Molecular Determinants of the Balance between Co-repressor and Co-activator Recruitment to the Retinoic Acid Receptor*

Szilvia Benko‡, James D. Love§, Marta Beládi‡, John W. R. Schwabe§¶, and Laszlo Nagy‡¶‖

From the ‡Department of Biochemistry and Molecular Biology, Research Center for Molecular Medicine, University of Debrecen, Medical and Health Science Center, Nagyerdei krt. 98, Debrecen H-4012, Hungary and the §Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, United Kingdom

The repressive and activating states of nuclear hormone receptors are achieved through the recruitment of cofactor 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 and in particular the intrinsic role of the ligand binding domain (LBD) in cofactor binding and release, we carried out extensive mutational analysis of surface residues of the LBD. As seen previously we found that co-repressor and co-activator molecules bind to overlapping docking sites on the surface of the retinoic acid receptor α LBD. Perturbation of this surface impaired both co-activator and co-repressor association resulting in a transcriptionally inert receptor. Unexpectedly mutation of two residues, Trp-225 and Ala-392, which lie outside the docking site, had opposite effects on co-activator and co-repressor binding. W225A was a constitutive repressor that failed to bind co-activator and exhibited an increased, and ligand-insensitive, interaction with co-repressor. A392R, on the other hand, had reduced affinity for co-repressors and increased affinity for co-activators and behaved as a constitutive, but still ligand-inducible, activator. Analysis of known structures showed 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 cofactor affinity and determine the basal activity of receptors.

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 signaling pathways (1, 2). Among these receptors the retinoic acid receptor (RAR)[1] 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 Refs. 9–11). In the presence of agonists, the receptor binds co-activator proteins (such as ACTR, DRIP, and steroid receptor coactivator-1) (12–14). These in turn associate with additional proteins forming large complexes that 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 and nuclear receptor co-repressor (N-CoR)) (18–20). Again these form multicomponent repression complexes with enzymatic activities such as histone deacetylation that result 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 cofactors with nuclear receptors. Co-activators contain between one and five 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 ligand binding domain (LBD) (24–26). In an analogous fashion, co-repressor proteins have been found to contain two or three motifs with a similar consensus sequence of LXX(I/H)IXXX(I/L) (27–31). This motif also adopts an amphipathic α-helical conformation but is 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

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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 cofactor·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 cofactor 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 cofactors, 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 ligand binding domain of the receptor, 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 was dramatically changed.

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 Dulbecco's modified essential medium supplemented with 10% fetal calf serum (Invitrogen), 2 mM glutamine, penicillin, and streptomycin (Sigma). The day before transfection 1×10^6 cells were plated into 48-well plates.

Transient Co-transfection and Luciferase/β-Galactosidase Assay—Cells were transfected at 60–80% confluency using 1,2-dioleoyl-3-trimethyl ammonium-propane (Avanti Polar-Lipids, Inc.) as described previously (18, 43). Transfection was carried out in Dulbecco's modified essential medium containing 5% charcoal-stripped, delipidated bovine calf serum (Sigma). After 6–8 h, the medium was changed to Dulbecco's modified essential medium containing the indicated ligands or vehicle. Cells were lysed and assayed for reporter expression 24 h after transfection. The luciferase assay system (Promega) was used according to the manufacturer'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 three to six times.

Plasmids and Mutagenesis—Mammalian expression vectors expressing Gal-hSMRT-ID-1, -ID-2, -ID-1+2, Gal-hRARα-LBD, VP-hRARα-

LBD, CMX-hRXRα-LBD, CMX-hRXRα-FL, CMX-hRARα-FL, GST-C-SMRT, pMH100-TK-luc, and 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 QuikChange site-directed mutagenesis kit (Stratagene). All constructs were verified by DNA sequence analysis (DNA sequencing kit, BigDye Terminator Cycle Sequencing version 2.0, and ABI 310 sequence analyzer). Plasmids were grown in *Escherichia coli* strain DH5α. DNA was extracted and purified using Qiagen Maxi or Midi columns or Promega MiniPrep kit.

Protein Expression and Purification—GST fusion proteins were expressed and purified as described previously (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 Biosciences) in buffer containing 50 mM Tris (pH 8.0), 1% Triton X-100, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride. Bound proteins were eluted with 10 mM glutathione in 50 mM phosphate-buffered saline.

Ligand Binding Assay—Ligand binding assays were performed using bacterially expressed GST-hRARα-LBD fusion proteins and radiolabeled 9-*cis*-[³H]retinoic acid. For the saturation binding analysis, purified receptor protein was incubated for 1 h at room temperature in binding buffer (20 mM Tris-HCl, 50 mM KCl, 1 mM MgCl₂, 1 mM dithiothreitol, pH 8.0) with increasing amounts of 9-*cis*-[³H]retinoic acid. Bound ligand was separated from free ligand on chromatography columns (Bio Spin 30 Tris columns, Bio-Rad). 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 (E)-4-[2-(5,6,7,8-tetrahydro-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 phosphate-buffered saline buffer containing 0.1% Triton X-100 and 1 mg/ml bovine serum albumin and resuspended in 2× Laemmli buffer (27). After centrifugation, the proteins were analyzed by SDS-PAGE and visualized/quantified using an image plate scanner (Typhoon, Amersham Biosciences).

