NIEMKIEWICZ	CHARACTERIZATION OF THE AF-1 ACTIVITY OF mPPARα AND mPPARγ2 SUBTYPES	M.Sc.

CHARACTERIZATION OF THE AF-1 ACTIVITY OF mPPARα AND mPPARγ2 SUBTYPES

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Abstract

Peroxisome Proliferator-Activated Receptors (PPARs) belong to the nuclear receptor superfamily of transcription factors. PPARs, like some other nuclear receptors, bind their cognate DNA binding sites as heterodimers with Retinoid X Receptor (RXR). Like most members of this superfamily, PPARs regulate transcription of target genes in response to specific lypophilic ligands. Given the fact, however, that many members of the nuclear receptor superfamily possess a ligand independent activation function (AF-1) in their amino-terminal A/B domains, we identified and characterized the AF-1 activity in the A/B domains of mPPARα and mPPARγ2. When fused to the GAL4 DNA Binding Domain (DBD), the A/B domains of both mPPARα and mPPARγ2 subtypes are transcriptionally active both in mammalian and yeast cells in the absence of cognate PPAR activators, indicating that both PPAR subtypes do in fact contain AF-1 activities in their respective A/B domains. Our deletion studies localizing the region in the A/B domains of the PPAR subtypes indicate that the amino-terminal region of A/B domains of both PPAR subtypes is necessary, but not sufficient, for the AF-1 activity.

After having identified and characterized the AF-1 activities of both mPPARα and mPPARγ subtypes, we proceeded to investigate how this activity is controlled. Generally, nuclear receptors, including PPARs, do not activate transcription when ligand is absent, suggesting that the ligand-independent AF-1 activity is somehow masked. We used the GAL4 DBD to investigate this phenomenon. When fused to GAL4DBD, the full-length mPPARα receptor is not active in mammalian cells in absence of ligand

despite the presence of the AF-1 activity in the A/B domain. Only when the PPAR activator Wy-14 is added, does the receptor become active. Carboxy-terminal deletion of the fusion at the junction of the DBD (C domain) and the Hinge region (D domain) results in partially active protein whose activity does not depend on the presence of ligand, suggesting a role of the hinge region in the masking of the AF-1 activity.

The transcriptional activity of PPARs is ligand independent when PPARs are expressed as GAL4 DBD fusions or allowed to bind reporter genes as dimers with RXR. Yeast do not possess nuclear receptors or any homologues of known accessory proteins which interact with nuclear receptors. Given our observations and those of others made in yeast and mammalian cells, we suggest that the masking of the AF-1 activity occurs in the inactive state of the receptor, and that mammalian cells contain unidentified factor(s) which is able to maintain PPARs in the inactive state, thus enabling the masking of the AF-1 activity.

Nuclear receptors including PPARs are known to interact with a host of accessory proteins which modulate their activity and are thought to act as a bridge between the receptors and the basal transcription machinery. One of these proteins is RIP140 whose function has not been determined. Our studies suggest that RIP140 is a co-activator for the PPAR/RXR heterodimer, as well as for the AF-1 activity of mPPARα and mPPARγ2.

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List of Abbreviations

ACTR	Activator of the thyroid and retinoic acid receptor
AF-1	Activation Function 1
AF-2	Activation Function 2
AOx	Acyl-CoA oxidase
AP-1	Activator Protein 1
bHLH	basic helix-loop-helix
CBP	CREB binding protein
COUP-TF	chicken ovalbumin upstream-promoter transcription-factor II
CREB	cAMP response element binding protein
CTE	Carboxy-terminal extension
DBD	DNA Bbinding Domain
DR	Direct Repeat
ER	Estrogen Receptor
GR	Glucorticoid Receptor
GRIP	glucocorticoid receptor-interacting protein
HAT	Histone Acetylase
HD	Hydratase Dehydrogenase
HDAC	Histone Deacetylase
HDL	High Density Lipoprotein
LBD	Ligand Binding Domain
LDL	Low Density Lipoprotein

LXR	Liver X Receptor
NcoA	Nuclear receptor co-activator
N-CoR	Nuclear Receptor Corepressor
NR	Nuclear Receptor
P/CAF	p300/CBP associated protein
P/CIP	p300/CBP co-integrator associate protein
PBP	PPAR-binding protein
PGC-1	PPARγ co-activator
PPAR	Peroxisome Proliferator-Activated Receptor
PPRE	Peroxisome Proliferator Response Element
PR	Progesterone Receptor
PUFA	Polyunsaturated fatty acid
RAR	Retinoic Acid Receptor
RE	Response Element
RIP140	Receptor Interacting Protein 140
RXR	Retinoid X Receptor
SMRT	silencing mediator for RXR and TR
SRC	steroid receptor coactivator
STAT	signal transducer and activator of transcription
TBP	TATA binding protein
TF	transcription factor
TIF	transcription intermediary factor

TNF	Tumor Necrosis Factor
TR	Thyroid Hormone Receptor
TZD	thiazolidinedione
VDR	Vitamin D Receptor
VLDL	Very Low Density Lipoprotein

CHAPTER ONE

1. Introduction

1.1. PPARs as members of the nuclear receptor superfamily

Peroxisome Proliferator-Activated Receptors (PPARs) belong to the nuclear receptor superfamily which includes many proteins such as the estrogen receptor and the thyroid hormone receptor (Schoonjans et al., 1996b). Members of this superfamily act as ligand-activated transcription factors which regulate a wide assortment of genes. They share a ~~common~~ modular structure composed of several domains, namely the A/B, C, D and the E/F domains, arranged from the amino to the carboxy-terminal of the proteins in the order listed (Fig. 1) (Giguere et al., 1986; Rusconi and Yamamoto, 1987; Danielsen et al., 1987; Mangelsdorf et al., 1995). The A/B domain of many nuclear receptors contains an autonomous ligand-independent transcription activation function (AF-1). The C domain serves as the DNA binding domain (DBD). The D domain, also known as the hinge region, acts as a flexible linker between the DBD and the ligand binding domain as well as binds accessory regulatory proteins. The E/F domain, also known as the ligand binding domain (LBD), binds cognate ligand and contains the ligand-dependent transcription activation function (AF-2).

The DNA binding sites for nuclear receptors are referred to as response elements (REs) (Wahli and Martinez, 1991; Glass, 1996). They are composed of the idealized half-site sequence AGGNCA, where N can either be a T or an A residue. The actual half-sites found in natural REs diverge from the idealized sequence. While some receptors are capable of monomeric binding, most bind their cognate REs as homo- or

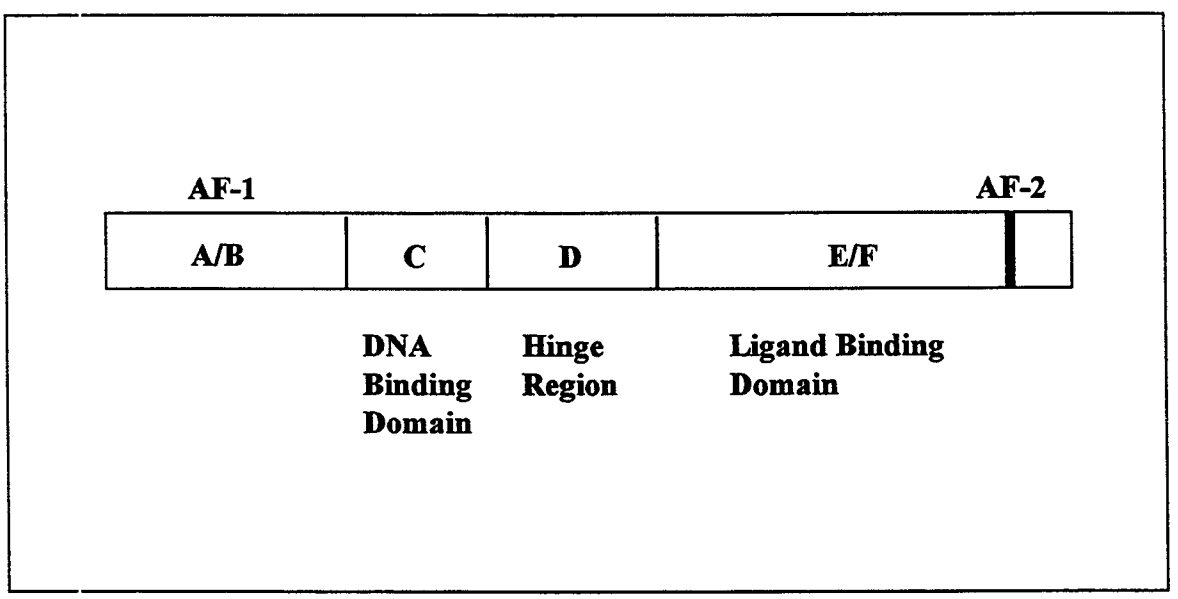


Figure 1. Modular structure of nuclear receptors.

heterodimers. Likewise, while monomeric binding sites do exist, most sites are composed of half-sites arranged as either inverted repeats (IRs), everted repeats (ERs), or direct repeats (DRs), separated by a specific number of nucleotides.

Nuclear receptors which bind to DNA as dimers do so either as homodimers or heterodimers with another receptor (Mangelsdorf et al., 1991; Mangelsdorf et al., 1995). Homodimer binding is generally observed for the steroid receptors such as GR and ER to palindromic DNA elements (inverted and everted repeats). In the case of heterodimeric DNA binding, retinoid X receptor (RXR), the receptor for 9-*cis* retinoic acid, serves as the heterodimerization partner for many receptors, including TR, RAR, VDR, and PPAR.

Nuclear receptors act as ligand responsive transcription factors (Wahli and Martinez, 1991). Ligand binding and ligand-dependent trans-activation are carried out by the LBD (Moras and Gronemeyer, 1998). While specific ligands have been discovered for many receptors, no ligand is known for many, due to which such receptors are often referred to as orphan receptors.

Two general classifications of NRs have been proposed based on DNA binding and dimerization properties. The first classification distinguishes NRs on the basis of half-site sequence preference dictated by a structural motif called the P-box in the DBD (Wahli and Martinez, 1991). NRs which recognize AGGACA half site sequence via the GSCKV P-box amino acid sequence fall into the GR-like category, while NRs which recognize the AGGTCA half site sequence via the (E/D)GCKX P-box sequence fall into the ER-like category. A second, more comprehensive classification divides NRs into 4 classes (Mangelsdorf et al., 1995). Class I receptors include steroid hormone receptors

which bind to palindromic elements as homodimers; class II receptors bind direct repeat elements as heterodimers with RXR; class III receptors bind primarily as homodimers to direct repeats; and finally, class IV receptors bind to half-sites as monomers. PPARs bind direct repeat elements as dimers with RXR and hence are class II receptors.

Several closely related proteins may serve as receptors for the same molecule. The term 'receptor subtypes' refers to products of individual genes, for instance PPARα and PPARγ, while the term 'receptor isoforms' refers to products of the same gene which differ for instance due to differential splicing and promoter usage, for instance PPARγ1 and PPARγ2 (Mangelsdorf et al., 1995).

1.2. Cloning and expression pattern of PPARs

The name Peroxisome Proliferator-Activated Receptor stems from the fact that the first subtype cloned, mouse PPARα, is activated by a family of compounds collectively known as peroxisome proliferators (Issemann and Green, 1990). Peroxisome proliferators form a structurally diverse group of compounds which induce an increase in the size and the number of peroxisomes in rodents, mainly in the liver (Osmundsen et al., 1991; Moody et al., 1991). The majority of these compounds are hypolipidemic clofibrate-like agents, while other types include phthalate ester plasticizers, industrial solvents, herbicides, food flavors, leukotriene D4 receptor antagonists, and the adrenal steroid dehydroepiandrosterone (Reddy and Chu, 1996). Direct involvement of PPARα in peroxisome proliferation is demonstrated by the fact that the PPARα knock-out mouse is not responsive to peroxisome proliferators (Lee et al., 1995).

Since the initial cloning of PPARα, two other PPAR subtypes have been cloned from several species, namely PPARγ and PPARδ, the latter also being known as NUC I, FAAR, or PPARβ (Zhu et al., 1993; Amri et al., 1995; Dreyer et al., 1992; Xing et al., 1995; Elbrecht et al., 1996; Chen et al., 1993; Kliewer et al., 1994; Schmidtet al., 1992; Sher et al., 1993; Greene et al., 1995; Tontonoz et al., 1994a). Two isoforms of PPARγ are known to be expressed, namely γ1 and γ2, with the latter containing extra N-terminal 30 amino acids (Tontonoz et al., 1994a)

In the rat, PPARα is expressed in the liver, heart, gut, and the proximal tubule cells of kidney, PPARγ is expressed in the fat tissue and the immune system, and PPARβ is expressed ubiquitously (Braissant al., 1996). In the mouse, PPARα is primairly expressed in the liver, kidney, heart, and brown adipose tissue (Schoonjans et al., 1996b). Low levels of expression can be detected in the intestine, muscle, testis, and thymus. While PPARγ1 is primarily expressed in the liver, kidney, and heart, with low expression in the muscle, testis, thymus and lung, PPARγ2 isoform is predominantly expressed in adipose tissue, with lower levels observed in the heart, spleen, adrenal gland, kidney, brain, and liver (Schoonjans et al., 1996b). PPARδ appears to be expressed ubiquitously (Schoonjanset al., 1996b). The expression pattern is different in Xenopus, where xPPARα and β are expressed in many tissue while the expression of PPARγ is restricted to fat (Dreyer et al., 1992; Dreyer et al., 1993).

1.3. Physiological Role of PPARs

Since the cloning of PPARα as peroxisome proliferator-responsive receptors, PPAR subtypes α and γ have been shown to play regulatory roles in lipid metabolism and energy homeostasis in the body. Various mitochondrial, peroxisomal, cytoplasmic and extracellular proteins involved at different stages of lipid metabolism appear to be regulated by PPARs (reviewed by Schoonhans et al., 1996b; Lemberger et al., 1996). PPARα appears to regulate genes involved with lipid utilization, especially during the time of starvation, as PPARα null mouse exhibits an aberrant, in cases lethal, phenotype when starved (Leone et al., 1999). PPARα activation mediates stimulation of fatty acid entry into cells, stimulation of β-oxidation, and a reduction of fatty acid synthesis (Schoonjanset al., 1996b). On the other hand, one of the main functions of PPARγ is the induction of adipocyte differentiation (Tontonoz et al., 1994a; Tontonoz et al., 1994b). Little is known about the function of the PPARδ subtype, but it appears to be involved in the regulation of high density lipoprotein (HDL) metabolism and oligodendrocyte maturation (Gelman et al., 1999a; Granneman et al., 1998).

1.3.1. Physiological role of PPARα

PPARα is abundantly expressed in the liver. Liver plays a central role in energy metabolism in the body. When energy supplies are high, excess triglycerides are secreted into the blood stream as VLDL destined for storage in fat tissue. In time of starvation, when circulating fatty acids are high and glucose is low, gluconeogenesis takes place in the liver and fatty acid oxidation in the liver leads to the production of ketone bodies which serve as fuel for other tissues (reviewed in Lemberger et al., 1996). Under fed

conditions, PPARα knock-out mouse appears normal except for accumulation of lipid droplets in the liver (Lee et al., 1995), however when examined, the enzymatic activity in the liver of the knock-out mouse reveals deficiencies in activities of many enzymes involved in lipid utilization (Aoyama et al, 1998). Furthermore, the PPARα null mouse appears to be unable to utilize fat stores and exhibits an abnormal response to even a short-term starvation (Leone et al., 1999). Under starvation, PPARα null mice fails to control fatty acid levels in the blood stream, accumulates fat in both the heart and the liver presumably due the failure to induce fatty acid oxidation, exhibits hypoglycemia and a poor ketogenic response.

In view of above and the fact that fatty acids act as PPAR ligands, it is not surprising that a number of enzymes and proteins found at various steps of fatty acid utilization have been shown to be regulated by PPARα (Schoonjans et al., 1996b; Lemberger et al., 1996). The first genes identified as targets of PPARα regulation were the genes involved in the peroxisomal β-oxidation. Expression of all three genes, namely acyl-CoA oxidase, enoyl-CoA hydratase/dehydrogenase, and keto-acyl-CoA thiolase, has been found to be up-regulated by PPARα (Dreyer et al., 1992; Tugwood et al., 1992; Zhang et al., 1992; Zhang et al., 1993; Marcus et al., 1993). Microsomal cytochrome P-450 IV enzymes are also induced by PPARα. These enzymes carry out ω-hydroxylation of fatty acids, leukotrienes and prostaglandins to produce dicarboxylic acid derivatives which are in turn oxidized in peroxisomes (Muerhoff et al., 1992; Aldridge et al., 1995; Johnson et al., 1996) Components of mitochondial β-oxidation have also been found to be regulated by PPARα. Carnitine palmitoyl transferase I, a protein responsible for the transport of

long-chain fatty acids into the mitochondria, has been found to be regulated by PPARα (Brady et al., 1989; Foxworthy et al., 1990; Yu et al., 1998; Brandt et al., 1998; Mascaro et al., 1998). An enzyme involved in the mitochondrial β-oxidation itself, medium-chain acyl-CoA dehydrogenase as well as hydroxymethylglutaryl-CoA synthase, which controls the rate-limiting step of ketone body production, have been shown to be controlled by PPARα (Gulick et al., 1994; Rodriguez et al., 1994). Several cytosolic proteins involved in fatty acid metabolism have been identified as PPARα targets as well, namely the long chain fatty acid acyl-CoA synthase (Schoonjans et al., 1995), palmitoyl-CoA hydrolase (Berge et al., 1989), the malic enzyme (Gilmour et al., 1993), and fatty acid binding proteins (Tontonoz et al., 1994a; Vanden Heuvel JP et al., 1993; Nakagawa et al., 1994). Uptake of fatty acids also appears to be regulated by PPAR, as lipoprotein lipase (LPL) which hydrolyses fatty acids from circulating triglycerides (Lemberger et al., 1996) and fatty acid transporters (Schoonjans et al., 1996b) are upregulated by PPARα. Interestingly, a key enzyme in gluconeogenesis, phosphenolpyruvate carboxykinase also appears to be regulated by PPAR (Tontonoz et al., 1995).

1.3.2. Physiological role of PPARγ

Expression of PPARγ, especially the PPARγ2 isoform, in adipocytes has led to the identification of PPARγ as a key determinant of adipogenesis (Tontonoz et al., 1994a; Gelman et al., 1999a; Fajas et al., 1998). Ectopic expression of PPARγ2 has been shown to induce adipogenesis of fibroblast and muscle cell lines (Tontonoz et al., 1994b; Hu et al., 1995). Other factors, namely CCAAT-enhancer binding proteins (C/EBP α, β and δ)

as well as ADD-1/SREBP-1 act in concert with PPARγ to stimulate and maintain adipogenesis. ADD-1/SREBP-1 appear to co-operate with PPARγ in adipogenesis by a production of a yet undefined PPARγ ligand (Kim et al., 1998; Fajas et al., 1998).

Since the initial identification of PPARγ's function as a key determinant of adipogenesis PPARγ has been shown to have a number of other functions. A great interest was generated in the role of PPARγ in energy homeostasis when it was discovered that it is the molecular target for the thiazolidinedione (TZD) class of insulin sensitizing drugs (Lehmann et al., 1995). A host of effects mediated by PPARγ are likely to contribute to the anti-diabetic effects of these drugs (Kliewer and Willson, 1998). In white fat, activation of PPARγ leads to a generation of a greater number of small adipocytes which are metabolically more active than large ones as well as to the attenuation of the production of cytokines such as TNFα whose secretion may lead to insulin resistance. In the brown fat, and perhaps muscle where PPARγ is also expressed, PPARγ is able to induce the expression of uncoupling proteins by utilizing a novel and specific co-activator protein (Puigserver et al., 1998).

Consistent with its role in differentiation, PPARγ has been shown to mediate the withdrawal from the cell cycle (Altiok et al., 1997; Tontonoz et al., 1997). The finding that several types of cancers such as liposarcoma (Tontonoz et al., 1997), breast (Mueller et al., 1998) and colon cancer (Dubois et al., 1998) express PPARγ prompted attempts to target PPARγ as means of arresting tumor growth by the induction of terminal differentiation and cell cycle withdrawal (Tontonoz et al., 1997; Mueller et al., 1998).

PPARγ also appears to play a role in inflamatory responses. Activation of PPARγ leads to a suppression of cytokine production in activated macrophages (Ricote et al., 1998; Jiang et al., 1998). PPARγ has also been shown to play a role in monocyte differentiation and the formation of foam cells which are found in atherosclerotic lesions (Nagy et al., 1998; Tontonoz et al., 1998). Activation of PPARγ leads to an increased uptake of oxidized LDL via an increased expression of CD36 surface scavenger receptor. Two components of oxidized LDL, 9- and 13-hydroxyoctadecadienoic acid (HODE) serve as PPARγ ligands, suggesting a possible role for PPARγ in the development of atherosclerosis.

1.4. Molecular mechanisms of transcription activation mediated by PPARs and other nuclear receptors.

A number of processes are involved in the regulation of transcription by nuclear receptors (reviewed in Torchia et al, 1998; Schoonjans et al., 1996b). To regulate transcription, nuclear receptors, usually as dimers, must bind to specific DNA sequences upstream of the target gene. When unoccupied by ligand, the receptor complex is inactive, but activates transcription once the appropriate ligand binds the LBD.

A number of accessory proteins interact with nuclear receptor in the ligand-unoccupied and ligand-occupied states (McKenna et al., 1999). Proteins known as co-repressors interact with nuclear receptors in the ligand-unoccupied state, while proteins known as co-activators interact with the nuclear receptors in the ligand-occupied state. These are thought to serve as intermediary actors between the nuclear receptors and basal

transcription machinery. Furthermore, most nuclear receptors contain two separate regions capable of activation of transcription. As is described in some detail below, the A/B domain of many nuclear receptors contains an autonomous ligand-independent transcription activation function referred to as AF-1. The LBD contains a ligand-dependent transcription activation function referred to as AF-2 (Moras and Gronemeyer, 1998). While the mechanisms of function of the AF-2 are well studied, a lot less is known about the mechanisms which regulate the AF-1 activity and through which the AF-1 functions.

1.4.1. The Peroxisome Proliferator Response Element (PPRE)

PPAR has been demonstrated to require RXR to binds to its cognate response elements (Palmer et al., 1995; Gearing et al., 1993; Kliewer et al., 1992; Keller et al., 1993). The first element responsible for PPAR/RXR binding was found upstream of the acyl-CoA oxidase (AOx) gene, whose product carries out the first step in the peroxisomal β-oxidation pathway (Dreyer et al., 1992; Gearing et al., 1993; Kliewer et al., 1992; Marcus et al., 1993; Tugwood et al., 1992; Zhang et al., 1993). The AOx PPRE was found to be composed of an imperfect direct repeat of the AGGTCA sequence separated by one nucleotide (DR1 elements). Since, similar DR1 PPREs have been found upstream of many genes involved in lipid homeostasis (Schoonjans et al., 1996b). Since DR1 bind several receptors, such as RXR, COUP-TF1, ARP-1 and NHF-4 homodimers and PPAR/RXR, RAR/RXR, COUP-TF1/RXR, ARP-1/RXR heterodimers (Nakshatri and Chambon, 1994; Nakshatri and Bhat-Nakshatri, 1998; Sladek et al., 1990;Ladias and

Karathanasis, 1991; Tugwood et al., 1992, Miyata et al., 1993) the mechanism for maintaining specificity of PPAR signaling is not clear. The sequence immediately 5' to the DR1, the 5' extended site region of many PPREs resembles the extended binding site of monomeric receptors such as Rev-ErbAα, NGF1-β (Wilson et al., 1993; McBroom et al., 1995; Palmer et al., 1995). From earlier studies it was observed that this region, appears to facilitate binding of PPAR/RXR heterodimers (Palmer et al., 1995; Ijpenberg et al., 1997). Half-sites found in known PPREs are more divergent in sequence than those found in response elements of other receptors. Some have suggested that having an additional DNA binding site determinants in the 5' region imparts specificity for PPAR binding while excluding binding of other receptors to the poorly conserved half sites of the DR1. Accordingly, DNA sequence immediately 5' of the DR1 was found to play a role in PPAR recognition (Palmer et al., 1995; Ijpenberg et al., 1997; Juge-Aubry et al., 1997). The proposed ideal 5' extended sequence was proposed to be C(A/G)(A/G)A(A/T)CT. In the context of natural PPREs, the match to the 5' extended site consensus sequence rather than match of the half-site sequence to the idealized AGGTCA sequence was found to determine the relative binding efficiencies of PPAR subtypes to various PPREs (Juge-Aubry et al., 1997). Accordingly, this sequence was find to be more important PPAR/RXR binding to imperfect DR1s than to idealized DR1s. Furthermore, the 5' extended site sequence in combination with the poor conservation of half-site sequence within PPREe appear to inhibit RXR homodimer binding, as RXR monomers bind poorly to half-sites found in PPREs, and the presence of the extended 5' site diminishes RXR homodimerization (Palmer et al., 1995).

