Molecular determinants of the balance between co-repressor and co-activator recruitment to the retinoic acid receptor

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SUMMARY

The repressive and activating states of nuclear hormone receptors are achieved through the recruitment of co-factor proteins. The binding of co-repressors and co-activators is believed to be mutually exclusive and principally regulated by ligand binding. To understand the molecular determinants of the switch induced by ligand in the retinoic acid receptor (RAR), and in particular the intrinsic role of the ligand binding domain (LBD) in co-factor binding and release, we have carried out extensive mutational analysis of surface residues of the LBD. As seen previously we find that co-repressor and co-activator molecules bind to overlapping docking sites on the surface of the RARα LBD. Perturbation of this surface impairs both co-activator and co-repressor association resulting in a transcriptionally inert receptor. Unexpectedly, mutation of two residues, W225 and A392, which lie outside the docking site, have opposite effects on co-activator and co-repressor binding. W225A is a constitutive repressor that fails to bind co-activator and exhibits an increased, and ligand-insensitive, interaction with co-repressor. A392R, on the other hand, has reduced affinity for co-repressors and increased affinity for co-activators and behaves as a constitutive, but still ligand-inducible, activator. Analysis of known structures shows that these mutations lie in the proximity of helix 12 (H12) and their effects are likely to be the result of perturbations in the behavior of H12. These data suggest that residues in the close vicinity of H12, regulate co-factor affinity and determine the basal activity of receptors.
INTRODUCTION

Nuclear hormone receptors are ligand-activated transcription factors that regulate gene expression as monomers, homodimers or heterodimers, either by directly interacting with DNA response elements or through “cross-talk” interactions with other signalling pathways (1,2). Among these receptors the retinoic acid receptor (RAR) has a central role in a number of cellular and physiological events such as epithelial and myeloid cell differentiation, reproduction, embryonic and adult development, and also regeneration of several tissues and organs (3-6). RAR heterodimerizes with the retinoid X receptor (RXR) (7) and induces repression or activation of target genes in response to receptor-specific ligands (8). Both the repressive and active states of the receptor appear to be critical for the biological functions of RAR.

In common with other nuclear receptors, RAR regulates gene expression in response to ligands through the direct recruitment of co-regulator proteins (for reviews see (9-11)). In the presence of agonists, the receptor binds co-activator proteins (such as ACTR, DRIP, SRC-1) (12-14). These in turn associate with additional proteins forming large complexes which activate transcription through interaction with basal transcription factors and chromatin remodeling enzymes (e.g. histone acetyltransferases and methyltransferases) providing the mechanistic and enzymatic requirements for transcriptional activation (15-17). In the absence of agonists, RAR binds co-repressor proteins (such as SMRT, N-CoR) (18-20). Again, these form multi-component repression complexes with enzymatic activities such as histone deacetylation that results in transcriptional repression (21,22).

Recent studies have shown that both co-activator and co-repressor proteins contain short sequence motifs (termed Interaction Domains - IDs) that are necessary
and sufficient to mediate the association of these co-factors with nuclear receptors.

Co-activators contain between 1 and 5 IDs with a consensus sequence of LxxLL (23). These motifs are able to form short amphipathic α-helices that bind to the surface of the nuclear receptor LBD (24-26). In an analogous fashion, co-repressor proteins have been found to contain 2 or 3 motifs with a similar consensus sequence of LxxI/HIxxxI/L (27-31). This motif also adopts an amphipathic α-helical conformation, but somewhat longer than the co-activator motif (32).

