THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D)

Molecular Determinants of Co-regulator Binding and Transcriptional Activity of Retinoic Acid Receptor - Retinoid X Receptor Heterodimers

Szilvia Benkő

Supervisor:
Laszlo Nagy, M.D., Ph.D

University of Debrecen
Medical and Health Science
Department of Biochemistry and Molecular Biology
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1. INTRODUCTION

1.1. Nuclear receptors as transcription factors

In a multicellular organism the different cell types differ dramatically in both their shape and function. The reason of this difference is that they synthesize and accumulate different sets of protein molecules without altering the sequence of their DNA. Although there are many steps in the pathways leading from DNA to protein and all of these steps can be regulated, one of the most important points of control is the initiation of gene expression.

The eukaryotic genome is structurally organized into nucleosomes to form chromatin, which regulates gene expression, in part, by controlling the accessibility of regulatory factors. When packaged into 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 important characteristic that distinguishes NRs from other transcription factors is that they are able to bind small, lipophilic ligands, mainly hormones or metabolites.

The study of steroid hormones started centuries ago, but scientists started focusing on the nuclear receptors that mediate effects of these molecules only in the middle of the 1900s. The important role of lipophilic hormones and metabolites in human health led to the fast increase of the knowledge on the role, structure, the interacting and regulatory proteins of nuclear receptors, as well as about the ligands that influence their function and the target genes and pathways that they influence.

Today we know that the nuclear receptor superfamily is a large group of molecules that have sequence, structural and functional relationships and members of this family can be grouped into 6 subfamilies according to their sequence alignment and phylogeny. They are universal among the metazoans but different species possess different number of nuclear receptor genes. They can also be grouped according to the source and characteristics of the ligand they bind. These ligands can be agonists or

antagonists and beside the discovery of natural ligands the number of the synthetic ligands is also increasing dynamically. Nuclear receptors bind to consensus sequences on their target gene promoter as monomers, homodimers or heterodimers.

Nuclear receptors mediate their effect by recruiting coregulator molecules, depending on the absence or the presence of their ligands. Generally, in the absence of ligand they bind corepressor molecules that recruit complexes that contain proteins with histone deacetylase (HDAC) activity. The activity of HDACs results in hypoacetylated chromatin that is assumed to be transcriptionally silent. In contrast, in the presence of agonistic ligand, nuclear receptors bind co-activator protein that either themselves posses intrinsic histone acetyltransferase activity (HAT) or recruit proteins harbouring such activity. The function of these molecules lead to the hyperacetylation of regions of chromatin and thus becomes permissive for transcription initiation.

1.2. Retinoids and their receptors

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 morphogenesis, organogenesis, growth, cellular differentiation and homeostasis. For a long time the physiological functions of retinoids were mainly inferred from studies on vitamin A deficient (VAD) animals. Since the discovery of a retinoic acid receptor (RARα1) in 1987 several other combinatorial effects emerged that still broadens our molecular understanding how retinoids could exert their pleiotropic effects.

1.2.2. Retinoid receptors. The retinoic acids exert their multiple effects through two classes of NRs, the retinoid X receptor (RXRs) and retinoic acid receptors (RARs). Each of these two classes consists of three isotypes (α, β, γ). Their distinct spatiotemporal expression pattern in the developing embryo and various adult tissues enhance the combinatorial effect of their function.

1.2.2. Retinoids. From the several forms of natural retinoic acids, the all-trans retinoic acid (ATRA) and 9-cis retinoic acid (9-cisRA) appear to exert the most important

effects among these ligands. While 9-cisRA seems to be the most potent ligand for RXR, RAR can be activated with both ATRA and 9-cisRA. Although retinoids are widely used therapeutic agents, their teratogenic and toxic effect led to the development of synthetic, receptor selective ligands such as LG268, AM580, TTNPB. The synthesis and the metabolism of the ligands can be cell-specifically modulated, thus they represent an additional level of control and complexity.

