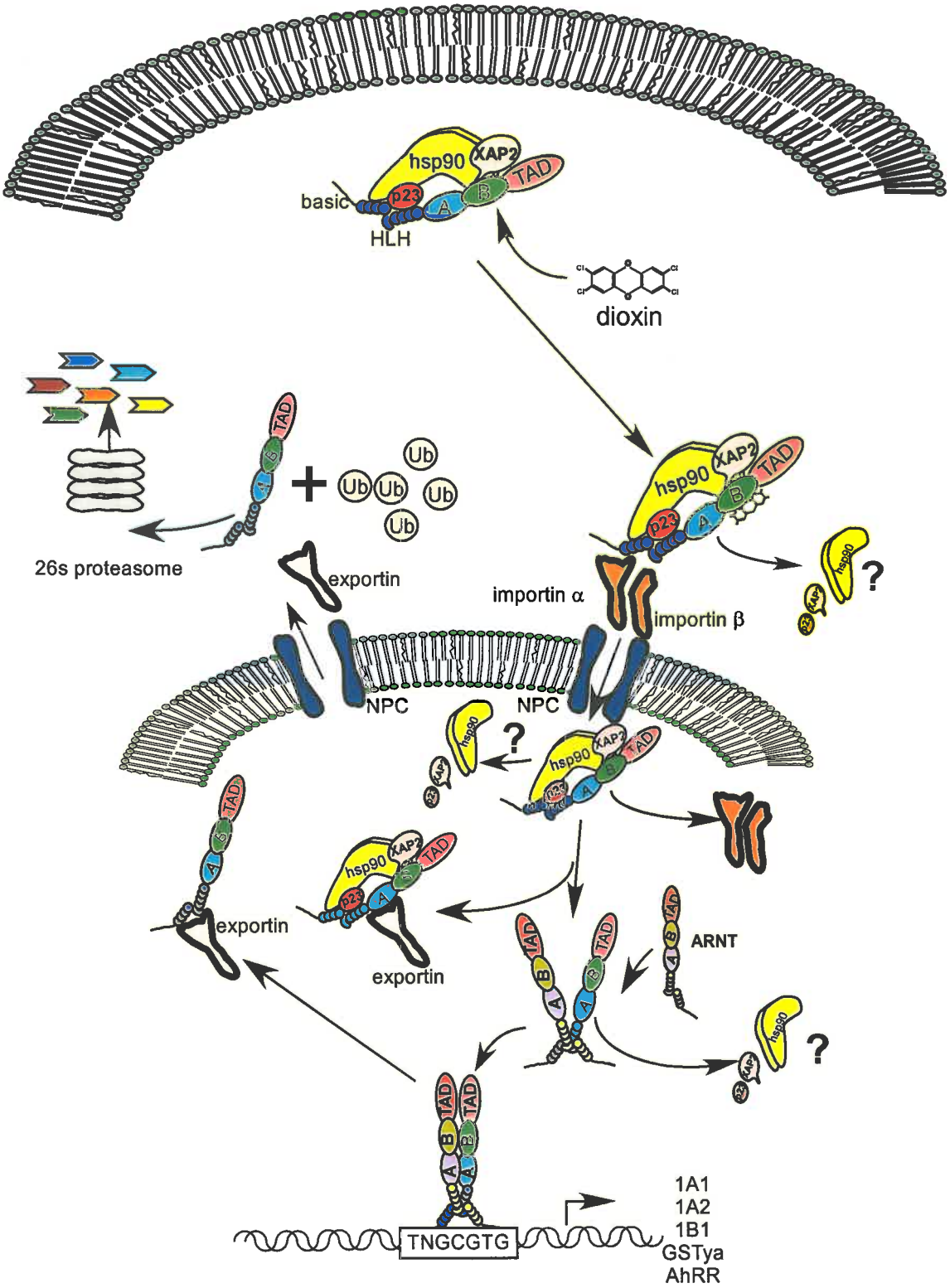


Figure 1.5. *The DR pathway.* The DR resides in the cytosolic compartment of the cell, bound to the molecular chaperones hsp90, XAP2 and p23. Ligand activation of the DR invokes nuclear translocation via an N-terminal NLS, transporting the DR into the nucleus by interaction with importinα and moves through the Nuclear Pore complex (NPC). Once in the nucleus, the DR heterodimerises with the nuclear partner factor ARNT to upregulate expression of xenobiotic metabolising enzymes in addition to the AhR repressor protein. During the activation process, hsp90 is shed from the DR in a poorly understood mechanism. Following ligand activation the DR is targeted for degradation via the addition of ubiquitin (Ub) and 26S proteasome mediated degradation. Shuttling between compartments also occurs.

The DR as a bHLH/PAS protein

The DR is so far unique amongst the bHLH/PAS family in that it is the only member of the family activated by a ligand. DR activation is regulated at several different levels (Figure 1.5). Primarily the DR is kept latent by its cytoplasmic localisation (Pollenz *et al* 1994) and hence isolated compartmentally from the partner factor Arnt. Ligand activation is believed to invoke a nuclear translocation event, initiated by a conformational change in the DR exposing a nuclear localisation sequence located C-terminally adjacent to the bHLH region (Ikuta *et al* 1998). Upon entry into the nucleus several events occur. HSP90 is shed and heterodimerisation with the Arnt partner factor occurs, however the sequence of these events in this process is poorly understood (Hoffman *et al* 1991, McEwan *et al* 1997). Subsequent to Arnt dimerisation, DNA binding to cognate XRE DNA sequences (Xenobiotic Response Elements) occurs. The XRE core motif was predicted from analysis of the CYP1A1 enhancer and has subsequently been demonstrated *in vitro* by site selection studies (Swanson *et al* 1995), mutational analysis (Shen and Whitlock 1992) and immunoprecipitation studies using α-Arnt antibodies to co-precipitate the DR/Arnt heterodimer complexed with various substituted DNA probes (Bacsi *et al* 1995). Taken together these studies suggest that the XRE core sequence is an asymmetric recognition sequence comprising TNGCGTG where N is any nucleotide with preference for a C or T. Within this sequence is a 3' GTG element, predicted to be occupied by Arnt (Swanson *et al* 1995, Bacsi *et al* 1995). Upon binding XRE's the DR/Arnt complex recruits transcriptional coactivators to upregulate transcription of target genes, the model gene being CYP1A1 which is upregulated in an attempt to metabolise the activating ligand. Upon activation by ligand the DR becomes a target for ubiquitin mediated degradation (Davarinos and Pollenz 1999, Roberts and Whitelaw 1999, Ma and Whitlock 2000), however whether this degradation occurs in the nucleus (Roberts and Whitelaw 1999) or is exported to the

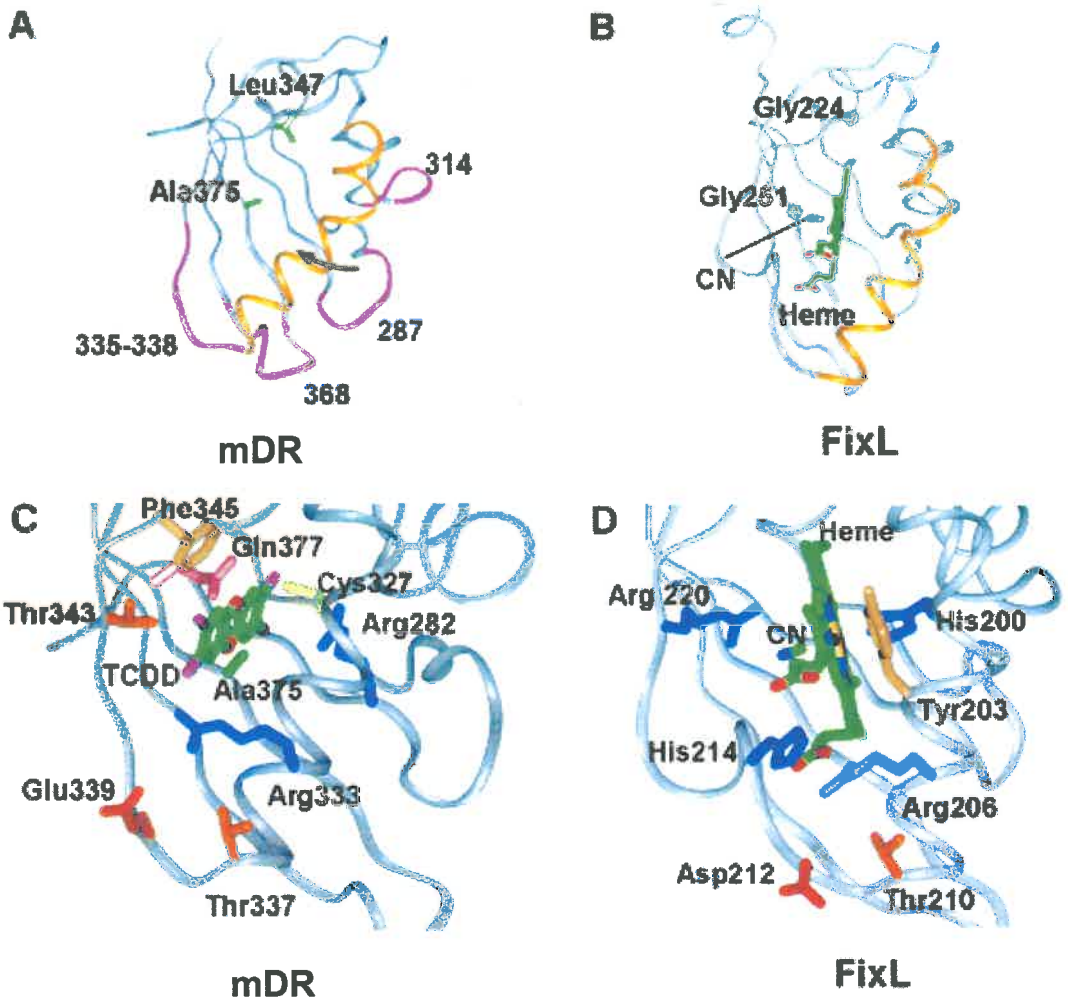


Figure 1.6. *Modelling the DR LBD*. Presented is the theoretical structure of the DR LBD based on modelling studies using the PAS domain of the heme binding protein FixL (This figure was presented in the study by Procopio *et al* 2002). (**A**) and (**B**) The model predicts a B-sheet backbone (grey) upon which the ligand or heme (green) resides and is locked in by a helical connector (orange) for the mDR and FixL respectively. (**C**) and (**D**) are close up views of the respective proteins, of interest is the position of Ala375, which is predicted to reduce the size of the ligand binding cavity when present as a polymorphic valine residue.

cytoplasm via a Nuclear Export Sequence (Ikuta *et al* 1998) and then targeted for degradation is still unclear (Davarinos and Pollenz 1999).

The ligand binding domain of the DR sets it apart from the other members of the bHLH/PAS family, it is a key region of regulation for the DR and understanding this regulation is of particular interest. Recently the Ligand Binding domain has been modelled on the bacterial O_2 sensing FixL PAS family member (Procopio *et al* 2002). A loose model was proposed based on predicted structure inferred from primary sequence analysis, as no real consensus exists in terms of overall amino acid homology between these proteins. The model predicts a (hydrophobic) B-sheet backbone in which the ligand resides and is locked in place by the hydrophobic face of a closing alpha helix (Figure 1.6). This model also takes into account the observations that Ala 375 is a critical amino acid in terms of the ligand binding domain for the DR (Ema *et al* 1994). Interestingly, another mutation which leads to an extension of the mDR by 43 amino acids also leads to a decreased affinity for dioxin (Ema *et al* 1994). How this relates to the proposed model of the ligand binding domain is unclear.

DR mediated transactivation

Analysis of the CYP1A1 promoter *in vivo* demonstrates that in the uninduced state the CYP1A1 promoter is nucleosome bound which accounts for the low basal activity of the CYP1A1 gene in uninduced cells (Wu *et al* 1992). Upon treatment with TCDD the promoter undergoes major structural changes and nucleosomal displacement as evidenced by increased accession of DNA to micronuclease treatment (Okino *et al* 1995), these changes are induced by binding of up to 8 DR/Arnt heterodimers to the genomic CYP1A1 enhancer sequence (Whitlock 1999). The ability of the DR to confer these alterations in genome structure are reliant on the C-terminus of the DR (Ko *et al*

1996). The dynamics of DR/Arnt transactivation are complex and appear to be dependent on promoter context (Ko *et al* 1996, Whitelaw *et al* 1994). For example, ligand dependent induction of a reporter gene driven by XRE sequences appears to be dependent on the C-terminal transactivation domain (CTAD) of Arnt as full transcriptional competency can be achieved with C-terminal deletions of the DR which remove the proposed transactivation domain (Whitelaw *et al* 1994). However it appears that for transcription of endogenous CYP1A1 the CTAD of Arnt is dispensable and induction is dependent on the CTAD of the DR (Ko *et al* 1996). Several co-activator proteins have been demonstrated biochemically to interact with the C-terminal region of the DR including the coactivator Rip140 via an LXXLL independent mechanism (Kumar *et al* 1999), in contrast to the majority of steroid hormone receptors which have been shown to complex with RIP140 or similar co-activators through the LXXLL motif of the co-activator (Westin *et al* 1998, Nolte *et al* 1998). Also shown to interact with the DR transactivation domain is the chromatin remodelling protein BRG1 (Wang and Hankinson 2002). This interaction is reliant on the Q-rich region in the DR transactivation domain. BRG1 restoration in BRG-1 mutant cell lines restores CYP1A1 inducibility to these cells whilst an ATPase mutant failed to do so. However this mutant was demonstrated to associate with the CYP1A1 promoter by a chromatin immunoprecipitation assay to the same degree as the wild type coactivator, demonstrating the requirement of the ATPase catalytic domain and suggesting the involvement of chromatin remodelling factors (Wang and Hankinson 2002). In addition, the Q-rich region of the DR has been shown to interact with SRC-1 (a bHLH/PAS protein itself) in an LXXLL dependent manner, (Kumar and Perdew 1999). The C-terminal region of the DR has also been shown *in vitro* to interact with the basal transcription factor TFIIB, an interaction which may stabilise the DR/Arnt DNA binding complex (Swanson and Yang 1998). Recently, an interaction has been demonstrated biochemically between the C-terminal Acidic activation domain (amino

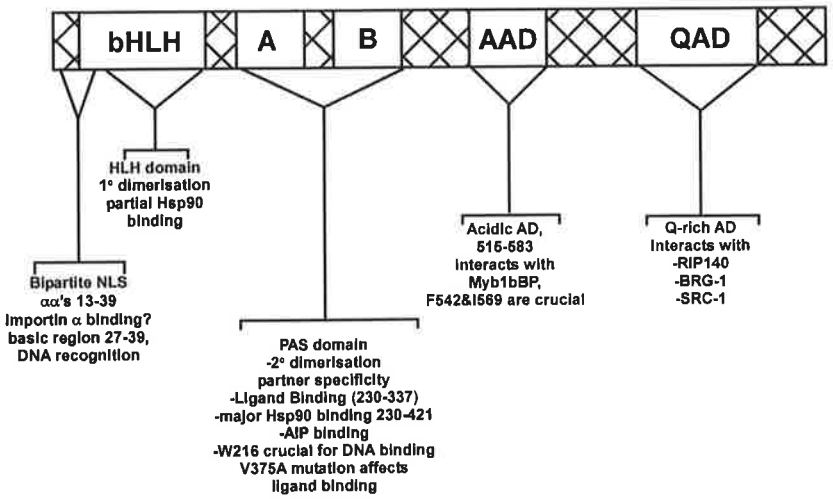


Figure 1.7. *Key regions which modulate DR function.* Indicated are some of the major protein/protein interaction regions which regulate DR activity and control its transcriptional output. See text for details. AAD, Acidic rich activation domain; QAD, Glutamine rich activation domain.

acids 515-583) of the DR and Mybbp1a (Myb binding protein 1a), however this interaction was lost when F542 and I569 of the DR were mutated to alanine (Jones *et al* 2002). Transfection of Mybbp1a cDNA augmented the DR mediated response from a luciferase reporter gene (Jones *et al* 2002). However the role that Mybbp1a plays in DR activation is unclear and one could posit a role for nuclear/cytoplasmic shuttling. Thus the C-terminal region of the DR is complex and only loosely characterized, and involves the multiple interplay of several coactivator proteins, potentially acting within a cell specific context. Some of the key regulatory regions which contribute to DR regulation and where these regulatory regions map to are presented in Figure 1.7.

Physiological role for the DR

Three independent laboratories have created targeted disruptions of the DR locus (Fernandez-Salguero *et al* 1995, Schmidt *et al* 1996, Mimura *et al* 1997). The first of these described the most dramatic phenotype including reduced lymphocyte numbers, reduced organ size, and DR mice were smaller than wild type litter mates (Fernandez-Salguero *et al* 1995). The second study (Schmidt *et al* 1996) observed a reduction in liver size, which was corrected after several weeks into development. The most compelling studies using the KO mice demonstrated failure of dioxin to induce cleft palate and a complete lack of a carcinogenic response to Benzo(a)pyrene exposure (Mimura *et al* 1997, Shimuzu *et al* 2000). Wild type mice convert BP to a carcinogenic metabolite which is reliant on DR upregulation of P4501A1 leading to gross tumour formation, whilst DR null mice display a complete lack of either fibrosarcoma, rhabdomyosarcoma or squamous cell carcinomas over the course of the exposure (Shimuzu *et al* 2000). DR null mice (born from DR null mothers) also experience higher levels of neonatal lethality, the cause of which is poorly characterised but it is proposed that DR null mothers fail to provide sufficient nourishment to pups in uterine

or during lactation (Abbott *et al* 1999). The aberrant immune response initially observed (Fernandez-Salguero *et al* 1995) could not be repeated in a more comprehensive follow up study which investigated the capacity of these mice to generate both a humoral and cell mediated immune response (Vorderstrasse *et al* 2001). The only consistent phenotypes between the three null strains is a decrease in bodyweight/size and decreased liver size. This decrease is a result of Porto systemic shunting (the shunting of the blood supply from the liver to the heart) which is a result of abnormal vasculature structure in the liver of KO mice, leading to a 35% reduced hepatocyte size and a correlative decrease in the overall liver size (Lahvis *et al* 2000). Other vasculature abnormalities were observed in both the eye and kidney (Lahvis *et al* 2000).

Alternate mechanisms of activation of the DR

The observation that mice with a targeted disruption of the DR gene display aberrant liver development leads to the tantalising possibility that there either exists an as yet unidentified endogenous ligand for the DR, or alternatively, there exist other yet to be explained mechanisms of activation of the DR which occur during murine development. Increasing expression of Arnt has been shown *in vitro* to override the obligation for ligand activation in cell culture systems (J. McGuire 1997). Thus potentially, the DR might be regulated during development or in specific tissues by increasing expression of Arnt. Another alternate, yet poorly understood activation mechanism, is based on the observation that cells which are exposed to hydrodynamic shear activate the DR leading to increased expression from both XRE driven reporter genes and endogenous target genes such as CYP1A1 (Sadek and Allen-Hoffmann 1994a, Sadek and Allen-Hoffmann 1994b, Mufti *et al* 1995).

Molecular Chaperones

Molecular chaperone proteins play an essential part of normal physiological function in addition to participating in cellular recovery from cellular stress. The best characterized of the molecular chaperones are the heat shock proteins (hsp's), which are abundantly expressed under standard physiological conditions and constitute approximately 2% of cellular protein. The role of hsp's in protein folding varies for a given substrate, for instance the interaction of hsp70 with its substrates is generally considered a transient association to prevent aggregation of exposed hydrophobic patches on the protein surface whilst the nascent polypeptide is completing translation, enabling the protein to adopt its correct three dimensional structure (Young *et al* 2001, Hartl and Hartl 2002). Additionally these chaperones respond to conditions of cellular stress, such as exposure to heat shock whereby proteins become either partially or fully denatured, exposing hydrophobic patches within the protein which are ordinarily buried internally within the protein, leading to protein aggregation (Hartl and Hartl 2002). The major chaperone system to exist is the hsp70 system which binds to newly synthesized polypeptide upon release from the ribosome, in combination with several other co-chaperone proteins, including NAC, Hsp40 and Prefoldin (Wiedmann *et al* 1994, Vainberg *et* al 1998). Proteins that require additional assistance in folding can be shunted to the TriC/Chaperonin system, which acts to isolate the nascent polypeptide from the crowded molecular environment of the cell so that the entire protein or specific domains of the protein can form the correct three dimensional structure. This system acts as a cylinder and closes the peptide from the cytosol and in an energy dependent process, undergoes multiple rounds of chaperone association and release until the correctly folded conformation has been obtained. The TriC system in eukaryotes is remarkably well conserved with the well characterized GroEL/GroES system of prokaryotes

(Leroux and Hartl 2000, Dunn *et al* 2001). In addition to these chaperone systems, some proteins, including steroid hormone receptors and protein kinases in addition to the DR require a more permanent association with the hsp90 chaperone (eg Raf-l kinase (Stancato *et al* 1993), Androgen Receptor (Fang *et al* 1996), Mineralocorticoid Receptor (Bruner *et al* 1997), Glucocorticoid Receptor (Rexin *et al* 1991) and the Progesterone Receptor (Kosano *et al* 1998)). Hsp90 is perhaps best known for its ability to increase the affinity of steroid hormone receptors for their relative hormones/ligands (Picard *et al* 1990, Bohen and Yamamoto 1993). Both hsp70 and hsp90 chaperone systems utilise energy via an ATPase domain within each of these proteins (Grenert *et al* 1999, Morishima *et al* 2001, Richter *et al* 2001).

A critical protein-protein interaction domain mediating the interactions of the various chaperone proteins with their respective substrates is the tetratricopeptide repeat (TPR) domain. TPR domains form a structural unit composed of a pair of anti parallel alpha helices able to recognize a specific substrate motif (Das *et al* 1998, Scheufler *et al* 2000). For example the hsp70/hsp90 organizing protein Hop/p60 has two critical TPR domains. TPR2A recognizes the C-terminal pentapeptide MEEVD motif of hsp90 (Young *et* al 1998, Scheufler *et al* 2000, Brinker *et al* 2002). Additionally there is a separate TPR motif of hop, TPR1, which has been shown to recognize a nearly identical motif located at the C-terminal region of hsp70 PTIEEVD (Brinker *et al* 2002). In this manner, by virtue of Hop containing two separate heat shock protein interaction motifs, Hop brings the substrate polypeptide to hsp90 by virtue of hsp70 having the first association with the substrate (Hartl and Hartl 2002).

The molecular chaperone system acts at the crossroads of protein folding. Proteins can either be chaperoned further in an attempt to achieve a correctly folded substrate or alternatively the protein can be targeted for destruction by means of the 26s proteasome.

Central to this research has been the identification of the BAG-1 (Bcl-2 associated athanogenes) and CHIP (Carboxy terminus of hsp70-interacting protein). CHIP was initially identified in a yeast two hybrid screen in a search for TPR interacting proteins (Ballinger *et al* 1999). It is predicted that CHIP contains three TPR domains which mediate interactions with both hsp70 and hsp90 (Ballinger *et al* 1999, Connell *et al* 2001). In addition to the TPR containing region, CHIP contains a C-terminal region of homology with the U-box domain from the yeast E4 ubiquitin chain elongation factor UFD2. It is proposed that this U-box is structurally similar to RING-finger domains found in a sub-class of E3 ubiquitin ligases targeting substrate proteins for 26S proteasomal degradation (Aravind and Koonin 2000). The current model posits that CHIP acts as the first protein to induce ubiquitination of the substrate which then switches to the BAG-1 proteins which further facilitate substrate degradation (Demand *et al* 2001). However the processes which dictate the decision to target a chaperone substrate for degradation or persist with folding processes are poorly understood.

An additional factor in chaperone systems is a smaller cochaperone protein p23. This protein is an auxiliary factor for hsp90 mediated chaperoning of steroid hormone receptor substrates including the estrogen receptor (Knoblauch and Garabedian 1999), glucocorticoid receptor (Hutchison *et al* 1995) and progesterone receptor (Johnson *et al* 1994). p23 is expressed highly in the majority of murine tissues (Freeman *et al* 2000), however it has been shown that p23 and a related protein (transcript similar p23) were alternately expressed in different tissues (Freeman *et al* 2000). In both mammalian cell culture systems and a yeast reconstitution systems it was shown that the two forms of p23 can act in either an identical or opposing fashion, depending on the steroid receptor substrate (Freeman *et al* 2000). The role of p23 in hsp90 mediated chaperone processes is poorly understood but is proposed to facilitate the ATPase activity of hsp90, however this is not due to an increase in ATPase activity of hsp90 rather p23 is proposed to

transmit the ATPase induced conformational change within hsp90 more efficiently (Young and Hartl 2000). It appears that for several steroid hormone signalling systems p23 is actually dispensable, as has been demonstrated *in vivo* for the estrogen receptor in a p23 depleted yeast strain (Knoblauch and Garabedian 1999).

The DR/Chaperone complex

The DR exists in the cytosol as a complex of several proteins which have so far been very poorly characterised (Perdew and Whitelaw 1991, Chen and Perdew 1994, Carver and Bradfield 1997, Ma and Whitlock 1997, Meyer *et al* 1998). Early biochemical studies using in vivo labelling of cells demonstrated the DR could be immunoprecipitated as a complex comprising several proteins (Perdew and Whitelaw 1991, Chen and Perdew 1994) including the molecular chaperone hsp90. The association of the DR with hsp90 is thought to act severalfold. Firstly it masks the N-terminal nuclear localisation sequence (amino acids 13-39, Ikuta *et al* 1998), retaining the DR in cytoplasm (Fukunaga *et al* 1995). Secondly chaperone association is thought to maintain the DR in a form competent to bind ligand. Evidence for this is provided by the fact that the major hsp90 binding region of the DR co-localises with the ligand binding region (Burbach *et al* 1992, Whitelaw *et* al 1993, Coumailleau *et al* 1995). In addition to this, yeast strains that have regulatable levels of hsp82 (the yeast homologue of hsp90, Picard *et al* 1990) have been shown to require hsp90 to restore DR signalling in yeast (Carver *et al* 1994, Whitelaw *et al* 1995). These studies act twofold, first and foremost they demonstrate the requirement of hsp90 for DR signalling, and secondly they depict the LBD as a repression domain, which can be relieved by ligand binding.

Immunophilin like proteins

In addition to hsp90, the DR has been shown by yeast two hybrid and biochemical analysis to interact with an immunophilin type protein termed XAP2 (Hepatitis Virus B X associated protein 2, simian clone), AIP (Aryl Hydrocarbon Receptor Interacting Protein, murine clone)/Ara9 (Aryl Hydrocarbon Receptor Associated Protein 9, human clone) (Meyer *et al* 1998, Ma and Whitlock 1997, Carver and Bradfield 1997, respectively). Sequence alignment displays a loose homology of this protein with the FKBP52 immunophilin associated with several steroid hormone receptor complexes (Carver and Bradfield 1997). The homology suggests the presence of two notable domains. The first of these is the peptidylprolyl isomerase domain, and secondly, within the C-terminus, XAP2 is predicted to contain 3 Tetratricopeptide repeat (TPR) domains (Carver and Bradfield 1997).

Immunophilin proteins are commonly found in steroid hormone receptor complexes (Schmitt *et* al 1993, Owens-Grillo *et al* 1995, Pratt and Toft 1997), they are so named due to the fact they contain PeptidylProlyl Isomerase (PPIase) domains which are inhibited by the immunosupressants rapamycin or FK506, or in the case of the cyclophilin class of PPIase proteins cyclosporin A. They are also referred to as FK506 Binding Proteins (FKBP) due to their affinity for the FK506 immunosuppressant. Commonly immunophilins also possess a TPR domain in either a single or multicopy fashion, aiding the recruitment of the immunophilin to substrate proteins by acting as a docking motif (Young *et* al 1998, Scheufler *et al* 2000). It has been postulated that immunophilins modulate nuclear trafficking/docking of the glucocorticoid receptor (Czar *et al* 1995, Owens-Grillo *et al* 1996, Pratt *et al* 1999). The most common immunophilin proteins are FKBP51 and FKBP52 (FK506 Binding Proteins with molecular mass of 51kDa and 52kDa), which are components of both the GR and PR

systems (Czar *et al* 1995, Milad *et al* 1995). PPIase domain containing proteins typically utilise this domain in the isomerisation of proline residues from the cis to trans position in substrate proteins aiding in the maturation process of the substrate or refolding of a denatured protein (Shaw 2002). Isomerisation of target proline residues may rely on phosphorylation or other post translational modification of a substrate domain (Yaffe *et al* 1997). It has been suggested based on sequence homology (in the amino terminus Ara9 shares around 28% identity with FKBP12, Carver and Bradfield 1997) that XAP2 could posses PPIase activity, however this appears not to be the case as the putative PPIase domain lacks the ability to bind FK506, an inhibitor of PPIase enzymatic activity (Carver *et al* 1998). The PPIase domain of FKBP52 has recently been shown to be required for association with the molecular motor dynein even if the PPIase activity of this domain has been inhibited by FK506 (Silverstein *et al* 1999, Gagligniana *et al* 2001).

The role of XAP2 and p23 in DR signalling

The role that XAP2 plays in DR signalling is unclear. Transient transfection studies demonstrate an approximate twofold increase in XRE driven reporter gene activity which (Carver and Bradfield 1997, Ma and Whitlock 1997, Meyer *et al* 1998), which correlates to an approximate two-fold increase in DR expression (Meyer and Perdew 1999, Lapres *et al* 2000). To this end XAP2 was shown to decrease ubiquitinated forms of the DR in transient overexpression experiments (Kazlauskas *et al* 2000). Furthermore DR chimeric proteins fused to a fluorescent tag suggest a role for XAP2 in cytosolic retention for this protein (Petrulis *et al* 2000, Kazlauskas *et al* 2001). A common feature of the DR system is that transient overexpression of the DR, leads to increased nuclear accumulation of the DR and a subsequent increase in reporter gene activity in a ligand independent fashion. This was generally explained by an overriding of some unknown

cytoplasmic retention system. By co-expressing XAP-2 it was shown that XAP2 could overcome this ligand independent nuclear accumulation of DR-FP (Petrulis *et al* 2000, Kazlauskas *et al* 2001), intimating that the factor being out titrated was actually XAP2. Recently it has been proposed that XAP2 acts in concert with hsp90 and p23 to mediate maturation of the DR complex. Treatment of the DR/hsp90/XAP2/p23 complex in vitro with Geldanamycin destabilizes this complex such that p23 and XAP2 are lost from the complex (Kazlauskas *et al* 2001), furthermore, treatment of COS-1 cells transiently transfected with a DR-GFP fusion protein and XAP2 with Geldanamycin resulted in the ablation/amelioration of XAP2 enhanced cytoplasmic retention (Kazlauskas *et al* 2001). Taken together these results suggest that XAP2 stabilizes the DR/hsp90/p23 complex, preventing transient unmasking of the N-terminal nuclear localisation sequence which would prevent ligand independent nuclear accumulation of the DR. In addition this stabilization of the latent chaperone complex protects the DR from proteasome mediated degradation (Kazlauskas *et al* 2000).

Further to this it was shown that p23 is required to remain associated with the DR complex in order for the DR to be able to functionally associate with the nuclear import protein, Importinα/Pendulin. Treating the DR complex with the hsp90 binding agent Geldanamycin, which prevents ATP hydrolysis by hsp90 (Stebbins *et al* 1997) and stalls the maturation process of hsp90 with its substrates at the p60/Hop associated phase of substrate maturation, renders the DR incapable of associating with Importin α (Kazlauskas *et al* 2001).