Electrophoretic Mobility Shift Assays—Full-length hRARα and hRXRα receptors were produced using the T7 Quick TNT *in vitro* transcription/translation kit (Promega). The electrophoretic mobility shift assay probe DNA (RARE-DR5) was prepared by annealing complementary oligonucleotides and was labeled with [³²P]dCTP using reverse transcriptase. The RARE-DR5 probe (5′-CGTTGGCGCC**GGGTCA**C CGAA**AGGTCA**GAATTAG-3′) was incubated with the receptors and bacterially expressed SMRT in binding buffer (20 mM HEPES, pH 7.5, 75 mM KCl, 0.1% Nonidet P-40, 7.5% glycerol, 2 mM dithiothreitol) in the absence or presence of receptor-specific ligands for 20 min at room temperature. Samples were analyzed using a 10- × 10-cm, 0.7% agarose gel buffered in 0.5× Tris borate. Bold, underlined residues indicate the consensus binding site. The gel was dried and visualized using an image plate scanner (Typhoon, Amersham Biosciences).

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, ID-1 and ID-2 (44). To determine whether 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. Fig. 1 shows that SMRT ID-1+2 interacts strongly with the RAR-LBD and that this interaction is abolished by treatment with AM580, an RARα selective agonist. The isolated SMRT ID-2 essentially recapitulated the ligand-dependent behavior of SMRT ID-1+2, whereas the assay failed to detect any interaction with the isolated SMRT ID-1 alone.

To test whether the domain specificity remains in the context of the combined interaction domain, which includes both ID-1

A

B
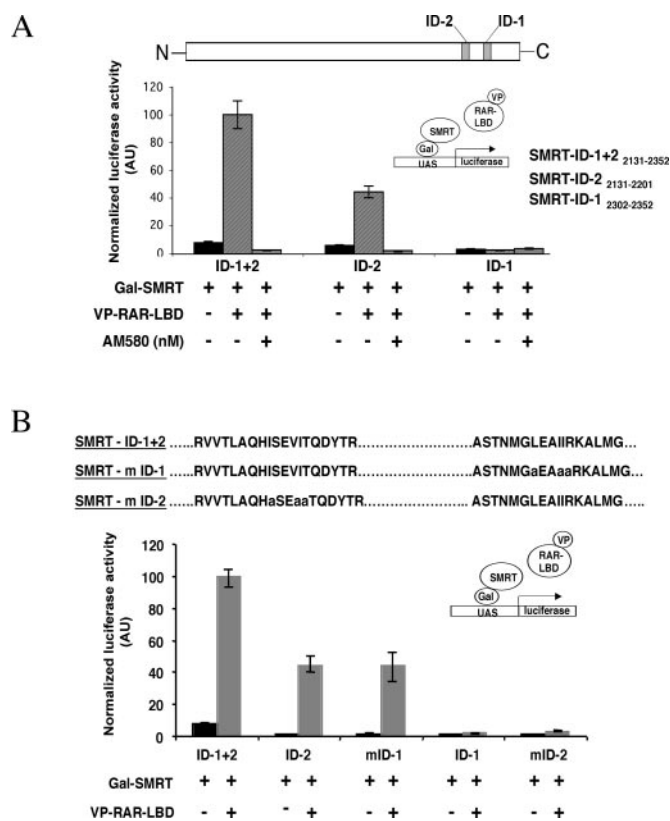
FIG. 1. **Interaction of SMRT IDs with RAR-LBD.** Interaction analysis of SMRT IDs and RAR-LBD was carried out using mammalian two-hybrid assays as described under "Materials and Methods." The strength of interactions is expressed as normalized luciferase activity. The mean of at least three independent measurements ± S.D. 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 (100 nM). *B*, *upper panel*, shows the amino acid sequence of SMRT ID-1+2 interaction domains; *lowercase* indicates the mutations in ID-1 (*mID-1*) and ID-2 (*mID-2*). The *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. *AU*, arbitrary units.

and ID-2, we used point mutations to remove conserved hydrophobic amino acids from the individual IDs (27). SMRT ID-1+2 harboring 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 RID domain (SMRT ID-1+2) showed 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, although it does not appear to facilitate direct binding to the LBD. These experiments also established that ID-2 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 residues lie on helices 3 and 4 (Trp-225, Ser-229, Ser-233, Ile-236, Val-240, Lys-244, Gly-248, Phe-249, Thr-250, Ile-254, Gln-257, Ile-258, and Leu-261), helix 5 (Cys-265 and Leu-266), and helix 11 (Ala-392 and Val-395) (Fig. 2, *A* and *B*). Mutations in several of these residues have been analyzed in previous studies and serve as a reference in our analysis (Fig. 2, *A* and *B*). Mutations were

generated by standard mutagenesis technology, then sequenced, and tested in mammalian two-hybrid interaction assays for interaction with SMRT ID-2. 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, and C265A behaved very similarly to the wild type receptor with a strong interaction in the absence of ligand that 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, and L266A showed very little or no interaction at all. One particular mutant, W225A, showed an interaction with co-repressor protein equally as strong 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 IDs. Fig. 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 rescreened the RAR-LBD mutants in the heterodimer assay system (Fig. 3*B*). Only three mutants, G248A, L261A, and L266A, behaved differently from the assay with the RAR-LBD alone (compare Fig. 3*B* to Fig. 2*C*). These mutants regained wild type-like activity suggesting that RXR may stabilize RAR-LBD·SMRT-RID interactions leading to wild type-like cofactor 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 pulldown experiments were performed using bacterially expressed GST-SMRT ID-1+2 protein and radiolabeled RAR-LBD. In this assay (Fig. 3*C*) 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 ligand-sensitive interaction with the co-repressor than did wild type.

