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Differential Action on Coregulator Interaction Defines Inverse Retinoid Agonists and Neutral Antagonists

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SUMMARY

Retinoic acid receptors (RARs) are ligand-dependent transcription factors that control a plethora of physiological processes. RARs exert their functions by regulating gene networks controlling cell growth, differentiation, survival, and death. Uncovering the molecular details by which synthetic ligands direct specificity and functionality of nuclear receptors is key to rational drug development. Here we define the molecular basis for (*E*)-4-[2-[5,6-Dihydro-5,5-dimethyl-8-(2-phenylethynyl)naphthalen-2-yl]ethen-1-yl]benzoic acid (BMS204,493) acting as the inverse pan-RAR agonist and define 4-[5,6-Dihydro-5,5-dimethyl-8-(quinolin-3-yl)naphthalen-2-carboxamido] benzoic acid (BMS195,614) as the neutral RARα-selective antagonist. We reveal the details of the differential coregulator interactions imposed on the receptor by the ligands and show that the anchoring of H12 is fundamentally distinct in the presence of the two ligands, thus accounting for the observed effects on coactivator and corepressor interactions. These ligands will facilitate studies on the role of the constitutive activity of RARs, particularly of the tumor suppressor RARβ, whose specific functions relative to other RARs have remained elusive.

INTRODUCTION

Three retinoic acid receptors (RARα [NR1B1], RARβ [NR1B2], and RARγ [NR1B3]) mediate biological processes regulated by retinoids (Chambon, 1996; Germain et al., 2006; Mark et al., 2006). Mechanistic studies of RARs and other members of the nuclear receptor (NR) superfamily have demonstrated that ligand binding modulates the ability of the receptor to "communicate" with its intracellular environment, which entails essentially receptor-protein and receptor-DNA/chromatin interactions. Upon binding, a ligand allosterically alters receptor surfaces required for interaction with coactivator (CoA) and corepressor (CoR) complexes that are central to the control of target gene transcription. RAR agonists facilitate the exchange between CoR and CoA complexes by destabilizing CoR and stabilizing CoA-RAR interfaces. The subsequent recruitment of epigenetically active and/or chromatin-modifying complexes leads to chromatin alterations that facilitate activation of target gene expression by the basal transcriptional machinery. In the absence of agonists, RARs can actively repress gene transcription as nonliganded RAR establishes CoR complexes comprising repressive factors, which are believed to promote chromatin compaction (for recent reviews see Gronemeyer et al., 2004; Lonard and O'Malley, 2007; Rosenfeld et al., 2006). The physiological impact of CoRs in NR signaling has been recently revealed by gene deletion studies (Astapova et al., 2008; Nofsinger et al., 2008).

This active transcriptional silencing mediated by RARα in complex with its retinoid X receptor (RXR) heterodimeric partner is a direct consequence of their association with CoRs such as silencing mediator for retinoid and thyroid hormone receptors (SMRT)/T3 receptor-associating cofactor (Chen and Evans, 1995; Jepsen et al., 2007; Sande and Privalsky, 1996) or nuclear receptor corepressor (NcoR)/receptor interacting protein 13 (Horlein et al., 1995; Seol et al., 1996; Zamir et al., 1996). These cofactors exhibit modular structures facilitating the assembly of high molecular weight complexes and serve also as CoRs for other nuclear orphan receptors (Zamir et al., 1996) and transcription factors (see, for example, Evert et al., 2006; Melnick et al., 2002). Among others, Sin3A and histone deacetylases are factors found in these complexes (Alland et al., 1997; Heinzel et al., 1997; Nagy et al., 1997). The formation of a CoR complex with associated histone deacetylase activity at the promoters of NR target genes is believed to cause transcriptional silencing through histone deacetylation and chromatin condensation at receptor-targeted loci. Binding of an agonist to RAR induces a conformational switch of the ligand-binding domain (LBD), leading to the release of CoRs and concomitant association of CoA complexes.

Our understanding of how ligand binding leads to the activation of NRs has been greatly advanced by structural studies of

NR LBDs and their interactions with CoA- and CoR-derived peptides (Bourguet et al., 2000b). Together with biochemical analyses, these studies demonstrated that the activation function 2 in helix 12 (AF-2 H12) of the LBD is critically involved in NR activation. Indeed, in the case of RARs, agonistic ligands establish a direct contact to H12 residues and maintain the helix in a position that generates a surface with increased affinity for the LxxLL motifs (also called NR box), which are located in the NR-interacting surface of CoA proteins. In all reported cases, the LxxLL peptide forms a short α helix, which is bound to the hydrophobic groove generated by the carboxy-terminal part of H3, the loop L3-4, and H4 of the LBD.

Correlative analyses of biochemical data and sequences provided evidence that CoA and CoR recruitment share a similar molecular basis. Evaluating CoR binding to mutants in the CoA binding site of the thyroid hormone receptor α (TRα, NR1A1) demonstrated that mutations that impair activation and CoA recruitment also decrease repression and CoR binding, thus indicating that NCoR binds to a NR surface topologically related to that involved in CoA interaction (Hu and Lazar, 1999). Mapping of the NR determinants of NCoR binding using a series of mutations introduced in TRα indicated that the groove formed by residues from helices H3 and H4 corresponds to the primary CoR binding site but that H11 and H12 are not involved.

The C-terminal NR-interacting region of SMRT and NCoR is composed of two or three independent interacting subdomains (IDs) (Cohen et al., 2001; Seol et al., 1996; Zamir et al., 1996). Examination of the two interaction domains ID1 and ID2 in SMRT and NCoR revealed sequences (CoRNR1 and CoRNR2 by analogy to NR boxes of CoAs; note that the numbering of CoRNR1 to CoRNR2 is from N to C terminus and that the ID1/2 and CoRNR1/2 nomenclature is used synonymously) similar but not identical to the LxxLL motif of CoAs, which were also predicted to adopt an amphipathic helical conformation (Hu and Lazar, 1999; Nagy et al., 1999; Perissi et al., 1999). Chimeric CoRNR box peptides were designed to show that although the consensus hydrophobic core ΦxxΦΦ is necessary and sufficient for CoR binding, sequences flanking the CoRNR box strengthen the interaction and determine NR specificity. In addition, within CoR, the two CoRNR boxes are not equivalent. CoRNR box 1 interacts almost exclusively with RAR/TR and CoRNR box 2 binds to both heterodimeric partners. Perissi et al. (1999) further extended the analogy to the helical CoA LxxLL motifs. They proposed a model in which the CoRNR box motif would fold in a significantly longer LxxI/HIxxxI/L helix when compared to the CoA LxxLL NR box motif. This model is supported by the crystal structure of a ternary complex containing the PPARα-LBD bound to the SMRT CoRNR2 motif (Xu et al., 2002). In the absence of agonists, the CoRNR box helix could interact with the H3-H4 hydrophobic groove displayed by unliganded receptors. However, in the presence of an agonist, the repositioning of the activation helix H12 in its active (holo) conformation would prevent this interaction. Indeed, in contrast to the unliganded NR case, the length of the helix that can be accommodated by the H12-containing groove is strictly defined by the presence of the charge clamp that specifically recognizes helices of the NR box type (Nolte et al., 1998). Together, these results showed that, unexpectedly, CoAs and CoRs use similar mechanisms of interaction with distinct NR conformations. Discrimination by NR H12 helix of the lengths of CoA and CoR interaction helices occupying partially overlapping binding sites may constitute the molecular basis of ligand-mediated coregulator exchange on NRs.