1.2.3. RXR heterodimers. Among nuclear receptors RXR has a unique role since it is able to form heterodimers with other members of the superfamily. Based on the ligand induced activity of these heterodimers they can be devided into non-permissive heterodimers (that activation requires the presence of the ligand of the partner receptor) and permissive heterodimers (that can be activated only by RXR ligand and the activation does not require the presence of the partner receptor's ligand).

1.2.4.Regulator molecules. As many other NRs, RARs and RXRs also interact with multiple putative coregulators that also can have cell and tissue specific expression and distribution, increasing the combinatorial effects that underlie the pleiotropic effects of retinoids.

1.3. Structure and function of nuclear hormone receptor domains

All nuclear receptors consist of structurally and functionally different regions/domains. The N-terminal, variable A/B region has a ligand-independent activation function (AF-1). It is likely to be responsible for mediating isoform-specific responses. The DNA-binding domain (DBD) is the most conserved region. It comprises two zinc fingers. This domain is responsible for high-affinity DNA-binding, for the recognition of specific hormone response elements (HREs) of the target gene, and it also serves as a weak dimerization site. The hinge region serves as a connection between the DBD and Ligand-binding domain (LBD), allowing rotation of the DBD. The LBD of all NRs shows a canonical, three-layer antiparallel α-helical sandwich comprising 11-13 α-helices. The C-terminal helix 12 (H12) possess ligand-dependent activation function (AF-2) and can adopt multiple conformations, depending on the nature of the bound ligand. This motif is conserved in most members of the NR superfamily.

1.4. The function of the ligand-binding domain (LBD)

The ligand-binding domain (LBD) is a multifunctional domain possessing four main functional regions. One of the key regions of the LBD comprises a strong dimerization interface. It also posses the ligand-binding pocket (LBP) that has evolved remarkably down to the single-residue level to recognize and bind specific ligands. It varies greatly in size, from 0 (nil)(Nurr1) to 1400Å (PPAR). The specificity of ligand binding is also determined by the shape of the ligand-binding cavity and by the hydrophobic / hydrophilic nature of the pocket surface. The highly conserved subregion within the distal C-terminus of the LBD, termed the AF-2 domain or H12 harbours the ability of ligand-dependent transcriptional activation. The fourth functional region is the hydrophobic cleft on the surface of the LBD that serves as binding site for co-regulator (co-activators and co-repressor) proteins. The precise positioning of the AF2 helix, which is controlled by the binding of ligands to the LBD, determines the binding of the specific co-regulator molecule.

1.5. Co-regulators

Nuclear receptor coregulators 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.

1.5.1. DRIP205 / TRAP220 as co-activators. The DRIP/TRAP complex was initially identified in separate biochemical screens for proteins recruited by NRs in a ligand dependent manner. 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) via a receptor interacting motif (ID, LXXLL-motif,) identical to that found in other coactivator molecules.

1.5.2. SMRT/NcoR as co-repressors. Since many components of the 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), that are related both structurally and functionally, were isolated by a yeast two-hybrid screening of a human lymphocyte cDNA library with unliganded NRs. SMRT have also been implicated as co.repressor for a variety of unrelated transcription factors, which regulate cellular processes.

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 with LXXLL consensus sequence. Similar interaction domains were identified for co.repressors, but analysis of IDs revealed that the L-X-X-X-I-X-X-X-I/L motif of co.repressors is predicted to form an extended, one helical turn longer α-helix than the co.activator motif. Analysis of the LXXLL motifs has revealed that in the amphipatic α-helices that they form the leucines creates a hydrophobic surface on one face of the helix. These motifs are critical for assembling the ligand-dependent nuclear receptor – co.regulator complexes.