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, and I258A abolished or significantly reduced co-repressor binding. Two mutations, W225A and A392R, lie outside this region. W225A interacted with SMRT ID-2 more strongly than did wild type receptor, and this interaction was 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

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A

hRARα	207	-YTTNNSSEQRVSLD[IDLWDKFSELSTKCIIKTVEFAK]QLPGFTTLT[IA]	256
hRXRα	251	-EANMGLNP---SSPNDPVTNICQAADKQLFTLVEWAKRIPHFSELPLD	297
hTRβ	247	-QAPIVNAPEGGKVDLEAFSHFTKIITPAITRVVDFAKKLPMFCELPCE	296
hPPARγ	264	FKHITPLQEQSKEVAIRIFQGCQFRSVEAVQEITEYAKSIPGFVNLDLN	314

H3

hRARα	257	[DQITLLKAACLDILILRICTR]YTPEQDTMTFSDGLTLN[RTQMHN]AGFGPLT	305
hRXRα	298	DQVILLRAGWNELLIASFSHRSIAVKDGILLATGLHVHRNSAHSAGVGAIF	346
hTRβ	297	DQIILLKGCCMEIMSLRAAVRYDPESETLTLNGEMAVIRGQLKNGGLGVVS	345
hPPARγ	315	DQVTLLKYGVHEIIYTMLASLMNKDGVLISEGQGFMTREFLKSLRKPFGDF	363

H4 H5

hRARα	355	[LEALKVYVRKR]RSPRPH[MFPKMLMKITDLRSISAK]GAERVITLKMEIP-G	403
hRXRα	397	YASLEAYCKHKYPEQPGRFAKLLLRLPALRSIGLKCLEHLFFFKLIGD-T	445
hTRβ	395	LLAFEHYINYRKHHVTHFWPKLLMKVTDLRMIGACHASRFLHMKVECPTE	444
hPPARγ	411	LQALELQLKLNHPESSQLFAKLLQKMTDLRQIVTEHVQLLQVIKKTETDM	460

H9 H10 H11

FIG. 2. **Interaction of RAR-LBD mutants with SMRT IDs.** *A*, sequence alignment of the LBD of different nuclear receptors (hRARα, hRXRα, hTRβ, and hPPARγ). *Gray boxes* show the amino acid residues that were mutated. The secondary structure elements (α-helices (*H*)) are *boxed in black*. Amino acids mutated by others are indicated by *stars* (27–29). *B*, surface and ribbon representation of the RARγ-LBD (Protein Data Bank code 2LBD). Residues on helix 3, helix 4, helix 5, and helix 11 mutated in this study are shown in *red* and labeled. The ligand ATRA is shown in *green*. Helix 12 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 (100 nM). The strength of interactions is expressed as normalized luciferase activity. The mean of at least three independent measurements ± S.D. is presented. *AU*, arbitrary units.

B

C
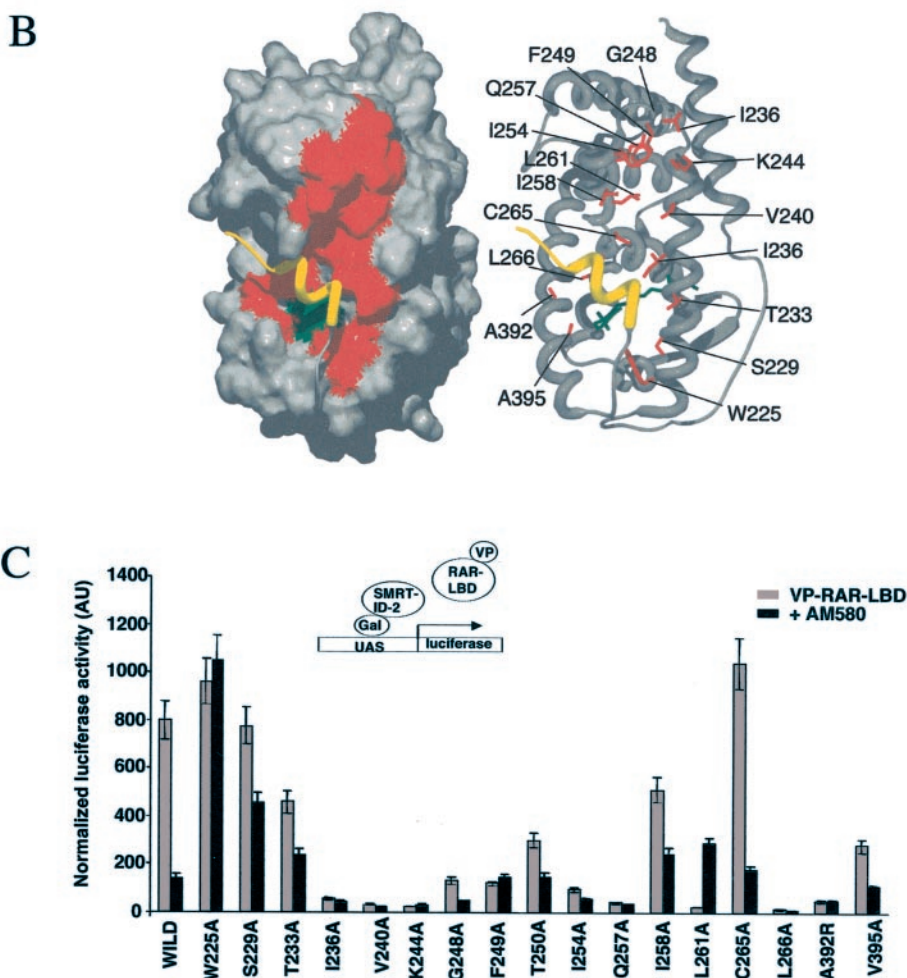
interaction domains of two co-activators: ACTR and DRIP205/TRAP220. These have both been shown to directly interact with RAR, and they represent distinct classes of co-activator proteins (45). Mammalian two-hybrid analyses were performed using Gal4-DBD fusions of the receptor interacting domains from 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 (Fig. 4*A*). It is interesting to note that in all cases the binding of the RAR·RXR heterodimer is ~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 with wild type, shows an increased co-activator binding even in the absence of ligand, which further increases almost 2-fold in the presence of ligand (Fig. 4*B*). 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 cofactor binding profile as shown in the diagram on

