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Retinoic Acid Receptors β and γ Do Not Repress, But Instead Activate Target Gene Transcription in Both the Absence and Presence of Hormone Ligand

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Retinoic acid receptors (RARs) are important mediators of retinoid signaling in morphogenesis, development, and cell differentiation. Three major isotypes of RARs, denoted α, β, and γ, have been identified, each encoded by a distinct genetic locus. Although RARα, RARβ, and RARγ share many structural and functional features, these three isotypes are known to play unique, as well as overlapping, roles in physiology and development. We report here that the three RAR isotypes display different transcriptional properties in the absence of hormone ligand; under these conditions, RARα is a strong repressor of target gene expression, whereas both RARβ and RARγ fail to repress and instead are able to mediate substantial levels of hormone-independent transcriptional activation. These differing transcriptional properties appear to reflect the differing abilities of the three RAR isotypes to interact with the SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) corepressor protein: RARα binds to SMRT strongly both *in vitro* and *in vivo*, whereas RARβ and RARγ interact only weakly with SMRT. The ability to repress or to activate transcription in the absence of hormone maps predominantly to isotype-specific differences in the sequence of helix 3 within the hormone binding domain of the RARs, and the transcriptional properties of one isotype can be exchanged with that of another by exchanging portions of helix 3. The different transcriptional properties of RARα, RARβ, and RARγ in the absence of hormone contribute to the distinctive biological functions of these proteins and provide a rationale for the strong conservation of the three distinct isotypes during the vertebrate evolutionary radiation. (*Molecular Endocrinology* 17: 373–385, 2003)

NUCLEAR HORMONE RECEPTORS are a large family of ligand-regulated transcription factors that play important roles in organismal development, differentiation, and homeostasis (1–8). The nuclear hormone receptors include the steroid receptors, thyroid hormone receptors, vitamin D receptors, peroxisome-proliferator activated receptors, retinoid X receptors, and retinoic acid receptors (RARs), all of which are defined by the presence of a characteristic central DNA binding domain joined to a more C-terminal, hormone-binding domain (1–8). Nuclear hormone receptors regulate transcription by binding to DNA enhancer elements, referred to as hormone response elements, located within or adjacent to target gene promoters (9). Notably, many nuclear hormone receptors display bimodal transcriptional properties and can either repress or activate target gene transcription depending on the concentration and chemical nature of the hormone ligand, the nature of the target promoter, and the influence of additional signal transduction pathways operative in the cell (10, 11). These bipolar transcriptional responses reflect the ability of the nuclear hormone receptors to recruit a panel of auxiliary proteins, denoted corepressors and coactivators, which mediate the actual molecular events necessary for repression or activation of gene transcription (12–19).

Nuclear hormone receptors typically bind to corepressors, such as SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) or its paralog, N-CoR (nuclear hormone receptor corepressor), in the absence of hormone agonist (10, 11). SMRT and N-CoR tether, in turn, additional components of the corepressor holocomplex, including TBL-1 (transducin β-like protein-1) and a series of histone deacetylases (HDACS) that result in transcriptional repression (12–19). Conversely, the binding of hormone agonist to a nuclear hormone receptor is believed to induce a conformational change that dissociates the corepressor complex and promotes the recruitment by the receptor of one or more coactivators, such as SRC-1 (steroid receptor coactivator protein-1)/GRIP-1(glucocorticoid receptor-interacting protein 1)/ACTR (activator of thyroid hormone and retinoic acid receptors), CBP (cAMP-responsive transcription factor binding protein)/p300, and the DRIP (vitamin D receptor interacting protein)/TRAP (thyroid hormone receptor associated protein) complexes, leading to target gene

Abbreviations: ATRA, All-trans retinoic acid; DBD, DNA binding domain; GST, glutathione-*S*-transferase; N-CoR, nuclear hormone receptor corepressor; RAR, retinoic acid receptor; RARE, RAR response element; RXR, retinoid X receptor; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor.

activation (10, 11). Corepressors and coactivators can modulate gene expression by multiple mechanisms, including covalent modifications of the chromatin complex and interactions with the general transcriptional machinery (20–26).