Mapping DR/XAP2 interactions

Several studies have attempted to demonstrate the processes underlying DR/XAP2 interactions, and in part they have mirrored the processes which occur in steroid hormone receptor systems. Intriguingly it was shown that XAP2 only interacts with the

DR from species which are able to bind to dioxin or dioxin like chemicals, linking the immunophilin with the construction/integrity of a functional LBD (Bell and Poland 2000). This study showed that the last 5 amino acids which are predicted to form an alpha helix are crucial in the interaction between XAP2 and the DR in vitro. It is noteworthy that this putative alpha helix lies outside of the predicted TPR motifs of XAP2. Hsp90 is required for DR/XAP2 binding and the binding of DR to XAP2 stabilises the DR/XAP2/hsp90 interaction. A mutation in one of the predicted TPR domains of XAP2 abrogates XAP2/hsp90 interactions suggesting that the interaction between XAP2 and hsp90 is in fact a TPR mediated process (Bell and Poland 2000). XAP2 was also shown to prevent ubiquitination of the DR (Kazlauskas et al 2000), although whether this is a symptom of the overexpression studies used to arrive at this conclusion or is a function of XAP2 enhancing cytosolic localisation and hence preventing degradation cues which are initiated in the nucleus from occurring is unclear.

Nuclear import and Export pathways

Many transcription factors are cytoplasmically localised and thus to fulfil their role as a transcriptional regulator must translocate from the cytoplasm to the nucleus. Often this cellular compartmentalisation is crucial for regulating the specific signalling pathway involved (Carmo-Fonseca 2002). An ideal example of this is the circadian rhythm members of the PAS family which shuttle between the cytosol and nucleus in response to light cues, which is regulated by multiple phosphorylation events (Kloss *et al* 2001, Akashi *et al* 2002). Entry into the nucleus is restricted, small molecules (less than 40kDa) are able to passively diffuse through the Nuclear Pore Complex (NPC), a large macromolecular structure which traverses the double membrane of the nuclear envelope. However, for molecules larger than this an energy dependent process has evolved. This process utilises a region of enriched basic amino acids which functions as a Nuclear Localisation Sequence (NLS) on the target protein which recognises proteins

of the Importin α/β /Karyopherinα/Pendulin class which mediates entry of the protein into the nuclear compartment in a RanGTP dependent process (Jans *et al* 2000). NLS motifs can be classified as classical, such as the SV40 large T-antigen which is highly basic in nature or non-classical such as the Bi-partite NLS sequences which are also basic in nature but two basic regions are separated by a spacer of 10-12 amino acids (Jans *et al* 2000). The current model for importin mediated nuclear translocation suggests that Importinα (the NLS recognition component) recognises the NLS of target proteins once the Importinβ subunit has bound to Importinα. An opposing system exists to traffic the export of proteins from the nucleus. This system uses exportin proteins, the best characterised of which is Crm1 (Stade *et al* 1997) but also includes CAS and the recently identified exportin 4 (Ossareh-Nazari *et al* 2001), to bind to a target protein and translocate the protein from the nucleus to the cytoplasm. Classical Nuclear Export Sequences (NES) are leucine rich and are typified by the NES of the rev protein of HIV (Wen *et al* 1995). However a novel exportin protein (CRT) which does not recognise classical leucine rich sequences but instead recognises a critical phenylalanine/phenylalanine doublet between the zinc finger DNA binding domain nuclear hormone receptors has recently been isolated (Black *et al* 2001), raising the possibility that a specific exportin exists for the nuclear hormone receptor superfamily. Exportins are RanGTP binding proteins and upon translocation through the NPC RanGTP is hydrolysed to RanGDP in a RanGAP dependent process and the Exportin/cargo complex dissociates (Ossareh-Nazari *et al* 2001).

Nuclear import and export of the DR

The DR contains a novel bipartite nuclear localization sequence between amino acids 13-39 (Ikuta *et al* 1998). This minimal region when fused to GFP as a purified recombinant protein and microinjected into the cytoplasm of cells, was sufficient to

localize the GFP in a ligand independent manner (Ikuta *et al* 1998). Further *in vitro* experiments have confirmed that the DR can interact with PTAC58/importin α/karyopherin α (Kazlauskas *et al* 2001). In addition the DR has a Nuclear export sequence (NES) located in Helix 2 of the HLH in a leucine rich region located around amino acids 63-73 (Ikuta *et al* 1998). The region encompassing aa's 55 and 75 was sufficient to translocate a BSA fusion protein microinjected into the nucleus into the cytoplasm but not mutant chimeras which had critical leucines (Leu 70 and 72 in the mDR) mutated according to an alignment with other proteins shown to interact with the Crm1 nuclear export machinery (Wen *et al* 1995, Stade *et al* 1997 and Fornerod *et al* 1997). However, the corresponding mutations in the native DR failed to exhibit nuclear retention (Davarinos and Pollenz 1999) in the absence of ligand, but once cells were treated with ligand, a greater proportion of cells demonstrated nuclear localization (Davarinos and Pollenz 1999). Several studies have shown that the DR shuttles between the cytosolic and nuclear compartments in the absence of exogenous ligand treatment (Ikuta *et* al 2000 and Kazlauskas *et al* 2001). Presumably, these mutant proteins have a deficiency in binding to the nuclear export proteins Crm1, as the DR export pathway has been shown to be Leptomycin B sensitive (Davarinos and Pollenz 1999, Ikuta *et* al 2000) suggesting a requirement for the Crm1 nuclear export pathway, however this was not demonstrated biochemically in this study. Additionally, a second putative NES has been identified which lies between the PAS A and PAS B regions of the DR and the authors propose that this NES acts as a shuttling NES in the absence of ligand (Berg and Pongratz 2001).

The AhRR

In addition to being actively exported out of the nucleus the DR has an additional level of control. One of the target genes upregulated by the DR encodes a protein which is homologous to the N-terminal region of the DR but diverges after the PASA domain. This protein has the ability to heterodimerise with Arnt and bind a radiolabelled XRE probe and has been termed the AhR repressor (Mimura *et al* 1999). This protein has been shown to be upregulated both *in vitro* and *in vivo* upon exposure to the DR ligand 3-MC (Mimura *et al* 1999, Baba *et al* 2001). Further analysis of the AhRR promoter and adjacent regulatory sequence reveal the presence of several XRE like sequences which when mutated in combination in transient transfection assays reduce the inducibility of the reporter gene, implying that the repressor is indeed a bona fide target gene of the DR (Baba *et al* 2001). Overexpression of the AhRR in cell culture systems ablates TCDD mediated reporter gene induction (Mimura *et al* 1999). The model posits that, upregulation of the AhRR switches off DR mediated gene induction by sequestering Arnt and directly competing for genomic XRE target sequences. In addition it has been proposed that the human AhRR can recruit corepressor complexes with HDAC activity (Fujii-Kuriyama unpublished data). Recently, patients with the undermasculinizing syndrome known as micropenis have been shown to have a higher incidence of homozygosity for a Pro185Ala mutation within the AhRR, this mutation would lie within the C-terminally adjacent to the PASA region and would potentially disrupt DNA binding, however this remains to be demonstrated biochemically (Fujita *et al* 2002).

Cell Cycle

Several models implicating the DR in cycle control have been proposed by various groups. There exists somewhat contradictory data surrounding this area of research, for instance TCDD treatment of thymocytes *in vivo* leads to increased apoptosis (McConkey *et al* 1998), whilst TCDD treatment of hepatocytes has been shown to both increase (Moolgavkar *et al* 1996) and decrease (Wiebel *et al* 1991) proliferation rates. In a similar rein, TCDD has been shown to paradoxically both induce proliferation of keratinocytes (Milstone and LaVigne 1984) as well as lead to keratinocyte terminal differentiation (Choi *et al* 1991, Gaido *et al* 1992). The first model is based on the observation that a DR deficient cell line C12, a chemically mutagenised derivative of the model DR signalling cell line Hepa 1c1c7, which has approximately 10% DR levels compared to the parent cell line (Miller *et al* 1983), has a slower cell cycle progression, in the absence of exogenous ligand treatment. It was identified that these cells spend twice as long in the G1 phase of the cell cycle. This aberration was attenuated by reinstating a functional DR back into these cells such that they resume a cell cycle which resembles the wild type cell line (Ma and Whitlock 1996). In apparent contrast to this it has been demonstrated that the DR binds to the Retinoblastoma protein via an LXCXE domain located at amino acids 325-329, in addition to a second interaction domain located within the glutamine rich region of the transactivation domain (Ge *et al* 1998, Puga *et al* 2000, Elferink *et al* 2001). The retinoblastoma protein has the capacity to stall the cell cycle by negatively regulating genes that promote cell cycle progression (Hickman *et al* 2002). Furthermore, the DR has been shown to directly upregulate expression of the cell cycle gene p27KIP1, which also stalls the cell cycle (Kolluri *et al* 1999). Taken together these observations imply that the DR may act to stall the cell cycle whilst there is a presence of potentially hazardous DNA damaging xenobiotics. By stalling the cell cycle this gives xenobiotic metabolising enzymes in the cell a

chance to remove the inducer and DNA damaging chemicals in addition to repairing any mutations that may have occurred as a result of the barrage from the xenobiotic, prior to DNA synthesis, thus minimising the chance of mutation accumulation.

2

Materials and Methods

Chapter 2 MATERIALS AND METHODS

Materials

The concentrations of chemicals used for treatments is indicated in the text. Suppliers for chemicals are the following, MG132 (Biomol) was diluted in ethanol to 1000x concentration from a 50 mM stock in DMSO and then delivered to cells. Staurosporine (Sigma) was stored as a 100μM stock in DMSO, TCDD was kept as a 10 μM stock in DMSO, Bisindolylmalemeide I (calbiochem) 200μM stock in DMSO, 3-4-methoxy-4-nitroflavone (Steven Safe, Texas A&M) 10μM stock in DMSO, Genistein (Sigma) 100mM stock in DMSO, Puromycin (Sigma) 10mg/ml stock in H_2O, Glutathione and Ni/NDA agarose were from Zymetrix, NiNta agarose was from Qiagen, Dual Luciferase reporter assay kit from Promega, Broad range prestained molecular weight markers from New England Biolabs, 1kB DNA ladder from Life technologies, Thrombin from Sigma. Radiolabelled chemicals containing ^{32}P was purchased from Geneworks. Hypoxia treatments were performed by placing anaerobe sachets (Oxoid) into air tight boxes and incubated for the indicated periods

Plasmids

DR/IRES/puro–Constructed by subcloning an MluI/XbaI fragment from AhR/CMV4 into pEF/IRES/puroV.

DR-NLS/IRES/puro- An oligonucleotide encoding the nucleoplasmin nuclear localisation sequence (Bold type, (Kang *et al* 1994) and Haemagglutinin epitope (underlined) **KRPAATKKAGQAKKKK** RYPYDVPDYA was inserted in duplicate into an XhoI site generated at the 3' end of the coding sequence of the murine dioxin receptor. A hexa-histidine tag was also incorporated at the extreme 3' end of the coding sequence. The C-terminally modified DR was then subcloned into both the pCIN4

expression vector (Rees *et al* 1996), generating the plasmid pDR-NLS/CIN4 and the pEF/IRESpuro vector (Steven Hobbs IRC, London) to generate the plasmid pEF/DR-NLS/IRESpuro.

DRΔLBD/IRES/puro -The DRΔLBD construct has recently been described elsewhere (McGuire *et al* 2001). Briefly this construct is a deletion mutant lacking amino acids residing between 287-421 of the mDR. This construct was subcloned as an MluI/XbaI fragment from DRΔLBD/EFBOS into pEF/IRES/puro.

DRbHLH/HIF/EFBOS -This construct generates a chimeric protein containing the bHLH (amino acids 1-82) of the DR fused to the HIF-1α protein lacking the bHLH region (amino acids 74-826), and was subcloned as a BamHI fragment from DRbHLH/HIF-1α, into BamHI/pEF/BOS.

XIXI and T81 -The XRE-Tk-Luciferase reporter plasmid pXIXI and control Tk-Luciferase (T81) have been described previously (Berghard *et al* 1993).

pRL-TK (Promega) -encodes the luciferase gene from *Renilla reniformis* and was used as an internal control in transient transfection experiments

asXAP2/EFBOS –this vector was generated by subcloning a partial EcoRV/BamHI digest from Ara9/sport, such that the BamHI site at position 1060 was used to subclone in the reverse orientation into pEF/BOS.

XAP2/EFBOS –a KpnI/XbaI fragment from Ara9sport (Chris Bradfield, Madison Wisconsin) was subcloned into KpnI/XbaI EFBOS

XAP2myc -was a gift (Ben Roberts, University of Adelaide) and contains the human XAP2 sequence fused to a 6myc epitope tag.

pcDNA3/CHIP -was a gift (Cam Patterson, Chapel Hill, North Carolina) and contains the cDNA sequence for human CHIP.

pEF/CHIP/IRES/puro -was generated by subcloning an XbaI fragment from pcDNA/CHIP into XbaI pEF/IRES/puro.

GSTLBDpuro -The GST sequence was PCR amplified from pGEX4T3 (promega) using GSTfwdXho1 primer (CCGCTCGAGCGACCATGTCCCCTATACTAGG) and GSTrevMlu1 (TCGACGCGTGGATCCACGCGGAAC), and Pfu polymerase. The cycling conditions were 94°C, 30s; followed by 35 cycles of (94°C for 30s denaturing, 60°C for 30s annealing, 72°C for 2min extension) and a final 2 min extension at 72°C. The PCR fragment was subcloned into the Xho1/Mlu1 pEF/IRES/puro. The LBD fragment of the DR was PFU PCR amplified using the mDRfwMlu1230 primer (CGACGCGTGCAATGAATTTCCAAGGG) and DR421myc-rev primer (GCTCTAGAGATCTTCTTCAGAAATCAACTTTTGTTCTAGGGGATCCATTATG GG) which contained the minimal myc epitope of EQKLISEEDV, a stop codon and an Xba1 restriction site for subcloning. PCR cycling was performed as for the GST primer pairs, and following amplification the fragment was subcloned into Mlu1/Xba1 digested pEF/GST/IRES/puro vector. Constructs were verified by sequencing.

pEF/HisLBDmyc/IRES/puro -This construct was generated by using the Xho230fwdHIS primer (CCGCTCGAGCGACCATGCATCATCATCATCATCATG CAAATGAATTTCCAAGGG), encoding an ATG start site and 6xHIS tag upstream of the mDR sequence beginning at amino acid 230 with the DR421myc_rev primer (see above). PCR conditions are identical to above. The PCR product was inserted as an XhoI/XbaI fragment into XhoI/XbaI digested pEF/IRES/puroVI.

Antibodies

Hsp90 (Transduction laboratories). RPT1 (Garry Perdew, Penn. State, Pennsylvania). HIF-1α and Arnt (generated in our laboratory). P23 (David Toft, Mayo Clinic Rochester, MN). 12CA5 (αHaemagglutinin, murine clone). 9E10 (anti myc) were purchased from the antibody generating facility IMVS Adelaide. 3F10

(αHaemagglutinin, Rat clone, Boehringer). CHIP (R. Takahashi, Riken Brain institute, Japan). 1A1 (J. Hardwick Rootstown, Ohio).

Methods

Cell culture

Mouse adrenal Y1 cells, mouse hepatoma Hepa1c1c7 cells and the human embryonic kidney transformed cell line 293T were routinely grown in Dulbecco's modified Eagles medium (Gibco/BRL) supplemented with 10% foetal calf serum and streptomycin 100U/ml and gentamycin 100 U/ml.

Transfection based experiments

Stable cell lines

Y1 cells (2×10^6) were transfected with 10 μg of either pDR-NLS/CIN4 or pCIN4 using standard electroporation procedures (Kerry *et al* 1996). Cells were then seeded into 10cm dishes and allowed to recover for 24 hours before addition of G418 at an initial concentration of 250 μg/ml. After 2-3 weeks of selection single G418 resistant colonies were expanded in medium containing 1.5 mg/ml G418. For generation of Hepa 1c1c7 and 293T stable cell lines, cells were transfected in 60mm dishes at 70% confluency using either DOTAP or Lipofectamine-2000 according to the manufacturers instructions. Following a 24 hour transfection period, 50% of the cells were transferred to a 100mm dish and the remainder of the cells were reserved for analysis by Western blotting to verify construct expression. Cells were grown for a further 24 hours before addition of puromycin at an initial concentration of 1μg/ml. Typically, pools of cells were harvested following a 10-14 day selection period and were subsequently passaged in complete medium with the appropriate concentration of puromycin. Following

selection of cell pools at 1μg/ml puromycin, cells were routinely selected in higher levels of puromycin (up to 25 μg/ml), to encourage higher selection of the gene of interest.

Transient Transfection assays

For reporter assays performed in results chapter 3, Hepa1c1c7, Y1/DR-NLS, Y1/Neo-Ctrl or 293T stable cell lines were seeded at a density of 1.5 x 10^5 cells into wells of a 24 well tray and grown for 24 hours. Duplicate wells were transfected with 200ng of pXIXI firefly luciferase reporter and 50 ng of pRL-TK via the DOTAP transfection method (Boehringer). Following 12 hours transfection, cells were induced with tetrachlorodibenzo-*p*-dioxin (TCDD, 1nM) or vehicle alone (0.1% DMSO) for 30 hours unless otherwise stated. For geldanamycin (Gibco/BRL) and MG132 (Biomol) treatments, cells were incubated with combinations of geldanamycin (1μg/ml), MG132 (7.5μM) and TCDD (1 nM) for 16 hours. These combinations of chemicals yielded no signs of toxicity to Y1 cells over this period. For reporter gene assays performed for results chapters 4 and 5, reporter assays were undertaken as above except transfections were performed in triplicate and transfections were performed for 24 hours followed by an overnight treatment with the appropriate chemical/ligand. For antisense experiments, DNA content was normalised using the empty pEFBOS vector to ensure equal transfection efficiency. No abnormal differences in renilla luciferase counts were observed due to increased levels of the antisense vector. Cells were assayed for luciferase activity using the DLR luciferase kit (Promega) according to the manufacturer's instructions. For expression assays, cells were transfected using Lipofectamine-2000 according to the manufacturers instructions for a period of 36 hours.

Immunofluorescence

Cells were seeded onto coverslips and grown for 48 hours before being fixed by washing twice with PBS and immersion in methanol for 2 minutes at room temperature. Cells were rehydrated in PBS for 15 minutes, and then incubated with 3F10 rat anti-haemagglutinin monoclonal antibody (Boehringer, 0.2 µg/ml) for 2 hours at room temperature. The coverslips were washed three times with PBS and subsequently incubated with a 1/30 dilution of fluorescein isothiocyanate (FITC) conjugated goat anti-rat mAb (Sigma) for 45 minutes at room temperature, followed by a wash in PBS before incubation with bisbenzimide stain (Hoechst 33258, 10 µg/ml, Sigma) for 1 minute. Following another two washes in PBS, the coverslips were dried, mounted onto slides with glycerol and sealed. Cells were viewed with a Ziess microscope.

RT-PCR

Total RNA was prepared from cells using RNAzolB (TEL-TEST INC) according to the manufacturers instructions. To generate cDNA from total RNA, one microgram of total cellular RNA was incubated with 0.5 µl of 100 µM dN_6 primer (Geneworks) and DEPC water was added to a total volume of 8 µl. The mixture was incubated at 70°C for 5 minutes and then chilled on ice. AMV Reverse Transcriptase was diluted in a master mix to 3.5 Units/µl in 10% glycerol, 10 mM potassium phosphate pH 7.4, 0.2% Triton X-100 and 2 mM DTT and the appropriate amount of DEPC water and incubated on ice for 30 minutes. 3 µl of this master mix was used for each cDNA synthesis in addition to 2 µl of first strand buffer, 2 µl 0.1 mM DTT, 4 µl 5 mM dNTP mixture, 0.2 µl (1mg/ml) BSA, 0.5 µl RNAsin (40 units/µl). A 20 µl reaction volume resulted when the 8 µl annealing mix was added to 12 µl of the enzyme master mix. cDNA synthesis was carried out at 37°C for 2 hours, followed by heating for 5 minutes at 95°C. 1 µl of the

cDNA reaction was used for PCR. Primers 130N (GATGCCTTCCTCTTCTATG) and 443C (ATCCTTACTTGGGGTTGAC).

Immunoblotting

Whole cell extracts were prepared as previously described (Whitelaw *et al* 1993). Cytosolic and nuclear extracts for immunoblotting were prepared as follows. Cells were harvested with TNE (40 mM Tris-HCl (pH 7.4), 150 mM NaCl and 10 mM EDTA), washed with PBS and pelleted. Cells were resuspended in 2.5 pellet volumes of hypotonic + NP40/Ficoll lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.4% NP-40, 10% Ficoll-400, 1 mM DTT, 1 mM PMSF, 2 μg/ml apoprotinin, 4 μg/ml bestatin, 5 μg/ml leupeptin and 1 μg/ml pepstatin) and incubated on ice for 5 minutes. The cells were then centrifuged for 30 minutes at 14,000 rpm at 4°C. The supernatant was used as the cytosolic fraction. The pellet was resuspended in 1.5 pellet volumes of nuclear extract buffer (20 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 20% glycerol, 0.42 M KCl, 1 mM DTT and the above protease inhibitors), incubated with shaking for 45 minutes on ice and then centrifuged at 14,000 rpm at 4 °C to provide the supernatant nuclear fraction. Protein concentrations were determined by Bradford assay and samples were subjected to 7.5% SDS-PAGE then transferred to nitrocellulose in a semi-dry blotter (Hoeffer). Proteins were detected using the appropriate primary antibody and visualised using enhanced chemiluminescence.

Stripping and Reprobing of membranes with a second primary antibody

Membranes to be reprobed with a second primary antibody were incubated in stripping buffer (100 mM 2-mercaptoethanol, 62.5 mM Tris-HCl pH 6.7 and 2% SDS) at 50°C for 30 minutes with occasional agitation. The stripped membrane was washed 2x 10

minutes in PBS-T with shaking, blocked overnight at 4°C in blocking buffer and then incubated with the appropriate primary antibody as previously described.

Immunoprecipitations

Y1/DR-NLS cells were treated with combinations of TCDD (1 nM), geldanamycin (1 μg/ml) and MG132 (7.5 μM) for 2 hours. Nuclear extracts were prepared as described above and immunoprecipitations using polyclonal antisera directed against the C-terminus of Arnt were performed as previously described (Sogawa *et al*, 1995). Immunoprecipitates were boiled in SDS sample buffer, separated by 7.5% SDS-PAGE and then transferred to nitrocellulose. The DR-NLS protein was detected by the 12CA5 anti-Ha mAb. Immunoprecipitations using the 3F10 anti-Ha mAb (Boehringer) were performed as follows: whole cell extracts (1 mg) were incubated with 50 μl of a 1:1 slurry of protein G agarose (Boehringer) for 1 hour at 4°C and centrifuged for 1 minute at 10,000 rpm. The supernatant was removed and diluted 2-fold with hypotonic buffer and 3F10 mAb (5 μg) was added and incubated for 3 hours with shaking, after which time 100 μl of protein G agarose was added and incubated overnight. The immunoprecipitated complexes were washed 4 times with Buffer A (10 mM Tris pH 7.5, 0.1% Trition X-100, 2 mM EDTA pH 8.0, 120 mM mM KCl, 2% milk powder and 1 mM PMSF).

Electrophoretic mobility shift assays

Protein extracts from stable cells for use in gel shift assays were generated by swelling of pelleted cells in hypotonic buffer followed by one freeze thaw cycle and centrifugation (30 min/14,000 rpm/4°C). The supernatant was kept as the cytosolic fraction while the nuclear extract was obtained by shaking the pellet in one pellet volume of hypotonic buffer + 0.42 M KCl for 30 min on ice. *In vitro* transformation

reactions using Hepa1c1c7, 293T/puro or 293T/DR cytosolic extracts were performed at room temperature for 2 hours with the indicated combinations of TCDD (10 nM), geldanamycin (10 μg/ml), or vehicle alone (0.2% DMSO) or 30 minutes with staurosporine (1 μM or 100 μM). Conditions for DNA binding with the ^{32}P labelled XRE1 sequence from the rat cytochrome P4501A1 promoter and subsequent non-denaturing electrophoresis were as previously described (Hapgood *et al* 1989).

Nickel affinity purification of DR/NLS

Whole cell extracts from 293T and 293T DR/NLS-puro cells were used for purification of the DR/NLS protein by utilising the hexahistidine tag. Protein (1.5 mg) from untreated whole cell extracts or extracts from cells cotreated with MG132 -/+ geldanamycin (1 μg/ml for 30 minutes) were incubated with 5 mM imidazole binding buffer (500 mM NaCl, 20 mM Tris pH 7.5 and 0.1% Triton X-100) and 400 μl of 1:1 Ni-Nta Resin (Qiagen) for 1 hour. The column was washed with 5x 1 ml fractions of 5 mM Binding Buffer followed by successive washes with 300 μl of 10, 20, 30, 40 and 50 mM imidazole in binding buffer followed by elution with 300 μl of 150 mM imidazole in Binding buffer. 500 μg of BSA was added as a carrier protein and the protein was precipitated with acetone overnight at 4°C. Following centrifugation the pellet was resuspended in SDS sample buffer and separated by SDS-PAGE as described.

Partial Trypsin digestion reactions

For partial proteolysis of Y1/DR-NLS whole cell extracts, 100 μg of extract was incubated with 150 ng of trypsin (Boehringer) at 37°C for 20 minutes. For proteolysis of immunoprecipitated complexes, Y1/DR-NLS cells were treated with the indicated ligand and MG132 as described above and whole cell extracts were taken and

immunoprecipitated also as described above (with the exception that no PMSF was present in the wash buffer). Immunoprecipitates were collected by centrifugation at 3,000 rpm for 5 minutes and resuspended in 30 µl of Tris (10 mM, pH7.5) containing 25 ng of trypsin and incubated at room temperature for 15 minutes. Inactivation of the trypsin was achieved by boiling in SDS sample buffer for 5 minutes after which time the digests were separated by SDS Page and visualised by Western analysis using the 12CA5 mAb.

In vivo labelling

Cells for labelling were passaged from a confluent T75cm^2 flask to 60mm dishes. The next day cells were washed 3 times with phosphate free medium (Gibco), followed by the addition of 1.5 ml Phosphate free medium supplemented with 10% phosphate free foetal calf serum (phosphate free FCS was generated by dialysing FCS against 2 changes of distilled sterile water for a period of 6 hours) and ^{32}Pi at a final concentration of 660µCi/ml. Following a 6 hour labelling period, cells were treated with the appropriate chemical for a further 2 hours, after which time the cells were washed with cold PBS and harvested in TEN buffer (plus PMSF and 1x phosphatase inhibitors, sodium vanadate 1 mM and sodium fluoride 10 mM) and centrifugation to pellet the cells. The pellet was resuspended in 200µl of whole cell extract buffer and cells were lysed and the soluble fraction was collected by centrifugation at 14,000 rpm for 15 minutes in an Eppendorf centrifuge following a 15 minute lysis period at 4°C with shaking. The lysate was incubated with a 30µl slurry of glutathione agarose for 1 hr after which time the pellet was rinsed twice with a 1 ml volume of PBS (500 mM NaCl) + phosphatase inhibitors and PMSF, followed by a third rinse with PBS (500 mM NaCl) + phosphatase inhibitors without PMSF. The agarose pellet was then incubated with 40 µl thrombin (0.25 units/ml final thrombin concentration), for 15 minutes at 37°C. The cleavage reaction was stopped by addition of 10µl 5x SDS load buffer and incubation

for 5 minutes at 100°C. Following centrifugation, the supernatant was separated by 12.5% SDS-PAGE for exposure of the dried gel to film, and an aliquot was run separately for assessing purification efficiency by means of Western analysis.

Results

3

The results presented in this chapter represent published work entitled,

"Multiple roles of ligand in transforming the dioxin receptor to an active basic helix-loop-helix/PAS transcription factor complex with the nuclear protein Arnt."
Molecular and Cellular Biology, 1999 Aug;19(8):5811-22.,
Lees MJ, Whitelaw ML.

Chapter 3. Multiple roles of ligand in transforming the Dioxin Receptor to an active basic helix-loop-helix/PAS transcription factor complex with the nuclear protein Arnt.

Introduction

The Role of ligand in Dioxin Receptor activation

Despite many investigations into the process by which the latent dioxin receptor becomes transformed into an active heterodimer, this mechanism is still poorly understood. Classic models suggested that ligand binding of the receptor led to dissociation of hsp90 in the cytoplasm, allowing dimerisation with Arnt, which subsequently translocated the receptor to the nucleus (Hoffman *et al* 1991). However, immunohistochemistry has recently shown Arnt to be a nuclear protein (Pollenz *et al* 1994, Hord and Perdew 1994), and a nuclear localisation signal has been mapped in the N-terminus of Arnt (Eguchi *et al* 1997), rendering this scenario unlikely. More recent dioxin receptor activation models posit that either the ligand activated receptor moves to the nucleus free of hsp90, in a manner similar to that proposed for hormone activation and translocation of the glucocorticoid receptor (McEwan *et al* 1997), or that hsp90 remains bound to the receptor throughout the nuclear translocation process. In vitro evidence suggests that Arnt plays a role in dissociating hsp90 from the ligand bound dioxin receptor, indicating that the transformation may occur in the nucleus (McGuire *et al* 1994). Further in vitro experiments suggest that ligand independent transformation of the hsp90 bound dioxin receptor to the heterodimeric form with Arnt is also possible (Pongratz *et al* 1992), thereby complicating models seeking to understand the mechanistic role of ligand in the transformation process.

To investigate the mechanisms which regulate bHLH/PAS factor heterodimerisation and explore the role of ligand in dioxin receptor activation the following work studied the function of mutant and modified bHLH/PAS proteins. Here we have added a heterologous nuclear localisation signal at the C-terminus of the dioxin receptor and generated two stable cell lines which express a constitutively nuclear dioxin receptor. This nuclear receptor remains in its latent form, requiring addition of exogenous ligand for activation. Analysis of dioxin receptor signalling in these novel cell lines has provided insights into the multifunctional roles ligand plays during formation of the active DR/Arnt transcription factor complex. In addition to the well documented initiation of nuclear translocation of the dioxin receptor, ligand is critical for invoking Arnt heterodimerisation and maintaining a conformation of the DR/Arnt complex competent for initiating transcription. These data have also produced intracellular evidence that hsp90 release from the dioxin receptor occurs within the nucleus in a concerted mechanism with Arnt dimerisation.

AIMS

The aims of this chapter are to investigate the role of ligand in the activation process of the DR, specifically is the sole purpose of ligand merely to translocate the DR from the cytosolic compartment of the cell to the nuclear compartment whereby once in the nucleus, the subsequent processes occur independently of ligand? Alternatively, ligand could play a more defined role in DR activation and be involved in processes such as release of hsp90/Arnt dimerisation/DNA binding/transactivation. To address this question, a form of the DR was generated that bypasses the ligand induced nuclear translocation step.