FIG. 3. **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* and *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 ± S.D. is presented. *A*, interaction of Gal-SMRT-ID-1+2 with VP-RAR-LBD ± RXR-LBD and AM580 (100 nM). *B*, interaction of Gal-SMRT-ID-1+2 with mutant RAR-LBDs in a heterodimer with RXR-LBD in the presence and absence of AM580 (100 nM). *C*, GST pull-down analysis showing interaction of RAR-LBD mutants with GST-SMRT-ID-2 in the presence or absence of (E)-4-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphtylenyl)-1-propenyl] benzoic acid (TTNPB) (100 nM). The *lower panel* shows the specific binding of *in vitro* translated, [^{35}S]methionine-labeled receptors on GST-SMRT-ID-1+2 matrix analyzed by SDS-PAGE (as described under "Material and Methods"), visualized by autoradiography, and then analyzed by densitometry (*upper panel*). Results are presented as the specific binding to GST-SMRT-ID-2 in arbitrary units (*AU*). 10% of the labeled protein input is shown in the *lower panel*.
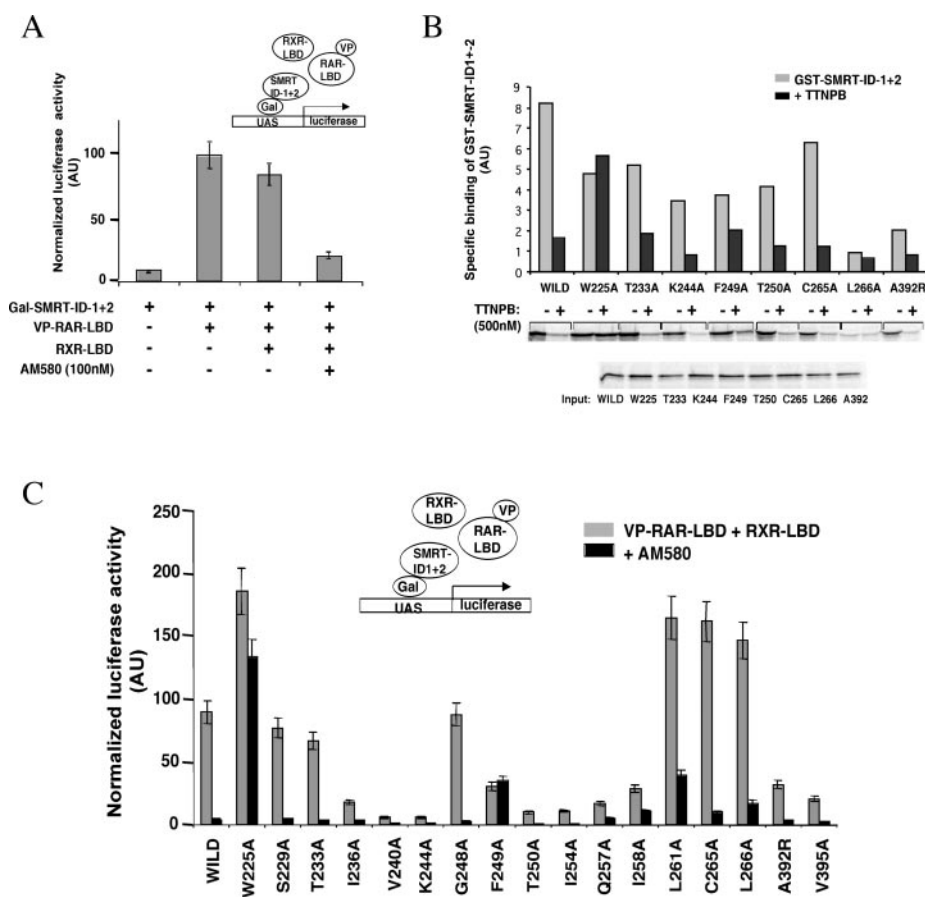
Fig. 4*C*. The majority (ten) of the mutants showed reduced co-repressor binding, and six of them showed a combined co-repressor co-activator binding deficiency. There were a few mutants (I236A, T250A, and V395A) that showed a reduced ability to bind co-repressor, and there was only one (L261A) with solely reduced co-activator binding. The existence of these mutants (*i.e.* mutants that 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 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 cofactors we believe that these two mutants do not alter the binding site *per se* but represent mutations of an intrinsic regulatory site.