Our understanding of how ligand-binding leads to the activation of nuclear receptors has been greatly advanced by structural studies of nuclear receptor ligand-binding domains and their interactions with co-activator and co-repressor peptides (24-26,32-34). The ligand-binding domain is a predominantly α-helical domain with a large non-polar cavity, which can accommodate lipophilic ligands. This cavity is capped by the C-terminal helix of the receptor (helix 12). In the active position, helix 12 adopts a conformation such that it lies across helices 3 and 10/11. This position favors binding of the α-helical co-activator in a perpendicular, largely non-polar, groove on the receptor surface (24-26). A conserved glutamate within helix 12 contributes to a charge clamp interaction with the co-activator helix dipole. Importantly, antagonists appear to function through preventing helix 12 from adopting this active position (26,33,35) and in some cases favor co-repressor binding to the ligand-binding domain in a position very similar to that of co-activator (32) but without the active conformation of helix 12. The structural studies of co-factor:receptor interactions have been supported by various biochemical approaches, particularly for TR and RXR (36-39). For these receptors cofactor binding sites were determined using site-directed mutagenesis, mammalian two hybrid analysis and in vitro interaction assays.
These biochemical and structural studies have given us a good understanding of the mechanisms of co-factor recruitment. However, one critical question that remains to be answered is what determines the balance between repression and activation. It is clear that this varies enormously between different nuclear receptors and, since these various receptors share a common group of co-factors, understanding how this different balance is achieved is of great biological significance. RAR and TR lie at one extreme of this balance since they strongly repress gene expression in the absence of ligand. (18-20,40). Receptors such as PPARγ lie at the other extreme and exhibit a high basal level of activity in the absence of ligand (41,42).

To investigate how the balance between repression and activation is encoded in the receptor’s ligand binding domain, we used a mutagenesis approach to look for residues that differentially affect co-repressor and co-activator recruitment to RAR. We characterized the interaction of these mutant receptors with co-activators and co-repressors using a mammalian two-hybrid approach and in vitro interaction analyses such as GST-pull-down assays and DNA bandshift assays. The inherent transcriptional activity of the mutant receptors was also determined using transient transfection-reporter gene assays. We found that mutation of the majority of the residues within the proposed co-repressor and co-activator docking sites reduced the binding of both co-activator and co-repressor. These can be viewed as simple docking site mutations. Two mutations behaved very differently in that the effects on co-repressor and co-activator binding were reversed and the basal activity of the receptor is dramatically changed.
FOOTNOTE

ACTR – activator for thyroid hormone and retinoid receptors,

AF-2–activation function-2, ATRA - All-trans retinoic acid,

CBP – CREB-binding protein, DRIP – vitamin D receptor-interacting protein,

GST – glutathion-S-transferase, HAT – histone acetyltransferase, HDAC – histone deacetylase, ID – interaction domain, LBD – ligand binding domain,

LG100268 - 6-[1-(3, 5, 5, 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 ,

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

TR – thyroid hormone receptor, TRAP – thyroid hormone receptor-associated protein
MATERIALS AND METHODS

Cell culture and reagents. The CV-1 (Green monkey kidney fibroblast) cell line was used for all transfection studies. Cells were maintained in DMEM (Dulbecco’s Modified Essential Medium), supplemented with 10% FCS (Invitrogen), 2mM glutamine, penicillin and streptomycin (Sigma). The day before transfection 1x10^6 cells were plated into 48-well plates.

Transient cotransfection and luciferase/β-galactosidase assay. Cells were transfected at 60-80% confluency using DOTAP (AVANTI, Avanti Polar-Lipids, Inc) as described previously (18,43). Transfection was carried out in DMEM containing 5% charcoal stripped, delipidated calf serum bovine (Sigma-Aldrich). After 6-8 hours, the medium was changed to DMEM containing the indicated ligands or vehicle. Cells were lyzed and assayed for reporter expression 24 hours after transfection. The Luciferase Assay System (Promega) was used according to the manufacture’s instruction. The β-galactosidase activity was determined as described previously (27). Measurements were made using a Wallac, Victor-2, Multilabel Counter. Luciferase activity of each sample was normalized according to the β-galactosidase activity. Each transfection was carried out in triplicate and repeated 3-6 times.

Plasmids and mutagenesis. Mammalian expression vectors expressing Gal-hSMRT-ID-1, -ID-2, -ID1+2, Gal-hRARα-LBD, VP-hRARα-LBD, CMX-hRXRα-LBD, CMX-hRXRα-FL, CMX-hRARα-FL, GST-C-SMRT, pMH100-TK-luc, pCMX-β-galactosidase were described previously (18). The Gal-DRIP-ID-1+2 expression
plasmid contained residues 527-775 of DRIP cloned into the vector pCMX-Gal4-DBD.

