Molecular Determinants of Co-regulator Binding and Transcriptional Activity of the Retinoic Acid Receptor – Retinoid X Receptor Heterodimer

Thesis for the Degree of Doctor of Philosophy (Ph.D.)

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ABBREVIATION LIST

ACTR – activator for thyroid hormone and retinoid receptors

AF-2– activation function-2

AM580 - (4-[(5,6,7,8-tetrahydro- 5,5,8,8-tetramethyl-2-naphthalenyl)carboxamido]benzoic acid)

APL – acut promielocytic leukemia

ATRA - All-trans retinoic acid

CBP – CREB-binding protein

DR5 – direct repeat

DRIP – vitamin D receptor-interacting protein

FL – full length

GST – glutathion-S-transferase

HAT – histone acetyltransferase

HDAC – histone deacetylase

ID – interaction domain

LBD – ligand binding domain

LG100268 - 6-[1-(3, 5, 5, 8-8-pentamethyl-5, 6, 7, 8-tetrahydronaphthlen-2-yl)-cyclopropyl]-nicotinic acid

N-CoA – nuclear receptor co-activator

N-CoR – nuclear receptor co-repressor

NR – nuclear receptor

PPAR - peroxisomal proliferation activated receptor

9-cisRA- 9-cis Retinoic Acid

RAR – retinoic acid receptor

RARE – retinoic acid receptor response element

RID – receptor interacting domain

RXR – retinoid X receptor

SDS-PAGE – SDS- polyacrylamide gel electrophoresis

SMRT – silencing mediator of retinoic acid and thyroid hormone receptor

THR - thyroid hormone resistance

TR – thyroid hormone receptor

TRAP – thyroid hormone receptor-associated protein

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1. INTRODUCTION

1.1. NUCLEAR RECEPTORS AS TRANSCRIPTION FACTORS

1.1.1. Function of nuclear receptors

The different cell types in a multicellular organism differ dramatically both in their structure and function. The reason of this difference is that they synthesize and accumulate different sets of RNA and protein molecules without altering the sequence of their DNA. There are many steps in the pathway leading from DNA to protein, such as transcription, RNA processing and transport, translation and posttranslational modification of proteins. Although all of these steps can be regulated, one of the most important points of control is the initiation of transcription.

The DNA transcription is highly selective and only 1% of the DNA sequence is copied into functional RNA sequence in most mammalian cells. The enzyme that catalyzes DNA transcription is the RNA polymerase. The eukaryotic RNA polymerase II (Pol II) binds to the core promoter elements of the DNA that determines the start site of transcription. One of the most important characteristics of eukaryotic RNA-polymerases is that they can bind their core promoter elements only in the presence of additional proteins. These trans-acting proteins are called general transcription factors (GTF). They are necessary for the initiation of RNA synthesis and control transcription by binding at cis-acting regulatory DNA-sequences. However, the control of gene transcription requires other transcription factors that behave as sequence specific transcription regulators. They can act even when they are bound to DNA hundreds of nucleotide pairs away and either stimulate or repress the recruitment of the GTF and Pol II to the promoter. There are several gene regulatory proteins have been identified. Although most of these proteins have unique feature, they consist of one or more activation domain and a DNA-binding domain that recognize DNA through one of a small number of structural motifs, including helix-turn-helix, the leucine zipper motif, the helix-loop-helix motif and the zinc finger motif [15]. One of these zinc finger motif containing regulatory factors are the nuclear receptors [7].

The eukaryotic genome, which is structurally organized into nucleosomes to form chromatin, regulates gene expression in part by controlling the accessibility of sequence specific regulatory factors. When packaged as chromatin, many promoters are transcriptionally repressed, thus reducing the access of transcription factors to their binding

sites. However, nuclear receptors (NRs) are a group of transcription factors that have the ability to access their binding sites in this repressive chromatin structure leading to the regulation of gene expression. One of the most important characteristics that distinguish NRs from other transcription factors is that they are able to bind small, lipophilic ligands, mainly hormones and metabolites that regulate the activity of NRs, hence providing a mechanism to directly regulate pathways of gene expression within cells. NR activities are not confined to cognate target genes since some NRs can also "crosstalk" in a ligand-dependent fashion with other signalling pathways, leading to mutual interference with the transactivation potentials of the involved factors.

The regulation of gene expression by transcription control is required for many cellular events for the proper development of any organism. The importance of NRs in maintaining the normal physiological state is illustrated by the enormous pharmacopoeia that has been developed to combat disorders that have inappropriate NR signalling as a key pathological determinant (Table 1.1.) [30,85,100,114,165]. These disorders affect nearly every field of medicine, including reproductive biology, inflammation, cancer, diabetes, cardiovascular disease and obesity. For this reason, characterization of these mammalian hormone receptors in normal physiology and abnormal disease processes is one of the major goals of biomedical research.

NUCLEAR RECEPTOR	ASSOCIATED DISEASE
- Androgen Receptor(AR)	- prostate cancer
- Estrogen Receptor (ER)	- breast cancer, osteoporosis Alzheimer disease
- Glucocorticoid Receptor (GR)	- inflammatory diseases
- Liver X Receptor (LXR)	- cardiocascular disease inflammatory disease
- Nuclear receptor related-1 (Nurr1)	- Parkinson disease
- Peroxisome Proliferator Activated Receptor (PPAR)	- type 2 diabetes, obesity atherosclerosis
- RAR, RXR	- leukemia, acne, psoriasis hepatocarcinoma APL, melanome
- Thyroid hormone Receptor (TR)	- several cancers, THR
- Vitamin D Receptor (VDR)	- ricket

Table 1.1. Diseases associated with nuclear receptor functions

1.1.2. Studies on nuclear receptors

In the 16th century it was already known by Chinese alchemists that there are "particles that are important to keep the body healthy". In 1915, Kendall was able to crystallize thyroid hormone and in the following years the increasing knowledge about the endocrine organs and their physiological functions led to the discovery of adrenal hormone, sex steroids and vitamin D, as well as to the realization that they have importance in normal development and physiology (Fig.1.1.). As hormones became more in the focus of scientific research, researchers wanted to know how these molecules could function. Intense efforts led to the discovery of the first steroid nuclear receptor, the oestrogen receptor (ER) in 1967 [180]. Using isotope labelled ligands in the 1970s the sites of hormone action were identified and it turned out that they occur in the nucleus, close to the chromatin [76]. The development of high affinity synthetic ligands allowed the purification of several nuclear receptors during the 1980s [77,153,207]. Biochemical studies of these purified receptors revealed that they could bind both ligand and DNA. The two functions could be separated by limited proteolysis and this led to the idea that these molecules might have domain structures [62,186,202].

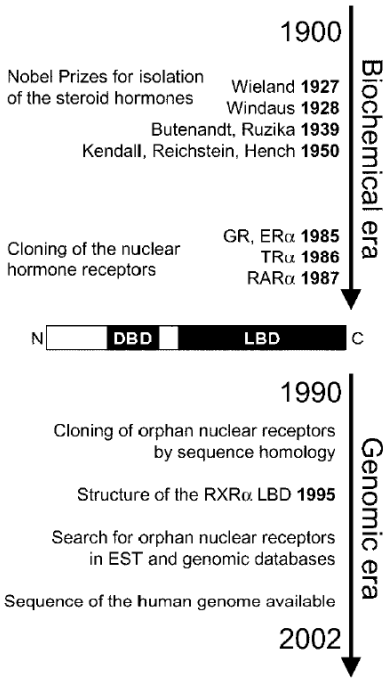


Figure 1.1. The eras of nuclear receptor research. Figure taken from Willson and Moore, 2002 [199].

The realization that these receptors bind DNA led to identification of several hormone response elements on the DNA, and it turned out that several of these elements have dyad symmetry which suggested that these receptors might form dimers upon DNA binding [10,18,29].

The expression cloning of glucocorticoid receptor (GR) in 1985 revealed that a segment of its sequence is very similar to the v-erbA oncogene, and this led to the discovery of the c-erbA locus as the thyroid hormone receptor [158,197]. The presence of a highly conserved DNA element initiated searches for other cryptic receptor genes that resulted in the identification of new receptors like estrogen receptor (ER), progesterone receptor (PR), vitamin D receptor (VDR) [8,39,59-61,69,92,115,196,197] and also the retinoic acid receptor (RAR) in 1987[55,144,154]. All these findings led scientists to solidify the hypothesis of the existence of a large group of molecules that has structure/function and sequence relationships among the member: the nuclear receptor superfamily. These studies described several gene products that appeared to belong to the nuclear receptor family based on sequence similarity but had no identified ligands, thus they were called "orphan receptors" [119,121,137]. The cloning of orphan receptors led to the era of "reverse endocrinology" [87]. While in classical endocrinology the purified hormone was used to identify its receptor, in reverse endocrinology the orphan receptors are used to find previously unknown hormones. The successful application of reverse endocrinology led to the discovery of 9-cis Retinoic Acid (9-cisRA) as a high affinity ligand for RXR receptors [109]. The expanding field of NRs, and the development of novel molecular biological tools led to the fast increase of our knowledge in this field. With sequencing methods scientists found isoforms, with site-directed mutagenesis, recombinant cloning techniques and with knockout (KO) animal studies scientists obtained detailed information about the normal and pathological characteristics of the receptors thus they could associate several diseases with these molecules.

The increase in the knowledge about the function of nuclear receptors also increased the need to know more about their structural characteristics. The first crystal structure showing the unliganded retinoid X receptor (RXR-LBD) was published in 1995 [23], then within the following two years several other NRs were crystallized including liganded (ligand binding domains) LBDs, and DNA-bound DNA-binding domains (DBDs) [24,50,151,152,162]. Using yeast-two hybrid screening systems several interacting proteins, so called nuclear receptor co-regulators (co-activators, co-repressors) were identified [28,47,211]. It was also revealed that most of them behave in a ligand-dependent manner. Soon it became more obvious that these co-regulators are parts of other complexes that basically serve as bridges between nuclear receptors and the basal transcription machinery [125]. In 1998, the first NR structure with a short peptide of a co-activator molecule was published [136], that was followed with several other similar structures [38,166]. These cocrystallizations identified the binding surface of LBDs for co-activators. At the meantime

there were several attempts to determine the co-repressor-binding site of the receptors using mutagenesis screening and biochemical methods [111]. With these approaches several independent studies (including our one) showed that co-activators and co-repressors are likely to bind to an overlapping surface on the LBD [17,53,122]. The first NR-co-repressor peptide structure was crystallized in 2002 [206]. Although it showed an antagonist bound receptor, so far this is the only structural evidence that shows the binding site for co-repressor.

1.2. THE NUCLEAR RECEPTOR SUPERFAMILY

Nuclear receptors are specific to metazoans since they were found in all the animal phyla but not in plants, fungi, algae or protozoa [58,171]. The superfamily has been grouped into 6 subfamilies based on sequence alignment and phylogenetic tree construction [2,99] (Fig.1.2.). Subfamily I includes many of the well-characterized ligand-activated receptors such as thyroid receptor (TR), Retinoic Acid Receptor (RAR), Vitamin D Receptor (VDR) and Peroxisome Proliferator-Activated Receptors (PPARs), as well as different orphan receptors. The second subfamily is formed by the Retinoid X Receptor (RXR) together with Chicken Ovalbumin Upstream Stimulators (COUPs) and Hepatocyte Nuclear Factor 4 (HNF4). The RXR has a unique role among NRs since it serves as a general heterodimerization partner for most of the receptors belonging to subfamily I. The third subfamily contains all the steroid receptors like Glucocorticoid Receptors (GR), Androgen Receptors (AR) and Estrogen Receptors (ER). The subfamily IV, V and VI are small families of orphan receptors, like NGFI-B and GCNF. Most subfamilies appear to be ancient since they have an arthropod homolog, with the exception of steroid receptors that has no known homologs.

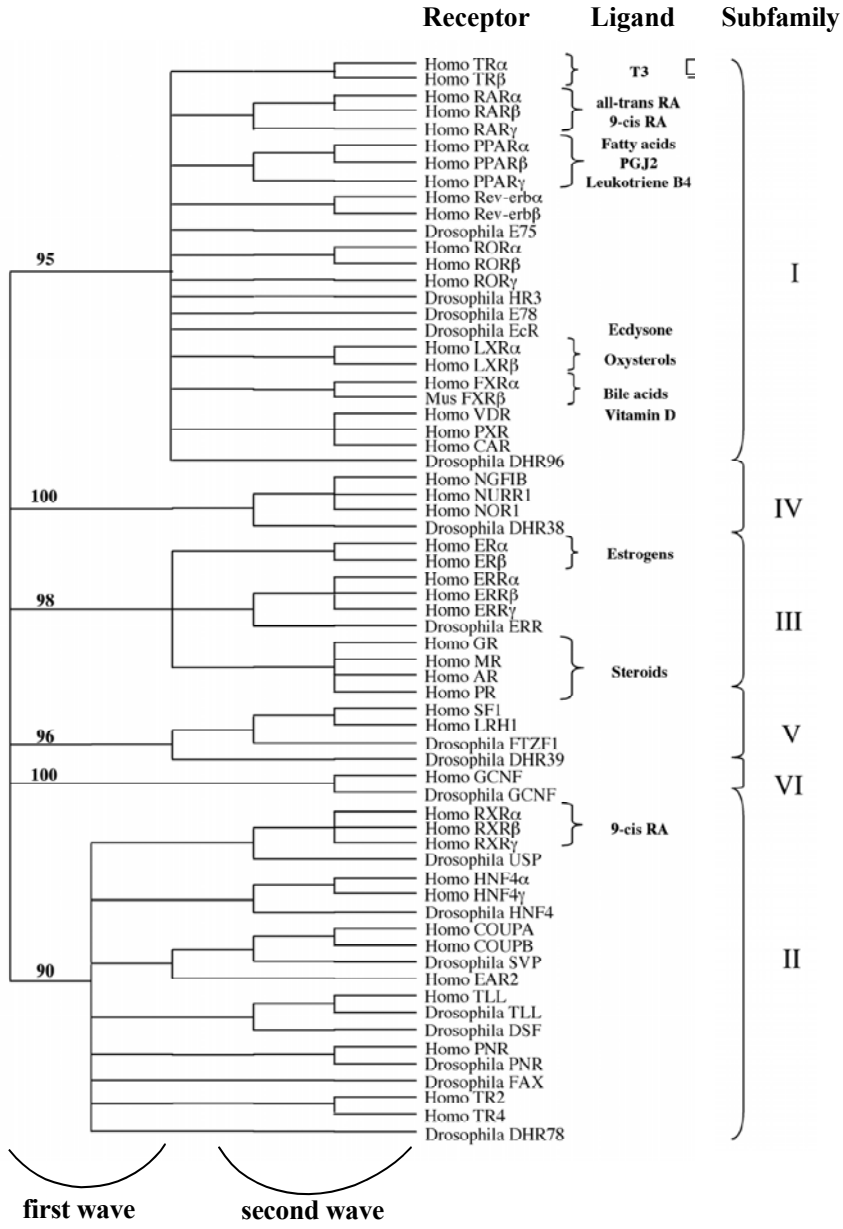


Figure 1.2. Nuclear receptor superfamily. Figure partially taken from H, Escriva et al., 2004, *in press*.

The discovery of the first insect receptor [135,140] and the identification of the ecdysone receptor as the member of the nuclear hormone receptor superfamily [91] suggested the likely universal nature of these receptors in animals. In the last few years, complete genome sequences have been determined for different [1,3,98,192]. This provides a unique opportunity to study the whole set of NRs for a given organism. By today we know that the different species possess different number of genes coding NRs. Invertebrates like *Ciona intestinalis* have 18 NR genes (including RAR, TR, PPAR). *Caenorhabditis elegans* and *Drosphila melanogaster* possess more than 270 and 21 respectively, but both are missing RAR, TR and PPAR. Among vertebrates the mouse genome has 49, while the human genome

contains 48 NRs. These genes are randomly dispersed in the complete genomes and no functional relation between gene position and function or phylogeny of NRs is known.

The diversity of NRs was generated by two waves of gene duplication during evolution (Fig.1.2.). The first occured very early during metazoan evolution, and resulted in the diversification into six subfamilies, while the second were specifically in vertebrates, produced the different paralogs within each subfamily. It is generally assumed that many genes have two to four paralogs in vertebrates, all orthologous to a unique invertebrate gene. In agreement with this observation, a majority of NRs have two to four paralogs in land vertebrates, with a unique ortholog in Drosophila. Thus it is also assumed that the number of NRs appears to be a good marker of large-scale events of genome duplication thus in genome evolution. [44].

NRs can also be grouped according to the characteristics and source of the ligand they bind. (Fig.1.3.). One group contains the receptor for steroid hormones (Fig.1.3. blue box). These ligands are synthesised exclusively in endocrine organs and reach their target tissues through circulation in the body. The steroid hormone receptors usually function as monomers or heterodimers. They are largely cytoplasmic in the absence of ligand and they are held in the cytoplasm in complex with heat-shock proteins [173]. The high affinity ligand binding (K_d= 0,01-10 nM) helps the receptors to shed these proteins, move into the nucleus, dimerize, and interact with the appropriate hormone response element. In such a scheme, the unliganded receptor does not bind co-repressor and cannot behave as transcriptional repressor, as it is held in the cytoplasm, away from the DNA. However, they are able to bind co-repressor when they bind an antagonist ligand [27]. The degree of the nuclear versus cytoplasmic localization of unliganded steroid receptors varies with different receptors and in different cells, so the effect of the unliganded receptor on transcription will depend on the cell and response in question.

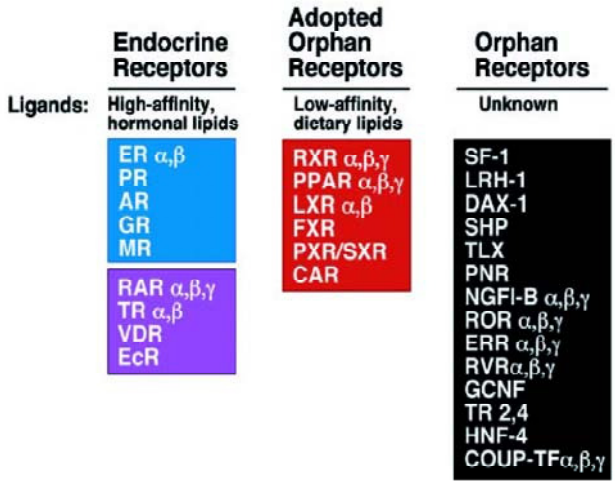


Figure 1.3. Nuclear receptor groups. Nuclear receptors grouped depending on the source and type of their ligands. Figure taken from Chawla et al., 2001 [30].

Another group is represented by the non-steroid hormone receptors (Fig.1.3. purple box). Their ligands are derived from dietary lipids (like vitamin A or cholesterol) or require exogenous elements for the synthesis (like sunshine for vitamin D or iodine for thyroid hormone) and regulate endocrine or lipid-sensing pathways. Several of the non-steroid receptors function as heterodimers with RXR. In contrast with the steroid receptors, in the absence of ligands they localize in the nucleus and the DNA-bound receptors bind co-repressors thus they repress transcriptional activity [147]. However, from studying the nuclear events and protein movements in real-time it has now become clear that most transcription factors are highly mobile within the eukaryotic nucleus, and exists in a rapid and dynamic equilibrium with multiple targets within the nuclear compartments. This rapid mobility appears to be a common feature of NRs independent of their functional classification [63].

A third group contains the adopted orphan receptors that also function as heterodimers with RXR (Fig.1.3. red box). Orphan receptors become "adopted" when they are shown to bind a physiological ligand mainly derived from dietary lipids. The receptor binds the ligand with low affinity (K_d=1-10 μM), which is comparable to their physiological concentration. These receptors are considered lipid sensors and regulate genes involved in lipid metabolism, storage, transport and elimination, thus they maintain nutrient lipid homeostasis.

The orphan receptors that belong to the fourth group still do not have known ligand or target genes (Fig.1.3. black box). The orphan receptors are not clustered in a specific subfamily, but spread through the whole phylogenetic tree of the NR superfamily. This situation could be explained by independent gain of ligand-binding capacity several times during NR evolution, specifically in each branch of the tree. This hypothesis implies that the

ancestral NR was an “orphan” activator or repressor, which acquired ligand-binding ability during evolution [45]. On the contrary, DNA-binding and dimerization functions coevolved with NRs.

1.3. RETINOIDS AND THEIR RECEPTORS

The principal function of vitamin A (retinol) as a chromophore in the visual process has been recognized many decades ago. Since then both clinical and experimental approaches have shown that vitamin A and its biologically active derivates (collectively referred to as retinoids), most notably retinoic acids (RAs), play central roles in many essential biological processes and exert a wide variety of profound effects on vertebrate physiology like morphogenesis, organogenesis, growth, cellular differentiation and homeostasis. The significance of retinoid signal is also reflected in the teratogenic effect of retinoids in humans and other animals [130] and in the successful use of retinoids in the treatments for some skin diseases and some forms of cancer [6,116,129,170,172]. (Table 1.2.)

Functions of retinoids in embryonic development
- development of CNS, eyes, face, ear, limb, urogenitals, skin, lung, heart, hematopoetic system, body axis development
Functions of retinoids in adults
- control of epithelial growth and differentiation - proper function of male and female reproductive organs - influencing growth and differentiation of various hematopoetic progenitor cells - antioxidant agent - growth, development
Functions of retinoids in therapeutical treatments
- in dermatological hyperkeratinosis (acne, psoriasis) - chemoprevention and differentiation therapy of several cancers (skin, head, breast, cervix, leukemia) - antidepressant

Table 1.2. Functions of retinoids

For a long time the physiological functions of retinoids were mainly inferred from studies on vitamin A deficient (VAD) animals [127]. These studies showed that vitamin A (retinol) is required during pre- and postnatal development. VAD can cause a number of

malformation of the embryo of all vertebrates (CNS, eye, face, dentions, ear, limb, urogenitals, skin, lung, heart, hematopoetic system, body axis development). RAs can prevent or reverse the effects of a postnatal VAD diet, with the exception of night blindness and photoreceptor degeneration [179]. Since the discovery of a retinoic acid receptor (RARα1) in 1987 [55,144,154] several other combinatorial effects emerged that still broadens our molecular understanding as to how retinoids could exert their pleiotropic effects. The most important aspects are the followings:

1.3.1. The retinoid receptors

Retinoic acids exert their multiple effects through two classes of NRs, Retinoid X Receptors (RXRs) and Retinoic Acid Receptors (RARs). Each of these two classes consists of three isotypes (α, β, γ) [4,26,55,74,93,144,215] encoded in separate genes and there are several isoforms among the isotypes that arise from the differential usage of promoters and by alternative splicing showing the expression profile of the different isoforms). The distinct spatiotemporal expression pattern of RAR [16,40,55,74,149] (Table1.3.) and RXR in the developing embryo and various adult tissues suggests that each RAR and RXR type and isoform might perform unique functions thus involves the possibility of the combinatorial effects [123].