RESULTS

Generation of a stable cell line expressing a constitutively nuclear dioxin receptor

Of the bHLH/PAS factors thus far analysed for location within the cell, Sim and Trachealess have been reported to be nuclear, while the latent dioxin receptor and HIF-1α are cytoplasmic. Arnt is generally found in the nucleus, but has also been reported as cytoplasmic in some cell types at distinct embryonic stages (Abbott and Probst 1995), while a Drosophila homologue, Tango, is mostly cytoplasmic (Ward *et al* 1998). In the case of HIF-1α, a hypoxic regulated nuclear localisation signal in the C-terminus induces nuclear translocation at low oxygen levels (Kallio *et al* 1998). In contrast, constitutively nuclear Sim and Trachealess seemingly operate in the absence of any environmental signals. In vitro experiments have shown that Sim, Trachealess and HIF-1α all form strong heterodimers with Arnt in the absence of exogenous inducers (McGuire *et al* 1995, Swanson *et al* 1995; Gradin *et al* 1996, Ema *et al* 1996, Sonnenfeld *et al* 1997, Hogenesch *et al* 1997). While interaction of the dioxin receptor with Arnt is strictly ligand dependent in common cell lines, some in vitro studies have observed varying degrees of ligand independent dimerisation upon mixing of protein fractions containing the dioxin receptor and Arnt (Numayama-Tsuruta *et al* 1997, J.McGuire, personal communication), suggesting that an extranuclear location of the dioxin receptor may be important for maintenance of its latent form. One hypothesis regarding the role of ligand in dioxin receptor activation posits that the sole purpose of ligand might be to invoke transport of the receptor into the nucleus, whereupon transformation of the receptor to an active heterodimer with Arnt could ensue irrespective of the presence of ligand. To investigate the possibility that ligand independent activation of the dioxin receptor might be achieved by artificially translocating it to the nucleus, we modified the receptor to include a nuclear localisation

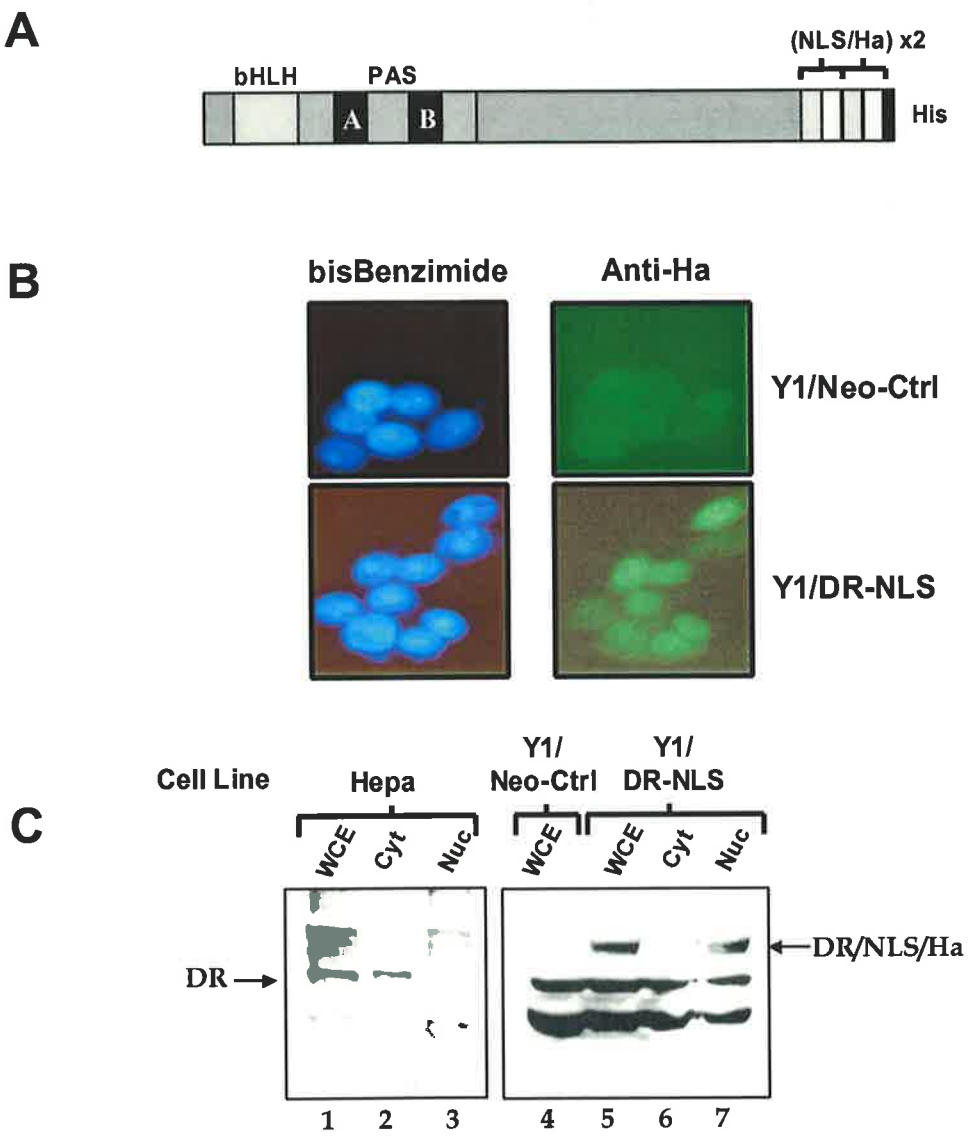


Figure 3.1. *Generation of a stable cell line expressing a nuclear localised dioxin receptor.* (**A**) Schematic representation of the C-terminally modified dioxin receptor containing duplicate sequences of the nucleoplasmin Nuclear Localisation Sequence (NLS) and the Haemagglutinin epitope (Ha) followed by a hexahistidine tag. bHLH, basic-Helix-Loop-Helix; PAS, Per/Arnt/Sim homology region (**B**) The Y1/DR-NLS stable cell line expresses a constitutively nuclear dioxin receptor. Y1/Neo-Ctrl and Y1/DR-NLS cells were seeded onto coverslips and fixed with methanol. Cells were incubated with the rat mAb 3F10 directed against the Ha epitope, followed by incubation with a FITC conjugated, goat anti-rat secondary Ab. Nuclei were visualised by bisbenzimide (blue) staining (**C**) Immunoblot analysis of the dioxin receptor in cell extracts. Protein extracts (100 μg) from whole cells (WCE), cytosol (cyt), and nuclei (nuc) of non-treated Hepa1c1c7, Y1/Neo-Ctrl and Y1/DR-NLS cells were separated by 7.5% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with the Rpt 1 mAb (specific for the native DR, lanes 1 to 3) or the 12CA5 mAb (specific for the Ha tag, lanes 4 to 7). Positions of the native and NLS tagged versions of the protein are shown.

signal at its C-terminus. The 3' end of the mouse dioxin receptor cDNA was extended to contain a duplicate of an oligonucleotide encoding the nuclear localisation signal (NLS) from nucleoplasmin and the haemagglutinin epitope (Ha) recognised by the 12CA5 and 3F10 monoclonal antibodies (Figure 3.1a).

The mouse adrenal Y1 cell line lacks endogenous dioxin receptor as determined by immunoblotting and RT-PCR (data not shown), and was therefore chosen as a background free model system in which to study activity of the constitutively nuclear dioxin receptor. Y1 cells were transfected with an expression vector encoding the NLS modified dioxin receptor and the neomycin resistance gene, which were linked by the encephalomyocarditis virus internal ribosome entry site. Several clonal lines revealed stable expression of the NLS/Ha modified dioxin receptor following G418 selection. These lines exhibited similar characteristics in terms of acquired dioxin signalling, and one was selected for further analysis, hereafter termed Y1/DR-NLS. A G418 resistant control cell line was also isolated which contained stable integration of the blank expression vector, hereafter called Y1/Neo-Ctrl. Immunofluorescence using a mAb directed against the Ha epitope demonstrated that the modified dioxin receptor was constitutively localised to the nucleus in Y1/DR-NLS cells (Figure 3.1b). The slight cytosolic staining observed is non-specific as Y1/Neo-Ctrl cells exhibited a similar cytosolic staining pattern, while there was no nuclear staining in the Y1/Neo-Ctrl cells (Figure 3.1b). In standard dioxin receptor expressing cell lines such as the mouse hepatoma Hepa1c1c7, immunohistochemistry has demonstrated the dioxin receptor to translocate from the cytoplasm to nucleus upon treatment with ligands (Pollenz *et al* 1994). As expected for a cell line containing a constitutively nuclear receptor, the immunofluorescence pattern of the Y1/DR-NLS cells was not altered upon TCDD treatment (data not shown). Immunoblot analysis of whole cell extracts from Y1/DR-NLS lines confirmed the presence of the modified DR, which was absent from the

Y1/Neo-Ctrl cells (Figure 3.1c compare lanes 4 and 5). Consistent with the immunofluorescence observations, cell fractionation of untreated Y1/DR-NLS cells allowed recovery of the modified dioxin receptor in the nuclear extract, whereas it was absent from the cytosolic extract (Figure 3.1c, compare lanes 6 and 7). In total contrast, extracts from untreated Hepa1c1c7 cells showed the native dioxin receptor to be recovered in the cytosolic but not the nuclear fraction (Figure 3.1c, compare lanes 2 and 3). These experiments clearly demonstrate that we have derived a cell line which exhibits stable expression of a constitutively nuclear dioxin receptor. Western analysis using DR specific antibodies revealed that levels of the modified receptor in Y1/DR-NLS cells is lower than wild type receptor in Hepa1c1c7 cells, thus obviating any potential aberrant signalling due to overexpression (data not shown). A second stable cell line, derived from human embryonic kidney HEK 293T cells, was also generated and termed 293T/DR-NLS. As the Y1/DR-NLS cell line is free of endogenous dioxin receptor, it provides a unique model system to investigate the role of ligand in the dioxin receptor activation process. While the following experiments report the full characterisation of the Y1/DR-NLS line, similar results have been obtained in the independent 293T/DR-NLS line.

A constitutively nuclear dioxin receptor is not constitutively active

The dioxin receptor is a ubiquitous protein which resides in untreated cells as a latent complex with the molecular chaperone hsp90 and a 38kD immunophilin like protein (Ma and Whitlock 1997, Carver and Bradfield 1997, Meyer *et al* 1998). In close analogy to the glucocorticoid receptor, the hsp90 complex is thought to have critical roles in maintaining cytoplasmic retention of the dioxin receptor as well as chaperoning its ligand binding conformation (for reviews see Poellinger 1995, Schmidt and Bradfield 1996). In well established model systems such as Hepa1c1c7 cells, ligand

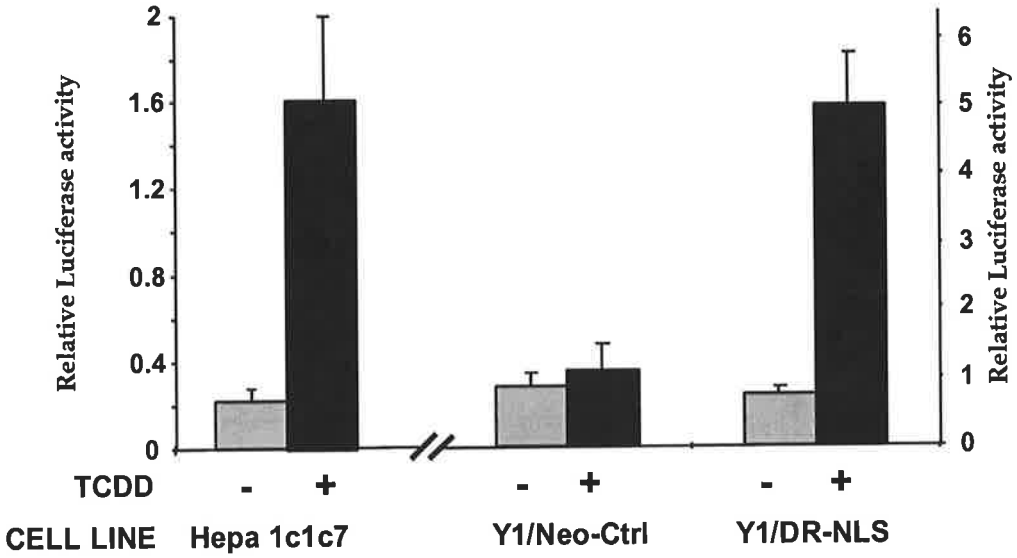


Figure 3.2. *The nuclear localised dioxin receptor requires ligand to activate transcription in Y1/DR-NLS cells.* Hepa1c1c7, Y1/Neo-Ctrl and Y1/DR-NLS cells were transiently transfected with an XRE-Luciferase reporter gene and the renilla luciferase internal control vector pRL-TK. Cells were treated with dioxin (TCDD, 1 nM, dark bars) or vehicle alone (0.1% DMSO, light bars) for 24 hours (Hepa1c1c7) or 30 hours (Y1/Neo-Ctrl and Y1/DR-NLS). Luciferase activity was normalised against the internal control, and is an average -/+ SE of 6 transfection experiments. The left hand Y-axis pertains to the Hepa1c1c7 transfections, while the right hand Y-axis relates to transfections in the modified Y1 cell lines.

treatment initiates nuclear translocation of the dioxin receptor (Pollenz *et al* 1994), although the mechanics of this process are not well understood. It has been generally proposed that ligand treatment invokes dissociation of hsp90 to allow free receptor to pass into the nucleus. Other bHLH/PAS proteins shown to interact with hsp90 are HIF-1α (Gradin *et al* 1996, Hogenesch *et al* 1997) and Sim (McGuire *et al* 1995), although the relevance of hsp90 association with these factors, and any influence of this interaction on their cellular location, is unclear at this time.

To test whether our constitutively nuclear dioxin receptor behaves like other nuclear bHLH/PAS proteins and undergoes non-stimulated transformation into an active transcription factor, we assessed reporter gene activity in the Y1/DR-NLS cell line. Transfection of an XRE driven luciferase reporter gene into Y1/Neo-Ctrl cells gave a low level of background activity which remained unaltered by dioxin treatment (Figure 3.2), consistent with Y1 cells being deficient for the dioxin receptor. Transfection of the XRE reporter gene into untreated Y1/DR-NLS cells gave a similar level of background activity to the Y1/Neo-Ctrl cells, indicating that there is little or no active DR-NLS/Arnt complex in non-stimulated cells. Strikingly, ligand treatment of Y1/DR-NLS cells resulted in an approximate 6 to 8 fold increase in XRE reporter gene activity (Figure 3.2). A similar ligand induction of reporter gene activity was observed in 293T/DR-NLS cells (Figure 4.2b, 5.1a-b). These increases were similar to that observed when Hepa1c1c7 cells were transfected with the reporter gene and treated with ligand (Figure 3.2). These results demonstrate that the constitutively nuclear localised dioxin receptor remains in a latent form, and importantly, as ligand is necessary to activate the constitutively nuclear dioxin receptor, indicate that the role of ligand in dioxin receptor activation is more complex than merely initiating the nuclear translocation of a cytosolic receptor.

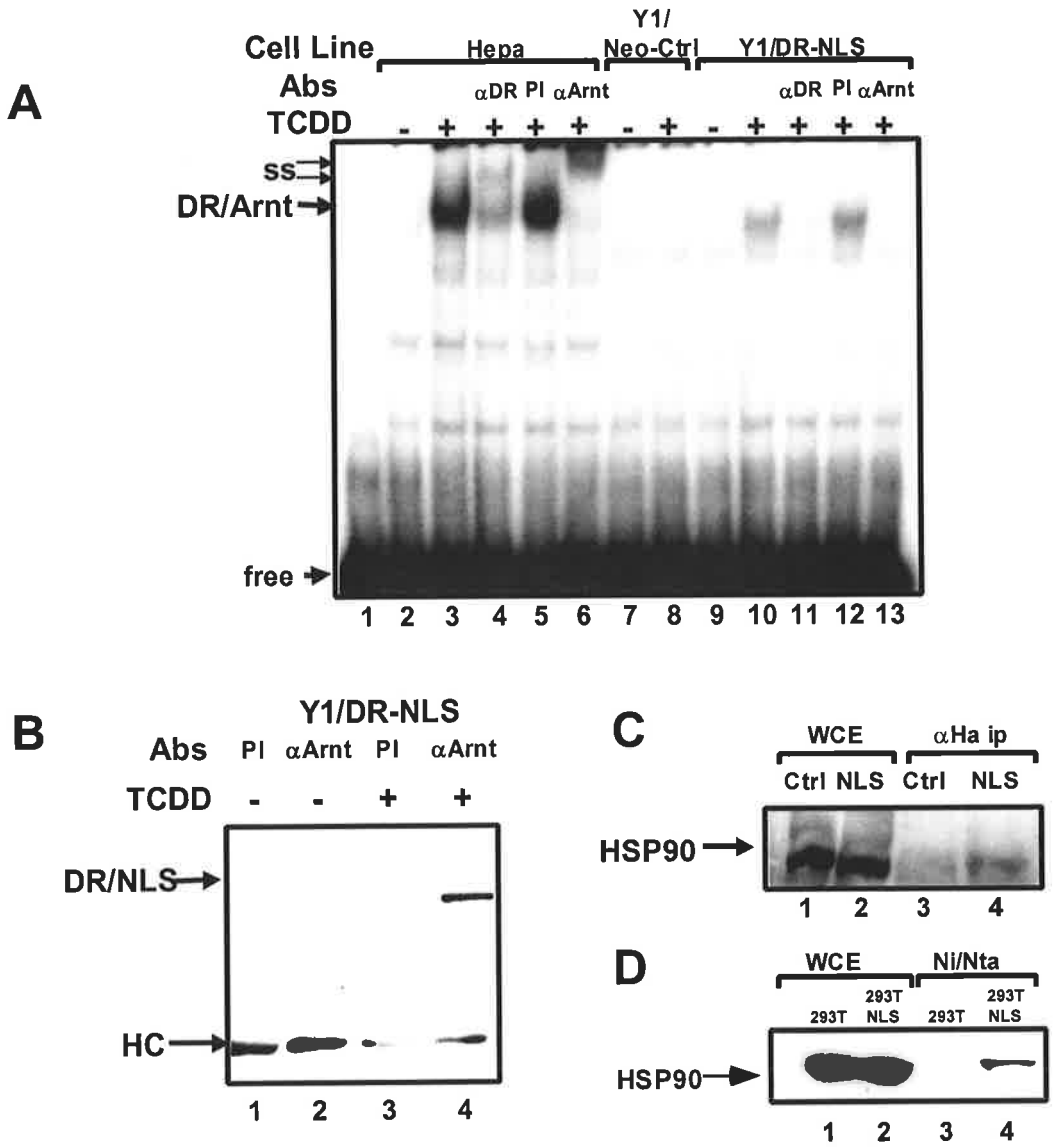


Figure 3.3. *The nuclear localised dioxin receptor requires ligand to heterodimerise with Arnt and bind DNA.* (**A**) The DR-NLS protein from Y1 cells is in a non-DNA binding form in the absence of ligand. Nuclear extracts (15 mg) from Hepa 1c1c7 cells (lanes 2 to 6), Y1/Neo-Ctrl cells (lanes 7 and 8) or Y1/DR-NLS cells (lanes 9 to 13) treated with 1 nM TCDD or vehicle alone (0.1% DMSO) for 4 hours were incubated with a 32P-labelled XRE probe and separated by non-denaturing 5.5% PAGE. The position of the DR/Arnt band and free XRE probe are indicated. ss refers to the supershifted bands generated by incubation with antibodies directed against either the DR (αDR), Arnt (αArnt) or pre-immune sera (PI). (**B**) The nuclear localised DR requires ligand to heterodimerise with Arnt. Y1/DR-NLS cells were treated with 1 nM TCDD or vehicle alone (0.1% DMSO) for 2 hours. Nuclear extracts (100 μg) were immunoprecipitated with antiserum raised against Arnt (αArnt) or pre-immune serum (PI), separated by 7.5% SDS-PAGE and immunoblotted with the 12CA5 αHa mAb. Positions of the DR-NLS protein and Ig heavy chain (HC) are indicated. (**C**) and (**D**), The nuclear localised DR remains bound to hsp90. (**C**) Whole cell extracts from Y1/Neo-Ctrl or Y1/DR-NLS cells were immunoprecipitated using the rat 3F10 anti-HA mAb, separated by SDS-PAGE and immunoblotted with an antibody specific for hsp90. (**D**) Whole cell extracts from 293T or 293T/DR-NLS cells were purified using nickel affinity chromatography and separated by SDS-PAGE before immunoblotting with an hsp90 specific mAb. The position of hsp90 is indicated with an arrow.

Ligand treatment is needed for the nuclear dioxin receptor to interact with Arnt.

While the native dioxin receptor is cytoplasmic in untreated cells, immunofluorescence has revealed Arnt to be an exclusively nuclear protein in cultured cell lines (Pollenz *et al* 1994; Hord and Perdew 1994). As the constitutively nuclear dioxin receptor was dependent upon ligand to become transcriptionally active, we wished to assess whether this nuclear dioxin receptor was in fact in a heterodimeric form with Arnt which lacked transcriptional activity due to the absence of ligand, or whether it remained in a latent complex typical of the cytosolic receptor. Electrophoretic mobility shift assays were used to determine if the untreated nuclear dioxin receptor was capable of binding the XRE target DNA sequence. As expected, nuclear extracts from untreated Hepa1c1c7 cells or control Y1 cells did not harbour DNA binding DR/Arnt complexes (Figure 3.3a, lanes 2 and 7). Nuclear extracts from ligand treated Hepa1c1c7 cells showed the established XRE mobility shift which is diagnostic for the DR/Arnt complex (Figure 3.3a, lane 3; Poellinger 1995). This XRE bound complex could be supershifted and partially depleted with antibodies specific for both the DR and Arnt, but was unaffected by preimmune sera (Figure 3.3a compare lane 3 with lanes 4-6). No TCDD inducible band was generated from nuclear extracts of ligand treated Y1/Neo-Ctrl cells, again demonstrating a lack of endogenous dioxin receptor in the parent cell line (Figure 3.3a, lane 8). Nuclear extracts from untreated Y1/DR-NLS cells lacked the characteristic DR/Arnt band in this assay, which appeared in extracts from ligand treated cells (Figure 3.3a, compare lanes 9 and 10). In a fashion similar to the wildtype DR, antibodies specific for both the DR and Arnt could supershift and partially deplete this TCDD inducible complex whilst preimmune sera had no effect (Figure 3.3a compare lane 10 with lanes 11-13). These experiments reveal that the DR-NLS protein is not present in a DNA binding form in untreated Y1/DR-NLS cells, indicating that it is unlikely to be in a constitutive heterodimeric complex with Arnt. Lower intensities of the XRE gel shift

bands from Y1/DR-NLS extracts reflect the relatively low expression of DR-NLS as compared to the wild type receptor in Hepa 1c1c7 cells.

To assess directly whether or not the nuclear localised dioxin receptor interacts with Arnt independently of ligand, co-immunoprecipitation experiments were performed with antibodies raised against the C-terminus of Arnt. Proteins immunoprecipitated from nuclear extracts of Y1/DR-NLS cells were subjected to Western blot analysis by probing with the 12CA5 monoclonal antibody directed against the Ha epitope. Anti-Arnt immune sera failed to co-precipitate the DR-NLS from untreated Y1/DR-NLS cells, (Figure 3.3b, lane 2). However, the immunoprecipitation protocol showed a clear interaction between Arnt and DR-NLS in nuclear extracts from ligand treated cells (Figure 3.3b, lane 4), establishing that generation of the heterodimeric complex between the nuclear dioxin receptor and Arnt is ligand dependent. Control precipitations with pre-immune sera failed to show any background in this assay (Figure 3.3b, lanes 1 and 3), confirming the specificity of the anti-Arnt immune sera. To determine if hsp90 was bound to the latent nuclear dioxin receptor, immunoprecipitation assays using an anti-Ha monoclonal antibody were performed with cell extracts from untreated Y1/Neo-Ctrl and Y1/DR-NLS cells. Western blot analysis using an antibody directed against hsp90 revealed no hsp90 in immunoprecipitates from the Y1/Neo-Ctrl cell line, whereas hsp90 was coimmunoprecipitated with the Ha tagged dioxin receptor from extracts of Y1/DR-NLS cells (Figure 3.3c, compare lanes 3 and 4). As expected, hsp90 levels were identical in the two cell lines (Figure 3.3c, compare lanes 1 and 2). In a second analysis, purification of the hexahistidine tagged DR-NLS from 293T/DR-NLS cells by nickel affinity chromatography showed that hsp90 co-purified with the DR-NLS (Figure 3.3d, lane 4). An identical purification procedure using protein from control 293T cells established there was no background hsp90 adsorbed to the nickel affinity resin during this assay (Figure 3.3d, lane3). Data from these two co-precipitation methods using

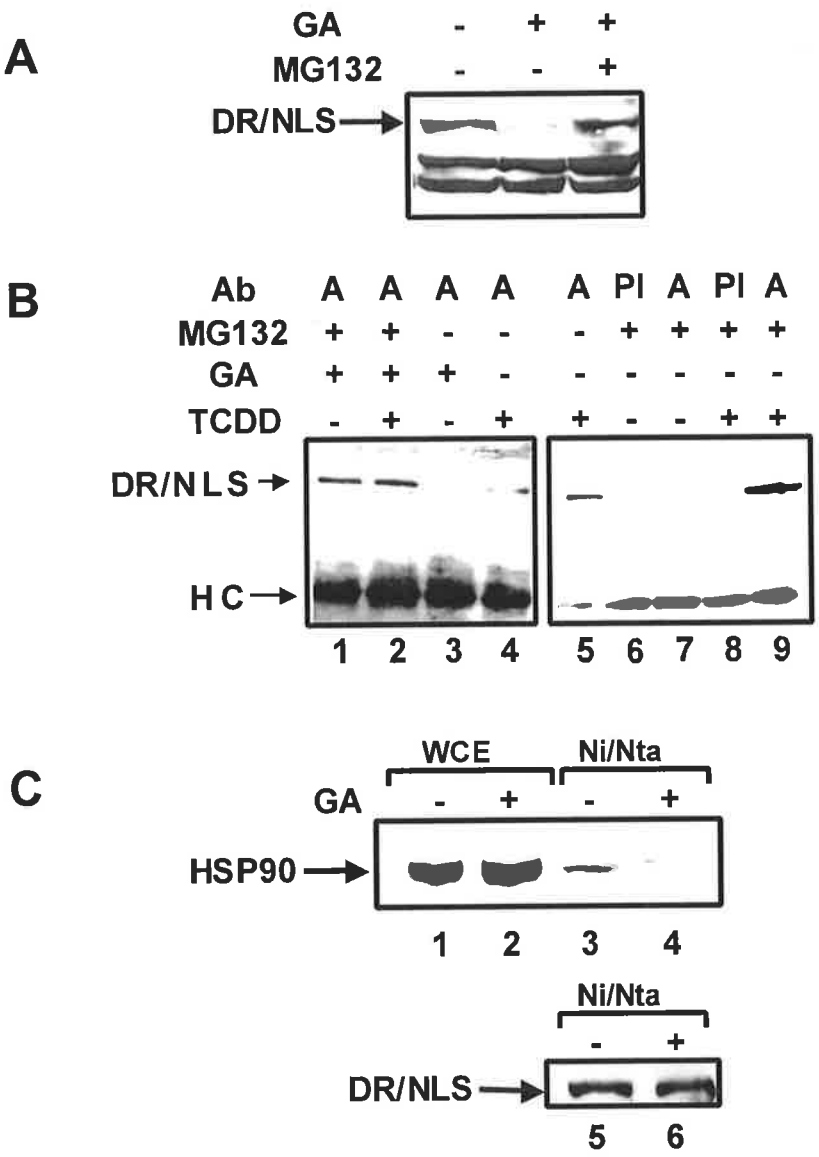


Figure 3.4. *The hsp90 binding agent geldanamycin can induce formation of DR/Arnt heterodimers.* (**A**) Geldanamycin (GA) treatment of Y1/DR-NLS cells stimulates dioxin receptor degradation which can be inhibited by the proteasome inhibitor MG132. Whole cell extracts from Y1/DR-NLS cells treated with vehicle alone (0.1% DMSO, lane 1), GA (1 μg/ml, lane 2), or GA (1 μg/ml)+ MG132 (7.5 mM) (lane 3) for 2 hours were analysed for the presence of the DR-NLS protein by immunoblotting with the 12CA5 anti-Ha mAb. The position of the DR-NLS protein is indicated, the two lower bands representing background proteins detected by 12CA5. (**B**) Geldanamycin can induce a DR/Arnt heterodimer in the Y1/DR-NLS cell line. Y1/DR-NLS cells were treated with the indicated combinations of GA (1 μg/ml), TCDD (1nM) and MG132 (7.5 μM) for 2 hours. Nuclear extracts of treated cells were immunoprecipitated with polyclonal antisera raised against Arnt (A) or pre-immune serum (PI), separated by 7.5% SDS-PAGE and immunoblotted with the 12CA5 anti-Ha mAb. Location of the DR-NLS protein and Ig heavy chain (HC) are indicated. (**C**) Geldanamycin destabilises DR-NLS/hsp90 complexes. Whole cell extracts from 293T/DR-NLS cells treated for 30 minutes with DMSO (0.1%) or GA (1 μg/ml) in the presence of MG132 (7.5μM), were purified using Ni-NTA resin prior to immunoblotting with an hsp90 mAb. 10% of the eluted protein was run on a separate gel and immunoblotted with the RPT1 aDR mAb (lanes 5 and 6). Lanes 1 and 2 contain aliquots of the extracts prior to purification. The positions of hsp90 and DR/NLS are indicated.

extracts from two independent cell lines firmly establish that the latent DR-NLS protein is bound to hsp90. These results show that the constitutively nuclear dioxin receptor is bound to hsp90 and undergoes a ligand induced transformation process which is indistinguishable from that which occurs for the latent cytosolic receptor (Poellinger 1995), indicating that nuclear compartmentalisation per se does not invoke any part of this transformation.