In Fig. 4*D* the various mutations that affect both co-activator and co-repressor binding on the structure are color-coded. Residues that, when mutated, reduced both co-activator and co-repressor binding are shown in *blue* and represent the cofactor binding surface or docking site. Residues in *cyan* reduced co-repressor binding but not co-activator binding. The *magenta* residue Leu-261, if mutated to alanine, reduced co-activator binding without altering co-repressor binding. Mutation of Trp-225 (in *red* on the *right*) increased co-repressor binding and diminished co-activator binding, while Ala-392 (in *red* on the *left*), if mutated to arginine, reduced co-repressor binding but enhanced 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 helix 12, differentially influence co-activator and co-repressor binding.
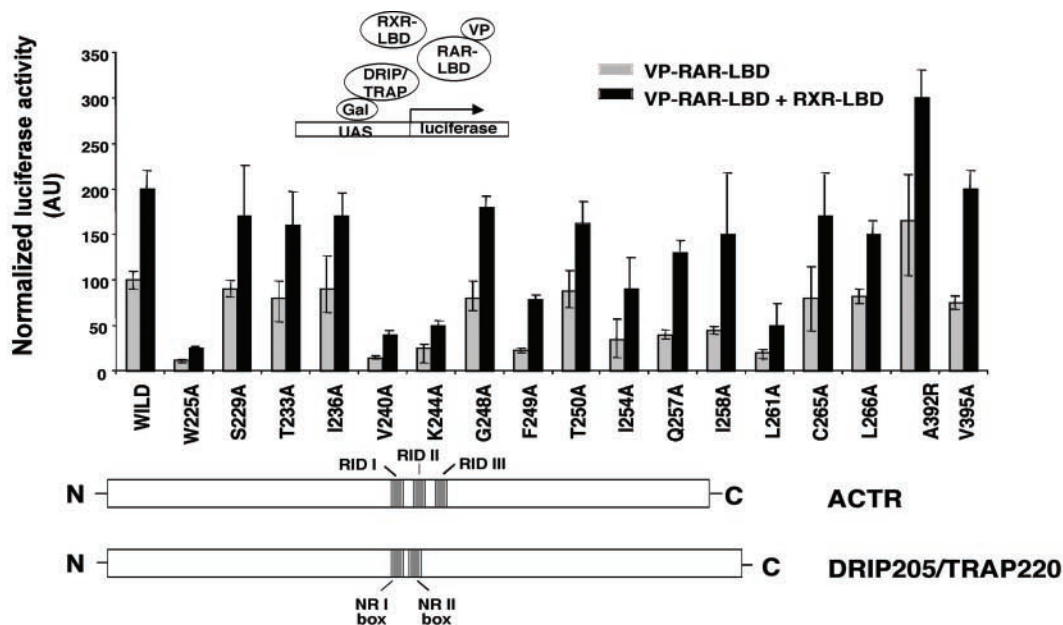
Transcriptional Activity of the Mutant Receptors—It is important to understand whether the transcriptional activity of a nuclear receptor is determined simply from the cofactor bind-