Retinoids and rexinoids (RXR-selective ligands) have significant promise for the treatment and prevention of cancer and other diseases (Altucci and Gronemeyer, 2001; Altucci et al., 2007; Shulman and Mangelsdorf, 2005). As the natural ligand all-*trans* retinoic acid (atRA) acts as pan-RAR agonist and 9-*cis* retinoic acid activates both RARs and RXRs, synthetic medicinal chemistry efforts have been directed to the generation of RXR and RAR subtype-selective ligands with the goal to improve therapeutic indices by eliminating adverse effects possibly caused by activating/repressing the action of other receptor subtypes. Consequently, many synthetic retinoids have been generated that exhibit a distinct pattern of agonistic/antagonistic activities with the three RARs (for reviews see Dawson, 2004; de Lera et al., 2007).

Here we report on the comparative mechanistic analysis of two distinct types of retinoid antagonists, BMS204,493 and BMS195,614 (hereafter referred to as BMS493 and BMS614; for chemical structures see Figure 1A). We define BMS493 as inverse agonist because it strongly enhances NCoR interaction by selectively stabilizing the ID1/CoRNR1 interface and destabilizing ID2/CoRNR2 association in the context of the RAR-RXR heterodimer. In a striking contrast, BMS614 acts as a neutral antagonist, as it does not significantly affect NCoR binding and moderately decreases SMRT binding to RAR; importantly, it does not support CoA interaction but rather antagonizes agonist-induced CoA recruitment. Probing the ligand-induced conformational changes revealed that the anchoring of H12 is fundamentally distinct in the presence of BMS493 and BMS614, thus accounting for the divergent effects on CoA and CoR interaction. This study illustrates the potential of NR-based drug design and reveals that subtle changes in the chemical structure of the ligand translate in very selective effects on the communication potential of the receptor with the promise of a drug-directed fine tuning of the cognate signaling pathways.

RESULTS

Inverse Agonists Modulate CoR Binding to RAR

Transient transactivation-based ligand competition assays derived from challenging 10 nM TTNPB pan-RAR agonist with an increasing concentration of BMS493 revealed that BMS493 is a powerful pan-RAR antagonist (Figure 1B). Two-hybrid analyses performed in Cos cells with chimeras containing the GAL4 DNA binding domain fused to the NCoR or SMRT (termed Gal-NCoR and Gal-SMRT) and the LBD of either RARα, β, or γ fused to the activation domain of VP16 (termed RAR-VP16) showed not only that RAR-CoR interaction was maintained in the presence of BMS493 but, moreover, that NCoR interaction was strongly (NCoR; Figure 1C) or moderately (SMRT; Figure 1D) enhanced in the presence of BMS493 (see figure legend for a more detailed explanation of the two-hybrid results). Due to this reinforcement of CoR interaction with RARs, we reasoned that BMS493 should increase the repression function of all three RARs in intact cells. Indeed, BMS493 efficiently decreased the basal level of a retinoic acid-responsive chimeric gene ($RARE_3$-tk-Luc) in transient transfections with all three RARs in HeLa cells (Figure 2A). Moreover, in the same cell line, BMS493 decreased both the expression of

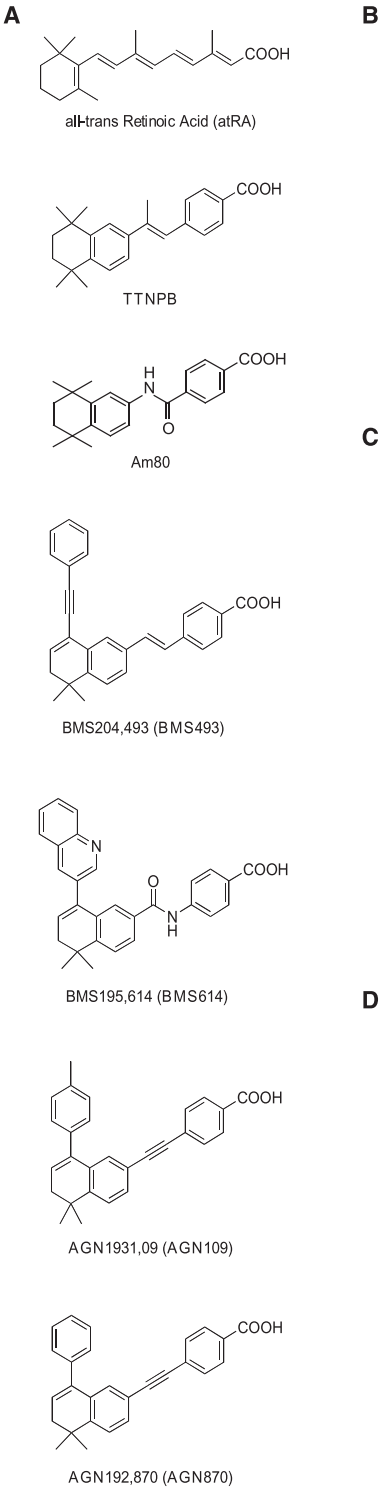

Figure 1. RAR Antagonist Potential of BMS493

(A) Chemical structures of RAR ligands.

(B) To assess antagonistic activities of BMS493, HeLa cells were cotransfected with reporter (17 m)5x-G-Luc and Gal-RARα (closed squares), Gal-RARβ (open triangles), or Gal-RARγ (closed circles). The reporter was activated by 10 nM TTNPB (100%) and increasing concentrations of BMS493 were added, as indicated.

(C and D) Mammalian two-hybrid assays with (17 m)5x-G-Luc and Gal-NCoR (C) or Gal-SMRT (D) as bait and VP16-RARs as prey were performed in HeLa cells to assess the influence of indicated synthetic retinoids at 1 μM on interaction between all three RARs and both CoRs NCoR and SMRT in a cellular context. 100% corresponds to reporter gene transcription induced in the absence of ligand (RAR apo forms). In the two-hybrid assays, a chimeric luciferase-based reporter gene ((17 m) 5x-G-Luc) is transfected together with two expression vectors. One expresses a fusion protein (Gal-NCoR or Gal-SMRT) that binds through the Gal DNA binding domain (Gal) to the pentamer of the "17m" DNA recognition site in the reporter gene and contains the C-terminal region of the CoR, which can interact with RARs. Note that this region does not recruit HDAC repressor complexes. The other vector expresses a second fusion protein composed of the VP16 acidic transcription activation domain, a domain that confers constitutive transcription activation if it is brought close to a promoter and the LBD of RAR. If a ligand induces RAR-CoR interaction this results in (indirect) recruitment of the VP16 acidic transcription activation domain to the promoter of the luciferase reporter gene, thereby inducing transcription and luciferase protein production that is quantitated in the luminometer.

endogenous *CYP26* (Figure 2B), a RAR target gene known to harbor a retinoic acid response element in its promoter, and the chromatin acetylation status of the *CYP26* promoter in vivo (Figure 2C). Thus, BMS493 is a bona fide pan-RAR inverse agonist.