A ligand for hsp90 can stimulate the nuclear dioxin receptor to form a heterodimer with Arnt

The antitumour agent geldanamycin has recently been shown to act as a ligand for hsp90 (Stebbins *et al* 1997), complexing at the ATP binding site within the N-terminus (Prodromou *et al* 1997). Binding of geldanamycin has been shown to prevent or disrupt interaction of hsp90 with a number of its substrates, including steroid hormone receptors (Segnitz and Gehring 1997), the tyrosine kinase v-Src (Whitesell *et al* 1994), and c-Raf-1 (Schulte *et al* 1995). As a means to further explore the role of ligand in dioxin receptor activation, we were interested to see whether geldanamycin treatment of Y1/DR-NLS cells could affect transformation of the nuclear dioxin receptor to the heterodimeric complex with Arnt. Previously it has been reported that geldanamycin treatment of Hepa 1c1c7 cells produced a loss of the DR, supposedly because a conformational change within the DR/hsp90 complex increased susceptibility of the receptor to degradation (Chen *et al* 1997). We investigated the stability of the nuclear dioxin receptor upon exposure to geldanamycin by performing Western blot analyses. Following a 2 hour geldanamycin treatment of Y1/DR-NLS cells, Western blots of whole cell extracts revealed that the receptor had almost completely disappeared (Figure 3.4a, compare lanes 1 and 2). As our laboratory has previously observed that turnover of the native dioxin receptor can be inhibited by the proteasome inhibitor MG132 (Roberts

and Whitelaw 1999), Y1/DR-NLS cells were cotreated with geldanamycin and MG132 for 2 hours before subjecting whole cell extracts to Western analysis. The proteasome inhibitor completely inhibited the loss of the DR-NLS protein during geldanamycin treatment (Figure 3.4a, lane 3). Intriguingly, when the Y1/DR-NLS cells were cotreated with MG132 and geldanamycin, immunoprecipitation assays with αArnt antibodies demonstrated a clear heterodimerisation of the DR-NLS protein with Arnt (Figure 3.4b, lane 1). Cotreatment of Y1/DR-NLS cells with dioxin, geldanamycin and MG132 provided levels of DR-NLS/Arnt heterodimer that were similar to levels seen from cells treated with geldanamycin and MG132, or from cells treated with dioxin and MG132, indicating that geldanamycin induced transformation of the dioxin receptor was of similar efficiency to that of dioxin induced transformation (Figure 3.4b, compare lanes 1,2 and 9). Treatment of cells with MG132 alone produced negligible amounts of co-precipitated DR-NLS, establishing that MG132 does not in itself stimulate receptor transformation (Figure 3.4b, lane 7). The ability to generate a heterodimer was dependent upon MG132 treatment, as geldanamycin treatment alone produced a minimal interaction between DR-NLS and Arnt (Figure 3.4b, lane 3), which is consistent with the DR-NLS protein being degraded upon exposure to geldanamycin. These results imply that geldanamycin has the ability to disrupt DR/hsp90 complexes, resulting in dramatically increased lability of the DR. To investigate if hsp90 is released from the DR upon geldanamycin exposure, the successful nickel affinity purification of the DR-NLS/hsp90 complex from 293T/DR-NLS cells was repeated as described in Figure 3.3d. Following cotreatment of 293T/DR-NLS cells with MG132 and geldanamycin, or DMSO vehicle alone for 30 minutes, whole cell extracts were Ni/Nta purified and separated by SDS-PAGE. Geldanamycin + MG132 treatment led to a decrease in the level of hsp90 which copurified with the DR-NLS protein (Figure 3.4c, compare lanes 3 and 4). Importantly, this treatment had no effect on the amount of DR-NLS protein purified (Figure 3.4c, compare lanes 5 and 6), ruling out that lower hsp90

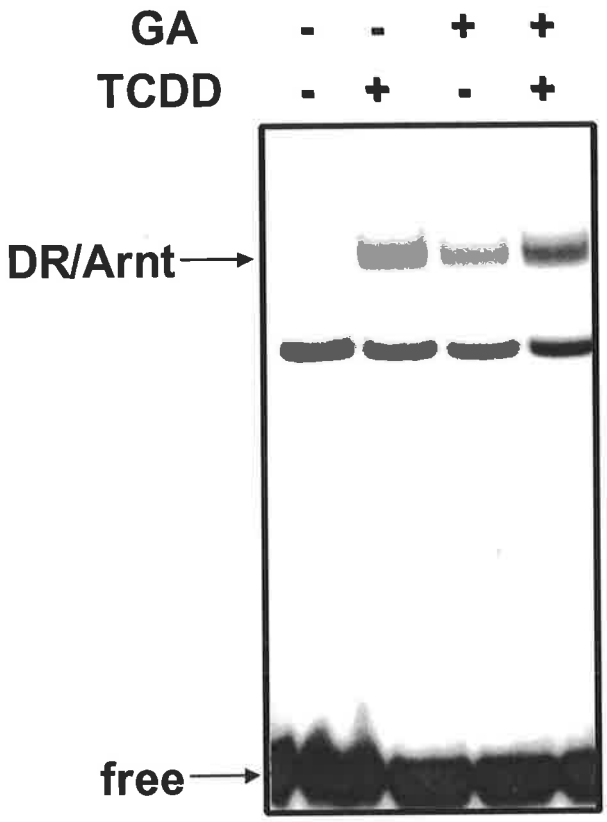


Figure 3.5. *Geldanamycin induced DR/Arnt heterodimers are capable of binding DNA.* Cytosolic extracts (15 μg) from Hepa1c1c7 cells were treated with DMSO vehicle (lane 1), TCDD (10 nM, lane 2), GA (10 μg/ml, lane 3) or a combination of TCDD (10 nM) and GA (10 μg/ml) (lane 4) for 2 hours at room temperature, followed by incubation with a 32P-labelled XRE probe prior to separation by 5.5% non-denaturing PAGE. Positions of the DR/Arnt band and free probe are indicated.

copurification was a result of lower receptor levels. Furthermore, geldanamycin had no detrimental effect on hsp90 levels present in the whole cell extracts (Figure 3.4c, compare lanes 1 and 2).

The geldanamycin induced dioxin receptor/Arnt heterodimer is not transcriptionally active.

To ascertain that the geldanamycin induced dioxin receptor/Arnt heterodimer seen in MG132 treated Y1/DR-NLS cells is not an isolated phenomenon, we investigated the ability of geldanamycin to transform the native dioxin receptor *in vitro*. Hypotonic cytosolic extracts of Hepa1c1c7 cells typically contain both the latent dioxin receptor and Arnt, the presence of the latter due to leakage from the nucleus during the hypotonic fractionation procedure. Dioxin treatment of Hepa1c1c7 cytosol is a well established method to transform the receptor into a heterodimeric complex with Arnt (Poellinger 1995), as detected by electrophoretic mobility shift assay with an XRE probe (Figure 3.5, compare lanes 1 and 2). Treatment of Hepa1c1c7 cytosolic extracts with geldanamycin resulted in transformation of the dioxin receptor with only slightly lower effectiveness to that seen with dioxin (Figure 3.5, compare lanes 2 and 3). Cotreatment with dioxin and geldanamycin gave a similar level of transformation to that of dioxin alone (Figure 3.5, lane4). Importantly, the experiments of Figure 3.5 show that an artificial transformation of the dioxin receptor, using a ligand which binds hsp90 rather than the receptor, can produce a heterodimer with Arnt which maintains its ability to bind the XRE cognate DNA sequence.

As the geldanamycin induced dioxin receptor/Arnt heterodimer maintains its DNA binding ability, it was investigated whether this complex was functional as a transcription activator. Y1/DR-NLS cells were transfected with the XRE-luciferase

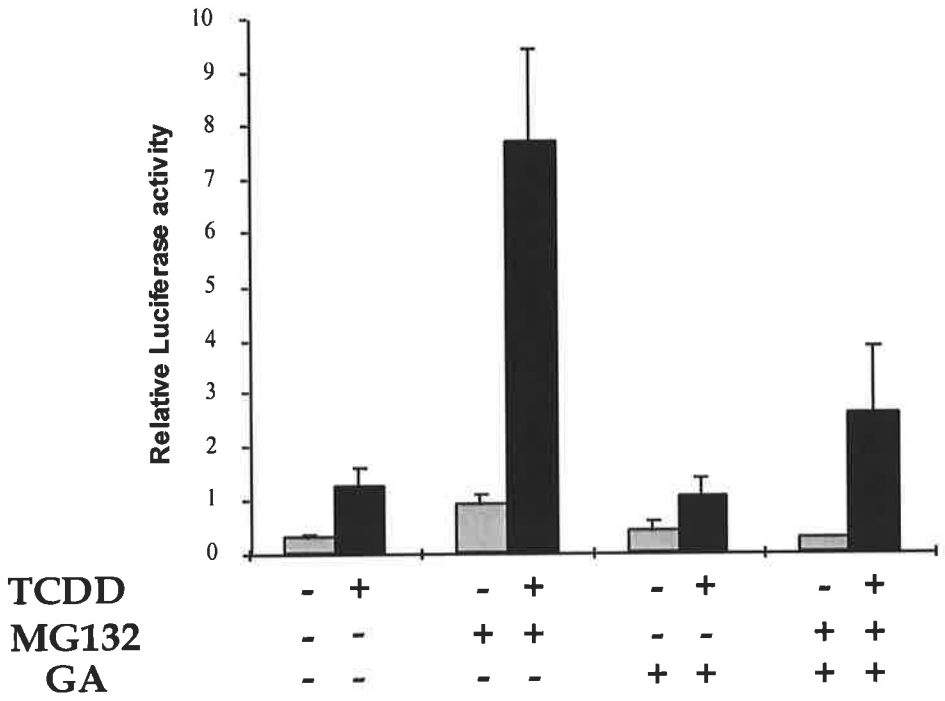


Figure 3.6. *The DR/Arnt complex induced by geldanamycin does not activate transcription.* Y1/DR-NLS cells were cotransfected with the XRE-luciferase reporter gene and the renilla luciferase internal control vector pRL-TK. Cells were then treated with the indicated combinations of DMSO vehicle alone, TCDD (1 nM, dark bars), Geldanamycin (1 μg/ml, light bars) and MG132 (7.5 μM) for 16 hours. Luciferase activity was normalised against the internal control and is an average -/+ SE of 4 transfection experiments.

reporter gene and treated with geldanamycin in the presence or absence of MG132. Geldanamycin alone failed to activate the reporter gene (Figure 3.6), as was expected considering the rapid proteolysis of the receptor observed upon geldanamycin treatment of Y1/DR-NLS cells. Upon cotreatment of geldanamycin and MG132, conditions which result in DR-NLS stabilisation and DR-NLS/Arnt heterodimer formation, the reporter gene remains totally inactive. Surprisingly, the artificially induced heterodimer does not function as a transcription factor. In total contrast, cotreatment of Y1/DR-NLS cells with dioxin and MG132 provided a 5 to 6 fold increase in reporter gene activity over that seen with dioxin treatment alone, and a 20 to 30 fold increase over activity in nontreated cells (Figure 3.6). These experiments establish that MG132 treatment does not interfere with the transcription activating potency of the DR-NLS/Arnt heterodimer, but in fact enhances it, presumably due to increased receptor stability. The inability of the geldanamycin and MG132 cotreated Y1/DR-NLS cells to show reporter gene activity cannot therefore be due to a non-specific detrimental effect of MG132. Cotreatment of Y1/DR-NLS cells with TCDD and geldanamycin gave a marginally lower response than treatment with TCDD alone, while cotreatment with TCDD, geldanamycin and MG132 provided reporter gene activity midway between TCDD treated and TCDD + MG132 cotreated cells (Figure 3.6). These last results are consistent with geldanamycin competing with TCDD for transformation of the nuclear dioxin receptor, in which case a mixture of active and inactive DR-NLS/Arnt heterodimers would be formed.

Intriguingly, an artificial activation of the latent NLS-dioxin receptor to the DR-NLS/Arnt heterodimeric form results in a non-functional transcription factor complex. These results imply that for the ligand bound dioxin receptor, the ligand may play a structural role in creating a receptor competent for communication with the basal transcription machinery or transcription mediating cofactors. Treatment of

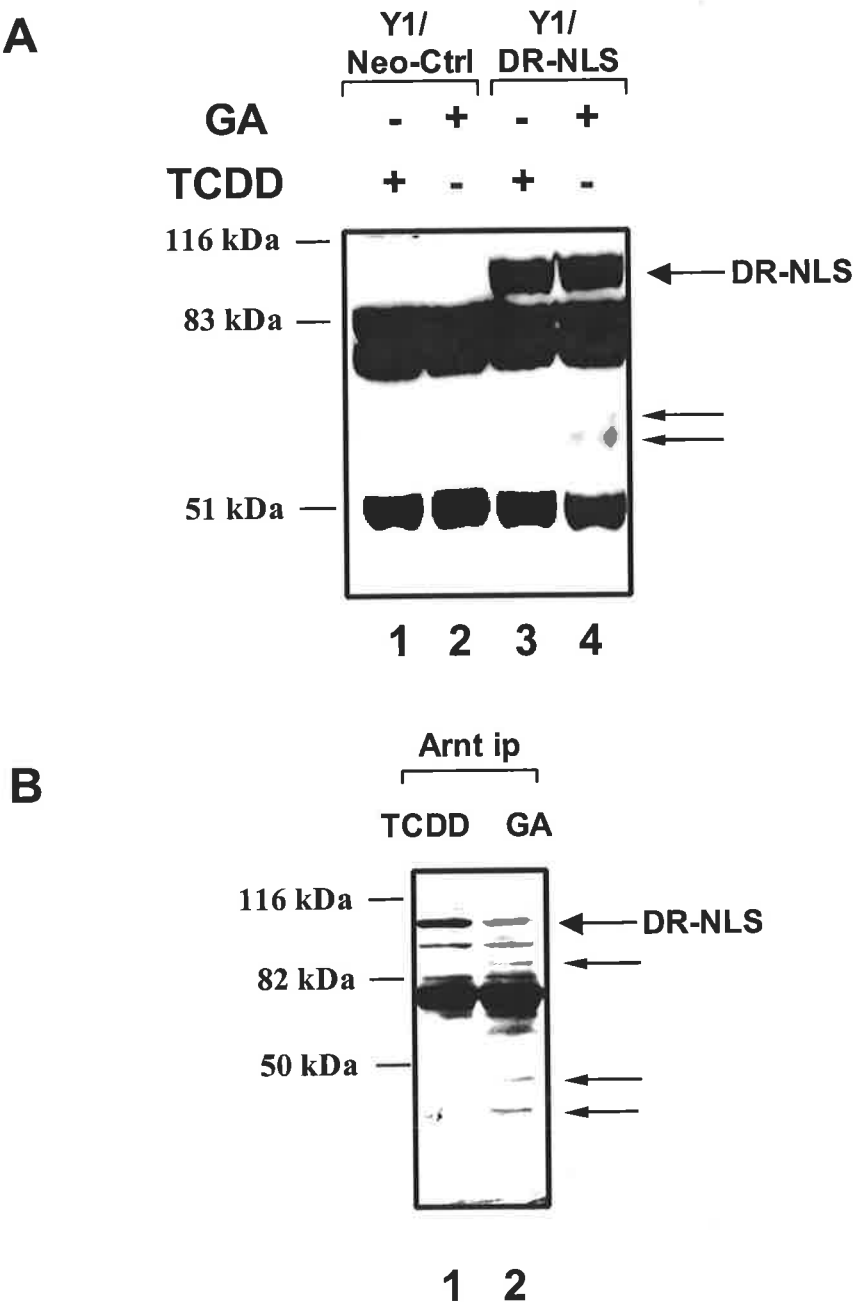


Figure 3.7. *The DR/Arnt complex induced by geldanamycin differs in conformation from heterodimers induced by dioxin.* (**A**) Whole cell extracts (100μg) from Y1/Neo-Ctrl or Y1/DR-NLS cells treated for 2 hours with TCDD (1nM) or GA (1μg/ml) in the presence of 7.5μM MG132 were incubated with 150ng of trypsin (20 min, 37° C), separated by 10% SDS-PAGE and immunoblotted with the 12CA5 anti-Ha mAb. (**B**) Whole cell extracts from Y1/DR-NLS cells treated as in (A) were immunoprecipitated with αArnt antibodies and digested with 25ng of trypsin (15min, 25° C) whilst bound to protein A sepharose. Proteolytic fragments were separated by 12.5% SDS PAGE and detected by immunbloting with the 12CA5 αHa mAb. GA specific bands are indicated with small arrows.

geldanamycin allows an unnatural release of the dioxin receptor from the molecular chaperone hsp90 (Figure 3.4c), which presumably results in an aberration of receptor structure and renders it unable to activate transcription. Consistent with this model, studies of dioxin receptor activity in yeast have shown that in strains where hsp90 levels can be reduced to approximately 5% of endogenous levels, the receptor signalling pathway ceases to function (Carver *et al* 1994, Whitelaw *et al* 1995).

DR/Arnt heterodimers induced by dioxin differ structurally from heterodimers induced by geldanamycin

Following the observation that DR/Arnt heterodimers induced by geldanamycin are transcriptionally inactive it was next investigated whether this might be due to conformational differences between the two heterodimeric forms. To gain evidence for differences in conformation between dioxin and geldanamycin transformed receptors limited proteolytic digestion assays were performed. Following treatment of Y1/DR-NLS cells with either TCDD or geldanamycin for a two hour period in the presence of MG132, whole cell extracts were obtained and subjected to short incubations with trypsin. Western blotting with the 12CA5 anti-Ha mAb revealed proteolytic fragments in the digests from geldanamycin treated cells which were not observed in digested extracts from TCDD treated cells (Figure 3.7a, compare lanes 3 and 4). No corresponding fragments could be detected in digested extracts from the Y1/Neo-Ctrl cell line treated with either TCDD or geldanamycin (Figure 3.7a lanes 1 and 2), indicating that these fragments are derived from the DR-NLS protein. To confirm that unique bands could be produced by proteolysis when the DR was complexed with Arnt, Y1/DR-NLS cells were treated with either geldanamycin or TCDD in the presence of MG132 and protein extracts immunoprecipitated with anti-Arnt antibodies before being subjected to trypsin digestion and Western analysis. As observed for partially digested

whole cell extracts, geldanamycin specific DR-NLS proteolytic fragments were observed upon digestion of the Arnt coimmunoprecipitates (Figure 3.7b). An estimation of the fragment sizes generated from whole cell extracts and immunoprecipitated complexes suggests that the geldanamycin transformed dioxin receptor is being primarily cleaved in a region approximately 40-60 kDa from the carboxy terminus, which would locate the cleavage region within the ligand binding domain. This observation is consistent with the notion that a destructuring of the ligand binding domain occurs upon geldanamycin induced release of hsp90.

DISCUSSION

Signal Regulated bHLH/PAS Proteins

It is evident that some of the bHLH/PAS proteins respond to specific signalling pathways, and it has been postulated that the PAS domain is a general sensing domain for oxygen, redox, or light reception in a diverse array of organisms including mammals, insects, plants, fungi and bacteria (Zhulin *et al* 1997). Within the mammalian bHLH/PAS factors, the dioxin receptor and HIF-1α have been demonstrated to respond to specific but quite distinct intracellular stress signals. For the dioxin receptor, the well established binding of xenobiotics functions to initiate a multistep activation pathway, while low oxygen tension induces a rapid increase in protein levels of HIF-1α.

Activation and nuclear translocation of the dioxin receptor

The PAS domain is inhibitory for nuclear translocation of the unliganded dioxin receptor (Ikuta *et al* 1998), consistent with hsp90 being an agent of cytoplasmic retention. Upon ligand binding, it has been proposed that hsp90 is released from the dioxin receptor to stimulate activity of a bipartite nuclear localisation signal in the N-terminus of the receptor (Ikuta *et al* 1998). In this manner, the nuclear translocation mechanism of the dioxin receptor is strongly reminiscent of models proposed for nuclear translocation of the glucocorticoid receptor. In the case of the glucocorticoid receptor, hsp90 also interacts with the ligand binding domain, and upon ligand binding the receptor translocates to the nucleus and forms a homodimer with DNA binding activity (Picard and Yamamoto 1987). While hsp90 is critical for keeping both the GR and DR in their latent forms, it has not previously been determined whether these receptors shed hsp90 before or after entering the nucleus. As with the dioxin receptor,

small immunophilin molecules are found associated with the GR/hsp90 complex and are postulated to have a role in nuclear targeting of the GR (Pratt and Toft 1997). One scenario suggests that the complete GR/hsp90 complex may pass through to the nucleus. It has been shown that such a complex is not restricted from passing through the nuclear pore in a study where the nucleoplasmin NLS was attached to hsp90 to allow co-translocation of NLS mutant glucocorticoid and progesterone receptors (Kang *et al* 1994).

The results in this chapter show that constitutive nuclear localisation of the DR can be achieved by placing exogenous NLS signals at the extreme C-terminus. The natural bipartite NLS of the DR is in the N-terminus, incorporating basic residues within the bHLH region (Ikuta *et al* 1998). Interestingly, the N-terminal bHLH region is a secondary, weaker site for hsp90 interaction (Antonsson *et al* 1995a), suggesting that the NLS may be sterically masked in the unliganded state. It has been found here that in untreated cells the DR-NLS protein translocates to the nucleus with hsp90 attached (Figures 3c and 3d), avoiding cytoplasmic retention due to the freely available NLS at the C-terminus. Once in the nucleus, the DR-NLS protein remains in a latent state, needing the presence of ligand to initiate heterodimerisation with Arnt (Figure 3.3b).

Treatment with geldanamycin, a ligand for hsp90 that is known to disrupt hsp90 interaction with a number of substrates, renders the constitutively nuclear receptor extremely susceptible to degradation. This phenomenon has also been seen during studies of other hsp90 binding factors such as steroid hormone receptors, Src and Raf (Chen and Perdew 1997). In the case of steroid hormone receptors, geldanamycin has been proposed to prevent association of hsp90 with the receptors rather than dissociate existing receptor/hsp90 complexes (Segnitz *et al* 1997). Contrary to this we present evidence that geldanamycin has the ability to influence pre-existing DR/hsp90

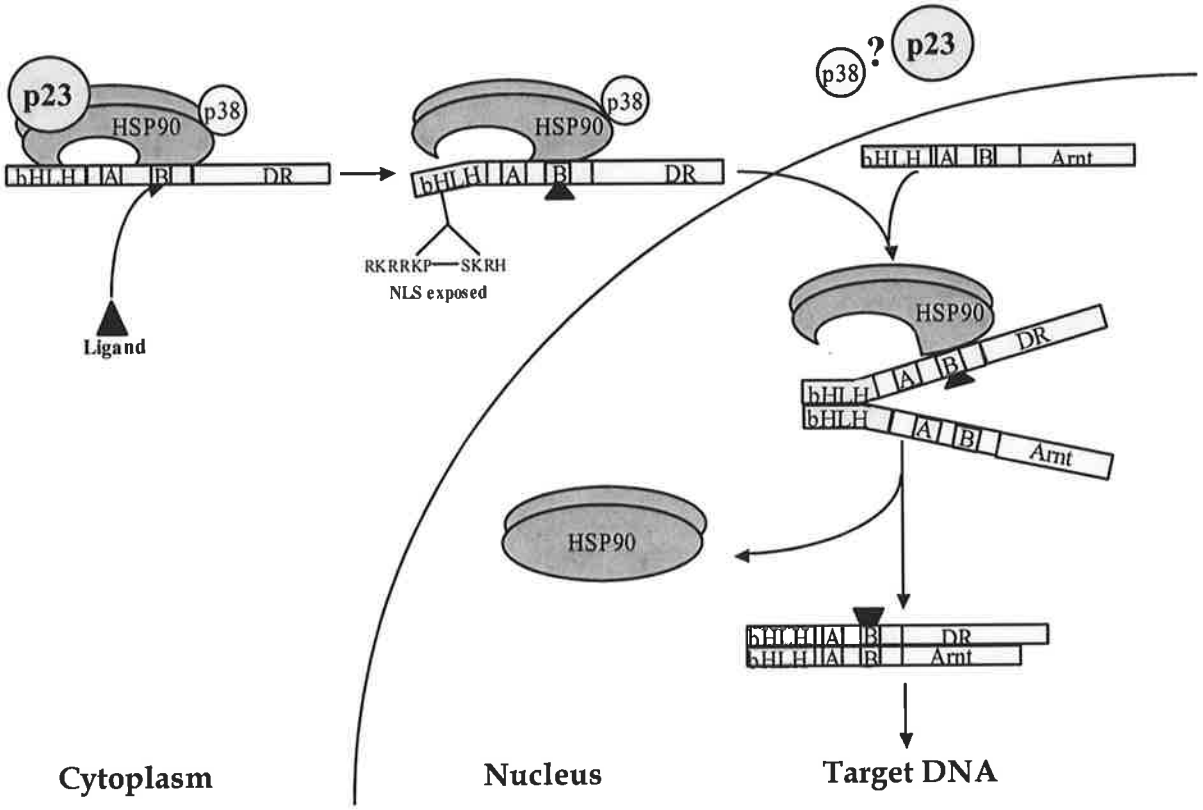


Figure 3.8. *Model for ligand induced transformation of the cytosolic dioxin receptor to the nuclear dioxin receptor/Arnt heterodimer.* Ligand binding stimulates release of hsp90 from the N-terminus, exposing the nuclear translocation signal to promote nuclear import of the PAS B bound hsp90/DR complex. Once in the nucleus, interaction of the free bHLH domain with Arnt initiates concomitant release of hsp90 and formation of the mature DR/Arnt heterodimer. See text for details.

complexes, as geldanamycin was able to both disrupt the ability of hsp90 to copurify with the DR-NLS protein (Figure 3.4c) as well as generate an in vitro DNA binding DR/Arnt complex from Hepa cytosol (Figure 3.5). Whether Geldanamycin acts by completely disrupting pre-existing DR/hsp90 complexes or instead by inducing conformational changes in the DR/hsp90 complex and thus rendering the DR competent for heterodimerisation with Arnt, we have yet to conclusively determine.

The extreme lability of the dioxin receptor upon geldanamycin treatment of Y1/DR-NLS cells indicates that it is highly improbable that an inadequately chaperoned form of receptor can exist within the cell. Therefore, how does ligand treatment process the native dioxin receptor, which is cytoplasmic and hsp90 bound, into the nuclear heterodimeric complex with Arnt? Upon ligand binding of the native cytosolic receptor, it is envisaged that a conformational change taking place within the DR to release hsp90 from its weak interaction with the bHLH region, exposing the NLS. The results here show that it is extremely unlikely that hsp90 is totally released from the dioxin receptor when bound by ligand in the cytosol. In this scenario, the native dioxin receptor would translocate to the nucleus bound with both ligand and hsp90, and upon entry into the nucleus interact with Arnt via the exposed N-terminal bHLH region. Following this initial interaction, it is envisaged that a concerted mechanism whereby the dioxin receptor and Arnt PAS domains form a strong association concomitant with full hsp90 release from the dioxin receptor PAS B region (Figure 3.8), thus avoiding any presence of non partnered, labile dioxin receptor. Importantly, this mechanism is consistent with recent in vitro studies of dioxin receptor activation. Ligand treatment of Arnt free cytosolic extracts, or in vitro translation mixtures containing the hsp90/DR complex, was shown to be highly inefficient at disrupting hsp90/DR complexes. Addition of Arnt in conjunction with ligand resulted in release of hsp90 as shown by the loss of co-precipitated DR with anti-hsp90 antibodies (McGuire *et al* 1995). Taken together, these

data indicate that within cells a nuclear mechanism involving concerted exchange of hsp90 for Arnt is a key step in the dioxin receptor activation process.

The role of ligand in dioxin receptor activation

Results here demonstrate that a constitutively nuclear dioxin receptor remains in its latent state, needing stimulation with ligand to form an active heterodimer with Arnt. Importantly, this observation reveals that interaction with ligand has functions beyond merely initiating nuclear translocation of the dioxin receptor. This is emphasised by the fact that ligands for either hsp90 or the dioxin receptor can induce DR/Arnt heterodimers capable of recognising the cognate XRE sequence, although only the dioxin stimulated heterodimer can activate transcription. As cotreatment with the proteasome inhibitor MG132 is necessary to obtain a stable receptor from geldanamycin treated Y1/DR-NLS cells, it is possible that the receptor is modified by ubiquitination, resulting in its lack of activity. However, MG132 cotreatment with dioxin results in an increase of reporter gene activity as compared to treatment of dioxin alone in the Y1/DR-NLS cells, arguing against this mechanism. Another possibility for the lack of transcriptional activity of the geldanamycin transformed receptor is that the receptor needs a defined structure of the ligand binding domain, normally produced by interaction with ligand, to be transcriptionally active. In the case of steroid hormone receptors, ligand plays a critical role in defining the structure of transcriptionally active receptors. For example, crystal structures show that binding of estradiol to the estrogen receptor positions the AF-2 transactivation domain correctly for induction of transcription, while antiestrogens produce a conformation which misplaces this domain. (Brzozowski *et al* 1997). Interestingly, both estrogens and antiestrogens can derepress the inhibitory function of the estrogen receptor ligand binding domain when it is attached to heterologous proteins such as the FLP recombinase (Nichols *et al* 1998),

indicating that derepression and activation can be distinct mechanistic processes, and illustrating that ligands can play multifunctional roles in transformation of nuclear receptors to active transcription factors.

We and others have previously shown that the C-terminal transactivation domain of the dioxin receptor functions autonomously when attached to a heterologous DNA binding domain such as that of GAL4 or the glucocorticoid receptor zinc finger (Jain *et al* 1994, Whitelaw *et al* 1994, Ma *et al* 1995). Therefore, the presence of ligand is not essential to the intrinsic activity of the dioxin receptor transactivation domain. However, when portions of the ligand binding domain were included in these chimeras they repressed activity of the transactivation domain. As the ligand binding domain coincides with the primary hsp90 binding region, hsp90 is a logical candidate for the agent of repression, and these chimeras were therefore analysed in a yeast system where hsp90 levels were dramatically lowered. The chimeras were also repressed in the low hsp90 environment, revealing that the unchaperoned ligand binding domain maintained its repressive activity (Whitelaw *et al* 1995). Moreover, it has recently been found that a deletion mutant of the dioxin receptor which lacks the ligand binding control region can activate transcription in the absence of any inducer (J.McGuire *et al* 2001 and Figure 4.3b). Thus, the ligand binding domain functions as a potent repression domain, which can be counteracted by interaction with ligand.

What is the function of ligand during conversion of the dioxin receptor to the Arnt heterodimeric complex? We favour a mechanism where ligand is important to maintain the structural integrity of the PAS B ligand binding/hsp90 binding region. The geldanamycin induced DR/Arnt heterodimer provides a non functional complex due to the ability of the unchaperoned ligand binding domain to disrupt transactivation function of the dioxin receptor/Arnt C-terminal complex, in a similar fashion to that

previously observed during our analysis of GR/DR chimeric proteins in low hsp90 yeast (Whitelaw *et al* 1995). It is notable that in the nuclear receptor superfamily, examples of RXR heterodimers exist where ligand binding to one subunit can influence the structure and function of a transactivation domain in the other subunit (Peet *et al* 1998). Data presented here are consistent with a role for dioxin receptor ligands in providing a derepressed structure to the PAS B region during transformation to the Arnt heterodimer. This hypothesis is also consistent with the PAS B region forming the core ligand and hsp90 binding domain of the dioxin receptor (Whitelaw *et al* 1993), as well as being a key region for interaction with Arnt (Whitelaw *et al* 1995). It has recently been proposed that intracellular dioxin receptor ligands also exist (Chang *et al* 1998), which may help structure this region during potential endogenous activation mechanisms. It will now be important to assess whether the PAS B domains of other bHLH/PAS proteins, such as HIF-1a, form key sites for hsp90 and Arnt interaction, and whether chaperoning of these regions is critical during formation of their respective transcription activating heterodimers.

Results

Regulation of DR activity by molecular chaperones

4

Chapter 4. Regulation of DR activity by molecular chaperones

Introduction

Regulation of Receptor activity by TPR proteins

Ligand induced activation of the DR involves processes reminiscent of those observed for members of the nuclear hormone receptor superfamily, specifically the Glucocorticoid receptor. For instance the GR has been isolated from cells in a complex with a dimer of hsp90 together with a TPR containing protein such as FKBP52, FKBP51, Cyp40 or protein phosphatase 5 (Silverstein *et al* 1997). These TPR proteins compete for interaction with the C-terminal 5 amino acids of hsp90, namely MEEVD (Silverstein *et al* 1999, Scheufler *et al* 2000, Brinker *et al* 2002) with the result being that within a given cell or tissue the GR can be isolated in complexes of varying composition (Silverstein *et al* 1997). TPR proteins also typically contain a peptidyl prolyl isomerase domain, which in terms of GR signalling has been poorly characterised. For FKBP52 this domain has been demonstrated to interact with the molecular motor protein dynein (Silverstein *et al* 1999, Gagligniana *et al* 2001) and overexpression of the PPIase domain of FKB52 inhibits microtubule directed movement of the GR into the nucleus (Gagligniana *et al* 2001). However this function is independent of the catalytic activity of the PPIase domain as treatment with FK506 or rapamycin (PPIase inhibitors) do not abrogate the rapid hormone induced movement along the microtubules (Gagligniana *et al* 2001). Furthermore, the latest model of the GR indicates that there is an initial hormone induced switching event whereby the FKBP51 immunophilin is replaced by FKBP52, which is required to mediate GR movement into the nucleus (Davies *et al* 2002). The GR also interacts with a small highly acidic molecule, p23, which is poorly characterised but has been suggested to

mediate maturation of the GR/hsp90 complex (Hutchison *et al* 1995). For the GR, it has been proposed that p23 facilitates substrate release from a chaperone complex (Young and Hartl 2000, Freeman *et al* 2000). Recently it has been proposed that p23 acts as a recycling agent for nuclear hormone receptors such as the thyroid hormone receptor, serving to disassociate "experienced" nuclear hormone receptors (ie receptors which have been activated by ligand and performed a role in transcriptional upregulation of a target gene) from DNA (Freeman and Yamamoto 2002). The hypothesis is such that this maintains the receptor in a constant state of cellular sampling and to ensure that transcription of target genes is switched off efficiently upon hormone withdrawal.