Three different loci encode retinoic acid receptors in vertebrates: RARα, RARβ, and RARγ. These three RAR isotypes (2, 3, 5) are similar but not identical in sequence and structure to one another, and they are expressed in distinctive tissue-specific and developmentally regulated patterns (2, 5, 27). Although gene disruption studies in mice have revealed some functional redundancy between the different RAR isotypes, each RAR isotype also performs unique functions in development and differentiation that cannot be replaced by the actions of the other isotypes; the use of isotype-selective retinoid ligands confirms these conclusions and provides additional evidence that each RAR isotype exerts a mixture of overlapping and specific actions in target gene regulation (28–30). Therefore, each of the RAR receptors plays a specific function in the organism that is likely to account for the high degree of conservation of the three isotypes throughout vertebrate evolution.

In contrast to their distinct biological functions *in vivo*, the different RAR isotypes display generally similar DNA and hormone binding properties *in vitro* (2, 3). However, we have previously noted that the RARα, RARβ, and RARγ isotypes do differ in terms of their ability to bind to the corepressor SMRT: RARα efficiently binds to SMRT, whereas RARβ and RARγ bind to SMRT much more weakly (31). The biological consequences of this isotype-specific corepressor interaction were not determined in our prior work, leading us to subsequently investigate the transcriptional regulatory properties of the different RAR isotypes. We report here that all three isotypes are able to activate reporter gene transcription in the presence of hormone agonist. Notably, however, although RARα can repress target gene transcription in the absence of hormone, RARβ and RARγ fail to repress and instead mediate transcriptional activation under the same conditions. The divergent transcriptional properties of RARα, RARβ, and RARγ in the absence of hormone parallel the ability of these isotypes to interact with SMRT and map primarily to isotype-specific amino acid differences in helix 3 within the hormone binding domain of these receptors. Only three amino acids differ between the relevant helix 3 domains of RAR, RARβ, and RARγ: Ile 224, Asp 225, and Ser 234 in RARα are replaced by Leu, Gly, and Ala, respectively, in RARβ and RARγ. Notably, exchanging the amino acids in these three positions can exchange the ability of these receptors to bind to corepressor *in vitro* and to mediate transcriptional repression in transfected cells. We conclude that the different SMRT binding properties of RARα, RARβ, and RARγ result in distinct transcriptional regulatory properties that contribute to the divergent biological roles of these different isotypes.

RESULTS

Unlike RARα, the RARβ Isotype Fails to Repress and Functions Instead to Activate Transcription in the Absence of Hormone

As noted in our prior study (31), RARα and RARβ displayed different abilities to bind to SMRT corepressor in a glutathione-*S*-transferase (GST)-pulldown/protein interaction assay: RARα readily bound to an immobilized SMRT construct under these conditions, whereas RARβ interacted with SMRT with only low efficiency (Fig. 1A, *filled bars*). This marked difference in the ability of RARα and RARβ to bind to GST-SMRT was maintained over a 10-fold range of receptor inputs and over a 12-fold range of GST-SMRT concentrations (data not shown). A similar difference was observed in the ability of RARα and RARβ to interact with SMRT in a mammalian two-hybrid assay (31). We therefore sought to investigate the effects of this isotype-specific corepressor recruitment on RAR-mediated transcriptional regulation in cells. We first tested whether RARα and RARβ differ in their abilities to repress transcription in hormone-striped culture medium. Figure 1B displays the results from a transient transfection of CV-1 cells. In the absence of hormone, RARα repressed the expression of a luciferase reporter containing RAR-response elements (pTK-Luc-RARE$_3$) compared with the same reporter lacking a response element (pTK-Luc empty; Fig. 1B, *filled bars*). In contrast to these results with RARα, introduction of RARβ did not repress the pTK-Luc-RARE$_3$ reporter in the absence of hormone, but instead increased expression of the reporter to levels significantly above those observed in the absence of an RARE (Fig. 1B, *filled bars*). This difference in the transcriptional properties of the unliganded RARα and RARβ isotypes was observed over a range of expression vector concentrations (data not shown). As anticipated from prior reports (10, 11, 32), all-*trans* retinoic acid (ATRA), a pan-agonist for RARs, induced the dissociation of SMRT *in vitro* (Fig. 1A, *open bars*) and permitted both RARα and RARβ to function as strong transcriptional activators of the pTK-Luc-RARE$_3$ reporter in transfected cells (Fig. 1B, *open bars*). The expression of a pCH110-lacZ reporter, used as an internal transfection control and lacking response elements, or of the pTK-Luc empty reporter itself, was not altered by expression of either RAR isotype, plus or minus hormone (Fig. 1B, and data not shown).