1.4.2. Mechanism of DNA recognition and binding by PPARs and nuclear receptors

Most functions pertaining to response element recognition and binding are carried out by the DNA Binding Domain (DBD) of nuclear receptors. The DBD, also known as the C domain, is the most conserved domain among the nuclear receptors. The DBD contains two Zinc fingers and makes contacts with DNA half-sites which comprise a DNA binding site. The DBD alone dictates the specificity of binding of nuclear receptors to their cognate response elements (Green and Chambon, 1987; Kumar et al., 1987; Kumar and Chambon, 1988; Mader et al., 1993).

The crystal structures of DBDs of several receptors bound to DNA binding sites have been solved. From these as well as biochemical studies several regions with distinct functions within the DBD have been characterized. The P-box, which is comprised of amino acids within the carboxy region of the first Zn finger, has been shown to direct DNA half-site sequence specificity of the nuclear receptor DBDs by dicating specificity according to the identity of bases at positions 3 and 4 within the half-site (Umesono and Evans, 1989; Schwabe et al., 1993; Luisi et al., 1991; Nelson et al., 1995a; Nelson et al., 1995b; Smit-McBride and Privalsky, 1994; Nelson et al., 1994; Zilliacus et al., 1991; Mader et al., 1989).

Another region within the DBD located in the N-terminal region of the second Zn finger, called the D-box, has been shown to be crucial for the formation of protein-protein interaction surface within the receptor dimer. The co-operative binding of GR to its appropriately spaced response element (Hard et al., 1990) is mediated by protein-protein

1993; Towers et al., 1993; Predki et al., 1994; Zechel et al., 1994a; Rastinejad et al., 1995).

The CTE may also contribute to the specificity of binding by imposing conformational restrictions on dimer formation. In the crystal structure of the TR and RXR DBDs bound to a DR4 element, the CTE region contacts the four nucleotide spacer sequence, suggesting that this region is also responsible for the half-site spacing restriction of TR/RXR heterodimer binding (Gronemeyer and Moras, 1995; Rastinejad et al., 1995). The CTE overlaps the hinge region to an extent, which connects the DBD and the LBD. Unlike the DBDs, the LBDs appear to make symmetric contacts in a receptor dimer, which requires a degree of rotational flexibility between the DBD and the LBD. In this respect, the hinge region, including the CTE, is thought to dictate the extent of rotation between the DBD and the LBD (Kurokawa et al., 1993).

While the CTE of RXR may limit the spacing between the two half-sites to one nucleotide by comparison to the TR/RXR heterodimer, the CTE of PPAR plays a role in restriction of binding sites in a slightly different way (Hsu et al., 1998). As mentioned in the previous section, the 5' extended site have been shown to play a role in PPAR binding consistent with PPAR binding the 5' half-site. Subsequently, the CTE region of PPAR was shown to be responsible for 5' extended site requirement for PPAR binding (Hsu et al., 1998). It was shown that the DBDs of PPAR with an intact CTE region were able to bind as monomers to DR1 sites with the appropriate 5' extended site. The CTE region of PPAR also seems to prevent homodimerization on DR1 elements. When fused to the DBD of RXR, it prevented the otherwise observed homodimerization of RXR

DBDs on such elements, likely because the CTE of PPAR in the 3' position is incompatible with one nucleotide spacing on the DR1 element.

Areas of the receptors outside of the DBD have also been found to direct binding characteristics of the receptors. Full length PPAR requires RXR as its dimerization partner for PPRE binding (Palmer et al., 1995). A similar RXR requirement is also observed for related receptors, such as TR, RAR, and VDR (Mangelsdorf et al., 1995). Residues in the DEF region (hinge region plus ligand binding domain) have been shown to form interaction surfaces with RXR homodimers (Zhang et al., 1994; Bourguet et al., 1995). In the case of TR and RAR binding in a heterodimer with RXR, the respectable DBDs were sufficient for heterodimer formation, provided the A/B domains were not present (Leid et al., 1992). The LBD of both RXR and TR or RAR were required for heterodimerization on DNA when the A/B-DBD fragments are expressed (Leid et al., 1992). In the case of the PPAR, however, no heterodimer formation was observed with only the DBDs of PPAR and RXR on DR1 elements (Hsu et al., 1998). Instead, PPAR DBD seems to bind a DR1 element as a monomer, and formation of a dimeric complex with RXR requires the presence of PPAR and RXR LBDs, underlining the importance of interaction with RXR via the LBD for DNA binding. Similarly to the RAR and TR, the PPAR construct deleted in the LBD (which contains only the A/B domain and the DBD) was not able to bind a PPRE neither as a monomer nor a dimer, indicating an inhibitory function of the A/B domain on DNA binding.

1.4.3. PPAR ligands

Nuclear receptors including the PPAR bind their cognate ligands in a ligand-binding pocket formed by the fold of the LBD (Bourguet et al., 1995; Moras and Gronemeyer, 1998; Renaud et al., 1995; Nolte et al., 1998). PPARs appear to have the ability to bind and be activated by an unusually high number of structurally different ligands in comparison to other nuclear receptors. The ability to bind a large repertoire of ligands appears to be due to a large ligand-binding pocket observed in the LBD of PPARγ in comparison to other receptors (Nolte et al., 1998). Physiologically, this likely allows the receptors to elicit a general response to a variety of related compounds, such as fatty acids, which are metabolized through common pathways.

Although no physiological ligand of PPARs has been agreed on, several synthetic compounds, natural fatty acids and eicosanoids have been found to bind to PPAR ligand binding domain. Several hypolipidemic agents, such as Wy-14643, ciprofibrate and clofibrate, which are known to act as peroxisome proliferators and PPAR activators, have been shown to bind PPARα (Forman et al., 1997). As polyunsturated fatty acids (PUFA) have been observed to have hypolipidemic effects similar to those of known PPAR activators, the possibility that endogenous fatty acids may be ligands for PPAR has been investigated (Schoonjans et al., 1996a). Long chain saturated and several unsaturated fatty acids have been shown to activate PPARs (Gottlicher et al., 1992; Keller et al., 1993). Subsequently, long chain (C16) saturated chain fatty acids as well as oleic, petroselenic, linolenic, linoleic and arachidonic acids have been shown to bind to PPARα at estimated physiological concentrations (Forman et al., 1997; Kliewer et al., 1997). Products of arachidonic acid metabolism with a role in the inflamatory response, the

eicosanoid 8(S)-hydroxyeicosatetraenoic acid (8(S)-HETE) and leukotriene B4 have been shown to activate and bind PPARα (Yu et al., 1995; Kliewer et al., 1997; Devchand et al., 1996). A possibility exists that activation of the microsomal ω-hydroxylation and peroxisomal β-oxidation may lead to the degradation of signaling molecules and attenuation of the inflammatory response (Devchand et al., 1996)

At least one arachidonic acid metabolite has been identified as PPARγ activator and ligand. 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 has been shown to induce adipocyte differentiation, activate and bind PPARγ (Tontonoz et al., 1994b; Forman et al., 1995b; Kliewer et al., 1995). Several memebers of the thiazolidinedione class of anti-diabetic drugs are thought to act via PPARγ. Pioglitazone an BRL49653 have been found to specifically activate and bind PPARγ (Forman et al., 1995b; Lehmann et al., 1995). More recently, two component of oxidized LDL, 9- and 13-hydroxyoctadecadienoic acid (HODE) have been shown to activate and bind PPARγ with the dissociation constant in low μM range (Nagy et al., 1998). As oxidized LDL is thought to be one of the key agents in atherosclerotic plaque formations and PPARγ is expressed in differentiated monocytic cells called foam cells found in atherosclerotic lesions, these findings strongly implicate PPARγ to have a role in atherosclerosis (Nagy et al., 1998 Tontonoz et al., 1998).

Of interest is the observation that knock-out mice in the peroxisomal β-oxidation acyl- CoA oxidase gene exhibits phenotype similar to peroxisome proliferation as well as the induction of expression of PPARα responsive genes, suggesting that metabolites

upstream of the AOx enzyme may act as PPARα ligands. (Fan et al., 1998),. Similarly, the adipogenic ADD1/SREB1 factors were shown recently to cooperate with PPARγ during adipogenesis by production of a yet unidentified PPARγ activator (Kim et al., 1998).

1.4.4. Role of co-activators in ligand-dependent activation of transcription.

Ligand binding and ligand-dependent trans-activation, also referred to as activation function 2 (AF-2), is carried out by the LBD (reviewed in Moras and Gronemeyer, 1998). A number of crystallographic and biochemical studies have identified a region in the carboxy-terminus of nuclear receptors which is necessary for AF-2 activity. Mutational studies showed the AF-2 to reside in a conserved and transferable motif which consists of an invariant glutamic acid residue flanked by two pairs of hydrophobic residues in the carboxy terminal of the LBDs of nuclear receptors. Mutation of this motif abolish ligand-induced activity without altering other properties of receptors, such as ligand or DNA binding (Danielian et al., 1992; Tone et al., 1994; Barettino et al., 1994; Durand et al., 1994) Ligand binding is thought to induce a transition of the receptor from an inactive to an active state. The structures of the LBD of RXR without bound ligand as well as the ligand-bound LBDs of RAR and TR (Bourguet et al., 1995; Renaud et al., 1995)provided insight into how the AF-2 region ligand may effects such transition. The LBDs of all three receptors have been found to follow similar overall fold of a twelve-helix anti-parallel sandwich, which creates a ligand-binding pocket on one side. The AF-2 motif was found to lie in a carboxy-terminal α helix (helix 12) whose conformation

differed strikingly between the liganded and unliganded structures. In the unliganded structure, the helix projects outward into the solvent (OFF state), whereas in both the liganded structures it is tucked over the ligand-binding pocket (ON state). Interestingly, in the structure of ligand-unoccupied LBD dimer of PPARγ, the AF-2 of one dimer partner is in the ON state while the AF-2 belonging to the other partner is in the OFF state, suggesting that perhaps both conformations are possible in the unliganded state (Nolte et al., 1998).

Although nuclear receptors have been shown to interact directly with components of the basal transcriptional machinery such as TFIIB (Ing et al., 1992; Tsai et al., 1987; Baniahmad et al., 1993), TBP (Sadovsky et al., 1995) and TFIID (Jacq et al., 1994), indirect interactions via accessory proteins collectively known as co-activators appear to play a crucial role in nuclear receptor-mediated trans-activation (reviewed in Torchia et al., 1998; Shibata et al., 1997; McKenna et al., 1999; Xu et al., 1999). Existence of such cofactors has first been proposed from the observation that overexpression of a given nuclear receptor may inhibit or 'squelch out' signaling via another nuclear receptor, presumably by competition for such co-activators (Meyer et al., 1989). A series of genetic and biochemical screens have identified a number of co-activator proteins. Initially, ligand-dependent and AF-2 dependent interaction has been identified between the LBD of ER and two groups of proteins of molecular weights 140 and 160 kDa (termed p140 and p160) present in mammalian cell extracts (Halachmi et al., 1994; Cavailles et al., 1994) A multi-protein complex termed TRAPs (TR-associated proteins) has been shown to associate with TR in ligand-dependent manner and to be required to

TR mediated transcription (Fondell et al., 1996). Also, proteins which interact with the GR termed GRIPs (GR-interacting protein) (Eggert et al., 1995) and VDR (DRIPs) (Rachez et al., 1998) have been identified.

A number of co-activator proteins have been cloned. Three related groups of co-activator proteins, namely SRC-1/NCoA-1; TIF2/GRIP1/NcoA-2 and p/CIP/ACTR/AIB1/ RAC3/TRAM-1 (Onate et al., 1995; Kamei et al., 1996; Voegel et al., 1996; Hong et al., 1996; Torchia et al., 1997; Blobel, 1997; Li et al., 1997; Takeshita et al., 1997; reviewed in Torchia et al., 1998) appear to be a part of the protein group identified as the p160 co-activators through biochemical studies (Cavailles et al., 1994). Also identified as co-activators (or putative coactivators in the case of RIP140) were TIF1 (Le et al., 1995), CBP/p300 (Torchia et al., 1997), p/CAF (Yang et al., 1996; Chen et al., 1997) and RIP140 (Cavailles et al., 1995). Of these, CBP/p300 appears to be required not only by nuclear receptors but also by other transcription factors such CREB, AP-1 and STATs, and is thought to be a bridge through which distinct signaling pathways may communicate (Horvai et al., 1997).

The proteins considered to belong to the group of p160 co-activators share a number of functional and structural features. All have been shown to interact with the LBD of a number of nuclear receptors in a ligand and AF-2 dependent manner (Onate et al., 1995; Kalkhoven et al., 1998; Yao et al., 1996; Takeshita et al., 1996). Generally they are able to augment ligand-dependent trans-activation in co-transfection experiments. They possess a conserved domain structure, containing notably a basic helix-loop-helix (bHLH) motif and a period/aryl hydrocarbon receptor/single minded

(PAS) domain in the amino-terminus, nuclear receptor interaction domains, a CBP/p300 interaction domain which overlaps a stronger activation domain, and a weaker activation domain in the carboxy terminus.

CBP/p300 protein has been shown to interact with nuclear receptors in an AF-2 and ligand-dependent manner and augment ligand-dependent activity of many receptors (Kamei et al., 1996; Chakravarti et al., 1996; Smith et al., 1996; Yao et al., 1996; Hanstein et al., 1996; Korzus et al., 1998). Distinct interaction domains have been identified in CBP/p300 for a number of transcription factors and co-activators, consistent with its co-activator function for a number of signaling pathways (Kurokawa et al., 1998). CBP/p300 contains distinct domains for interaction with nuclear receptors, p/CAF, p160 co-activators as well as TFIIB and TBP among other factors. Of note is the fact that CBP/p300 can act as a co-activator for nuclear receptors without a functional nuclear receptor interaction domain, indicating that direct interaction is not required (Kurokawa et al., 1998).

A major fraction of cellular CBP/p300 has been found to be associated with p/CIP (Torchia et al., 1997). P/CIP has been found to interact with several receptors in ligand-dependent manner and has been found to be necessary for the function of CBP/p300-dependent transcription factors, indicating that the two cofactors associate in a functional complex (Torchia et al., 1997; Kurokawa et al., 1998). While both p/CIP and NcoA-share sequence homology with other p160 co-activators and are required for nuclear receptor signaling, only p/CIP and not NcoA-1 is required for the action of other

CBP/p300 transcription factors (Torchia et al., 1997). Although related, p/CIP and other p160 proteins appear to function via separate pathways (Torchia et al., 1997).

Another protein thought to play a role in nuclear receptor signaling is p/CAF. This protein associates with CBP/p300 (Yang et al., 1996), and co-immunoprecipitates with NcoA-1, SRC-1 and p/CIP (Korzus et al., 1998). P/CAF appears to associate with nuclear receptors in an AF-2 and ligand-independent manner (Korzus et al., 1998), however, it is required for ligand-dependent activation via RAR, TR and ER,

The AF-2 and ligand-dependent interaction between nuclear receptors and co-activator proteins have been shown to be carried out by a LXXLL structural motifs present in co-activator proteins (Heery et al., 1997). Most co-activators possess a number of such motifs, each capable of an interaction with the LBD. Presence of multiple LXXLL motifs within a single protein may afford a level of combinatorial selectivity for nuclear receptor interaction (Torchia et al., 1997). The precise mechanism of binding via this motif is illustrated by the crystal structure of ligand-occupied LBD of mPPARγ bound to a fragment of SRC-1 containing such two such motifs. The SRC-1 fragment binds both LBDs in the homodimer via its two LXXLL motifs. The E471 in the AF-2 region which is in the ON conformation in the structure, together with K301 present in helix 3, forms a 'charge-clamp' structure which defines the orientation and placement of the LXXLL motif (Nolte et al., 1998).

Both CBP/p300 and p/CAF possess histone acetylase (HAT) activity (Yang et al., 1996; Schekman, 1996; Bannister and Kouzarides, 1996). The related SRC-1 and ACTR have also been shown to possess a weaker HAT activity (Chen et al., 1997; Spencer et al.,

1997). Histone acetylation has been implicated with de-repression of chromatin and activation of transcription, suggesting that co-activators may exert its effects on transcription through alteration of chromatin structure (Grunstein, 1997; Wolffe and Pruss, 1996). This apparent redundancy of HAT activity may be resolved by the observation that different transcription factors require HAT activities belonging to different co-activators (Korzus et al., 1998).

The evidence gathered so far points to the presence of a multi-component co-activator complex necessary for trans-activation by nuclear receptor and other transcription factors. Some co-activators are nuclear receptor-specific while others appear to be shared with other transcription factors. The presence of multiple co-activators with multiple interaction surfaces and redundant HAT activities allows for a combinatorial assembly of a specific co-activator complex in accordance of the requirements of a specific transcription factor (Kurokawa et al., 1998; Korzus et al., 1998).

1.4.5. Role of co-repressors in silencing of transcription.

Several related proteins termed co-repressors have been cloned which seem to mediate silencing of transcription by unliganded receptors (reviewed in McKenna et al., 1999; Torchia et al., 1998; Xu et al., 1999). Several receptor heterodimers, such as TR/RXR and RAR/RXR, are able to repress transcription below the basal level when bound to their cognate response elements in the absence of ligand (Baniahmad et al., 1992; Baniahmad et al., 1995). Repression is thought to be mediated at early steps of

formation of the pre-initiation complex (Fondell et al., 1993). Early studies suggested that such repression is mediated by a soluble limiting factor or factors, as repression could be 'squelched out' by cotransfection with an unliganded receptor (Baniahmad et al., 1995; Tong et al., 1996). Further studies localized the repression domain to the LBD and the hinge region of TR or RAR (Baniahmad et al., 1992; Baniahmad et al., 1995; Tong et al., 1996). Dominant negative mutants of TR which repress transcription in both the absence or presence of ligand exist (Baniahmad et al., 1992) Such mutants lack a functional AF-2 helix, indicating a role for this domain in relieving repression (Baniahmad et al., 1992; Baniahmad et al., 1995).

Two-hybrid yeast screens with unliganded LBD of TR or RXR led to the cloning of two related proteins, N-CoR (Horlein et al., 1995) and SMRT (Chen and Evans, 1995), believed to act as co-repressors for nuclear receptors. Both proteins associate with TR/RXR and RAR/RXR heterodimers on their cognate response elements in the absence of ligand, and are released upon binding of ligand (Horlein et al., 1995; Chen and Evans, 1995; Chen et al., 1996). Dominant-negative mutants of TR require the binding of co-repressors and are generally defective in their release in response to ligand (Safer et al., 1998; Nagaya et al., 1998).

The amino-terminal region of the LBD including the hinge region of TR as well as carboxy-terminal regions of the LBD have been shown to be required for N-CoR binding (Horlein et al., 1995). More specifically, a structural motif in the hinge region called the CoR box was identified in the hinge region of several receptors and was found to be required for binding of the co-repressors (Horlein et al., 1995). Binding of N-CoR

to some orphan receptors, however, was found to occur independent of the CoR box, indicating a possibility of different modes of interaction of corepressors and nuclear receptors (Zamir et al., 1996).

Earlier studies in yeast implicated two proteins, Sin3 and RPD3, to be required for transcriptional repression (Vidal and Gaber, 1991; Nasmyth, 1993). RPD3 was later shown to possess histone deacetylase activity (Rundlett et al., 1996). Mammalian homologs of Sin3 (mSin3) and RPD3 (mRPD3 or HDAC 1 and HDAC 2) have been identified (Taunton et al., 1996; Yang et al., 1996) Presence of both mSin3 and mRPD3 is required not only for nuclear receptors but also for a number of other repressors of transcription (Hassig et al., 1997; Laherty et al., 1997; Nagy et al., 1997). Complexes containing N-CoR, mSin3 and mRBD3 proteins have been isolated, indicating the involvement of a multi-component complex in transcriptional repression (Heinzel et al., 1997; Alland et al., 1997). As alteration of chromatin structure through deacetylation of histones is associated with transcriptional repression, these findings provide a mechanistic link between co-repressors and chromatin structure.

The general model for the role of co-repressors and co-activators in the role of trans-activation by nuclear receptors is that in the absence of ligand, nuclear receptor heterodimer bound to DNA represses transcription via a recruitment of the co-repressor complex. Following ligand binding to the LBD, due to allosteric changes in the LBD of the receptor involving the AF-2 helix, the co-repressor complex is released, following which the co-activator complex is recruited, leading to the activation of transcription

(Horlein et al., 1995; Kurokawa et al., 1995; Chen and Evans, 1995; Nagy et al., 1997; Lin et al., 1997; DiRenzo et al., 1997).

1.4.6. Interaction of PPARs with co-repressors and co-activators.

Association of PPARs with co-repressors

The role of co-repressors in PPAR signaling has never been firmly established. PPAR do not appear to repress transcription in unliganded state and do not appear to possess a functional CoR box which has been shown to be required for interactions with co-repressors for TR and RAR. Binding of PPARg to SMRT and N-CoR has been shown in solution, however, no binding could be detected in the context of a heterodimer with RXR (Zamir et al., 1997; DiRenzo et al., 1997). Nonetheless, microinjections of anti-SMRT but not N-CoR antibody has been shown to counteract the repressive effects of phosphorylation of PPARγ on Ser112 (Lavinsky et al., 1998), and the presumed sequestration of repressors with unliganded TR has been shown to increase the activity of the AF-1 domain of mPPARα (Juge-Aubry et al., 1999). Furthermore, a recent two-hybrid screen with the LBD of mPPARα isolated N-CoR as a PPAR-interacting protein (Dowell et al., 1999). In GST pull-down assays, PPAR did require a region corresponding to the N-CoR box of the TR for interaction with N-CoR, but rather a region immediately carboxy-terminal to the DBD. Out of the ligands tested, only Wy-14 was able to abolish the interaction between N-CoR and PPAR. Although N-CoR was shown to suppress PPAR activity in the one case reported, complexes of N-CoR with

PPAR on DNA were not reported as they have been for other receptors in agreement with previous observations (DiRenzo et al., 1997).

Association of PPARs with co-activators

Several co-activator proteins have been shown to associate with PPARs. While most proteins reported to date are the p160 co-activators, CBP/p300 and RIP140 proteins, some appear to be novel and unique to PPARs.

Both SRC-1-like and CBP/p300 proteins have been identified as co-activators for the PPAR. Ligand-dependent interaction with p160 and p140 proteins as well as SRC-1 has been shown for PPARγ (DiRenzo et al., 1997). In a different report, SRC-1 was shown to enhance ligand-dependent activity of PPARγ, and to bind PPARγ independently of ligand (Zhu et al., 1996). The two conflicting results may due to use of different SRC-1 clones or different experimental conditions. TIF-2 has been shown to interact with mPPARα in ligand-dependent manner (Leers et al., 1998). Similarly ARA70 has been shown to interact in ligand-enhanced manner with PPAR and enhance ligand-dependent activation by PPAR (Heinlein et al., 1999). CBP/p300 has been shown to interact with PPAR in AF-2 and ligand-enhanced fashion and was able to enhance the activity of PPAR in transfections (Gelman et al., 1999b; Schulman et al., 1998; Dowell et al., 1997). More recently, CBP/p300 has been shown to interact with the A/B domain of PPARγ in ligand-independent manner and with the hinge region-LBD of PPARγ in AF-2 and ligand-dependent manner possibly via two distinct regions (Gelman et al., 1999b).

A protein whose role in PPAR and nuclear receptor signaling in general is not clear is RIP140. RIP140 has been cloned initially as an ER-interacting protein and has been shown to interact with ER, TR, RAR and RXR ligand-dependent manner (Cavailles et al., 1995; Horset et al., 1996). RIP140 possesses a high number of LXXLL motifs (Heery et al., 1997) and is capable of interaction with nuclear receptors via two receptor interaction domains (Horset et al., 1996). In transfection experiments, however, it was shown to repress ER-mediated signaling. Our laboratory identified RIP140 as a PPARα-interacting protein in a two-hybrid screen. The interaction of RIP140 with PPARα was shown to be ligand-independent, and it was shown to inhibit PPAR signaling in transfections. Interestingly, interaction of RIP140 with nuclear receptors has been shown to be dependent on the AF-2 region, even in the case of PPAR, where ligand independent interaction has been observed (Cavailles et al., 1995; Horset et al., 1996; Joyeux et al., 1997; Collingwood et al., 1997; Henttu et al., 1997; White et al., 1997; Treuter et al., 1998; Miyata et al., 1998). As RIP140 posses intrinsic trans-activation potential and was shown to augment transcriptional activity of ER (Cavailles et al., 1995) and PPARα (see results) in yeast cells, it is not clear what function it serves.