Transient transactivation studies with all three RARs revealed that two other synthetic retinoids, AGN192,870 and AGN193,109 (hereafter referred to as AGN870 and AGN109; for chemical structures see Figure 1A), displayed divergent receptor-selective activity (Figure 2A). While AGN870 is a weak agonist for RARα and β, it acts as a weak inverse agonist for RARγ, in keeping with its ability to stabilize NCoR binding to RARγ, which is significantly higher than to RARα or β (Figure 1C). Notably, SMRT binding was rather decreased in the presence of AGN870; this effect was most pronounced for RARβ (Figure 1D). This demonstrates that AGN870 modulates RAR activity in a receptor subtype and CoR-selective manner and that the acquired pattern of coregulator interaction apparently accounts for its activity as a RARα/β agonist and (weak) RARγ inverse agonist.

Contrary to AGN870, AGN109 is a weak inverse agonist for RARα and β (Figure 2A), which is apparently due to its greater

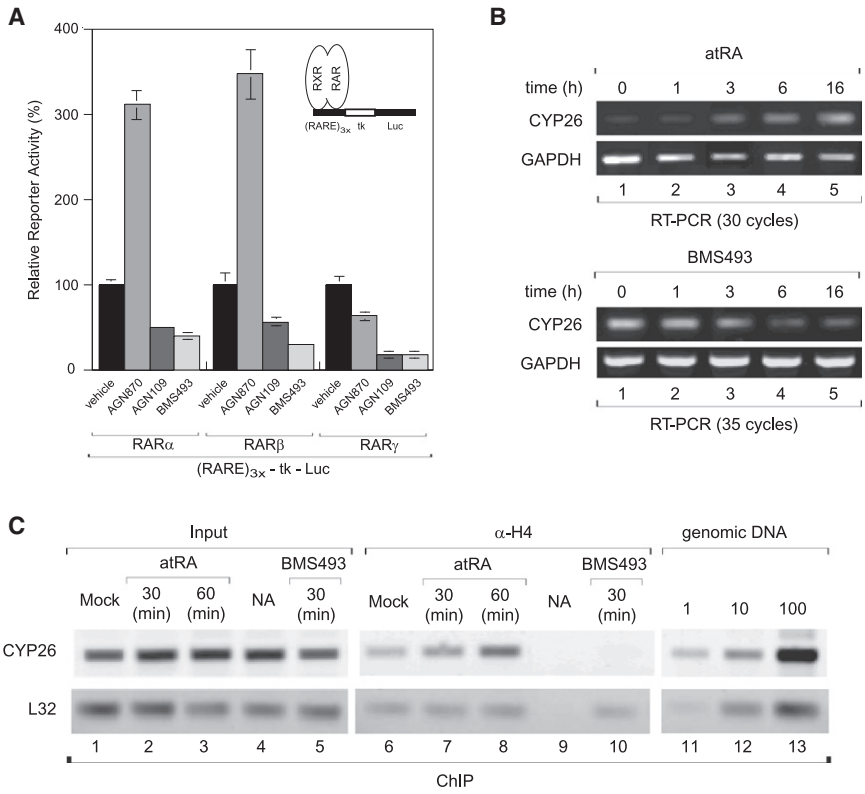

Figure 2. RAR Inverse Agonist Potential of BMS493-Inducing Transcriptional Repression in HeLa Cells

(A) HeLa cells were cotransfected with the reporter (RARE)3x-tk-Luc and RARα, β, or γ in the presence of the indicated synthetic retinoids at 1 μM or in the absence of ligands (100%, basal transcriptional activity).

(B) Kinetics of *CYP26* expression investigated by semiquantitative RT-PCR performed with primers specific for retinoic acid-responsive *CYP26* or *GAPDH* (used as control) mRNAs using total RNA of HeLa cells either treated with atRA or BMS493 at 1 μM, as indicated.

(C) ChIP assays using antibodies directed against acetylated histones H4 and HeLa cells treated with either atRA or BMS493 at 1 μM for 30 or 60 min. Immunoprecipitated chromatin was analyzed by PCR using primers specific for *CYP26* or *L32* (used as control) promoters. NA, ChIP assays were performed using no antibody.

ability to enhance NCoR binding (Figure 1C). No significant effect on SMRT binding was seen for RARα and β, but the interaction of both SMRT and NCoR with RARγ was strongly stimulated by AGN109 (Figures 1C and 1D). Relative to BMS493, the effect of AGN109 on the interaction with RARγ was more pronounced for NCoR than for SMRT. Consequently, AGN109 is another inverse pan-agonist with a particular RARγ-selective effect on NCoR recruitment. Together the above results demonstrate that synthetic retinoids can be designed that modulate RAR-CoR interaction in a RAR subtype and CoR-selective fashion.

BMS493 Stabilizes NCoR-RAR Binding by Targeting CoRNR Box 1

To assess whether one or more of the three receptor IDs of NCoR mediate the enhancing effect of BMS493, we studied its effects on the interaction of the previously defined CoRNR box peptides 1 and 2 (CoRNR1 and CoRNR2) (Hu and Lazar, 1999) with the RARα LBD by electrospray ionization mass spectrometry (ESI-MS) under nondenaturing conditions. Note that we have not observed any significant interaction of ID3 with RARs or RAR-RXR heterodimers (see below). In the absence of ligand, ESI-MS revealed two approximately equally abundant populations of molecules that correspond to the RARαLBD and the RARαLBD-CoRNR1 complex (Figure 3A, top). Addition of BMS493 resulted in the disappearance of these species and the appearance of a single novel peak with a mass corresponding to the RARαLBD-BMS493-CoRNR1 complex (Figure 3A, bottom). No such effect was observed with the CoRNR2 peptide even though both the LBD and the LBD-CoRNR2 complex efficiently bound BMS493, as is obvious from mass shift of these species, which corresponds to the added mass of the retinoid (Figure 3B).

We then addressed whether the features of BMS493 were affected by heterodimerization. By using mass spectrometry we studied the binding efficiencies of the synthetic peptides encompassing CoRNR1 and CoRNR2 in the context of the purified RXRα LBD-RARαLBD heterodimer. In the absence of ligand, equal amounts of heterodimers bound either CoRNR1 or CoRNR2 (45% and 40%, respectively; Figures 3C and 3D, top panels; note that the peptides were added in molar excess over the heterodimer). Both peptides dissociated quantitatively in the presence of the RAR agonist Am80 (Figures 3C and 3D, bottom panels). No tetrameric RAR-RXR-CoRNR1-CoRNR2 complex was observed in experiments where the two peptides were added simultaneously (data not shown). As with RAR monomer, the inverse agonist BMS493 strongly stabilized the interaction between the heterodimer and CoRNR1 (Figure 3C, middle panel). However, in a striking contrast to the RAR monomer results, the heterodimer-CoRNR2 complex was significantly destabilized upon binding of this inverse agonist (Figure 3D, middle panel), such that the most abundant species (≥80%) is the RXR-RAR/BMS493-CoRNR1 complex. Therefore, other than in the case of the RAR monomer (see above), the BMS493-bound heterodimer does not apparently contain a binding surface that is accessible for CoRNR2 of NCoR.