Receptor	Expression
RARα	all tissues
RARβ	dermis
RARγ	skin (dermal, epidermal)
RXRα	liver, skin, intestine
RXRβ	ubiquitous
RXRγ	heart, muscle

Table 1.3. Expression profile of RAR and RXR

The most direct way to investigate the physiological function of a given retinoid receptor is to mutate the corresponding gene by homologous recombination. The use of transgenic mouse technology to generate null mutations and isoform specific knockout mice of these receptors provided some insight into RXR/RAR function at the whole organism level [83]. Single and compound knockouts of RXRα, RARα and RARγ genes established the functionality of RXR/RAR heterodimers and demonstrated that different combination of RXR and RAR isotypes are differentially involved in the control of RA-induced cellular responses [25,33]. This revealed that several symptoms are identical with the characteristic symptoms of VAD-mice. The most characteristic defects that could be found in both VAD-mice and KO mice (Table-1.4.) include poor viability, growth deficiency and male sterility [83,123].

Studies of RAR null mutations revealed that compensatory activity of other RARs might occur when one RAR is not produced, since most of the defects was not exhibited by single RAR isotype mutant. This indicates that there is a marked functional redundancy between RARs. Then genetic crosses between different RAR null mutations have been used to dissect the functions of the RARs. RARα/β, RARα/γ, RARβ/γ double null mutants recapitulated all the VAD-induced defects characteristics of the foetal VAD syndrome that can be cured or prevented by RA treatment.

Abnormalities in VAD syndrome	**Similar abnormalities in RAR and RXR knock-out mice**
respiratory tract defect	RARα/β
"spongy" myocardium	RARα/γ
heart abnormalities	RARα/β, RARα/γ, RXRα
ureter abnormalities	RARα/β
genital tract abnormalities	RARα/β, RXRβ
ocular abnormalities	RARα/γ, RARβ/γ, RXRα

Knock-out mice	
RARα normal	RXRα growth def.cardiac and occular defects
RARβ normal	RXRβ normal *
RARγ normal	RXRβ/γ normal
RARα/RXRα VAD syndrome	
RARβ/RXRα VAD syndrome	
RARγ/RXRα VAD syndrome	

*deficiency caused by the impared function of the heterodimerizing partner, PPAR

Table 1.4. Characteristic symptomes of VAD- and KO mutant mice

RXRα appears to be functionally the most important during development, since RXRβ/RXRγ null mutant develop normally, while severe developmental defects are synergistically generated in double mutants in which an RAR (α,β,γ) mutation is associated with an RXRα but not an RXRβ or RXRγ mutation. These observations strongly supported the notion that RXR:RAR heterodimers are the functional units that transduce retinoid signal *in vivo*.

1.3.2.Retinoids

Retinoids are bioactive metabolites of vitamin A. Vitamin A (retinol) is obtained in the diet as retinyl ester, mostly in animal products such as liver, eggs, milk,.and as a carotenoid precursor in plant products, particularly from green leafy vegetables. Retinoic acid (RA) is a natural product derived by oxidation of vitamin A. Retinol is transported in the blood as a complex with retinol-binding protein (RBP), while retinoids travel in the blood and within different cellular compartments as non-covalent complexes with various proteins, such as albumin. The RA concentration in the blood is two orders of magnitude lower than that of retinol (10^{-8} versus 10^{-6}). In the cell cellular retinol binding protein (CRBP) and cellular retinoic acid binding (CRABP) is responsible for RA transport.

There are several forms of natural retinoic acids, like ATRA, 9-cisRA, all-trans 3-4 didehydro RA, 4-oxo-RA, 11-cis RA, 13cis-RA produced by the organisms. To our knowledge, the ATRA and 9-cisRA has the most important effects among these ligands, however, they show receptor specific affinity and specificities (Fig.1.4.). While 9-cisRA seems to be the most potent ligand for RXR, RAR can be activated with both ATRA and 9-cisRA [68,109]. Although 9-cisRA was the first natural ligand to be discovered for the NR superfamily following the characterization of the active form of vitamin D [109], there are still debates about the physiological role of 9-cisRA. While ATRA is easily detectable in many mammalian tissues, detection of 9-cisRA, as originally reported, has not been confirmed.

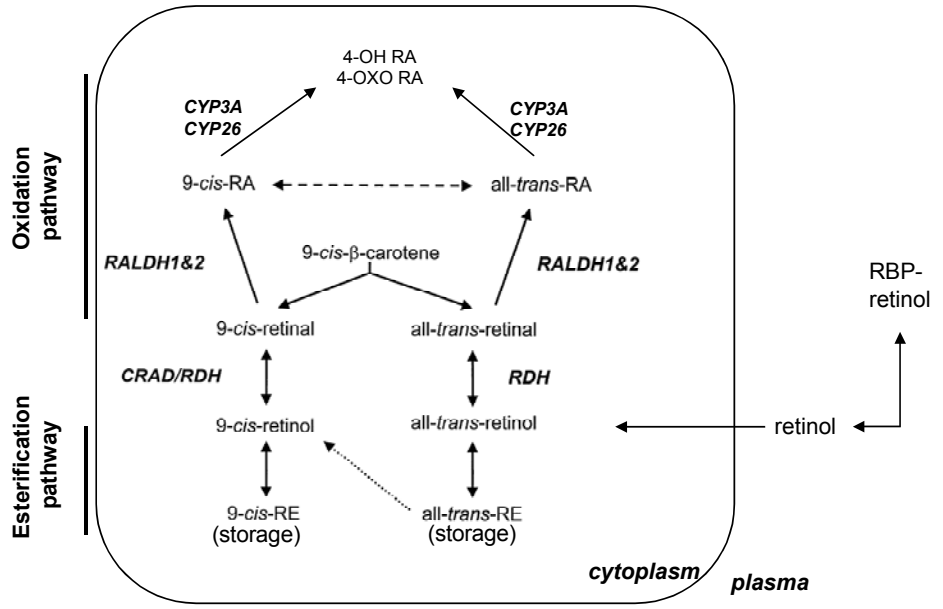


Figure 1.4. Possible route of ATRA and 9-cisRA metabolism. Figure taken with changes from Napoli et al., 1999 [134].

Biochemical studies indicated that both isomers of RA can be generated *in vitro* from vitamin A (Fig.1.5.), by cytosolic alcohol-dehydrogenases and microsomal short chain dehydrogenases, each catalyze oxidation of all-trans-retinol and 9-cis-retinol to the corresponding retinaldehyde derivates, followed by cytosolic retinaldhyde-dehydrogenase (Raldh1 and -2), which catalyzes further oxidation of both all-trans retinaldehyde and 9-cis-retinaldehyde to produce ATRA and 9-cisRA [134,182]. The isomerization of ATRA to 9-cisRA was also demonstrated [21,141,175,185].

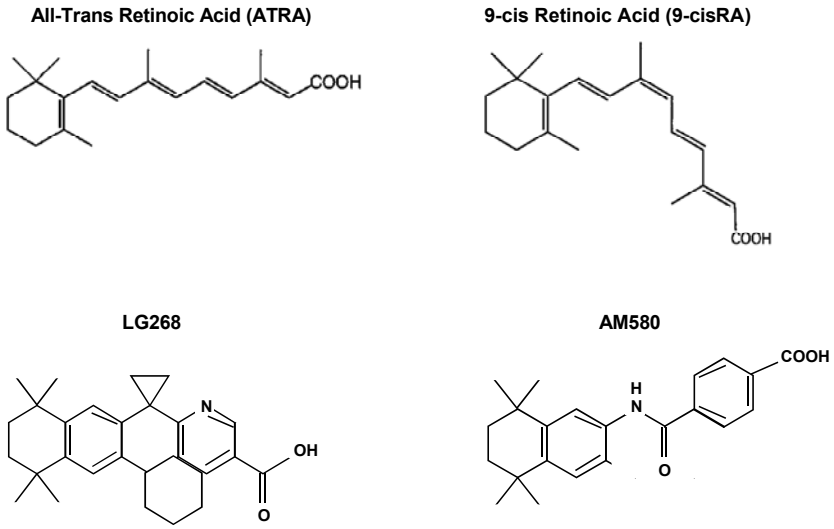


Figure 1.5. Structure of RAR and RXR ligands. ATRA: natural RAR agonist, 9-cisRA: natural panagonist, LG268: synthetic RXR agonist, AM580: synthetic RAR agonist.

Because of these pleiotropic actions, it is not surprising that plasma and tissue levels of RAs are kept under tight homeostatic control. To contribute to this homeostatic control, the inactivation of RAs through oxidative metabolism of the retinoids is also important. It is initiated by the 4-hydroxylation of RA to form 4-hydroxy-RA and 4-keto-RA. This process is carried out by microsomal cytochrome P450 (CYP) isoenzyme system. Several isoenzymes have been shown to be capable of metabolising RA *via* this reaction, but CYP26 appears to be the most likely candidate [19].

Although retinoids are widely used as therapeutic agents [64,178], the teratogenic and toxic effect of RAs led to the need of development of synthetic ligands, which proved to be therapeutically beneficial similar to the natural ligands without severe side effects [54,159,177]. (Fig.1.4.)

1.3.3. The RXR heterodimers

Although RXR and RAR form stable heterodimers required for high affinity binding to DNA, RXR is also able to form heterodimers with other members of the superfamily. With the discovery of RXR in 1990 [120], it became clear that this is the missing protein partner required for efficient transcriptional activation of TR, VDR and PPAR, RAR [88,89,106,107].

QuickTime™ and a TIFF (LZW) decompressor are needed to see this picture.

Figure 1.6. Non-permissive and permissive heterodimers.

These RXR heterodimers can be divided into two groups, based on their ligand-induced activity (Fig.1.6.). The so called non-permissive heterodimers (RAR:RXR, TR:RXR) can be activated only by the partner's ligand but not by an RXR ligand alone. In this case the ligand-induced transcriptional activity for RXR is suppressed when complexed with the partner, thus RXR is said to be a "silent partner" [49,95]. On the other, hand permissive heterodimers (like PPAR:RXR, LXR:RXR) can be activated by ligand of either RXR or its partner receptor, and in the presence of both ligands they show synergistic activity [52,143,200]. Since the heterodimerizing partner of RXR can also have different isotypes, can bind diverse ligands and moreover they can also show tissue specific expression and promoter specific activity, these aspects widely increase the complexity and pleiotropic effects of the retinoids.

1.3.4. Co-regulator molecules

As many other NRs, RARs and RXRs also interact with multiple putative co-regulators that also can have cell and tissue specific expression and distribution, increasing the combinatorial effects that underlie the pleiotropic effects of retinoids. The more detailed overview about co-regulators can be found in Chapter 1.5.

1.4. STRUCTURE AND FUNCTION OF NULCEAR HORMONE RECEPTORS

All nuclear receptor consists of structurally and functionally different regions, like A/B region, DBD, hinge region and LBD with AF-2 (Fig.1.7.).

Figure 1.7. Schematic representation of nuclear receptor domains. Numbering is according to the hRARα receptor.

1.4.1. The A/B region

A typical NR contains a variable so called *A/B region* in its very N-terminus. In many cases this region contains an AF-1 (activation function-1) domain that shows ligand-independent activity. From structural studies it appears to be in an unfolded state and does not closely resemble one another in sequence, location, or size [94]. Its promoter- and cell-specific activity [20], also to be target for posttranslational modifications, mainly for phosphorylation, suggests that this region is likely to be responsible for mediating isoform-specific responses [164].

1.4.2. DNA-binding

1.4.2.1. The DNA-binding domain (DBD)

The DNA-binding domain (DBD), that comprises about 75-90 amino acids, is the most conserved region of nuclear receptors. Among the RAR types there is 94-97% similarity while among the RXR types the similarity is 91-97%. This domain comprises two zinc fingers and each contains four conserved cysteins coordinating a zinc ion. The zinc finger region contains two α-helices, the first fits specifically into the major groove of the DNA helix at the response element [118,162] (Fig.1.8.). The second helix is thought to be the interface for stabilizing heterodimerization on DNA-binding. Overall, the DBD is responsible for high-affinity DNA-binding, for the recognition of specific target hormone response elements (HREs) [14,46] and it also serves a weak dimerization site [119].

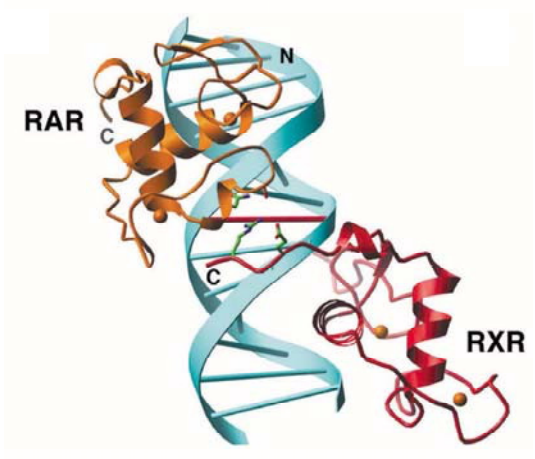


Figure1.8. Structure of the RAR-RXR-DBD heterodimeric complex with DR1. Figure taken from Rastinejad et al., 2001 *[150]*.

1.4.2.2. The hormone response element (HRE)

HREs are located close to the core promoter, or in some cases they are present in enhancer regions several kilobases upstream of the transcriptional initiation site. These recognition elements contain a 6 bp core recognition motif that represents an idealized consensus sequence: AGG/TTCA [56]. The dimeric receptors bind dimeric HREs, and the half-sites can be configured as palindromes (Pal), inverted palindromes (IPs), or direct-repeats (DR) (Fig1.9.) [7,183].

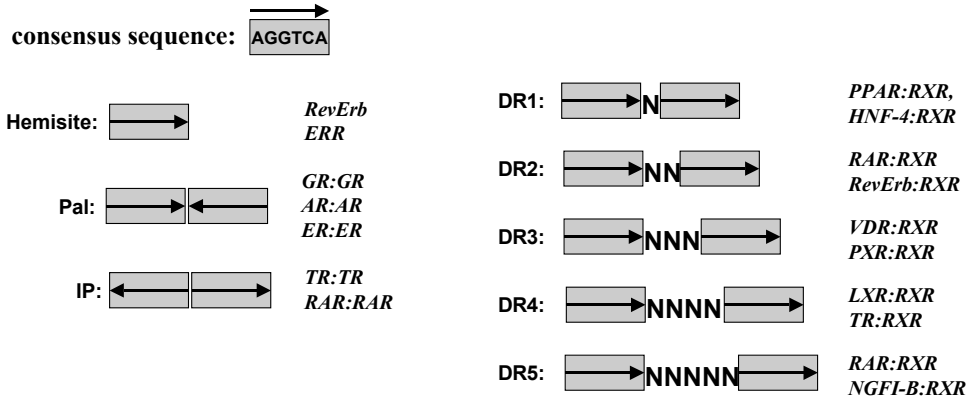


Figure 1.9. The different orientation of the consensus sequences in the hormone response elements.

Steroid hormone receptors typically bind to palindrome sequences, while the most potent HREs for non-steroid receptors are configured as DRs. The length of the spacer region between the half sites is an important determinant of the specificity of hormonal responses. Thus in most cases the number of the bases between the half sites varies between 1-5 (this is the so called 1-5 rule) (i.e., DR1, DR2, DR3, etc.). The most frequent spacing observed for RAR-RXR heterodimer corresponds to 5bp (DR5), but DR1 and DR2 have also been found.

1.4.3. The hinge region

The hinge region is not well conserved among the different receptors and serves as hinge between the DBD and LBD, allowing rotation of the DBD. In many cases this region harbours nuclear localization signals.

1.4.4. The ligand binding domain (LBD)

1.4.4.1.The structure of LBD

All NR LBD structures determined so far contained 11-13 α-helices that are arranged into a three-layer antiparallel α-helical sandwich [204] (Fig.1.10.) The three long helices (helix 3,7,10) form the two outer layers of the sandwich [119].

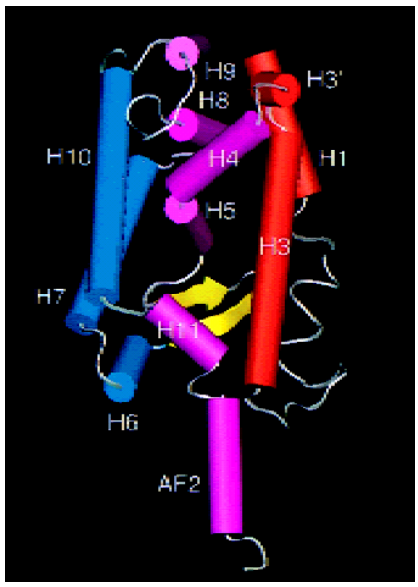


Figure 1.10. Structure of RXR-LBD crystallized by D.Moras in 1995. Cylinders indicate α-helix and arrows indicate β-strands. Figure taken from Gampe et al., 2000 [51].

The middle layer of helices (helix 4,5,8,9) is present only in the top half of the domain, but is missing from the bottom half, thereby creating a cavity for ligand binding in most of the receptors. Structural comparison reveals that the top half of the domain is highly similar among various LBDs, suggesting that the helix sandwich fold is evolutionarily selected for the binding of small molecules in most receptors. The ligand binding site, in the bottom half of the LBD shows differences between receptors required for the recognition of unique hormones and ligands. In particular, the number of B-strands (between H5 and H6) coupled with the presence or absence of helix 2 (RXR has H2, PPAR has H2 and H2', RAR has no H2) contributes to the variability in the ligand-binding site [152].

The C-terminal activation region also forms an α-helix (H12) that harbours the ligand-dependent activation function-2 (AF-2), which can adopt multiple conformations, depending on the nature of the bound ligand [84,128]. This region possesses a high homology over a very short region from which the consensus motif, ffXEff (f being a hydrophobic amino acid) can be derived, preceded by a loop of varying from 8 to 12 amino acid that is variable in sequence and composition. The region comprising the conserved sequence adopts an

amphipathic α-helical conformation with the two well conserved pairs of hydrophobic residues pointing toward the core of the LBD and negatively charged residues exposed on its surface [142]. This motif is conserved in most members of the NR superfamily. In the case of RARs and RXRs the sequence homology of their LBDs is 84-90% and 88-95%, respectively. The conservation of the LBD is not higher among the RARs and the RXRs than between these receptors and many other members of the superfamily, which suggests that RARs and RXRs have evolved independently and that the appearance of RXR-like receptors predates the divergence in the invertebrates and vertebrates.

1.4.5. The functions of LBD

The ligand-binding domain or LBD is a multifunctional domain possessing four main functional regions.

1.4.5.1 dimerization

One of the key regions of the LBD comprises a strong dimerization interface along the outside part of helix 10 [48] (Fig. 1.11.). Upon dimerization the receptors form a typical butterfly shape. Homodimers show C2 symmetry around this interface, whereas RXR heterodimers form an asymmetric interface with additional contributions from amino acids of helix 9 [23,27,104].

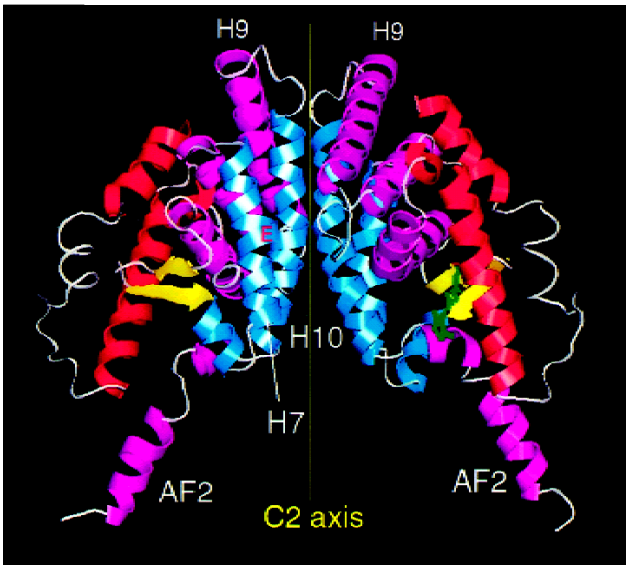


Figure 1.11. The dimer interface. The butterfly shape of an RXR-LBD dimer is presented in a ribbon presentation. Figure taken from Gampe et al., 2000 [51].

1.4.5.2 The ligand binding

Comparison of the structures of the LBDs shows that they have evolved to serve a remarkable variety of functions within their capacity as ligand sensors. The ligand-binding

pocket varies greatly in size, from 0 (nil)(Nurr1) - 30A(DHR38) [9,191] to 1400A (PPAR). The variation seems to be consistent with the biology mediated by these receptors. The large pocket (in PXR and the PPAR) allows these receptors able to bind to diverse metabolites promiscuously and with low affinity [136,184,193,194]. In contrast, the small pocket renders these receptors to recognize a specific ligand with high affinity [27,190]. Such high affinity and specificity of ligand recognition may be required for these receptors to mediate their physiological roles.

The specificity of ligand binding is also determined by the shape of the ligand-binding cavity, which also varies greatly among receptor subtypes to accommodate a variety of functions mediated by these receptors. The large pocket seen in PPARs has a distinct three-arm Y-shape, allowing it to bind ligands with multiple branches (phospholipids, synthetic fibrates) or to bind single branched ligands, such as fatty acids, in multiple conformations. In contrast, PXR has an elliptical shape pocket with a volume of 1200A, allowing binding to the cholesterol-lowering drug in three different conformations [205]. Beside the shape and the size of the ligand binding pocket (LBP) the characteristics of the ligand can also influence of its own binding. Although the LBD of the RAR and RXR have only modest sequence identity (30%) and according to the crystal structures the shape of their LBPs are different, they are able to effectively bind the same ligand, 9-cisRA. The reason is the flexibility of 9-cisRA that allows it to adopt different conformations in RARγ and RXRα (Fig.1.12.). The β-ionin ring of 9-cisRA exhibits a rotation of about 90^{o} therefore in RXR it points to the bottom of the LBD (away from H12), while in RAR it makes hydrophobic contacts with H12. Thus in RXR 9-cisRA exhibits a bend, whereas in RAR its shape is closer to the ATRA-bound shape [86]. In these forms, the ligand-binding cavity of RXR is less occupied than in the case of RAR that leads to RXR making firmer hydrophobic contacts with the ligand. This is the explanation of the lower affinity of RXRα for 9-cisRA (K_d=2 nM) compared to RARγ (K_d=0,2-0,8 nM for ATRA and 9-cisRA) [42].

Thus beside the size and the shape, hydrophobic/hydrophilic nature of the pocket surface also plays a determining role in ligand binding specificity. Taken together, NRs have evolved remarkably down to the single-residue level to recognize specific ligands by changing the

a b

9cisRA in RXRα **9cisRA in RARα**

size, shape and polar/non-polar nature of their ligand-binding pockets. (E.Xu, 2003) The adaptation of ligands to the protein leads to an optimal number of interactions for binding and selectivity, and justifies modelling approaches for ligand design [128].

Figure 1.12. The bound 9-cisRA in RXRα and RARγ. Figure taken from Egea et al., 2000 [43].

1.4.5.3. Activation function (AF-2 or H12)

Early mutational analysis led to the observation that a highly conserved subregion within the distal C-terminus of the LBD, termed the AF-2 domain or H12 was involved ligand-dependent transcriptional activation [12,37]; Durand, 1994 #141; Tone, 1994 #142}. The precise positioning of H12 is controlled by the binding of ligands to the LBD, which allows small molecules to regulate the transcriptional activity of the receptor. Studies on H12 deletion mutant NRs showed that the deletion results in loss of activation ability and moreover it makes the receptor dominant-negative over the wild-type receptor.

1.4.5.4. Co-regulator binding

The fourth functional region is the hydrophobic cleft on the surface of the LBD that serves as the docking site for co-activators and co-repressor proteins. See in more detail in Chapter 1.6.