Some proteins isolated based on PPAR specifically have been isolated. A two-hybrid screen with the LBD of PPARγ led to the cloning of PPAR-binding protein (PBP) containing two LXXLL motifs with no homology to other known co-activators, which shows ligand-enhanced interaction with PPARγ, PPARα, RAR and TR (Zhu et al., 1997). PBP also enhances activity of PPARγ in transfections and its receptor-interacting fragment abolishes the activity of PPARγ.

Another two hybrid screen with the LBD of mPPARγ has led to cloning of a protein termed PGC-1 which is thought as a cold-inducible brown-fat specific co-activator for PPARγ (Puigserver et al., 1998). PGC-1 appears to be required for the induction of the uncoupling protein 1 (UCP-1) promoter by PPARγ. Induction of UCPs is consistent with the thermogenic response in response to cold, since they are mitochondrial proteins which dissipate the proton gradient leading to a generation of heat. PGC-1 contains one LXXLL motif and interacts with PPARγ in ligand-independent manner, however, its binding to RAR and TR appears to be ligand enhanced.

The A/B domain of PPARγ has been shown to carry the adipogenic potential of PPARγ (Castillo et al., 1999). A two hybrid screen using this domain led to the isolation of a novel protein termed PGC-2 (Castillo et al., 1999) which interacts with the A/B domain of mPPARγ and the full length mPPARγ ligand-independent manner. PGC-2 does not possess intrinsic trans-activating capability and does not augment the transcriptional activity of the A/B domain of PPARγ. It does however have an expression pattern similar to that of PPARγ and augments the transcriptional activity of PPARγ and not α or δ subtypes, and hence it is thought to serve as an adipogenic adapter protein.

1.4.7. Role of the A/B domains in PPAR and nuclear receptor function

The role of the A/B domains and of the ligand-independent trans-activation AF-1 region they contain in nuclear receptor signaling is not well understood. These domains are the least conserved domains in the nuclear receptor superfamily (Schoonjans et al., 1996b). No apparent sequence homology exists between the A/B domains of either the

different members of the nuclear receptor superfamily or between subtypes of the same receptor.

The A/B domains of several steroid and nuclear receptors have been characterized in most cases by deletion studies in the context of the wild type full-length receptors and as GAL4 DBD fusions. Despite of the apparent lack of amino acid sequence homology between the A/B domains of various receptors, common roles and mechanisms of action such as synergism with AF-2, context dependence, phosphorylation and association with regulatory proteins have been described for several receptors.

No motifs responsible for transcriptional activity of the AF-1 common to several receptors have been identified. Several such however has been identified for individual receptors. A VP-16 like acidic activation domain was identified in the N-terminal region of RARβ2 (Folkers et al., 1995), a structural PYTCYSP motif was found to be responsible for activity of TRβ2 AF-1 (Sjoberg and Vennstrom, 1995), and a structural KRKRK motif in cTRα was found to be responsible for activity of AF-1 and TFIIB binding in cTRα (Hadzic et al., 1995).

Although in most cases the AF-1 region possesses ligand-independent activity when fused to a heterologous DNA binding domain, the region has also been shown to be required for ligand-dependent trans-activation and full activity (synergism with AF-2 region) for TRβ1 (Wilkinson and Towle, 1997), ER (Pierrat et al., 1992; Metzger et al., 1995; McInerney and Katzenellenbogen, 1996), chicken TRα (Hadzic et al., 1995), and the AR (Ikonen et al., 1997). Moreover, the AF-1 region has been shown to function via targets different from AF-2 region for RARβ2 by squelching studies (Folkers et al.,

1995). The two activation functions of ER were shown to act via different factors, at least some of which are required for function of VP16 acidic activation domain (Tasset et al., 1990).

The activity of the AF-1 region has been shown to be dependent on cell and promoter context for AR (Ikonen et al., 1997), RARβ2 (Folkers et al., 1995), TRβ2 (Sjoberg and Vennstrom, 1995), estrogen receptor (ER) in yeast (Pierrat et al., 1992) and mammalian cells (Metzger et al., 1995). A study also linked agonistic function of the estradiol antagonist tamoxifen on the ER receptor to cell and reporter context activity of the AF-1 region (Berry et al., 1990).

A/B domains of several receptors have been shown to be phosphorylated. Phosphorylation of Ser118 has been shown to increase activity of the ER receptor, which effect may be cell dependent (Metzger et al., 1995). In a recent study the A/B domain of the RARα was shown to be phosphorylated by the CDK7 component of the basal transcription factor TFIIH, resulting in transcriptional activation of the receptor (Rochette-Egly et al., 1997).

PPARγ has been shown to be phosphorylated on Ser112 by a MAP kinase pathway resulting in a reduction of both its transcriptional activity and adipogenic potential (Reginato et al., 1998; Hu et al., 1996). A study by Lavinsky et al (Lavinsky et al., 1998) suggested that phosphorylation may result in the recruitment of SMRT co-repressor and hence the attenuation of its activity. A recent study suggests that this phosphorylation in fact results in a decrease for ligand affinity, indicating a degree of communication between the A/B domain and the LBD (Shao et al., 1998). Serine phophorylation of the

A/B domain of mPPARα has been shown to increase the activity of that domain, possibly via the disruption of association of the domain with co-repressors (Juge-Aubry et al., 1999)

Insights into the molecular mechanism of AF-1-mediated trans-activation have come from studies of interaction of the A/B domain with its possible targets. Functional association between the ABCD and EF fragments of the ER has been observed to be estradiol dependent in the context of the mammalian two hybrid system (Kraus et al., 1995). Tamoxifen was shown to produce an association between these domains, albeit not a transcriptionally active one (Kraus et al., 1995). The association between the A/B and the LBD domains has also been shown for the androgen receptor and has been shown to be facilitated by SRC-1 and CBP in the presence of testosterone (Berrevoets et al., 1998; Doesburg et al., 1997; Ikonen et al., 1997). These two activators were also shown to potentiate testosterone dependent activation (Ikonen et al., 1997). RIP140, which was shown to activate the receptor, was shown to disrupt the association of the two domains (Ikonen et al., 1997). SRC-1, CBP and RIP140 were able to activate the AR lacking the ligand binding domain (Ikonen et al., 1997). Physical interaction between the A/B and LBD was also observed in vivo and in vitro for the PR in response to agonists but not antogonists (Tetel et al., 1999). This interaction was shown to be direct and did not require the presence of SRC-1 and CBP/p300 co-activators (Tetel et al., 1999).

A/B domain have been shown to interact with several proteins. The activity of the AF-1 region was shown to be enhanced by an interaction with TFIIB in the case of cTRα (Hadzic et al., 1995). SRC-1 contains a domain capable of interacting with the A/B

domain of several receptors in addition to surfaces used for interaction with the AF-2 of nuclear receptors and other proteins (Onate et al., 1998). A study reported that SRC-1 binds the AF-1 region of the AR (Ma et al., 1999) via a region different from that used for AF-2 binding. SRC-1 fragment containing this interaction domain was able to enhance the transcriptional activity of the A/B domain and full length AR (Ma et al., 1999). SRC-1 has also been found to bind the AF-1 region ER via a region distinct from that required for AF-2 binding (Webb et al., 1998). Subsequently, SRC-1 was shown to be recruited to the AF-1 of ER in response to Ser phosphorylation of this domain via the MAPK pathway (Tremblay et al., 1999). More recently, the A/B domain and the LBD of PPARγ has been shown to interact with CBP/p300 in ligand-independent and –dependent manner respectively possibly via two binding sites (Gelman et al., 1999b).

A protein termed p68 was found to specifically interact with the A/B domain of ER (Endoh et al., 1999). Phophorylation of A/B domain of ER on Ser118 was shown to be required for activity of ER, and this phosphorylation enhanced the interaction of A/B domain with p68. P68 furthermore potentiated activity of AF-1 but not AF-2 in cell type specific manner, but not of other receptors tested. P68 was shown to bind p300 in vitro, possibly acting as a bridge protein between the AF-1 and the co-activator complex.

1.4.8. Other factors affecting nuclear receptor signaling

The model of co-repressor release and co-activator release in response to ligand binding serves as the current paradigm for nuclear receptor mechanism of action

((Torchia et al., 1998; Shibata et al., 1997; McKenna et al., 1999; Xu et al., 1999). This model does not account for the role of the ligand-independent AF-1 activity present in the A/B domain of nuclear receptors. A number of other complications appear to be superimposed on the current model. Factors such as response element architecture, polarity of binding, the identity of heterodimerization partner, outside signaling via protien kinases as well as the nature of ligand itself may affect the exchange of co-repressors and co-activators and hence the trans-activation output of a given receptor.

Estrogen is the natural ligand and activator of the ER dimer. Tamoxifen is an estrogen antagonist and in contrast to estrogen, promotes the association of co-repressors with the ER. Tamoxifen may however exhibit a partial agonist activity, which has been linked to unavailability of functional co-repressors in the cell (Jackson et al., 1997; Lavinsky et al., 1998). Similar situation is observed for the PR partial agonist RU486 (Zhang et al., 1998). Phosphorylation of ER on Ser118 found in the A/B domain has been shown to be required for activity (Bunone et al., 1996; Ali et al., 1993). This phosphorylation requirement may be explained by the fact that Ser118 phosphorylation decrease interaction of ER with co-repressors (Lavinsky et al., 1998). Similarly, inhibition of repression mediated by unliganded TR observed upon epidermal growth factor-dependent phosphorylation of TR appears to be mediated via disruption of interaction with a co-repressor (Hong et al., 1998).

While RAR/RXR heterodimers bind to DR5 elements and activate transcription in response to RAR ligands (Lehmann et al., 1992), they form transcriptionaly inactive complexes on DR1 elements (Kurokawa et al., 1994). Polarity of RAR/RXR complex is

reversed on the DR1 element in comparison to DR5 element, with RAR occupying the 5' position on the DR1 element (Kurokawa et al., 1994). RAR/RXR complexes are transcriptionaly inactive on DR1 elements because while the RAR/RXR dimer on DR5 elements is able to dissociate co-repressors in response to ligand and recruit p160 and p140 coactivators, RAR/RXR dimer on DR1 is not able to remove the co-repressors in response to ligand (Kurokawa et al., 1995). This illustrates ability of dimer polarity dictated by DNA binding site to influence ability to interact with cofactors.

PPAR forms heterodimers with RXR on DR elements similarly to TR, RAR, and VDR. As heterodimers with RXR, TR, RAR, and VDR (Forman et al., 1995c) prevent signaling via the RXR lignad, 9-cis retinoic acid while retaining the ability to signal via their cognate lignads (Lehmann et al., 1992). RXR is however responsive to its own ligand in the context of a heterodimer with PPAR (Forman et al., 1995c; Kliewer et al., 1992) LXR (Willy et al., 1995) and FSR, (Forman et al., 1995a) as well as in the context of a RXR homodimer (Heyman et al., 1992; Mangelsdorf et al., 1991; Levin et al., 1992). The blocking of RXR signaling has been shown to be due to the allosteric inhibition of ligand binding to RXR by the LBD of the heterodimerization partner (Forman et al., 1995c; Kurokawa et al., 1994), likely via interaction between their respective LBDs in the dimer. The CoR box of RAR, previously described to be required for association with co-repressors, has been shown to be required for allosteric inhibition of ligand binding to RXR (DiRenzo et al., 1997). Not surprisingly, RXR can bind its ligand in the context of PPAR/RXR heterodimer bound to DR1 element (DiRenzo et al., 1997). PPAR/RXR heterodimers recruit p160/p140 coactivators in response to either ligand

(DiRenzo et al., 1997). Interestingly, while the AF-2 of RXR appear to be dispensable, only the AF-2 helix of PPAR is required for activation of PPAR/RXR and recruitment of CBP/p300 by either ligand, indicating nevertheless an allosteric interaction between the two proteins (Schulman et al., 1998) This is in turn in contrast to the situation observed with RAR/RXR heterodimers, where the AF-2 regions of both partners were shown to be required for full activity even in the presence of only one ligand
(Botling et al., 1997).

The RAR/RXR dimer is irresponsive to 9-cis retinoic acid even though RAR can bind both 9-*cis* (RXR ligand) and *all-trans* retinoic acids. It seems however that all-trans retinoic acid is much more efficient than 9-cis retinoic acid at affecting co-repressor release from RAR (Hong and Privalsky, 1999), providing further explanation why only all-trans retinoic acid can activate RAR/RXR heterodimers.

It is interesting however that RAR/RXR can be activated by both subunits, providing that RAR ligand exists at sub-optimal concentrations (Botling et al., 1997). Consistent with this observation, RXR can bind its ligand when dimerized with RAR provided RAR is occupied. This is however different from the situation observed with the TR, where the presence of a TR ligand does not relieve the block of RXR ligand binding (Forman et al., 1995c).

While a finding mentioned above suggests that the allosteric inhibition RXR ligand binding by RAR depends on the presence of the CoR box of RAR, a recent study based on structural and biochemical studies suggests that this inhibition is due to the interaction of AF-2 helix of RXR with the co-activator binding site of RAR (Westin et al., 1998)

Such interaction in unliganded receptor dimer would prevent the AF-2 of RXR from undergoing the conformational shift required for ligand binding observed with ligand-bound LBDs. A RAR ligand binding followed by recruitment of a co-activator such as SRC-1 would then displace the AF-2 helix of RXR from the LBD of RAR and free it to bind ligand. It is interesting that the AF-2 fragment RXR has a poor affinity for the coactivator binding site of PPAR, which is consistent with lack of inhibition of RXR ligand binding by PPAR.

1.5. Project Overview

As outlined above, the A/B domains of nuclear receptors (Fig. 1) carry a number of discrete functions. One of these function is ligand-independent transcription activation function (AF-1), which is responsible for transcriptional activity of the A/B domains when they are independently targeted to DNA. Both mPPARα and mPPARγ2 subtypes, whose function is described above, have been studied in our laboratory. Given that the A/B domains of other nuclear receptors have been found to play a role in trans-activation by virtue of the presence of the AF-1, our investigation focussed on identifying and characterizing the AF-1 activity of the A/B domains of mPPARα and mPPARγ2 subtypes.

A motif common to several receptors responsible for conferring the AF-1 activity has not been identified. The AF-1, nonetheless, is carried by the A/B domains of many nuclear receptors despite the fact that the A/B domains are the most divergent domains of the of nuclear receptors. Even subtypes of an individual receptor, such as in the case of PPAR subtypes, exhibit no apparent homology in their A/B domains (Fig. 2). The first

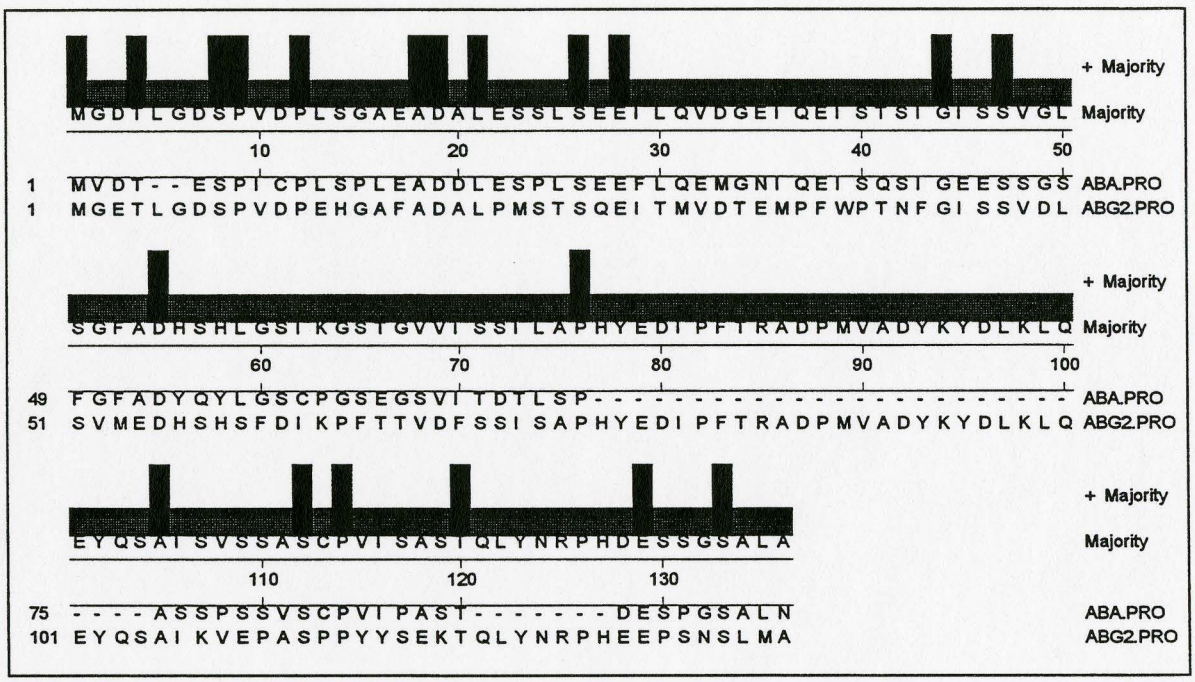


Figure 2. Sequence alignment of A/B domain of mPPARα and mPPARγ2. Sequences were aligned using DNA Star software. Tall bars indicate conserved regions.

part of our study was aimed at identifying regions in the A/B domains of mPPARα and mPPARγ2 responsible for the AF-1 activity.

Having shown that PPARs do in fact possess an AF-1 activity in their A/B domains, we then investigated how this activity is controlled. PPARs, much like other members of nuclear receptor superfamily, act as ligand activated molecular switches which control transcription of target genes (Schoonjans et al., 1996b). In the absence of ligand, PPARs do not activate transcription, despite the fact that their A/B domain contain the AF-1 activity. Our studies conclude that regions outside of the A/B domains, notably the hinge region and the DBD, 'mask' the AF-1 activity in the absence of ligand.

Lastly, we investigated the role of that RIP140, a potential nuclear receptor co-activator, may have in relation to the AF-1 activity of mPPARα and mPPARγ2. We conclude that RIP140 is a potential co-activator protein for the AF-1 activity of mPPARα and γ2 subtypes, although not as strong as other co-activators in the cell.

CHAPTER TWO

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals and Reagents

Agarose	Life Technologies
Acrylamide	GibcoBRL
Agar	Becton Dickinson and Company
Amino acids	Sigma
Ampicillin	Sigma
ATP	GibcoBRL
Bacto-peptone	Difco Laboratories
BioRad Protein Assay	BioRad
Bovine Serum Albumin	Life Technologies
Calf thymus DNA	Sigma
Calcium Chloride Dihydrate	Life Technologies
Charcoal, Dextran coated	Sigma
Chloroamphenicol	Sigma
Coenzyme A	Sigma
Deoxynucleotide triphosphates (DNTPs)	Pharmacia Biotech
Dithiothreitol (DTT)	Sigma
Dimethylsulphoxide (DMSO)	Caledon Laboratories
Glucose	BDH Inc.

L-Glutamine	Life Technologies
Glycerol	BioShop
HEPES (N-[2-Hydroxyethul]piperazine-N'-[2-ethanesulfonic acid])	Sigma
Hoescht 33258 dye	Polysciences Inc.
Lithium Acetate	Sigma
Luciferin	Biosynth
Magnesium Sulphate, heptahydrate	BDH Inc.
Manganese Chloride, tetrahydrate	BDH Inc.
Molecular weight markers	
1 Kb (Plus) DNA ladder	Life Technologies
Pre-stained BenchMark markers	GibcoBRL
N,N'-methylenebisacrylamide	Life Technologies
MOPS (3-[N-Morpholino]propanesulfonic acid)	BioShop
ONPG (o-nitrophenyl β-D-Galactopyranoside)	Sigma
PEG4000 (polyethelyne glycol)	Sigma
Potassium Acetate	DBH Inc
5x Reporter Lysis Buffer	Promega
Rubidium Chloride	Sigma
Salmon Sperm DNA	Sigma
Serum, calf	Life Technologies
Serum, fetal bovine	Sigma

Sodium Acetate Trihyrate	BDH Inc.
Sodium Carbonate	BDH Inc.
Sodium Chloride	ACP
Sodium Deoxycholate	Sigma
Sodium Dodecyl Sulphate (SDS)	Sigma
Sodium EDTA (ethylenediaminetetraacetate)	BDH Inc.
Sodium Hydroxide	BDH Inc.
Sodium phosphate, dibasic	BDH Inc.
Sodium phosphate, monobasic	BDH Inc.
Tris base	BioShop
Triton X-100	Sigma
Tween20	Sigma
Wy-13,643	ChemSyn Laboratories
X-Gal (5-bromo-4-chloro-3-indoyl-β -D-galactoside)	Life Technologies
Yeast Extract	Becton Dickinson Company
Yeast Nitrogen Base	Difco Laboratories

2.1.2. Enzymes

Calf intestine alkaline phophatase (CIP)	New England Biolabs
Restriction Endonucleases	New England Biolabs
RNase A	Pharmacia Biotech

T4 DNA ligase	New England Biolabs
Klenow fragment	New England Biolabs
VentTM DNA polymerase	New England Biolabs
Pfu DNA polymarase	Stratagene
Polynucleotide kinase (PNK)	New England Biolabs

2.1.3 Kits and other materials

ECL kit	Amersham Life Sciences
Six-well tissue culture plates	NUNC Brand Products
X-Omat Blue XB-1	Kodak
Qiagen Maxi Plasmid Kit	Qiagen
QiaexII Gel Extraction Kit	Qiagen

2.1.4 Antibodies

Anti-GAL4DBD mouse monoclonal antibody	Santa Cruz Biotechnology
Anti-mouse Ig, horseradish linked whole antibody (from sheep)	Amersham Life Sciences

2.1.5 Oligonucleotides

All primers used for sub-cloning purposes were made by the Central Facility of the Institute for Molecular Biology (MOBIX) facility at McMaster University, Hamilton, Ontario, and are listed below 5' to 3'.