To date, the DR has been found associated with the TPR protein XAP2 (Carver and Bradfield 1997, Ma and Whitlock 1997, Meyer *et al* 1998) and hsp90 and appears to undergo chaperone mediated receptor maturation in a similar fashion to the GR which results in the association with p23 (Kazlauskas *et al* 2001). Reconstitution studies in yeast utilising a p23 deletion strain demonstrate that p23 augments the DR response but is not essential for DR signalling (Cox and Miller 2002). It is not yet clear what role p23 plays in DR signalling in mammalian cells. Studies focussing on the DR have shown that p23 is associated with the DR both *in vitro* and *in vivo* as the DR is immunoprecipitated with antibodies directed against p23 using either *in vitro* translated DR (Kazlauskas *et al* 1999) or using cell extracts (Kazlauskas *et al* 2001). The current model for the DR chaperone complex posits that p23 is initially recruited to the DR complex with early chaperones such as hsc/hsp70 and hop and remains stably associated with the mature hsp90/XAP2 complex. Potentially, this association acts by precluding the DR from interacting with nuclear import machinery as it has been demonstrated that removal of p23 coincides with the ability to heterodimerise with both the importins and Arnt (Kazlauskas *et al* 2001).

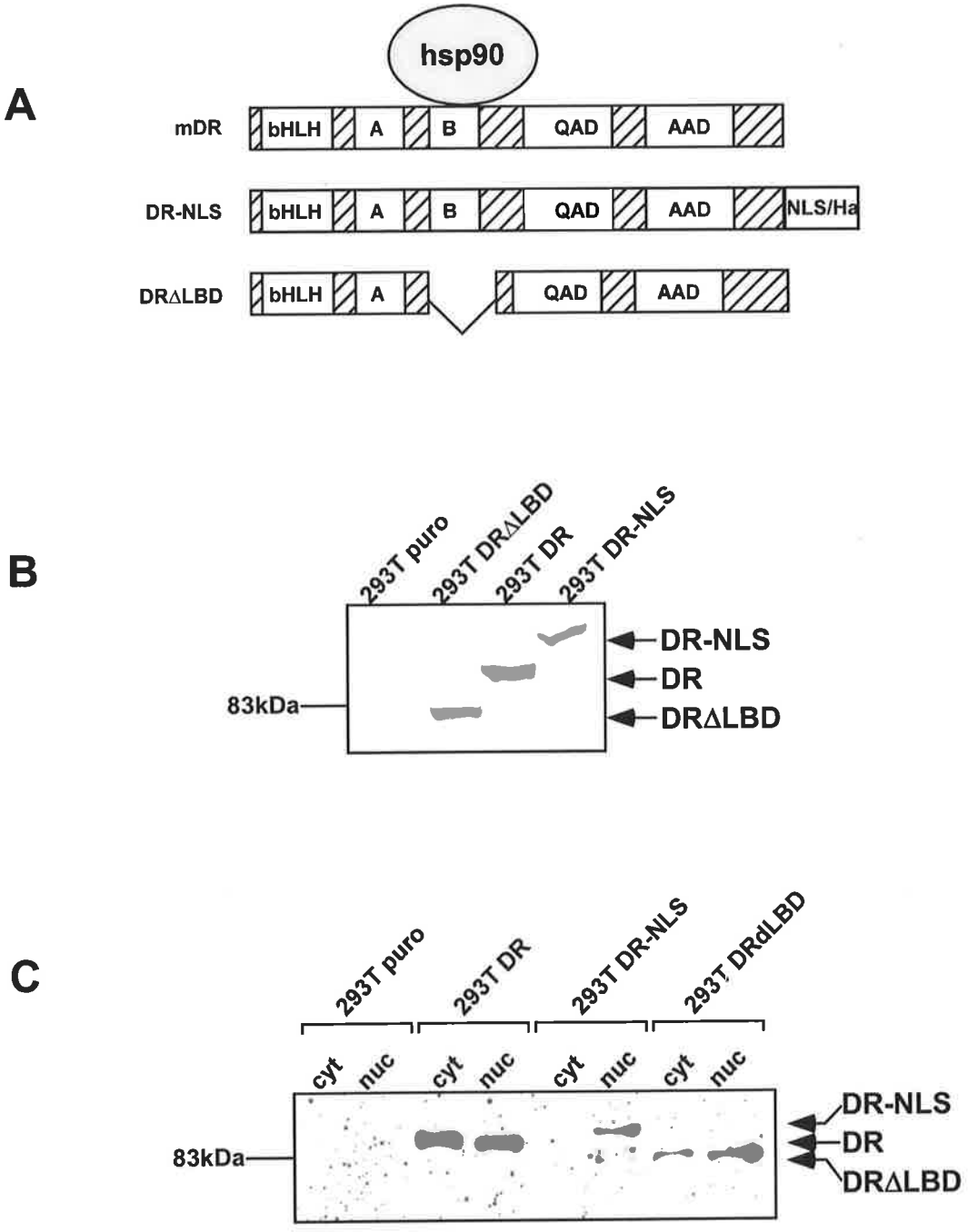


Figure 4.1. *Schematic representation, expression and cellular localisation of DR, DR-NLS and DRΔLBD constructs.* (**A**) Schematic representation of the DR, DR-NLS and DRΔLBD constructs. The DRΔLBD construct lacks the major hsp90 interaction region (amino acids 287-421). (**B** and **C**) Expression of DR constructs from stable cell lines. (**B**) Whole cell extracts (30μg) or (**C**) Cytosolic (cyt) and Nuclear (nuc) extracts (30μg of each) were separated by 10% SDS-PAGE prior to immunoblotting with an αDR mAb. The location of the relevant DR constructs is indicated.

Aims

As proper chaperoning of the DR ligand binding domain appears to be critical for its function (Chapter 3), this chapter aims to investigate the role of the XAP2 accessory protein in DR signalling. This was approached by examining the effects of XAP2 depletion on signalling by the nuclear localised DR construct, to ascertain whether XAP2 has roles beyond aiding cytosolic localisation of the DR. In addition, a potential role for the chaperone linked E3 ubiquitin ligase protein CHIP in DR degradation will be discussed.

Results

Generation and characterisation of modified DR stable cell lines

As a means of assessing the requirement of chaperone proteins in DR signalling, a series of stable cell lines were utilised which expressed modified forms of the DR that mimic progressive stages of the signalling pathway. These constructs include an unmodified mouse DR, a C-terminally modified DR which contains two copies of the nucleoplasmin nuclear localisation sequence and a haemagglutinin epitope in addition to a hexahistidine tag, and a deletion mutant (lacking amino acids 287-421) which lacks the ligand/hsp90 binding repressive PASB region (Figure 4.1a). The constructs were transcribed from a vector that links expression of a puromycin resistance cassette to the cDNA of interest by production of a bicistronic message. Puromycin resistant pools of transfected 293T cells were expanded and tested for expression and functionality of the DR proteins. The 293T human embryonic kidney cell line was chosen as it is amenable to transfection based experiments and has low levels of endogenous DR (Figure 4.1b), thus minimising complications contributed by signalling through the endogenous DR.

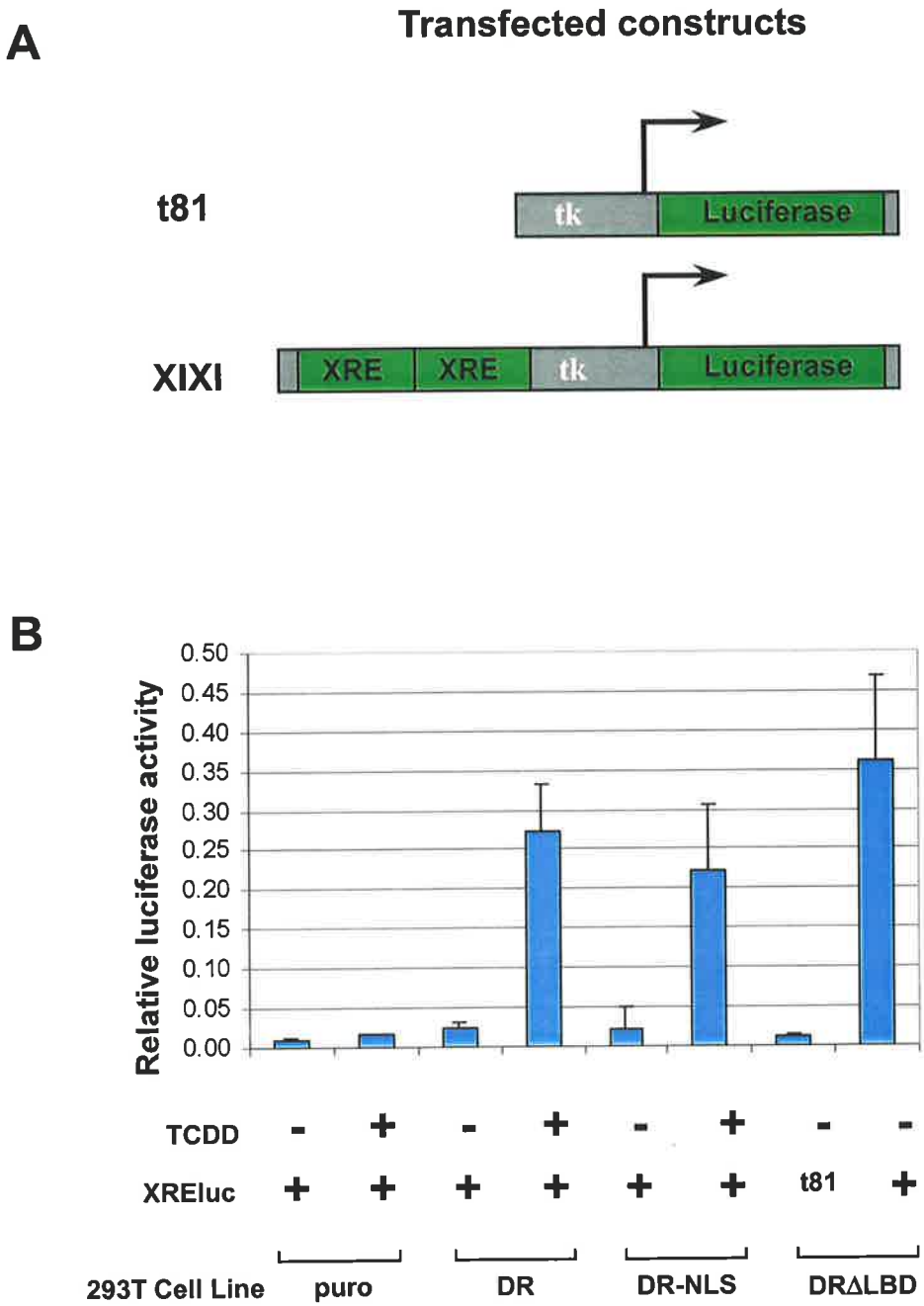


Figure 4.2. *Transcriptional activity of the DR, DR-NLS and DRΔLBD constructs.* (**A**) Schematic representation of XIXI and t81 reporter constructs. t81 is a minimal thymidine kinase promoter driving luciferase expression, XIXI contains two XRE sequences from the Cyp1A1 promoter. (**B**) 293T stable cell lines were transfected with the XRE luciferase reporter plasmid for 24 hours followed by treatment with TCDD (10nM) or DMSO control for 12 hours as indicated. To control for XRE-luciferase activity in the DRΔLBD line which displays ligand independent activity, cells were transfected with the control plasmid lacking XRE sequences. Shown is a representative experiment -/+ SE performed in triplicate a minimum of three times.

Western blots of whole cell extracts from the three DR containing cell pools and 1 control line, followed by probing with an antiDR antibody raised against the N-terminal region of the mouse receptor demonstrated the presence of a DR of the predicted size (approximately 89kDa, 95 kDa and 74 kDa for the wtDR, DR/NLS and DRΔLBD respectively) (Figure 4.1b). The heterologous NLS has previously been shown to be functional by localising the DR to the nucleus in the mouse adrenal cell line Y1 in the absence of ligand by both immunofluorescence and cell extraction techniques (Figure 3.1b-c). To confirm this in the 293T cells, cytosolic and nuclear extracts were generated from each of the stably transfected cell pools. Using this technique the wild type DR was isolated from both the cytosolic and nuclear fractions (Figure 4.1c). The presence of the wild type DR in the nuclear compartment could be a function of overexpression of the DR leading to an overriding of the cytosolic retention mechanism, or may simply be due to the recent observation that the DR shuttles between the cytosolic and nuclear compartments in the absence of exogenous ligand in a variety of cell types (Singh *et al* 1996). In contrast to the wild type DR, the DR-NLS construct was isolated consistently from the nuclear fraction (Figure 4.1c). The DRΔLBD construct could be isolated from both compartments of the cell (Figure 4.1c), consistent with recently reported localisation data using a GFP fusion with this protein (McGuire *et al* 2001).

Following confirmation of expression of each of the constructs in the stable lines the cells were characterised for their responsiveness to the prototypical DR ligand, TCDD. Cell lines were transfected with the XIXI luciferase reporter plasmid containing XRE sequences from the CYPIA1 promoter and treated with TCDD overnight. The stable cell lines containing either the wild type or nuclear localised DR gave a robust response to ligand on the XRE driven reporter (Figure 4.2). The activity of the stable cell line expressing the DRΔLBD construct showed ligand independent XRE driven reporter activity when compared to the parent reporter vector lacking XRE sequences, consistent

with the removal of a repressive region of the DR. In addition the basal activity of the luciferase reporter was not significantly different between the wt DR and the DR-NLS construct reaffirming the inability of the DR-NLS construct to act in a ligand independent manner (Figure 3.2). In addition, both the DR and DR-NLS constructs require ligand to generate a DNA binding complex, whilst the DRΔLBD construct clearly binds DNA in a ligand independent fashion (Figure 5.3).

Analysis of DRΔLBD activity in the context of an endogenous gene

Reporter gene analysis demonstrates the ability of the DRΔLBD to function in a ligand independent manner on an artificial promoter. From work in different laboratories, it appears using artificial reporters that the transactivation domain of the DR is dispensable and can be compensated for by the transactivation domain of Arnt (Whitelaw *et al* 1994, Ko *et al* 1996). However in the context of an endogenous gene it appears that this may not be true as the transactivation domain of the DR is necessary to upregulate expression of the CYP1A1 target gene (Ko *et al* 1996). To test the function of the DRΔLBD construct in the native chromatin setting it was necessary to stably transfect this construct into the model cell line, Hepa 1c1c7, which exhibits TCDD inducible CYP1A1 expression. This experiment was not able to be performed in the 293T background, like most cell lines they lack inducible expression of cytochrome CYPp450 genes. Hepa 1c1c7 cells were stably transfected with the puromycin resistant DRΔLBD expression construct, but in contrast to the 293T DRΔLBD cells, DRΔLBD expression could not be detected by either immunoblotting or upregulation of an XRE containing reporter gene. Expression of the DRΔLBD construct could only be detected by RT-PCR of cDNA generated from RNA extracted from the cell lines using primers spanning the ligand binding domain (17N and 443C), which showed the presence of a fragment of the expected size of 876 bp and matching the size of the plasmid control

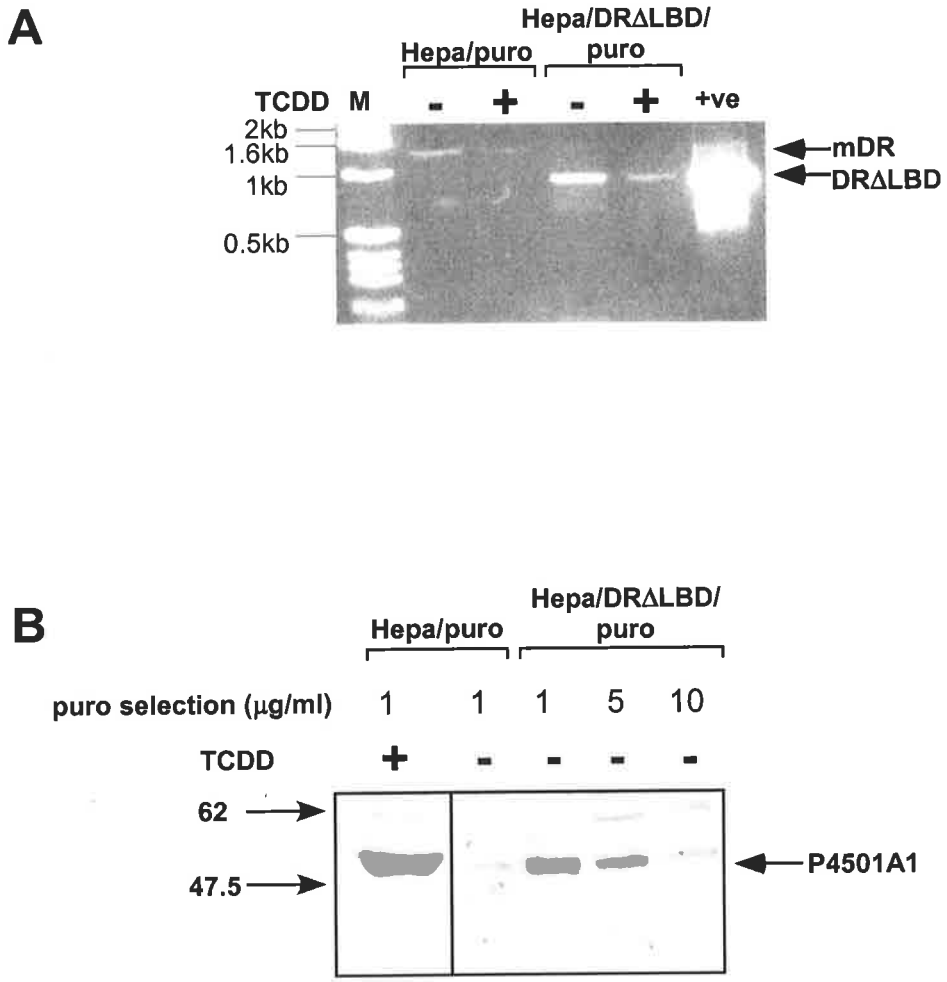


Figure 4.3. *Expression of DRΔLBD construct in Hepa1c1c7 cells and ligand independent induction of the CYP1A1 protein.* (**A**) Stable expression of the DRΔLD construct in Hepa cells. RT-PCR from cDNA derived from Hepa/Puro or Hepa/DRΔLBD cells using primers 17N and 433C spanning the LBD, positions of full length mDR and DRΔLBD construct are indicated. (**B**) 1A1 protein is expressed in Hepa/DRΔLBD cells independently of ligand. Whole cell extracts (30μg) from Hepa/Puro or Hepa/DRΔLBD cells, selected at the indicated concentrations of puromycin, were separated by 10% SDS-PAGE and immunoblotted with an antibody directed against P4501A1. TCDD treated Hepa/Puro cells are included as a positive control and the position of P4501A1 is indicated.

(Figure 4.3a). Brighter exposure of the gel revealed the presence of full length DR of the expected size of 1278 bp in the Hepa/ DRΔLBD line (Figure 4.3a). Strikingly, however, these cells demonstrated upregulation of CYP1A1 protein in a ligand independent fashion (Figure 4.3 b). In our laboratory, the pEF/IRES/puro vector system has the capacity to generate pools of cells expressing higher levels of the protein of interest if the level of puromycin selection is increased (M. Kleman unpublished observation). The theory supporting this is that under higher levels of antibiotic selection, cells are selected that express higher levels of the gene of interest due to its linkage with the antibiotic resistance marker via the internal ribosome entry site system. Strikingly, selection of cells expressing the DRΔLBD construct at higher levels of puromycin lead to an inverse relationship to the level of ligand independent CYP1A1 protein expressed (Figure 4.3b), suggesting that the DRΔLBD construct is unable to be expressed at high levels in Hepa1c1c7 cells and potentially these cells have a mechanism to select against the high expression of constitutively active DR. These experiments demonstrate the functionality of the DRΔLBD protein using both *in vitro* reporter constructs (293T cells) and *in vivo* (P4501A1 protein upregulation) techniques. In addition, two recent publications using the same construct have since supported these results in terms of the ability of the DRΔLBD to be predominately nuclear localised (McGuire *et al* 2001) and activate both reporter genes (McGuire *et al* 2001) and endogenous target genes (Andersson *et al* 2002) in the absence of ligand.

The role of the ligand binding domain in DR activation

The results presented in Chapter 3 propose a role for the correct chaperoning of the ligand binding domain in order to achieve a transcriptionally active DR/Arnt heterodimer. Thus, it is of interest to note that the DRΔLBD construct displays DNA binding and transcriptional activity which is independent of ligand treatment. That is,

removal of the repressive LBD control region resulted in successful heterodimerisation and transcriptional upregulation on an artificial reporter gene. Importantly, however, this construct is also able to transactivate the endogenous target gene CYP1A1 (Figure 4.3b) and (Andersson *et al* 2002), suggesting that the transactivation domain of the DR is correctly structured and able to interact with transcriptional coactivators. This implies that the presence of the LBD is not essential for activities in other portions of the protein (i.e. dimerisation, DNA binding and transactivation). Rather, the LBD is a repression domain important for latency, which must be derepressed in a specified manner, eg by ligand binding, to produce a functional receptor. Improper derepression, such as by geldanamycin treatment, leads to a malfolded LBD which then inhibits the activity of the DR presumably by misfolding of the transactivation domain.

XAP2 depletion abrogates DR signalling through DR degradation

The development of the stable cell lines containing different forms of the DR provided a unique opportunity to understand several facets of DR signalling. XAP2 is known to associate with the latent DR complex (Carver and Bradfield 1997, Ma and Whitlock 1997, Meyer *et al* 1998). These initial reports have all demonstrated that overexpression of XAP2 leads to an approximate twofold increase in DR responsive reporter genes in both cell culture systems (Carver and Bradfield 1997, Ma and Whitlock 1997, Meyer *et al* 1998) and yeast (Lapres *et al* 2000). To address the role of XAP2 in DR signalling, an antisense system was employed utilising the cell lines expressing the cytoplasmic and nuclear localised forms of the receptor to investigate the effect that XAP2 depletion has on the signalling output from both of these proteins. The antisense system utilised an expression vector, which generated the complement strand covering the first 1060 nucleotides of the XAP2 message. The fragment was subcloned into the powerful mammalian expression vector pEF/BOS, and transiently transfected into the cell lines in combination with the XRE driven luciferase reporter gene. Cells were transfected for 24

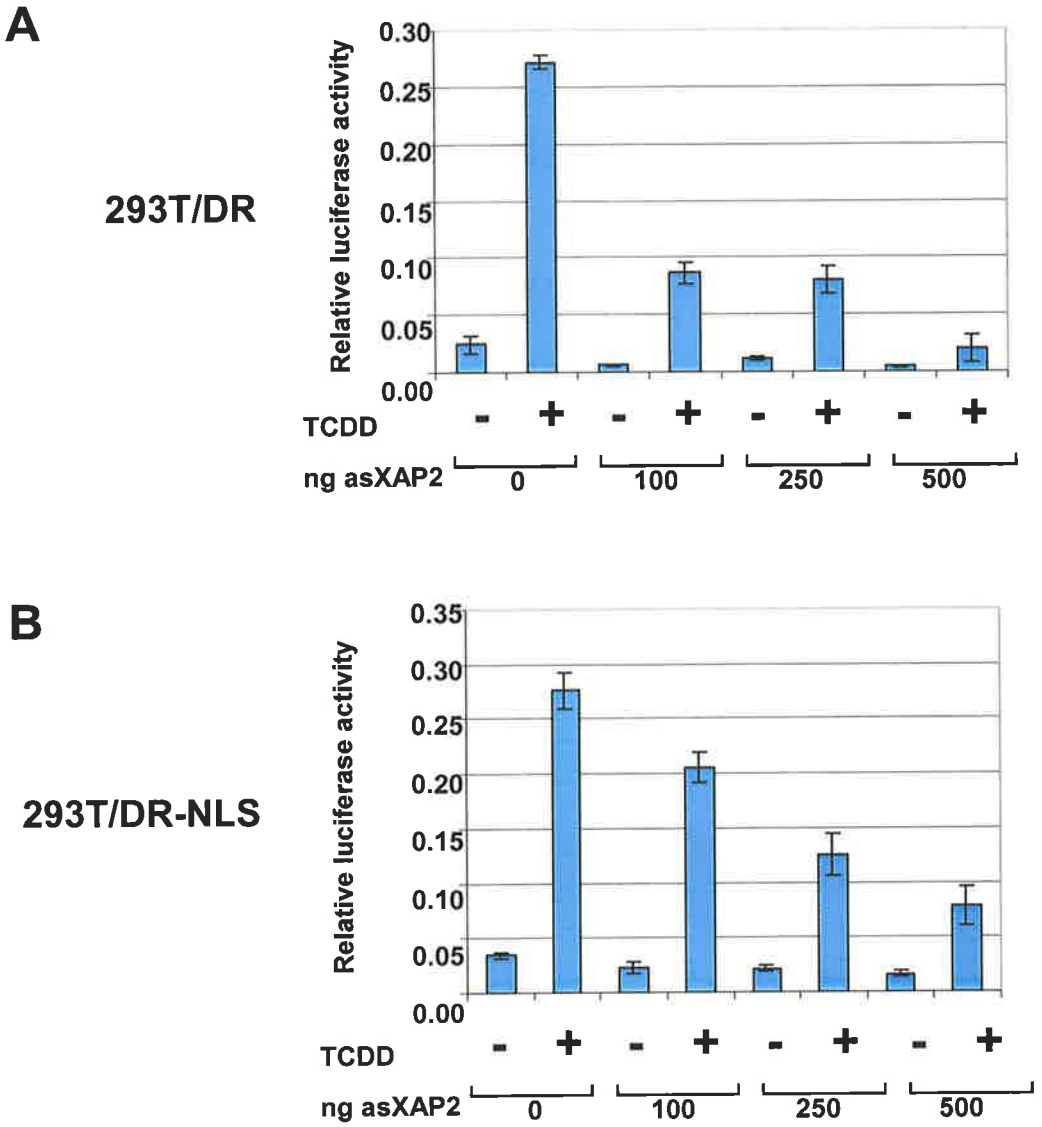


Figure 4.4. *An antisense XAP2 construct diminishes DR and DR-NLS signalling and abrogates XAP2myc expression.* (**A** and **B**) DR or DR-NLS stable cell lines were transfected with the XRE-luciferase reporter and indicated amounts of the antisense XAP2/EFBOS construct (asXAP2) in addition to 20ng of XAP2myc tracer for 24 hours prior to treatment with TCDD (10nM) or DMSO as indicated for 12 hours. (**C**) Extracts from duplicate wells from B were separated by 10% SDS-PAGE and immunoblotted with an the 9E10 αmyc mAb. The position of the XAP2myc construct is indicated. Displayed is a representative experiment -/+ S.D. performed in triplicate. Transfected DNA content washeld constant by addition of pEFBOS vector.