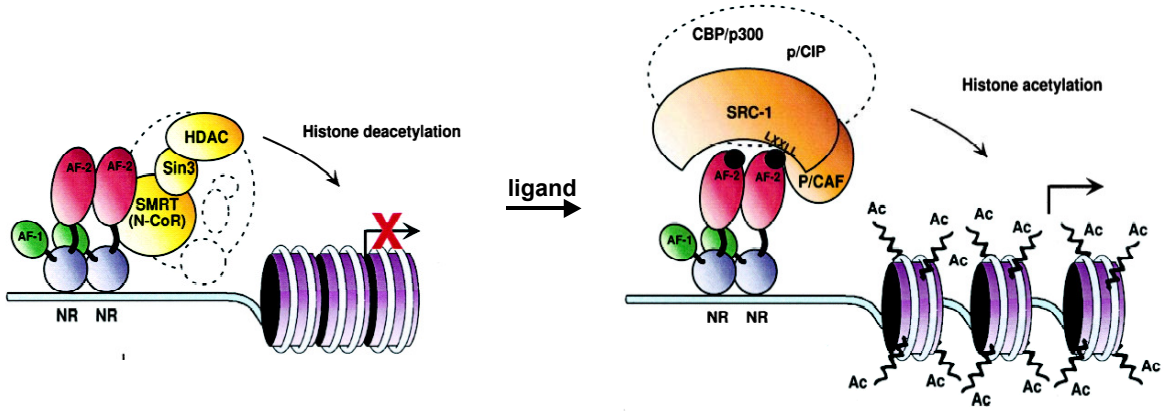
1.5. CO-REGULATORS

1.5.1. The general function of co-regulators

The covalent modifications of histone tails and DNA itself involves several different posttranslational modifications like methylation, phosphorylation and acetylation [65,174]. The acetylational state of chromosomal histones has long been known to correlate strongly

with transcriptional status. Thus hyperacetylated regions of chromatin frequently contain active transcription units while hypoacetylated chromatin is transcriptionally silent [189]. The relative level of histone-acetylation is assumed to be determined by the enzymatic activities of both HATs and HDACs. The binding of NRs directs these modifications of the local chromatin structure through a number of co-regulatory proteins which serve as bridges between nuclear receptors and general transcription factors (GTFs) [15]. Thus NR co-regulators can be broadly defined as cellular factors recruited by NRs that complement their function as mediators of the cellular responses to endocrine signals. Biochemical and expression cloning approaches have been used to identify a large number of co-regulators that interact with NRs in either a ligand-independent or ligand-dependent manner. Many of these factors have been demonstrated to be capable of directly potentiating NR activity while others appear to function as components of large, multiprotein complexes [78,125,155].

In common with other nuclear receptors, RAR and RXR regulate gene expression in response to ligands through the direct recruitment of co-regulator proteins. In the presence of an agonist, co-activator proteins (such as ACTR, DRIP, SRC-1) bind to the receptor (review:



[105,126]). These co-activator proteins in turn associate with additional proteins forming large activation complexes. These activate transcription by interacting with basal transcription factors as well as chromatin remodelling enzymes (e.g. HAT, methyltransferase) and provide the mechanistic and enzymatic requirements for transcriptional activation {Korzus, 1998 #27; Ogryzko, 1996 #21; Spencer, 1997 #17}. In the absence of agonists, receptors bind co-repressor proteins (such as SMRT, N-CoR) and form multi-component repression complexes that harbour enzymatic activities opposite to that of co-activators (such as histone deacetylation (HDAC)) which mediate transcriptional repression [27,67,71,128].

Figure 1.13. Co-activator and co-repressor complexes in NR transcription. Co-repressor complex repress transcription through deacetylation of histone tails. The function of co-activator complex leads to transcriptional activation through the acetylation of histone. Figure taken from Mc Kenna et al, 1999 [125].

Many co-regulators normally show tissue specific distribution, and their level can vary dramatically among specific cell types. Such differences in expression levels might indicate cell specificity of NR-mediated transcription regulation, and might partially explain how the same gene can be regulated differentially in different cell types.

1.5.2. DRIP205 / TRAP220

Since the identification of the first co-activator molecule (SRC-1 in 1995) till to date there are several co-activator complexes have been characterized and been group into families upon sequence and domain similarities. The best characterized co-activators are p300, CBP [28,82,138], the p300/CBP-associated factor (P/CAF) and the members of p160 family, including SRC-1, GRIP1/TIF-2/SRC-2 and ACTR/AIB1/RAC3/SRC-3, PGC-1, TRAP/DRIP/SMCC/ARC/CRSP/NAT/Mediator complex [31,70,108,139,188]. The DRIP/TRAP complex was initially identified in separate biochemical screens for proteins recruited by TR and VDR in a ligand dependent manner [47,148]. Subsequent studies have shown TRAP to be capable of enhancing the function of other receptor types (like RARs), and this has been interpreted as evidence of a general role of the complex in NR-mediated

signalling. Consistent with such role in NR-mediated transcriptional regulation, null mutation of the gene encoding TRAP subunit results in embryonic lethality attributed to a variety of pleiotropic abnormalities, including defects in cell cycle regulation and increased apoptosis [75]. The complex is composed of 14-16 proteins that range in size from 70 to 230 kDa and is recruited to the core AF-2 receptor region in response to ligand binding through a single subunit (DRIP205/TRAP220/PBP) via a receptor interacting motif (ID, LXXLL-motif,) identical to that found in other co-activator molecules [210]. This subunit anchors the other proteins comprising the TRAP/DRIP complex, which presumably preformed in the cell. Since DRIP205/TRAP220 does not have intrinsic HAT activity the proteins in the complex that DRIP associates with posses this activity.

Figure 1.14. Schematic representation of co-activator (DRIP205/TRAP220) and co-repressors (SMRT). Darker regions show that functional domains that are involved in mediating interactions with other proteins. ID: interaction domain, RD: repression domain.

1.5.2. SMRT /NCoR

Similarly to co-activators a number of co-repressor proteins such as NCoR, SMRT, RIP140 and LCoR, SUN-CoR may also be recruited to the receptors, dependent on the ligand bound, and help repress transcription [32,71,96,212]. Since many component of chromatin can non-specifically repress transcription, an important component in the activity of co-repressors is their targeting to the receptor. The silencing mediator for retinoic acid and thyroid hormone receptors (SMRT) and nuclear receptor co-repressors (NCoR) were isolated by a yeast two-hybrid screening of a human lymphocyte cDNA library with unliganded TR in 1995 [32,103,157]. They are related both structurally and functionally and share close to 40% amino-acid identity. SMRT is a ubiquitously expressed 270 kDa protein and a component of a variety of multiprotein complexes that contain histone deacetylases, that appear to repress transcription by deacetylating lysine residues in the N-terminal tail of histones. Although cloned on the basis of their interaction with unliganded RAR and TR, SMRT appear to confer transcriptional repression on many nuclear receptors (COUP-TF, Rev-Erb, PPARγ [101,167,213]). Moreover, SMRT has also been implicated as co-repressors for a variety of unrelated transcription factors, which regulate divers cellular processes. SMRT interacts with and can repress transcription by serum response factor (SRF), activator protein-1 (AP-1), and nuclear factor-κB (NFκB), which are all transcription factors involved in stimulation of cell proliferation.

Domain analysis of SMRT has revealed that it contains several functional domains. In the N-terminal, it contains four repression domains that recruit a complex containing Sin3, HDAC and several additional proteins [5,67,132,208]. SIN3 interacts with a diverse collection of nuclear proteins like Mad, Ume6, Ski, Ikaros, p53, PLZF [90]. The size and complexity of Sin3 suggests multifunctionality and regulatory control over co-repressor composition and activity. Thus its main function appears to be to serve as an intermediary between the receptor and histone deacetylase itself [97,201]. The C-terminal domain contains two interaction domains (IDs) that mediate the connection between the co-repressor and the receptors [35,111].

1.5.3. LXXLL motif

Inspection of the amino acid sequence of the NR interaction domains of co-activators revealed the presence of leucine-rich motifs of the consensus sequence LXXLL [38,66,102,181]. The concept that LXXLL motif represents a general structure for NR recognition is supported be the observations that nearly all factors that have been cloned by virtue of their ability to interact with NRs in a ligand dependent manner contain one or more copies of this motif. Similar interaction domains were identified for co-repressors but detailed and comparing analysis of IDs revealed that the L-X-X-X-I/H-X-X-X-I/L motif of co-repressors [72,133,142,195], is predicted to form an extended, one helical turn longer α-helix than the co-activator motif.

A **B**

Figure 1.15. The LXXLL motif. (A) Conserved amphypatic helices in co-repressor IDs, H12 of different nuclear receptors and the co-activator LXXLL motif. Hydrophobic residues indicated by gray shading. Figure taken from Nagy et. al., 1999, [133]. (B) Ribbon diagram of the co-repressor extended helix (in red). The binding of the shorter co-activator helix of GRIP-1 to the same pocket is represented in green, H12 is shown in yellow. Figure taken from Perissi et al., 1999 [142].

Analysis of the LXXLL motifs has revealed that in the amphipatic α-helices that they form the leucines create a hydrophobic surface on one face of the helix. The structure of these receptor interacting motifs is reminiscent of that of H12 in the receptors, which is required for ligand-dependent interaction with co-activators, and also forms an amphipatic α-helix (Fig.1.15.). It is conceivable that these motifs have evolved to provide a critical mode of assembling the ligand-dependent nuclear receptor – co-ativator complexes [124].

It was shown that receptor stoichiometry is a crucial determinant of transcriptional repression and activation [214]. These studies proved that a single peptide of the ID of co-activator or co-repressor associates with one receptor. The structures of the co-crystals also indicates that two LXXLL motifs from a single co-activator molecule interact with the H12 of

both dimmer partners and that each member of the homo- or heterodimer can properly recruit one molecule of co-activator or co-repressor [136].

Though the sequence encompassing the LXXLL motifs is sufficient for NR-co-regulator interactions, amino acids flanking the LXXLL motif appear to make additional contacts with the LBD. In the case of co-repressors it is due to specific sequences in the L-X-X-X-I-X-X-X-I/L motif that SMRT shows preferency for RAR, while NCoR has preferency for TR [34]. These residues are conserved among different co-activators and may play roles in determining the specificity of NR-co-activator interaction, possibly specifying which co-activator will bind to a particular NR dimer or heterodimer with highest affinity [36,38,203]. These interactions are potentially influenced by structurally distinct physiologic ligands for a particular NR that might induce distinct conformations.

1.6. DETERMINANTS OF CO-REGULATOR BINDING

1.6.1. Allosteric coupling of LBD functional regions

Since the first set of LBD structure of the apo-RXR and ligand-bound RAR and TR were published in 1995, more than two-dozen LBD structures have been determined for the classic receptors. These structures are obtained with various LBDs in complex with agoinst or antagonist, some with fragments of co-activators and one with co-repressors. The rich information provided by these structures has made it possible to develop a global view of the molecular basis of ligand binding and ligand mediated regulation of nuclear receptors.

The obtained information made clear that nuclear receptor activity requires a complex allosteric interaction between all four LBD functional surfaces. For example, ligand binding induces a conformational change in the co-regulator binding site and H12 that leads to exchange of co-repressors for co-activators. In nuclear receptors that form heterodimers with RXRs, ligand binding also affects the stability and propagation of signals across the heterodimerization interface indicating that the partners of the heterodimers in a way communicate with each other [169]. The “cascade” of intramolecular changes that is usually triggered by ligand binding and results in the co-regulator exchange is mediated by the different positioning of H12.

1.6.2.The positioning of H12 upon agonist binding and the “mouse trap” model

The crystal structure of unliganded and agoinst bound LBDs for several NRs have confirmed the hypothesis that the H12 undergoes a ligand-dependent conformational change. In the crystal structure of unliganded RXR, H11 is almost perpendicular to H10 and points towards the ligand-binding pocket. The H12 extends away from the core LBD, pointing away from the dimer axis at an angle of about 45 [23]. In contrast, in the agonist bound RARγ, TRα, PPARγ and RXRα- LBD structures, H11 is repositioned in the continuity of H10, and the concomitant swinging of H12 unleashed the omega loop (between H2 and H3) which flips over underneath H6, carrying along the N-terminal part of H3. Thus upon agonist binding H12 helix moves in a 'mouse trap' being tightly packed against helix 3 and 4, seals the ligand binding cavity, in some cases making direct contacts with the ligand that further stabilizes ligand binding [128]. It is worth to note that there are receptors of that H12 change is much less marked as in RAR or RXR. Such as apo-PPARγ structure is very similar to its holo-structure since its H12 occupies a position, which is much closer to the position of the ligand bound receptor. These ligand-induced structural changes result in the formation of a surface that facilitates co-activator binding.

Figure 1.16. The conformational changes induced by ligand binding in hRXRα-LBD. The superposition of unliganded (blue) and liganded (green) hRXRα monomer. The arrows show the showing the main conformation changes that effect H3,H6, H11 and H12. Figure is taken from Egea et al., 2000 [43] .

1.6.3. Charge clamp and co-regulator exchange

Co-crystallization of several NRs together with theirs cognate agonist and the LXXLL motif /fragment of co-activators revealed the structural basis of NR – co-activator interaction [38,136,166]. The structures obtained indicate that a conserved glutamate residue in H12 and the conserved lysine residue at the COOH terminus of H3 make hydrogen bonds to leucines 1

and 5 of LXXLL. Together the glutamate and lysine residues form a charge clamp that positions the LXXLL helix to allow the leucine side chains to pack into the intervening hydrophobic cavity. These structures suggest that the structural basis of ligand-dependent activation is the closure of The H12 to form the charge clamp. The length and orientation of the LXXLL helical motif is vital for proper backbone interactions with both residues and for the proper positioning of co-activator IDs [57].

The LXXLL binding pocket comprises two parts: the constant part (H3, H3', H4 and H5) and the variable part (H12). The constant part adopts essentially the same conformation in all the LBD structures, and does not change with the binding of different ligands. In contrast the H12 adopts different conformations, depending on the nature of the bound ligand [112].

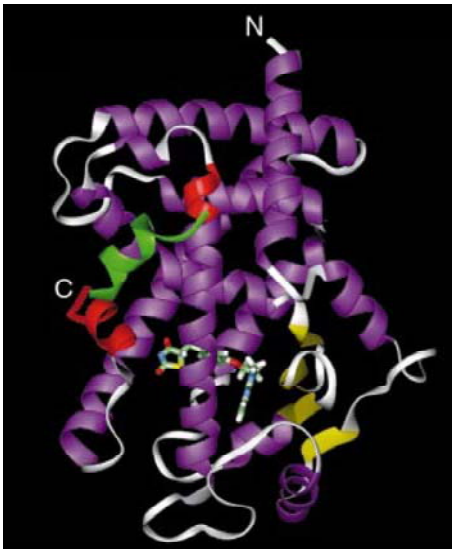


Figure 1.17. Charge clamp. Ribbon diagram showing agoinst bound PPARγ-LBD with LXXLL helix domain of SRC-1 coactivator. Residues on H3 and H12 that form the 'charge clamp' are shown in red, SRC-1 helix is shown in green. Figure taken from Nolte et al., 1998[136]..

The LXXLL co-activator motif adopts a two-turn a-helix with its three-leucine side chains fitting into the hydrophobic pocket between the two charge clamp residues, which further stabilize the co-activator helix by capping both helical ends. The high degree of amino acid sequence conservation in the co-activator-binding pocket suggests that this mode of co-activator binding represents a general mechanism for activation of nuclear receptors (Fig.1.18.).

Figure 1.18. Conservation of the charge clamp pocket. Sequence alignment of coactivator binding pocket in nuclear receptors. Blue: positively charged residues, Red: negatively charged residues, green: polar residues, black: hydrophobic residues. Arrows indicate the position of the charge clamp. Figure taken from Li et al., 2003 [112].

On the other hand, the position of the H12 also plays a key role in recruiting co-repressors such NCoR, SMRT. The co-repressors bind to LBD via the LXXLL motives, which are similar to the LXXLL co-activator motif but has an N-terminal extension. In comparison to co-activators, the longer co-repressor motif adopts a three-turn a-helix instead of two turns for the co-activator motif, and modelling of these binding sites hypothesized that they bind to the overlapping site as for the LXXLL. [17,72,122,133,142,195,206]. The additional turn of the co-repressor helix extends into space that would normally be occupied by the H12 when it is in the active conformation. Thus the binding of co-repressor and the active H12 conformation is mutually exclusive. The H12 must shift to some alternative position to accommodate the larger co-repressor helix. In the case of PPARα, rearrangement of the H12 is achieved by an antagonis, which pushes the H12 from its active position to provide additional space for binding of co-repressor helix (Fig.1.19.). The arrangement of H12 from its active position also allows the co-repressor helix to dock closer into the charge clamp pocket. The binding mode of co-repressors, similar to that of co-activators, is also highly conserved among nuclear receptors. It seems clear that binding of co-activators and co-repressors is tightly modulated by the position of the H12 helix. The conformational flexibility of this helix allows it to sense the presence of the bound ligand, either an agonist or an antagonist, and to recruit the co-activators or co-repressors that ultimately determine the transcriptional output of NRs [112].

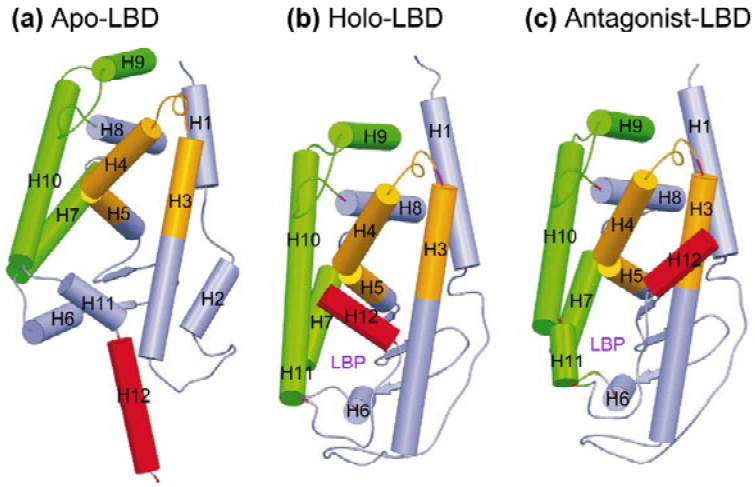


Figure 1. Schematic representation of three different conformational states of NR-LBDs. Figure taken from Bourguet et al., 2000 [22].

To summarize, in the absence of ligand H12 would adopt the apo position and going from the apo to the agonist position necessitates conformational changes, which are most likely to be responsible for disrupting interactions with the co-repressor, thus facilitating the ligand induced co-regulator exchange [32].

1.6.2.The positioning of H12 upon antagonist binding

Today there are also some structures of antagonist bound NRs (ERα-raloxifen, ERa-4-hydroxy-tamoxifen, RARa-BMS614) [27] have been reported. All these structures are very similar to the holo-NR-LBDs with the exception that H12 is unable to adopt the holo-position and form the charge-clamp required for co-activator binding. The general characteristic of these antagonists is that they possess a bulky side chain that cannot fit to the agonist-binding pocket thus sterically prevent H12 positioning. Thus in the antagonist conformation, the unwinding of the C-terminus of H11 leads to the lengthening of the loop L11-12. This enables H12 to make a 120^{o} rotation and adopt a second low-energy position by binding to the co-activator recognition site, in the groove formed by H5 and the C-terminal of H3. In addition to the complete or full antagonists, partial agonist-antagonists have also been reported [145,187]. The major difference between pure and partial antagonists lies in their steric properties. Though the partial antagonists do not have bulky extensions, they still induce unwinding of H11 and positioning of H12 in the antagonist groove, thus in this respect the effect of these ligands are similar to the antagonists.

2. AIMS OF OUR STUDIES

The biochemical and structural studies on NRs and their co-regulators have given us a better understanding of the mechanisms of their behaviour. It appears that NR activity requires a complex allosteric interaction between all four functional surfaces of LBD. A critical question that remains to be answered is what determines the balance between repression and activation. It is clear that this enormously between different NRs and since these various receptors share a common group of co-regulators, understanding how this different balance is achieved is of great biological significance [160].

In order to gain further insight into these regulatory mechanisms studying RAR-RXR heterodimers we tried to find the answers on the following questions:

1) Do co-repressors and co-activators bind to the same or overlapping surface on the RAR LBD?

The finding that both co-repressors and co-activators contain similar LXXLL motives in their IDs emerged the possibility that these co-regulators bind to overlapping surface on the ligand-binding domain of the receptors. With the help of the information obtained from structures of co-activator domain co-crystallized with receptor-LBD, we used a mutagenesis approach to identify the binding site for a co-repressor on the surface of RAR-LBD. We also wanted to compare the binding sites for co-repressors with co-activators and we were interested in finding residues that differentially affect co-repressor and co-activator recruitment to RAR.

2) How RAR mutations influence transcriptional activity of the RAR-RXR heterodimer? How is it related to co-regulator binding?

If we assume that the NR activity is a sum outcome of the co-regulator binding we sought to understand how the activity of the mutant receptors correlates with their co-regulator binding.

3) How RXR-H12 influences co-repressor and co-activator binding?

It has been known that RAR/RXR heterodimer is a non-permissive heterodimers and RXR-selective ligands cannot activate them. While it seems that RAR clearly plays the major role in defining the transcriptional property of the RAR/RXR heterodimer by directly mediating repression and activation, the role of RXR (other than facilitating DNA binding) is not well characterized. We examined the potential modulatory effect of RXR-H12 on co-repressor and co-activator binding using H12 deletion mutant RXR molecules.

4) How H12-deleted mutant RXR influences the activity of RXR heterodimers?

To study the effect of the H12 deletion of RXR on the activity of the heterodimer we performed transactivation studies in transiently transfected cells, and we made attempts to establish a virus infected cell line that stably expresses the mutant RXR.

3. MATERIALS AND METHODS

3.1. Cell cultures and reagents

The CV-1 (green monkey kidney fibroblast) and 293T cell lines were used for the transient transfection studies. Cells were maintained in DMEM, supplemented with 10% FCS (Fetal Calf serum), 2mM glutamine, penicillin and streptomycin. MonoMac6 macrophage cell line was used for virus infection following the stable expression of RXR-ΔH12-LBD. This cell line was maintained in RPMI, supplemented with 10% FCS (Fetal Calf serum) (Invitrogen), 2mM glutamine, penicillin and streptomycin.

3.2. Transient co-transfection and luciferase/beta galactosidase assay.

The day before transfection 10^6 cells were plated onto 48-well plates. Cells were transfected at 60-80% confluency using DOTAP (AVANTI, Avanti Polar-Lipids, Inc) according to the manifacture's instruction. Transfection was carried out in DMEM containing 5% charcoal stripped, delipidated calf serum (Sigma-Aldrich). After 6-8 hours medium was changed to DMEM containing the indicated ligands or vehicle. Cells were assayed for reporter expression 36 hours after transfection using Luciferase Assay System (Promega). Cells were lysed in 140 ul lyses buffer and from the lysate luciferase and beta-galactosidase activity was determined [166]. For the Luciferase measurement we used Luciferine substrate and measured the intensity of the emitted light of the samples in a white colour, 96-well microtiter plate. For the b-galactosidase activity measurement we used ONPG as substrate and measured the light absorption of the product on 405 nm wavelength in a transparent, 96-well plate. For the luminometric and photometric measurements a Wallac, Victor-2, Multilabel Counter was used. Luciferase activity of each sample was normalized for the level of beta-galactosidase activity. Each transfection was carried out in triplicate and repeated 3-6 times.