Mutations within the VP-hRARα-LBD, VP-hTRβ-LBD, Gal-hRARα-LBD and CMX-hRARα-FL were obtained using the methods described in the QuickChange Site-Directed Mutagenesis Kit (Stratagene). All constructs were verified by DNA sequence analysis (DNA Sequencing Kit, BigDye Terminator Cycle Sequencing v2.0 and ABI 310 Sequence analyzer). Plasmids were grown in E. coli strain DH5α. DNA was extracted and purified using Qiagene Maxi or Midi Columns or Promega MiniPrep Kit.

**Protein expression and purification.** GST-fusion proteins were expressed and purified as previously described (27). Briefly, proteins were expressed in E. coli strain BL21. 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 8.0), 1% Triton-X-100 and 1 mM AEBSF. Bound proteins were eluted with 10 mM glutathione in 50 mM PBS.

**Ligand-binding assay.** Ligand-binding assays were performed using bacterially expressed GST-hRARα-LBD fusion proteins and radiolabelled ³H-9-cis-RA. For the saturation binding analysis, purified receptor protein was incubated for 1 hour at room temperature in binding buffer (20 mM Tris-Cl, 50 mM KCl, 1 mM MgCl₂, 1 mM DTT, pH 8.0) with increasing amount of ³H-9-cis-RA. Bound ligand was separated from free on chromatography columns (Bio Spin 30 Tris Columns, Bio-Rad Laboratories). Binding affinities were calculated using Scatchard analysis from three separate experiments on independently generated protein samples.
**GST-pull-down.** hRARα-LBD mutant proteins were synthesized using a T7 Quick TNT in vitro Transcription/Translation Kit (Promega) with [35S] methionine. 35S-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 twice with PBS buffer containing 0.1% Triton-X-100 and 1 mg/ml BSA and resuspended in 2x Laemmli buffer (27). After centrifugation, the proteins were analyzed by SDS-PAGE and visualized / quantified using an image plate scanner (Typhoon, Molecular Dynamics, Amersham Bioscience).

**Electrophoretic mobility shift assays.** Full-length hRARα and hRXRα receptors were produced using the T7 Quick TNT *in vitro* Transcription/Translation Kit (Promega). The EMSA probe DNA (RARE-DR5) was prepared by annealing complementary oligonucleotides and was labeled with 32P-dCTP using reverse transcriptase. The RARE-DR5 probe (5'-CGTTGGCGCCGGTCA CGAAAGGTCAGAATTAG–3') was incubated with the receptors and bacterially expressed SMRT in binding buffer (20mM HEPES pH 7.5, 75mM 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 analyzed using 10 x 10 cm, 0.7% agarose gel buffered in 0.5x TB. The gel was dried and visualized using an image plate scanner (Typhoon, Molecular Dynamics, Amersham Bioscience).
RESULTS

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, ID1 and ID2 (44). 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 the Gal4 DNA binding domain and challenged with the RAR-LBD fused to a VP16 activation domain. Figure 1 shows that SMRT ID1+2 interacts strongly with the RAR-LBD and that this interaction is abolished by treatment with AM580, an RARα selective agonist. The isolated SMRT ID2 essentially recapitulates the ligand-dependent behavior of SMRT ID1+2, whereas the assay failed to detect any interaction with the isolated SMRT ID1 alone.

To test if the domain specificity remains in the context of the combined interaction domain, which includes both ID1 and ID2, we used point mutations to remove conserved hydrophobic amino acids from the individual ID’s (27). SMRT ID1+2 harboring mutations in ID1 behaves exactly like the isolated ID2 and similarly, SMRT ID1+2 with mutations in ID2 behaves like the isolated domain of ID1. Interestingly, the combined RID domain (SMRT ID1+2) shows a stronger interaction, than ID2 alone, either as an isolated domain or as an ID1 mutant. This suggests that ID1 may contribute to ID2:RAR-LBD interaction even though it does not appear to facilitate direct binding to the LBD. These experiments also establish that ID2 is the primary site on SMRT for interaction with RAR-LBD and that it is necessary and sufficient for ligand sensitive interaction.
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 the RAR-LBD. 17 residues were selected as being potentially important for the co-repressor-receptor interaction by inspection of the available crystal structures and analysis of the conservation between different receptors. 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 2A and B). Mutations in several of these residues have been analyzed in previous studies and serve as a reference in our analysis (figures 2A &B). Mutations were generated by standard mutagenesis technology, then sequenced and tested in mammalian two hybrid interaction assays for interaction with SMRT ID2. The mutant receptors can be grouped into different categories based on the strength of their interaction with SMRT ID2 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 a significantly reduced, ligand sensitive interaction. Several others such as I236A, V240A, K244A, Q257A, L266A showed very little or no interaction at all. 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. In contrast the L261A mutant exhibited increased interaction with SMRT in the presence of ligand.
Interaction of RAR-LBD:RXR-LBD heterodimers with SMRT RID