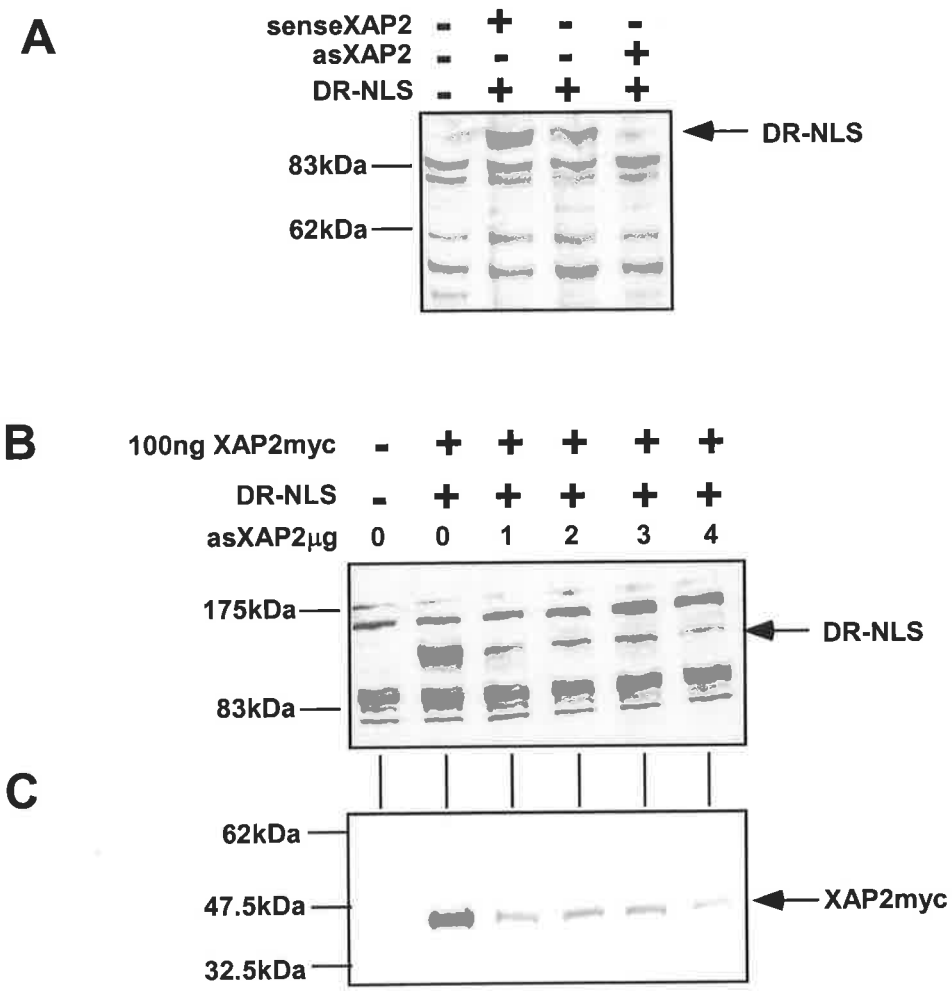


Figure 4.5. *XAP2 depletion decreases DR-NLS protein expression.* (**A**) 293T cells in 6 well trays were transfected for 36 hours with the DR-NLS/RcCMV expression construct alone or in combination with the XAP2/EFBOS sense construct (1μg) or antisenseXAP2/EFBOS construct (4μg). Whole cell extracts (50μg) were separated by 7.5% SDS-PAGE and DR-NLS expression was detected with the 12CA5 αHa mAb. (**B** and **C**) 293T cells in 6 well trays were transfected for 36 hours with the DR-NLS/RcCMV construct, XAP2myc tracer construct and the indicated amount of antisenseXAP2/EFBOS. Whole cell extracts (50μg) were separated by 7.5% SDS-PAGE for immunoblotting with the 12CA5mAb (**B**) or 10% SDS-PAGE for XAP2myc expression (**C**). Total DNA content was normalised using empty pEFBOS vector. The positions of DR-NLS and XAP2 proteins are indicated.

hours with the antisense expression vector and the luciferase reporter, followed by overnight treatment with TCDD or vehicle alone. In the absence of the antisense expression vector, TCDD gave a robust induction for both the DR and DR/NLS constructs (Figure 4.4a & 4.4b). Strikingly, as the amount of antisense XAP2 expression vector increased, the reporter gene output diminished significantly (Figure 4.4a & 4.4b). As a means of verifying that the antisense vector was capable of depleting the cell of XAP2, 293T cells were cotransfected with trace amounts of a XAP2 sense construct, which contained a C-terminal 6myc epitope. Cell extracts from the transient transfections were separated by SDS-PAGE followed by immunostaining with the 9E10 α-myc mAb. Lanes from duplicate transfection experiments displayed a consistent dose dependent decrease in levels of the tracer XAP2 myc construct (Figure 4.4c). Several possibilities exist as to the mechanism behind XAP2 depletion leading to a decreased reporter response, including poor nuclear uptake of the DR leading to diminished transcriptional signalling. However, as the nuclear localised DR also displayed a decreased response we reasoned that XAP2 depletion was acting at a step beyond nuclear translocation, as the exogenous NLS is located at the C-terminus and should be impervious to the presence or absence of a DR specific cochaperone, which acts within the LBD (Bell and Poland 2000). An alternative possibility to effects on DR localisation is that the depletion of XAP2 affects the stability of the DR constructs, which takes into account the reported observations that overexpression of XAP2 leads to an increase in DR signalling (Carver and Bradfield 1997, Ma and Whitlock 1997, Meyer *et al* 1998). To this end, we investigated the stability of the DR-NLS construct in a transient transfection assay. In an analogous fashion to previous reports (Meyer and Perdew 1999, Kazlauskas *et al* 2000, LaPres *et al* 2000), cotransfection of an XAP2 sense construct stabilised DR-NLS levels approximately two fold (Figure 4.5a), showing further similarities between the nuclear localised and cytosolic proteins. Following co-transfection of the DR-NLS/RcCMV construct with the antisense XAP2 construct, DR-

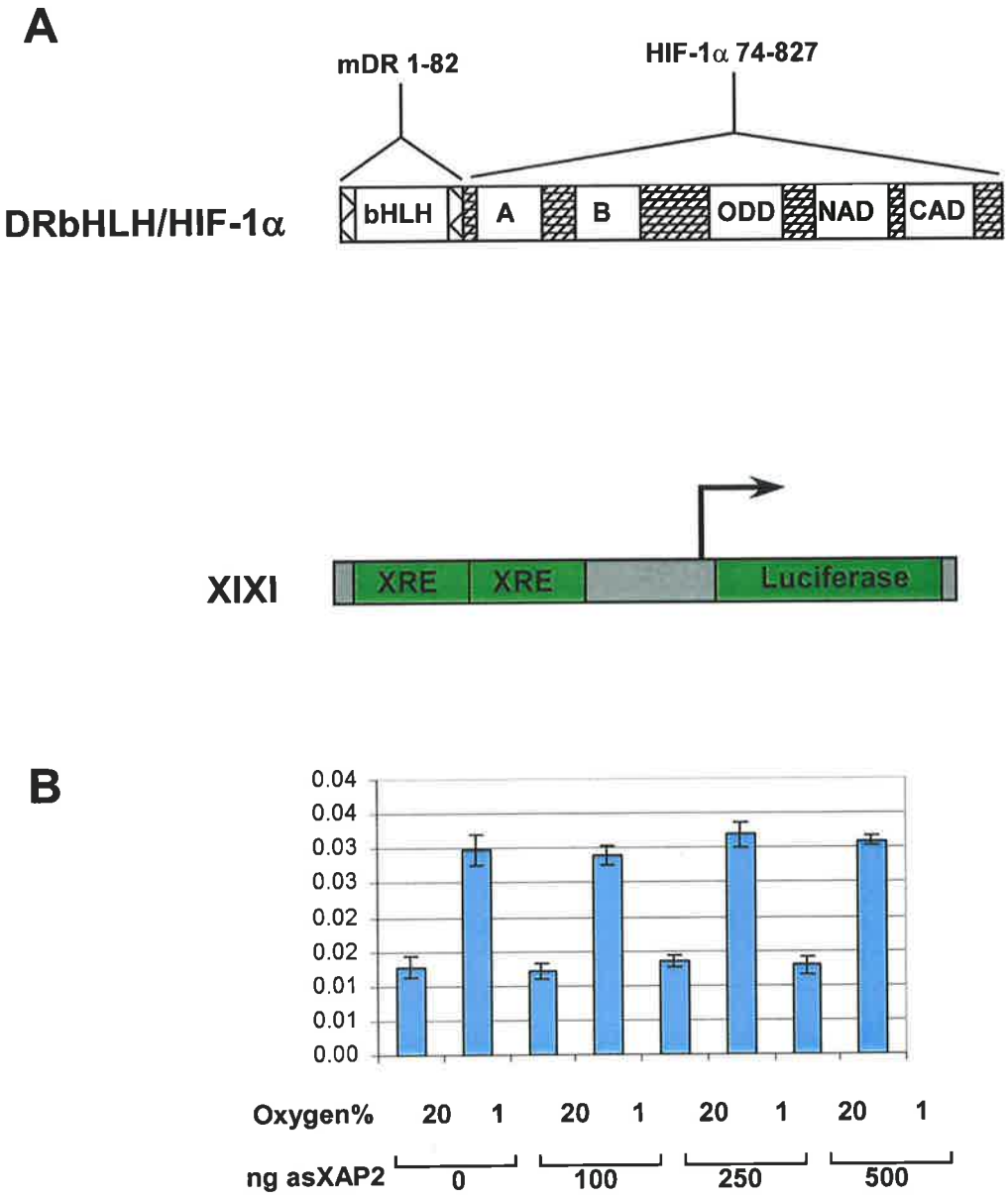


Figure 4.6. *A DRbHLH/HIF-1α construct drives XRE driven luciferase activity and is not sensitive to XAP2 depletion.* (**A**) Schematic representation of the DRbHLH/HIF-1α chimeric protein which regulates XRE-luciferase activity in a hypoxia inducible fashion. (**B**) 293T cells were transfected with the DRbHLH/HIF-1α construct in combination with the XRE-luciferase reporter and the indicated amount of asXAP2 for 24 hours prior to incubation in a hypoxic chamber for a 12 hour period. Data represents an typical experiment -/+ S.D. performed in triplicate three times.

NLS protein expression was noticeably depleted in the presence of the antisense construct compared to transfection with the control vector (Figure 4.5a), suggesting that depletion of XAP2 leads to destabilisation of the DR resulting in the subsequent decreased output in the luciferase reporter assay. In a similar fashion to the reporter assays, this effect was somewhat dose dependent as increasing levels of the antisense construct led to depleted levels of both DR-NLS protein (Figure 4.5b) and XAP2myc tracer (Figure 4.5c), although this effect was most dramatic at the lower concentrations of the antisense construct added. To verify that these observations were specific for the DR, a chimeric protein which fuses the bHLH region of the DR to the PAS/C-terminal portion of HIF-1a was employed (Figure 4.6a). This chimeric construct is subject to hypoxic regulation and regulates gene output via XRE sequences instead of HRE sequences (Figure 4.6b). In transient transfection experiments, which were identical to those performed for the DR constructs, the DRbHLH/HIF chimeric protein was not regulated by a depletion in XAP2, demonstrating that this effect is not a global influence on transcriptional output and is specific for a region of the DR C-terminal to the bHLH region.

These results demonstrate that depleting the cells of XAP2 by using an antisense expression vector diminishes transcriptional output from an XRE driven reporter gene as a result of depletion of DR from the cell. This effect occurs for both the cytosolic and nuclear forms of the DR suggesting a stabilisation role for XAP2 in either the cytoplasm or the nucleus.

A Role for CHIP in DR degradation?

Recently, it has been shown that the GR can be degraded by the ubiquitin proteasome system in a process mediated by CHIP (C terminal hsp70/90 Interacting protein). CHIP has been proposed to be either an E3 (Demand *et al* 2001, Jiang *et al* 2001, Murata *et al*

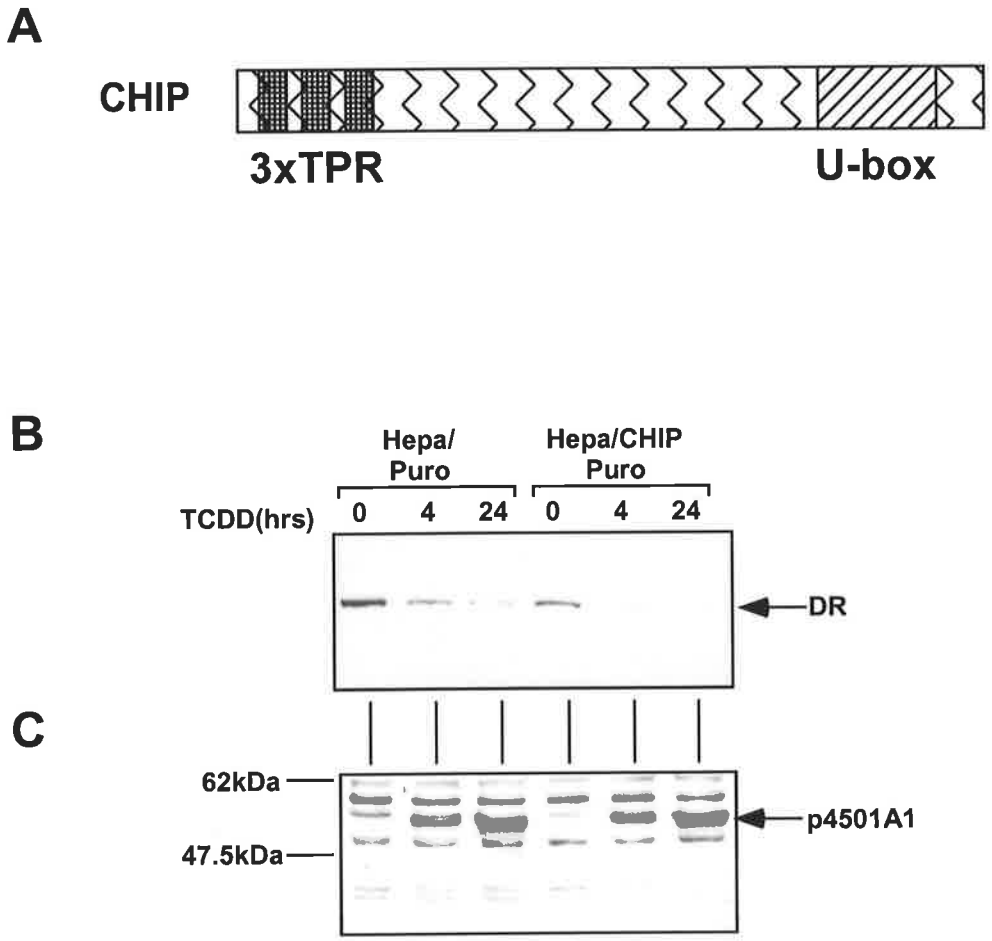


Figure 4.7. *Chip, a U-box and TPR containing protein induces DR degradation in Hepa/CHIP cells.* (**A**) Schematic representation of CHIP, depicting the N-terminal TPR domains and C-terminal U-box domain. (**B** and **C**) Hepa/puro or Hepa/CHIP stable cell lines were treated with TCDD (10nM) for the indicated times or DMSO control for 24 hours. Whole cell extracts (20μg) were separated by 7.5% SDS-PAGE followed by immunoblotting with an αDR mAb (**B**) or 10% SDS-PAGE and immunoblotting with an αP4501A1 polyclonal Ab (**C**). The positions of the DR and 1A1 proteins are indicated.

2001, Cyr *et al* 2002) or E4 ubiquitin ligase protein based on homology to the yeast E4 protein UFD2. CHIP is a TPR containing protein with an additional U-box subdomain (shown schematically in Figure 4.7a), which has been demonstrated to be absolutely required for its ubiquitin conjugating activity (Jiang *et al* 2001). U-box containing proteins are part of the new wave of E3 type ubiquitin ligases and the U-box domain is predicted to be structurally similar to the more firmly established RING finger family of ubiquitin ligases which are involved in receiving activated ubiquitin from an E2 ligase (Joazeiro *et al* 2000, Aravind and Koonin 2000). E3 ubiquitin ligases are fairly well characterised and provide substrate recognition to a target protein whereupon they utilise E1 and E2 components to promote the incorporation of ubiquitin moieties onto the substrate and promote degradation by the 26s proteasome (Conaway *et al* 2002). E4 ligases, however, are less well characterised and are proposed to modulate polyubiquitination of a target protein (Koegl *et al* 1999). As an initial approach to investigate the possible role of CHIP in DR degradation, transient transfection experiments were performed in 293T/DR cells in a reporter assay as a rapid means of testing the ability of CHIP to influence DR signalling. This approach however was unfeasible as increasing levels of CHIP lead to a decrease in both firefly and renilla luciferase, complicating data interpretation. The TPR domain alone of CHIP has been demonstrated to inhibit luciferase refolding *in vitro* (Wu *et al* 2001). In order to circumvent this problem, once again the pEF/IRES/puro vector was employed and a stable cell line was generated expressing CHIP. Following puromycin selection, extracts from Hepa/CHIP cells were separated by SDS-PAGE and probed with an anti DR monoclonal antibody. Extracts from these cells consistently revealed a decrease in DR levels in the absence of ligand (Figure 4.7b). Following extended ligand treatment, levels of DR within the cell become almost undetectable in the Hepa/CHIP line, presumably due to lower levels of starting material (Figure 4.7b). To ascertain whether the decrease in DR levels disrupted DR signalling, Hepa/CHIP cells were treated with

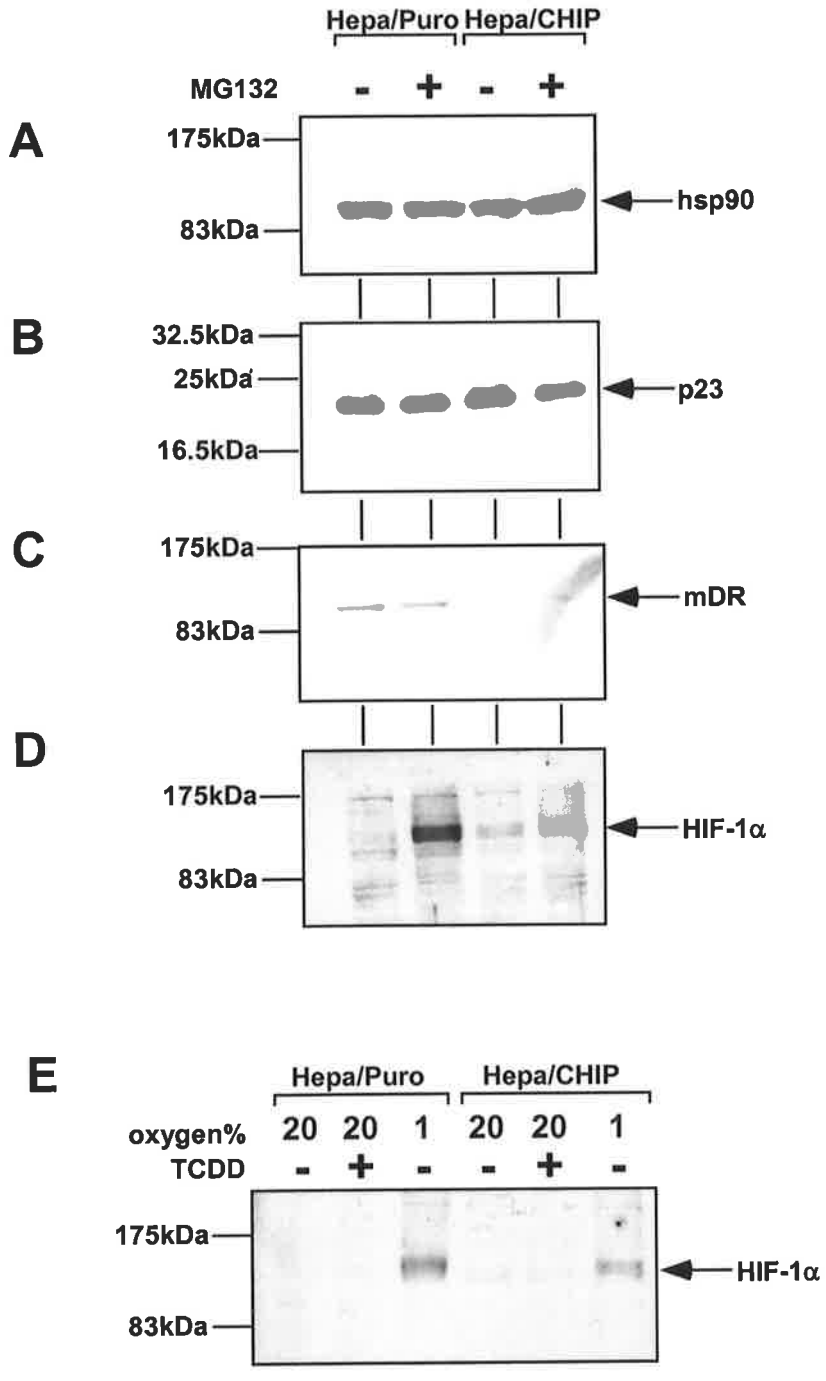


Figure 4.8. *The proteasome inhibitor MG132 does not prevent CHIP induced degradation.* Hepa/Puro or Hepa/CHIP cells were treated with 25μM MG132 or with ethanol vehicle for 6 hours after which time whole cell extracts (20μg) were analysed using antibodies specific for Hsp90 (**A**), p23 (**B**), DR (**C**) or HIF-1α (**D**). The immunoblot in (**E**), depicts HIF-1α expression in whole cell extracts (20μg) from cells exposed to hypoxia or TCDD (10nM) for 4 hours or left untreated. The positions of the relevant proteins are indicated.

TCDD or vehicle control for the indicated times. As shown in Figure 4.7c, no appreciable difference was observed in the inducibility of CYPIA1 protein between the two cell lines, demonstrating that despite the Hepa/CHIP cells containing less DR they are still able to induce an endogenous target gene with equal efficiency. This unexpected result can be explained by the fact that in Hepa 1c1c7 cells, the DR is not the limiting factor but rather other factors such as Arnt are limiting. This is in agreement with quantitative data from the laboratory of Richard Pollenz that suggests that there are approximately 10 times more DR molecules per cell than Arnt (Holmes *et al* 1997).

Evidence suggests that CHIP can promote the degradation of a variety of both hsp90 and hsp/hsc70 substrate proteins including the GR, raf-1 kinase (Demand *et al* 2001), Cystic Fibrosis transmembrane receptor (Meacham *et al* 2001) and PaelR (Imai *et al* 2002). In addition however, there is some evidence that proposes that CHIP also targets the chaperone proteins Hsc70 and Hsp90 for ubiquitination whilst not altering the steady state levels of the chaperones (Ballinger *et al* 1999, Jiang *et al* 2001). Furthermore, one of the partners in CHIP mediated degradation, Bag-1, has also been proposed to be a target for CHIP mediated ubiquitylation which is theorised to enable a novel form of targeting to the proteasome (Alberti *et al* 2002). Figure 4.8a and 4.8b show that hsp90 and p23 levels remain unchanged in Hepa/CHIP cells respectively. To ascertain if ubiquitination is the means of CHIP mediated DR degradation, Hepa/CHIP cells were treated with the proteasome inhibitor MG132. Previously, MG132 has been demonstrated to prevent ligand induced degradation of the DR (Davarinos and Pollenz 1999, Roberts and Whitelaw 1999, Ma and Whitlock 2000) and successfully prevents CHIP induced degradation of the GR, Pael-R and CFTR (Connell *et al* 2001, Imai *et al* 2002, Meacham *et al* 2001). Hepa/CHIP cells were treated with 25μM MG132 for up to 6 hours, and whole cell extracts were separated by SDS-PAGE. However, these conditions failed to stabilise the DR as assessed by Western blot analysis with an

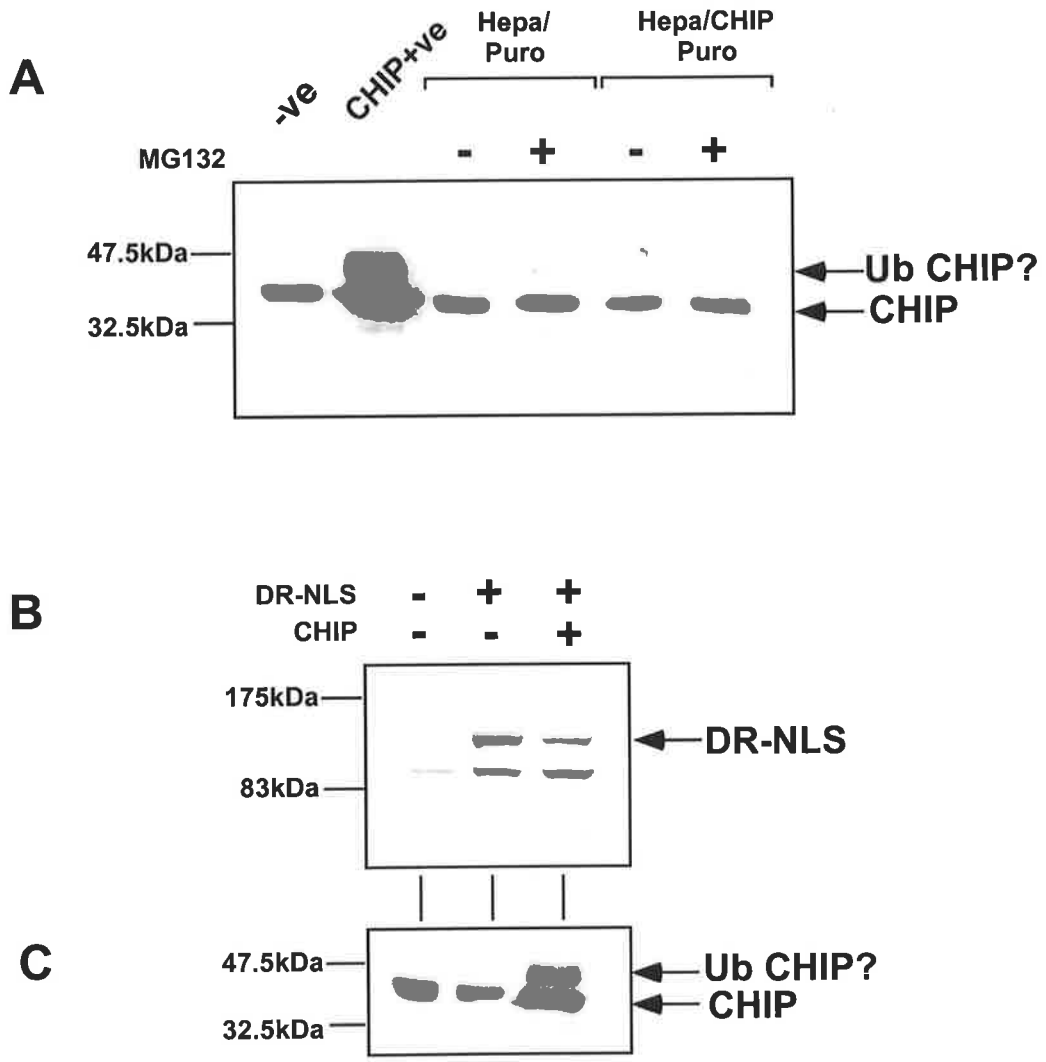


Figure 4.9. *CHIP is not overexpressed in Hepa/CHIP stable cells but can be overexpressed in 293T cells to destabilise the DR-NLS protein.* (**A**) Hepa/puro or Hepa/CHIP cells were treated for 6 hours with MG132 (25μM) and whole cell extracts (20μg) were separated by 10% SDS-PAGE followed by immunoblotting with an αCHIP polyclonal Ab. Included is a transient transfection of 293T cells transfected with the pEF/CHIP/IRES/puro construct (CHIP+ve) and the position of overexpressed CHIP and slower migrating forms of CHIP are indicated. (**B** and **C**) 293T cells were transiently transfected with pcDNA or pcDNA/CHIP in combination with DR-NLS/RcCMV as indicated. 20μg of whole cell extracts were separated by 7.5% SDS-PAGE and analysed for DR-NLS expression with the αHA mAb (B) or 10% SDS-PAGE and analysed by the αCHIP polyclonal Ab, the positions of the relevant proteins are indicated.

antibody directed against the DR (Figure 4.8c). As a positive control for the MG132 treatment, extracts were assayed for HIF-1α expression which has been well characterised in its ability to be stabilised by proteasome inhibitors including MG132 (Salceda and Caro 1997, Kallio *et al* 1999). Figure 4.8d shows that MG132 stabilisation of HIF-1α was successful in both cell lines, yet intriguingly HIF-1α levels were decreased in the Hepa/CHIP line in a reproducible fashion. HIF-1α levels were also depleted in Hepa/CHIP cells exposed to 4 hours hypoxia (Figure 4.8e). Thus whilst serving as a useful control for MG132 treatment, the observation that HIF-1α levels are depleted in the Hepa/CHIP line raises interesting questions regarding the role of the hsp70/hsp90 chaperone pathway in HIF-1α stability and signalling.

CHIP/IRES/puro cells display decreased levels of the DR, however unexpectedly we were unable to demonstrate in preliminary experiments that this degradation is mediated by the E3 ubiquitin ligase activity of CHIP, as MG132 treatment was unable to stabilise the DR. Further experiments need to be undertaken to examine this effect more closely, including the use of ubiquitin mutant constructs in transfection based experiments which prevent polyubiquitination of the substrate protein and thus increasing the likelihood of visualising the ubiquitinated species.

To determine whether Hepa/CHIP cells overexpress CHIP, extracts were separated by SDS-PAGE and blots were probed with an anti-CHIP antibody. Despite the fact that Hepa/CHIP cells display decreased levels of DR, no significant increase in CHIP protein levels could be observed (Figure 4.9a). To confirm that the CHIP/IRES/puro construct could in fact express CHIP protein, 293T cells were transiently transfected with the construct and analysed for CHIP protein levels. Figure 4.9a shows that the construct can drive CHIP expression in a transient assay system. It has been proposed that CHIP like other E3 ligases can autoubiquitylate (Jiang *et al* 2001), this is supported

by the higher migrating forms of CHIP following transient expression (Figure 4.9a) which could represent ubiquitinated or as yet unidentified post-translationally modified forms of CHIP by either autoubiquitylation or other means. This observation provides a potential mechanism for the steady state levels of CHIP in a stable cell line. CHIP has been proposed to be lowly expressed in relation to the folding chaperones (Wiederkehr *et al* 2002), the suggestion being that the decision between protein folding and degradation is a tightly controlled mechanism which could be influenced by small changes on either side of the chaperone ledger. However, we failed to observe slower migrating forms of CHIP in the Hepa/CHIP line in response to MG132 treatment. Future experiments should be directed towards stable expression of an epitope tagged version of CHIP to ensure that the protein can be stably expressed.

To ascertain whether CHIP can promote the degradation of the hsp90 bound nuclear localised form of the DR, transient transfection assays were performed using 293T cells and the DR-NLS/RcCMV construct in the presence or absence of co-expressed CHIP. This system demonstrates that DR-NLS levels are moderately reduced (Figure 4.9b) in the presence of overexpressed CHIP in 293T cells (Figure 4.9c). However, we cannot formally rule out that this degradation occurs in the cytoplasm before the DR reaches the nucleus.

Future interest will lie in the interplay between CHIP and the other TPR containing proteins which interact with the DR chaperone complex namely HOP and XAP2. The possibility exists that the mechanism of XAP2 mediated stabilisation of the DR lies solely in the ability of XAP2 to prevent the association of the DR with the E3 ubiquitin ligase CHIP via a competition mechanism through the TPR domains of CHIP and XAP2 for the TPR acceptor site of hsp90.

Discussion

Interplay between TPR containing proteins

In an attempt to define a role for XAP2 in DR signalling, it was demonstrated that a depletion in XAP2 led to a depletion in levels of DR signalling for both the wild type DR in addition to the nuclear localised form of the DR (Figure 4.4). XAP2 depletion leads to a corresponding decrease in DR-NLS protein levels, arguing a case for XAP2 in a role beyond cytoplasmic retention and for a role in DR stabilisation. Recently, there have been several studies which have investigated the role of XAP2 in DR signalling such that XAP2 has been demonstrated to play two roles, that being promotion of cytoplasmic localisation and DR stabilisation by preventing ubiquitination of the DR (Kazlauskas *et al* 2000). This is consistent with *in vitro* data showing XAP2 stabilisation of the DR/hsp90 complex (Bell and Poland 2000) by virtue of XAP2 having the capacity to bind both hsp90 and the DR. Taken together this suggests that XAP2 stabilisation of the DR/hsp90 chaperone complex promotes cytosolic localisation of the DR, possibly by a decreased occurrence of transient unmasking of the N-terminal NLS when XAP2 is present in the complex. However, this does not fully explain the observation that XAP2 also prevents ubiquitin mediated degradation of the DR. Previously, XAP2 has been the only TPR containing protein found to be associated with the DR/chaperone complex. So far we have preliminary evidence for an association of the TPR containing protein CHIP with the DR, provided by the observation that a stable cell line expressing human CHIP destabilises the endogenous DR from Hepa1c1c7 cells (Figure 4.7). We have yet to show a direct association between the DR and CHIP in biochemical assays. However this may be a reflection of the transient nature of the interaction between these two proteins. That is, once CHIP associates with the DR

complex it is rapidly targeted for degradation which complicates capturing the interaction.

What is the relevance of an hsp90 mediated degradation mechanism in regards to DR signalling? CHIP is proposed to mediate protein triage decisions for hsp70/hsp90 client proteins that may be considered problematic (and therefore require a molecular chaperone to prevent aggregation within the cell). To this end, these proteins are continually tested for suitability and either maintained in a chaperoned state or are passed on to the proteasomal machinery using the CHIP pathway (Connell *et al* 2001). It will be intriguing to investigate further the role that XAP2 plays in this process and whether the stabilisation effect that XAP2 affords the DR acts through a competition effect between the TPR containing proteins XAP2 and CHIP. Little is known about the selection process for client proteins at this triage decision making process. Perhaps in this context the decision as to whether or not the DR is selected for degradation is based solely on the presence of XAP2 in the hsp90 chaperone complex through competition of the TPR acceptor site of hsp90.

CHIP and HIF-1α degradation

The observation that HIF-1α levels were depleted in the Hepa/CHIP cell line raises interesting possibilities concerning HIF-1 α degradation. Some elegant and convincing work has greatly enhanced our understanding of hypoxia mediated degradation of the HIF-1 α subunit, which links oxygen availability to a hydroxylation event which in turn links VHL degradation machinery to a region located within the oxygen dependent degradation domain (Ivan *et al* 2001, Jaakola *et al* 2001, Yu *et al* 2001, Min *et al* 2002, Hon *et al* 2002). An alternative pathway has also been proposed for HIF-1 α degradation based on the observation that HIF-1 α appears to bind hsp90 (Minet *et al* 1999, Isaacs *et al* 2002), and that in a VHL deficient line HIF-1 α degradation is

accelerated following treatment with geldanamycin in a similar manner to the DR and other hsp90 substrates (Chapter 3, Chen *et al* 1997, Isaacs *et al* 2002). It is well accepted that the heat shock proteins are employed during cellular stress events, whether hsp90 chaperoning is involved in a hypoxic stress event and indeed in the normal cellular context remains to be seen. Thus, this control experiment to ensure successful MG132 treatment supports the notion of an hsp90 mediated HIF-1 α folding event.

Alternative roles for XAP2

There is some evidence that the DR shuttles from the cytoplasm to the nucleus. If this is the case, what is the purpose of nuclear/cytoplasmic shuttling? Does it provide a more comprehensive surveillance mechanism by sampling both the cytosolic and nuclear compartments of the cell? Is shuttling a function of low levels of endogenous ligand, or control by ligand independent means? Shuttling between the cytoplasm and the nucleus has been described for other PAS factors, particularly the PER proteins and this appears to be a major step in the mechanism of gene regulation by these proteins (Kloss *et al* 2001, Lee *et al* 2001, Akashi *et al* 2002). However, these proteins act independently of ligand and thus shuttling is a feasible method of regulation in combination with phosphorylation and other potential post translational modifications. However, for the DR it becomes a little more complicated as ligand is required for steps subsequent to nuclear localisation (Chapter 3), in order to achieve transcriptional competency. Potentially, the DR can act as a shuttling protein itself, to piggyback other proteins into the nucleus. Candidate proteins to utilise this mechanism include the retinoblastoma protein and Myb1bBP, both of which are very poorly characterised in relation to DR signalling (Ge *et* al 1998, Elferink *et al* 2001, Jones *et al* 2002). It is tempting to speculate that this shuttling phenomenon could be regulated by the availability of the

co-chaperone XAP2 in a spatial and temporal manner, as XAP2 has been shown to enhance DR cytosolic localisation (Petrulis *et* al 2000, Kazlauskas *et al* 2000).