1.6. Determinants of co.regulator-binding

1.6.1. The positioning of AF-2 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 AF-2 region undergoes a ligand-dependent conformational change. Upon agonist binding H12 helix moves as a 'mouse trap', seals the ligand binding cavity, in some cases making direct contacts with the ligand further stabilizing ligand binding. These ligand-induced structural changes result in the formation of a surface that facilitates co.activator binding.

1.6.2. The charge clamp and co-regulator exchange. From structural studies it is also known 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 motif of the co-activator. Together the glutamate and lysine residues form a charge clamp that positions the LXXLL helix. 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. On the other hand, the

position of the AF-2 helix also plays a key role in co-repressor binding. The co-repressors bind to LBD *via* the LXXLL motifs. In comparison to co-activators, the longer co-repressor motif adopts a three-turn α-helix instead of two turns for the co-activator motif. The additional turn of the co-repressor helix extends into space that would normally be occupied by the AF-2 helix when it is in the active conformation. Thus the binding of co-repressor and the active AF-2 conformation is mutually exclusive.

1.6.3. Allosteric coupling of LBD functional regions. Several structures have been determined with various LBDs in complex with an agoinst or an antagonist, some with fragments of co-activators or co-repressors. The rich information provided by these structures has made it possible to develop a global view on the molecular basis of ligand binding and ligand-mediated regulation of NRs. It appears that NR activity requires a complex allosteric interaction between all four functional surfaces of LBD. For example, ligand binding induces a conformational change in the co-regulator-binding site and in the H12 that leads to exchange of co-repressors for co-activators. 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.

The regulation of gene expression by transcriptional 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. These disorders affect every field of medicine, including reproductive biology, inflammation, cancer, diabetes, cardiovascular diseases and obesity. For this reason, characterization of the mechanism of NR behaviour in normal physiology and abnormal disease processes is one of the major goals of biomedical research.

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-factors, understanding how this different balance is achieved is of great biological significance.
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 corepressors and coactivators 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 recepors. 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 coactivators and we were interested in finding residues that differentially affect co-repressor and co-activator recruitment to RAR.

2) How RAR mutations influence basal activity of the RAR-RXR heterodimer and if these are in correlation with the altered co-regulator binding?

If we assume that the NR activity is a sum outcome of the co-regulator binding we wanted to know how the activity of the mutant receptors would correlate with the co-regulator binding they possess.

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 the 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 cotransfection 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. After 6-8 hours the cells were treated with the indicated ligands or vehicle. Cells were assayed for reporter expression 36 hours after transfection using a luciferase assay system. For the assay cells were lysd in 140 μl lysis-buffer and from the lysate luciferase and beta-galactosidase activity was determined. 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, -ID1+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 kindly provided by Dr. R. Evans.

All the mutants were generated by site directed mutagenesis using the appropriate oligonucleotide pairs. The VP-hRXRa-LBD-dH12, Gal-hRXRa-LBD-dH12 and CMX-hRXRa-LBD-dH12 expression plasmids were constructed with oligonucleotide primers that build STOP - codon in place of D443. The VP-hRARa-LBD-dH12, Gal-hRARa-LBD- dH12 and CMX-hRARa-LBD- dH12 expression plasmids were constructed with oligonucleotide primers that build a STOP - codon in place of K443. Plasmids pMDL,

RSV, CMV and PG for virus production were kindly provided by Dr. D.Trono. PG-hRXRa-FL-dH12 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, growth of bacteria, purification and detection of plasmids

Plasmids used for transient transfection and virus production were transformed into DH5α ultracompetent *E. coli* host. Bacteria containing plasmids for transient transfection were plated onto ampicillin-containing agar plates and were grown on 37 ^{0}C overnight in ampicillin-containing LB media. Bacteria containing plasmids for virus production were plated onto carbenicillin-containing agar plates and were grown on 37 ^{0}C for 24 hours in carbenicillin containing TB (Terrific Broth) media. DNA was extracted and purified using purification kits.