ing 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 whether, 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 cofactor binding and release profile has a predictive value on overall transcriptional activity. Six mutants were compared: three were deficient in both co-activator and co-repressor binding (V240A, K244A, and I254A), and three other ones differentially affected cofactor association (W225A, A392R, and V395A).
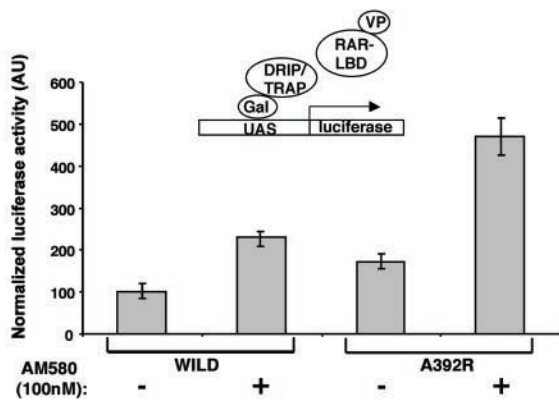
Fig. 5*A* shows the activity of wild type and mutant receptor LBDs (fused to the Gal4-DBD) in the absence of exogenous ligand. In this experiment wild type receptor repressed transcription of the reporter gene by ~50%. W225A repressed transcription significantly more strongly than wild type (~95%). The 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 to assess the ligand-inducible transcriptional activity (Fig. 5*B*). The wild
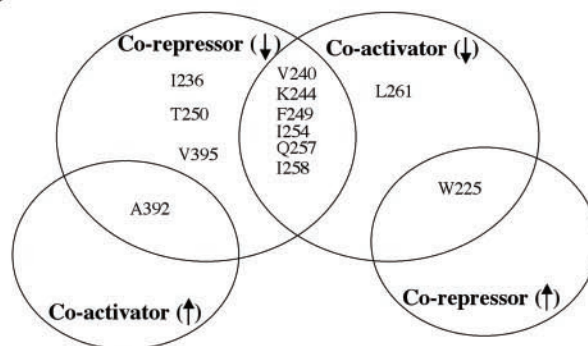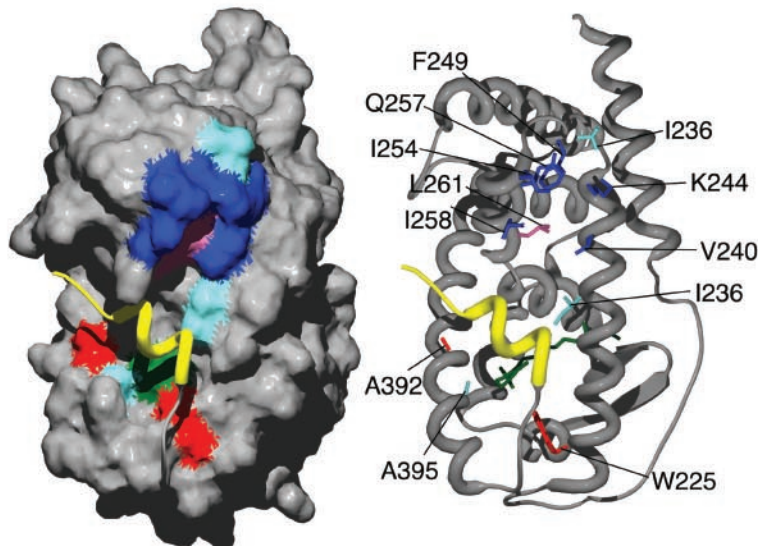
FIG. 4. **Characterization of co-activator binding by monomer and heterodimer mutant RAR-LBDs.** Interaction analysis of Gal-DRIP205/TRAP220 fusion proteins and wild type and mutant VP-RAR-LBD chimeric receptors was carried out in mammalian two-hybrid assays. The strength of interactions is expressed as normalized luciferase activity. The mean of at least three independent measurements ± S.D. is presented. *A, upper panel*, shows interaction of Gal-DRIP205/TRAP220-RIDs with VP-RAR-LBD mutants in monomer (*gray column*) and heterodimer (*black column*) studies in the presence of AM580 (100 nM). Binding is expressed as the percentage of binding observed for the wild type
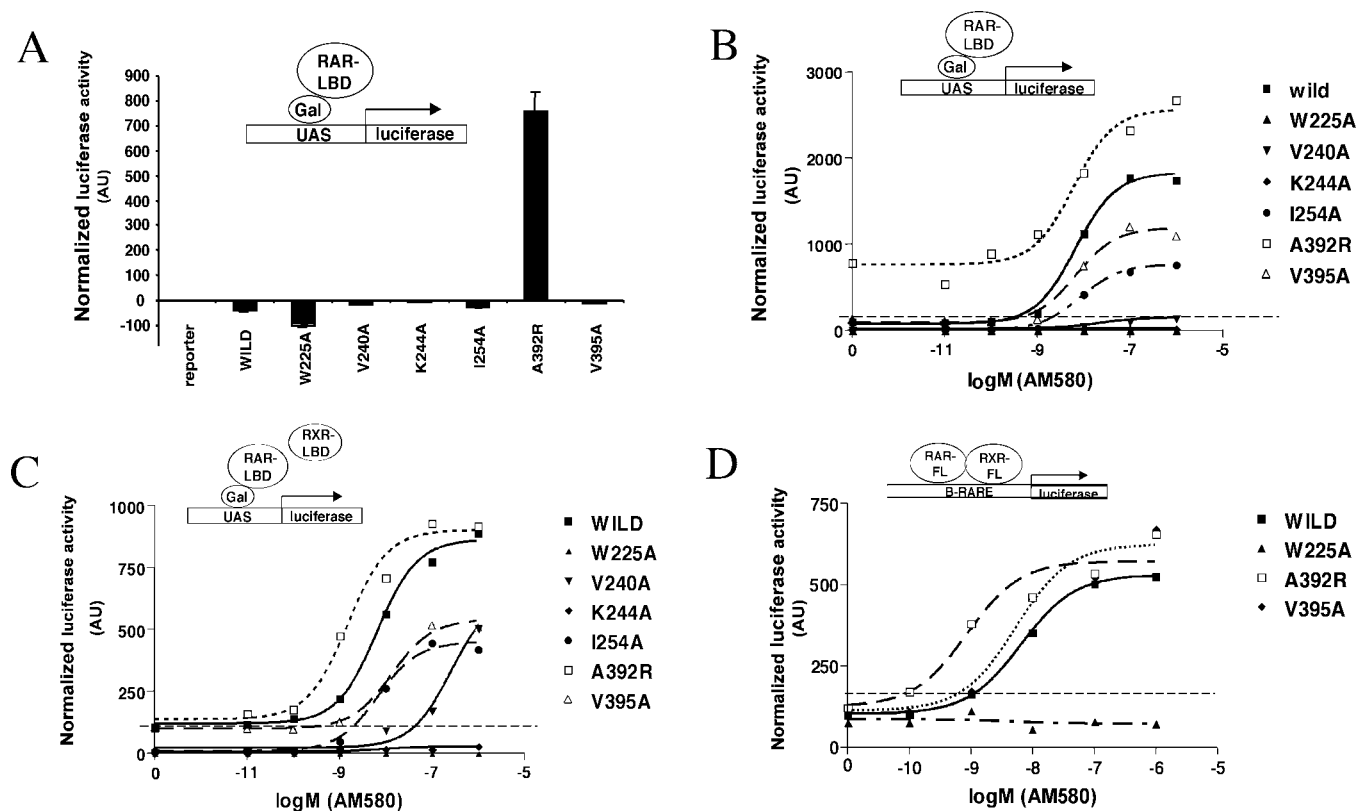
FIG. 5. **Transcriptional activity of mutant receptors.** The transcriptional consequences of selected mutations were determined using transient transfection assays. Transient co-transfection assays were carried out with the indicated plasmid constructs, and transcriptional activity is expressed as normalized luciferase activity. Curves were fitted by Prism (GraphPad Prism Software, Inc.). *A*, transcriptional repression or activation of unliganded RAR mutants. The *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. The *broken line* indicates the basal activity of the reporter construct. *D*, dose-response curves of full-length RAR·RXR heterodimers co-transfected with β RARE-TK-LUC upon AM580 treatment. The *broken line* indicates the basal activity of the reporter construct. *AU*, arbitrary units.