Results

Investigating the role of post translational modifications on DR activation

5

Chapter 5. Investigating the role of post translational modifications on DR activation

Introduction

Post translational modifications are well known to control the activity of transcription factors and regulate their activity at multiple levels. Phosphorylation of proteins can regulate their cellular localisation, dimerisation, affinity for DNA and transactivation (Steegenga *et al* 1996, Decker and Kovarik 2000, Moustakas *et al* 2001, Wang *et al* 2002, Itoh *et al* 2002). Alternate modifications include the hydroxylation events of the HIF's which regulate their stability and recruitment of co-activators (Ivan *et* al 2001, Jaakkola *et* al 2001, Lando *et al* 2002a, Lando *et al* 2002b). Recently, protein acetylation, traditionally thought to be restricted to histones, has also been found to control protein activity at the level of cellular localisation, dimerisation, DNA binding and stability (Kouzarides 2000). The significance of acetylation is exemplified by the emergence of deacetylases, implying that this mode of regulation can be controlled in a reversible fashion (Kouzarides 2000). In addition, protein modification by Sumo (small ubiquitin like molecule) has been demonstrated to function at several levels of protein regulation including cellular localisation and protein stability (Seeler and Dejean 2001, Wilson and Rangasamy 2001).

Post translational modifications occurring on the DR have yet to be conclusively shown. Previous studies have focussed on phosphorylation of the DR using a battery of protein kinase inhibitors and analyses of the effects each one has on the inducibility of either the CYP 1A1 target gene or an XRE driven reporter gene (Carrier *et al* 1992, Berghard *et al* 1993, Gradin *et al* 1994). Using an approach such as this is complicated by the fact that there are multiple steps in the DR activation pathway which could possibly be

regulated by any number of post-translational modifications. However, this has been circumvented by isolating the DR and Arnt via *in vitro* translation or immunoprecipitation and analysing the effects of kinases and phosphatases on each of the subunits in terms of heterodimerisation/DNA binding assays (Berghard *et al* 1993, Gradin *et al* 1994). The most direct evidence for post translational modification of the DR have been performed using *in vivo* labelling experiments, which demonstrated the latent DR to be a phosphoprotein (Berghard *et al* 1993, Mahon and Gasiewicz 1995). This has been extended using crude mapping analysis and a combination of hydroxylamine and cyanogen bromide cleavage to identify a phosphorylated region between amino acids 368-605, which incorporates the C-terminal region of the ligand binding domain (Mahon and Gasiewicz 1995). It appears that a decrease in net phosphorylation of the DR occurs upon heterodimerisation with Arnt (Perdew 1991).

A summary of these studies suggests that phosphorylation in the DR activation pathway is complex. When nuclear extracts from ligand treated cells were incubated with potato acid phosphatase, DNA binding was abrogated without a corresponding decrease in DR/Arnt heterodimerisation (Mahon and Gasiewicz 1995). Furthermore, by treating nuclear extracts with phosphatase inhibitors, DNA binding was actually enhanced suggesting a role for an endogenous phosphatase acting on the DR complex to inhibit DNA binding. However, if the DR and Arnt are *in vitro* translated in isolation and treated with potato acid phosphatase the results are somewhat different, dephosphorylation of DR prevents DNA binding but not heterodimerisation, whilst dephosphorylation of Arnt prevents DR/Arnt heterodimerisation and consequently DNA binding as well (Berghard *et al* 1993). Treatment of several cell lines with a variety of protein kinase inhibitors has demonstrated the susceptibility of components of the DR pathway to Protein Kinase C inhibitors (including staurosporine, calphostin C, H7 Dihydrochloride and the PKC depleting agent TPA) to induce CYPIA1 mRNA (Carrier

et al 1992, Berghard *et al* 1993). In one study, staurosporine has been suggested to act as a ligand for the DR based on the observation that cytosolic extracts from guinea pig liver treated with staurosporine have a reduced capacity to bind [^{3}H] TCDD, in addition to possessing the ability to induce an XRE binding species in the absence of TCDD (Schafer *et al* 1993). The structure of staurosporine does not conform to a typical DR ligand due to the bulky side groups and lack of planarity (Figure 4.2) arguing against this suggestion. One possibility as to how staurosporine negatively affects DR signalling is based on the observation that staurosporine induces degradation of the DR in a dose dependent manner (Singh and Perdew 1993). This could be indicative of the notion that staurosporine is a ligand for the DR. However, this same study showed that the receptor that remained possessed full TCDD binding capacity when the amount of degradation induced by staurosporine was accounted for (Singh and Perdew 1993), indicating that staurosporine is probably not acting as a competitive ligand for the DR. Previous experiments (Pongratz *et al* 1992) showed it is possible to artificially strip the DR of hsp90 and generate a DR/Arnt heterodimer which loses the capacity to bind TCDD. That is, during a non-ligand activation process of the DR, hsp90 is lost concomitantly with the ability to bind ligand. The above results might be somewhat reconciled in a model where staurosporine initiates the process of DR activation and that upon loss of hsp90, the LBD alters conformation and loses the capacity to bind ligand and the DR gets degraded.

A role for a tyrosine kinase in DR signalling has also been proposed as genistein inhibits DR function (Gradin *et al* 1994), and more recently anti-phospho tyrosine antibodies show that the DR is phosphorylated on a tyrosine residue (Park *et al* 2000). Genistein abrogated TCDD induced CYPIA1 in human keratinocytes by inhibiting the ability of the DR to form a DNA binding complex. Using chimeric proteins containing portions of the DR, a genistein sensitive region was mapped to amino acids 83-593 of

the DR (Gradin *et al* 1994). The authors proposed that this was due to an inability of the DR to dissociate from hsp90 following ligand treatment, although not demonstrating this formally. However, this study differs from others which demonstrated no significant difference in the capacity to generate a DNA binding complex in Hepa-1 cells (Carrier *et al* 1992) in addition to the observation that Rat4IIE cells are resistant to genistein abrogation of TCDD induction of CYPIA1, measured by mRNA or protein levels (Backlund *et al* 1997). Interestingly, one group has presented preliminary evidence to suggest that c-src is a component of the latent DR complex, and the activity of this kinase is increased in response to TCDD activation (Enan and Matsumura 1996).

In addition to a role for phosphorylation mediating heterodimerisation and DNA binding, it has been indirectly demonstrated that phosphorylation may increase turnover of the DR. This idea arises from the observation that treatment with the phosphatase inhibitor calyculin dramatically accelerates degradation of DR-NLS, the constitutively nuclear, hsp90 bound form of the DR (Roberts and Whitelaw 1999).

AIMS

The aim of this chapter was to analyse the DR, DR-NLS and DRΔLBD 293T stable cell lines for responses to treatment with kinase inhibitors. In addition to this, potential TCDD induced changes on the phosphorylation status of the DR were investigated. Furthermore, an attempt to map the location of potential phosphorylation changes and to initiate the development of a system for large-scale expression and purification of the DR for analysis by more direct means such as mass spectrometry was performed.

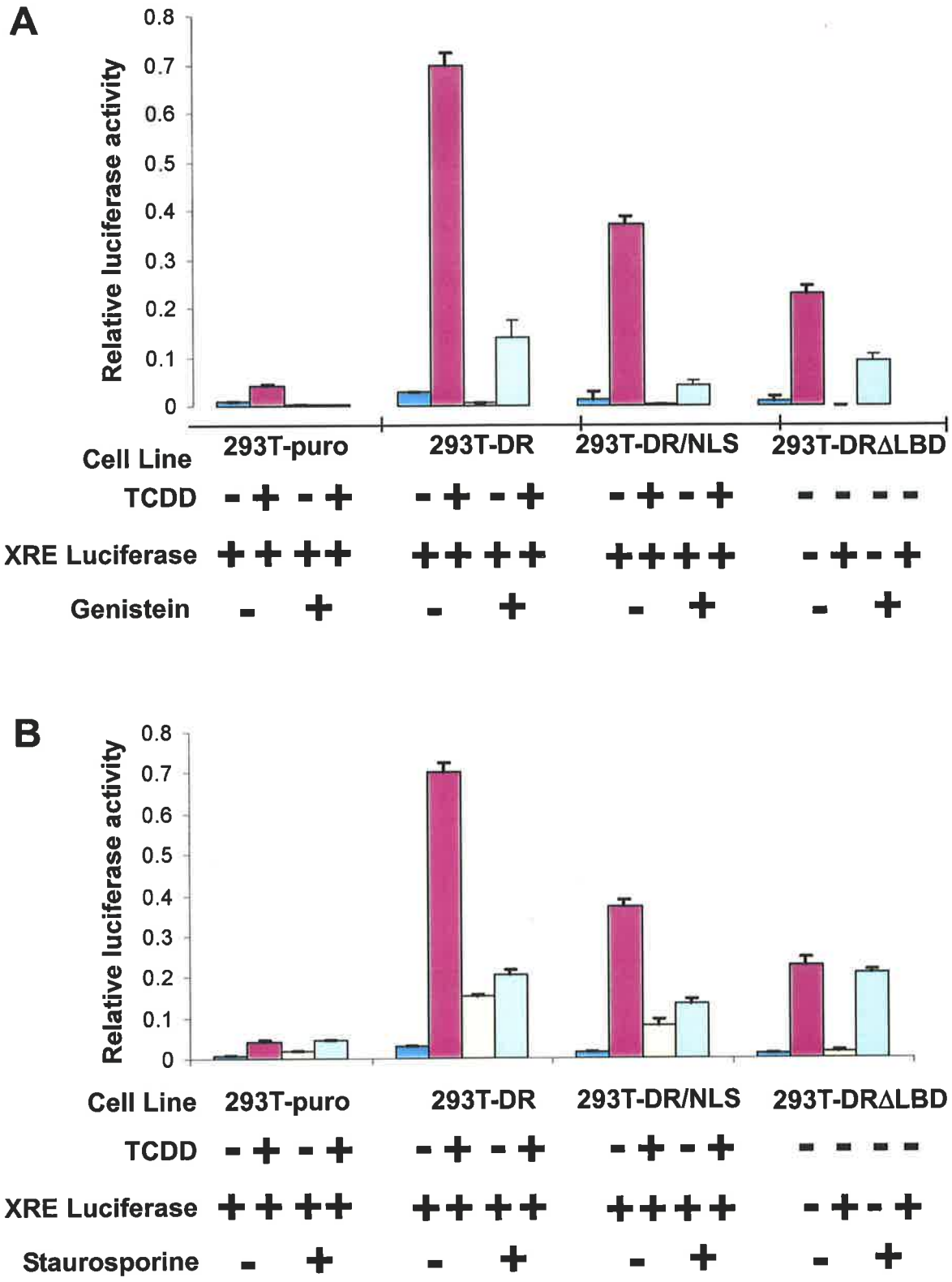


Figure 5.1. *Protein kinase inhibitor treatment of 293T stable cell lines.*
Stable cell pools were transfected with an XRE luciferase reporter or the minimal luciferase reporter lacking XRE sequences for 24 hours, followed by co-treatment with TCDD (10nM) or DMSO control in addition to genistein (100μM) **A**, or staurosporine (100nM) **B**, for 12 hours as indicated. Data presented are a ratio of relative firefly luciferase activity: renilla luciferase activity and are a representative experiment performed a minimum of 3 times in triplicate. Data are expressed as the mean -/+ S.E.

Results

Kinase inhibitor treatment of DR, DR-NLS and DRΔLBD stable cell lines

To address the relevance of phosphorylation in DR signalling we decided to utilise the stable cell lines expressing forms of the DR that mimic progressive stages of activation (Figure 4.1a). The rationale behind this approach was that having forms of the DR at progressive stages of activation would be useful in identifying phosphorylation changes pertinent to particular aspects of activation. For example, a phosphorylation event that is required to induce nuclear translocation in the unmodified DR would be bypassed by the DR-NLS (by means of an exogenous Nuclear localisation sequence). Thus, inhibiting this phosphorylation event by exposure to a kinase inhibitor would abrogate wtDR signalling but not DR-NLS signalling. In addition, using this approach it is theoretically possible to identify phosphorylation events required to dissociate the chaperone machinery from the DR by comparing differences between the DR-NLS construct and the DRΔLBD construct. Using such an approach it was hoped to identify differences in activities of the modified DR's following treatment with a range of protein kinase inhibitors.

The results presented in Figure 5.1 demonstrate that treatment with the tyrosine kinase inhibitor genistein diminishes DR signalling in all three cell lines (Figure 5.1a). This is in agreement with previous studies using keratinocytes, which demonstrated that genistein similarly affects the inducibility of both CYP1A1 and reporter constructs (Gradin *et al* 1994). The results presented in Figure 5.1 show that the DRΔLBD construct is affected by genistein treatment to a comparable level as the full length receptors. This differs somewhat from the model proposed by Gradin et al (Gradin *et al* 1994) which posits that genistein prevents release of hsp90, in which case the non-

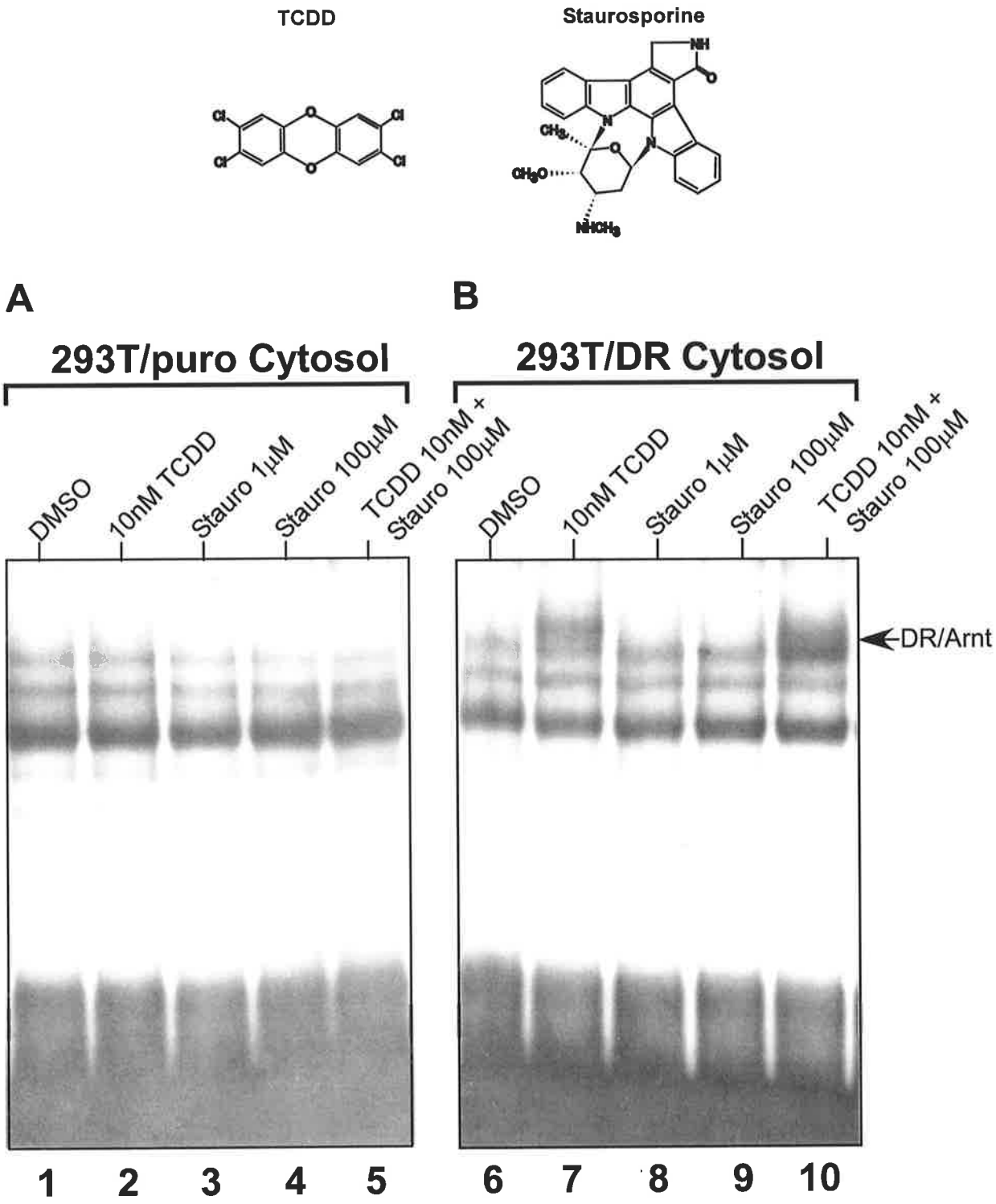


Figure 5.2. *Staurosporine does not transform the mDR into a DNA binding form in vitro.* Cytosolic extracts from 293T cells (**A**) or 293T/DR cells (**B**) were treated with DMSO control, TCDD (10nM), staurosporine (1μM or 100μM) or a combination of TCDD and staurosporine (10nM and 100μM, respectively) as indicated for 30 minutes prior to incubation with a ^{32}P labelled XRE probe and separation via 5.5% non-denaturing PAGE. The structures of TCDD and staurosporine are provided.

hsp90 binding DRΔLBD would be impervious to this problem. Strikingly however, when cells were treated with the broad spectrum kinase inhibitor staurosporine, both the DR and DR-NLS constructs increased reporter gene output in a TCDD independent fashion (Figure 5.1b). Upon co-treatment with staurosporine and TCDD, the reporter output was attenuated and did not reach the levels of cells treated with TCDD alone, rather the luciferase output reached identical levels as for staurosporine treatment alone. In parallel experiments, the DRΔLBD construct did not display the same attenuated reporter output in response to staurosporine treatment. This implies that staurosporine does not prevent phosphorylation events important for nuclear translocation, DNA binding or transactivation, but somehow inhibits the full ligand induced transformation of the DR, whilst being able to partially activate the DR in the absence of ligand.

In order to address whether staurosporine is acting as a ligand for the DR, cytosolic extracts from 293TDR cells were mixed with vehicle, 10nM TCDD, either 1μM or 100nM staurosporine or 1μM staurosporine in combination with 10nM TCDD. Incubation of the cytosolic extracts with TCDD alone produced the characteristic DR/Arnt/XRE band (Figure 5.2b compare lanes 6 and 7). Treatment with staurosporine at either 1μM or 100 μM failed to produce an inducible DNA binding species, indicating that staurosporine does not act as a conventional ligand for the DR. Treatment with TCDD in combination with staurosporine generated the same DNA binding species as TCDD alone. Identical conditions to those described above were used from cell extracts from 293T control cells, which as expected failed to produce the observed DNA binding species with any of the treatments (Figure 5.2a).

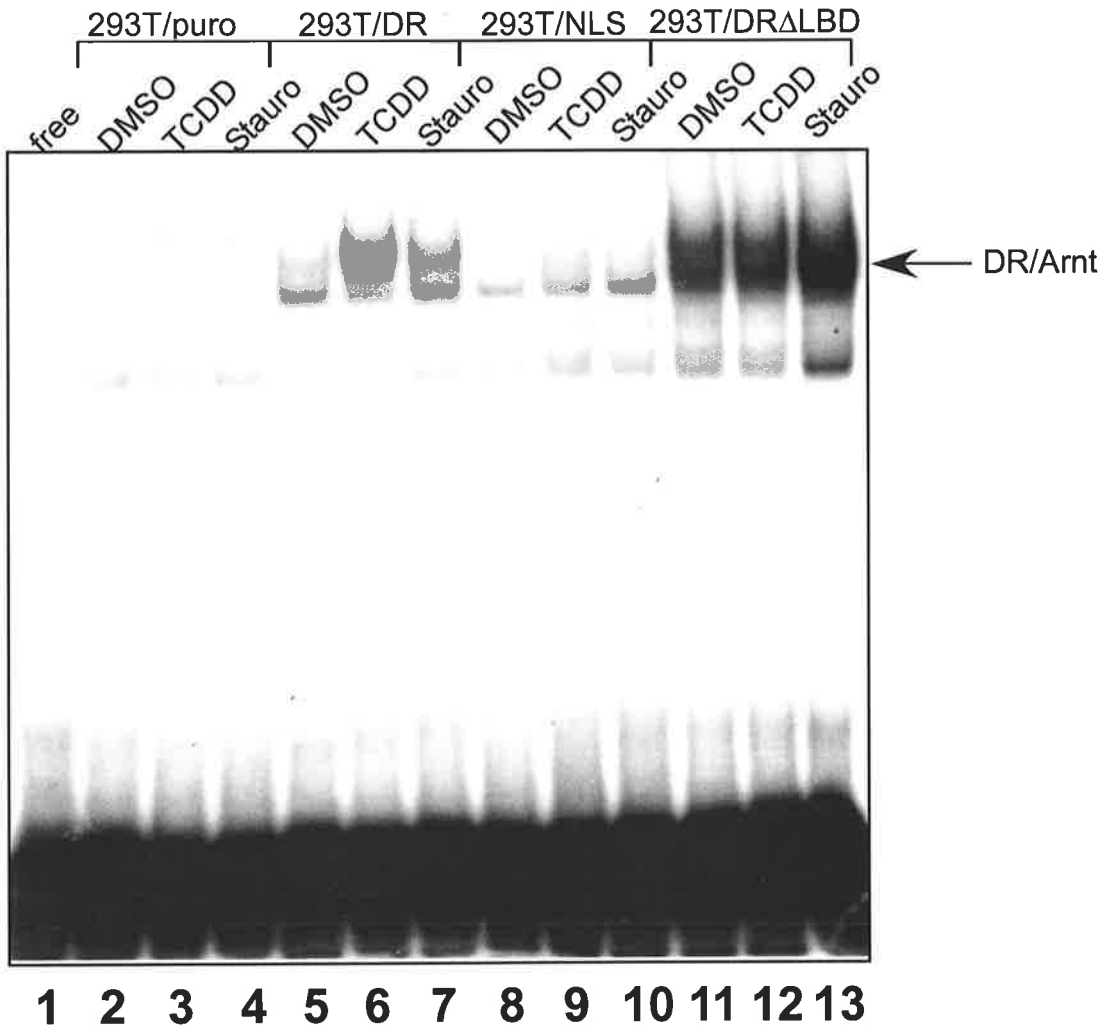


Figure 5.3. *Staurosporine transforms the DR and DR-NLS constructs to a DR/ARNT binding heterodimer but does not effect the DRΔLBD construct.* Nuclear extracts from the indicated stable cell lines treated for 2 hours with DMSO, TCDD (10nM) or staurosporine (100nM) were incubated with a ^{32}P labelled XRE and separated by 5.5% non-denaturing PAGE. The position of the relevant DR/ARNT constructs is shown.

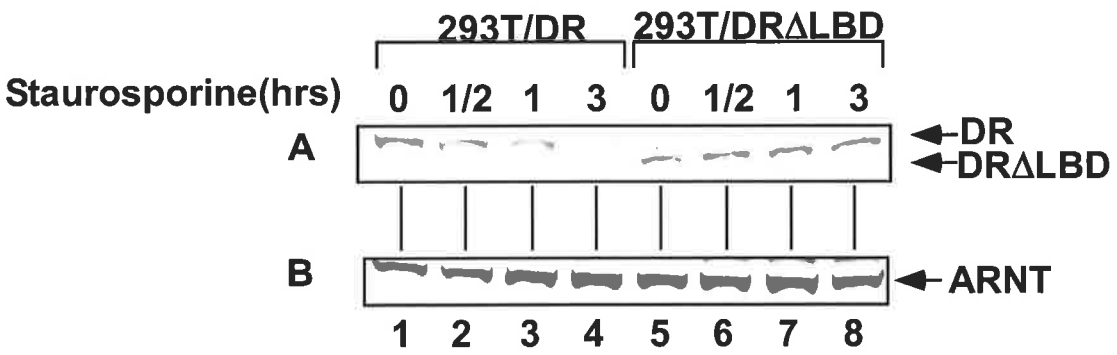


Figure 5.4. *Staurosporine induces degradation of the DR but not DRΔLBD construct.* 293T/DR or 293T/DRΔLBD stable cells were treated for the indicated time with 100nM staurosporine. Whole cell extracts (50μg) were separated by 10% SDS-PAGE and immunblotted with antibodies directed against the DR (**A**) or blots were stripped and reprobed with αARNT antibodies (**B**) as a loading control. The position of the relevant DR constructs and ARNT are indicated.

Staurosporine induces a DR/Arnt DNA binding complex in cells

To further investigate the response following staurosporine treatment, gel shift assays were performed, using nuclear extracts from staurosporine treated cells. Figure 5.3 demonstrates that as a result of staurosporine treatment of the 293T-DR cell line, a weak XRE DNA binding complex is induced which is identical in mobility to the complex induced with TCDD (Figure 5.3 compare lane 5 to lanes 6 and 7), confirming that several of the features of the activation of the DR pathway namely nuclear translocation, Arnt dimerisation and DNA binding are preserved with protein kinase inhibitor treatment. In an identical fashion, the 293T/DR-NLS generated a DNA binding complex from TCDD or staurosporine treated cells (Figure 5.3 compare lanes 9 and 10 to lane 8), albeit at a reduced level of binding. It is unclear as to why this is the case as the expression levels between the cytosolic and nuclear proteins appears identical (Figure 4.1b). Neither TCDD nor staurosporine had any effect on DNA binding by the DRΔLBD construct (Figure 5.3 lanes 11-13). Treatment of the 293T parental cell line failed to induce either a TCDD or staurosporine induced XRE binding complex (Figure 5.3 lanes 2-4).

Staurosporine induces degradation of the DR but not DRΔLBD construct

A further hallmark of DR activation is a decreased half-life compared to the latent DR (Roberts and Whitelaw 1999, Ma and Baldwin 2000). To explore whether staurosporine activation of the DR undergoes a similar fate, a time course experiment was performed which monitored DR protein levels following staurosporine treatment. Western analysis using an αDR antibody shows that the DR is degraded following addition of staurosporine to the culture medium (Figure 5.4a compare lanes 1-4), consistent with previous reports using murine Hepa1c1c7 cells (Singh and Perdew 1993). In contrast

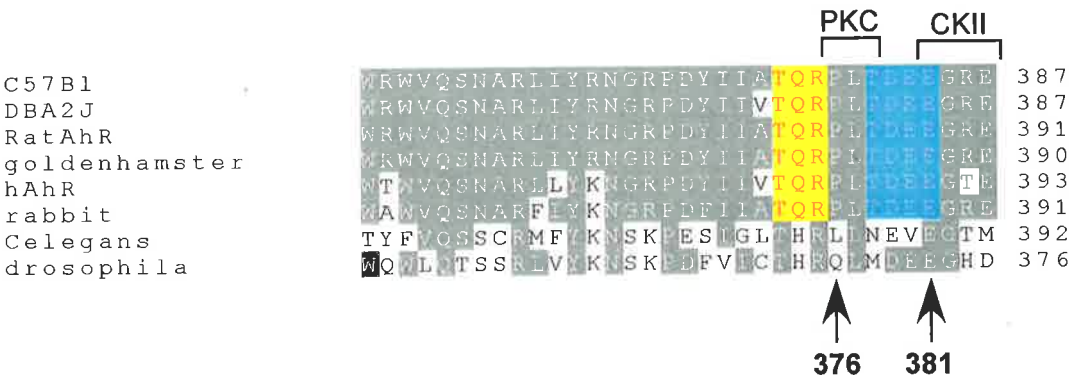


Figure 5.5. *Species conservation of putative protein kinase sites between ligand binding DR's.* The consensus site for Protein Kinase C is X-S/T-X-R/K where S/Tis the phosphorylated residue, whilst the consensus for Casein Kinase II is X-S/T-X-X-D/E + acidic residues at +1,+2 (C-terminal to phosphorylated S or T residue). The Threonine residues mutated using site directed mutagenesis (376 and 381) are indicated.

however, levels of the DRΔLBD construct remained constant for the identical time points observed for the wt DR construct (Figure 5.4a compare lanes 5-8). The blots were stripped and reprobed with αArnt Antibodies to act as a control to ensure equal protein loading, and also to investigate whether any of the observed effects could be attributed to altered Arnt levels. Figure 5.4b demonstrates that Arnt levels remain unchanged throughout the timecourse. Taken together with the transient transfection data, these results suggest that the DR can be weakly activated by treatment with a protein kinase inhibitor and as *in vitro* gelshift assays failed to generate an XRE binding complex, the effect of staurosporine is presumably due to the protein kinase inhibitory effects of this chemical.

Taken together these results imply that staurosporine can mimic ligand activation of the DR. This activation however is only partial and could not recapitulate full TCDD induced activation of the DR in reporter assays. Furthermore, co-treatment with staurosporine in combination with TCDD dampened TCDD signalling. This is consistent with a model whereby staurosporine partially activates the DR by inhibiting an unidentified kinase event either in the DR itself or upstream of the DR pathway.