3.3. Plasmids and mutagenesis.

Mammalian expression vectors expressing Gal-SMRT-ID-1, -ID-2, -ID-1+2, Gal-hRARalpha-LBD, VP-hRARalpha-LBD, CMX-hRXRalpha-LBD, CMX-hRXRalpha-FL, CMX-hRARalpha-FL, GST-C-SMRT, pMH100-TK-luc, pCMX-beta-galactosidase were described previously [32] and kindly provided by DR. R. Evans.

Gal-DRIP-ID-1+2 expression plasmid was constructed with a PCR amplified DRIP fragment (527-775 aa) as a template. The resulting PCR product was cloned into pCMX-

Gal4-DBD using restriction sites BamHI and EcoRI. CMX-RAR-LBD expression plasmid was constructed with PCR amplified RAR-LBD fragment (210-460 aa) as a template. The resulting PCR product was cloned into a pCMX-plasmid using Asp718 and BamHI restriction sites.

All the mutants were generated by site-directed mutagenesis using the appropriate oligonucleotid pairs. Mutations within the VP-hRARα-LBD, VP-hTRβ-LBD, Gal-hRARα-LBD and CMX-hRARα-FL were obtained according to the methods described in the QuickChange Site-Directed Mutagenesis Kit (Stratagene).

The VP-hRXRα-LBD-ΔH12, Gal-hRXRα-LBD-ΔH12 and CMX-hRXRα-LBD-ΔH12 expression plasmids were constructed using oligonucleotide primer that builds STOP - codon in place of D443. The VP-hRARa-LBD-ΔH12, Gal-hRARα-LBD-ΔH12 and CMX-hRARα-LBD-ΔH12 expression plasmids were constructed with oligonucleotide primer that builds STOP - codon in place of K443 site.

Plasmids pMDL, RSV, CMV and PG for virus production were kindly provided by Dr. D.Trono. PG-hRXRα-FL-ΔH12 expression plasmid was generated by blunt-end cloning.

All constructs from cloning and mutagenesis were verified by DNA sequence analysis (DNA Sequencing Kit, BigDye Terminator Cycle Sequencing v2.0 and ABI 310 Sequence analyzer).

3.4. Transformation and growth of bacteria, and purification and detection of plasmids

Plasmids used for transient transfection and virus production were transformed into DH5α ultracompetent E. coli host with $CaCl_2$ heat shock method. Bacteria containing plasmids for transient transfection were plated onto Ampicillin containing agar plates and were grown on 37^0C overnight in Ampicillin containing LB media. Bacteria containing plasmids for virus production were plated onto Carbenicillin containing agar plates and were grown on 37^0C for 24 hours in Carbenicillin containing TB (Terrific Broth) media.

DNA was extracted and purified using Qiagene Maxi or Midi Columns, or Promega Wizard Plus SV MiniPrep Kit according to the manufacturer's instruction.

Prior to transfection, we determined the concentration of the purified plasmids and ran them on agarose gel to check their purity and proper conformation.

3.5. Protein expression and purification

Expression plasmids for GST-fusion proteins were kindly provided by Dr. J.W.R.Schwabe. They were transformed and grown in E. coli BL21 Gold host. The cells were

lysed by sonication and the protein was purified using a glutathione-Sepharose 4B affinity column (Amersham Pharmacia Biotech) in buffer containing 50 mM Tris (pH 8), 1% Triton-X-100 and 1 mM AEBSF. Bound proteins were eluted with 10 mM glutathione in 50 mM PBS (phosphate-buffered saline). The concentration of the eluted protein was measured according to Bradford and (equal amounts of each protein) was run on SDS-PAGE.

3.6. GST-pull-down.

hRARα-LBD mutant proteins were synthesized using a T7 Quick TNT in vitro Transcription/Translation Kit (Promega) according to manufacturer's instruction with [^{35}S] methionine. ^{35}S-labeled proteins were incubated with GST-fusion protein (bound to glutathione-Sepharose 4B resin) in the presence or absence of TTNPB ((E)-4-(2-(5,6,7,8-terahydro-5,5,8,8.tetramethyl-2-(naphtylenyl)-1-propenyl) benzoic acid) (2.5 μM), for 2 h at room temperature. After centrifugation, the beads were washed twice with PBS buffer containing 0.1% Triton-X-100 and 1 mg/ml BSA (bovine serum albumin), and resuspended in 2x Laemmli buffer. After centrifugation, the proteins were analyzed by 15% SDS-PAGE. After drying, gels were visualized and quantified using an image plate scanner (Typhoon, Molecular Dynamics, Amersham Bioscience).

3.7. Electrophoretic mobility shift assays.

Full-length hRARalpha and hRXRalpha receptors were produced using T7 Quick TNT in vitro Transcription/Translation Kit (Promega). The EMSA probe DNA (RARE-DR5) was labelled with ^{32}P-dCTP using reverse transcriptase after annealing and purifying of the two complementary oligonucleotides. The following RARE-DR5 sequence was used as probe: 5'-CGTTGGCG CC**GGGTCA**C CGAA**AGGTCA**GAATTAG–3'. The labelled probe was incubated with the receptors and bacterially expressed SMRT protein in binding buffer (20mM HEPES, pH 7.5, 75 mM KCl, 0.1% Nonidet P-40, 7,5 % glycerol, 2mM DTT) in the absence or presence of receptor-specific ligands for 20 min at room temperature. Samples were analysed using 10 x 10 cm, 0.7% agarose gel buffered in 0.5x TB (Tris-borate) at 4 ^{0}C at 30 mA. The gel was then dried and visualized using an image plate scanner (Typhoon, Molecular Dynamics, Amersham Bioscience).

3.8. Virus production and infection of MonoMac6 cell line

For virus production we used 293T fibroblast cells. Virus was generated as previously described [41]. According to this 293T cells were transfected with the four plasmids

responsible for virus generation, package and integration using Lipofectamine (Invitrogen). The media containing the produced virus over the transfected cells were changed and collected each day for 3 days. Then the collected media was filtered and concentrated with ultracentrifugation, at 20,000 rpm, 20 ^{o}C, for 2 hours. The pellet containing the virus was resuspended in media and was frozen in aliquots at –70 ^{o}C. The number of virus particles was determined by p24 Elisa kit (Beckman Coulter) according to the manufacturer's instruction.
10^5 MonoMac6 cell were infected with virus and cells were grown 10-14 days meantime changing the cell culture media as required.

3.9. RNA extraction and real-time quantitative PCR

Total RNA was isolated from cells using Trizol Reagent. Transcript quantitation was performed by quantitative real-time reverse transcriptase (RT) polymerase chain reaction (PCR) using Taqman probes. Transcript levels were normalized to the level of 36B4.

4. RESULTS

4.1. Analysis of SMRT RID RAR-LBD interactions

Previous domain mapping studies of the co-repressor SMRT have shown that the protein contains two receptor interaction domains, ID-1 and ID-2 [166]. In order to determine if one or both IDs are necessary for interaction with the ligand-binding domain of RAR, a mammalian two-hybrid assay was established in CV-1 cells. SMRT interaction domains were fused to a Gal4 DNA binding domain and challenged with the RAR-LBD fused to a VP16 activation domain. Figure 4.1. shows that SMRT-ID-1+2 interacts strongly with the RAR-LBD and that this interaction is abolished by treatment with AM580, an RAR□ selective agonist. The isolated SMRT-ID-2 essentially recapitulates the ligand–dependant behaviour of SMRT-ID-1+2, whereas the assay failed to detect any interaction with the isolated SMRT-ID-1 alone.

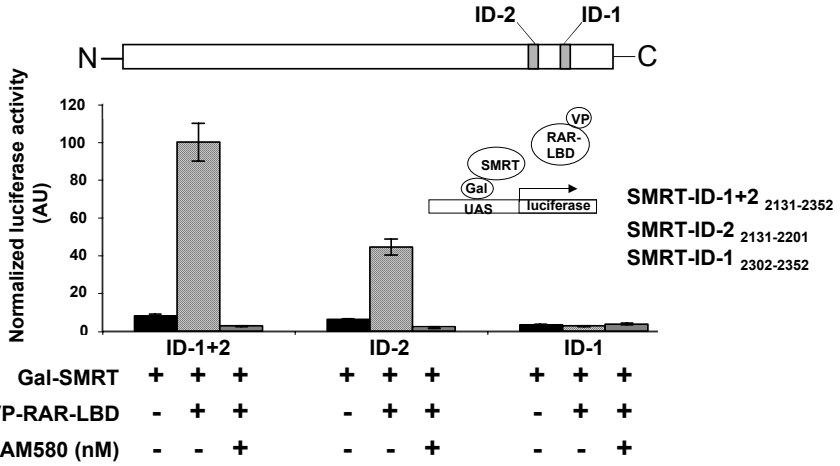


Figure 4.1. **Interaction of SMRT-IDs with RAR-LBD.** Interaction analyses of SMRT-IDs and RAR-LBD was carried out using mammalian two hybrid assays. Interactions of Gal- SMRT-ID-1+2, ID-2 and ID-1 with VP-RAR-LBD in the presence and absence of AM580 (100nM).

To test if the domain specificity remains in the context of the combined interaction domain, which includes both ID-1 and ID-2, we used point mutations to remove conserved hydrophobic amino acids from the individual ID's [128] (Fig.4.2.). SMRT-ID-1+2 harbouring mutations in ID-1 behaved exactly like the isolated ID-2, and similarly, SMRT-ID-1+2 with mutations in ID-2 behaved like the isolated domain of ID-1. Interestingly, the combined ID domain (SMRT ID-1+2) shows a stronger interaction, than ID-2 alone either as an isolated domain or as an ID-1 mutant. This suggests that ID-1 may contribute to ID-2:RAR-LBD interaction, even though it does not appear to bind to the LBD itself. These experiments also establish that ID-2 is the primary site on SMRT for interaction with RAR-LBD and it is necessary and sufficient for ligand sensitive interaction.

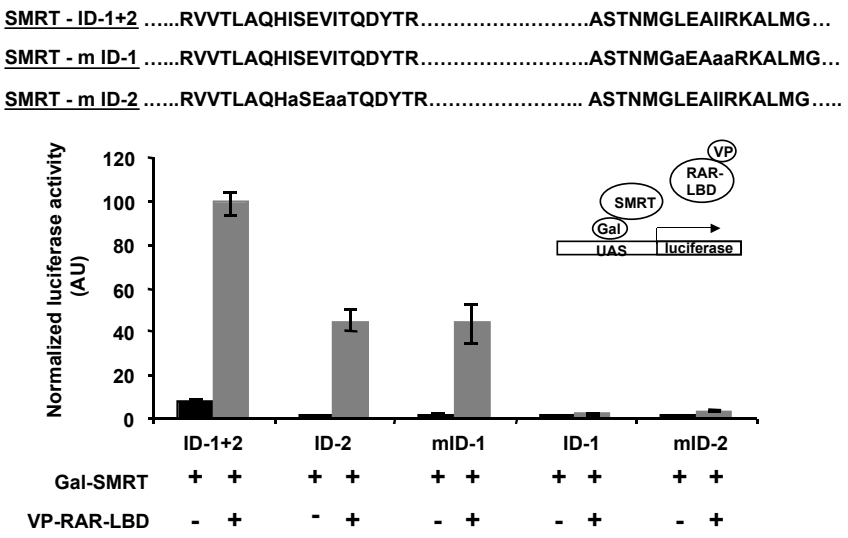


Figure 4.2. Interaction of mutant SMRT-IDs with RAR-LBD. *Upper panel* shows the aminoacid sequence of SMRT-ID-1+2 interaction domains lower case indicates the mutations in ID-1 (mID-1) and ID-2 (mID-2). *Lower panel* shows the comparison of the strength of interactions of wild type and mutant forms of Gal-SMRT-ID-1+2 with VP-RAR-LBD.

4.2. Mutational analysis of RAR-LBD co-repressor interactions

To gain insight into the molecular determinants of co-repressor binding on the surface of the RAR-LBD we made a series of specific point mutations of surface residues of RAR-LBD. Through inspection of the available crystal structures and analysis of the conservation between different receptors, 17 residues were selected for mutation. These lie on helices 3 and 4 (W225, S229, S233, I236, V240, K244, G248, F249, T250, I254, Q257, I258, L261), helix 5 (C265, L266) and helix 11 (A392, V395) (Figure 4.3.A and B). Several of these mutants have been analyzed in previous studies and serve as references in our analysis [142].

A

B

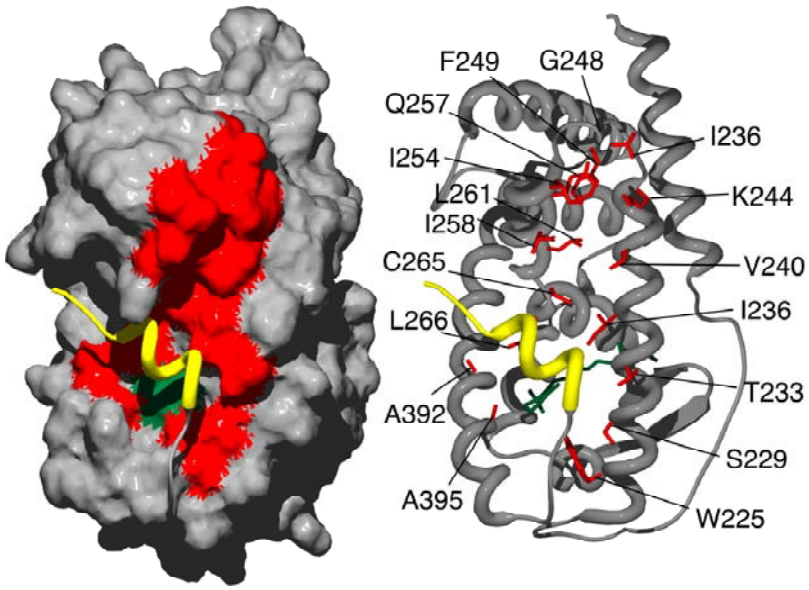


Figure 4.3. Interaction of RAR-LBD mutants with SMRT-IDs. (A) Sequence alignment of the ligand-binding domain (LBD) of different nuclear receptors (hRARα, hRXRα, hTRβ, hPPARγ). *Grey boxes* show the amino acid residues, which were mutated. The secondary structure elements (α-helices) are *boxed in black*. Amino acids mutated by others are indicated by *stars* [53,66,128]. (B) Surface and ribbon representation of the RARγ-LBD (Protein Data Babk code: 2LBD). Residues on H3, H4, H5 and H11 mutated in this study are shown in *red* and labeled. The ligand ATRA is shown in *green*. Helix H12 is shown in *yellow*. The mutated residues form a cluster on the surface of RAR-LBD.

Mutantions were generated by standard mutagenesis technology, then sequenced and tested in mammalian two hybrid interaction assays for interaction with SMRT ID-2 (Fig.4.4.).

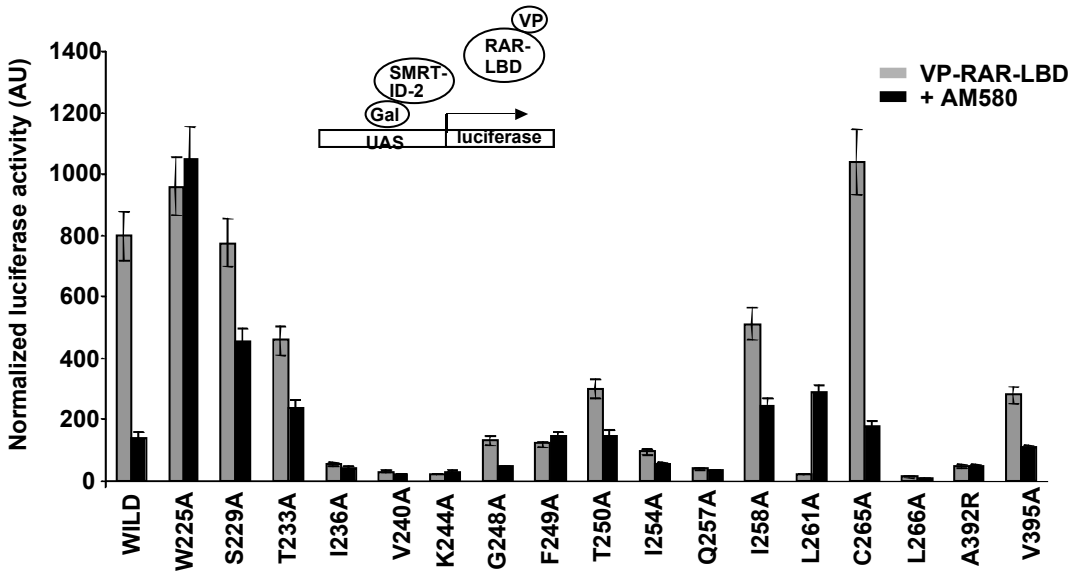


Figure 4.4. Interaction of SMRT-ID-2 with the mutant hRARα-LBDs. Mammalian two-hybrid analysis of the interaction of VP-RAR-LBD mutants with Gal-SMRT-ID-2 in the presence or absence of AM580 (100nM).

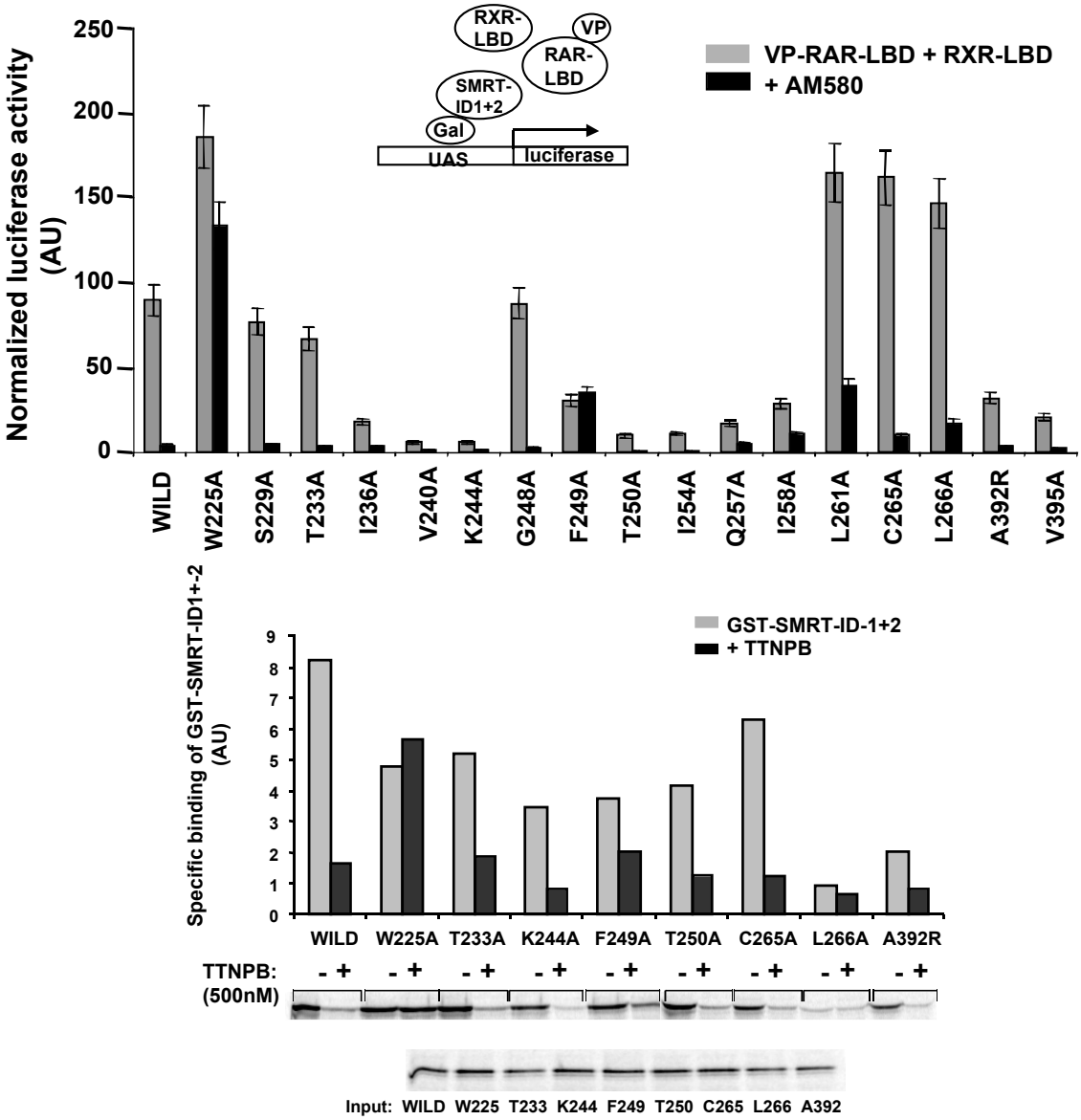
The mutant receptors can be grouped into different categories based on the strength of their interaction with SMRT-ID-2 in the presence or absence of a RARα selective ligand. Mutants such as S229A, T233A, I258A, C265A behaved very similarly to the wild type receptor with a strong interaction in the absence of ligand which was abolished on addition of ligand. The majority of the mutants (G248A, F249A, T250A, I254A, A392R and V395A) showed significantly reduced ligand sensitive interaction, and several others such as I236A, V240A, K244A, Q257A, L266A showed very little or no interaction at all, suggesting that in these cases the mutation reduced or eliminated co-repressor receptor interaction. One particular mutant, W225A, showed equally strong interaction with co-repressor protein as the wild type receptor, but was completely unable to release the co-repressor upon ligand binding.

4. 3. Interaction of RAR-LBD:RXR-LBD heterodimers with SMRT-ID

RAR functions as a heterodimeric complex with the retinoid X receptor (RXR). To recapitulate the SMRT:RAR interaction in a more physiological context, we examined the effects of the RAR mutations in the presence of RXR and both SMRT ID's. We re-screened the RAR-LBD mutants in the heterodimer assay system (Figure 4.5). Only two mutants, G248A and L266A, behaved differently in this assay than in the RAR-LBD alone assay (compare Figure 4.5.A to Figure 4.4.). In both cases these mutants regained wild type like activity suggesting that RXR may stabilize RAR-LBD:SMRT-RID interactions leading to

wild type-like co-regulator binding activity in these mutants. This also means that using heterodimers is a more stringent assay for assessing the effects of mutants on co-repressor binding since it is likely that some folding instability generated by the mutation get corrected this way.

Figure 4.5. Interaction of RAR-LBD mutants with SMRT-ID-1+2 in the presence of RXR-LBD. Interaction between SMRT-ID-1+2 and RAR-LBD mutants in the presence of RXR-LBD was determined using mammalian two-hybrid analysis (A) or GST pull-down assay (B). (A) Interaction of Gal-SMRT-ID-1+2 with mutant RAR-LBDs in heterodimer with RXR-LBD in the presence and absence of AM580 (100nM). (B) GST-



pull down analysis showing interaction of RAR-LBD mutants with GST-SMRT-ID-2 in the presence or absence of TTNPB (100nM). *Lower panel* shows the specific binding of in vitro translated, [^{35} S] methionine–labeled receptors on GST-SMRT ID-1+2 matrix run on SDS-polyacrylamide gel. *Upper panel* shows the densitometrical measurements of the visualized autoradiography. Results are presented as the specific binding to GST-SMRT-ID-2 in arbitrary unit. 10% input of the wild type and the mutant labeled proteins were run and are shown at the bottom.