type RAR-LBD showed a dose-response curve with a half-maximal induction (EC_{50}) at 7 nM AM580. The I254A and V395A mutants have an EC_{50} 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 (Fig. 5*C*). Interestingly the A392R mutant showed an altered activity in this assay: its constitutive activity in the absence of ligand was significantly reduced, and it showed an unexpected increased potency for AM580 ($EC_{50} = 1.5$ 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 (Fig. 5*C*).

Finally, to examine the effect of mutations in the most phys-

iological 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 showed a dose-dependent transactivation profile with an EC_{50} of 6.7 and 5.5 nM, respectively (Fig. 5*D*). In the same assay the W225A mutant showed no significant transactivation, while 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 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 radiolabeled β RARE probe (Fig. 6). Addition of SMRT ID-1+2 (expressed in bacteria) gave a clear supershift. As expected, the co-repressor was not dissociated by the RXR-specific ligand LG100268 but was readily dissociated on addition of AM580 (Fig. 6). Compared with the wild type

RAR-LBD (in the presence of ligand) in the same assay. The *lower panel* is the schematic representation of the two nuclear receptor box motifs (*NRI* and *NRII*) in the full-length DRIP 205/TRAP220 (amino acids 1–1566) 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 (100 nM). *C*, Venn diagram showing the altered co-activator and co-repressor interactions of RAR mutants. *D*, surface and ribbon representation of the RARγ-LBD (Protein Data Bank code 2LBD). Residues on helix 3, helix 4, helix 5, and helix 11 mutated in this study are colored according to their effect on cofactor 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 cofactor binding. The ligand ATRA is shown in *green*. Helix 12 is shown in *yellow*. *AU*, arbitrary units.
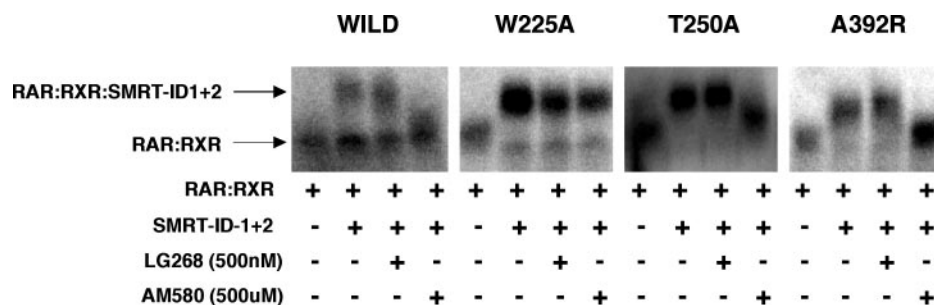
Mutations Biasing Cofactor Balance

FIG. 6. **Electrophoretic mobility shift analysis of wild type and mutant full-length RAR·RXR heterodimers.** Electrophoretic mobility shift analysis 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.

receptor, W225A bound SMRT ID-1+2 more strongly, and this interaction was not dissociated by either ligand. T250A and A392R showed co-repressor binding activity similar to that of wild type, consistent with the results obtained in previous experiments (Fig. 5).

Homologous Mutations in hTRβ Have Effects Similar to Those in hRARα—Finally we reasoned that, if the two residues (Trp-225 and Ala-392) 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 (F264A and A431R) were made in VP16 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, while A431R showed a somewhat reduced interaction (75% of the wild type, Fig. 7). These results were comparable to the equivalent mutations in RAR. Moreover, upon ligand (triiodothyronine) treatment, the interaction of the F264A mutant with SMRT was hardly affected by ligand. In contrast A431R was more sensitive to ligand than was 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.