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 both SMRT ID’s. Figure 3A shows that for the wild type receptor there is no significant difference between monomer and heterodimer in the interactions with SMRT ID 1+2. Neither the strength of interaction, nor its ligand sensitivity was altered suggesting that RXR does not contribute to or alter the RAR-LBD SMRT interaction per se. Based on these findings we re-screened the RAR-LBD mutants in the heterodimer assay system (Figure 3B). Only three mutants, G248A, L261A and L266A, behaved differently from the assay with the RAR-LBD alone (compare Figure 3B to Figure 2C). These mutants regained wild type like activity suggesting that RXR may stabilize RAR-LBD-SMRT-RID interactions leading to wild type-like co-factor binding activity. This also means that the heterodimer assay is more stringent for assessing the effects of mutations on co-repressor binding since it is likely that some folding instability generated by certain mutations may be overcome by RXR acting as a chaperone.

While mammalian two-hybrid analysis is a sensitive and quantitative way of measuring interactions, we sought to confirm these results in an in vitro interaction assay. GST-pull down experiments were performed using bacterially expressed GST-SMRT ID1+2 protein and radiolabeled RAR-LBD. In this assay (Figure 3C) 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 ID1+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.
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 (32,36,38). Mutations on this surface of the LBD such as I236A, V240A, K244A, F249A, T250A, I254A, Q257A, I258A abolished or significantly reduced co-repressor binding. Two mutations W225A and A392R lie outside this region, W225A interacts with SMRT ID2 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.

**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 sought to establish a co-activator binding assay using the previously identified receptor interaction domains of two co-activators: ACTR and DRIP205/TRAP220. These have both been shown to directly interact with RAR and they represent distinct classes of co-activator proteins (45). Mammalian two hybrid analyses were performed using Gal4-DBD fusions of the receptor interacting domains from DRIP205/TRAP220 and ACTR (12,46,47) and 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 4A). It is interesting to note that in all cases the binding of the RAR:RXR heterodimer is approximately 2-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, whereas 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 further
increases almost two fold in the presence of ligand (Figure 4B). Note that 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.

To summarize the various mutants, we grouped them based on their co-factor
binding profile as shown in the diagram on Figure 4C. The 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 solely 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 that the co-activator and co-repressor binding surfaces do not completely
overlap. Intriguingly, we have found two mutations, W225A and A392R, each of
which inversely affects co-activator and co-repressor binding. Since both of these
mutants are located outside of the mapped and proposed docking site for co-factors
we believe that these two mutants do not alter the binding site per se, but represent
mutations of an intrinsic regulatory site.

In Figure 4D the various mutations are color-coded that affect both co-
activator and co-repressor binding on the structure. Residues which, when mutated,
reduce both co-activator and co-repressor binding are shown in blue and represent the
co-factor binding surface or docking site. Residues in cyan reduce co-repressor
binding but not co-activator binding. The magenta residue L261, if mutated to
alanine, reduces co-activator binding without altering co-repressor binding. Mutation
of W225 (in red on the right) increases co-repressor binding and diminishes co-activator binding, while A392 (in red on the left), if mutated to arginine, reduces co-repressor binding but enhances co-activator binding. In general it appears that mutations within the binding/docking site reduce both co-activator and co-repressor binding. Mutations outside this area, in close proximity of H12, differentially influence co-activator and co-repressor binding.