AB14132	GCGGATCCAATTACAAGAAATGGAAAACATT
AB14131	GCATCTCGAGGTTCAGGGCACTGCCGGGGGA
AB16946	GCGGATCCAACTCTCCCCACTGGAGGCAGAT
AB16121	GAGCCCCATCTGTCCTTGATCCCCACTGGAGGCA
AB16122	TGCCTCCAGTGGGGATCAAGGACAGATGGGGCT
AB16509	GCGGATCCAAATGGTTGACACAGAGATGCCA
AB14133	GCGGATCCAAGGAATCAGCTCTGTGGACCTC
AB14037	GGATCTCGAGGGCCATGAGGGAGTTAGAAGGTTC
AB16944	CACAATGCAATTCGCTGAGCAAGAATGCCAAGATC
AB16945	GATCTTGGCATTCTTCCTCAGCGAATTGCATTGTG
AB13152	CTACCCGGGGATGGTGGACACAGAGAGCCCCATC
AB13153	GCTAGGATCCTTAGTTCAGGGCACTGCCGGGGGACTC
AB12641	GGTAGAATTCATGGGTGAAACTCTGGGAGAT
AB12612	GCTAGGATCCTTAGGCCATGAGGGAGTTAGAAGGTTC

2.1.6 Plasmids

Vectors constructed for the project.

pSG424:ABα

The XhoI/BamHI fragment containing part of GAL4 DBD and the A/B region of PPARα was excised from pGBT9:ABα and inserted into XhoI/BamHI sites of the pSG424 vector (Sadowski and Ptashne, 1989).

pSG424:ABα28-94

The DNA fragment containing amino acids 28 to 94 of PPARα was amplified from pSG5:mPPARα vector using primers AB14132 (5') and AB14131 (3') containing BamHI and XhoI sites respectively. The amplified and digested fragment was cloned into BamHI and SalI (compatible with XhoI) sites of the pSG424 vector (Sadowski and Ptashne, 1989).

pSG424:ABα11-94

The DNA fragment containing amino acids 11 to 94 of PPARα was amplified from pSG5:mPPARα vector using primers AB16946 (5') and AB14131 (3') containing BamHI and XhoI sites respectively. The amplified fragment was digested with BamHI and phosphorylated with T4 polynucleotide kinase and cloned into BamHI and blunted XbaI site of the pSG424 vector (Sadowski and Ptashne, 1989).

pSG424:1-10α

A stop codon was placed after the 10^{th} amino acid of mPPARα fused to GAL4 DBD using the QuickChange™ mutagenesis protocol using primers AB16121 and AB16122 and pSG424:mPPARα as template.

pSG424:1-27α

The EcoRI fragment containing amino acids 28 to the end of mPPARα was excised from the pSG424:mPPARα construct. The remaining vector was then religated resulting in

an in-frame fusion of amino acids 1 to 27 of mPPARα to the GAL4 DBD and containing two extraneous amino acids (N-Glu-Leu-C) at the carboxy terminus of the protein.

pSG424:1-46α

The SacI fragment containing amino acids 47 to the end of mPPARα was excised from the pSG424:mPPARα construct. The remaining vector was then religated resulting in an in-frame fusion of amino acids 1 to 46 of mPPARα to the GAL4 DBD and containing one extraneous amino acid (Arg) at the carboxy terminus of the protein.

pSG424:ABγ2

The XhoI/BamHI fragment was excised from the pGBT:ABγ2 construct and inserted into XhoI/BamHI sites of the pSG424 vector (Sadowski and Ptashne, 1989).

pSG424:ABγ1

The DNA fragment containing the A/B region of mPPARγ1 protein (amino acids 31-136 of mPPARγ2) was amplified from pSV-SPORT:mPPARγ2 using primers AB 16509 (5') and AB 14037 (3') containing BamHI and XhoI sites respectively. The DNA fragment was digested with BamHI and phosphorylated with T4 polynucleotide kinase and cloned into the BamHI and Klenow-blunted XbaI sites of the pSG424 vector (Sadowski and Ptashne, 1989).

pSG424:ABγ2.44-136

The DNA fragment containing amino acids 44 to 136 of mPPARγ2 was amplified using AB14133 and AB14037 primers containing BamHI (5') and XhoI (3') sites. The fragment was digested with BamHI and XhoI and inserted into the BamHI and SalI sites of the pSG424 vector (Sadowski and Ptashne, 1989).

pSG424:mPPARα (full length, amino acids 1-463)

The XhoI/BamHI fragment containing a part of GAL4 DBD orf fused to full length mPPARα was excised from pGBT9:mPPARα construct and inserted into XhoI/BamHI sites of the pSG424 vector (Sadowski and Ptashne, 1989).

pSG424:α1-401

The PmlI/XbaI fragment containing amino acids 402 to the end of mPPARα was excised from the pSG424:mPPARα construct. The XbaI site was blunted with Klenow, and the remaining vector was then religated resulting in an in-frame fusion of amino acids 1 to 401 of mPPARα to the GAL4 DBD and containing three extraneous amino acids (N-Leu-Asp-Lys-C) at the carboxy terminus of the protein.

pSG424:α1-221

The SmaI fragment containing amino acids 222 to the end of mPPARα was excised from the pSG424:mPPARα construct. The remaining vector was then religated resulting in

an in-frame fusion of amino acids 1 to 221 of mPPARα to the GAL4 DBD and containing seven extraneous amino acids (N-Gly-Ile-Arg-Ala-Leu-Asp-Lys-C) at the carboxy terminus of the protein.

pSG424:α1-167

A stop codon was inserted into the mPPARα orf at position 168 of mPPARα fused to GAL4 DBD resulting in an in-frame fusion of ABC domains of PPARα to GAL4 DBD domain. QuickChange™ protocol using primers AB16944 and AB16945 and pSG424:mPPARα was employed.

pSG424:mPPARγ2

The XhoI/SalI fragment containing a part of GAL4 DBD fused to mPPARγ2 was excised from pGBT9:mPPARγ2 vector and was inserted into XhoI/SalI sites of the pSG424 vector (Sadowski and Ptashne, 1989).

pGBT9:ABα

The DNA fragment encoding the AB domain of mPPARα (amino acids 1-94) was amplified from pSG5:mPPARα using AB13152 (5') and AB13153 (3') primers containing SmaI and BamHI sites respectively. The fragment was then digested with SmaI and BamHI and inserted into Klenow-blunted EcoRI and BamHI sites of the pGBT9 vector (Clonetech).

pGBT9:ABγ2

The DNA fragment encoding the AB domain of mPPARγ2 (amino acids 1-136) was amplified from pSV-SPORT:mPPARγ2 using AB12641 (5') and AB12642 (3') primers containing EcoRI and BamHI sites respectively. The fragment was then digested with EcoRI and BamHI and inserted into EcoRI and BamHI sites of the pGBT9 vector (Clonetech).

pGBT9:mPPARα

The EcoRI/BamHI fragment containing amino acids 28 up to carboxy terminus (aa 463) of mPPARα was excised from pSG5:mPPARα and inserted into EcoRI/BamHI sites of pGBT9:ABα.

pGBT9:mPPARγ2

The NcoI/MluI fragment containing partial orf of mPPARγ2 running from the NcoI site in the AB domain until the end of the protein was excised from pSV-SPORTmPPARγ2. The MluI site was blunted with Klenow, and the fragment was inserted into NcoI and Klenow-blunted BamHI site of the pGBT9:ABγ2 construct.

pSG424-GAL4

The GAL4 DBD was removed from the pSG424 vector (Sadowski and Ptashne, 1989) by removing the encoding DNA fragment using HindIII/BamHI sites and religating the empty vector.

pGK425:RIP140

RIP140 was excised from pBOS RIP140 (Cavailles et al., 1995) with SpeI and cloned blunt into the BglII site of PGK promoter/terminator sequence which was inserted into the HindIII site of pRS426 (Christianson et al., 1992). The PGK:RIP140 cassette was cloned as a SalI/SpeI fragment into same sites of pRS425 (Christianson et al., 1992).

Vectors obtained from other sources.

pRS313

Low copy centromeric yeast vector containing ampicilin and HIS3 markers used for expression of protein in yeast (Sikorski and Hieter, 1989).

pRS314

Low copy centromeric yeast vector containing ampicilin and TRP1 markers used for expression of protein in yeast (Sikorski and Hieter, 1989).

pRS315

Low copy centromeric yeast vector containing ampicilin and LEU2 markers used for expression of protein in yeast (Sikorski and Hieter, 1989).

pRS316

Low copy centromeric yeast vector containing ampicilin and URA3 markers used for expression of protein in yeast (Sikorski and Hieter, 1989).

pRS425

A high copy 2 μm yeast vector containing ampicilin and LEU2 markers (Christianson et al., 1992).

pRS426

A high copy 2 μm yeast vector containing ampicilin and URA3 markers (Christianson et al., 1992).

pGK313:mPPARα

mPPARα was excised from pSG5:mPPARα (Issemann and Green, 1990) with BamHI and cloned into the BglII site of PGK promoter/terminator sequence which was inserted into the HindIII site of pRS426. The PGK:mPPARα cassette was excised using BamHI/XhoI and cloned into pRS313 sites (Marcus et al., 1995).

pGpd313mPPARγ2

mPPARγ2 was cut out of pSPORT vector (Tontonoz et al., 1994a) with SpeI/MluI, blunted with Klenow and ligated into SmaI of pBluescript. A SnaBI site was introduced by site-directed mutagenesis along with optimal yeast Kozak sequence. The fragment

was cut out with XhoI/SnaBI, blunted and ligated into a blunted BamHI site of pUGpD (pRS316 into which 0.7 kb of the GpD promoter has been interted as an EcoRI/BamHI fragment.). The GpD:mPPARγ2 fragment was then excised with XhoI/SpeI and ligated into compatible sites of pRS313.

pRXRGpD314

The 1.4 kb region of RXR was amplified from pSRXR3-1 using primers with BglII overhangs, and inserted into SmaI site of pGEM7zf as a blunt undigested product. The RXR fragment was then excised with BglII and ligated into the BamHI site of p2uGpD, from which a 2.1 kb XhoI/SpeI fragment was removed and ligated into pRS314 (Marcus et al., 1995).

1AOxΔL1

A 2 μm yeast plasmid carrying ampicillin and URA3 markers containing 1x AOx element upstream of GAL1 promoter linked to lacI-lacZ gene.

1HDΔL1

A 2 μm yeast plasmid carrying ampicillin and URA3 markers containing 1x HD element upstream of GAL1 promoter linked to lacI-lacZ gene.

pGBT9

A commercially available (Clonetech) yeast 2 μm expression vector carrying ampicillin and TRP1 markers containing GAL4DBD (aa 1-147) upstream of a multiple cloning site for expression of GAL4DBD fusion proteins.

pSG424

A mammalian expression vector containing GAL4DBD (aa 1-147) upstream of a multiple cloning site for expression of GAL4DBD fusion protein (Sadowski and Ptashne, 1989).

pEF-BOS

An ampicillin resistant eukaryotic expression vector containing a EF-1α promoter and a G-CSF poly-A signal (Mizushima and Nagata, 1990). Gift from Dr. Parker.

pBOSRIP140

As above with full length RIP 140 insert, a gift from Dr. Parker (Cavailles et al, 1995).

pUAS(gal4): luc

A reporter plasmid containing yeast 5x UAS_{gal4} binding sites upstream of Ad Eb1 promoter driving a luciferase gene. A generous gift from Dr. Hassell.

pCH110

A plasmid containing a β-galactosidase expression cassette used to monitor transfection effeciency (Hall et al., 1983).

2.2 Methods

2.2.1 Polymerase Chain Reaction

The polymerase chain reaction (PCR) was carried out in Techne PNC-3 Thermal Cycler. Each reaction was carried out in 100 µl volume and comprised of 1x Thermopol Buffer, 2-8 mM MgSO4, 0.4 mM dNTPs, approximately 100 ng of template, 50 pmol of each primer, and 0.002 Units of VentTM DNA polymerase. A hot start method was employed for all reactions, where polymerase was added during a 5 min incubation at annealing temperature following a 10 min melting at 95°C and prior to elongation period at 72 C of determined time (1 min/kb). Following the first elongation, DNA was amplified for thirty cycles which consisted of melting at 5 min at 95°C, 2 min annealing at determined temperature and 72°C elongation for a determined time (1 min/kb). Anealing temperature used was the lower melting temperature of the two primers whose melting temperatures were determined according to the formula Tm = 57 + 41x(GC count/total base count) – (500/total base count).

2.2.2 PCR mutagenesis

Mutagenesis was carried out according to PCR-based QuickChange method as prescribed by Stratagene. Reactions were carried out in 50 µl which consisted of 1x reaction buffer, 50 ng of dsDNA template, 125 ng of each primer and 2 mM dNTPs.

Reactions were subjected to 30 sec melting at 95°C, followed by sixteen cycles of 30 sec melting at 95°C, 1 minute annealing at 55°C, and elongation of 2 min/kb of plasmid at 68 °C. Upon completion of thermocycling, the reaction was digested with 10 U of DpnI enzyme, and 10 µl of the digest was used to transform DH5α *E. coli.*

2.2.3 Sequencing

Sequencing has been carried out by MOBIX sequencing facility at McMaster University.

2.2.4 Bacterial strains and growth conditions

The DH5α *E. coli* strain is of F-,Φ80deltalacZΔM15 Δ(*lacZ*YA-*arg*F) U169 *deo*R *rec*A1 *hsd*R17 (r_k^-, m_k^-) phoA supE44 λ^- *thi*-1 *gyr*A96 *rel*A1 genotype was grown in liquid or solid agar 2YT media (1.6% bacto-tryptone, 1% bacto-yeast extract and 0.5% NaCl) was supplemented with 100 µg/ml ampicillin as required. Frozen stocks were made in 25 % glycerol and stored at –70 C.

2.2.5 Yeast strains and growth conditions

The YPH-500 strain is of MATα *ura3, lys2, ade2, trp1, his3, leu2* genotype. The Y190 strain is of MATa *leu*2-3, 112, *ura*3-52, *trp*1-901, *his*3-Δ200, *ade*2-101, *gal*4Δ*gal*80Δ URA3 GAL-*lac*Z, LYS GAL-HIS3, *cyh*^{r}, (Harper et al., 1993) genotype was obtained from Clonetech. The MaV103 strain is described in (Vidal et al., 1996) obtained from and is of MAT**a** *leu*2-3, 112, *ura*3-52, *trp*1-901, *his*3Δ200, *ade*2-

101, *gal*4Δ *gal*80Δ *can*1^R *cyh*2^R SPAL10::URA3 GAL1::HIS3 GAL1::LacZ genotype. Yeast were grown at 30°C in liquid or agar solid media of either YPD media (rich media composed of 1% bacto-yeast extract, 2% bacto-peptone and 2% glucose) or SC media (synthetic complete composed of 0.67% yeast nitrogen base, 2% bacto-agar, 2% glucose) supplemented with all amino acids except those being selected for. Frozen stocks were made in 25 % glycerol and stored at –70°C.

2.2.6 Cell lines

The BSC40 cell line adapted to 40°C growth from BSC-1 cell line derived from African Green Monkey kidney cells (Brockman and Nathans, 1974) and was obtained from the Massachusetts Institute of Technology.

The COS-1 cell line is derived from CV-1 cells which are derived from African Green Monkey kidney cells transformed with SV40 T antigen (Gluzman, 1981) and were obtained from American Type Culture Collection.

2.2.7 Purification of Plasmid DNA

Alkaline-lysis Miniprep

Alkaline lysis method was employed for small-scale preparation of DNA (mini-prep) as described by Maniatis et al. Briefly, 1.5 ml of overnight culture of DH5α strain harboring the desired vector and grown in 2YT medium in the presence of ampicilling (100 μg/ml) at 37°C was pelleted. The pellet was re-suspended in 100 μl of Solution 1 (25 mM TrisHCl pH 8.0, 10 mM NaEDTA pH 8.0, 0.9% glucose), following which 200

µl of Solution 2 (0.2 M NaOH, 1% SDS) were added. Following a 5 min incubation on ice, 150 µl of Solution 3 (3 M KAc, pH 5.5) were added, sample was incubated for 10 min on ice and centrifuged for 10 min at 4°C. The supernatant was recovered and DNA was precipitated with 2.5 volumes of 95% ethanol. The DNA was pelleted by a 20 min centrifugation at 14,000 rpm at 4°C, supernatant was removed, and the DNA pellet was dried. The pellet was then re-suspended in 25 µl of TE (10 mM TrisHCl pH 8.0, 1 mM EDTA) containing Rnase (100 µl/ml).

Qiagen Maxi-Prep

Qiagen Maxi-Prep method was followed for large-scale plasmid DNA purification. Briefly, 500 ml of DH5α *E.coli* culture carrying the plasmid of interest growth in 2YT medium supplemented with ampicillin (100 µg/ml) and subjected to chloroamphenicol amplification were pelleted by centrifugation at 6000 g for 15 min at 4°C. Chloroamphenicol amplification was carried out as prescribed by Sambrook et al., (1989). Briefly, 500 ml of 2YT supplemented with ampicillin (100 µg/ml) were inoculated mid-day with 25 ml of overnight culture of the desired strain grown at 37°C in 2YT supplemented with ampicilling (100 µg/ml). The 500 ml culture was then incubated at 37°C until the culture reached OD_{600} of 0.6 (2.5-3 h). At that time, chloroamphenicol was added to final concentration of 170 µg/ml, and the culture was incubated overnight, at which point it was pelleted. The pellet was re-suspended in 10 ml of P1 buffer (50 mM TrisHCl pH 8.0, 10 mM EDTA), following which 10 ml of P2 buffer (200 mM NaOH, 1% SDS) were added. Following a 5 min incubation at ambient temperature, 10

ml of P3 buffer (3.0 M KAc pH 5.5) were added, followed by a 20 min incubation on ice. The cell debris was spun down at 20,000 g for 30 min and again for 15 min following removal of the supernatant. The supernatant from the second spin was applied to the columns, following which the columns were washed and eluted according to protocol. The eluted DNA was precipitated with 0.7 volume of isopropanol, pelleted at 15,000 g for 30 min, and washed with ice-cold 70 % ethanol. The pellet was re-suspended in TE following drying.

2.2.8 Fluorometry

Plasmid DNA obtained from large-scale preparations was quantified using flourometry according to procedure prescribed for Hoefer and Hoefer Mini-Fluorometer (TKO 100). Quantification was carried out in 2 ml of TNE buffer (1 mg/ml Hoescht 33258 dye, 0.2 M NaCl, 10 mM Tris-HCl, 1 mM EDTA pH 7.4) relative to 1 μg of calf-thymus DNA.

2.2.9 Transformation into bacterial cells

Preparation of RbCl competent cells.

Chemically competent *E. coli* DH5α cells were prepared according to a modified RbCl method prescribed by Ausubel et al, 1997. 2YT medium (200 ml) were inoculated with 8 ml of overnight culture in 2YT (50 ml) grown from a single colony at 37°C and was grown to OD_{550} of approximately 0.3. At that the culture was chilled on ice for 15 min, and pelleted at 3000 g for 5 min at 4°C. The pellet was re-suspended in 16 ml of Transformation Buffer 1 (0.1 M RbCl, 46 mM MnCl2, 30 mM KAc, 10 mM CaCl2, 15% glycerol pH 5.8) and incubated for 15 min on ice. The cells were pelleted again as

before, re-suspended in 16 ml of Transformation Buffer 2 (10 mM MOPS pH 6.8, 13 mM RbCl, 75 mM CaCl2, 15% glycerol), then were aliquoted, frozen and stored at – 70°C.

Heat-shock transformation

DNA contained in a volume of TE or water up 10 µl was incubated on ice for 40 min with 100 µl of competent DH5α *E. coli* cells thawed on ice. Cells were then heat-shocked for 30 sec at 37°C and incubated on ice for another 5 min. 2YT medium (500 µl) were then added and cells were incubated at 37 C for 1 h. Cells were then pelleted, supernatant was removed except approximately 100 µl into which volume the cells were re-suspended, and the cells were plated on 2YT plates supplemented with ampicillin (100 µg/ul). Plates were then incubated overnight at 37°C.

2.2.10 Transformation into yeast cells

A version of the Lithium Acetate method was employed to introduce one or two vectors at a time. Approximately 1.5 ml of yeast culture grown from a single colony overnight at 30°C were spun down at 6,000 rpm for 5 seconds. Most of the supernatant was decanted except for 50-100 µl. 2 µg of carrier salmon sperm DNA (10 mg/ml) and approximately 1 µg of plasmid DNA (1-3 µl) per transformed plasmid were added, re-suspending cells with the tip after each addition. Following vortexing, 0.5 ml of PLATE (40% PEG4000, 100 mM LiAc, 10 mM TrisHCl pH 7.5, 1 mM EDTA) mix was added, suspension was vortexed again, and 20 µl of 1.0M DTT solution were added. Following vortexing, cells were incubated 6 to 16 hours at ambient temperature, and heat-shocked

for 10 min at 42°C prior to plating. 100 µl of cell suspension from the bottom of the tube were plated on minimal YNB plate supplemented with appropriate amino acids and incubated at 30°C.

2.2.11. **Yeast β-galactosidase assays**

Agarose overlay assay

The agarose overlay assay was performed to qualitatively assess reporter activity in yeast and was performed according to a modified protocol of Bohen and Yamamoto (1993). Melted warm assay solution (10 ml per plate) of 0.5% agarose, 0.1% SDS, 0.05 % X-gal in 0.5 M phosphate buffer pH 7.0 was poured over a plate with large (2-3 day) colonies of yeast strain of interest, allowed to solidify, and incubated at 37°C until blue color was observed.

Liquid quantitative β-galactosidase assay.

The liquid assay for yeast β-galactosidase activity was performed according to Ausubel et al 1997. Yeast were grown from a single colony at 30°C overnight to saturation in selective YNB media. In the evening, 100 µl of yeast were sub-cultured into 5 ml of fresh selective YNB media and grown overnight at 30°C to OD_{600} of 0.5-1.0. 1 ml of each culture was pelleted for 5 min at 2500 rpm and pellets were re-suspended in 1 ml of buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1.0 mM Mg_2SO_4, 50 mM β-mercaptoethanol pH 7.0). Cells were lysed by freeze-thawing in liquid nitrogen and ambient temperature water three times. Each lysate was vortexed and assayed for β-

galactosidase activity and protein content using the Bradford protocol. To measure β-galactosidase, 25 to 900 µl of lysate mixed with buffer Z to bring up to 1 ml total volume. 200 µl of ONPG solution (4 mg/ml) was added to each tube and reaction was incubated at 30°C until yellow color was observed at which time 0.5 ml of 1 M Na_2CO_3 solution was added to stop the reaction and absorbance at 420 nm of each sample was measured. Aborbance readings were corrected to volume of lysate used in reaction, amount of protein present, and incubation time.

2.2.12 Transfections

Transient transfections were carried out according to calcium phosphate precipitation protocol prescribed by Graham and Van Der Eb, 1973 (Graham and Eb AJ van der, 1973). Each well in a six well plate (NUNC Brand Products) was seeded with $2.5x10^5$ and $3.0x10^5$ BSC-40 or COS-1 cells respectively in 3 mL of DUL medium supplemented with either 10% CS or 10% FBS for (BSC40 and COS-1 cells respectively), 1% L-Glutamine, and 1% Pen-Strep. Cells were transfected the following day at 80-95% confluence. Prior to applying precipitates, media was replaced with fresh media of same kind, except for cases where transfections were used to measure drug induction of a reporter gene, where the monolayers were washed with PBS and the media was changed to DUL supplemented with 10% charcoal stripped FBS, 1 % L-Glutamine, 1% Pen-Strep and +/-Wy14 ,643 (100 µM final concentration) in DMSO (0.5% final concentration). To make Calcium-Phosphate precipitates, plasmid DNA was combined with salmon sperm DNA to total 14 µg of DNA per 350 µl of 0.25 M $CaCl_2$ solution (sterile). 300 µl

of this mixture was added dropwise to equal volume of 2x HBSS buffer (0.28 M NaCl, 50 mM HEPES, 1.5 mM $Na_2HPO_4.7H_20$, pH 7.12, sterile) and the resulting mix was incubated for 20 min at ambient temperature. For transfections where drug induction was measured, precipitates were made by mixing 24 μg of total DNA per 600 μl of 0.25 M $CaCl_2$ and dropwise addition of 550 μl of this mixture to an equal volume of 2xHBSS in order to account for +/- drug conditions. 250 ul of the resultant precipitates were applied per well. As a result, for transfections other than those used for Westerns, 1 μg of reporter and 1 μg of pCH110 vector was applied per well together with specified amount of plasmid of interest, and for transfections used for Westerns 3 μg of plasmid of interest was applied per well. Cells were then incubated for 16 h, at which point the media were changed as described above. Cells were incubated for an additional 24 hours prior to preparation of cell lysates.

To prepare cell lysates to measure luciferase reporter activity, monolayers were washed twice with phosphate-buffered saline prior to application of 300 μl per well of 1x Reporter Lysis Buffer. Plates were shaken vigorously on rotary shaker, frozen at –70°C for 16 h, thawed and shaken once more, following which the lysates were collected into microfuge tubes. Lysates were then spun for 2 min at 14,000 rpm and the supernatants were collected.

To prepare cell lysates for Western immunoblotting, 250 μl of RIPA (50 mM TrisHCl pH 7.2, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate) buffer were added per well, cells were scraped with a cell lifter and collected into microfuge tubes. The lysates were

then sonicated briefly to disrupt DNA, centrifuged for 2 min at 14,000 rpm and the supernatants were collected.

2.2.13 Bradford Assay

Protein concentrations were measured using the Bradford method using the Bradford reagent. Appropriate volume of lysates (less than 10 µl) was mixed with 1x BioRad Protein Assay solution and incubated for 15-30 min. Aborbance of each sample was measured at 595 nm.

2.2.14 Luciferase Assay

Luciferase activity was measured according to protocol prescribed by Promega using Lumat LB 9507 luminometer, which injects and mixes 100 µl of the assay buffer (470 uM luciferin, 270 uM coenzyme A, 530 uM ATP, 33.3 mM DTT, 20 mM Tricine, 1.07 mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 2.67 mM $MgSo_4$, 0.1 mM EDTA) with 10 ul of lysate placed in a test tube. Luciferase activity is recorded as relative light units (R.L.U.).

2.2.15 β-galactosidase Assay Used with Transient Transfection Luciferase Assays

To correct luciferase units for transfection efficiency, 100 µl of cell lysate was combined with 400 µl of buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1.0 mM Mg_2SO_4, 50 mM β-mercaptoethanol pH 7.0) and 150 µl of ONPG solution (4 mg/ml). The reactions were incubated at 30°C until yellow color was observed, at which

time 200 µl of 1 M Na_2CO_3 solution was added to stop the reaction and absorbance at 420 nm of each sample was measured.