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. Bound proteins were eluted with 10 mM glutathione in 50 mM phosphate-buffered saline (PBS). The concentration of the eluted protein was measured according to Bradford and analyzed SDS-PAGE.

3.6. GST-pull-down.

hRARalpha-LBD mutant proteins were synthesized using a T7 Quick TNT *in vitro* Transcription/Translation Kit. The ^{35}S-labeled proteins were incubated with GST-fusion protein (bound to Glutathione-Sepharose 4B resin) in the presence or absence of TTNPB (2.5 μM), for 2 h at room temperature. After centrifugation, the beads were washed 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.

3.7. Electrophoretic mobility shift assays.

Full-length hRARalpha and hRXRalpha receptors were produced using T7 Quick TNT in vitro Transcription/Translation Kit. 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 labelled probe was incubated with the receptors and bacterially expressed SMRT protein in binding buffer 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 buffer at 4 ^{0}C at 30 mA. The gel was then dried and visualized using an image plate scanner.

3.8. Virus production and infection of MonoMac6 cell line

For virus production we used 293T fibroblast cells were used. 293T cells were transfected with the four plasmids responsible for virus generation, package and integration. 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. The pellet containing the virus was resuspended in buffer and freezed in aliquots at –70 ^{o}C. The number of virus particles was determined by a p24 Elisa kit. 10^5 MonoMac6 cell were infected with virus and cells were grown 10-14 days 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 AND DISCUSSION

4.1. Mutational analysis of RAR-LBD co-repressor interactions and identification of a passive docking site for co-repressor binding on RAR

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). Several of these mutants have been analyzed in previous studies and served as references in our analysis.
Since RAR functions as a heterodimeric complex with 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 we re-screened the RAR-LBD mutants in the heterodimer assay system.

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 protein and radiolabelled RAR-LBD. In this assay we found that the mutants behaved essentially identically to the mammalian two-hybrid assay.

Altogether these analyses are consistent with previous studies on other receptors which show that the primary co-repressor binding site on RAR is a hydrophobic groove between helices 3 and 4.

4.2. The binding site for co-activator on RAR is identical with the site for co-repressor 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. Some of the mutants (S229A, T233A, I236A, G248A, T250A, C265A, L266A and V395A) behaved like wild type while W225A, V240A, K244A, F249A, I254A, Q257A, I258A and L261A showed reduced co-activator binding.

Comparing the results with the co-repressor binding results 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.

In the meantime, parallel with our studies there were separate biochemical and crystallographic studies made by independent reseach groups on other nuclear receptors (like TR, RXR, PPAR). The results of these works further support our findings about the overlapping binding sites

4.3. 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 helix 12. 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. Analysis of the structure of the homologus RARγ shows that W225 is in an der Waals contact with L398 (4.1Å), I402 (3.6Å), M406 (3.5Å) and P407 (3.9Å). 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 helix 12. This would in turn favour co-repressor interaction with the mutant receptor and reduce the efficiency of release on binding ligand. This mutation significantly tips the balance towards co-repressor binding with almost total loss of co-activator binding.

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. Examination of the RARγ structure shows that the larger Arg side chain can be readily accommodated at this position and 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 (3.3Å) L414 (4.2Å) as well as hydrogen bonds to the side chain of N416 in helix 12. Consistent with the observed activity of this mutant, the A392R mutation is likely to stabilize the active conformation of helix 12 in the absence of ligand.

4.4. 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 hypothetized 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 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 compared 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. Analysis of the transcriptional activity of these receptors showed that the mutants harbouring mutation in their docking site were not able to activate. The W225A mutant that showed strong co-repressor binding even upon ligand treatment and no co-activator binding could act as a constitutive repressor of transcription. In contrast, the A392R mutant that was able to bind co-activator even in the absence of its ligand, as expected, turned out 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.

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

It is known from our and other's previous results that in the absence of ligand RXR doesn't repress basal transcription, unlike several other NRs (like RAR, TR, LXR) that show strong repression of basal transcription. To see if the co-repressor binding of RXR correlates with the basal repression, we compared the co-repressor binding of these receptors with RXR. 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 RXR.