While mammalian two-hybrid analysis is a sensitive and quantitative way of measuring interactions, we sought to confirm our results in a cell-free, *in vitro* interaction assay. GST-pull down experiments were performed using bacterially expressed GST-SMRT-ID-1+2 protein and radiolabelled RAR-LBD. In this assay (Figure 4.5.B) we found that the mutants behaved essentially identically to the mammalian two-hybrid assay. Significantly, the mutant W225A, which showed wild type like interaction with SMRT, but no ligand-sensitive release of SMRT-ID-1+2 behaved exactly the same way in the *in vitro* assay. Mutant A392R on the other hand showed a much weaker than wild-type ligand sensitive interaction with the co-repressor.

Altogether these analyses are consistent with previous studies on other receptors [148,152,163], which show that the primary co-repressor binding site on RAR is a hydrophobic groove between helices 3 and 4. Mutations of this surface of the LBD such as I236, V240, K244, F249, T250, I254, Q257, I258 abolished or significantly reduced co-repressor binding.

Two mutations W225 and A392 lie outside this region, W225 interacts with SMRT-ID/2 more strongly than wild type receptor and this interaction is ligand insensitive. In contrast the A392R mutation showed a markedly reduced interaction with co-repressor.

4.4. Analysis of co-activator binding

The premise of our studies was that the co-repressor and co-activator binding sites are largely overlapping. Therefore we next wanted to establish a co-activator binding assay using previously identified interaction domains of two co-activators: ACTR and DRIP205/TRAP220. We used these two co-activators because both have been shown to directly interact with RARs and they represent two distinct classes of co-activators [138]. We generated Gal-DBD fusion proteins with the previously identified receptor interacting domains of DRIP205/TRAP220 and ACTR [79,168,209] and carried out mammalian two hybrid analysis with VP-RAR-LBD fusion proteins in the presence of co-transfected RXR-LBD. The strength of interaction of the various mutants with DRIP205/TRAP220 ID has been plotted relative to that of the wild type receptor (Figure 4.6.). It is worth to note that in all cases the binding of the RAR-RXR heterodimer about two fold higher than that of the VP-RAR-LBD alone. Some of the mutants (S229A, T233A, I236A, G248A, T250A, C265A, L266A and V395A) behaved like wild type, W225A, V240A, K244A, F249A, I254A, Q257A, I258A and L261A showed reduced co-activator binding.

Remarkably, one mutant (A392R) showed significantly increased DRIP205/TRAP220 binding. This mutant, if compared to wild type, shows an increased co-activator binding even in the absence of ligand, which increases almost two fold more than the wild type's in the presence of ligand (Figure 4.7.). Although the two co-activators ACTR and DRIP205/TRAP220 are not related, there was no significant difference in binding (data not shown) suggesting that the two co-activators are likely to bind to the same site on the receptor.

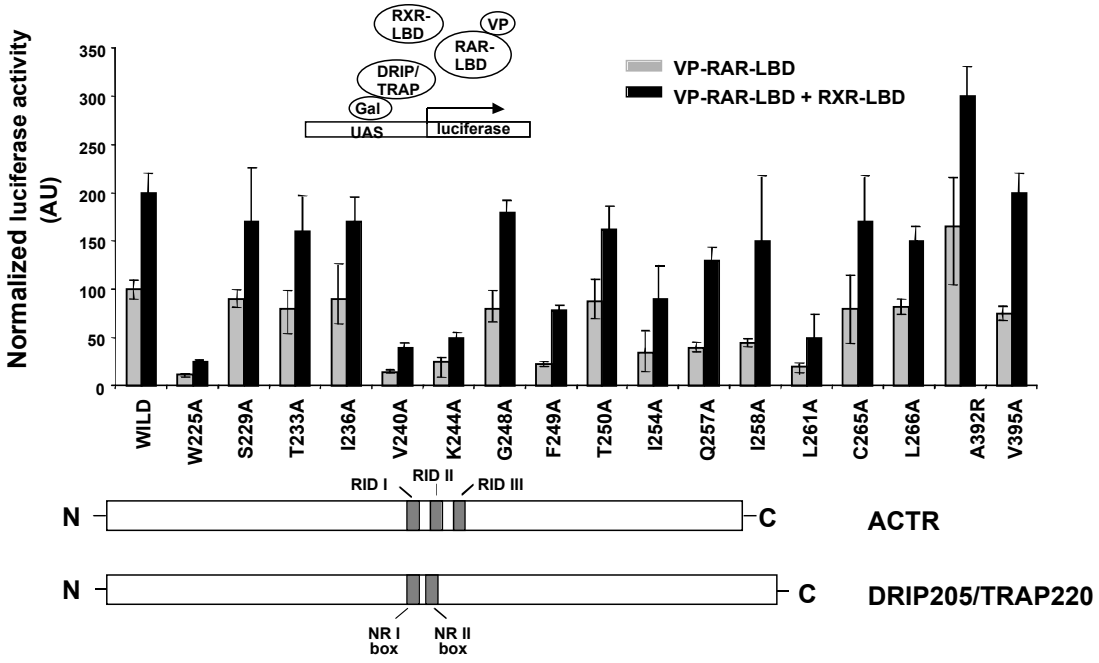


Figure 4.6. Co-activator-binding by monomer and heterodimer mutant RAR-LBDs. Interaction analysis of Gal-DRIP205/TRAP220 fusion proteins and wild type and mutant VP-RAR-LBD chimeric receptors were carried out in mammalian two-hybrid assay. *Upper panel* shows interaction of Gal-DRIP205/TRAP220-RIDs with VP-RAR-LBD mutants in monomer *(grey column)* and heterodimer *(black column)* studies in the presence of AM580 (100nM). *Lower panel* is the schematic representation of the two NR-box motifs (NR1 and NR2) in the full length DRIP 205/TRAP220 (amino acids 1 to 1566) and and IDs for ACTR (amino acids 1-1412) used in the two-hybrid assays.

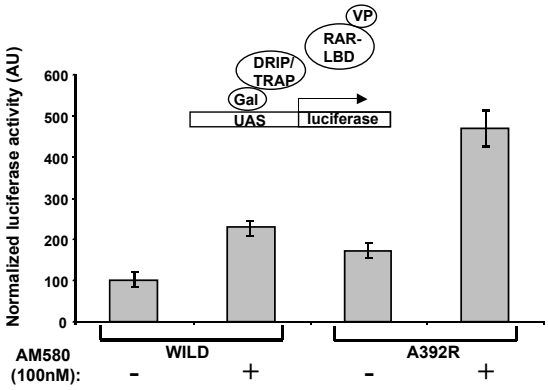


Figure 4.7. Interaction of DRIP205/TRAP220 with A392R mutant RAR. Interaction analysis of Gal-DRIP205/TRAP220 fusion proteins and wild type and mutant VP-RAR-LBD chimeric receptors were carried out in mammalian two-hybrid assay in the presence or absence of AM580 (100nM).

To summarize the various mutants, which showed altered co-activator or co-repressor binding and release, we grouped them based on their co-regulator binding profile as shown in the diagram on Figure 4.8. The vast majority (ten) of the mutants showed reduced co-repressor binding and six of them a combined co-repressor co-activator binding deficiency. There were a few mutants (I236A, T250A, V395A) that showed a reduced ability to bind co-repressor and only one (L261A) with reduced co-activator binding. The existence of these mutants (i.e. mutants which selectively reduce co-activator or co-repressor binding to the RAR-LBD) may be evidence for the not completely overlapping nature of the co-activator and co-repressor binding surface. Intriguingly, we have found two residues, W225 and A392, which if mutated to alanine or arginine, respectively, inversely affected co-activator and co-repressor binding. Since both of these mutants are located outside of the mapped and proposed docking site for co-regulators we believe that these two mutants do not alter the binding site *per se*, but represent mutations of an intrinsic regulatory site.

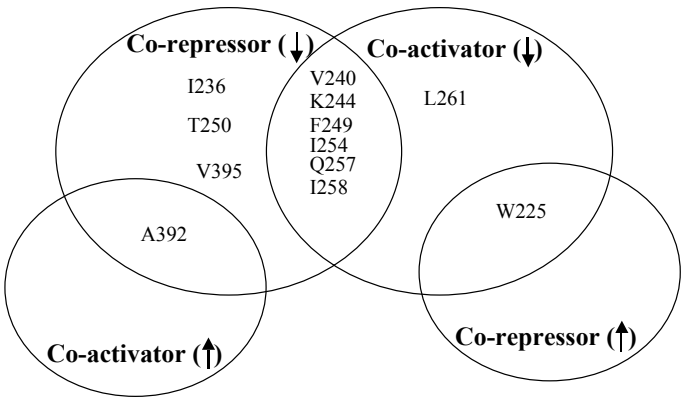


Figure 4.8. Summary of the altered co-regulator binding abilities of the different RAR-LBD mutants. (A) Venn diagram showing the altered co-activator and co-repressor interactions of RAR mutants.

We termed the site of the surface where the mutants affect both co-activator and co-repressor binding as docking site for co-regulators. Our results clearly show that mutations that affect the proposed binding/docking site reduce both co-activator and co-repressor binding consistent with the idea of a largely overlapping binding/docking site. While residues outside this area and in close proximity of H12 if mutated affect differently co-activator and co-repressor binding of the LBD.

4.5. Transcriptional activity of mutant receptors

It is critical to understand if a receptor LBD's transcriptional activity can be derived simply from co-regulator binding ability and profile. We hypothesized that receptors with combined co-activator/co-repressor mutations (docking site mutations) would become transcriptionally inert by losing some or most of their ability to repress as well as to activate but that their transcriptional activity would remain in the range defined by the wild type receptor. On the other hand regulatory mutations that affect the "set value" of co-activator and co-repressor association could produce receptors with significantly increased ability to repress and/or activate beyond the range of wild type. Therefore next we wanted to carefully examine if and how mutations affected the transcriptional activity of chimeric and full-length receptors. We considered a critical test of the above hypotheses that a determination of co-regulator binding and release profile has a predictive value on overall transcriptional activity, repression as well as activation. We decided to compare six mutants, three of these (V240A, K244A, I254A) showed deficiency in both co-activator and co-repressor binding and three other ones (W225A, A392R, V395A) affected co-regulator association differentially

We have generated mutations in Gal-DBD fusions of RAR-LBD and carried out transient transfection analysis of wild type and these mutant receptors in the absence or presence of increasing concentrations of AM580, an RARα selective retinoid. Figure 4.9. shows the activity in the absence of ligand as a measure of their ability to activate or to repress transcription in the absence of exogenous ligand. In this experiment wild type receptor repressed transcription of the reporter gene approximately 50 % and compared to wild type W225A repressed significantly (95 %) better. K244A and V395A showed no repressor activity at all. V240A and I254A mutants showed a reduced repressor activity (28 % and 14 % of the basal activity of the reporter gene, respectively) compared to wild type. A392R on the other hand produced very significant increase over basal activity of the TK-luciferase

reporter gene, showing that this mutant does not repress transcription but constitutively activates it.

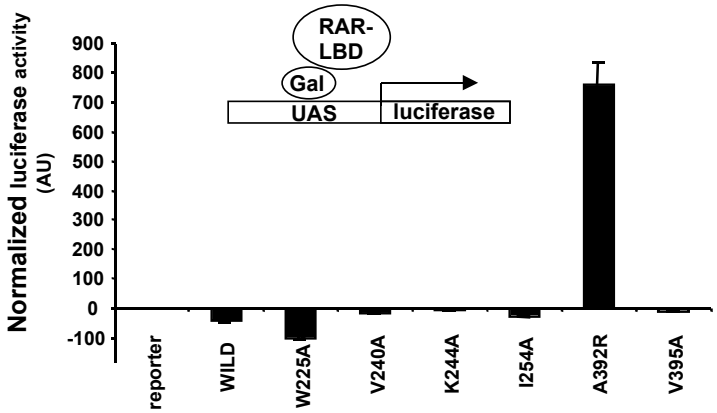
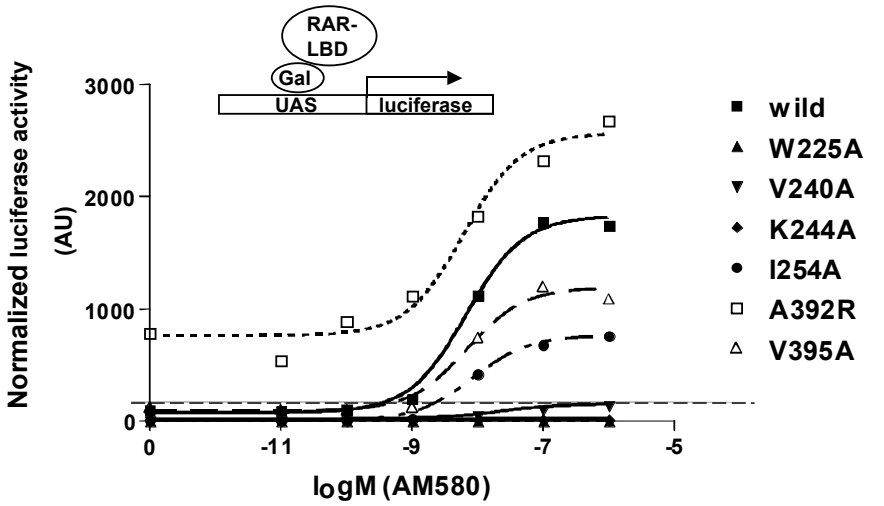


Figure 4.9. Transcriptional repression or activation of unliganded RAR mutants. The transcriptional consequences of selected mutations were determined using transient transfection assays. Representative experiments are presented. Repression is expressed as the percentage of basal activity of reporter plasmids.

Next we examined the response of the wild type and mutant chimeric receptors to an increasing dose of AM580 to assess ligand induced transcriptional activity (Figure 4.10A). Wild type RAR-LBD produced a dose response curve with a half maximal induction (EC_{50}) of 7 nM AM580 as expected. I254A and V395A had an EC_{50} similar to that of the wild type although the amplitude of induction was significantly lower on these mutants. W225A and K244A did not activate transcription to any significant degree, while A392R showed an increased basal activity and with this receptor AM580 also had a large amplitude of induction.

Next we wanted to see if co-transfection of RXR-LBD had an effect on the transactivation activity of mutant receptors (Figure 4.10B). Co-transfection of RXR-LBD effected only A392R. In the presence of co-transfected RXR the formed heterodimer regained some of its repressor activity and showed only a slightly increased transactivation in the absence of ligand, but showed an unexpectedly increased potency for AM580 (EC_{50}=1.5 nM). Mutant V240A, which was totally inactive in the monomer assay gained some transcriptional activity in the heterodimer assay, although this mutant was at least an order of a magnitude less sensitive to AM580 than the other mutants and wild type, as shown on Figure 4.10B.

A



B

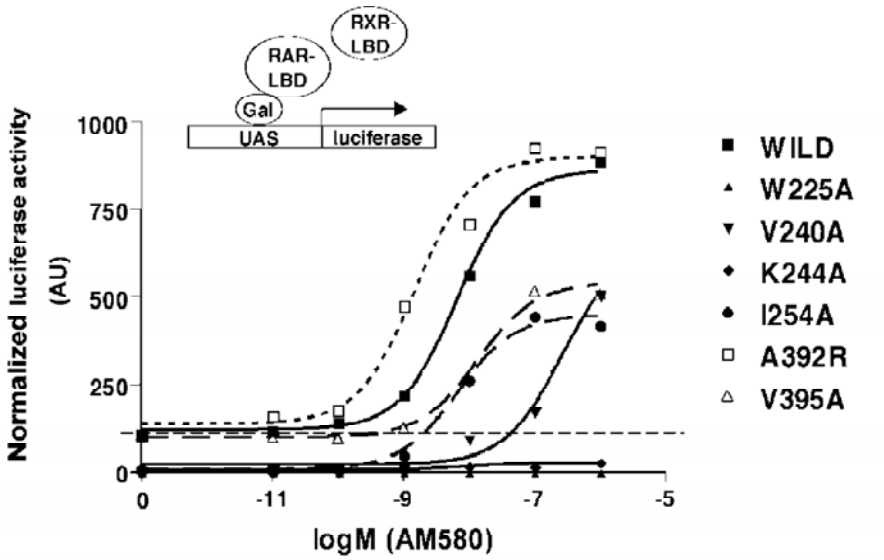
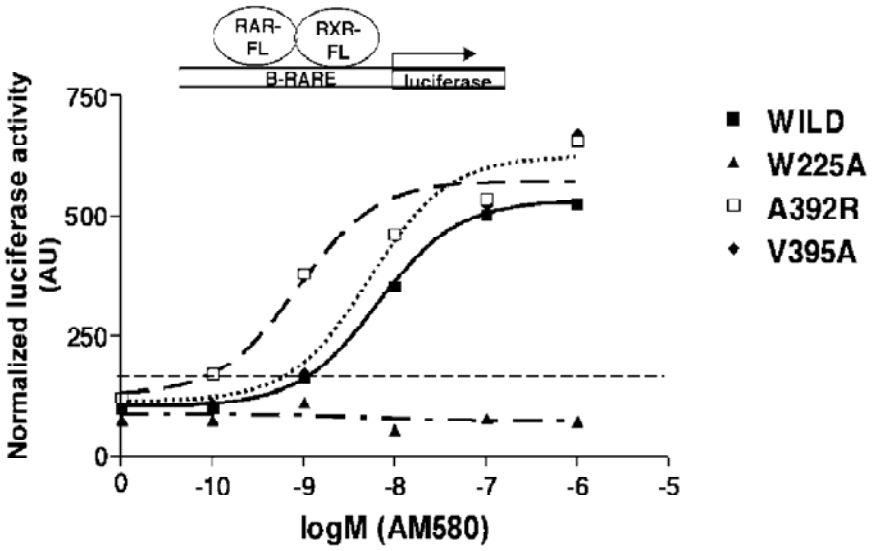


Figure 4.10. Transcriptional activity of mutant receptors. Transient co-transfection assays were carried out with the indicated plasmid constructs and transcriptional activity is expressed as normalized luciferase activity (A) Dose-response curves of mutant Gal-RAR-LBDs upon treatment with RAR-specific ligand, AM580. (B) Dose-response curves of mutant Gal-RAR-LBD: RXR-LBD heterodimers upon treatment with AM580. Broken line indicates the basal activity of the reporter construct. *Broken line* indicates the basal activity of the reporter construct. Curves were fitted by GraphPad Prism (from GraphPad Prism Software, Inc.).

Finally, we wanted to examine the effect of mutations in the most physiological setting available in transfection assays, on a non-chimeric, full-length receptor. Therefore we generated mutants in full length RAR and carried out transient transfection assays using beta RARE-TK-luc reporter genes in the presence of increasing amount of AM580. As shown on Figure 4.11 wild type and V395A receptors show a dose dependent transactivation profile with an EC_{50} of 6.7 nM and 5.5 nM respectively, W225A shows no significant transactivation

potential, while A392R remained more potent than wild type (EC_{50}=0.9 nM) as indicated by the dose response curve shifting to the left.

Figure 4.11. Transcriptional activity of full-lenth mutant receptors. Dose– response curves of full-length RAR:RXR heterodimers cotransfected with beta–RARE-TK-LUC upon AM580 treatment in transient co-transfection assays. Broken line indicates the basal activity of the reporter construct.



4.6. Electrophoretic Mobility Shift Assay analysis of mutant receptor

Electrophoretic mobility shift analysis (EMSA) was used to verify if the full-length receptors behaved the same way in a non-cellular, in vitro assay. In order to perform this experiment we made full-length receptors in an in vitro transcription translation system and bacterially expressed SMRT-D1+2. This experiment shows that wild type receptors form heterodimers, which readily bind co-repressor (Figure 4.12). The co-repressor is dissociated on addition ofAM580. Compared with wild type receptor, W225A binds SMRT ID-1+2 more strongly and this interaction is only slightly effected by addition of ligand. T250A and A392R showed EMSA activity similar to wild type, consistent with the results obtained in the chimeric and full-length receptor transactivation assays (Figure 4.11.). LG268 was used as a negative control and did not affect the complex.

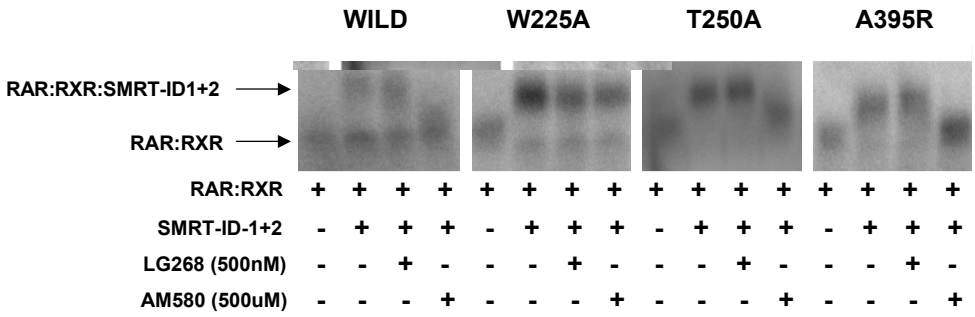


Figure 4.12. EMSA analysis of wild type and mutant full-length RAR:RXR heterodimers. Electophoretic Mobility Shift Analysis (EMSA) was performed in the presence or absence of the indicated ligands at a concentration of 500 nM. Autoradiographs are presented. Arrows indicate the position of the two specific complexes.

4.7. The homologous mutants of hRARα W225 and A392 have similar effects in hTRβ for co-repressor binding and release

Finally, we reasoned that if the two residues (W225 and A392) represent functionally conserved aminoacids contributing to the balance of co-repressor and co-activator binding to receptor LBDs, then mutations of the analogous amino acids in a different receptor may have similar effects. We chose hTRβ, because it is the closest homologoue of RAR and it is also a very strong transcriptional repressor. Analogous mutations (RAR-W225A=TRβ–F264A and RAR-A392R=TRβ-A431R) were made in VP fusions of hTRβ-LBD and transient transfection analysis was carried out with Gal-SMRT-ID-1+2. Wild type TRβ-LBD showed a strong interaction with SMRT ID-1+2, F264A showed an almost twice as strong interaction with the co-repressor while A431R showed a somewhat reduced interaction (75 % of the wild type) as expected based on the results with the analogous RAR mutants W225A and A392R, respectively (Figure 4.13). Moreover, upon ligand (T_3) treatment mutant F264A's interaction with SMRT was hardly affected by ligand, while mutant A431R's interaction was more sensitive to ligands if compared to wild type. We also noted that ligand failed to completely release SMRT-ID-1+2 from hTRβ-F264A. These data collectively suggest that analogous mutants in TR show a similar phenotype to those described in RAR, further underscoring the notion that the identified residues are functionally conserved.