2.2.16 Western immunoblotting

Proteins in COS-1 cell lysates (15 µg per lane in 1X SDS loading buffer, 50 mM TrisHCl pH 6.8, 10 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) were resolved by SDS-PAGE according to method by Laemmli (Laemmli, 1970) using stacking and separating gel composed of 3% 30:1 acrylamide:N,N'-methylenebisacrylamide, 125 mM TrisHCl pH 6.8, 0.1% SDS and 15% 30:1 acrylamide: N,N'-methylenebisacrylamide, 375 mM TrisHCl pH 8.8, 0.1% SDS. Electrophoresis was carried out at 200 V in Bio-Rad mini gel. Pre-stained markers were run concurrently to identify sizes (BenchMark, Gibco, BRL. Following electrophoresis, proteins were transferred to a HybonTM-C Pure nitrocellulose membrane in a mini-blot apparatus (BioRad) overnight at 23 Volts. The following morning, the membranes were blocked in 3 % skim milk solution in buffer for 1 hour and washed twice in TBST buffer (20 mM TrisHCl, 150 mM NaCl, 0.05% Tween20) for 15 min. The membrane was probed with primary mouse monoclonal anti GAL4DBD anitbody (0.1 µg/ml) in 1% skim milk solution in TBST buffer for 1 hour, was then washed with HBSS for 15 min three times, incubated with secondary sheep anti-mouse polyclonal antibody coupled to horseradish peroxidase diluted 1:20,000 in TBST, and washed as previously. ECL reagent was then prepared and applied according to manufacturer's specifications and the blot was exposed to X-Omat Blue XB-1 film for 20 to 40 minutes.

CHAPTER THREE

3. Results

3.1. PPARα and PPARγ2 subtypes possess an autonomous transcription activation function (AF-1) in the amino-terminal A/B domain which is active in both yeast and mammalian cells.

We set out to establish the presence of a ligand-independent transcription activation function (AF-1) in the A/B domains of mPPARα and mPPARγ2. Our approach was to tether each A/B domain to a heterologous DNA binding domain via which we could target each A/B domain to a reporter gene. We cloned the two A/B domains behind the GAL4 DBD in a yeast vector in order to assess their transcriptional activity in yeast cells. We chose to carry our initial characterization in yeast because yeast (Sauer and Tijan, 1997), sharing many components of the basal transcription machinery with mammalian cells, afford a simple and quick system for the assessment of the transcriptional activity of a given polypeptide. For this assay we used the Y190 yeast strain which contains $GAL4_{UAS}$ sites upstream of a β-galactosidase reporter gene (Harper et al., 1993). Our initial β-galactosidase agarose overlay assay indicated that the A/B domains of both mPPARα and mPPARγ2 do indeed possess an intrinsic transcriptional activity (data not shown) or an AF-1 activity.

Having been satisfied that the A/B domains contain an autonomous trans-activation function active in yeast, we undertook identification and characterization of that activity in mammalian cells. We cloned the A/B domains of mPPARα and γ2 into a mammalian GAL4DBD fusion vector. The reporter system used in these studies

consisted of a luciferase reporter vector containing $GAL4_{UAS}$ sites upstream of the Ad E1b promoter.

3.1.1. The AF-1 of mPPARα.

Transient transfection experiments were performed with GAL4DBD:ABα fusions (shown in Fig. 3) coupled with Western immunoblotting to characterize the AF-1 activity of the A/B domain of mPPARα. A titration of the GAL4DBD:ABα construct was performed to establish the presence of an AF-1 activity in the A/B domain of mPPARα as well as to determine a practical experimental range for future experiments and to measure the maximal transcriptional activity of the domain. Results of such titration indicated that the A/B domain of mPPARα does indeed possess an autonomous trans-activating function (Fig. 4). The GAL4DBD:ABα construct was found to be well expressed (Fig. 5A, lane 1). Further studies focussed on narrowing down the region in the A/B domain responsible for the trans-activating potential. Towards that goal a number of deletions fused to the GAL4DBD were constructed (Fig. 3). A titration of a protein deleted in the first 10 amino acids (GAL4: ABα 11-94, Fig. 6), as well as the absence of any transcriptional activity in the 10 amino acid amino-terminal fragment of the A/B domain of mPPARα fused to GAL4DBD (Fig. 9) despite its detectable expression (Fig. 5B, second lane), demonstrated that the first 10 amino acids are not required for the transcriptional activity. A further amino-terminal deletion of the A/B domain (total of 27 amino acids deleted, ABα 28-94) rendered the protein active to only about 5% of the full-length A/B domain (Fig. 9). The fusions exconding the full-length

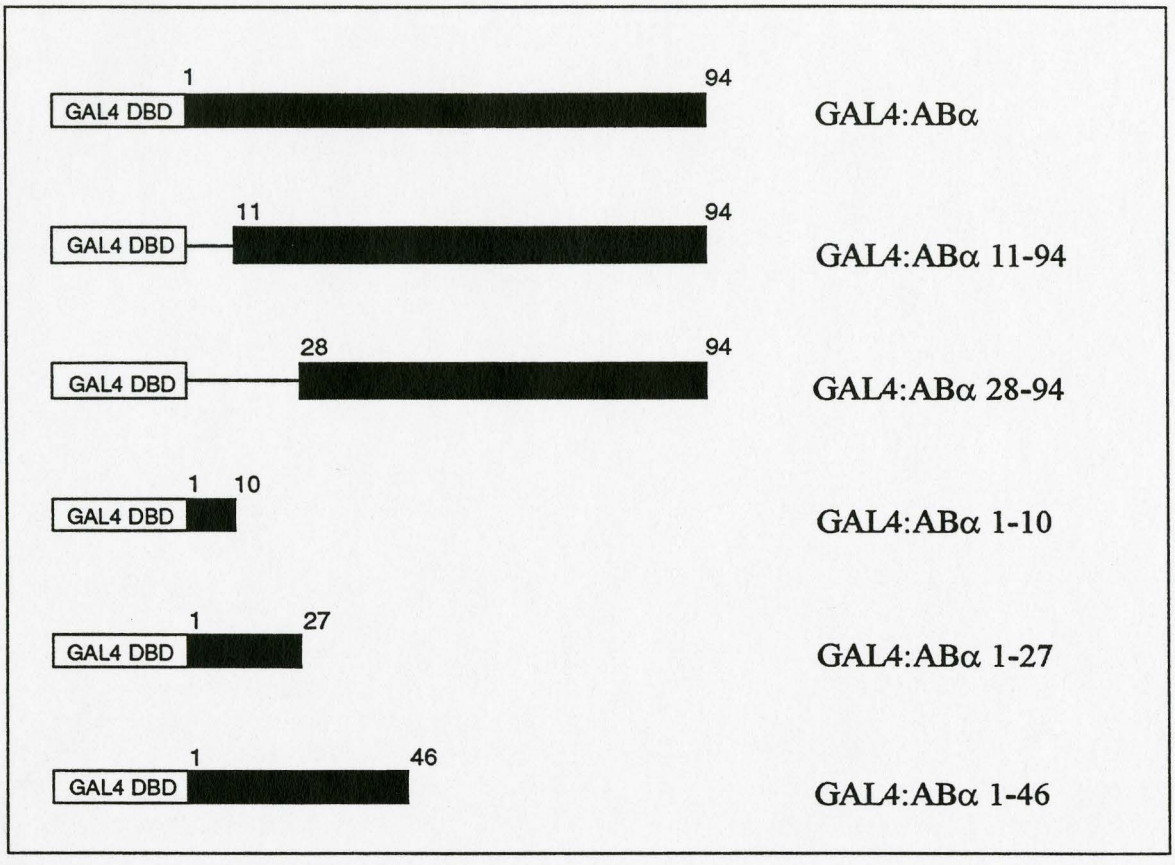


Figure 3. Fusions of GAL4 DBD to mPPARα A/B domain fragments used in experiments.

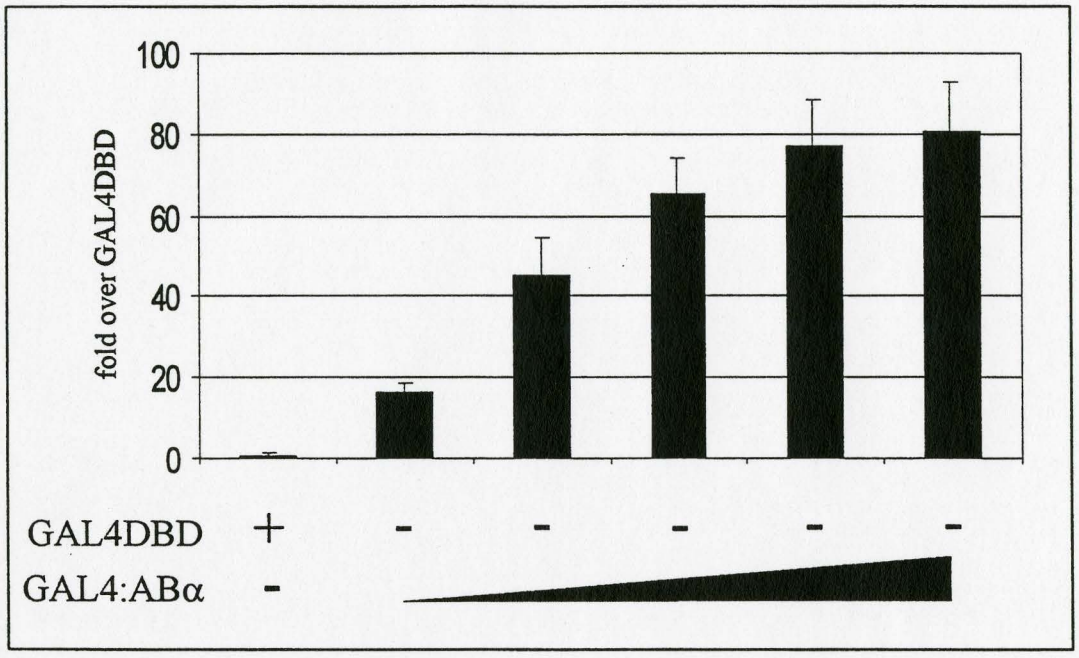


Figure 4. The A/B domain of mPPARα possesses a ligand-independent transcription activation function. BSC 40 cell line was transfected with increasing amounts of GAL4:ABα construct fusion (0.008, 0.03, 0.13, 0.5, and 1 μg) respectively. B-galactosidase activity was used to correct the reporter luciferase activity for transfection efficiency. Each value presented is a mean of three experiments (+/- SD) and expresses corrected reporter activity as a multiple of the corrected reporter activity observed with GAL4DBD alone.

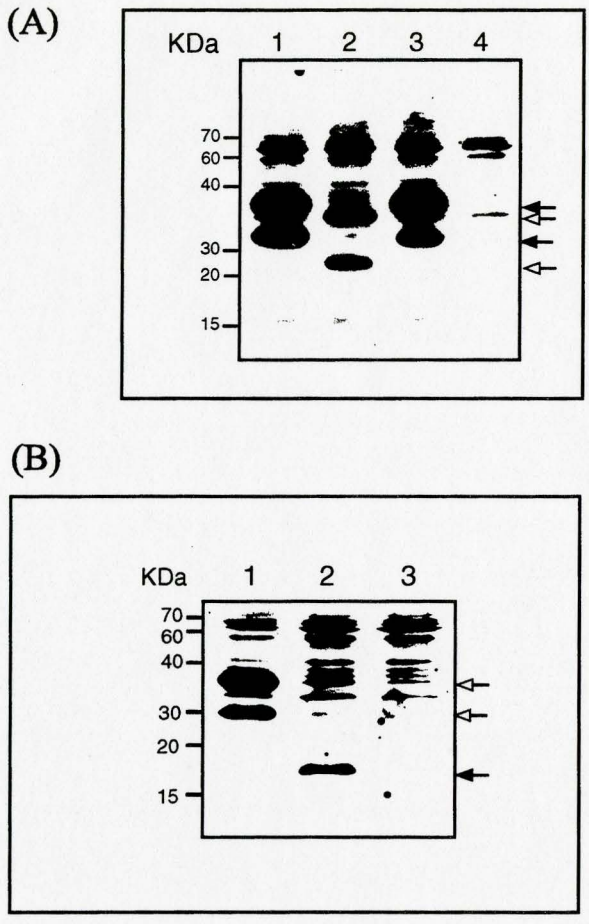


Figure 5. Expression of A/B domain of mPPARα GAL4DBD fusions.
COS-1 cells transfected with 3 μg of appropriate GAL4DBD fusion vector were lysed and 15 μg of each lysate were analysed by Western immunoblotting.
(A) Lane 1 shows the expression of ABα fusion (filled arrows), lane2 ABα 28-94 (open arrows), and lane 3 ABα 11-94 (filled arrows). All fragments run as doublets. Lane 4 represents expression of GAL4 DBD empty vector. (B) Lane 1 shows the expression of ABα fusion which is expressed as a doublet marked by open arrows, lane 2 ABα 1-10 marked by filled arrow, and lane 3 GAL 4 DBD alone. Each blot was detected with anti-GAL4 DBD mouse monoclonal antibody and anti-mouse antibody linked to horseradish peroxidase.

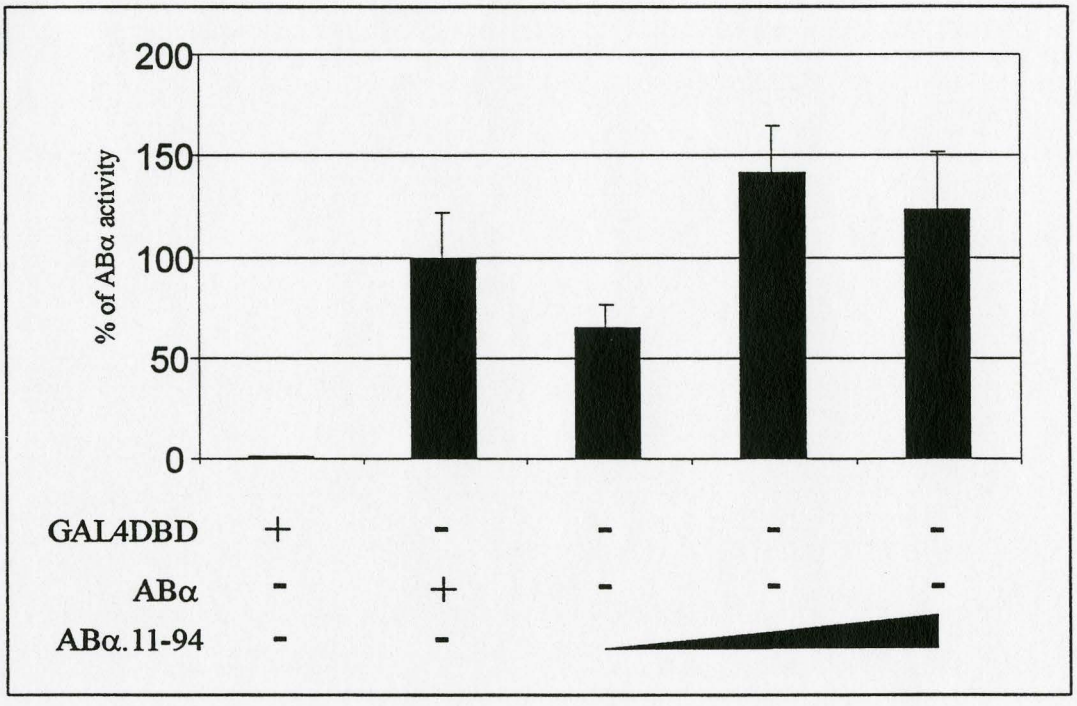


Figure 6. An amino-terminally deleted fragment of the A/B of mPPARα containing amino acids 11 to 94 is as active as the full length A/B domain. BSC 40 cell line was transfected with increasing amounts of GAL4:ABα 11-94 fusion (0.01, 0.1 and 1 μg respectively). β-galactosidase activity was used to correct the reporter luciferase activity for transfection efficiency. Each value presented is a mean of two experiments (+/- SD) and expresses the corrected reporter activity as a percentage of the corrected activity observed with GAL4:ABα.

A/Bα domain, A/Bα 11-94, and A/Bα 28-94 deletions were expressed. (Fig. 5A, lane 1, 3 and 2 resepectively).

A fusion of the first 27 amino acids to GAL4DBD was made and titrated, however, less than 5% of the activity of the full-length A/B domain was recovered (Fig. 7, Fig 9). Note, however, that the protein could not be detected by Western immunoblotting under the conditions used to detect the full length GAL4DBD:ABα fusion (not shown). About 20 % of the activity of the full length A/B domain were observed with the fusion of 46 amino-terminal amino acids to GAL4DBD (Fig. 8, Fig. 9), but again, the expression of this fusion could not be detected by Western immunoblotting (not shown). These results taken together might suggest that although required, the amino-terminal portion of the A/B domain is not sufficient for the full activity of the A/B domain of mPPARα. Recognizing, however, that the lack of a detectable expression of ABα 1-27 and ABα 1-46 GAL4DBD fusions presents a problem, the above statement is not conclusive.

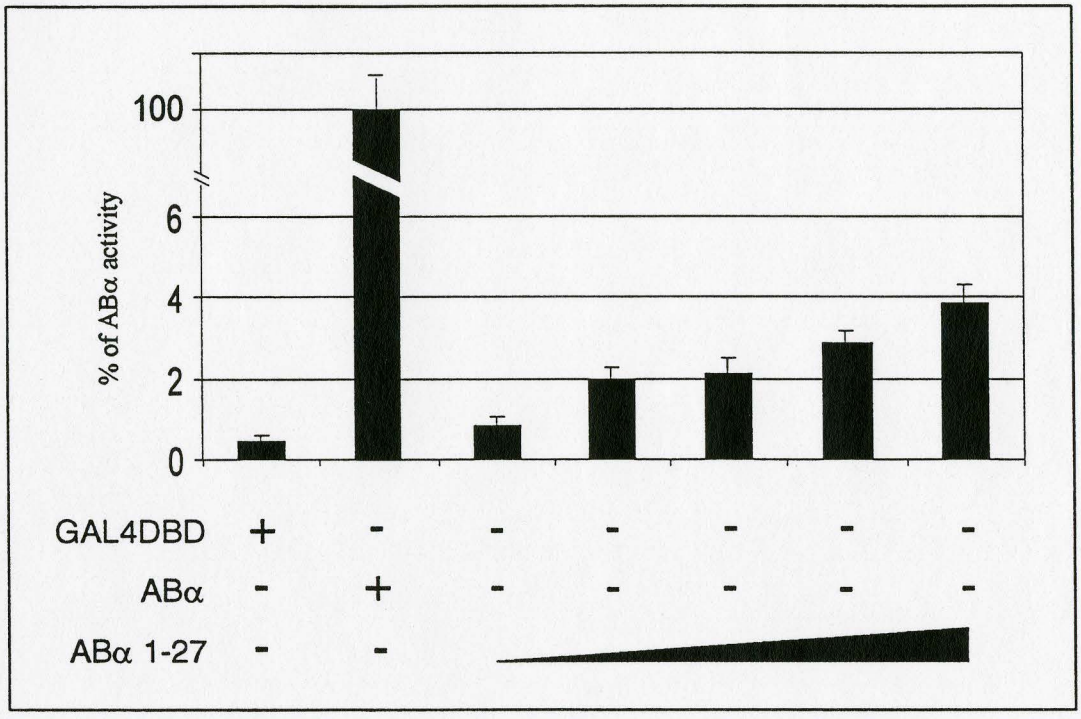


Figure 7. Amino-terminal fragment of the A/B domain of mPPARα containing amino acids 1-27 has little activity of the full length A/B domain. BSC 40 cell line was transfected with increasing amounts of GAL4:ABα 1-27 fusion (0.01, 0.05, 0.1, 0.5, and 1 μg respectively). β-galactosidase activity was used to correct the reporter luciferase activity for transfection efficiency. Each value presented is a mean of two experiments (+/- SD) and expresses the corrected reporter activity as a percentage of the corrected activity observed with GAL4:ABα.

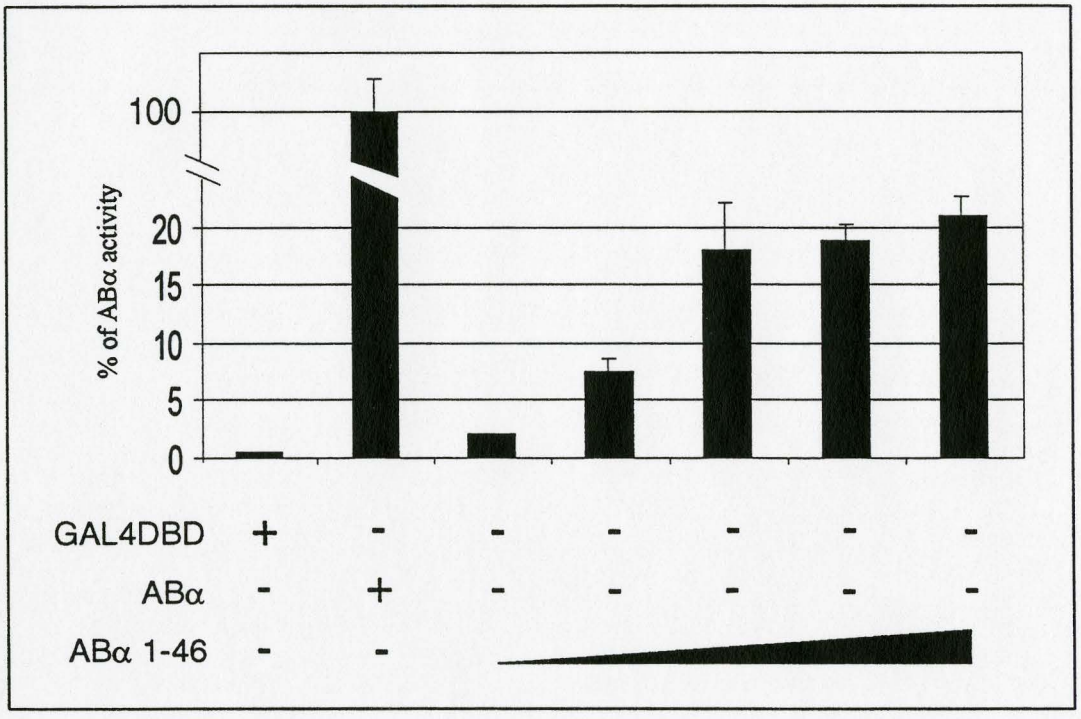


Figure 8. Amino-terminal fragment of the A/B domain of mPPARα containing amino acids 1-46 has some activity of the full length A/B domain. BSC 40 cell line was transfected with increasing amounts of GAL4:ABα 1-46 fusion (0.01, 0.05, 0.1, 0.5, and 1 μg respectively). β-galactosidase activity was used to correct the reporter luciferase activity for transfection efficiency. Each value presented is a mean of two experiments (+/- SD) and expresses the corrected reporter activity as a percentage of the corrected activity observed with GAL4:ABα.