Two-hybrid analyses with the previously reported chimeras, which encompass the Gal4 DNA binding domain and one of the three NCoR IDs (Gal-ID1, Gal-ID2, and Gal-ID3), fully supported the above in vitro results (Figure 4A). Gal-ID1 interaction with VP16-RARα was strongly stimulated by BMS493, whereas no ligand effect was seen with Gal-ID2. No significant interaction was seen with Gal-ID3. Mutations were introduced in both NCoR ID1 and ID2 to test the contribution of each ID in the context of the NCoR containing both IDs. Importantly, NCoR harboring a mutated ID1 or a mutated ID2 behaved like ID2 alone or ID1

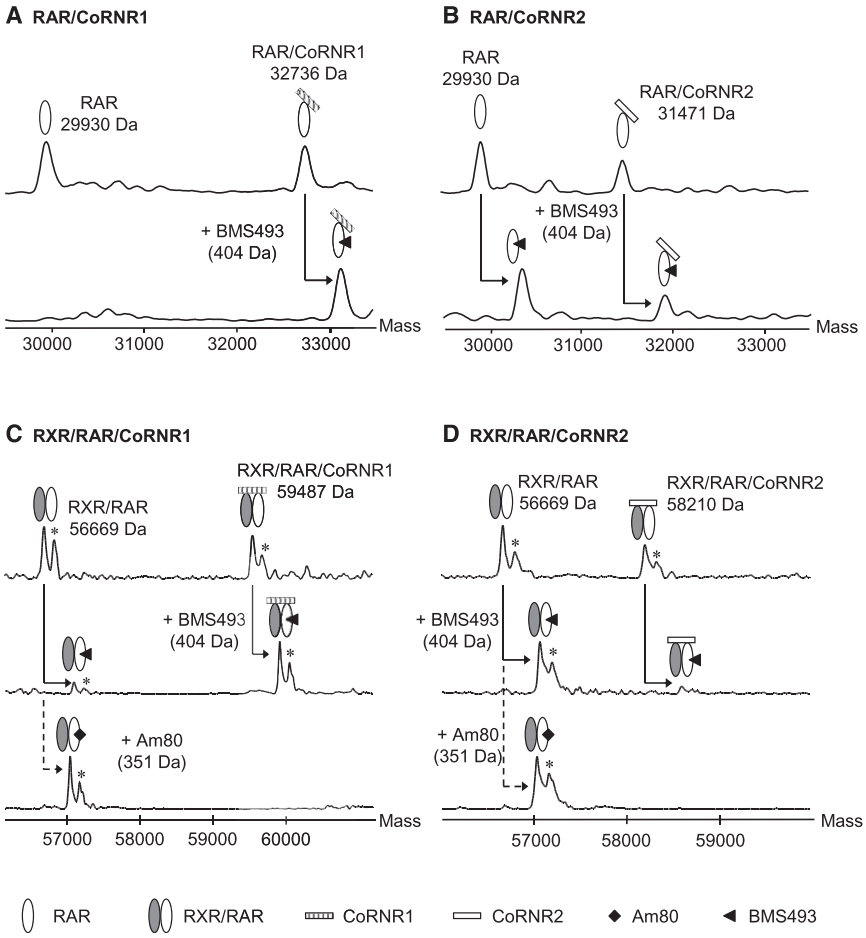

Figure 3. BMS493 Stabilizes NCoR CoRNR1 Peptide Binding to RARα

(A) Positive ESI mass spectra of RARαLBD-CoRNR1 complexes in the absence of ligand (top) or in the presence of 2-fold molar excess of BMS493 (bottom).

(B) Positive ESI mass spectra of RARαLBD-CoRNR2 complexes in the absence of ligand (top) or in the presence of 2-fold molar excess of BMS493 (bottom).

(C and D) Positive ESI mass spectra of RXRαLBD-RARαLBD-CoRNR1 complexes in the absence of ligand (top) or in the presence of 2-fold molar excess of BMS493 (middle) or Am80 (bottom), or RXRαLBD-RARαLBD-CoRNR2 complexes in the absence of ligand (top) or in the presence of 2-fold molar excess of BMS493 (middle) or Am80 (bottom). Note that the illustrations indicate the existence of a quaternary BMS493/RAR/RXR/CoRNR complex but are not meant to specify interface(s) of the CoRNR peptides with the heterodimer. Peaks labeled with an asterisk correspond to species with an additional N-terminal methionine. Please note that the numbering of CoRNR1 to CoRNR2 is from N to C terminus.

alone, respectively (Figure 4B). Together our above results demonstrate that the BMS493-enforced NCoR interaction with RARα is mediated specifically by ID1.

This conclusion was further supported by investigating NCoR interaction with the two other RARs. Two-hybrid assays with RARβ, which binds CoRs less efficiently than RARα, and NCoR ID mutants revealed that BMS493 strongly increased the interaction between NCoR and RARβ in an ID1 integrity-dependent manner (Figure 4C). Similar conclusions were made for RARγ (data not shown). Note that the weaker RARβ interaction with NCoR is apparently due to a weaker interaction of ID1 with RARβ relative to RARα. Overall our results demonstrate that BMS493-enhanced RAR-NCoR binding is due to a selective effect of the ligand on the interaction of the receptors with CoRNR1/ID1 and a destabilization of the CoRNR2/ID2 interaction seen for the RAR monomer.

Mechanism of Action of the Neutral Antagonist BMS614

Characterization of the molecular actions of other RAR antagonists, such as BMS614, led to the definition of "neutral retinoid antagonists" as compounds that destabilize CoR complexes but do not induce recruitment of CoAs. Two-hybrid analyses with chimeras composed of the Gal4 DNA binding domain and the NR ID of the CoA TIF2 (referred to as Gal-TIF2.5; this fusion protein harbors all three LxxLL NR boxes of TIF2) showed that Gal-TIF2.5 interaction with VP16-RARα was strongly stimulated by the agonist Am80, while no significant interaction was seen with BMS614 or BMS493; both antagonists efficiently counteracted Am80-induced interaction (Figure 5A). Fluorescence anisotropy experiments with a fluorescein-labeled NR box 2 peptide of SRC-1 fully supported the two-hybrid results (Table 1). In keeping with its agonistic activity, Am80 binding to RARα LBD monomer strongly enhanced the binding affinity of SRC-1 NR box 2 more than 17-fold (apparent K_d values are 0.47 μM and 8.18 μM for the Am80-RARα and apo-RARα LBDs, respectively). The BMS493-RARα LBD displayed a similar affinity for SRC-1 NR box 2 as the apo-RARα LBD, while BMS614 even reduced the affinity of RARα by 11-fold (apparent K_d = 92.70 μM).

Two-hybrid analyses in Cos cells showed not only that RAR-CoR interaction was enforced in the presence of BMS493 but, moreover, that BMS493 was able to induce CoR interaction even with the so-called RARα "AHT" mutant (RAR_{AHT} harbors the mutations A194G, H195A, and T198A in helix H1) (Horlein et al., 1995; Schulman et al., 1997), which was reported to be deficient for CoR binding (Figure 5B) (Vivat et al., 1997). Indeed, very weak binding was seen with apo-RAR_{AHT} but BMS493 is apparently able to restore CoR binding to an extent similar to that seen with wild-type apo-RAR. In a striking contrast, BMS614 is unable to induce such an effect. In the presence of this ligand, SMRT interaction with wild-type RAR was significantly decreased, while NCoR interaction was similar to that of apo-RAR. Moreover, BMS614 could not overcome the effect of the "AHT" CoR box mutation, indicating that this ligand is unable to generate a surface compensating for the effect of the AHT mutation. The SMRT interaction pattern was recapitulated by