**Transcriptional activity of the mutant receptors**

It is important to understand whether the transcriptional activity of a nuclear receptor is determined simply from the co-factor binding profile. If this is the case, we would predict that receptors with combined co-activator/co-repressor mutations (docking site mutations) would be transcriptionally inert as a result of losing some or most of their ability to repress as well as to activate but that their basal transcriptional activity would be similar to that of the wild type receptor. On the other hand, regulatory mutations that differentially affect co-activator and co-repressor association could produce receptors that can either repress or activate transcription significantly beyond the normal range of wild type receptor. To explore this possibility we carefully examined if, and how, selected mutations affect the transcriptional activity of the Gal4-DBD fused and full-length receptors. We considered this to be a critical test of the hypothesis that the co-factor binding and release profile has a predictive value on overall transcriptional activity. Six mutants were compared: three are deficient in both co-activator and co-repressor binding (V240A, K244A, I254A) and three other ones differentially affected co-factor association (W225A, A392R, V395A).
Figure 5A shows the activity of wild type and mutant receptor LBDs (fused to the Gal4-DBD) in the absence of exogenous ligand. In this experiment wild type receptor repressed transcription of the reporter gene by approximately 50%. W225A repressed significantly more strongly than wild type (c.95%). The K244A and V395A mutants showed no repression activity at all. The V240A and I254A mutants also showed a reduced repressor activity compared with wild type (28% and 14% of the basal activity of the reporter gene, respectively). Strikingly the A392R mutant produced a very significant increase in activity of the reporter gene. Thus, rather than repressing basal transcription, this mutant acts as a constitutive activator.

Next we examined the response of the wild type and mutant chimeric receptors to an increasing dose of AM580 so as to assess the ligand-inducible transcriptional activity (Figure 5B). The wild type RAR-LBD showed a dose response curve with a half maximal induction (EC₅₀) at 7 nM AM580. The I254A and V395A mutants have an EC₅₀ similar to that of the wild type, although the amplitude of induction was significantly lower. In contrast, W225A and K244A did not activate transcription to any significant degree. As expected, the A392R mutation showed an increased basal activity and interestingly exhibited a larger amplitude of induction by AM580.

To ascertain whether RXR has an effect on the transactivation activity of the mutant receptors, we co-transfected a plasmid that expresses the RXR-LBD (Figure 5C). Interestingly, the A392R mutant showed an altered activity in this assay: it’s constitutive activity in the absence of ligand was significantly reduced and it showed an unexpected increased potency for AM580 (EC₅₀=1.5 nM). The only other mutant to behave significantly differently in the presence of the RXR-LBD was V240A. This mutant was totally inactive as a monomer, but regained some transcriptional activity.
in the presence of RXR. However this mutant remains at least an order of a magnitude less sensitive to AM580 than the wild type receptor (Figure 5C).

Finally, to examine the effect of mutations in the most physiological setting possible, we examined the behavior of the full length RXR:RAR heterodimer bound to a natural response element. Transient transfection assays were performed, in the presence of increasing AM580, using full-length RAR (wild-type and mutant), full-length RXR and a β RARE-TK-luc reporter gene. Wild type and the V395A mutant receptors show a dose dependent transactivation profile with an EC$_{50}$ of 6.7 nM and 5.5 nM respectively (figure 5D). In the same assay the W225A mutant shows no significant transactivation, whilst the A392R remained more potent than wild type receptor as indicated by the dose response curve shifting to the left (EC$_{50}$=0.9 nM).

**Electrophoretic Mobility Shift Assay analysis of mutant receptors**

Electrophoretic mobility shift analysis (EMSA) was used to verify that the full-length receptors behave the same way in an *in vitro* assay. The full-length RAR:RXR heterodimer (expressed in an *in vitro* transcription/translation system) formed a specific complex with the radiolabelled β RARE probe (figure 6). Addition of SMRT ID1+2 (expressed in bacteria), gave a clear supershift. As expected, the co-repressor is not dissociated by the RXR specific ligand LG100268, but is readily dissociated on addition of AM580 (Figure 6). Compared with the wild type receptor, W225A binds SMRT ID1+2 more strongly and this interaction is not dissociated by either ligand. T250A and A392R showed co-repressor binding activity similar to wild type, consistent with the results obtained in previous experiments (Figure 5).
Homologous mutations in hTRβ have similar effects to those in hRARα