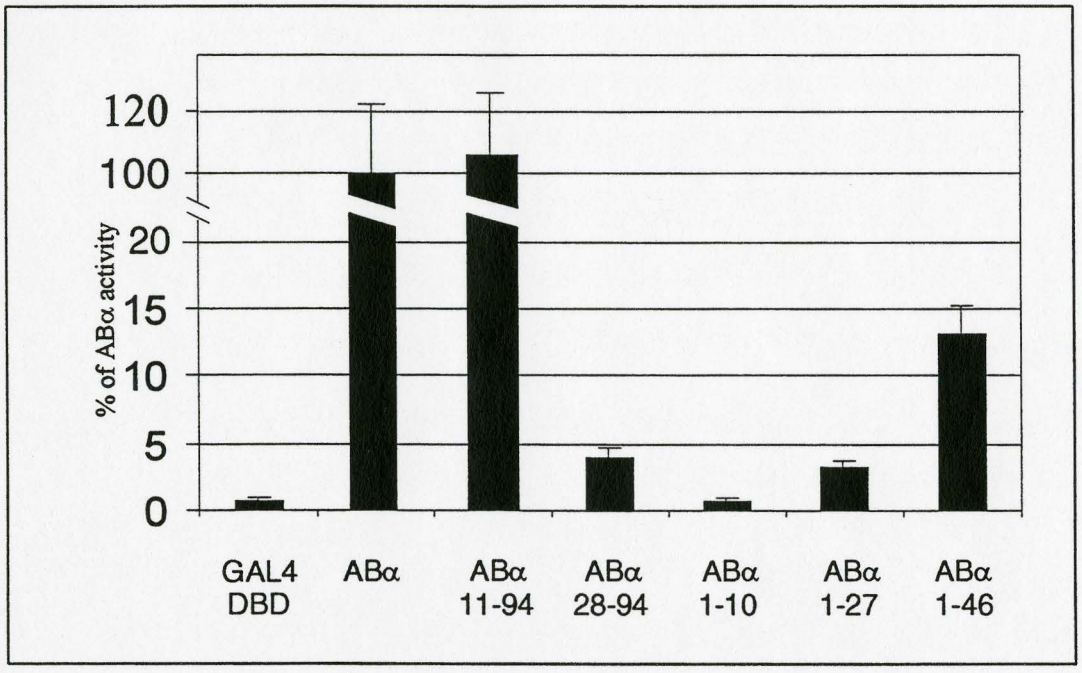


Figure 9. Deletion study of the A/B domain of mPPARα. BSC 40 cell line was transfected with specified fragments of the A/B domain of mPPARα (0.5 μg each). β-galactosidase activity was used to correct the reporter luciferase activity for transfection efficiency. Each value presented is a mean of three experiments (+/- SD) and expresses the corrected reporter activity as a percentage of the corrected activity observed with GAL4:ABα.

3.1.2. The AF-1 of mPPARγ2.

To assess the transcriptional activity of the A/B domain of mPPARγ2, several fusions with GAL4DBD were made as shown in Fig. 10. A titration of the A/B domain of mPPARγ2 fused to GAL4DBD confirmed that this domain is an activator of transcription in mammalian cells (Fig. 11), in agreement with previous findings (Werman et al, 1997). A GAL4DBD fusion to a portion of the A/B domain of mPPARγ2 amino-terminally deleted in 30 amino acids, containing amino acids 31-136 and being the same as the A/B domain of mPPARγ1, is transcriptionally less active than the A/B domain of mPPARγ2 (Fig 12). Further deletion of 43 amino acids from the amino terminus of the A/B (A/Bγ2 44-136) resulted in a protein with less than 10% of the activity of the full length A/B domain (Fig. 12). Both the full length A/B domain and fragment containing amino acids 44-136 were detected by Western immunoblotting (Fig. 13, lanes 1 and 3), while the fusion containing amino acids 31-136 was not detected (Fig. 13, lane 2). These results indicate that the amino-terminal portion of the A/B domain is required for trans-activating activity of the A/B domain of mPPARγ2.

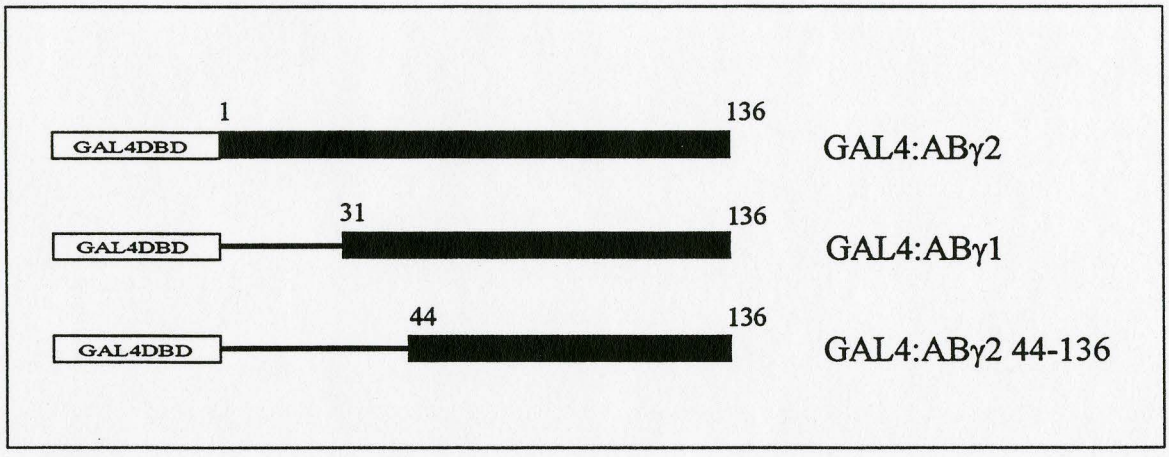


Figure 10. GAL4 DBD mPPARγ A/B domain fusions used in transfection experiments.

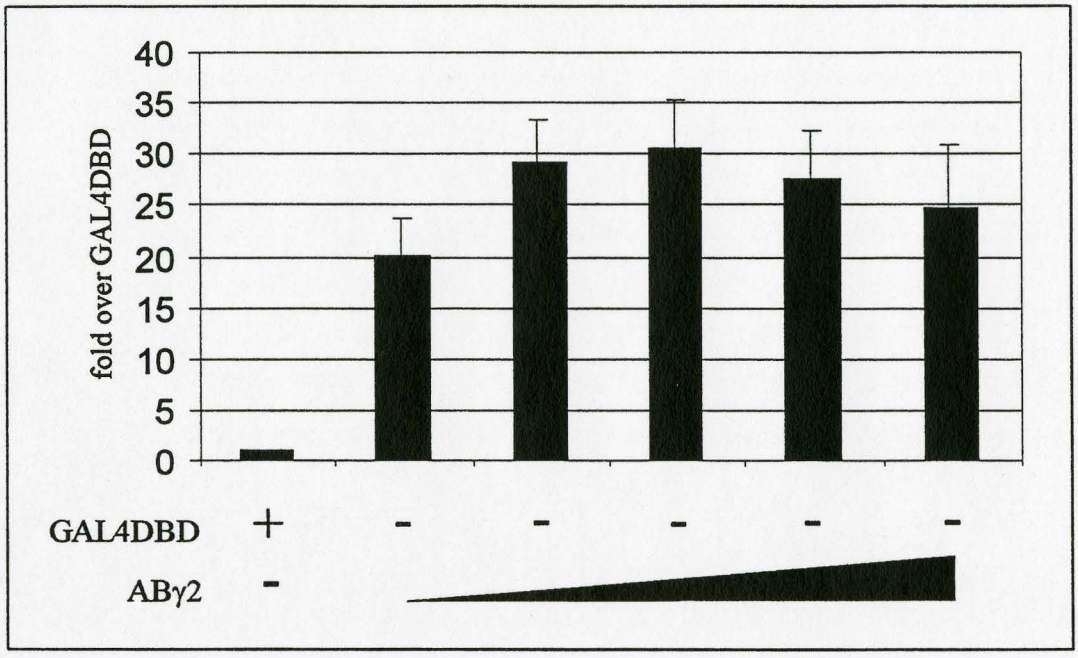


Figure 11. The A/B domain of mPPARγ2 possesses a ligand-independent transcription activation function. BSC 40 cell line was transfected with increasing amounts of GAL4:ABγ2 construct fusion (0.008, 0.03, 0.13, 0.5, and 1 μg) respectively. β-galactosidase activity was used to correct the reporter luciferase activity for transfection efficiency. Each value presented is a mean of three experiments (+/- SD) and expresses corrected reporter activity as a multiple of the corrected reporter activity observed with GAL4DBD alone.

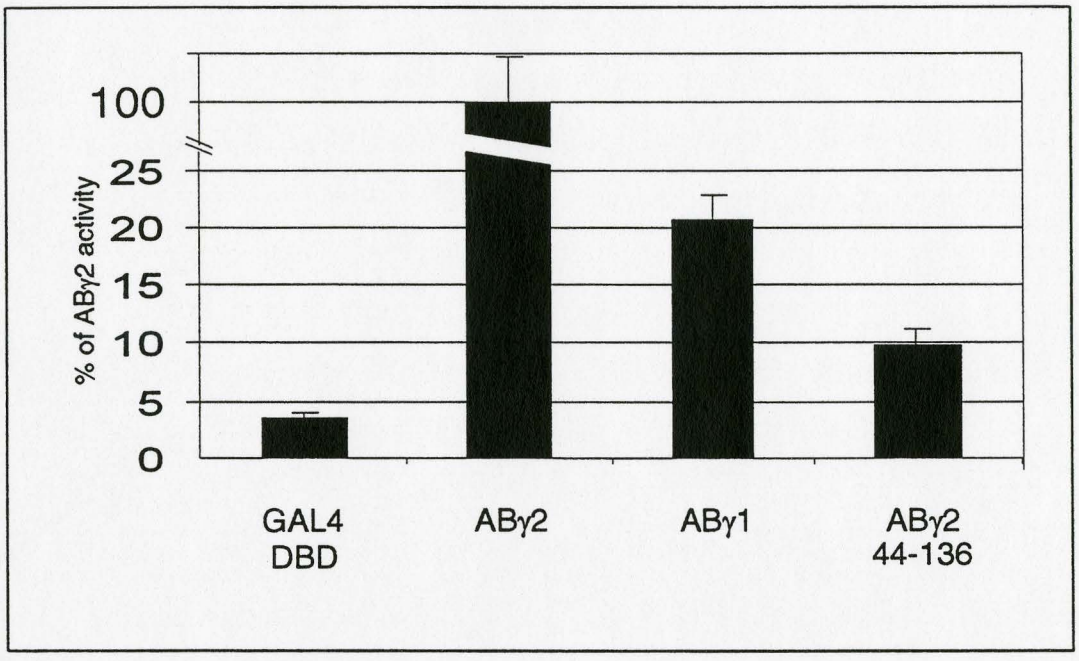


Figure 12. Progressive amino-terminal deletions abrogate the transcriptional activity of the A/B domain of mPPARγ2. BSC 40 cell line was transfected with specified GAL4:ABγ2 deletions (0.5 μg each). β-galactosidase activity was used to correct the reporter luciferase activity for transfection efficiency. Each value presented is a mean of three experiments (+/- SD) and expresses the corrected reporter activity as a percentage of the corrected activity observed with GAL4:ABγ2.

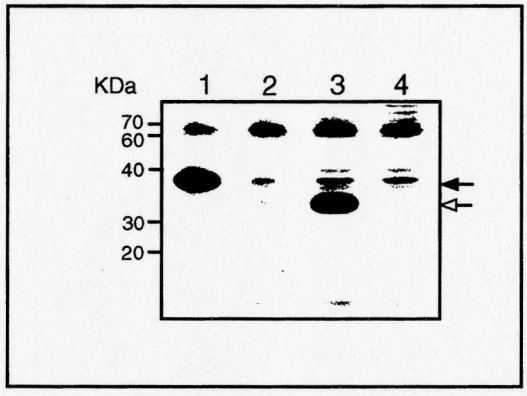


Figure 13. Expression of A/B of mPPARγ2 GAL4DBD fusion constructs. COS-1 cells tranfected with 3 μg of appropriate GAL4DBD fusion vector were lysed and 15 μg of each lysate were analysed by Western immunoblotting. Lane 1 shows expression of ABγ2 fusion (indicated by filled arrow), lane 2 shows inability to detect expression of ABγ1 fusion, lane 3 shows expression of ABγ2 44-136 fusion (indicated by open arrow), and lane 4 shows expression of an empty GAL4 DBD vector. The blot was detected with anti-GAL4 DBD mouse monoclonal antibody and anti-mouse antibody linked to horseradish peroxidase.

3.2. Control of the AF-1 activity in mPPARα

We established that the A/B domains of both mPPARα and mPPARγ2 subtypes contain a constitutive ligand-independent transcription activation function (AF-1) which is active in both yeast and mammalian cells. The transcriptional activity of PPARs and other receptors however, is dependent on the presence of ligand (Kliewer et al., 1992; Mangelsdorf et al., 1995). In fact, some receptors, such as the TR, actively repress transcription in the absence of ligand despite the presence of an AF-1 activity in their A/B domain (Baniashmad et al., 1992, 1995). The lack of a positive transcriptional activity in the absence of ligand is therefore a central feature which allows nuclear receptors to act as ligand-activated molecular switches. It follows then, that the AF-1 activity, if present, must be controlled or masked somehow within the context of the full-length receptor in the absence of ligand for the receptor to fulfill its function. As the mechanisms of such control have not been studied previously, in view of our findings of the AF-1 activity in the A/B domains of PPARs, we proceeded to investigate in some detail the mechanisms of regulation of the AF-1 activity found in the A/B domain of mPPARα.

3.2.1. mPPARα is active only in response to ligand in mammalian cells.

The PPAR/RXR heterodimer has previously been shown to be transcriptionally silent in the absence of ligand by our laboratory and as well as by other researchers, despite the presence of the AF-1 activity in the A/B domain of mPPARα (Kliewer et al., 1992; Zhang et al., 1992, Schoonjans et al., 1996b). Upon the addition of a mPPARα

ligand, such as WY-14, the heterodimer activates transcription. The activity of the AF-1, however, is not dependent on the presence drug when the A/B domain is detached, as shown in our studies presented above. In order to study the drug dependence of the AF-1 activity in the context of the full length receptor we chose to use the GAL4 DBD system used to study the AF-1 activities of the A/B domains of PPARs described above. In this system either the full-length PPAR peptide or fragments thereof were fused to GAL4 DBD and so targeted to $GAL4_{UAS}$ sites upstream of a basal promoter driving a luciferase reporter gene. A major advantage of such a system is that through its simplicity it eliminates the requirement of RXR for targeting of PPAR to DNA. The A/B domains for instance are incapable of DNA binding as are some deletions used in our study.

When the full length mPPARα was fused to GAL4 DBD (shown in Fig. 14), it was found to be transcriptionally silent in transfection experiments in the absence of ligand over the titration range tested (Fig. 15). The protein became active only upon the addition of its ligand. This behavior is in accordance with that of PPAR/RXR heterodimer bound to a PPRE, which is also silent in the absence of ligand. Titration was employed to assess the maximal activity of the receptor and to discount for a possibility that the AF-1 activity may only be active at certain concentrations beyond which possible factors responsible for transmitting the AF-1 activity are no longer available in adequate supply, or 'squelched out'. Our data show that, much like is the case with PPAR/RXR heterodimer, the full-length mPPARα as a GAL4 DBD fusion is active only in response to ligand, despite the presence of the AF-1 activity in the A/B domain. The GAL4 DBD system clearly demonstrates the masking of the AF-1 activity in the absence of ligand in

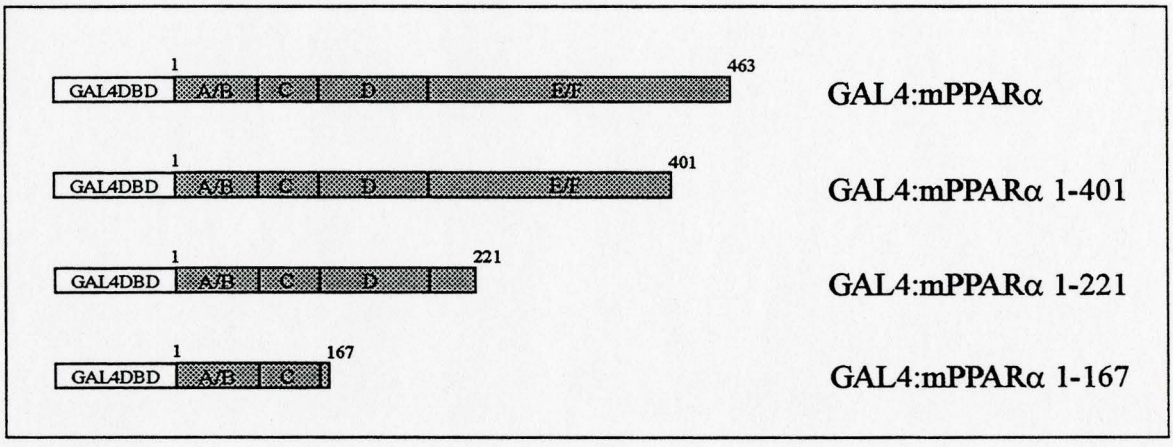


Figure 14. Carboxy-terminal deletion constructs of mPPARα GAL4DBD fusions used in experiments.

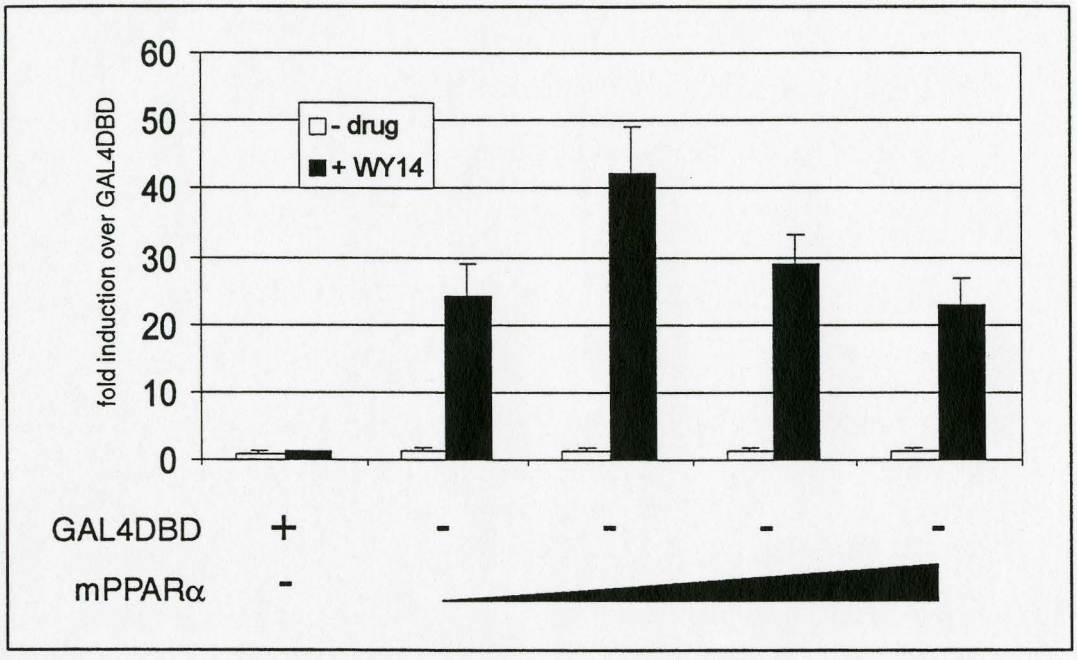


Figure 15. The full length mPPARα is activates transcription only in the presence of its activator when fused to GAL4DBD. BSC 40 cell line was transfected with increasing amounts of GAL4:mPPARα fusion construct (0.01, 0.1, 0.5, and 1 μg) respectively. Where indicated, Wy-14,643 was added to media at 100 μM concentration. β-galactosidase activity was used to correct the reporter luciferase activity for transfection efficiency. Each value presented is a mean of three experiments (+/- SD) and expresses corrected reporter activity as a multiple of the corrected reporter activity observed with GAL4DBD alone in absence of drug.

the context of the full-length receptor. Note that similar findings were made in the case mPPARγ2 GAL4 DBD fusion (data not shown).

3.2.2. The hinge region plays a role in silencing of the AF-1.

In order to locate the regions in the PPAR which play a role in masking of the AF-1 activity in the context of the full-length receptor, a series of carboxy-terminal mPPARα truncations were inserted behind the GAL4DBD (Fig. 14). Titrations were performed in order to determine maximal activity of each construct and to discount for differences in expression levels. Truncation of mPPARα after residue number 401, which truncates the protein in helix 8 of the LBD effectively removing the AF-2, while retaining the majority of the LBD, resulted in a transcriptionally inactive protein in the absence or the presence of ligand (Fig. 16). A truncation immediately carboxy terminal to position 221, which terminates the protein at the beginning of the LBD resulted in a weak constitutive activator of transcription, having under 10% of the activity of the A/B domain (Fig. 17 and Fig. 16). A further truncation of the protein, immediately carboxy-terminal to position 167, or at the start of the hinge D region, resulted in a ligand-independent activator of transcription recovering roughly a quarter of the activity observed with the A/B domain on its own (Fig. 18 and Fig. 16). Note that expression of the full-length mPPARα GAL4DBD fusion as well as that of truncation fusions was observed by Western immunoblotting (Fig. 19). This study indicated that the hinge region, and possibly the DBD, play a role in masking the AF-1 activity found in the A/B domain of mPPARα in the absence of ligand.

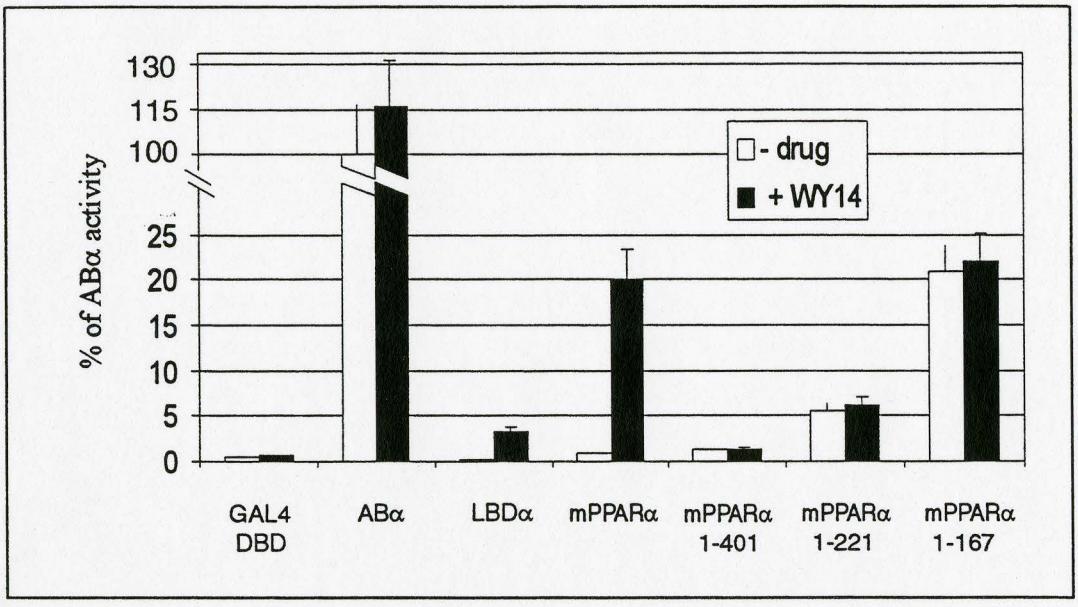


Figure 16. Progressive carboxy-terminal deletion of mPPARa fused to GAL4 DBD unmask the ligand-independent transcriptional activity of the A/B domain. BSC 40 cell line was transfected with specified mPPARα fragments and truncations fused to GAL4DBD (0.5 μg each). Where indicated, Wy-14,643 was added to media at 100 μM concentration. β-galactosidase activity was used to correct the reporter luciferase activity for transfection efficiency. Each value presented is a mean of two experiments (+/- SD) and expresses the corrected reporter activity as a percentage of the corrected activity observed for GAL4:ABα.

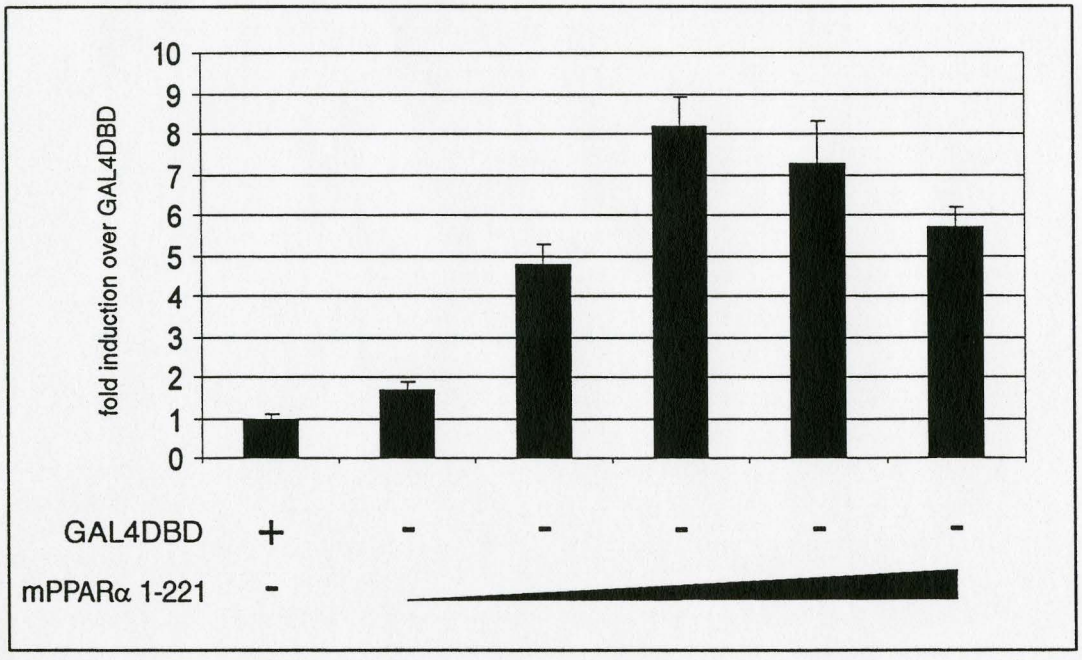


Figure 17. mPPARα fused to the GAL4DBD truncated at the start of the LBD (mPPARα 1-221) is a weak ligand-independent activator of transcription. BSC 40 cell line was transfected with increasing amounts of GAL4:mPPARα 1-221 fusion construct (0.001, 0.01, 0.1, 0.5, and 1 μg) respectively in presence of DMSO but in the absence of drug. β-galactosidase activity was used to correct the reporter luciferase activity for transfection efficiency. Each value presented is a mean of three experiments (+/- SD) and expresses corrected reporter activity as a multiple of the corrected reporter activity observed with GAL4DBD alone.