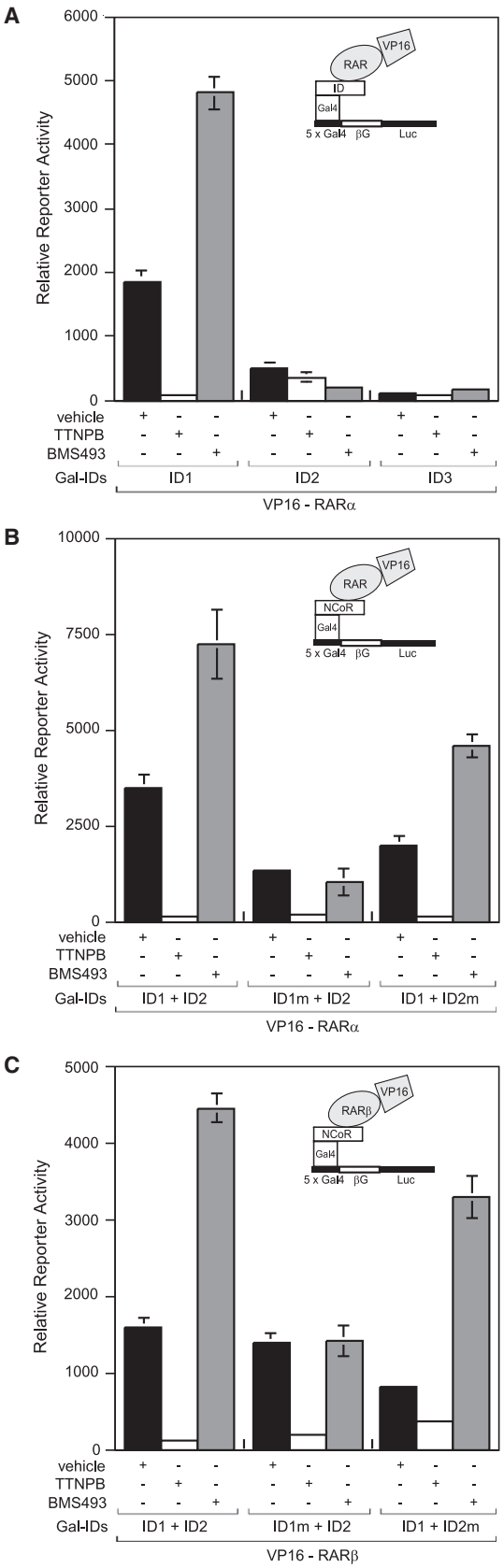

Figure 4. BMS493 Increases NCoR Interaction with RARs Specifically through ID1

Mammalian two-hybrid assays with (17 m)5x-G-Luc reporter were performed in HeLa cells as in Figure 1.

(A) Transient transfections with VP16-RARα and Gal-ID1, Gal-ID2, or Gal-ID3.

(B and C) Transient transfections with VP16-RARα (B) or VP16-RARβ (C) and Gal-NCoR harboring both IDs (ID1 + ID2), mutations in ID1 (ID1m + ID2), or mutations in ID2 (ID1 + ID2m). In all assays, both TTNPB and BMS493 are used at 0.1 μM and 1 μM, respectively. Please note that the numbering of CoRNR1 to CoRNR2 is from N to C terminus.

fluorescence anisotropy analyses with a peptide (CoRNR1) derived from SMRT ID1 (Table 1). The dissociation of the SMRT CoRNR1-apoRARα LBD complex (K_d = 0.20 μM) strongly increased upon Am80 binding (K_d = 3.80 μM) and was slightly reduced (K_d = 0.18 μM) by BMS493. Importantly, the association of BMS614 to the RARα LBD provoked a robust reduction of SMRT-CoRNR1 binding affinity (K_d = 2.88 μM), similar to values obtained for the agonist Am80. Overall our results demonstrate that based on their mechanism of action the two antagonists BMS493 and BMS614 have to be classified as inverse agonist and neutral antagonist, respectively.

Crucial Role of H12 Positioning in Inverse Agonist and Neutral Antagonist Action

It is well known that ligand binding induces a conformational change in NRs. Protease digestion studies can not only reveal such changes but also distinguish between distinct structural alterations induced by agonists and antagonists. We therefore examined the molecular basis of ligand-induced structural changes in RARα by limited proteolysis studies. Carboxypeptidase Y (CY) is a vacuolar serine carboxypeptidase from *Saccharomyces cerevesiae,* which catalyzes a stepwise removal of C-terminal amino acids from proteins. This exopeptidase has been used previously to reveal altered positioning by protease sensitivity of the C-terminal H12 of RARs in the presence of a particular ligand (Lin et al., 1997). Indeed, apo-RARα was highly sensitive to CY digestion, indicating that H12 is accessible to the enzyme, while TTNPB (agonist) or BMS614 (neutral antagonist) binding conferred resistance to CY (Figure 5C, top panel, lanes 5–7 and 11–13). These results are consistent with, and can be readily explained from, previously reported 3D LBD structures for agonist and BMS614-bound RARα (compare Figure 5D, left). In these structures, H12 is tightly packed against the body of the LBD in the presence of an agonist and embedded in a hydrophobic groove formed by H3 and H4 in the presence of BMS614; such structures are compatible with a poor susceptibility to CY attack. In a striking contrast to BMS614, the inverse agonist BMS493 did not confer any detectable resistance of RARα toward CY exopeptidase attack, as the proteolysis pattern of unliganded and BMS493-bound RARα were virtually indistinguishable (Figure 5C, top panel, compare lanes 2–4 and 8–10). This indicates that H12 is less tightly bound to the body of the LBD and thus more susceptible to proteolysis. Importantly, however, this difference is only seen with the exopeptidase. Indeed, there is a very similar protection of the LBD against endopeptidase attack when RAR is bound to BMS493, BMS614, or TTNPB, as revealed by virtually identical protection patterns seen with trypsin (Figure 5C). Thus, the three above ligands