Finally, we reasoned that if the two residues (W225 and A392) represent functionally conserved amino acids 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 should have similar effects. We chose hTRβ, because it is the closest homologue of RAR and is a strong transcriptional repressor. Analogous mutations in (F264A and A431R) were made in VP fusions of the hTRβ-LBD and interaction with Gal-SMRT-ID 1+2 was measured using transient transfection assays. F264A interacted with co-repressor almost twice as strongly as the wild type, whilst A431R showed a somewhat reduced interaction (75% of the wild type – Figure 7). These results were comparable to the equivalent mutations in RAR. Moreover, upon ligand (T₃) treatment, the interaction of the F264A mutant with SMRT was hardly affected by ligand. In contrast A431R was more sensitive to ligand than wild type. We also noted that ligand failed to completely release SMRT ID1+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.
DISCUSSION

Transcriptional activation by nuclear receptors depends critically upon the balance of co-factor 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 and by the availability of specific co-factors. The biological significance of this interplay of factors has been highlighted by Brown and colleagues who have elegantly shown that for the estrogen receptor the tissue selective response for SERMS (Selective Estrogen Receptor Modulators) is a consequence of the specific balance between co-activators and co-repressors in a particular cell type (48).

Structural studies have provided a static view of both co-activator and co-repressor complexes with various nuclear receptors (24-26,32). Xu et al. provided the first structural evidence that the co-repressor and co-activator binding sites are indeed largely overlapping (32). These studies combined with biochemical analyses suggest that the two types of co-factors bind in a very similar fashion to a conserved surface on the receptor ligand-binding domain. They also reveal that co-activator binding requires that helix 12 be in the active position and conversely that co-repressor binding requires that helix 12 is displaced from the active position. In some, but not all, receptors it is clear that ligand directly stabilizes the active conformation of helix 12 and therefore promotes co-activator recruitment. Whilst these studies have been very revealing, they leave unanswered the question of what determines the basal activity of different receptors. Why do some receptors such as TR and RAR strongly repress transcription in the absence of ligand and why do others such as PPARγ show
a basal transcriptional activity? In other words what are the intrinsic determinants of co-factor binding balance?

In order to explore this we first established a simple co-repressor and co-activator interaction assay using mammalian two-hybrid analysis. We demonstrated that ID2 of SMRT is necessary and sufficient to mediate interaction between the RAR-LBD and SMRT. We then sought to identify mutations that would alter the balance between co-repressor and co-activator binding and thus alter the intrinsic basal activity of the receptor.

**Identification of a passive docking site for co-factor binding**

Consistent with previous mutagenesis and structural studies, we find that mutations in helices 3 and 4 disrupt both co-activator and co-repressor binding. It appears therefore, that whilst the RAR-LBD interacts preferentially with SMRT ID2 (rather than ID1 like other tested receptors), the interaction is analogous to that between SMRT-ID1 and other receptors. 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. 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. We also noted that this mutant induced co-repressor interaction in the presence of RAR ligand. It is important to note however that these differences do not translate into substantive differences in transcriptional activity and suggest 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).
Residues that determine the basal activity of the receptor

Whilst 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. 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 homologous RARγ shows that W225 is in Van der Waals contact with residues in the loop between helices 11 and 12: L398 (4.1Å), I402 (3.6Å), M406 (3.5Å) and P407 (3.9Å) (Figure 8A) (49,50). It is generally difficult to predict the structural consequences of mutations and especially so given the dynamic character of nuclear receptor ligand binding domains (51). However, given the location of W225A, the dominant negative phenotype and the knowledge that co-repressor binding requires the displacement of helix 12, it is tempting to speculate that the W225A mutation may destabilize the active position of helix 12 as a consequence of removing the favorable contacts to the loop between helices 11 and 12. This would in turn favor co-repressor interaction with the mutant receptor and reduce the efficiency of release on binding ligand. The importance of the loop between helices 11 and 12 has also been suggested by others (52). Significantly the effects of this mutation are similar to those mutations in PPARγ
that result in a receptor with dominant negative repression activity (53) and which have been explicitly shown to destabilize helix 12 (54). 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 (125% of the wild type).