We wanted to know how co-repressor binding is affected when it binds to a heterodimer containing two receptors with very different co-repressor binding abilities, like RAR and RXR. We found that the presence of RXR attenuates the originally strong interaction between co-repressor and RAR by 35-50%. Although we expected 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.

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).

4.6. Deletion of RXR-H12 might open a cryptic binding site for corepressor

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 RXR weakly binds co-repressor since H12 sterically inhibits the binding. There appears to be at least two ways to induce co-repressor binding of RXR. One is to heterodimerize with receptors like RAR or TR that respositions 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. Thus this suggests 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.

4.7. Co-repressor release of RXR requires the presence of both the ligand and the co-activator

Previous studies already revealed that co-activator binding requires H12 to be in the active position and conversely, co-repressor binding requires H12 to be displaced from the active position. There are also arguments for that H12 as being important in facilitating co-repressor release as a result of hormone induced allosteric. According to this 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, helix 12 does not adopt the active conformation. This result was further supported in mobility shift assay and with mammalian two hybrid experiments. We explored the role of RXR-specific ligand in the regulation of co-repressor. These experiments show that, in contrast with other receptors (like TR and RAR) that are able to release co-repressor upon ligand treatment, RXR-LBD still interacts with SMRT in the presence of its specific ligand. However, we found that co-activator was able to compete for the receptor and abolishes the interaction between the co-repressor and the RXR that showed that the efficient displacement of co-repressor by H12 requires the presence of both ligand and co-activator.

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

We wanted to know what the effect of the ligand is on the co-repressor binding in the case of the mutant RXR. We found surprisingly that LG268 treatment decreased the interaction between the co-repressor and the mutant heterodimer. These results show that

in the case of mutant RXR co-repressor release does not require H12. The comparison of the effects of other RXR ligands shows that this effect is not characteristic only for synthetic agonist (LG1268) but natural agonist (9-cisRA) and synthetic antagonist (LG1208) also posses this ability.

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

Since H12 of the receptors is required for co-activator binding, also the finding that deletion of RXR-H12 changes co-repressor binding raises the question whether beside co-repressor binding it also has an effect on co-activator binding. We found that our results 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.

4.10. Deletion of RXR/H12 results in subordinated activation of partner receptor

The changed equilibrium in co-regulator binding led us to study the activity of mutant heterodimers. We have selected two types of system to study the transcriptional activity of the receptors. We have used cotransfection studies as well as attempted to establish a stably expressing cell line for the RXR-ΔH12. Results showed that the heterodimers that contain mutant RXR had subordinated responses even upon treatment with the ligand of the partner receptor.

4.11. Relatively small changes in co-factor binding translate into large changes in transcriptional activity

It is clear from the analyses of RAR- mutant 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. In this respect the LBD appears to function as a biological amplifier, where small structural changes affecting co-regulator 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.

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 helix 12 have a significant role in regulating transcriptional activity of nuclear receptors. Our results also confirm with the previous findings that show that helix 12 is required for transactivation, it contributes to the binding surface for co-activators and deletion or mutation of helix 12 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 helix 12 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.

This thesis is built on the following publications:

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) IF: 6,696

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
   J Biol Chem., 278(**44**): 43797-806 (2003) IF: 6,696

3. **Benko, S.**, Oros, M., Schwabe, J.W., Nagy, L.: Role of RXR-H12 in the equilibrium of coregulator binding and receptor activation
   In preparation

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.:
Transcriptional regulation of human CYP27 integrates retinoid, PPAR and LXR signaling leading to cholesterol efflux from macrophages
Submitted for publication

2. Balint,B. L., Gabor, P., **Benko, S.**, Szanto, A., Puskás, G.L., Davies, P.J.A., Nagy, L.:
Histone tail methylation provides epigenetic transcription memory for retinoid induced differentiation in myeloid cells
Submitted for publication