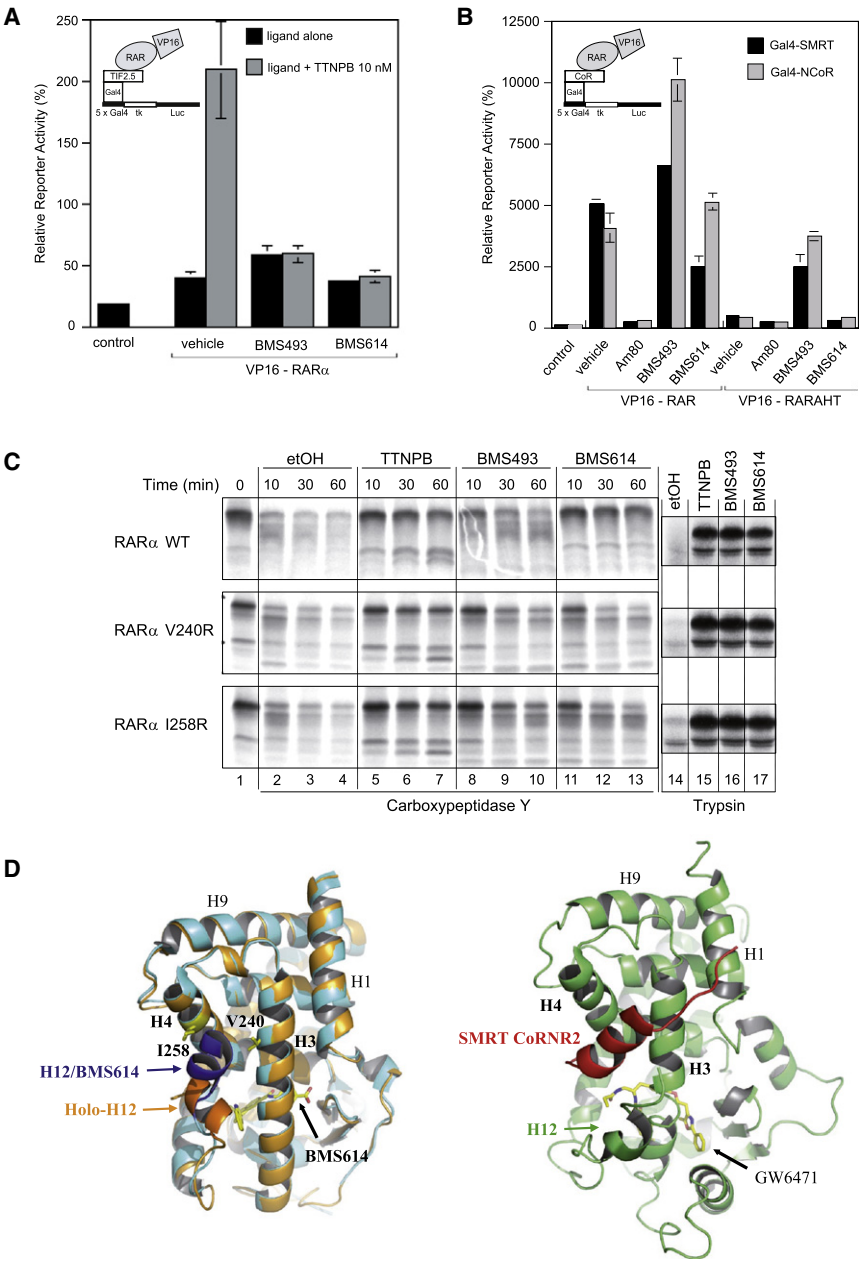

Figure 5. BMS493 and BMS614 Represent Two Different RARα Antagonist Classes

(A) Mammalian two-hybrid assays with (17 m)5x-G-Luc reporter were performed in HeLa cells as in Figure 1. Transient transfections with VP16-RARα and Gal-TF2.5 harboring the nuclear ID of TIF2.

(B) Transient transfections with Gal-NCoR, Gal-SMRT and VP16-RARα (VP16-RAR), or VP16-RARαAHT mutant (VP16-RARAHT). Only BMS493 is able to rescue VP16-RARAHT mutant for interaction with CoRs. Both BMS493 and BMS614 are used at 1 μM.

(C) Ligands differently alter protease sensitivity of RARα. Partial proteolysis maps of in vitro-translated RARs in the presence or absence either of TTNPB, BMS493, or BMS614. Radiolabeled RARs were exposed to CY for 10, 30, or 60 min as indicated. Top, wild-type RARα; middle, RARα V_{240}R mutant; bottom, RARα I_{258}R mutant. (right) Wild-type RARα, RARα V_{240}R mutant, or RARα I_{258}R mutant were exposed to trypsin for 10 min.

(D) Model of the structural basis of ligand action. (left) Schematic drawing of two different conformational states of RAR LBDs elucidated from crystal structures. Superposition of the LBDs of the agonist-bound (holo) atRA RARγ (orange) and the RARα-BMS614 complex (blue). Agonist ligands induce a conformation in which the holo position of H12 is stabilized. This active conformation provides a surface to which CoAs can bind via their NR boxes that contain LxxLL motifs. In contrast, BMS614 binding allows H12 to bind to the static part of the hydrophobic groove and then blocks by competition both CoA and CoR binding. (right) The crystal structure of the PPARαLBD (green) in a complex with the antagonist GW6471 and the CoRNR2 motif (red) of SMRT reveals that the binding of GW6471 to PPARα prevents the proper positioning of H12 in its agonistic site (Xu et al., 2002). H12 is stabilized in an alternative position that may generate an optimal interaction surface for CoRs.

apparently induce a common global allosteric transition of the LBD core as revealed by acquired resistance to trypsin but BMS493 differs from BMS614 and TTNPB in that it does not induce a tight packing of H12.

To gain additional direct insight into this differential packing of H12 we introduced mutations (RAR_{V240R} in H3 and RAR_{I258R} in H4; see Figure 5D) into RARα that correspond to those previously reported to impair both CoA and CoR interaction for TR and RXR (Hu and Lazar, 1999). These mutations disrupt the H3/H4 hydrophobic groove to which the CoR ΦxxΦΦ and CoA LxxLL motifs bind. Interestingly, neither of the mutations alters the CY protection pattern for RARα complexes with TTNPB or BMS493 (compare for each mutant the EtOH mock treatment with the ligand treatment in Figure 5C, middle and bottom panels, lanes 2–10). In contrast, on complexation with BMS614, both mutants exhibited a significantly increased sensitivity toward CY (lanes 11–13). This result supports a model in which residues V_{240} and I_{258} are required for tight interaction of H12 with the LBD core in the presence of BMS614. Importantly, none of the mutations altered the resistance of RARα to trypsin degradation, as identical proteolytic maps were observed for wild-type and mutant RARα irrespective of the bound ligand (Figure 5C).

DISCUSSION

In the present study, we report on the very different mechanistic and structural bases for the antagonism exerted by two retinoids, which we classify as neutral antagonist (BMS614) and inverse agonist (BMS493) due to the very different coregulator interaction patterns that these ligands impose onto RARs. Apart from the mechanistic insight in how subtle changes in the structure of

Table 1. Binding Affinities of Coregulator Peptides for RARα LBD

	Dissociation Constants (μM)	
Ligands	Fluo-SRC-1 NR2	Rho-SMRT CoRNR1
No ligand (apo)	8.18 ± 0.30	0.20 ± 0.02
Am80	0.47 ± 0.02	3.80 ± 0.41
BMS493	12.70 ± 0.80	0.18 ± 0.01
BMS614	92.70 ± 20.00	2.88 ± 0.35

Dissociation constants of SRC1 NR2 or SMRT CoRNR1 peptides from purified monomeric RARα LBD were determined using the fluorescein-labeled NR box 2 peptide of the CoA SRC-1 or the rhodamine-labeled CoRNR1 peptide of the CoR SMRT, respectively.

a NR ligand can exert fundamentally distinct responsivity of the receptor, which is discussed below, these compounds are also novel tools in understanding complex signaling pathways through pharmacological "dissection" with ligands that induce a defined subset of activities. It is, for example, unclear why RARβ exerts features of tumor suppressor (see Introduction for references), which is not the case for RARα or γ. One difference between the RARs is the rather high ligand-independent activity of RARβ. Compounds like BMS493, optimally subtype specific, allow us to decrease the constitutive activity of a RAR to study its loss-of-function consequences in suitable systems.

Our data suggest that BMS493 binding induces analogous conformational change in all three RARs, indicating that the positioning of BMS493 within the ligand-binding cavities is similar. This is apparently not the case for other previously described inverse agonists. Indeed, the mammalian two-hybrid assays reported here reveal that AGN109, which was originally described as inverse agonist (Klein et al., 1996), is generally less efficient than BMS493 at inducing the recruitment of CoRs and exerts convincing inverse agonist effects only on RARγ.