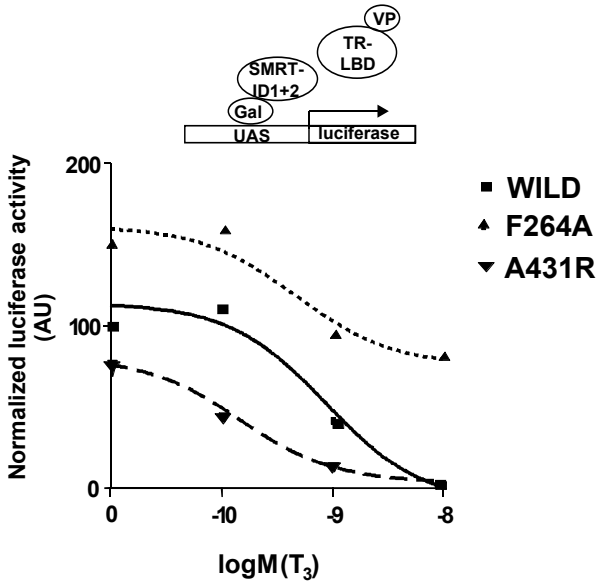


Figure 4.13. Interaction of SMRT-ID-1+2 with TR mutants containing homologous mutations to RAR-LBD-W225A and A392R. Transient transfections of VP-fusion hTRβ mutants were carried out in the presence of increasing amount of T_3. Curves were fitted by GraphPad Prism (from GraphPad Prism Software, Inc.).

4.8. Analysis of SMRT-ID RXR-LBD interactions

It is known from our and other's previous results that in the absence of ligand RXR doesn't repress basal transcription [160] as compared to the strong repression by several other NRs (like RAR, TR, LXR). To see if the co-repressor binding of these receptors correlates with basal repression, we carried out transient transfection studies where we co-transfected Gal-SMRT-ID-1+2 with VP-hRXRα-LBD, VP-hRARα-LBD or VP-mTRβ-LBD to compare their co-repressor bindings (Fig.4.14). We found that the co-repressor binding of RXR is much weaker than the very strong co-repressor binding of RAR and TR. These results show that there is a correlation between co-repressor binding and basal activity of nuclear receptors.

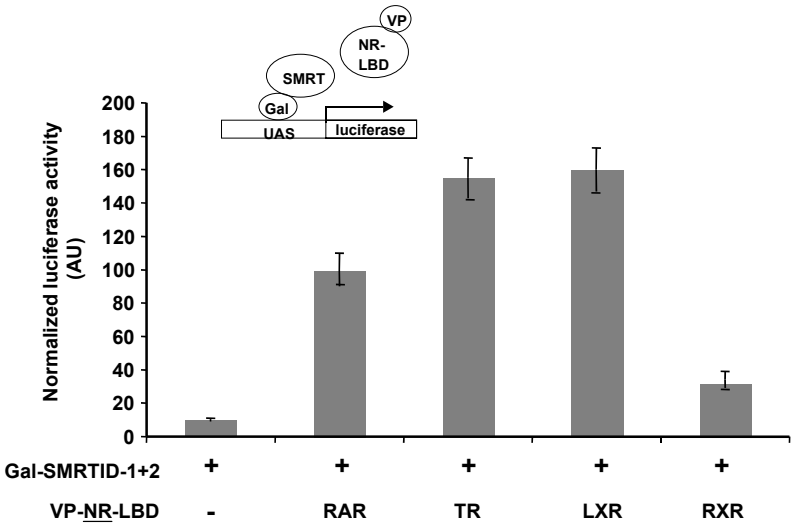


Figure 4.14. Co-repressor binding of the different nuclear receptor LBDs. Interactions between SMRT-ID-1+2 and the different LBDs were determined using mammalian two-hybrid analysis.

We also wanted to determine if RXR has specific binding to any of the IDs, like RAR preferencially binds to ID-2. We co-transfected Gal-SMRT-IDs with VP-fusion RXR-LBD. Fig. 4.15. shows that ID-2 has no interaction, while ID-1 shows interaction with RXR. These results suggest that while the co-repressor binds RAR though ID-2, the weak interaction between RXR and the co-repressor is mediated through ID-1.

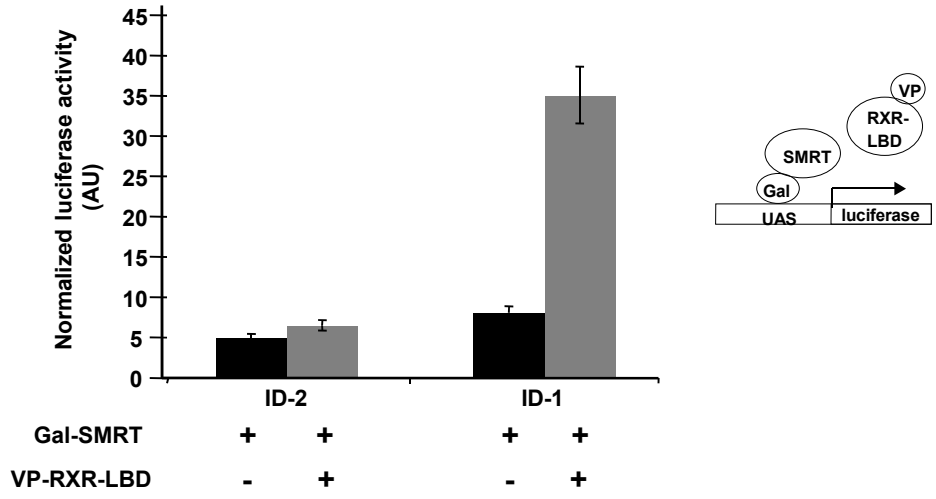


Figure 4.15. SMRT-ID binding of RXR-LBD. Interaction analysis of SMRT-IDs and RXR-LBD was carried out using mammalian two-hybrid assay.

4.9. Effect of RXR-H12 deletion on RAR co-repressor interaction

The previous results rose the question how co-repressor binding may change when it binds to a heterodimer containing two receptors with very different co-repressor binding abilities, such as RAR and RXR. To address this issue we transiently co-transfected Gal-SMRT-ID-1+2 and VP-hRARα-LBD alone or in combination with hRXRα-LBD (Fig.4.16). We found that the presence of RXR attenuated the originally strong interaction between the co-repressor and RAR (by 35-50%).

This means that although heterodimerization and co-repressor binding requires distinct surfaces of the receptor molecule, in using some unknown mechanism RXR influences the co/repressor binding of RAR.

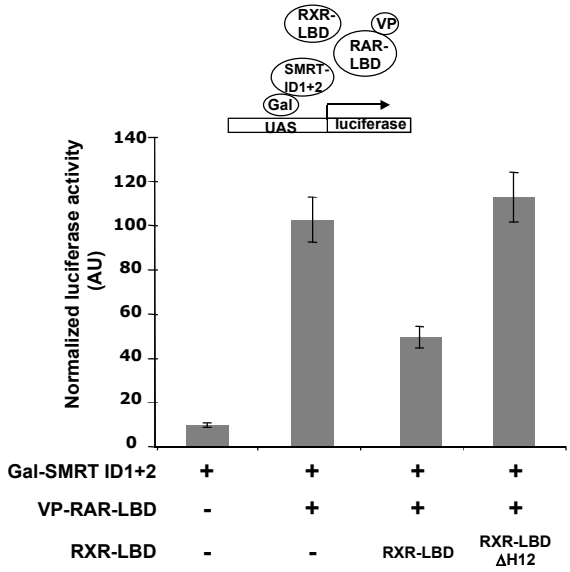


Figure 4.16. Effect of RXR on the interaction between co-repressor and RAR. Interactions between SMRT-ID-1+2 and the different LBDs were determined using mammalian two-hybrid analysis.

Structurally, H12 of the LBD harbours sufficient flexibility and mobility that makes it to be a good candidate for mediating the observed attenuation effect. This idea is supported by a model that suggests that in a RAR:RXR heterodimer the RXR-H12 protrudes and overhangs to RAR [198].

To find out more about the role of RXR-H12 in co-repressor binding we decided to examine if deletion of RXR-H12 has any effect on the attenuation of RAR - co-repressor interaction. In transient transfection studies we cotransfected Gal-SMRT-ID-1+2 with VP-RAR-LBD and RXR-ΔH12-LBD and compared the interaction to the wild-type heterodimer (Fig.4.16). We found that in contrast to the wild type RXR, that decreased the strong intraction between RAR and co-repressor, the mutant RXR did not have any effect on this interaction, strong binding was observed between RAR – ID-1+2 in the presence of RXR-ΔH12. This means that H12 of RXR is required for the attenuation of RAR – co-repressor interaction.

4.10. Analysis of the role of the separate co-repressor IDs in mediating attenuation

The results showed that RXR-H12 deletion reverses the attenuating effect of RXR, therefore we wanted to know which ID and which receptor mediates this change. Based on a model, that explains allosteric inhibitory effect of RAR [198], RXR-H12 binds to the co-activator binding region on the RAR-LBD. Since we showed that this surface overlaps with the co-repressor binding surface, we wanted to examine if there is a competition between ID-2 and RXR-H12 for the binding site on RAR-LBD, and the deletion of RXR-H12 would favour ID-2 binding. If that is the case, it could also explain our pervious findings where an almost completely abolished interaction between SMRT-ID-2 and RAR was observed in the presence

of RXR. At the same time, we also wanted to examine the effect of RXR-H12 deletion on RXR:ID-1 interaction.

In a transient transfection assay we co-transfected Gal-SMRT-ID-2 and VP-RAR-LBD in the presence or absence of RXR-LBD or RXR-ΔH12-LBD (Fig.4.17). In addition, in another experiment we co-transfected Gal-SMRT-ID-1 and VP-RXR-LBD or VP-RXR-ΔH12-LBD in the presence or absence of RAR-LBD. We found, surprisingly, that both RXR-LBD and RXR-ΔH12-LBD almost completely abolished ID-2:RAR interaction. This means that RXR-H12 deletion itself has no effect on RAR:ID-2 interaction and co-repressor ID-2 does not compete with RXR-H12 for binding site on RAR-LBD.

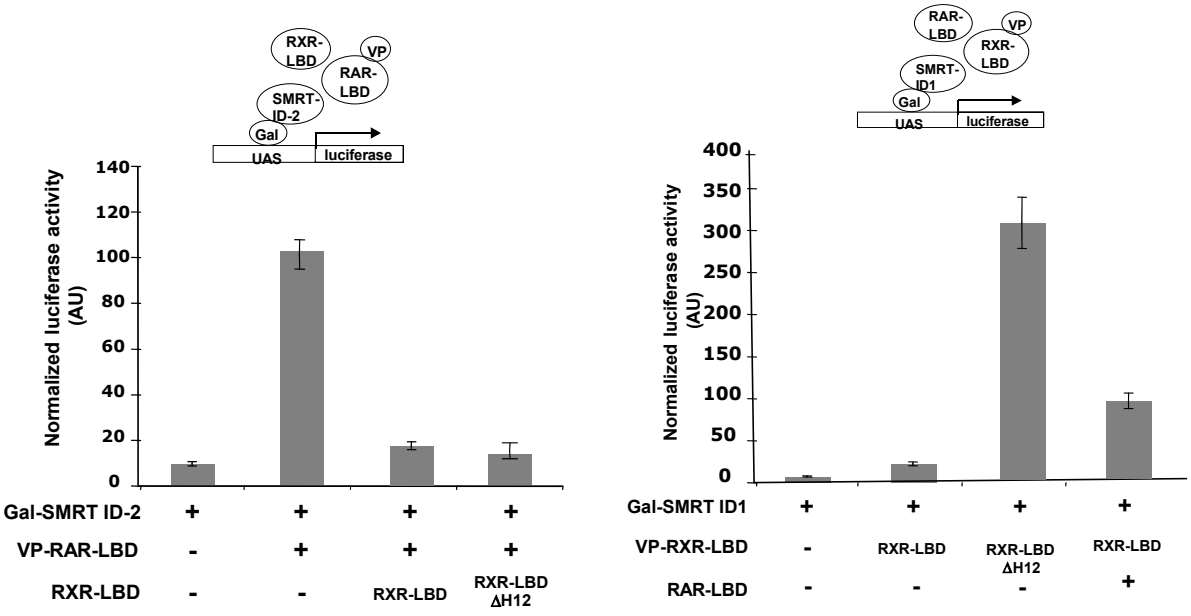


Figure 4.17. Effect of RXR-H12 deletion on the interaction between co-repressor and RAR. Interactions between (A) SMRT-ID-2 and RAR and (B) SMRT-ID-1 and RXR LBDs were determined using mammalian two-hybrid analysis.

Next we decided to examine how RXR – ID-1 interaction is affected by this mutation since the results showed that the deletion of H12 from RXR influences RAR – ID-1+2 interaction but it is not the ID-2 – RAR interaction that mediates this change. Thus in a transient transfection assay we cotransfected Gal-SMRT-ID-1 and VP-RXR-LBD or-VP-RXR-ΔH12-LBD (Fig.4.17.). We found that the deletion of H12 from RXR strongly increased the interaction between ID-1 and RXR. These co-repressor binding results together show that the increased interaction observed between the ID-1+2 and the mutant heterodimer is likely to be mediated through ID-1.

4.11. The co-repressor release of RXR

It was shown in previous experiments that repositioning of H12 is critical for the displacement of co-repressor proteins [11]. To explore the role of RXR-specific ligands in the regulation of co-repressor interaction we have used mammalian two-hybrid system and co-transfected Gal-SMRT-ID-1+2 with VP-NR-LBDs and treated the cells with their specific ligands (Fig. 4.18.). These experiments show that, in contrast to other receptors (such as TR and RAR) that were able to release co-repressor upon ligand treatment, VP-RXR-LBD still interacts with SMRT-ID-1+2 in the presence of its specific ligand. These results are further supported by our findings using an independent approach. The mobility shift assay shown in Figure 4.12. illustrates that upon LG268 treatment the co-repressor-receptor-DNA complex is intact. We decided to see if the presence of the co-activator ACTR has any effect on this complex. Thus we co-transfected the co-repressor and receptor plasmids with increasing amount of co-activator. We found that ACTR was able to compete for the receptor and abolished the interaction between the co-repressor and RXR (Fig.4.19.). This means that RXR requires the presence of co-activator and ligand at the same time to be able to make the conformation change that favours co-repressor release.

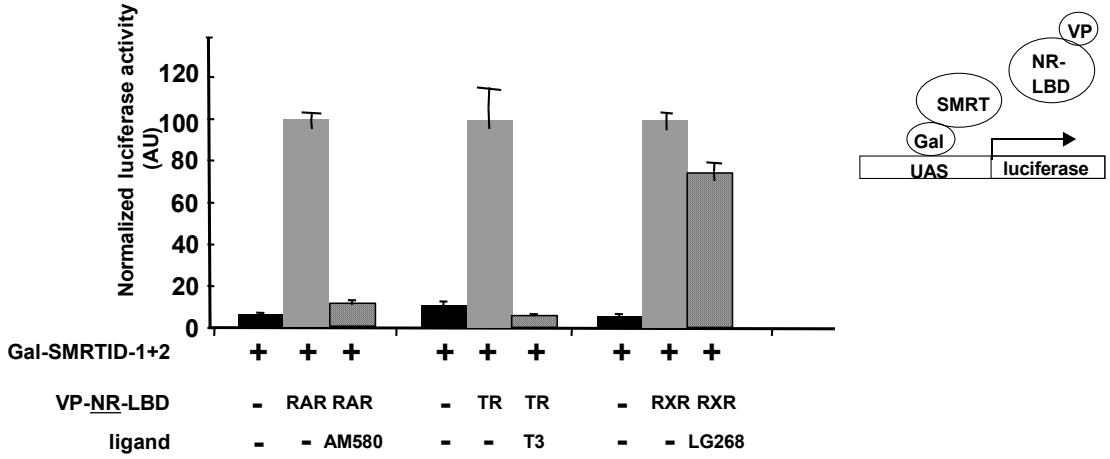


Figure 4.18. Effect of receptor specific ligands on the interaction between co-repressor and NRs. Interactions between SMRT-ID-1+2 and the different LBDs were determined using mammalian two-hybrid analysis.

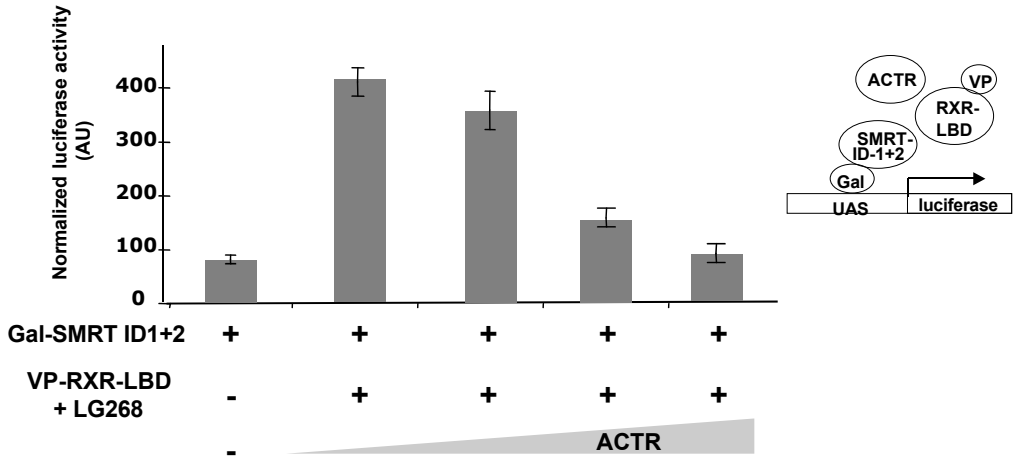
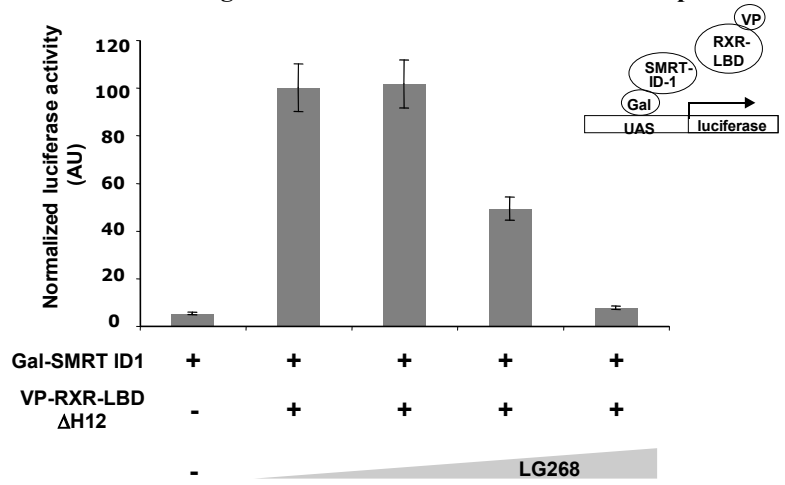


Figure 4.19. Co-repressor release by RXR-LBD. Interaction between SMRT-ID-1+2 and VP-RXR-LBD was determined using mammalian two-hybrid analysis.

4.12. Effect of LG268 treatment on the co-repressor binding of the mutant heterodimer

We wanted to know what was the effect of an RXR-specific ligand on the co-repressor binding in the case of the mutant RXR. We transiently co-transfected Gal-SMRT-ID-1 with VP-RXR-ΔH12-LBD and applied LG268 treatment (Fig.4.20). Surprisingly, LG268 treatment completely abolished the strong interaction between the molecules. To see if this effect is characteristic only for LG268 we applied other ligands (9-cisRA, LG1208) to the heterodimer. In each case we found similar results. These results show that in case of mutant RXR, the co-repressor release does not require H12 (data not shown).

Figure 4.20. Effect of the RXR ligand on the interaction between co-repressor ID-1 and RXR-ΔH12.



Interactions between SMRT-ID-1 and RXR-ΔH12-LBDs was determined using mammalian two-hybrid analysis.

4.13. Effect of RAR-H12 deletion on co-repressor binding

Based on the finding that ligand treatment abolished SMRT-ID-1 interaction with RXR-ΔH12, we decided to compare how RAR co-repressor binding is affected when its H12 is deleted. We decided to delete the H12 of RAR to see how RAR-specific ligand treatment effects SMRT-ID-2 – RAR interaction. In transient transfection studies we co-transfected Gal-SMRT-ID-2 with VP-RAR-LBD or VP-RAR-ΔH12-LBD in the absence or presence of RXR-LBD. We also treated the heterodimer co-repressor complex with RAR specific ligand to see its effect on the stability of the complex (Fig.4.21.). We found that, as in the case of RXR mutant receptor, both ID-1+2 and ID-2 had an increased, strong interaction with the mutant RAR, if compared to the wild type. Moreover, RXR was unable to decrease these interactions in this system. We also found that in contrast to the RXR mutant, RAR lacking H12 was unable to release the co-repressor even upon ligand binding. Together these results show that (RXR attenuation on the interaction of RAR to ID-2 requires the presence of RAR-H12.) Also, that RAR requires H12 to be able to release the bound co-repressor by ligand binding.

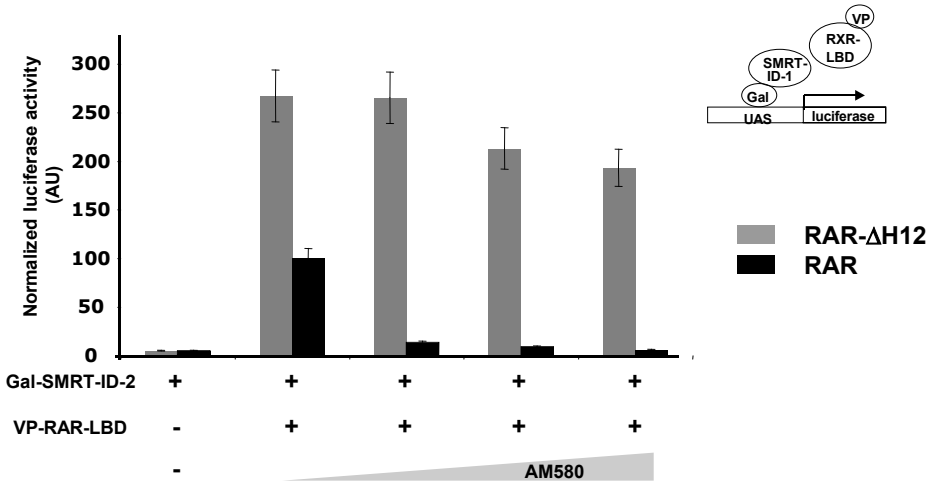


Figure 4.21. Effect of RAR-H12 deletion on the interaction between co-repressor and RAR. Interactions between SMRT-ID-2 and the RAR-LBDs was determined using mammalian two-hybrid analysis

4.14. The effect of H12 deletion on co-activator binding

It has been shown perviously that H12 of the receptors, in general, is required for co-activator binding. Also, recent studies have suggested that the surface for co-repressor binding partially overlaps with that of co-ativator binding and it has been confirmed also by us [17]. The finding that deletion of RXR-H12 changes co-repressor binding raises the question whether it also has an effect on co-activator binding.

To answer this question we co-transfected Gal-DRIP205 with VP-RAR-LBD and RXR-LBD or RXR-DH12-LBD and we treated the cells either with RXR-specific synthetic ligand (LG268) or RAR-specific synthetic ligand (AM580) since co-activator binding is facilitated by ligand-binding. We found that in the absence of ligand neither the wild type, nor the mutant heterodimer was able to bind to the co-activator. LG268 treatment of the wild type heterodimer resulted in an increased co-activator binding (as we have shown it earlier). In the case of the mutant heterodimer the co-activator binding was very weak as compared to the wild type. On the other hand, the treatment with AM580 resulted in a strong co-activator binding by the wild type heterodimer, while in the case of the mutant receptor this interaction was also very weak (Fig.4.22.). These results suggest that the mutation of RXR (that influences the co-repressor binding through the RXR part) has an effect not only on its own co-activator binding but it also affects the partner receptor's (RAR) co-activator binding.