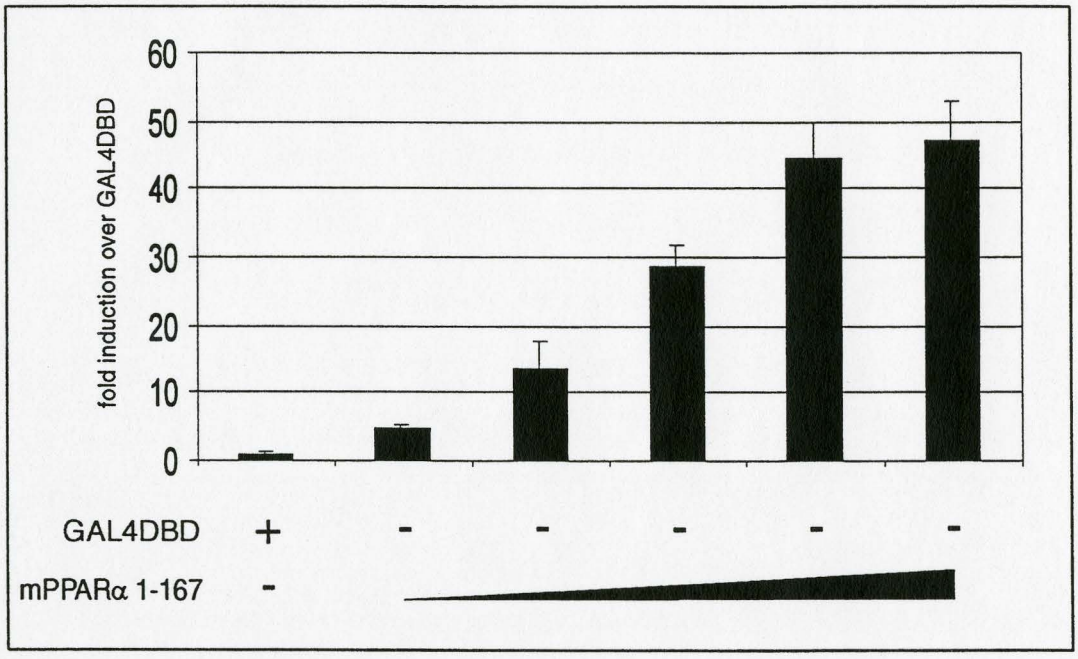


Figure 18. mPPARα fused to the GAL4DBD truncated at the start of the hinge region (mPPARα 1-167) is a ligand-independent activator of transcription. BSC 40 cell line was transfected with increasing amounts of GAL4:mPPARα 1-221 fusion construct (0.01, 0.05, 0.1, 0.5, and 1 μg) respectively in the presence of DMSO but in the absence of drug. B-galactosidase activity was used to correct the reporter luciferase activity for transfection efficiency. Each value presented is a mean of two experiments (+/- SD) and expresses corrected reporter activity as a multiple of the corrected reporter activity observed with GAL4DBD alone.

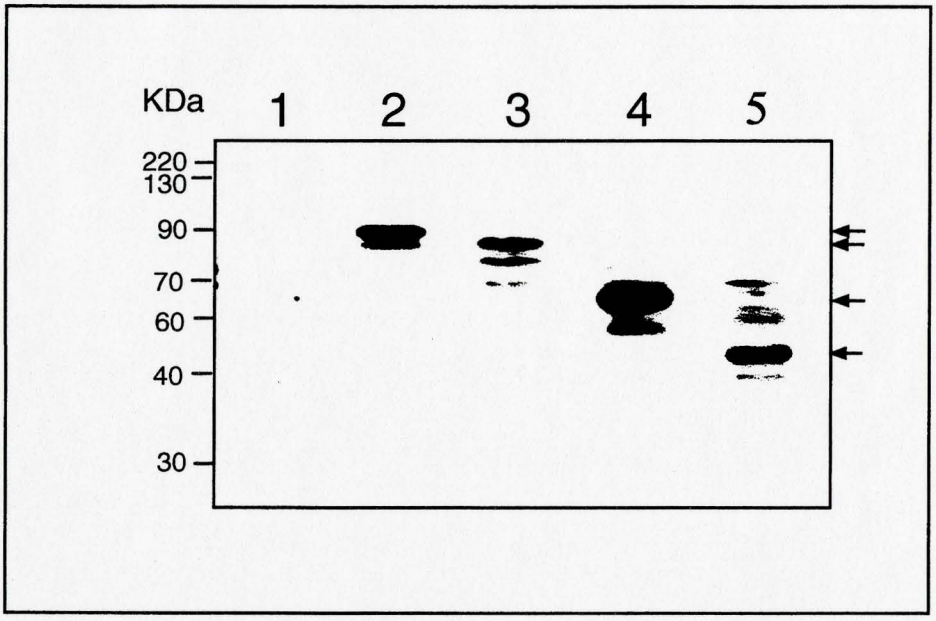


Figure 19. Full length and carboxy-terminally truncated mPPARα fused to GAL4DBD are expressed. COS-1 cells tranfected with 3 μg of appropriate GAL4DBD fusion vector were lysed and 15 μg of each lysate were analysed by Western immunoblotting. Lane 1 shows expression of empty GAL4 DBD vector, lane 2 full length mPPARα fusion, lane 3 mPPARα 1-401, lane 4 mPPARα 1-221, and lane 5 mPPARα 1-167. All fusions migrate as doublets, and the upper band of each is indicated by arrow. The blot was detected with anti-GAL4 DBD mouse monoclonal antibody and anti-mouse antibody linked to horseradish peroxidase.

3.3. Mechanism of masking of the AF-1 activity in the absence of ligand.

Having shown the presence of the AF-1 activity in the A/B domains of PPARs, the fact that it is masked in the context of the full length protein in the absence of ligand, and the fact that the hinge region plays a significant role in the regulation of that activity, we then turned to the investigation of the nature of the regulatory mechanism. The key question we wanted to answer is whether the mechanism of masking is allosteric in nature or if an accessory protein is involved.

3.3.1. PPARs are constitutively active in yeast cells.

Insights into the possible nature of this regulatory mechanism were afforded to us initially by our studies in the yeast system. Workers in our laboratory were previously able to express both PPARs and RXR in yeast and show that they form a transcriptionally active complex on PPREs in yeast cells (Marcus et al., 1995; Marcus et al, 1996). This yeast system employs a yeast strain which harbors episomal vectors expressing PPARs and RXR whose activity is assessed via a reporter vector where β-galactosidase gene is controlled by a PPRE linked to a basal promoter (Marcus et al., 1995, Marcus et al., 1996). In contrast to the findings from mammalian cells, where the activity of PPAR is dependent on ligand, in the yeast system both mPPARα and mPPARγ2 subtypes were found to be constitutively active in the absence of added ligand (Fig. 20). In order to help eliminate a possible influence of response element identity on the ligand independence of transcriptional activation, two different PPRE response elements were used in this study, namely the PPREs from the acyl-CoA oxidise (AOx) and the enoyl-CoA hydratase

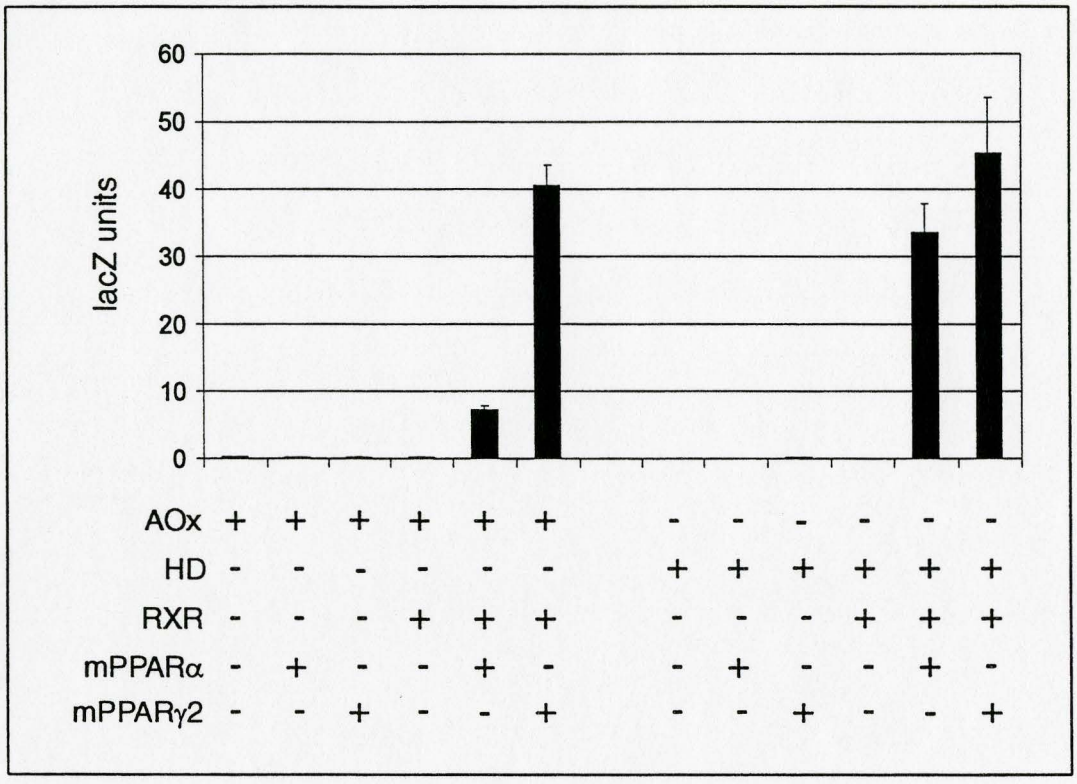


Figure 20. Transcriptional activity of mPPARα and mPPARγ2 via the AOx and the HD PPRE in yeast correlates to their relative binding affities to the AOx and HD PPREs. Yeast strain HF7c was transformed with the AOx or HD promoter and centromeric vectors expressing RXR, mPPARα and mPPARγ2 as specified. Values presented represent fifteen data points (+/- SD) and express β-galactosidase activity as lacZ units calculated by the formula (1000*OD420)/(time*μg of protein).

3-hydroxyacyl-CoA dehyrogenase (HD) genes (Fig. 21). These two genes carry out the fist and second steps in the peroxisomal β-oxidation and are a under positive control of PPARs (Bardot et al., 1995; Bardot et al., 1993; Chu et al., 1995; Dreyer et al., 1992; Gearing et al., 1993; Kliewer et al., 1992; Marcus et al., 1993; Tugwood et al., 1992; Zhang et al., 1992; Zhang et al., 1993). In both cases, despite the different architecture of the two elements, the activity of the PPAR subtypes was constitutive, correlating roughly with the relative binding efficiencies of the mPPARα/RXR and mPPARγ2/RXR heterodimers to the AOx and HD PPREs (previous results obtained by our laboratory; Judge-Aubry et al., 1997).

The constitutive activity of mPPARα and mPPARγ2 is likely due to the presence of the AF-1 activity in the A/B domains of the two mPPAR subtypes. In fact, it is likely that most if not all of the constitutive activity observed in yeast is due to the AF-1, and not the AF-2 activity. First, as shown above, the AF-1 activity is active in both mammalian and yeast cells. Furthermore, our observations and those of others, indicate that the LBDs (and hence the AF-2) of nuclear receptors are in general transcriptionally silent or very weak activators in yeast. Our results which support this hypothesis were obtained with the GAL4 DBD - $GAL4_{UAS}$ yeast system. In this system, both the full-length mPPARα and mPPARγ2 receptors and their respective A/B domains were fused to GAL4 DBD, much like was done in the case of the mammalian GAL4 DBD system. Both the full-length mPPARα and γ2 and their respective A/B domains were found to be active in this system without exogenous ligand added (Fig. 22). This observation is consistent with the observation made in Fig. 20, where the PPAR/RXR

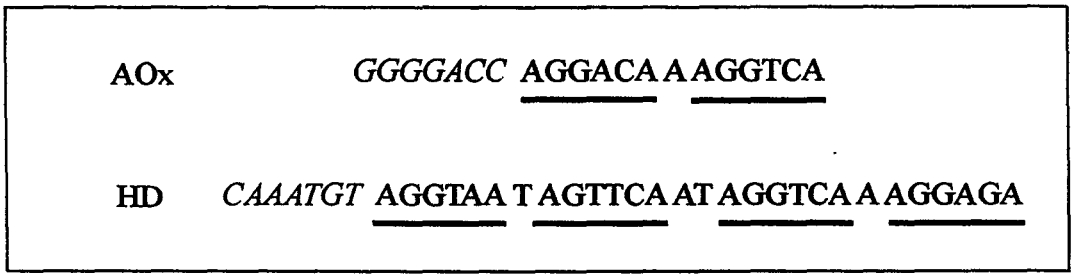
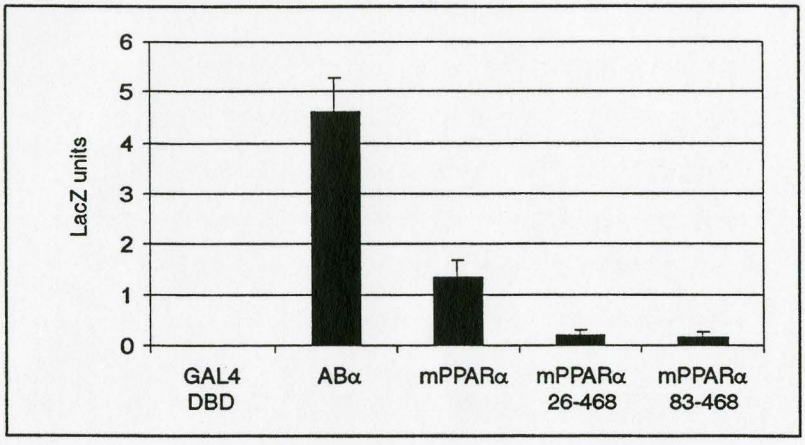


Figure 21. AOx and HD PPRE sequence and architecture. Half sites have been underlined. The 5' flanking sequences have been italicized.

(A)



(B)

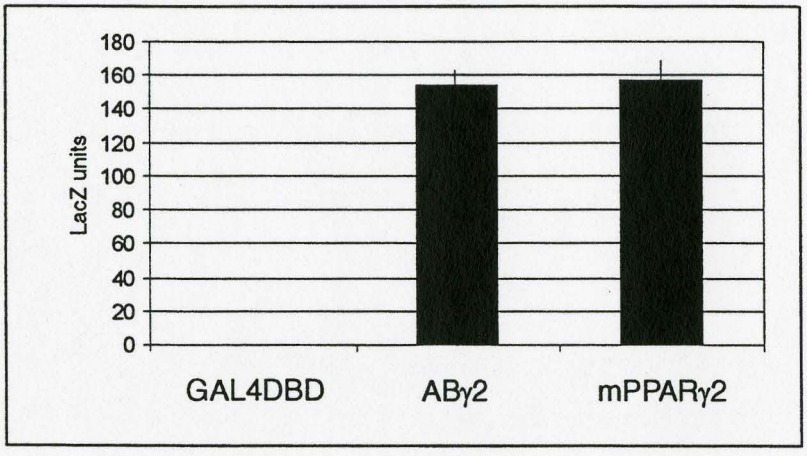


Figure 22. The A/B domain of both mPPARα and mPPARγ2 are transcriptionally active in yeast and are not masked in the context of full length receptors. Yeast strain Y190 was tranformed with 2 μm vectors expressing the A/B domain, full length receptor, or receptor deleted in the A/B domain of (A) mPPARα and (B) mPPARγ2 fused to GAL4DBD. Each value represents six data points (+/-) SD and expresses reporter β-galactosidase activity as lacZ units calculated by the formula $(10^5 * OD_{420})/(\text{time} * \mu g \text{ of protein})$.

heterodimer was found to be constitutively active in yeast. Most, if not all of the trans-activating activity of the full length PPAR GAL4DBD fusion appeared to come from the A/B domain, since mPPARα GAL4DBD fusions deleted in part of or in the A/B domain were inactive in yeast (Fig. 22, see mPPARα 26-468 and mPPARα 83-468), suggesting that the observed constitutive activity of PPARs in yeast is, in fact, due to an unmasked AF-1 activity.

3.3.2. Is a regulatory protein likely to mediate masking of the AF-1 activity in mammalian cells?

The masking of the AF-1 activity in the absence of ligand may be carried out by either an allosteric mechanism or by a recruitment of a regulatory protein in the absence of ligand. Based on our data obtained in our studies, the question as to which mechanism is correct can be best answered in consideration of results of others, and hence will be discussed in the discussion section.

3.4. RIP140 appears to function as a co-activator in yeast for the PPAR/RXR heterodimer and the AF-1 activity in the A/B domains of mPPARα and γ2 subtypes.

Little is known about the molecular mechanisms of trans-activation by the AF-1 activity found in the A/B domains of nuclear receptors. Much less is known about the nature of the postulated accessory protein(s) responsible for the attenuation of the AF-1 activity in the unliganded receptor. As outlined in the introduction, the interaction of the

A/B domains of several receptors with certain co-activators has been demonstrated previously. In order to help elucidate mechanisms of function of the A/B domains of mPPARα and γ2, we investigated the effect of a protein named RIP140 on the AF-1 activity.

In a two-hybrid screen, our laboratory isolated a protein previously cloned and referred to as RIP140, which has been shown to interact with and modulate the activity of other nuclear receptors (Cavailles et al, 1995). In the cases of the receptors tested, including mPPARα tested in our laboratory, RIP140 seems to activate the ligand-dependent transcription when transfected at low amounts, however, it represses the trans-activation when transfected at higher amounts (Cavailles et al, 1995, Miyata et al, 1998).

In view of the above facts, we tested the function of RIP140 within our PPAR/RXR yeast system. In the yeast, co-transformation of the RIP140 expression vector with PPARα/RXR vectors resulted in an augmentation of transcription via the AOx element (Fig. 23), suggesting that RIP140 may serve as a co-activators in PPAR signaling.

To study the role of RIP140 in modulation of the AF-1 activity of mPPARα and mPPARγ2, we initially took advantage of the yeast system. We observed that RIP140 enhances the transcriptional activity of A/B domain of both mPPARα and mPPARγ2 fused to GAL4DBD as well as that of the full- length GAL4DBD fusions of mPPARα and mPPARγ2 (Fig. 24). In mammalian cells, however, RIP140 attenuated the activity of the A/B domains both mPPARα and γ2 (Fig. 25), reflecting the results of K. Miyata

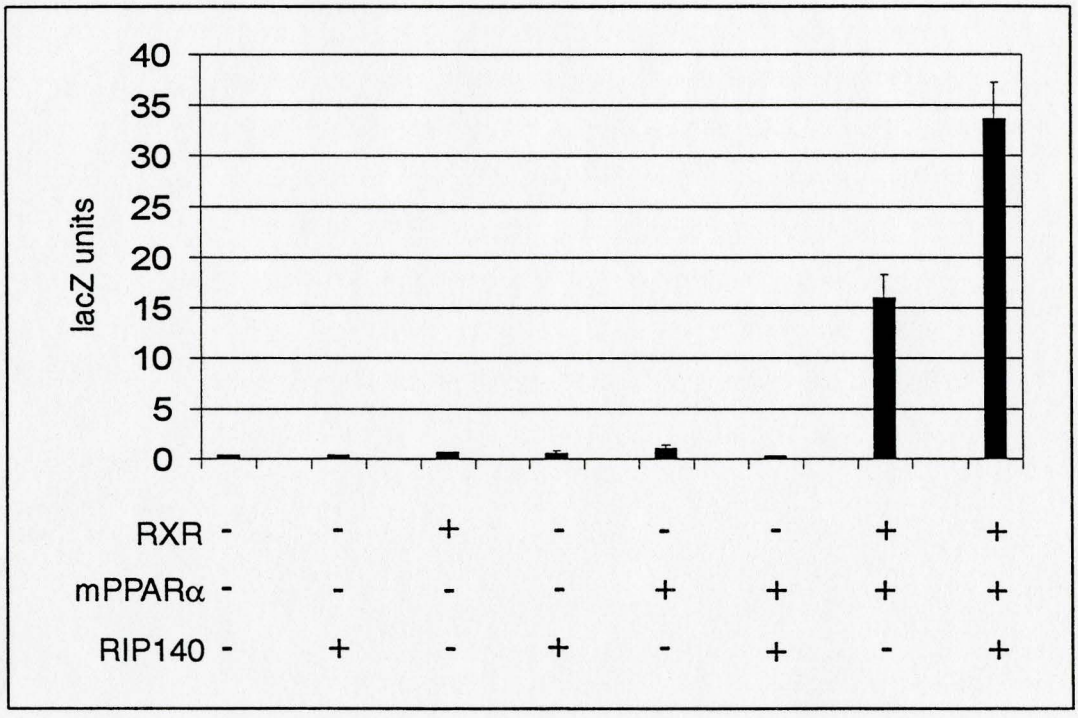
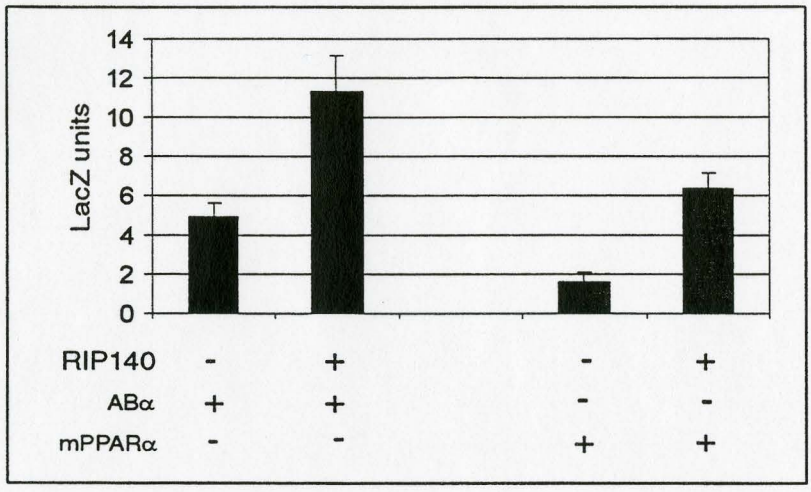


Figure 23. RIP140 acts as a co-activator protein for mPPARα in yeast. Yeast strain HF7c was transformed with the AOx reporter and centromeric vectors expressing RXR, mPPARα and RIP140 as specified. Values presented represent six data points (+/- SD) and express B-galactosidase activity as lacZ units calculated by the formula (1000*OD420)/(time*μg of protein).