This suggests a weaker overall repression potential of AGN109, possibly due to a positioning of AGN109 in the ligand-binding cavity, which generates a suboptimal CoR-binding interface. Conversely, AGN870 is a weak transcriptional activator capable of preventing CoR association with RARα and RARβ. As AGN870 differs from AGN109 only by the absence of a methyl group, these results reveal that relatively subtle ligand modifications can significantly alter the conformation of the LBD and H12 positioning, thereby generating distinct coregulator binding interfaces. Considering the divergent CoR interaction patterns induced by these compounds, they may possibly act in a cell type-selective manner. In contrast, BMS493 is expected to act as a strong global silencer of RAR-dependent gene activation programs. Cell type-specific ligand activities have indeed been reported for the class of so-called "selective oestrogen receptor modulators" (for a review, see Gronemeyer et al., 2004). In the case of selective oestrogen receptor modulators the tissue selective response is thought to be the consequence of differences in cell/tissue-specific coregulator levels. Similarly, the partial activity of RU486 with the progesterone receptor has been linked to differential CoR and CoA expression (Liu et al., 2002). Together our observations demonstrate that it is possible to generate RAR ligands with very selective profiles, in terms of coregulator binding interfaces generated and RAR-coregulator complexes formed, which can finally result in very different transcriptional outcomes.

A general feature common to all antagonists crystallized to date is the presence of a bulky side chain that cannot be accommodated within the agonist binding cavity. In our previous work we reported that in the RARαLBD-BMS614 complex (Bourguet et al., 2000c) the antagonistic quinolyl group of BMS614 points toward H12 and exits the binding pocket between H3 and H11. In contrast to RAR agonists, which stabilize the active conformation of holo-H12, thereby promoting CoA recruitment (as in the RARβLBD-9-cis-RA-LxxLL complex; Pogenberg et al., 2005), this particular position of BMS614 prevents the positioning of H12 in the active conformation. Furthermore, the RARαLBD-BMS614 structure reveals that H12 binds and occludes the CoA binding site by mimicking the hydrophobic interactions of the LxxLL motif of CoAs. This suggests that BMS614 may prevent interaction of CoA by inducing H12 stabilization in the hydrophobic cleft formed by H3, H4, and the H3/H4 loop, with the consequence that H12 competes with CoA binding. The reported fluorescence anisotropy and two-hybrid data support this view, as RARα interacts very poorly (even weaker than unliganded RARα) with CoAs upon BMS614 binding. Additional arguments in favor of a model in which H12 binds to the CoA interaction surface in the BMS614-RARα complex are provided by limited proteolysis study with exopeptidase CY. Overall, our study reveals that BMS614 acts as a pure RARα AF2 antagonist that prevents CoA binding most likely by stabilizing H12 in the hydrophobic cleft.

From a structural point of view, the crystal structure of a ternary complex comprising the PPARα-LBD, the antagonist GW6471, and a SMRT CoR motif (Xu et al., 2002) supports the model in which CoA and CoR binding sites are largely overlapping (Figure 5D, right). Conceptually, H12, CoA, and CoR could compete with each other for the hydrophobic groove of RARα, which is formed by H3, H4, and the loop LH3-H4. According to such a concept, BMS614 would reduce the affinities of both CoA and CoR for RARα, as is demonstrated by the fluorescence anisotropy experiments, due to H12 stabilization in this groove.

While all these studies have been very revealing, the question of what determines BMS493-mediated CoR interaction to RARs remains open. Obviously the differential recruitment of CoRs in the presence of BMS493 and BMS614 has to be a consequence of the allosteric effects on RARα. The induction of a stable positioning of H12 by the agonist or BMS614 directs the transcriptional readout from RARα by (1) enhancing (agonist) or reducing (BMS614) the affinity toward the LxxLL motif of CoAs and (2) simultaneous destabilization of CoR binding. Conversely, BMS493, which functions also as RAR antagonist, strongly stabilizes CoR interaction, with ID1 being both necessary and sufficient to fortify NCoR association. Therefore, helix H12 has to adopt in the BMS493-RARα complex an alternative position that favors CoR association specifically through ID1. As for BMS614, the antagonistic activity mediated by BMS493 has to be exerted through allosteric effects. Both BMS493 and BMS614 side chain substitutions are too long to be accommodated within the ligand-binding cavity and instead exit the pocket, interfere with H12 alignment, and thereby generate AF2 antagonism. Interestingly, our limited proteolysis assays imply an instability of the H12 conformation upon BMS493 binding, which is in stark contrast to H12 stabilization by BMS614; obviously a confirmation of this hypothesis has to await the establishment of a BMS493/RARαLBD/CoR structure.

It is tempting to speculate that BMS614 may prevent interaction of coregulators by inducing H12 stabilization in the hydrophobic groove with H12 acting as competitor precluding coregulator interaction, while BMS493 may bind to RAR such that it physically prevents H12 from adopting either its characteristic agonist (TTNPB) or AF2 antagonist (BMS614) orientation and thereby abrogates the interaction between H12 and the core of the LBD entirely. In this respect, BMS493 resembles the antiestrogen ICI164384. Indeed the crystal structure of the ERβLBD-ICI164384 complex revealed that, in contrast to other ER antagonists, H12 cannot adopt a defined position (Pike et al., 2001). The binding of this compound to ERβ completely abolishes the association between H12 and the remainder of the LBD. However, it cannot be excluded that the extensive flexible antagonistic substitution of ICI164384 interferes directly with coregulator binding; this is unlikely to occur in the case of BMS493, which has a very rigid structure and allows CoR recruitment. We note, however, that in the crystal structure of the GW6471-PPARα-LBD-CoRNR2 complex H12 is loosely packed against H3, leaving sufficient space to accommodate the CoR motif (Xu et al., 2002). Thus, under the influence of a ligand, H12 can be stabilized, albeit poorly, in a position that is different to the well characterized holo and AF2 antagonistic conformations (see Figure 5D, right). Overall our results suggest that the positioning of the BMS493 phenyl moiety and interference with H12 positioning are crucial features that generate RAR inverse agonism.

Reversible histone acetylation, governed dynamically by histone acetyl transferases and deacetylases, plays a pivotal role in regulation of gene expression through remodeling chromatin structure; histone deacetylation being associated with transcriptional repression of genes. The fact that the pan-RAR inverse agonist stabilizes the association of RARs with CoRs in vitro raises the possibility that it could be used not only to block the effect of agonistic retinoid signals but also to silence the constitutive expression levels of retinoid-responsive promoter. Indeed, BMS493 exposure reduced the luciferase levels seen in the absence of ligand from a RARE-luciferase transgene and, more importantly, also the basal expression level of the endogenous *CYP26* gene, which is known to harbor a functional RARE. Notably, BMS493 treatment also reduced basal histone acetylation of the *CYP26* promoter. Given that BMS493 enforces CoR binding to cognate promoters and that CoRs assemble complexes that contain histone deacetylases, we conclude that BMS493 stabilizes the entire CoR complex at RAR target sites in vivo, suggesting that chromatin histone deacetylation is (part of) the mechanism by which inverse agonists inhibit basal target gene transcription.