Taken together the co-regulator binding results, they suggest that deletion of RXR - H12 is likely to change the equilibrium of co-repressor - co- activator binding of the RAR:RXR heterodimer and tilt the balance to favour co-repressor binding and decreased co-activator binding.

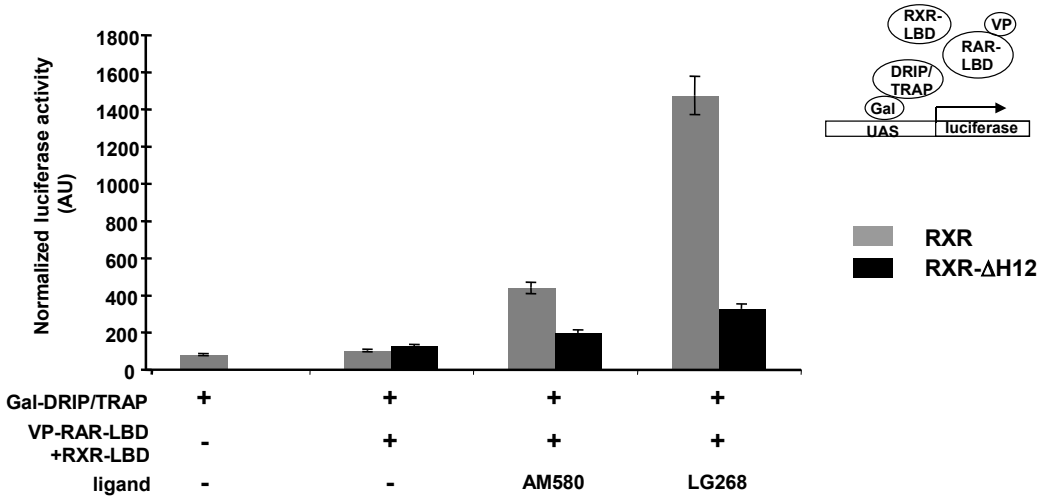


Figure 4.22. Effect of RXR-H12 deletion on the interaction between co-activator and RAR. Interactions between DRIP205/TRAP220 and RAR-RXR heterodimer were determined using mammalian two-hybrid analysis.

4.15. The effect of H12 deletion on the activity of RXR heterodimers

We wanted to know how the activity of the heterodimer is affected by the changed co-factor binding, thus we co-transfected Gal-RAR-LBD with RXR-LBD or RXR-ΔH12-LBD and treated the heterodimers with receptor-specific synthetic ligands (Fig.4.23.). We found that neither the wild type nor the mutant heterodimer was activated by RXR ligand, as it is expected in the case of a non-permissive heterodimers (and from the previous co-regulator binding). On the other hand, while the wild type heterodimer showed the expected activity profile upon RAR-ligand treatment, the activity of the mutant heterodimer was much weaker. In fact it did not go higher than the level of transcription detected in the absence of ligand.

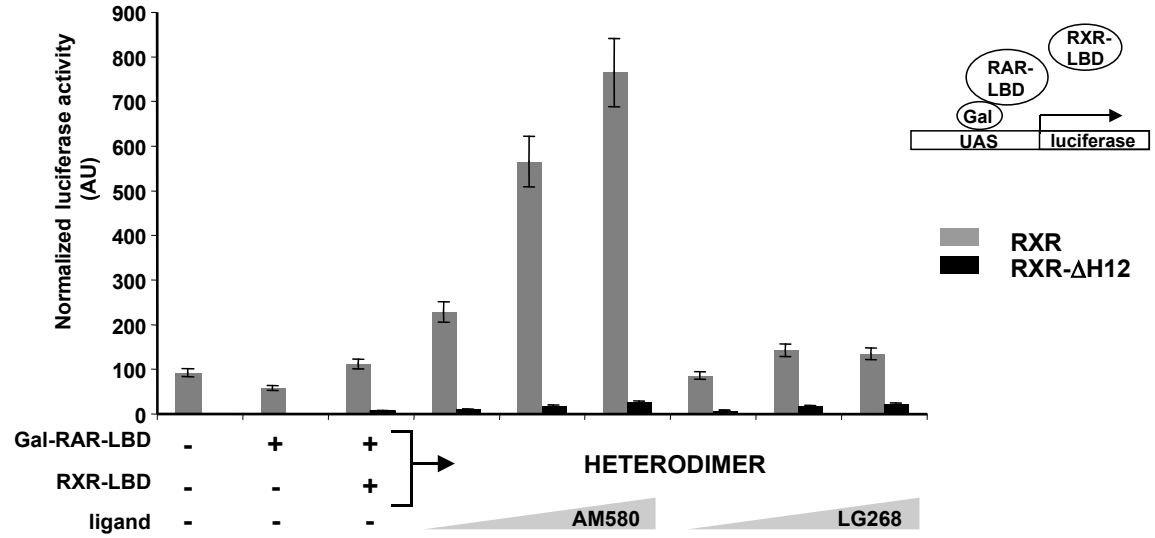
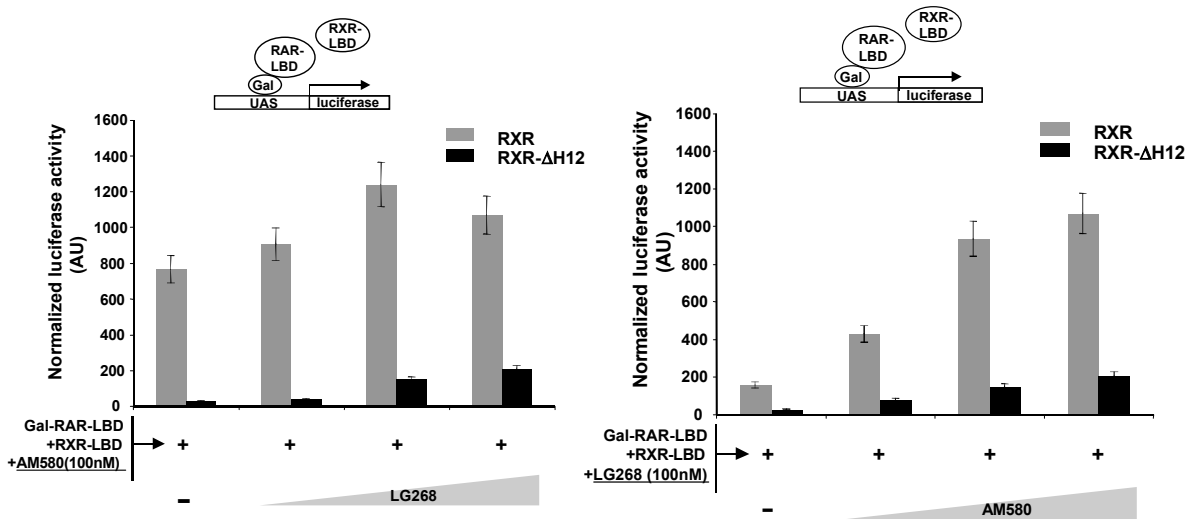


Figure 4.23. Transcriptional activity of RAR:RXR heterodimer. Dose-response curves of Gal-RAR-LBD:RXR-LBD heterosimer upon treatment with RAR-specific ligand, AM580 or RXR –specific ligand, LG268.

The activation of non-permissive heterodimers with RXR ligand can be detected only when the heterodimer is also treated with the partner receptor's ligand, and the double ligand treatment results in the synergistic activation of the heterodimer. To see how RXR-H12 deletion affects the activation of the heterodimer upon dual ligand treatment, we treated the heterodimers with the specific ligands (Fig.4.24.). In the wild type heterodimer we obtained synergistic activation both in the case of fixed amount of LG268 with increasing amount of AM580 treatment and in the case of fixed AM580 with increasing LG268 treatment. In the case of mutant heterodimer although responses were still subordinated, treatment with fixed amount of LG268 and increasing amount of AM580 activity showed 10 fold higher activity compared to the untreated heterodimer. Surprisingly, the mutant heterodimer treated with

fixed amount of AM580 and increasing amount of LG268 also showed the same increasing activity. These results show that the deletion of RXR-H12 influences not only the RXR response, but also the response of its partner receptor and leads to the subordinated response of RAR.

Figure 4.24. Effect of RXR-H12 deletion on RAR-RXR heterodimer transactivation. Transient co-transfection assays were carried out with the indicated plasmid constructs and transcriptional activity is expressed as normalized luciferase activity. Dose-response curves of Gal-RAR-LBD: RXR-LBD and Gal-RAR-



LBD: RXR-ΔH12-LBD heterodimers upon treatment with (A) fixed amount of AM580 and increasing amount of LG268 or (B) upon treatment with fixed amount of LG268 and increasing amount of AM580.

We wanted to know if we could find the same subordination response of the partner receptor in other heterodimers. Thus we decided to use LXR-RXR heterodimers where LXR, similarly to RAR, binds co-repressor strongly and repress basal transcription, and the presence of RXR attenuates LXR's co-repressor binding thus relieves basal repression [73]. We co-transfected Gal-LXR-LBD and RXR-LBD or RXR-ΔH12-LBD and treated the heterodimers with stable amount of LXR ligand (T09) and increasing amount of LG268 (Fig.4.25.). We found that the mutant heterodimer gave the same subordinated response upon ligand treatment as it was found in the case of the RAR-RXR heterodimer. These results together show that changing the equilibrium of co-repressor and co- activator binding by the deletion of RXR-H12 effects also the partner receptor and leads to the subordination of RXR partner receptors.

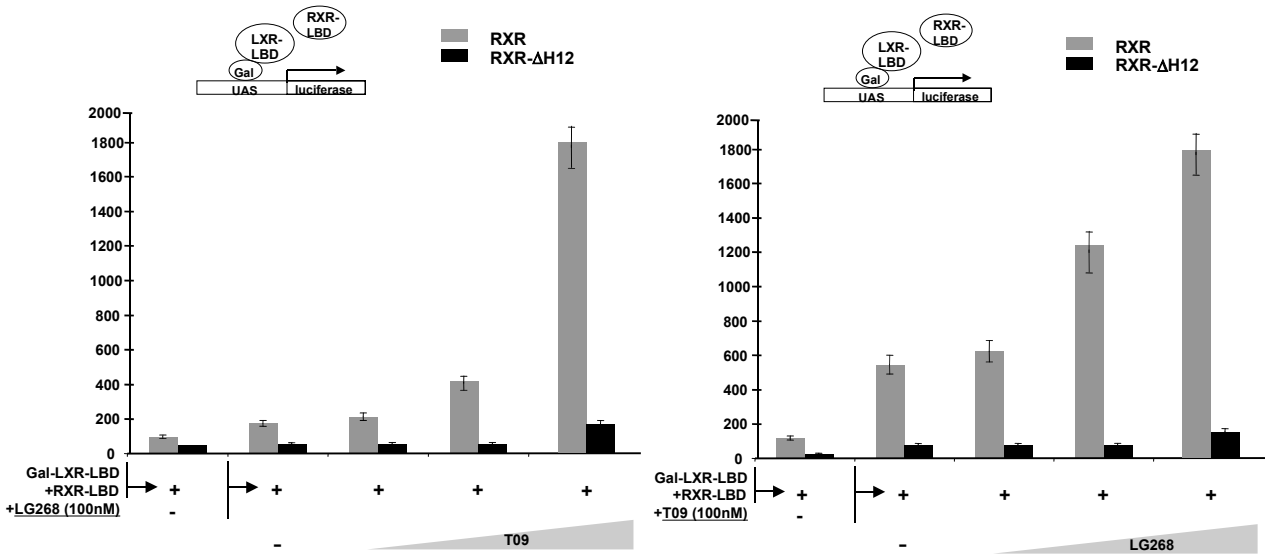


Figure 4.25. Effect of RXR-H12 deletion on LXR-RXR heterodimer transactivation. Transient co-transfection assays were carried out with the indicated plasmid constructs. Dose-response curves of Gal-LXR-LBD: RXR-LBD or RXR-ΔH12-LBD heterodimers upon treatment with (A) fixed LG268 and increasing LXR specific ligand, T09 or (B) with fixed T09 and increasing LG268.

4.16. *In vivo* studies of RXR mutants

We were curious about whether the effect of mutant RXR can be seen in a living system, thus we decided to carry out some *in vivo* studies about the effect of RXR-H12 deletion. Using lentiviral transduction system we established a stable cell line that over-expresses our mutant RXR. We used MonoMac6 (monocyte) cell line since the nuclear receptor levels and several target genes of them are well characterized by our groups. Using Q-PCR technique we determined the mRNA level of RXR before and after infection and we found that the level of RXR was approximately 5 fold increased in the infected cell line compared to the wild type cells (Fig.4.26.).

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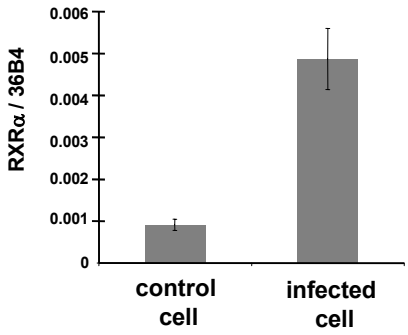

Figure 4.26. Q-PCR measurements of mRNA level of RXR. The mRNA level of MonoMac6 cell line was determined before infection (control) and after infection (infected) and was normalized for 36B4 as reference.

Then we treated the control and the infected cell lines for 48 hrs with stable amount of LXR ligand (T09) and increasing amount of LG268 or with stable amount of T09 and increasing amount of LG268 (Fig.4.27.)..

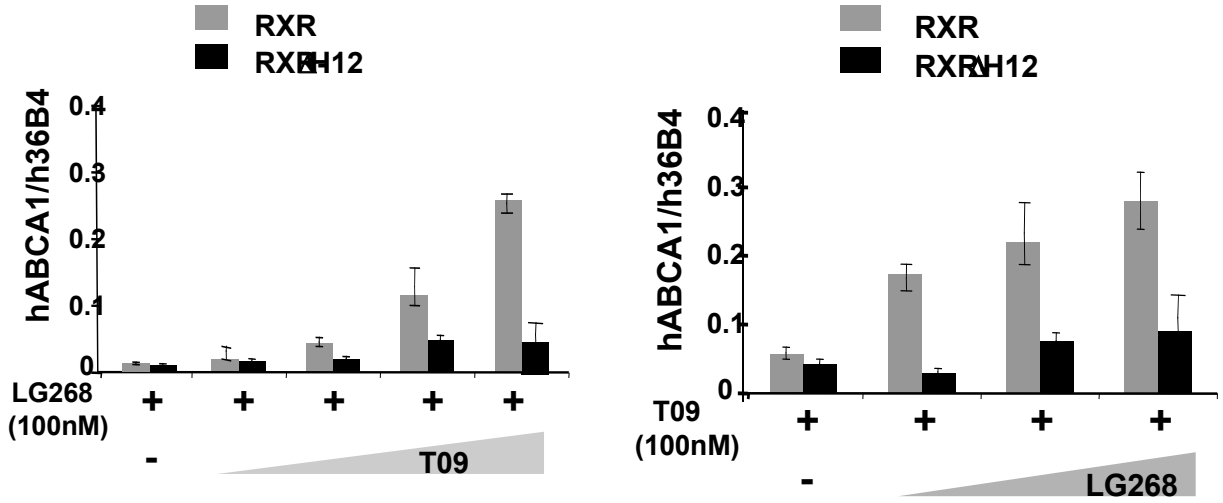

Figure 4.27. Q-PCR measurements of mRNA level of ABCA1 gene. Infected MonoMac6 cell lines were treated for 48hrs with the indicated ligands. Then the mRNA level of ABCA1, as LXR:RXR target gene was determined and normalized for h36B4 as reference.

We found that the mutant heterodimer gave the very similar subordinated response upon ligand treatment as it was found in the cotransfection studies These results indicate that the effect of H12 truncation is the same in *in vivo* as previously detected in *in vitro* experiments.

5. DISCUSSION

Transcriptional activation by nuclear receptors depends critically upon the balance of co-regulator recruitment. In the cellular environment multiple co-repressor and co-activator proteins are available to interact with nuclear receptors. It is the balance between the two that determines the transcriptional outcome. This balance is specific for each receptor and is controlled both by ligand, by the availability and biochemical characteristics of specific co-regulator and also by the heterodimerizing partners [160].

It has been known for a long time that heterodimerization with RXR differentially affects ligand-induced activity of the two involved partner. While the partner of RXR in a heterodimer is usually able to activate, RXR seems to depend on the nature of its partner. In an RAR:RXR heterodimer RAR clearly plays the major role in defining the transcriptional property by directly mediating repression and activation through controlling co-repressor and co-activator binding. This observation has led to the proposition of a silent partner role for RXR in these so called non-permissive heterodimers, where it is proposed that the allosteric control by RAR somehow prevents RXR from ligand binding [198]. Since a number of studies over the last several years have provided evidence that RXR in a RAR/RXR (and also in TR-RXR) heterodimer can bind its ligand [110], it rises the possibility that RAR controls the activity of RAR-RXR heterodimer in some other ways, perhaps allosterically influence the proper positioning of RXR-H12 and to adopt its active structure.

Structural studies providing a static view of both co-activator and co-repressor complexes with various nuclear receptors revealed that in the active structure H12 is required to be in a position that facilitates co-activator binding and conversely that co-repressor binding requires that H12 to be displaced from the active position. Together these studies combined with biochemical analyses also suggest that the two types of co-regulator bind in a very similar fashion to a conserved surface on the receptor ligand-binding domain. Xu et al. provided the first structural evidence that the co-repressor and co-activator binding sites are indeed largely overlapping [206].

While these studies have been revealing, they leave unanswered the question of what determines the basal activity of different receptors and what are the intrinsic determinants of co-regulator binding balance. Why do some receptors (such as TR and RAR) strongly repress transcription in the absence of ligand, others behave neutral (like RXR) and again others (such as PPARγ) show a basal transcriptional activity? Also, thought there have been several

attempts to model the behaviour and function of RXR in different heterodimers, its role and its effect on the co-regulator binding has not been characterized [161].

During my PhD studies, using molecular biological methods we aimed to study RAR-RXR heterodimer from both the RAR and RXR side. Using RARs that harbours point mutations and H12 deleted RXR we tried to characterise in more detail the factors that influence the co-regulator binding and exchange of RAR-RXR heterodimer, and find correlations between co-regulator binding and activation abilities of these receptors.

5.1. Identification of a passive docking site for co-regulator binding on RAR

With mutagenesis approach we found that mutations in helices 3 and 4 of RAR-LBD disrupt both co-activator and co-repressor binding. Significantly, the majority of mutations made on this co-factor binding surface of the receptor perturbed both co-activator and co-repressor binding. This clearly indicates that the binding sites are largely overlapping.

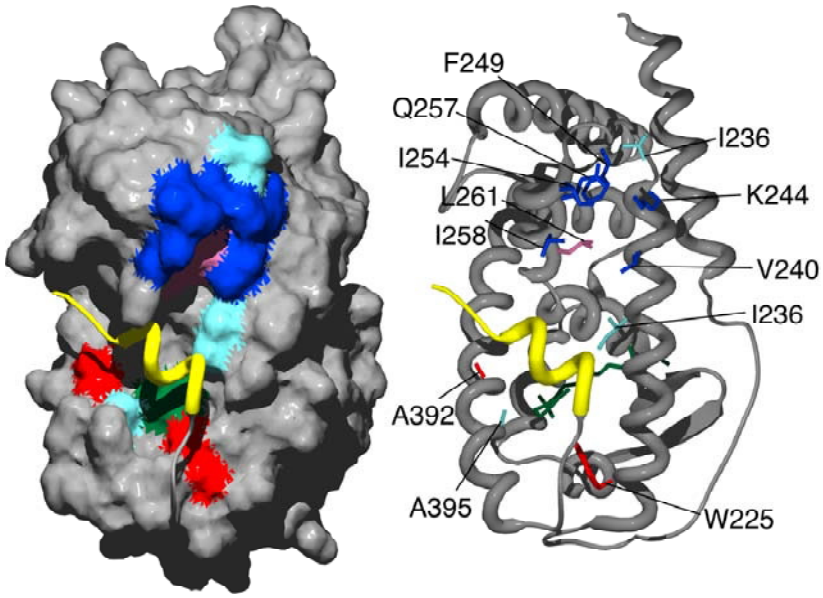

Figure 5.1. Binding sites for co-regulators. Surface and ribbon representation of the RARγ-LBD. Mutated residues on H3, H4, H5 and H11 are coloured according to their effect on co-regulator binding. *Blue*: impaired binding of co-activator and co-repressor; *cyan:* impaired binding of co-repressor only; *magenta* impaired binding of co-activator only; *red* residues that differentially influence co-regulator binding. H12 is shown in *yellow*.

Parallel with our studies there were other groups using mutagenesis approach to study different receptors (TR, RXR, PPAR) for co-repressor binding [72,122,133,142,195,206]. Moreover, there was the first crystal structure published in 2002 showing antagonist bound PPARγ with co-repressor peptide [206]. All of these findings support our results and conclude that the binding sites for co-repressor and co-activator are in overlapping surfaces on the LBD of these receptors. It appears therefore, that while the RAR-LBD interacts preferentially with SMRT ID-2 (rather than ID-1 like other tested receptors), the interaction is analogous to that between SMRT-ID-1 and other receptors.

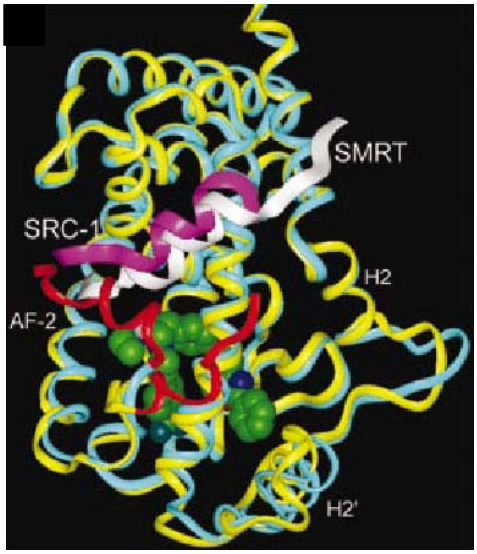

Figure 5.2. Structure of PPARα with co-repressor and co-activator peptide. Overlay of the PPARα-GW6471-SMRT and the PPARα-GW409544-SRC-1 complexes. PPARα-LBD is yellow in SMRT complex and blue in SRC-1 complex; SRC-1 is purple; SMRT is white. Figure taken from Xu et al., 2002 [206].

However a number of residues were found to selectively perturb co-repressor binding (I236, T250 &V395). In contrast, mutation of L261 reduced only co-activator binding without influencing co-repressor interaction. It is important to note however that these differences do not translate into substantive differences in transcriptional activity, and suggests that any mutation of this surface leads to transcriptionally inert receptor even if the co-repressors and co-activators are not evenly affected (i.e. V395A Figure 5). (Fig. 5.1.)