DISCUSSION

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

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.* (32) provided the first structural evidence that the co-repressor and co-activator binding sites are indeed largely overlapping. These studies combined with biochemical analyses suggest that the two types of cofactors 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
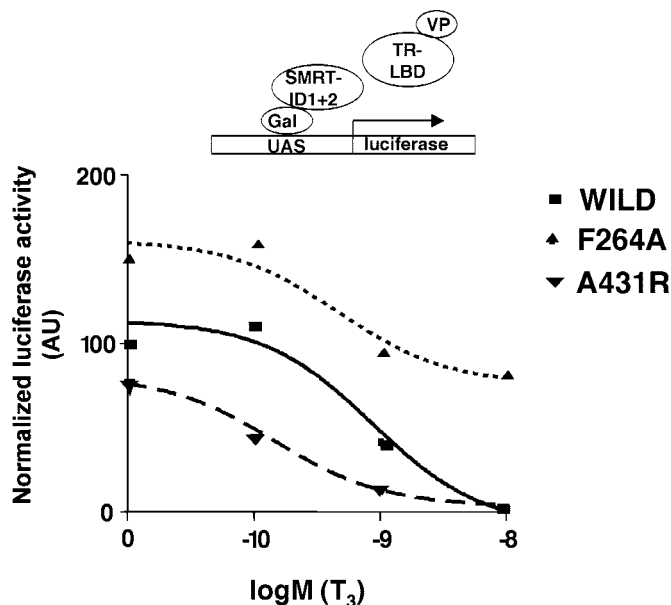

FIG. 7. **Interaction of SMRT ID-1+2 with TR mutants containing mutations equivalent to RAR-LBD W225A and A392R.** VP16 fusion proteins of hTRβ-LBD (wild type and F264A and A431R, equivalent to W225A and A392R in RARα) were tested for interaction with Gal-SMRT-ID-1+2 in mammalian two-hybrid analysis. Transient transfections of VP16 fusion hTRβ mutants were carried out in the presence of increasing amounts of triiodothyronine (T_3). The strength of interactions is expressed as normalized luciferase activity; representative experiments are shown. Curves were fitted by Prism (GraphPad Prism Software, Inc.). *AU*, arbitrary units.

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. While 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 cofactor binding balance?

To explore this we first established a simple co-repressor and co-activator interaction assay using mammalian two-hybrid analysis. We demonstrated that ID-2 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 Cofactor Binding—Consistent with previous mutagenesis and structural studies, we found that mutations in helices 3 and 4 disrupt both
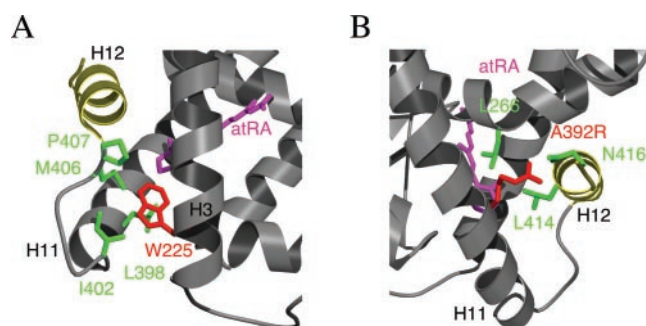
FIG. 8. **Residues Trp-225 and Ala-392 may influence the orientation of helix 12 (*H12*).** *A*, Trp-225 (*red*) is in Van der Waals contact with several residues (*green*) in the loop between helices 11 (*H11*) and 12. Mutation of this residue to alanine may disfavor the active position of helix 12 (*yellow*). The ligand ATRA is shown in *magenta*. The structure shown is that of RARγ-LBD (Protein Data Bank code 2LBD). *B*, mutation of Ala-392 to arginine (*red*) may stabilize the active position of helix 12 (*yellow*) through contacts to helix 12. *atRA*, all-*trans*-retinoic acid.

co-activator and co-repressor binding. 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. Significantly the majority of mutations made on this cofactor 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 (Ile-236, Thr-250, and Val-395). In contrast, mutation of Leu-261 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, Fig. 5).

Residues That Determine the Basal Activity of the Receptor—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 led to dramatic differences with respect to the different cofactors. 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 Trp-225 (in helix 3) to alanine resulted in a mutant with intrinsically high affinity for co-repressor binding combined with very low co-activator binding. This mutation significantly tips the balance toward co-repressor binding with almost total loss of co-activator binding. Analysis of the transcriptional activity of this receptor shows that, while 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 Trp-225 is in Van der Waals contact with residues in the loop between helices 11 and 12: Leu-398 (4.1 Å), Ile-402 (3.6 Å), Met-406 (3.5 Å) and Pro-407 (3.9 Å) (Fig. 8*A*) (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 that have been explicitly shown to destabilize helix 12 (54). It should be noted that Trp-225 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 changed the basal activity of RARα is A392R. This receptor showed minimal co-repressor binding in conjunction with significantly increased co-activator binding activity. Again the ligand binding affinity remained 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 (Fig. 8*B*) (49). The larger arginine side chain would, however, be in contact with a number of residues in helices 4 and 12. These include Van der Waals contacts to Leu-266 (3.3 Å) and Leu-414 (4.2 Å) as well as hydrogen bonds to the side chain of Asn-416 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 (Tyr-473, Leu-476, and Tyr-477) 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 Cofactor Binding Translate into Large Changes in Transcriptional Activity—It is clear from the analyses of these mutant receptors that the determination of cofactor 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 cofactor binding potential translates into a large shift in transcriptional activity (Fig. 5). In this respect the LBD appears to function as a biological amplifier where small changes affecting cofactor 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 cofactor balance.

A Critical Role for Helix 12 in Determining Cofactor 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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