SIGNIFICANCE

A major goal of drug development for an identified pharmaceutical target is the generation of modulators that display increased target selectivity and/or functional specificity in order to reduce potential side effects. The 48 members of the NR family that possess bona fide LBDs are attractive drug target because (1) they are master regulators of a large variety of major (patho)physiological processes and (2) their cognate ligands are small molecules that are convenient for chemical synthesis. Importantly, subtle changes in the structure of a cognate ligand can direct the activity toward a particular receptor subtype and various types of functional specifications, such as agonism/antagonism, (hetero)dimer selectivity, or cell/pathway selectivity like the one underlying the concept of NR modulators. In the present study we describe the molecular basis of two very distinct types of ligands, an inverse agonist (BMS204,493) and a neutral antagonist (BMS195,614) for the RAR family. We show that a differential interaction with coregulators is the basis of the different functionality of these two ligands. As the coregulators are platforms for complexes with epigenetic activities, such as histone methyl/acetyltransferases and deacetylases, such ligands can be used to regulate RAR-mediated gene programs in very different directions. We have previously shown that RARβ, which is generally considered a tumor suppressor, is unique in that it displays constitutive activity for transactivation and transrepression. The availability of ligands that are able to act as inverse agonists like BMS204,493 allows us to investigate the role of the constitutive activity of RARβ and may illuminate its function as a tumor suppressor.

EXPERIMENTAL PROCEDURES

Materials and Chemicals

The histidine-tagged LBD of human RARα1 (residues176–421 in pET15b vector) and the LBD of mouse RXRα1 (residues 227–467 in pET3a vector) were expressed in *Escherichia coli* BL21(DE3). Purification of the RXRαLBD-RARαLBD heterodimer was performed as described previously (Bourguet et al., 2000a). Peptides containing CoRNR1/ID1 and CoRNR2/ID2 sequences (Hu and Lazar, 1999) were synthesized and used as model peptides for the CoR proteins. CoRNR1/ID1 is a 24 amino acid peptide (THRLITLADHIC QIITQDFARNQV; 2806 Da) containing the consensus sequence that specifically interacts with the RAR subunit. CoRNR2/ID2 contains 14 amino acids (NLGLEDIIRKALM; 1541 Da). GAL-TIF2.5, Gal-NCoR (contains NCoR from 1629 to the C terminus), and Gal-SMRT (contains SMRT from 982 to the C terminus), as well as Gal-NCoR derivatives harboring both IDs (ID1 and ID2), mutations in ID1 (ID1m and ID2), or mutations in ID2 (ID1 and ID2m), have been described (Germain et al., 2002; Hu and Lazar, 1999; Voegel et al., 1998). The pSG5-based Gal-RARα and VP16-RARα expression vectors and the (17 m)5x-G-Luc reporter gene have been described previously (Nagpal et al., 1993). $(RARE)_3$-tk-Luc is a gift from P. Balaguer and has been described previously (Germain et al., 2004). Am80 was a generous gift of K. Shudo; BMS195,614 (Vivat et al., 1997) and BMS204,493 (Germain et al., 2002) were provided by Bristol-Myers Squibb; and AGN192,870 and AGN193,109 were provided by Galderma. TTNPB and atRA were purchased from Sigma.

Cell Culture and Transient Transfections

HeLa cells, cultured in Dulbecco's modified Eagle's medium/5% fetal calf serum, were transfected by using the standard calcium phosphate method as previously described (Vivat et al., 1997). Results were normalized to coexpressed β-galactosidase.

Chromatin Immunoprecipitation and RT-PCR

ChIP assays were carried out with the acetyl-histone H4 immunoprecipitation assay kit (Upstate Biotechnology). Briefly, approximately 10^6 HeLa cells were treated with ligands for 2 hr and ChIP assays were done using antisera against acetyl histone H4 as specified by the manufacturer. PCR of the *CYP26* gene promoter was performed with ChIP using the following oligonucleotides: *CYP26* forward, 5′-GCGGAACAAACGGTTAAAG; *CYP26* reverse, 5′-CCAG AGCTTGATCGCAG; *L32* forward, 5′-CCGTAGCTGGCGATTGGAAGAC; *L32* reverse, 5′-TTGACATATCGGTGTGACTGGTG. For RT-PCR, the following oligonucleotides were used: cyp26 forward, 5′- CGCGAGGCACTCGAATGC TACGTG; cyp26 reverse, 5′- CGCGTGAATGAGGTTCCGCGCCTT; GAPDH

forward, 5-CCTGGTCACCAGGGCTGCTTTTAAC; GAPDH reverse, 5′-GTC GTTGAGGGCAATGCCAGCC.

ESI-MS Analysis

Prior to any ESI-MS analysis, samples were desalted on Centricon PM30 microconcentrators (Amicon, Millipore) in 100 mM ammonium acetate (pH 6.5). Ammonium acetate presents the advantage not only to preserve the ternary and quaternary structures of proteins in solution but also to be compatible with ESI-MS experiments.

ESI-MS measurements were performed on an electrospray time-of-flight mass spectrometer (LCT, Waters). Purity and homogeneity of the retinoid receptors were verified by mass spectrometry analysis in denaturing conditions: proteins were diluted to 5 pmol/μl in a 1:1 water-acetonitrile mixture (v/v) acidified with 1% of formic acid. Mass spectra were recorded in the positive ion mode on the mass range 500–2500 m/z, after calibration with horse heart myoglobine diluted to 2 pmol/μl in a 1:1 water-acetonitrile mixture (v/v) acidified with 1% of formic acid. The following molecular weights were measured: 29,930 ± 1.8 Da for RARα and 26,735 ± 2.3 Da for RXRα with deletion of the N-terminal methionine and 26,867 ± 2.1 Da corresponding to RXRα. These results were in agreement with the molecular weights calculated from the known amino acid sequences.

The mass measurements of the noncovalent complexes were performed in ammonium acetate (50 mM; pH 6.5). Samples were diluted to 10 pmol/μl in the previous buffer and continuously infused into the ESI ion source at a flow rate of 6 μl/min through a Harvard syringe pump. Great care was exercised so that noncovalent interactions survive the ionization/desorption process. Especially the accelerating voltage (Vc), which controls the kinetic energy communicated to the ions in the interface region of the mass spectrometer, was optimized to 50 V in order to prevent ligand dissociation in the gas phase. ESI-MS data were acquired in the positive ion mode on the mass range 1000–5000 m/z. Calibration of the instrument was performed by using the multiply charged ions produced by a separate injection of horse heart myoglobine diluted to 2 pmol/μl in a 1:1 water-acetonitrile mixture (v/v) acidified with 1% of formic acid. The relative abundance of the different species present on ESI mass spectra were measured from their respective peak intensities, assuming that relative intensities displayed by the different species on the ESI mass spectrum reflect the actual distribution of these species in solution. The reproducibility of the determination of the relative proportions of the different species was estimated to be ±2%–3%.

Fluorescence Anisotropy Assays

Steady-state fluorescence anisotropy assays were performed with a BEACON 2000 polarization instrument (Panvera) regulated at 4°C as described previously (Pogenberg et al., 2005). Fluorescent peptides (fluorescein-RHKILHRLL QEGS corresponding to the NR box 2-binding motif of SRC-1 and rhodamine-RVVTLAQHISEVITQDYTR corresponding to the CoRNR1-binding motif of SMRT) were purchased from Neosystem.

Limited Proteolytic Digestion

In vitro-made ^{35}S-radiolabelled RARs (TNT kit, Promega) were used. Briefly, for trypsin, after incubating on ice for 1 hr, ligand receptor proteins were digested at 25°C for 10 min with 100 μg/ml trypsin as described previously (Germain et al., 2004). For CY, each time point contained 1 μl of TNT reaction products and 7 μl of 50 mM Tris-HCl (pH 6.8) and either 10 μM of ligand or an equivalent amount of ethanol carrier. After incubating on ice for 1 hr, ligand receptor proteins were digested at 25°C for either 10, 30, or 60 min with 2 μl CY (1 mg/ml; Sigma).

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