5.2. Residues that determine the basal activity of the RAR

While the majority of mutations resulted in only modest changes to the balance between co-repressor and co-activator binding, a second class of mutations lead to dramatic differences with respect to the different co-factors. It is striking that these mutations are not located in the proposed docking site but further away in the proximity of H12. Mutation of W225 (in helix 3) to alanine results in a mutant with intrinsically high affinity for co-repressor binding combined with very low co-activator binding. This mutation significantly tips the

balance towards co-repressor binding with almost total loss of co-activator binding. Analysis of the transcriptional activity of this receptor shows that, whilst it is still able to bind to ligand, it acts as a constitutive repressor of transcription. Analysis of the structure of the homologus RARγ shows that W225 is in van der Waals contact with L398 (4.1Å), I402 (3.6 Å), M406 (3.5 Å) and P407 (3.9 Å) [22,152]. Mutating this side chain to alanine would remove these favourable contacts to the loop between helices 11 and 12 and would likely destabilize the active position of H12 (Figure 5.3.). This would in turn favour co-repressor interaction with the mutant receptor and reduce the efficiency of release on binding ligand. Significantly the effects and mechanism of this mutation are similar to those of mutations in PPARγ that result in a receptor with dominant negative repression activity [13] that have been explicitly shown to destabilize H12 [81]. It should be noted that W225 makes only long range van der Waals contacts to the ligand, consistent with the observation that the W225A mutant retains ligand-binding ability (data not shown).

A second mutant that substantially changes the basal activity of RARα is A392R. This receptor shows minimal co-repressor binding in conjunction with significantly increased co-activator binding activity. Again the ligand binding affinity remains unchanged (data not shown). As expected, this receptor proved to be constitutively active, indicating that ligand binding *per se* is not required for transactivation, provided that there is an intrinsic ability to bind co-activator. Examination of the RARγ structure shows that the larger Arg side-chain can be readily accommodated at this position and in the model illustrated in Figure 5.3.B it is clear that the arginine would be able to make a number of favourable contacts to residues in helices 4 and 12. These include van der Waals contacts to L266 (3.3 Å) L414 (4.2 Å) as well as hydrogen bonds to the side-chain of N416 in H12. Consistent with the observed activity of this mutant, the A392R mutation is likely to stabilize the active conformation of H12 in the absence of ligand and hence promote co-activator binding, which absolutely requires h12 in the active position.

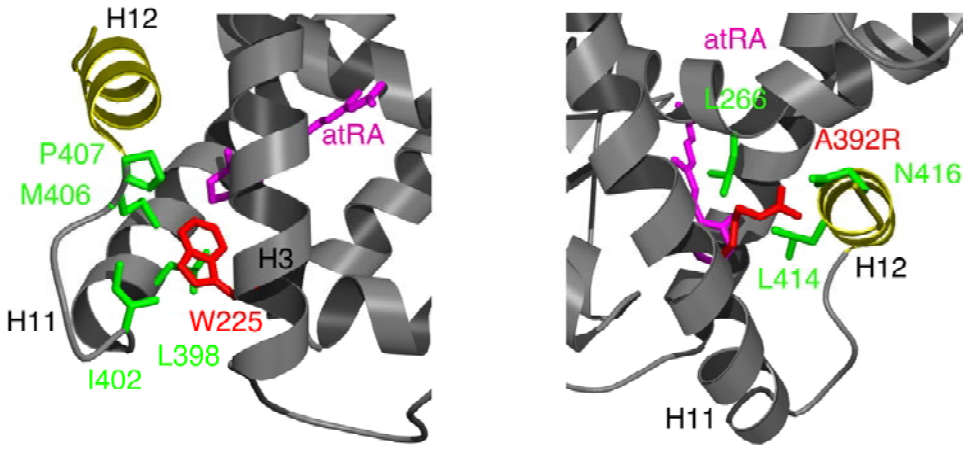

Figure 5.3. Residues W225 and A392 that may influence the orientation of H12.
A, Trp-225 (*red*) is in Van der Waals contact with several residues (*green*) in the loop between helices 11 (*H11*) and 12. Mutation of this residue to alanine may disfavor the active position of H12 (*yellow*). The ligand ATRA is shown in *magenta*. The structure shown is that of RARg-LBD (Protein Data Bank code 2LBD). *B*, mutation of Ala-392 to arginine (*red*) may stabilize the active position of H12 (*yellow*) through contacts to H12. *atRA*, all-*trans*-retinoic acid.

Significantly, in PPARγ the residue in this position is a valine. This sidechain makes multiple van der Waals contacts to residues in H12 of PPARγ (Y473, L476 and Y477) and thus stabilizes the active conformation of H12 in this receptor. This correlates well with the observation that PPARγ does not repress transcription in the absence of ligand, but rather exhibits a high basal transcriptional activity. As mentioned earlier this native behaviour of PPARγ closely matches the behavior of the A392R mutant RARα. Together these findings establish that intrinsic H12 positioning is a major determinant of co-regulator binding equilibrium.

5.3. RXR attenuates co-repressor binding of RAR and this requires RXR-H12

It is known from our (data not shown here) and others results that in contrast with several nuclear receptors (like TR, PPAR, LXR, VDR, RXR) that show specific SMRT-ID-1 binding, RAR seems to be unique with the characteristic that it shows clear specificity for SMRT-ID-2. In this way RAR-RXR heterodimer seems to be an ideal system to study co-repressor binding since each partner binds different domains of the co-repressor in contrast with heterodimers like LXR-RXR or TR-RXR where neither the function of SMRT-ID-2 nor the receptor dominancy for SMRT-ID-1 is not known. Although we would expect additive binding of the co-repressor IDs to the RAR-RXR, the results that show attenuated co-repressor binding of RAR-LBD in the presence of RXR-LBD enhance the hypothesis that there is an allosteric communication between the receptors and they have some kind of

cooperativity in the co-regulator binding (ref). Although it is known from structural studies and surface mapping that heterodimerization and co-repressor binding requires distinct surfaces of the receptor molecule, it is also believed that in non-permissive heterodimers like RAR-RXR one receptor can influence its partner's co-factor binding. Our results show that attenuation of RAR-co-repressor interaction requires H12 of RXR, thus it supports the idea that a possible candidate for mediating the cooperativity between the heterodimers is the H12 (of RXR) [216].

5.4. RXR-H12 does not compete for binding site with co-repressor on RAR surface

There are reports showing that the nature and liganded status of the dimerization partner might control the activity of H12 of RXR. According to this "silencing" model in the apo-RAR-RXR heterodimer RXR is unable to activate due to the allosteric inhibition of RXR-H12 positioning by RAR. This could be, at least in part, via the interaction of the RXR H12 with the co-activator-binding pocket of its dimeric partner. Crystal structure of receptor dimers indicates that the H12 of an apo receptor can interact with the LBD of a second receptor. Allosteric inhibition of RXR appears to result from a rotation of the RXR H12 that places it in contact with the RAR co-activator-binding site.

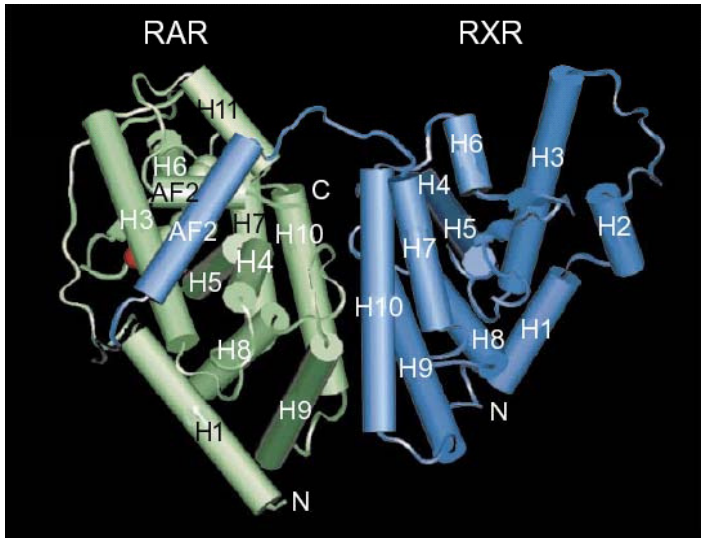

Figure 5.4. Model of a heterodimer of the RAR and RXR ligand-binding domains.
H12 of RXR can dock into the activation surface of the RAR ligand-binding domain. The coordinates of RXRα shown in blue, and RARγ shown in green, were superimposed. Figure taken from Westin et al., 1998 [198].

The binding of an RAR-ligand positions RAR-H12 in its active position and in the meantime releases RXR-H12 that is able/free to take up an active conformation upon binding of an RXR-ligand. If we assume that co-repressor and co-activator bind to overlapping sites

on the RAR-LBD it might be possible that RXR-H12 competes for binding sites with the co-repressor on the apo-RAR-LBD and this would be an explanation for the subordination that we observed in the presence of RXR. However, our results show that in the context of SMRT-ID-1+2 the deletion of RXR-H12 abolishes subordination, it does not influence ID-2 RAR interaction, but prefers increase ID-1 RXR interaction. These results mean that RXR-H12 does not compete for binding site with co-repressor. We have to note that this does not exclude the allosteric inhibition of RAR since it is still possible that RXR-H12 binds to other area of the RAR-LBD.

5.5. Deletion of RXR-H12 might open a cryptic binding site for co-repressor

There have been several attempts made over the years to build a model and explain the role of RXR and its H12 in co-regulator binding. According to the model of Lazar and his colleagues it is proposed that RXR weakly binds co-repressor since H12 sterically inhibits the binding. There appears to be at least two ways to make RXR bind co-repressor. One is to heterodimerize with receptors like RAR or TR that repositions RXR-H12 thus unmask the co-repressor binding sites on the surface of RXR-LBD. The other is to delete RXR-H12 that again opens a binding site for co-repressor [216]. Thus these results hypothesises that the two different conditions open the same co-repressor binding sites. However, from our results it seems that the co-repressor binding of the H12 deleted RXR and the heterodimerized RXR (where the H12 is probably repositioned) is different. This rise the possibility that deletion of H12 opens a cryptic binding site that although enables co-repressor to bind but this site is different from the site that was obtained with repositioning of H12 *via* heterodimerization.

5.6. RXR-H12 is not required for LG268 induced co-repressor release from RXR

It was earlier mentioned that previous studies already revealed that co-activator binding requires that H12 be in the active position and conversely that co-repressor binding requires that H12 to be displayed from the active position. There are arguments about that H12 is also important in facilitating co-repressor release as a result of hormone induced allosteric changes [11,113]. In this point of view it seems that LG268-bound RXR represents an exception among nuclear receptors since it was shown in structural studies that in the hRXRβ bound to the synthetic agonist LG268, H12 does not adopt the active conformation [117]. This result was further supported with mammalian two hybrid experiments that showed

that the efficient displacement of co-repressor by H12 requires the presence of both ligand and co-activator.

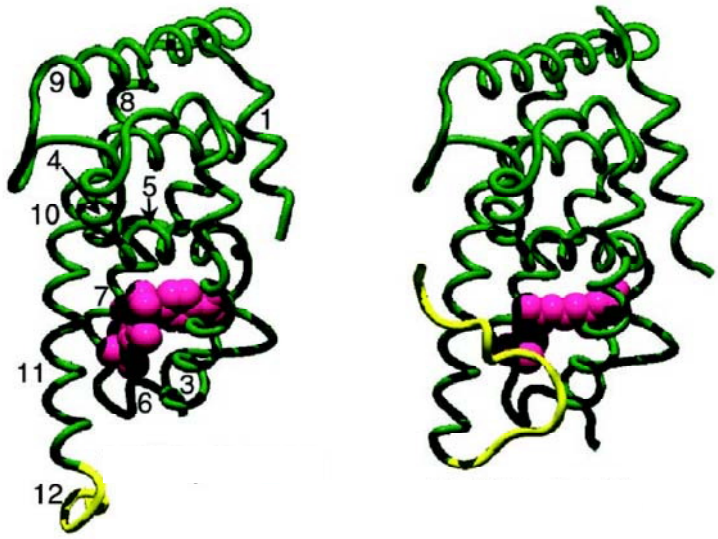

Figure 5.5. Comparison of hRXRβ-LG268 with hRXRα structures. *Left:* structure of hRXRβ with LG268. H12 is shown in yellow. *Right*: structure of hRXRα with 9-cis RA. Figure taken from Love et al., JBC, 2002 [117].

Together these mean that while in the case of RAR its liganded state clearly defines its co-repressor and co-activator binding property, thus its activation or repression, in the case of RXR there is a competition between the co-repressors and co-regulators for binding, and the amount and availability of co-regulators (that show tissue- and/or promoter-dependency) also the affinity of the different complexes for RXR together determines the balance of co-regulator binding and the activity of the receptor.

With these pieces of information it was surprising that the mutant form of RXR, that does not contain its H12 and shows strong interaction with co-repressor-ID-1, is able to release co-repressor upon ligand binding. The comparison of the effects of other RXR ligands shows that this effect is not characteristic for synthetic agonist (LG268) but natural agonist (9-cisRA) and synthetic antagonist (LG1208) also posses this ability. Although position of H12 in the agonist (ATRA) bound structure of RAR-LBD and 9-cisRA bound RXR shows the same positioning, it is striking that in the case of the H12 deleted RAR we did not observed ligand induced changes. Similarly to RXR-SMRT-ID-1 interaction the SMRT-ID-2 interaction with RAR-dH12 was increased, the RAR ligand was not able to release co-repressor binding.

5.7. Deletion of RXR-H12 changes the equilibrium of co-factor binding by RAR-RXR heterodimer and results in the subordination of RAR response

The central role of H12 in the regulation of co-regulator balance is further supported with the results that show impaired co-activator binding of the heterodimer containing the mutant RXR. On the other hand it is surprising that this effect was detected even upon RAR specific ligand treatment that means that in RAR-RXR heterodimers the functional RXR-H12 is required for efficient co-activator binding of liganded RAR side.

Taken together the co-regulator binding results that show increased co-repressor and decreased co-activator binding we can say that deletion of RXR-H12 is likely to change the equilibrium of co-repressor – co-activator binding of RAR-RXR heterodimer. This balance is pushed to the direction where co-repressor binding becomes more dominant over co-activator binding. Furthermore as our results show this changed dominancy is basically mediated through the ID-1-RXR interaction via a cryptic binding site for co-repressor binding.

5.8. Relatively small changes in co-regulator binding translate into large changes in transcriptional activity

It is clear from the analyses of RAR- mutants and RXR-H12 deleted mutant receptors that the determination of co-factor binding activity by mammalian two-hybrid analysis has a high predictive value on the transcriptional activity of the receptor. It is particularly striking that relatively small changes in the co-regulator binding potential translates into a large shift in transcriptional activity (Figure 5). In this respect the LBD appears to function as a biological amplifier, where small structural changes affecting co-factor affinity result in significant biological consequences (i.e. a constitutively active activator or repressor). This notion further underscores the significance and power of the intrinsic regulation of co-regulator balance.

5.9. A critical role for H12 in determining co-factor equilibrium

It is clear that structural studies by themselves do not explain the mechanism of co-regulator exchange by NRs. The results of biochemical and biophysical studies strongly support the assumption that ligand binding globally stabilizes the LBD that results in a more compact and rigid structure, and also leads to the active position of H12 [146]. However, it is also shown that the lower portion of the LBD is less rigid than the upper portion (Fig.5.6.), which means that the lower portion of the receptor is rather dynamic. This is especially true

for H12 that shows fast, independent and segmential motion [81,176]. The different NRs are likely to exhibit different degrees of dynamic abilities and this serves a good explanation for their different basal transcriptional activity. LRH-1 and RAR are good examples for these correlations. LRH-1 has constitutive activity and the crytsallization of the receptor shows stable structures in the absence of ligand, with the H12 being in the active position [156].

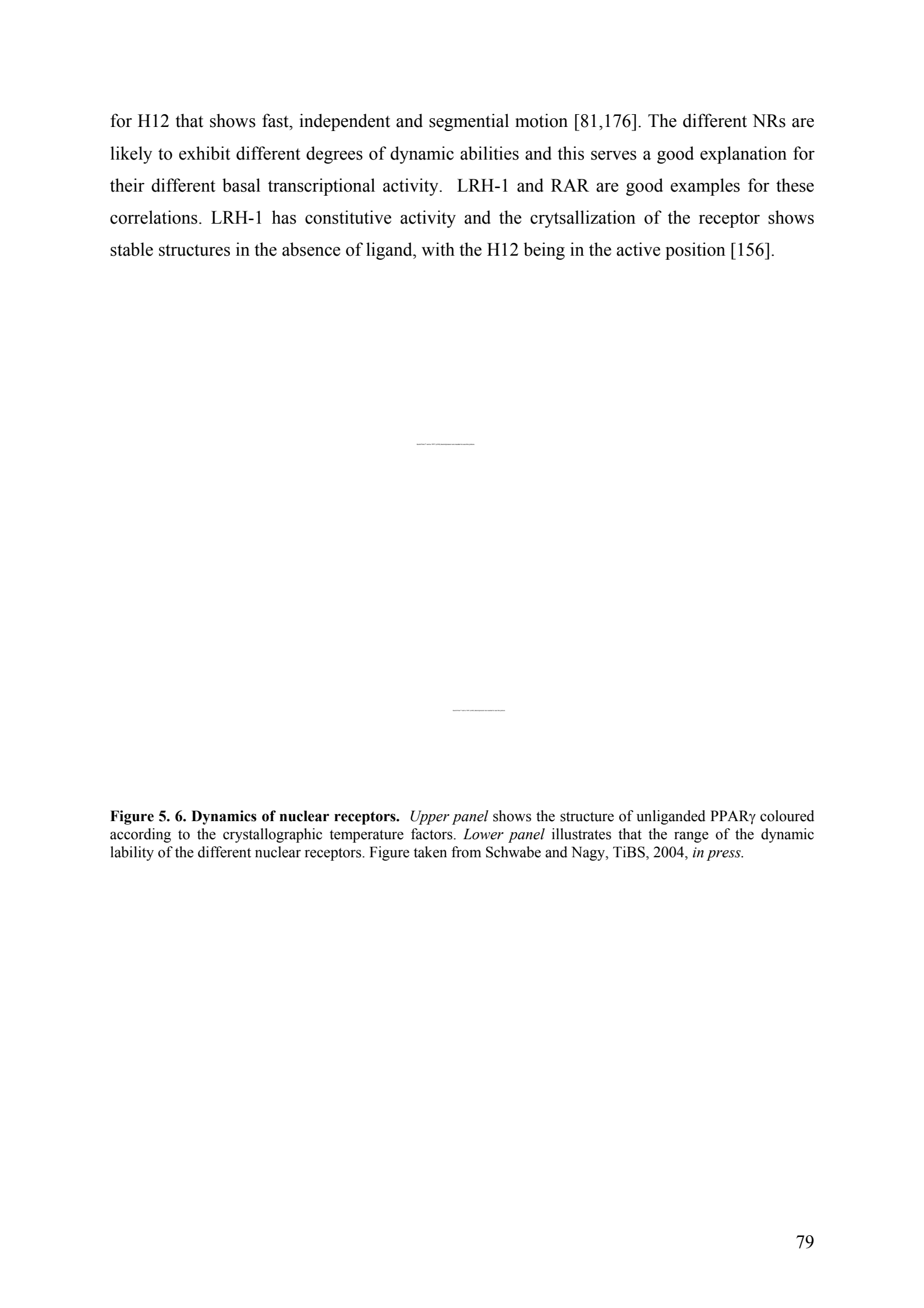

Figure 5. 6. Dynamics of nuclear receptors. *Upper panel* shows the structure of unliganded PPARγ coloured according to the crystallographic temperature factors. *Lower panel* illustrates that the range of the dynamic lability of the different nuclear receptors. Figure taken from Schwabe and Nagy, TiBS, 2004, *in press.*

On the other hand TR and RARα are strong repressors in the absence of ligand and crystallization attempts without ligand have been unsuccessful, consistent with the findings that they exhibit low melting temperature. PPARγ adopts an intermediate position between these extremes with a rather high basal activity, but still with the ability to be activated by ligand [79].

In conclusion, these studies contribute to the emerging view that the positioning, dynamics and stability of the position of H12 have a significant role in regulating transcriptional activity of nuclear receptors. It has been shown previously that H12 is required for transactivation [206], it contributes to the binding surface for co-activators [142] and deletion or mutation of H12 results in dominant negative receptors with increased co-repressor binding potential [13,80]. Our studies demonstrate that residues that influence the stability of the active position of H12 control the balance of the equilibrium between co-repressor and co-activator binding. These findings also explain the differences in basal activities between the various nuclear receptors and clearly have implications for our understanding of the evolution of nuclear receptors with rather diverse properties. Moreover, these results can be further exploited to design mutant receptors with a much wider range of altered transcriptional activity than was previously suspected.

6. SUMMARY

The regulation of gene expression by transcriptional control is required for many cellular events for the proper development of any organism. The biochemical and structural studies on nuclear receptors and their co-regulators have given us a better understanding of the mechanisms of their behaviour. The obtained information made clear that nuclear receptor activity requires a complex allosteric interaction between all four LBD function including dimerization, ligand binding, co-regulator binding and H12 positioning.

In my thesis work we aimed at to study the co-regulator binding of RAR and RXR receptors, the factors that determine and influence it, also we tried to find correlations between co-regulator binding and transcriptional activity of these receptors. During these studies I had the possibility to use several modern, molecular biological and biochemical methods. Utilizing these techniques (1) we determined the co-repressor binding area on the surface of RAR-LBD. Comparing the site for co-repressor binding with the site for co-activator binding (2) we found that these co-regulators bind to overlapping surfaces on the RAR-LBD. Despite the docking site for co-regulators (3) we also found residues that influence co-regulator binding through mediating the positioning of H12. Using H12 deletion mutant of RXR (4) we found that RXR attenuates the partner's co-repressor binding and this requires RXR-H12. (5) We also found that deletion of RXR-H12 changes the equilibrium of co-regulator binding and (6) this results in the subrodination of partner receptor response.

In conclusion, these studies contribute to the emerging view that the positioning, dynamics and stability of the position of H12 have a significant role in regulating transcriptional activity of nuclear receptors. Our results also confirm with the previous findings that show that H12 is required for transactivation [131], it contributes to the binding surface for co-activators and deletion or mutation of H12 results in dominant negative receptors with increased co-repressor binding potential. Our studies demonstrate that residues that influence the stability of the active position of H12 control the balance of the equilibrium between co-repressor and co-activator binding. Moreover, these results can be further exploited to design mutant receptors with a much wider range of altered transcriptional activity than was previously suspected.

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1. Love, J.D., Gooch, J.T., **Benko, S.**, Li, C., Nagy, L., Chatterjee, V.K., Evans, R.M., Schwabe, J.W.: The structural basis for the specificity of retinoid-X receptor-selective agonists: new insights into the role of helix H12.
   J Biol Chem., 277(**13**): 11385-91 (2002)

2. **Benko, S.**, Love, J.D., Beladi, M., Schwabe, J.W., Nagy, L.: Molecular determinants of the balance between co-repressor and co-activator recruitment to the retinoic acid receptor
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3. **Benko, S.**, Oros, M., Schwabe, J.W., Nagy, L.: Role of RXR-H12 in the equilibrium of coregulator binding and receptor activation
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Other publicatons:

1. Szanto, A., **Benko, S.**, Szatmari, I., Balint, B.L., Furtos, I., Rühl, R., Molnar, S., Csiba, L., Garuti, R., Calandra, S., Larsson, H., Diczfalusy, U., Nagy, L.:
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