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 (135% of the wild type). 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. Again, it is important to be cautious when trying to interpret the effect of this mutation in the context of the ligand binding domain structure – particularly given that this is a gain-of-function mutation. However, examination of the RARγ structure shows that the larger Arg side chain could be sterically accommodated at this position (Figure 8B) (49). The larger arginine sidechain would however be in contact with a number of residues in helices 4 and 12. These include Van der Waals contacts to L266 (3.3Å) and L414 (4.2Å) as well as hydrogen bonds to the side chain of N416 in helix 12. It is possible therefore, and would fit with the observed phenotype, that the A392R mutation might stabilize the active conformation of helix 12 in the absence of ligand and hence promote co-activator binding which absolutely requires helix 12 in the active position.

Significantly, in PPARγ the residue in this position is a valine. This side chain makes multiple Van der Waals contacts to residues in helix 12 of PPARγ (Y473, L476 and Y477) and thus stabilizes the active conformation of helix 12 in this receptor (24).
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 behavior of PPARγ closely matches the behavior of the A392R mutant RARα.

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

It is clear from the analyses of these 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-factor 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 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-factor balance.

**A critical role for helix 12 in determining co-factor equilibrium**

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 (54). It has been shown previously that helix 12 is required for transactivation (55,56), it contributes to the binding surface for co-activators (57) and deletion or mutation of helix 12 results in dominant negative receptors with increased co-repressor binding potential (58). Our studies demonstrate that residues with the potential to influence the stability of the
active position of helix 12 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.
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FIGURE LEGENDS

Figure 1. Interaction of SMRT-IDs with RAR-LBD. Interaction analyses of SMRT IDs and RAR-LBD was carried out using mammalian two hybrid assays as described in Materials and Methods. The strength of interactions is expressed as normalized luciferase activity. The mean of at least three independent measurements +/- SD is presented. (A) Interactions of Gal-RAR-LBD with VP-SMRT-ID-1+2, ID-2 and ID-1 in the presence and absence of AM580 (100nM). (B) Upper panel shows the amino acid 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.

Figure 2. 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 (27-29). (B) Surface and ribbon representation of the RARγ-LBD (PDB: 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. (C) 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 strength of interactions is expressed as normalized luciferase activity. The mean of at least three independent measurements +/- SD is presented.
Figure 3. Interaction of RAR-LBD mutants with SMRT-ID-1+2 in the presence of RXR-LBD. Interaction between SMRT-ID1+2 and RAR-LBD mutants in the presence of RXR-LBD was determined using mammalian two-hybrid analysis (A, B) or GST pull down assay (C). For the mammalian two-hybrid analysis the strength of interactions is expressed as normalized luciferase activity. The mean of at least three independent measurements +/- SD is presented. (A) Interaction of Gal-SMRT-ID-1+2 with VP-RAR-LBD +/- RXR-LBD and AM580 (100nM). (B) Interaction of Gal-SMRT-ID-1+2 with mutant RAR-LBDs in heterodimer with RXR-LBD in the presence and absence of AM580 (100nM). (C) 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, [35S] methionine–labeled receptors on GST-SMRT ID1+2 matrix analyzed by SDS-PAGE (as described Material and Methods) and visualized by autoradiography then analyzed by densitometry (upper panel). Results are presented as the specific binding to GST-SMRT-ID-2 in arbitrary units. 10% of the labeled protein input is shown in the lower panel.

Figure 4. Characterization of 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 assays. The strength of interactions is expressed as normalized luciferase activity. The mean of at least three independent measurements +/- SD is presented. (A) 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). Binding is expressed as the percentage of binding observed for the wild type RAR-LBD (in the presence of ligand) in the same assay. 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 RIDs for ACTR (amino acids 1-1412) used in the two-hybrid assays. (B) Interaction of DRIP205/TRAP220 with wild type and A392R mutant RAR in the presence or absence of AM580 (100nM). (C) Venn diagram showing the altered co-activator and co-repressor interactions of RAR mutants. (D) Surface and ribbon representation of the RARγ-LBD (PDB: 2LBD). Residues on H3, H4, H5 and H11 mutated in this study are colored according to their effect on co-factor binding. Blue indicates impaired binding of co-activator and co-repressor; cyan indicates impaired binding of co-repressor only, magenta indicates impaired binding of co-activator only and red indicates residues that differentially influence co-factor binding. The ligand ATRA is shown in green. Helix H12 is shown in yellow.