Mutational analysis of the DR LBD region

A comparison of either the non modified DR or the DR-NLS construct with the DRΔLBD construct indicated that a region within the LBD, (between amino acids 287-421) is sensitive to staurosporine treatment. In an attempt to identify potential sites of phosphorylation within this region of the DR, the primary amino acid sequence was subjected to analysis by Net Phos (http://www.cbs.dtu.dk/services/NetPhos/). From this, a putative protein kinase C site at 376 and a putative Casein Kinase II site at 381 in the mouse DR were identified (Figure 5.5) that were reasonably well matched to the

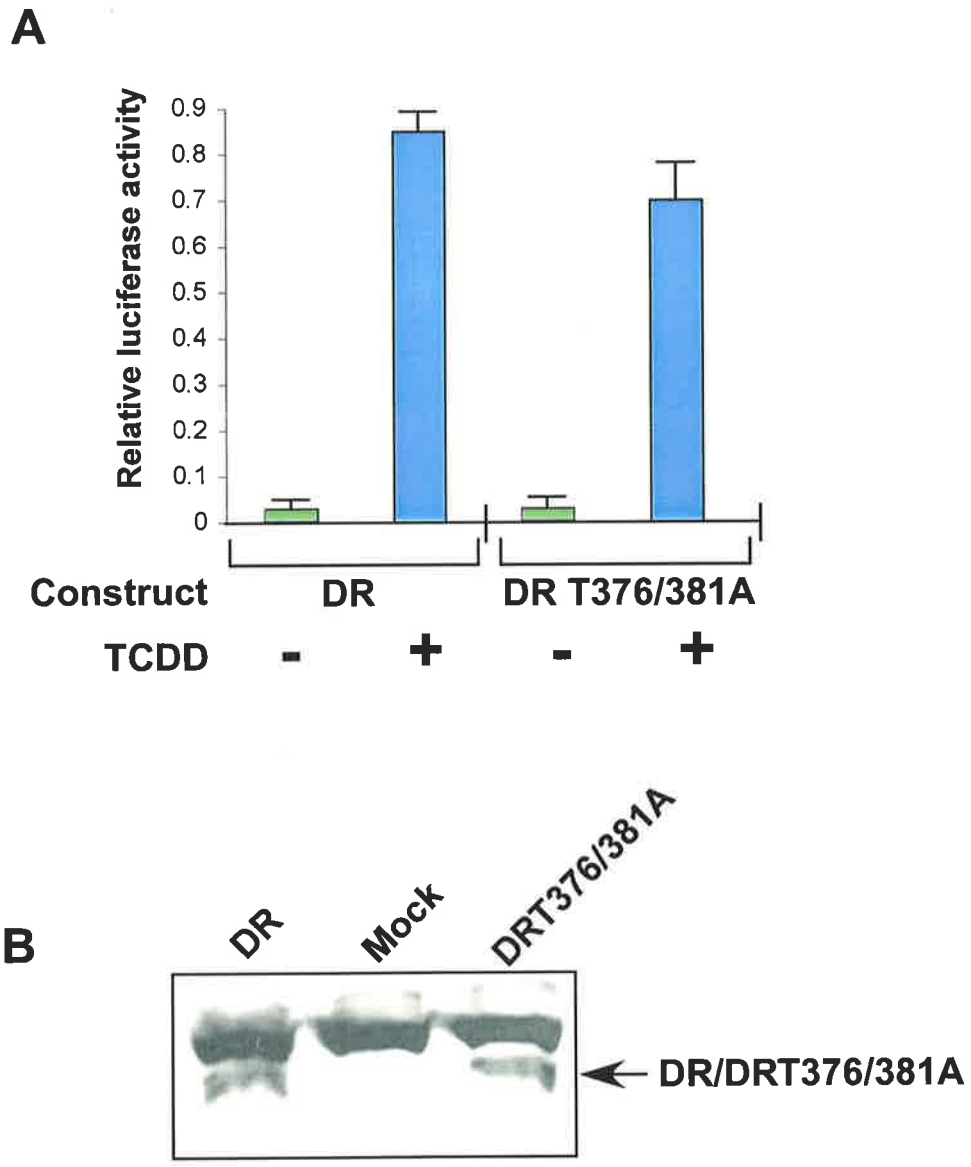


Figure 5.6. *Activity and expression of the DRT376/381A mutant protein.* (**A**) DR or DRT376/381A expression constructs were transiently transfected in addition to the XRE-luciferase reporter for 24 hours followed by treatment with DMSO or TCDD (10nM) for 12 hours. Data are presented as the average -/+ SE of three experiments performed in triplicate. (**B**) Whole Cell Extracts (30μg) from 293T cells either mock transfected or transfected with the DR or DRT376/381A mutant construct for 36 hours were separated by 10% SDS-PAGE followed by immunoblotting with an αDR antibody. The position of the DR or mutant protein is indicated.

consensus sequence, exhibiting scores of 0.911 and 0.75, respectively, whereby a score of 1 is considered a theoretically perfect consensus. The threonine residue at position 376 was of particular interest as this is adjacent to an important valine at position 375. Mutation of valine375 to alanine dramatically decreased the ligand binding properties of the DR (Ema *et al* 1994) and hence is positioned at a critical site in the DR which could be sensitive to structural changes (Procopio *et al* 2002). These two amino acids were interesting also as they are located within the cyanogen bromide/hydroxylamine cleavage fragment (368-605) identified previously to contain a phosphorylated residue(s) (Mahon and Gasiewicz 1995). The two threonine residues at these positions were mutated to alanines by site directed mutagenesis and the resulting construct was transiently transfected into HEK 293T cells followed by an overnight treatment with 10 nM TCDD or vehicle alone, and compared to the activity of the wildtype construct. Figure 5.6a demonstrates a similar reporter gene profile between the two constructs both in terms of ligand dependency and basal activity. Furthermore, Western analysis of the transfected constructs demonstrates no substantial difference between the level of expression between the two proteins (Figure 5.6b). Taken together, these results imply that the predicted kinase sites either do not function as kinase sites, or if they do they are not crucial to the DR activation process in the context of a transient transfection assay.

Traditionally staurosporine has been considered a protein kinase C inhibitor, but at the concentrations used in these assays, staurosporine could also be inhibiting CaM kinase (IC_{50}=20nM), myosin light chain kinase (IC_{50}=1.3nM), protein kinase A (IC_{50}=7nM), protein kinase G (IC_{50}=8.5nM) in addition to protein kinase C (IC_{50}=0.7nM). As a means of further exploring whether the effects of staurosporine on DR signalling are mediated through a PKC dependent mechanism, transient transfection assays were established in conjunction with Bisindolylmaleimide I, a highly selective protein kinase

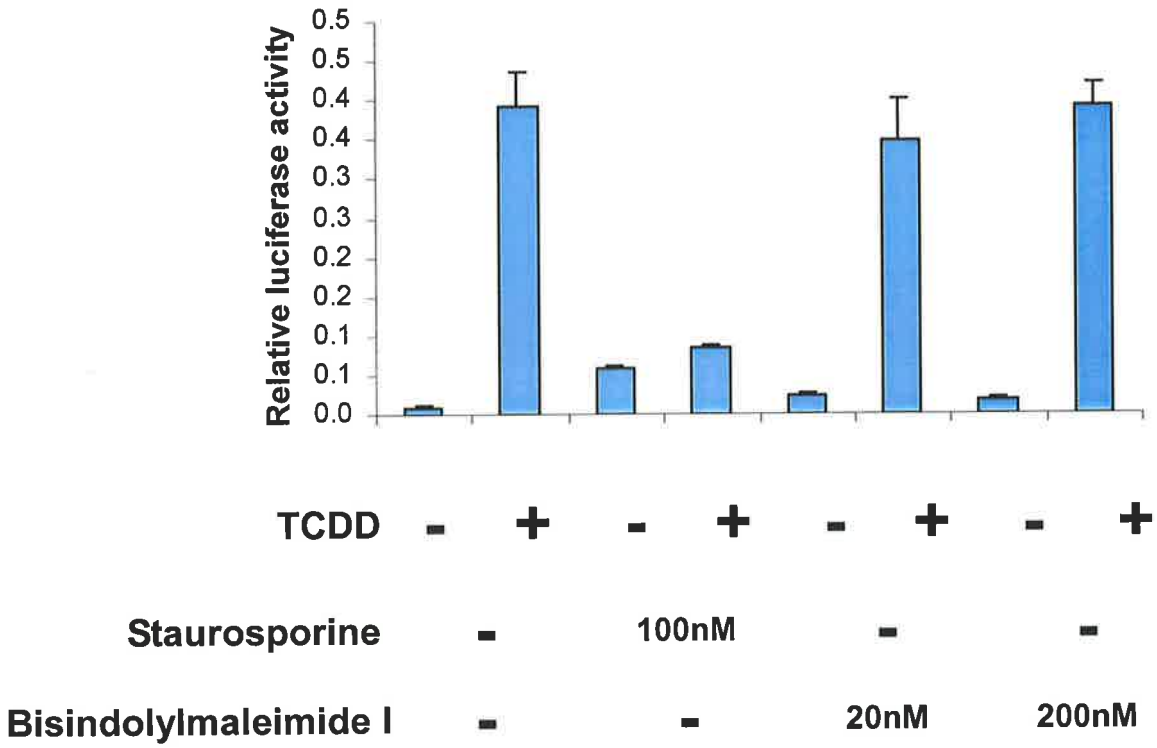


Figure 5.7. *The Selective PKC inhibitor Bisindolylmaleimide, fails to activate the DR.* 293T/DR cells were transfected for 24 hours with the XRE-luciferase reporter and reinella control, followed by overnight treatment with DMSO or TCDD (10nM) and the indicated kinase inhibitor at the indicated concentrations. Firefly luciferase activity was normalised to renilla luciferase activity and data are presented as relative activity. Experiments were performed in triplicate and data represents a typical experiment -/+ S.D., which was performed three times.

C inhibitor, which acts as a competitive inhibitor for the ATP binding site of this enzyme. Bisindolylmaleimide I is marketed by Calbiochem as showing high selectivity for PKC α, βI, βII, γ, δ and ε isoenzymes. This PKC inhibitor when used in transient transfection assays was unable to reproduce the effects of staurosporine (Figure 5.7), both in terms of providing a ligand independent activation of the reporter and also in the inability of this chemical to ameliorate TCDD induction of the luciferase reporter. These data in combination with the mutational analysis suggests that in 293T cells the effects of staurosporine are not mediated through a PKC dependent mechanism.

In order to define more directly the phosphorylated residues of the DR several alternative approaches were performed. Initial attempts were made to express and purify an LBD fragment, nominated as amino acids 230-421 (Coumailleau *et al* 1995) in a bacterial system. However, the protein product that was expressed as a thioredoxin fusion protein was insoluble and could only be recovered in strong denaturing conditions (8M urea), presumably this is due to a strong reliance of the LBD to be chaperoned by hsp90 and neither the bacterial hsp90 homologue HtpG nor chaperone protein homologues (DNA K and J) are able to perform an adequate chaperoning task. The protein product which was purified using Ni/Nta resin under denaturing conditions was resuspended in a kinase buffer supplemented with γ^{32}P-ATP and containing 293T cytosolic extracts and used in *in vitro* kinase assays in an attempt to identify potential phosphorylation events in both the presence and absence of ligand. This, however, was not a successful approach (data not shown), presumably because the LBD does not refold correctly on returning the LBD to non-denaturing conditions. Potentially the refolding process requires crucial interactions with the appropriate chaperone proteins with the nascent polypeptide chain, and the omission of these interactions precludes the bacterially expressed LBD fragment from being phosphorylated in an *in vitro* context.

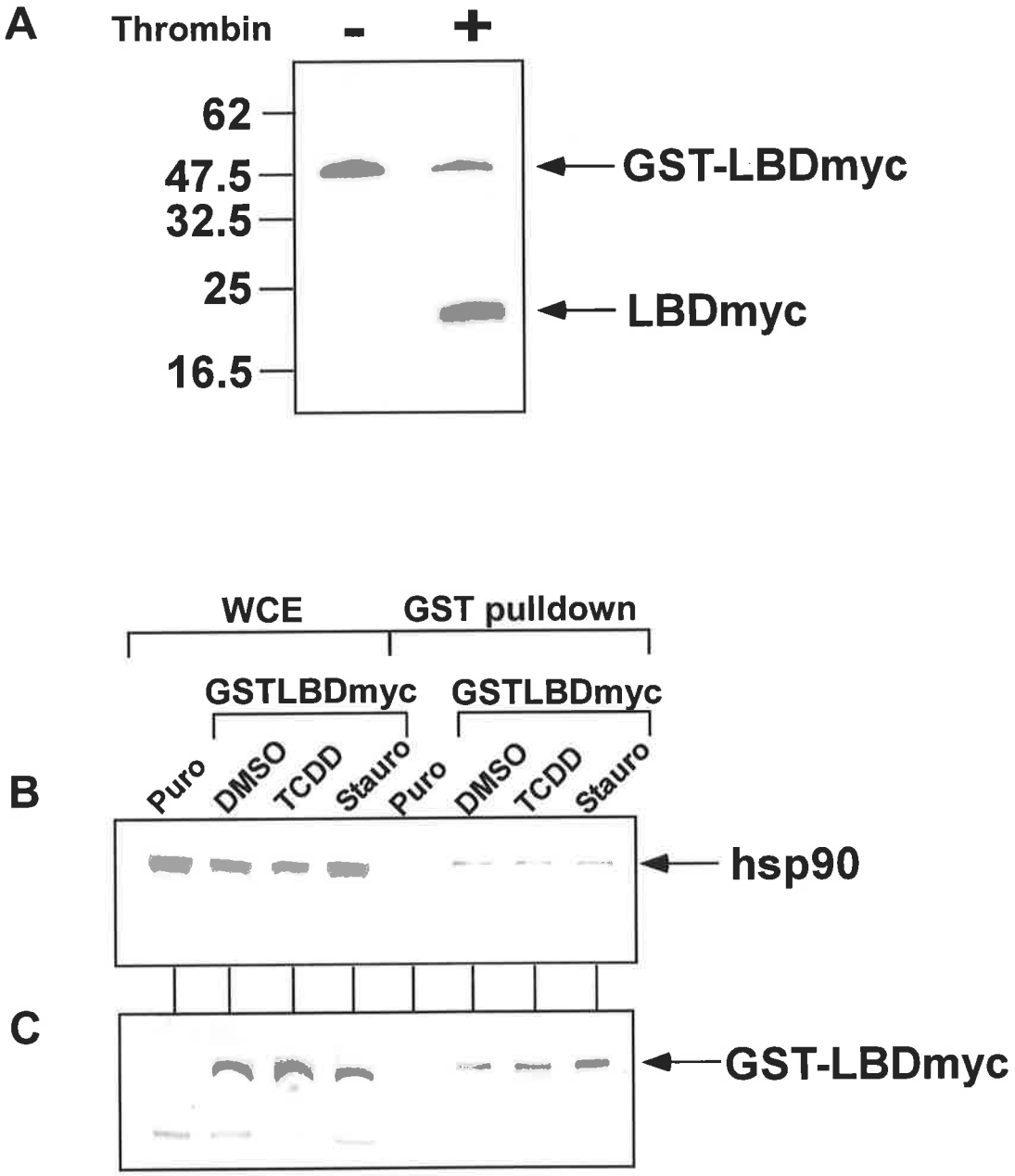


Figure 5.8. *Expression, purification, thrombin cleavage and Hsp90 binding characteristics of the GSTLBDmyc fusion protein.*(**A**) Whole cell extracts (50μg) from 293T/GST-LBDmyc cells were GST purified and incubated in the presence or absence of thrombin as indicated. Samples were released from the resin by boiling in SDS load buffer followed by immunblotting and analysis with the αmyc antibody. (**B**) Whole cell extracts (300μg) from 293T Puro cells or 293T/GST-LBDmyc cells treated with DMSO, TCDD (10nM) or Staurosporine (100nM) for 2 hours, were purified using glutathione-agarose, and eluted from the resin by boiling in SDS load buffer. 50% of the sample was separated by 10% SDS-PAGE in addition to 10 μg of whole cell extract for immunoblotting with an αhsp90 mAb (B) or an αmyc mAb (C). The position of hsp90 or the GSTLBDmyc fusion construct is indicated.

In vivo labelling of a minimal DR LBD fragment

To investigate by more direct means whether the phosphorylation status of the LBD alters in response to ligand, 293T-DR cells were stably transfected with a minimal LBD construct that has been previously demonstrated to bind TCDD and hsp90 *in vitro* (Coumailleau *et al* 1995) for analysis initially by *in vivo* labelling experiments using radiolabelled ^{32}P, and as a development towards a large scale purification system to perform analysis by mass spectrometry. The 230-421 fragment was fused in frame with the GST fusion protein along with a C-terminal myc epitope tag and then subcloned into the pEF/IRES/puro mammalian expression vector. Previously, the GST fusion system has been shown to be highly successful in the large scale purification of eukaryotic proteins which have been problematic to express in bacteria due to either size restrictions or the requirement for eukaryotic chaperones which are lacking in lower expression systems. 293T cells were again the cell system of choice, due to their suitability of having low levels of endogenous DR protein and are adequately able to restore ligand dependent DR signalling upon transfection of full length constructs into the cell line (Figure 5.1). Following puromycin resistance for a 10 day period a pool of cells was harvested and analysed for expression of the myc tagged construct (Figure 5.8a). Western analysis of whole cell extracts from the stable cell line purified by GST affinity chromatography demonstrated a polypeptide fragment of the predicted size of 47 kDa, which could be cleaved by thrombin to liberate the 22 kDa LBD fragment (Figure 5.8a) demonstrating the usefulness of this cell line in the purification of the LBD fusion protein in a native state. The 230-421 fragment has been defined previously as the minimal region necessary to obtain hsp90 binding characteristics *in vitro* in addition to displaying a virtually identical ligand binding profile as the full length DR (Coumailleau *et al* 1995). GST pull down assays followed by counterstaining with antibodies against hsp90 showed that hsp90 could indeed be co-precipitated with the

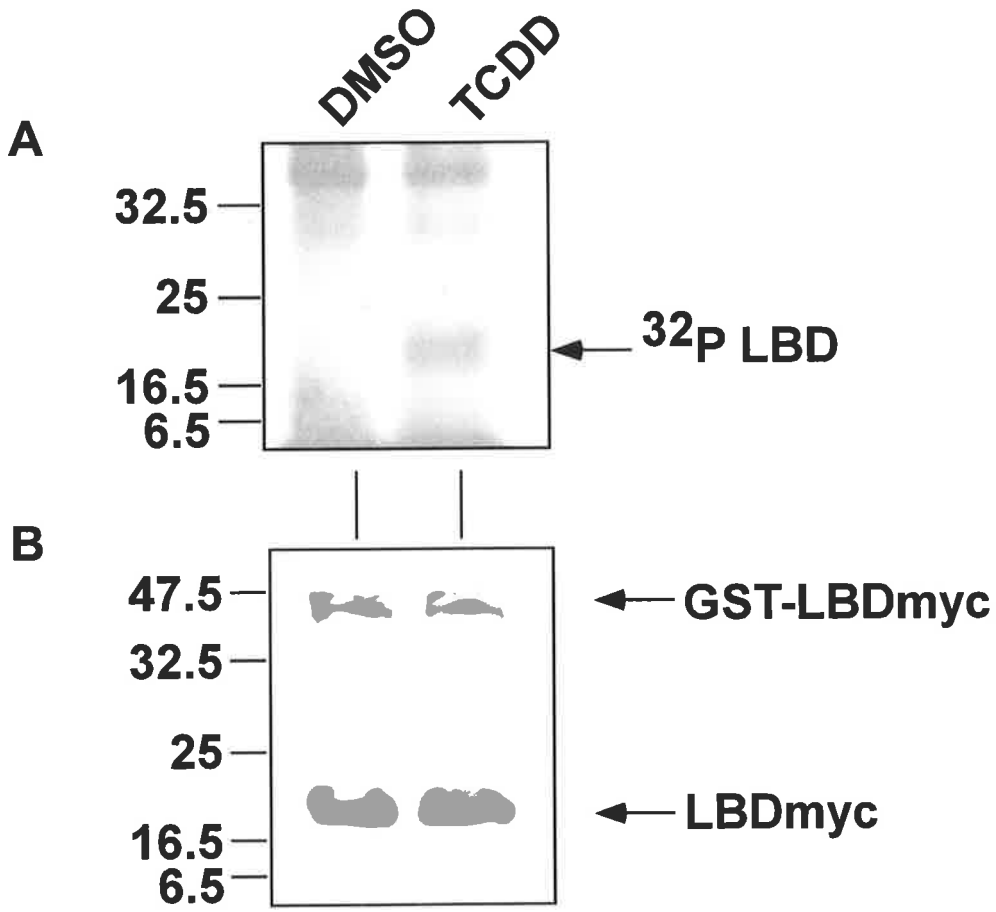


Figure 5.9. *In vivo labelling of the LBDmyc construct.* 293TGSTLBDmyc cells were *in vivo* labelled with ^{32}P inorganic phosphate for 6 hours prior to treating with DMSO or TCDD (10nM) for 2 hours. Whole cell extracts were purified using glutathione-agarose followed by thrombin cleavage and boiling in SDS load buffer prior to separation by 12.5% SDS-PAGE and autoradiography of the dried gel (**A**). Aliquots of the purification were separated by 12.5% SDS-PAGE and analysed by immunoblotting with the αmyc mAb to ensure equivalent purification (B). The position of the LBDmyc and GSTLBDmyc fusion proteins are indicated.

LBD fusion construct (Figure 5.8b), however this interaction was not affected by either TCDD or staurosporine treatment (Figure 5.8b). This is in good agreement with previous experiments which suggest that Arnt is required for a concomitant release of hsp90 through DR/Arnt heterodimerisation (Chapter 3, J. McGuire unpublished data). Likewise, the stability of the GST-LBD fusion construct was not affected by either of these treatments (Figure 5.8c), implying that whilst the LBD may mediate staurosporine induced degradation of the full length DR the LBD itself is not targeted directly for degradation. By developing a stable cell line expressing a form of the LBD which could be purified in a native form bound to the major chaperone required for ligand binding competency, it was hoped that several assays could be performed to analyse the effect of staurosporine and ligand induced post translational modifications within this fragment.

To explore whether this region of the DR was phosphorylated, *in vivo* labelling experiments were performed using the GST-LBD expressing cell line. Cells were labelled for 6 hours in phosphate free serum containing media in the presence of ^{32}P labelled inorganic phosphate, followed by a two hour treatment with 10nM TCDD or vehicle alone. Following treatment, whole cell extracts were made and subjected to GST purification and extensive washing. Subsequently, the purified fragment was cleaved with thrombin to remove the GST tag and release the LBD fragment. The agarose slurry was then boiled in SDS sample buffer and the eluate separated by 12.5% PAGE and the dried gel subject to autoradiography. An aliquot of the thrombin digest was analysed separately by Western analysis using the α-myc antibody to verify equal purification and loading. Figure 5.9a displays the resultant autoradiogram from the purification and a band of approximately 22kDa is observable following TCDD treatment. Western analysis (Figure 5.9b) in addition to background phosphorylation bands in the autoradiogram indicate that no appreciable differences existed during the

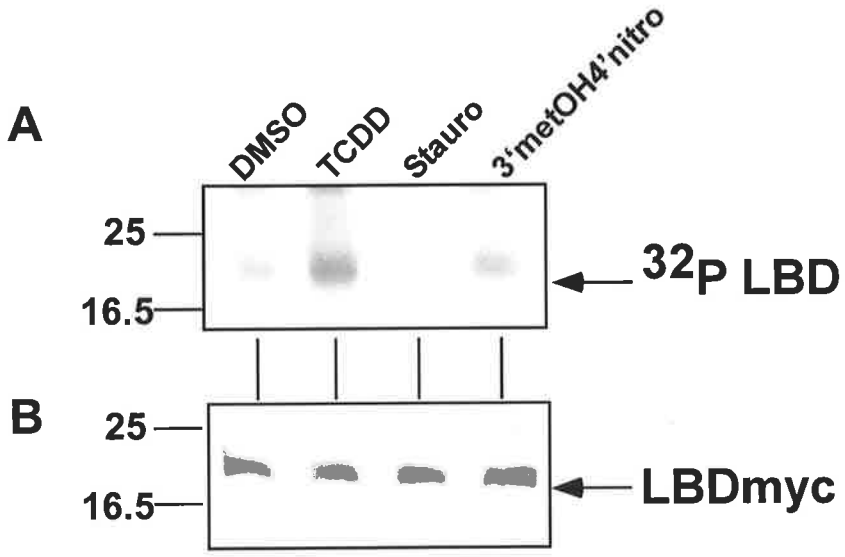


Figure 5.10. *Staurosporine inhibits in vivo labelling of the LBDmyc construct.* 293TGSTLBDmyc cells were *in vivo* labelled with ^{32}P inorganic phosphate for 6 hours prior to treatment with DMSO, TCDD (10nM), Staurosporine (100nM) or the DR antagonist 3'methoxy, 4' nitroflavone (10nM) for 2 hours. Whole cell extracts were purified using glutathione-agarose, followed by thrombin cleavage, boiling in SDS load buffer and separation by 12.5% SDS-PAGE for autoradiography (**A**) or an aliquot of the purification was analysed by immunoblotting with the αmyc mAb (B). The position of the LBDmyc construct is indicated.

extract procedure and that the difference in phosphorylation in the 22kDa band by TCDD is bona fide. To investigate the effect of staurosporine on this minimal LBD fragment the labelling procedure was repeated and cells were treated with staurosporine. Unsurprisingly, background labelling of cellular extracts was diminished upon treatment with the kinase inhibitor. In addition to this however, specific labelling of the 22kDa band which is observed in TCDD treated extracts (Figure 5.10a lane 2) but not untreated extracts (Figure 5.10a lanel) is also diminished. Several points can be made of this result. Firstly, if staurosporine is inhibiting a kinase event which leads to activation of the DR through changes in the LBD, then these changes appear to be distinguishable from the phosphorylation induced changes (as judged by increased incorporation of radiolabelled phosphate) induced upon TCDD treatment. Secondly, if in the unlikely scenario that staurosporine is acting as a ligand for the DR then it is unable to elicit the corresponding changes in phosphorylation in the DR as TCDD. To extend this further, the stable cell line was also treated with 3'methoxy 4' nitroflavone, a competitive antagonist for the DR, which is able to bind to the DR but is unable to induce transcriptional activation (Lu *et al* 1995, Lu *et al* 1996, Henry *et al* 1999). Figure 5.10a (lane 4) indicates that this antagonist can invoke similar changes in the phosphorylation status of the LBD as TCDD, providing further indirect evidence against staurosporine acting as a ligand for the DR. Aliquots of these purifications were analysed for efficiencies in purification procedure via western analysis, and this is verified in Figure 5.10b showing that no substantial differences in the level of protein purified occurred. These results indicate that a phosphorylation event occurs within the LBD as a result of ligand binding. Previous data have shown that the net phosphorylation status of the full length DR doesn't change in response to TCDD (Mahon and Gasiewicz 1995) or staurosporine treatment (Singh and Perdew 1993). However, this could reflect a balance between phosphorylation and dephosphorylation

kinase/phosphatase networks, a process which is not recapitulated by the expression of the smaller fragment of the DR.

Thus we have undertaken to develop a large scale purification strategy to analyse the DR and post translational modifications using more direct and contemporary means such as mass spectrometrical analysis. This approach has many advantages over the methods previously described in that it can precisely identify the amino acids that are modified in addition to having the capacity to identify post translational modifications in addition to phosphorylation. To this end the GST-LBD cell line is not suitable for such a purpose as the first purification step (ie a GST pulldown) fails to capture approximately 90% of the protein. This implies that whilst the LBD fragment that binds to the column is functional (as measured by its ability to bind hsp90 and undergo ligand induced phosphorylation changes), a large proportion of the protein contained in the extracts remains unfolded. To this end, the 230-421 construct has recently been expressed as a 6xHIS fusion construct to enable purification using strong denaturing conditions, which should facilitate large scale purification. Initial purification attempts indicate that this construct is more amenable to affinity purification than the GST fusion construct. However, subsequent purification steps have proved problematic (data not shown).

Discussion.

Whilst attempting to delineate post-translational modifications of the DR several interesting observations were made. The first of which was that the DRΔLBD construct was effected in a similar manner to the full length DR constructs in response to genistein treatment. The DRΔLBD construct does not bind hsp90 and thus as the activity of this construct is repressed by genistein this would suggest that genistein does

not effect DR signalling of the full length construct by inhibiting hsp90 release as previously proposed (Gradin *et al* 1994), but rather supports previously proposed models which predict that genistein effects a step subsequent to DNA binding (Carrier *et* al 1992, Park *et al* 2000). An unexpected result from these assays was that staurosporine induced nuclear translocation, DNA binding, transcriptional activation and DR degradation mimicking ligand activation of the DR. In our hands and others (Singh and Perdew 1993), staurosporine does not appear to be acting as a ligand for the DR, as it does not compete for [^{3}H]TCDD in ligand binding assays (Singh and Perdew) or promote DNA binding in *in vitro* assays. Mutational analysis in addition to reporter gene analysis in the presence of the specific PKC inhibitor Bisindolylmaleimide I suggests that staurosporine treatment is acting in a PKC independent manner. Potentially, staurosporine is acting to mimic ligand induction by interfering with a kinase/phosphatase pathway which normally maintains DR latency. This pathway, however, appears complex as *in vivo* labelling experiments indicate that this event is distinct from a ligand induced phosphorylation event (Figure 5.10). Ultimately, questions concerning the role of post translational modifications in DR activation will not be satisfactorily answered until comprehensive analysis of the protein by mass spectrometry techniques has occurred. Ideally this should be analysed in the context of the full length protein in the latent and ligand induced state, after which time the true effect that staurosporine has on DR activation can be defined. It was interesting that cytosolic extracts from 293T/DR cells were unable to generate a DR/Arnt DNA binding complex following treatment with staurosporine (Figure 5.2), in contrast to results obtained using Guinea Pig extracts (Schafer *et al* 1993). A potential explanation to account for these differences is that the experiments were performed using cytosolic extracts derived from different species. The guinea pig DR contains an extended C-terminus analogous to the human DR. Extending the C-terminal region of the mDR drastically alters its ligand binding properties (Ema *et al* 1994), such that ligand binding

affinity is reduced and the mDR acquires characteristics similar to the human DR. Thus it will be of interest to perform similar *in vitro* experiments using cytosolic extracts from human cell lines to test their responsiveness to staurosporine.

Final Summary

6

Chapter 6. Final Conclusions

The aim of this thesis was to investigate the activation mechanisms of the DR by addressing the following points. Firstly the role of nuclear compartmentalisation and whether nuclear localisation of a non ligand activated DR is sufficient to invoke DR activation. Furthermore by using the nuclear localised DR it was possible to investigate the effects of artificial transformation of the DR using the hsp90 inhibitor geldanamycin. Also under investigation was the XAP2 immunophilin type protein and its role as a chaperone in DR signalling. Finally, this thesis attempted to examine the effects of post translational modifications within the DR signalling pathway.

It was demonstrated that nuclear localisation of the DR in the absence of ligand is not sufficient to invoke DR activation. Ligand is required for steps beyond nuclear translocation, release of hsp90 being the preliminary step. Following publication of this work, similar findings were observed by Heid *et al* (Heid *et al* 2000), using a separate approach. This study utilised sodium molybdate, a chemical known to stabilise hsp90/substrate interactions. They demonstrated that stabilising the DR/hsp90 complex with molybdate treatment and then treating with agonist could invoke nuclear translocation of the DR but was insufficient to invoke hsp90/DR dissociation and DR/Arnt dimerisation. However by treating cells with geldanamycin we were able to show that by artificially removing hsp90, the DR forms a heterodimer with Arnt which is competent to bind DNA. However, this artificially invoked heterodimer was unable to activate transcription. This suggests that ligand plays a role in maintenance of functional conformation of the DR, presumably within the LBD, which is required to activate transcription. Interestingly, by treating with a kinase inhibitor, it was possible to partially activate DR induced transcription. Whether this is indicative of a key phosphorylation event within the LBD that can partially mimic an activated structural

integrity remains to be seen. Ultimately, these questions will not be answered until analysis of the protein by mass spectrometry has been performed in combination with structural data to understand the relevance of post-translational modifications. Until recently, this would have appeared an almost impossible task, given the problems of DR expression in prokaryote systems. However, the crystal structure of the GR has recently been solved by mutation of a single amino acid which dramatically improved solubility in a bacterial system (Bledsoe *et al* 2002).

The role of the DR specific co-chaperone XAP2 was also investigated in this study. Prior to the initiation of this work, XAP2 had been demonstrated to stabilize the DR and increase DR transcriptional output in transfection based experiments. Subsequently the model has been extended to include a role for XAP2 in cytosolic localisation of the DR. The present work supports the model of XAP2 stabilisation of the DR. However, by showing the susceptibility of the DR-NLS protein to XAP2 depletion, this suggests that the cellular localisation effect of XAP2 on the DR is secondary and that the primary function of XAP2 is to stabilise the DR. Combining the observation that XAP2 stabilises the DR/hsp90 complex, and artificial stripping of hsp90 from the DR leads to rapid destabilisation, this model appears feasible. Further to this, preliminary evidence suggests that the E3 ligase protein CHIP can destabilise the DR. This supports a model whereby CHIP and XAP2 compete for a docking site on hsp90 and the decision of whether the DR gets degraded or is maintained in an hsp90 chaperoned state is dictated by the presence of XAP2 in the complex. However, more experimental evidence needs to be provided to support this competition theory, including the demonstration that XAP2 can indeed out compete CHIP and perhaps the use of XAP2/TPR mutations known to abolish DR interaction to test the ability of these constructs to out compete CHIP. Furthermore, it will be crucial to examine whether CHIP promotes DR degradation via the ubiquitin proteasome pathway. Precedent with other CHIP

substrates would suggest that this might be the case, however, it remains to be demonstrated for the DR.

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Chapter 7. References

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