3. **Benko, S.**, Brazda, P., Szanto, A., Balint, B.L., XY, XY, Schwabe, J.W., Nagy, L.:
Characterization of a novel RXR antagonist
In preparation

Posters:

Brazda, P., **Benko, S.**, Nagy, L. (2004)
Characterization of a novel RXR antagonist
(in Hungrian, 9th Congress of the Society of Hungarian Biochemistry and Molecular Biology, Sopron, Hungary, 10-13 May)

Benko, S., Love,J., Schwabe, J.W.R., Nagy, L. (2003)
Molecular determinants of corepressor binding and release of nuclear hormone receptors (EMBO Conference on Biology of Nuclear Receptors, Villefranche-sur-Mer (Nice) France on June 4-7)

Széles, L.,* **Benko, S.,*** Nagy, L. (2003)

Studies of stably expressing nuclear receptors on cells generated by lentiviral transduction (in Hungarian, 8th Congress of the Society of Hungarian Biochemistry and Molecular Biology, Tihany, Hungary, 12-15 May)

Benko, S., Schwabe, J.W.R., Nagy, L. (2002)
The role of the transactivation helix (H12) of nuclear receptors in cofactor exchange (in Hungarian, 7th Congress of the Society of Hungarian Biochemistry and Molecular Biology, Hungary)

Benko, S., Love,J., Schwabe, J.W.R., Nagy, L. (2001)
Determinants of corepressor binding and release of nuclear hormone receptors (Keystone Symposia, Nuclear Receptor Superfamily, Snowbird, Utah, USA)

Benko, S., Love,J., Schwabe, J.W.R., Nagy, L. (2001)
Mapping of the cofactor binding region of retinoic acid receptor (EMBO, "Protein-protein interactions" course, Germany)

Benko, S., Love,J., Schwabe, J.W.R., Nagy, L. (2001)
Study of cofactor exchange of retinoic acid receptors by mutagenesis (in Hungarian, 6th Congress of the Society of Hungarian Biochemistry and Molecular Biology, Hungary)

Benko, S., Love,J., Schwabe, J.W.R., Nagy, L. (2001)
Molecular determinants of nuclear hormone receptor heterodimer interactions with coactivators and corepressor (EMBO Workshop, Nuclear Receptor Structure and Function, Erice, Italy)

Benko, S., Love,J., Schwabe, J.W.R., Nagy, L. (2000)
Characterization of nuclear receptor - corepressor interaction by RAR-mutants and receptor specific ligands (EMBO Lecture Course: Molecular and Cellular biology from Plant to Human Cells, Hungary)

Benko, S., Love,J., Schwabe, J.W.R., Nagy, L. (2000)
Molecular determinants of corepressor binding and release of nuclear hormon receptor (FEBS Advanced Lecture Course: New Dimensions in the Regulation of Gene Expression, Greece)

Presentations:

Benkő Szilvia, John W. R.Schwabe, Nagy László (2004)
Regulation of retinoid receptor
(in Hungarian, 9th Congress of the Society of Hungarian Biochemistry and Molecular Biology, Sopron, Hungary, 10-13 May)

László Nagy, **Szilvia Benko** (2002)

Molecular aspects of steroid hormone affects through protein-protein studies
Lecture on Promega-Bioscience Symposium, 2002. oct.4. Budapest, Hungary

Szilvia Benkö, László Nagy (2000)
"Determination of protein-protein interaction in mammalian-two-hybrid system with the help of *Lucifarese Assay System*" Lecture on Promega Workshop, 2000 (in Hungarian)

Szilvia Benkö, James Love, John W.R. Schwabe, László Nagy (2000)
Molecular determinants of corepressor binding and release of nuclear hormon receptor
(FEBS Advanced Lecture Course: New Dimensions in the Regulation of Gene Expression, 2000)