(A)



(B)

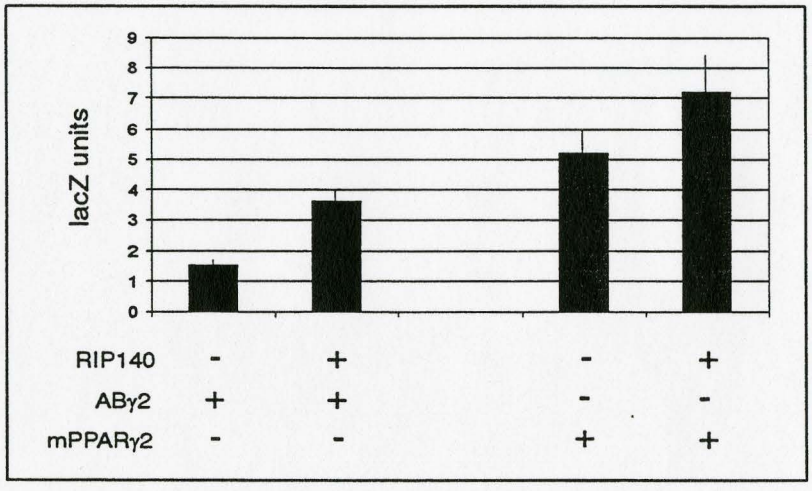


Figure 24. RIP140 serves as a coactivator in yeast for both A/B domains of and full length mPPARα and γ2 subtypes. Expression vectors for the A/B domain and mPPAR GAL4DBD fusions together with RIP140 expression vector were transformed into (A) Y190 for α and (B) MaV103 for the γ2 subtype. Each value represents six data points (+/- SD) and expresses reporter β-galactosidase activity as lacZ units calculated by the formula $(10^5 * OD_{420})/(time * \mu g$ of protein).

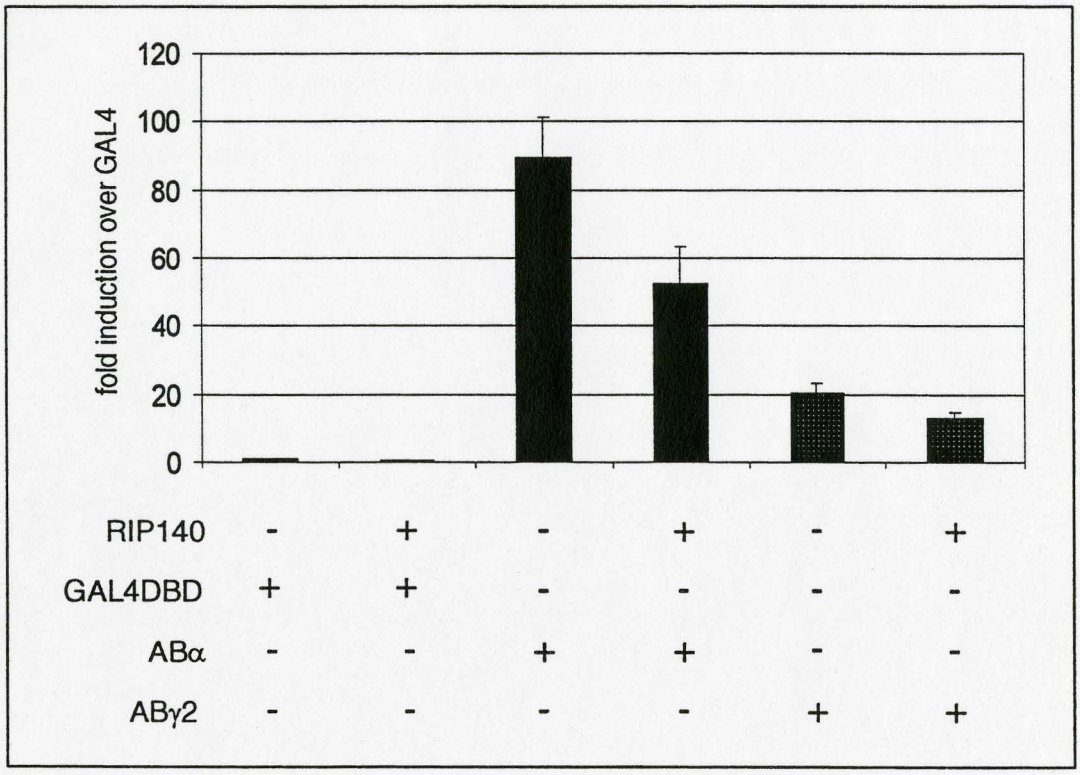


Figure 25. RIP140 appears to repress transcriptional activity of the A/B domain of mPPARs. BSC 40 cell line was transfected with constructs expressing the A/B domain of either mPPARα or mPPARγ2 fused to GAL4DBD in combination with RIP140. B-galactosidase activity was used to correct the reporter luciferase activity for the transfection efficiency. Each value presented is a mean of three experiments (+/- SD) and expresses the corrected reporter activity as a multiple of the corrected reporter activity observed with GAL4DBD alone.

and colleagues (Miyata et al, 1998) and others (Treuter et al, 1998) obtained with full length mPPARα/RXR heterodimer on the AOx response element. No good attempt at detection of binding of RIP140 to the A/B domains of PPARα and γ2 was made due to technical difficulties.

CHAPTER FOUR

4.0. Discussion

4.1. Identification of the AF-1 activity in the A/B domains of mPPARα and mPPARγ2.

The A/B domains of several nuclear receptors have been shown to contain an autonomous ligand-independent transcription activating function termed AF-1. In view of these findings, we set out to identify and characterize the AF-1 activity in the A/B domains of mPPARα and mPPARγ2. Our data indicate that both mPPARα and γ2 subtypes do in fact possess an AF-1 activity in their A/B domains which is active in both yeast (Fig. 22) and mammalian cells (Fig. 5 and Fig. 11).

Our results confirmed a previous study which indicated that the A/B domain of mPPARγ2 possesses an activation function when fused to GAL4 DBD (Werman et al., 1996). That study found the presence of the AF-1 activity in mPPARγ2 on the basis of results obtained in mammalian cells only. Our results extended those findings to showing the AF-1 activity to be functional in yeast cells as well. Another study, which was published while our work was in progress, confirmed our finding that the A/B domain of mPPARα contains an AF-1 region active in mammalian cells. That study also showed that phosphorylation of serine residues in the amino-terminal portion of the A/B domain of mPPARα is required for the AF-1 activity (Juge-Aubry et al, 1999). While we did not study the effects of phophorylation on the AF-1 activity of mPPARα, our results show that the AF-1 activity is active in yeast cells as well as mammalian cells, and, most importantly, is subject to regulation in the context of the full length receptor.

Our study was partly aimed at identifying regions in the A/B domain of mPPARα and mPPARγ2 responsible for the AF-1 activity of the proteins. As described in the introduction, a region responsible for conferring the ligand-dependent AF-2 activity has been identified and was found to contain a motif common to most, if not all, members of the nuclear receptor superfamily. An analogous common region or motif conferring the AF-1 activity has not been found and is likely not to exist, as the A/B domains are very divergent. The primary sequence of PPARs was hence of little help to our goal. We therefore conducted a deletion study in order to identify regions important for the AF-1 activity of mPPARα and γ2 subtypes. Our deletion study of the A/B domain of mPPARα indicated that the region between amino acids 11 to 27 is required for the AF-1 activity (Fig. 6, Fig. 9). We then assessed the transcriptional activity of amino-terminal fragments of the A/B domain of mPPARα in order to see whether the AF-1 activity is contained within the amino terminal portion of the A/B domain. We were able to measure a weak transcriptional activity of fragments consisting of amino acids 1 to 27 and 1 to 46 (Fig. 7 and Fig. 8 respectively). The lack of detectable expression of these fragments (not shown), however, prevented us from concluding whether or not the amino terminal portion of the A/B domain is sufficient for the AF-1 activity of mPPARα. Our results are consistent with results of the study of Juge-Aubry et al. (1999), which implicated this region of the A/B domain as important for AF-1 activity. That study found phophorylation of Ser12 and Ser21 found in this region to be required for the activity of the A/B domain mPPARα (Juge-Aubry et al, 1999).

All GAL4DBD:mPPARα constructs, except for the 1 to 10 amino acids fragment, migrated as doublets on Western immunoblots, with the relative difference in sizes of the bands in the doublet increasing as smaller fragments of mPPARα were expressed (Fig. 5, Fig. 19). The doublet may have been a result of protein degradation or phosphorylation status of the protein. The presence of a protease sensitive site in the protein would likely have led to the appearance of a band on the Western blot common to the various deletions. Since such band was not observed, protein degradation may be discounted, leaving protein phosphorylation as the likely cause of the appearance of doublets. The fact that the fragment (ABα 28-94) which does not contain the two previously identified phosphorylation sites (Juge-Aubry et al., 1999) still migrates as a doublet (Fig. 5A, lane 2) indicates that other modification sites may exist in the protein.

A more limited deletion study was carried out on the A/B domain of mPPARγ2. This study indicated that the A/B domain of mPPARγ2 is a stronger activator of transcription than the A/B domain of mPPARγ1, the latter being a 30 residue amino-terminal deletion (Fig. 12). Furthermore, it was found that the 43 amino-terminal amino acids are necessary for the transcriptional activity of the A/B domain of mPPARγ2 (Fig. 12). Although we did not show that the AF-1 activity is confined to the amino terminal portion of the A/B domain, it may be significant that the amino terminal portions of A/B domains of both mPPARα and γ2 are required for the AF-1 activity.

Our findings pertaining to the mPPARγ are in agreement with a previous study which showed that the A/B domain of PPARγ2 isoform is a stronger activator than the

A/B domain of PPARγ1 (Werman et al, 1997). In that study, the 30 amino acid extension was shown not to be able to activate transcription on its own. Furthermore, it was noted in that study that the presence of a region in the C-terminal end of the domain attenuates the activity of this domain. This region, incidentally, contains Ser112 whose phosphorylation has been shown to result in attenuation of the activity of the receptor (Reginato et al., 1998; Hu et al., 1996). These findings, similarly to the findings pertaining to the A/B domain of mPPARα described above (Juge-Aubry, 1999), may implicate serine phophorylation in the regulation of transcriptional activity of AF-1. Phosphorylation of Ser112, however, has only been shown to be relevant to the regulation of the activity of the entire PPARγ receptor. A study suggested that the recruitment of the co-repressor SMRT-1 in response to Ser112 phosphorylation is behind the attenuation of PPARγ2 transcriptional activity (Lavinsky et al., 1998). More recently, phophorylation at Ser112 has been linked to a decreased affinity for ligand (Shao et al, 1998). It is not at all clear how AF-1 activity or its regulation are related to the above effects.

4.2. Masking of AF-1 activity in the absence of ligand.

Even though the molecular mechanisms of the AF-1-mediated trans-activation are beginning to be slowly identified (see Introduction), it is not known how the AF-1 activity is controlled. Control via phosphorylation has been suggested for mPPARα (Juge-Aubry et al., 1999). Such mechanism, however, does not address how the AF-1 activity is controlled in response to ligand. When the A/B domains of both mPPARα and

γ2 subtypes are targeted to DNA using a heterologous DNA-binding domain, the AF-1 activity contained in those domains is observed in the absence of ligand (Fig. 4 and Fig. 11). Since PPARs, similarly to other receptors, function as ligand-activated molecular switches, clearly a mechanism must be in place to suppress this constitutive AF-1 activity in the context of the full-length receptor when ligand is not present.

The main part of our study was aimed at understanding the control of the AF-1 activity in the absence of ligand in the context of the full-length receptor. For the sake of consistency with the study of the AF-1 in the context of the A/B domains of mPPARs, we decided to tether the full-length mPPARα receptor to GAL4 DBD as a means of conveniently targeting PPAR to DNA. Normally, RXR is required for binding of PPAR to PPREs. Using GAL4DBD dispensed with the RXR requirement and provided a much simpler transfection system, and, as mentioned, allowed for targeting of receptor fragments which could not otherwise be targeted to DNA due to their inability to bind RXR. Furthermore, as RXR is a nuclear receptor in its own right, the GAL4DBD system allowed us to focus on PPAR alone.

In mammalian cells, both mPPARα (Fig. 15) and mPPARγ2 (data not shown) tethered to GAL4DBD were inactive in the absence of ligand and became active when ligand was added. As this mirrored the behavior of PPAR/RXR, these findings indicated to us that the GAL4DBD system is a viable model system. These results also provided a more striking evidence that the AF-1 activity carried in the A/B domains is in fact masked in the absence of ligand in the full length receptor in mammalian cells.

In order to characterize the region of mPPARα important for the masking of the AF-1 activity, the transcriptional activity of a number of carboxy-terminal deletions was assessed. Truncation of the protein which resulted in the removal of the AF-2 helix while leaving the majority of the LBD intact resulted in a silent protein irresponsive to ligand (Fig. 16). This is expected, as the AF-2 helix has been shown previously to be required for both the release of co-repressors and the recruitment of co-activators and hence trans-activation in response to ligand. AF-2 mutants of other receptors are transcriptionally silent (Moras and Gronemeyer, 1998). Deletion of the LDB which left the hinge region intact resulted in a protein with a low constitutive activity (Fig. 17 and Fig. 16) . Further truncation which deleted the hinge region of the protein resulted in a protein with considerable constitutive activity (Fig. 18 and Fig. 16), implicating that the hinge region is a key determinant of suppression of the AF-1 activity. The possible mechanisms of this asking will be addressed in the following section.

An interesting observation is that the A/B domains of several receptors appears to inhibit DNA binding of nuclear receptors to their cognate response elements (Leid et al, 1992, Hsu et al, 1998). This inhibition appears to be counteracted by the presence of LBDs. As DNA binding is carried out by the DBD as well as the CTE region which overlaps the hinge region to an extent, these observations lend support to some communication, either direct or indirect, between the A/B domain, which contains the AF-1, and the hinge region of nuclear receptors.

4.3. Possible mechanism of masking.

The masking of the AF-1 activity can conceptually be carried out via an allosteric mechanism or via the recruitment of an accessory protein. In the allosteric model, the full-length receptor in the absence of ligand is in the inactive conformation and masks the AF-1 activity in the absence of other regulatory proteins. In the accessory protein model, the AF-1 is not masked by the full-length receptors in the inactive conformation in the absence of ligand. Instead, a protein is recruited to the receptor in the absence of ligand to mask the AF-1 activity. Note that 'inactive conformation' is meant to refer to receptor whose LBD is in the inactive conformation as described in the introduction.

To answer the question as to which mechanism is the correct one, first it has to be stated that yeast do not contain endogenous nuclear receptors or homologs of any of the characterized mammalian regulatory proteins which are known to interact with nuclear receptors. This strongly suggests that it is in fact the presence of a regulatory mammalian factor which is responsible for the masking of the AF-1 activity in the absence of ligand observed in the mammalian but not yeast cells (compare Fig. 15 vs. Fig. 22). This does not, however, discount the allosteric mechanism of masking of the AF-1 activity.

Given our data, answering which one of the two mechanisms is the correct one will depend on what role in trans-activation one assumes the ligand to take. Traditionally, it has been thought that ligand binding causes the receptor to undergo a conformational change, in that the AF-2 helix moves from the inactive to active conformation in response to ligand binding (reviewed in Moras and Gronemeyer, 1998). This means that prior to ligand binding, the receptor exists in the inactive conformation, and after ligand binding, the receptor is in the active conformation.

Given this view on the effects of ligand binding, the allosteric mechanism of repression would only be likely in the event that yeast contain endogenous PPAR ligands, since the full length receptor is active in yeast. Presence of such ligands could account for the constitutive activity of PPAR/RXR heterodimer (Fig. 20) and the lack of repression of the AF-1 activity in yeast in the context of the full length PPAR GAL4DBD fusions in yeast (Fig. 22). As discussed below, yeast are not likely to contain PPAR ligands. Hence it is very likely then that mammalian cells contain a regulatory protein capable of masking the AF-1 activity in the absence of ligand.

There is, however, an alternate way of considering the role of ligand in transcriptional activation by nuclear receptors. It has been suggested previously (Schulman et al., 1996) that the receptor can exist in both the active and inactive conformations prior to ligand binding, and that ligand binding merely shifts this equilibrium between the two states towards the active state, or, in other words, stabilizes the active conformation. As some receptors are subject to phosphorylation and interact with a number of regulatory proteins, there may be other factors which affect this equilibrium.

The author of this work would like to postulate that whereas the equilibrium between the active and inactive states falls for a particular receptor will depend on that receptor, such that while most receptors prefer to be in the inactive conformation in the absence of ligand, PPARs are more free to move between the active and inactive states in the absence of ligand. The evidence for the above is summarized below.

As described in the introduction, the ligand-dependent activation of transcription in mammalian cells is mediated by the LBD domain through a conformational change in the AF-2 region (Moras and Gronemeyer, 1998). The equilibrium model is consistent with a crystallographic study of a dimer of LBDs of PPAR, where is was observed that one member of the dimer exists in the active conformation despite being unoccupied by the ligand (Nolte et al., 1998). The possibility that PPARs can adopt both the active and inactive states freely is further supported by the fact that the binding of many co-activators to PPARs is merely ligand-enhanced, while for other receptors it is strictly dependent on the presence of ligand (Zhu et al., 1997; Dowell et al., 1997; Heinlein et al., 1999; DiRenzo et al., 1997).

Now consider the observations in yeast. One striking feature of PPAR/RXR-mediated trans-activation in our yeast system is the independence of transcriptional activity on the presence of ligand (Fig. 22). In the past, attempts to further augment transcriptional activity of PPAR in yeast were not successful (Marcus et al., 1995). This is actually quite surprising, given that ligand inducible yeast systems exist for other receptors, such as TR, RAR, RXR, and ER (Privalsky et al., 1990; Hall et al., 1993; Sande and Privalsky, 1994; Heery et al., 1993; Allegretto et al., 1993; Mak et al., 1994; Heery et al., 1994; Metzger et al., 1988; Metzger et al., 1992).

Our observations are especially surprising, given that yeast are not likely to contain PPAR ligands. The evidence for this comes from the following study. As described in the introduction, co-activators are thought to interact with receptors which are in the active conformation while co-repressors are thought to interact with receptors

which are in the inactive conformation. The LBD of PPAR is capable of binding both co-activators such as SRC-1 and co-repressors such as N-CoR in yeast (Zhu et al, 1996; Dowell et al, 1999). The addition of PPAR ligand Wy-14 does in fact abolish interaction with N-CoR as expected (Dowell et al, 1999). These findings point to the absence of endogenous PPAR ligands in yeast, since these would likely bias the conformation towards the active state and exclude the co-repressor from binding.

Now consider that in the yeast the AF-2 of nuclear receptors is not functional, but the AF-1 is. In our system, the constitutive activity observed in yeast cells likely comes from the AF-1 located in the A/B domain and not AF-2 found in the C-terminus as is suggested by the inactivity of GAL4 DBD fusions with mPPARα deleted in the amino-terminus (mPPARα 26-468, mPPARα 83-468) in yeast (Fig. 22A). This is in agreement with several previous findings, which indicate that the LBD of PPAR as well as that of ER are transcriptionally silent in yeast cells (Zhu et al., 1997; Louvion et al, 1993, Berry et al, 1990).

Keeping the above in mind and the fact that yeast are not likely to contain regulatory proteins, the author would like to postulate that in the inactive conformation, the AF-1 is not functional, but that it is masked allosterically, and only becomes active when the receptor is in the active conformation. This postulate, in view of the equilibrium model, explains the fact that the receptors other than PPAR, as described, are inducible in yeast. The author would like to postulate that these receptors, based on the fact that their binding of co-activators is ligand dependent, naturally exist in the inactive state in the absence of ligand. The AF-1 is masked masked allosterically, and

these receptors are inactive in yeast in absence of their cognate ligands. PPARs however, which appear to be able to assume the active conformation in the absence of ligand more freely, do not mask their AF-1 effectively, and hence appear to have constitutive activity in yeast.

The activity of PPARs is not constitutive in mammalian cells, however. The AF-1 appears to be masked in the absence of a PPAR ligand. The author of this work would like to suggest that mammalian cells contain a factor or factors which indirectly mask the AF-1 activity by shifting PPAR's equilibrium between the active and inactive state towards the inactive state, or maintaining the PPARs the inactive state. Having such factor would provide a mechanism for maintaining PPARs in a silent state, while providing an additional level of control of the activity of the receptor. It must be stressed that no such factors have been found up to date.

Overall, it may be said after all that it is in fact the presence of a mammalian factor which mediates masking of theAF-1 activity in the absence of ligand. The analysis presented, however, makes the distinction between maintaining the receptor in an inactive state and the masking of the AF-1 activity. This distinction allows for a reconciliation of the fact that PPAR are not ligand inducible in yeast while other receptors are, the lack of activity of the LBD of nuclear receptors in general in yeast, the very likely lack of PPAR ligands in yeast, the fact that interaction of PPAR with co-repressors is generally ligand-enhanced rather than ligand-dependent, and finally, that the AF-1 activity is not masked in yeast and is in mammalian cells.

The identity this postulated cofactor is not known. The deletion study presented in section 3.3.2 suggests that the hinge region plays a role in the masking of the AF-1 activity. The known co-repressors, N-CoR and SMRT interact with nuclear receptors via the hinge region (Chen and Evans, 1995; Horlein et al, 1995). A study of the A/B domain of mPPARα indirectly implicated N-CoR and SMRT as regulators of the AF-1 activity. In this study, phosphorylation of the AB domain was shown to up-regulate the AF-1 activity (Juge-Aubry et al, 1999). Co-transfection of an unliganded TR, which is known to interact with the N-CoR and SMRT co-repressors and can effectively sequester them, seemed to abolish the phophorylation requirement. This suggests that the co-repressors mask the AF-1 activity in the absence of phophorylation and phosphorylation of the A/B domain results in a release of these co-repressors. Since the TR possesses an AF-1 region of its own (Hadzic et al, 1995; Sjoberg and Vennstrom, 1995; Wilkinson et al 1997), however, it is possible that the unliganded TR sequesters a different factor common to both PPARs and the TR. Lack of involvement of N-CoR and SMRT in PPAR siganaling is further supported by the fact that neither N-CoR nor SMRT have been observed to form complexes with PPAR/RXR bound to DNA (Zamir et al, 1997).

The fact that two different processes are likely involved in repression of transcription and suppression of the AF-1 activity also argue against the involvement of N-CoR and SMRT as the indirect regulators of the AF-1 activity. N-CoR and SMRT are thought to mediate repression of transcription by such receptors as unliganded TR, which are not merely transcriptionally silent but repress transcription to below basal levels (Chen and Evans, 1995; Horlein et al, 1995). No such repression of transcription has

been observed with unliganded PPARs, which rather appear to be transcriptionally silent in the absence of ligand. Different factors are therefore likely to mediate repression of transcription below basal levels and indirect silencing of the AF-1 activity of the receptors themselves.

4.4. RIP140 acts as a co-activator of the AF-1 activity.

RIP140 appears to repress the ligand-dependent activity of the nuclear receptors tested, including PPARα tested in our laboratory, when it is titrated in a co-transfection with a particular nuclear receptor (Treuter et al, 1998, Cavailles et al, 1995, Miyata et al, 1998). We observed however RIP140 to enhance trans-activation by PPARa/RXR via the AOx element in our yeast system (Fig. 23). This observation is in agreement with previous findings where RIP140 was shown to strongly enhance transcription in the case of the ER (Joyeux et al., 1997). We also observed RIP140 to enhance the trans-activation of GAL4DBD fusions of full-length mPPARα and mPPARγ2 subtypes as well as that of their respective A/B domains (Fig. 24). In contrast to the yeast results, RIP140 attenuated the AF-1 activity of both mPPARα and mPPARγ2 in mammalian cells (Fig. 25).

The repressive effect obtained in transfections (Miyata et al., 1998) and the enhancement of transcription in yeast suggest that RIP140 may be an activator protein for nuclear receptors, albeit a weaker one than activators such as SRC-1. Plausibly in the yeast, as there is no other co-activator proteins present, RIP140 enhances transcription. In mammalian cells, at higher transfection levels, RIP140 competes for and displaces the

more potent activators, such as SRC-1. This hypothesis is supported by the fact that in competition binding experiments, RIP140 appears to effectively compete with SRC-1 for binding to PPAR (Treuter et al., 1998; Leers et al., 1998).

Our data from mammalian and yeast cells furthermore indicate that RIP140 may act as a molecular target for the AF-1 activity of PPARs. As described in the introduction, the A/B domains of several members of the nuclear receptor superfamily have been shown to associate with known co-activators such as SRC-1 or CBP/p300, to associate with the LBD in response to ligand, and to modulate interaction with ligand or possibly interaction with co-repressors such as SMRT. Our results indicate that RIP140 may serve as a molecular target via which the signal of the AF-1 activity is transmitted, and may suggest that RIP140 is a co-activator for the AF-1, albeit a weaker one than others found in a cell, similarly to the situation with the full length receptor described above. In order to provide further evidence that RIP140 is a molecular target for the A/B domains of PPARs, a binding assay was performed in an attempt to detect any interaction between RIP140 and the A/B domains. This assay however was unsuccessful due to very poor in vitro translation of the individual A/B domains. More experimental evidence is needed before the role and significance of RIP140 in nuclear receptor signaling is understood.

5.0. References

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