**Figure 5. Transcriptional activity of mutant receptors.** The transcriptional consequences of selected mutations were determined using transient transfection assays. Transient co-transfection assays were carried out with the indicated plasmid constructs and transcriptional activity is expressed as normalized luciferase activity. Curves were fitted by Prism (GraphPad Prism Software, Inc.). (A) Transcriptional repression or activation of unliganded RAR mutants. Broken line indicates the basal activity of the reporter construct. Representative experiments are presented. Repression is expressed as the percentage of basal activity of reporter plasmids. (B) Dose-response curves of mutant Gal-RAR-LBDs upon treatment with RAR-specific
ligand, AM580. (C) 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. (D) Dose-response curves of full-length RAR:RXR heterodimers cotransfected with β RARE-TK-LUC upon AM580 treatment. Broken line indicates the basal activity of the reporter construct.

**Figure 6. EMSA analysis of wild type and mutant full-length RAR:RXR heterodimers.** Electrophoretic Mobility Shift Analysis (EMSA) was performed with *in vitro* translated RAR-FL, RXR-FL and bacterially expressed GST-SMRT-ID-1+2, These were bound to $^{32}$P-labeled RARE-DR5 oligonucleotide in the presence or absence of the indicated ligands at a concentration of 500 nM. Arrows indicate the position of the two specific complexes.

**Figure 7. Interaction of SMRT ID-1+2 with TR mutants containing mutations equivalent to RAR-LBD W225A and A392R.** VP16-fusion proteins of hTRβ-LBD (wild type, F264A and A431R – equivalent to W225A and A392R in RARα) were tested for interaction with Gal-SMRT ID1+2 in mammalian two hybrid analysis. Transient transfections of VP-fusion hTRβ mutants were carried out in the presence of increasing amount of T₃. The strength of interactions is expressed as normalized luciferase activity, representative experiments are shown. Curves were fitted by Prism (GraphPad Prism Software, Inc.).
Figure 8. Residues W225 and A392 may influence the orientation of helix H12.

(A) W225 (red) is in Van der Waals contact with several residues (green) in the loop between helices H11 and H12. Mutation of this residue to alanine may disfavor the active position of helix H12 (yellow). The ligand ATRA is shown in magenta. The structure shown is that of RARγ-LBD (PDB: 2LBD). (B) Mutation of A392 to arginine (red) may stabilize the active position of helix H12 through (yellow) through contacts to helix 12.
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Fig. 1

A

B

SMRT - ID-1+2 RVTLAQHISEVITQDYTR ......................ASTNMGLEAIIRKALMG...
SMRT - m ID-1 RVTLAQHISEVITQDYTR ......................ASTNMGeAaaRKLAMG...
SMRT - m ID-2 RVTLaHSEaTQDYTR ......................ASTNMGLEAIIRKALMG....

[Diagram showing normalized luciferase activity]
Fig. 5

A

B

C

D

Normalized luciferase activity (AU)

Normalized luciferase activity (AU)

Normalized luciferase activity (AU)

Normalized luciferase activity (AU)

logM (AM580)

logM (AM580)

logM (AM580)

logM (AM580)

wild  
W225A  
V240A  
K244A  
I254A  
A392R  
V395A

wild  
W225A  
V240A  
K244A  
I254A  
A392R  
V395A

wild  
W225A  
V240A  
K244A  
I254A  
A392R  
V395A

wild  
W225A  
V240A  
K244A  
I254A  
A392R  
V395A

RAR- 
LBD

(RAR- 
LBD)

UAS  luciferase

UAS  luciferase

UAS  luciferase

UAS  luciferase
Fig. 6
Fig. 7
Molecular determinants of the balance between co-repressor and co-activator recruitment to the retinoic acid receptor
Szilvia Benko, James Love, Marta Beladi, John W.R. Schwabe and Laszlo Nagy

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