We also examined the corepressor interaction and transcriptional regulatory properties of RARγ, the third major RAR isotype. RARγ interacted with SMRT corepressor relatively weakly (Fig. 1A) and did not repress, but rather enhanced expression of the pTK-Luc-RARE$_3$ reporter in the absence of hormone (Fig. 1B, *filled bars*). As noted for RARβ, this hormone-independent activation by RARγ required the presence of a functional response element in the reporter and was not detected for either the pTK-Luc empty reporter or

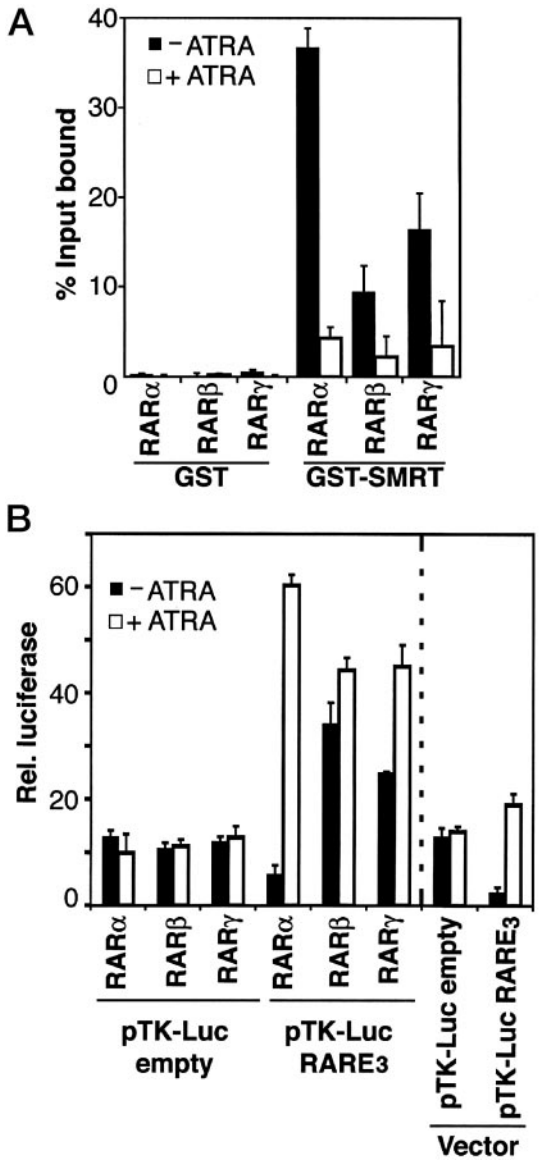

Fig. 1. Various RAR isotypes differ in their ability to bind to SMRT corepressor *in vitro* and to regulate reporter gene expression in cells. A, RARα, but not RARβ or RARγ, binds SMRT corepressor efficiently *in vitro*. Nonrecombinant GST, or a GST-SMRT fusion construct, was immobilized on a glutathione-agarose matrix and was incubated with radiolabeled RARα, β, or γ as indicated. Incubations were performed both in the absence (*filled bars*) and presence (*open bars*) of 1 μM ATRA. Radiolabeled receptors remaining bound to the GST- or GST-SMRT matrix after repeated washing were eluted, were resolved by SDS-PAGE, and were quantified by PhosphorImager analysis. The amount of each receptor bound to the GST- or GST-SMRT matrix, expressed as a percentage of the input, is presented. The average of duplicate experiments and the SD are shown. B, Unlike RARα, RARβ and RARγ activate reporter gene expression in both the absence and presence of ATRA hormone. CV-1 cells were transiently transfected with a pSG5 vector containing the RARα, β, or γ coding sequence, or an empty pSG5 expression vector (Vector), as indicated. Included in the transfection was a pTK-Luciferase reporter construct either lacking response elements (pTK-Luc empty) or containing three RAREs arranged in tandem (pTK-Luc $RARE_3$). A pCH100-lacZ reporter was included in all transfections as an internal normalization control. The cells were subsequently incubated in the absence (*filled bars*) or presence (*open bars*) of 400 nM ATRA, harvested, and assayed for luciferase and β-galactosidase activity. The relative luciferase activity was calculated as the ratio of absolute luciferase to β-galactosidase. The average of duplicate experiments and the SD are shown.

the internal lacZ transfection control (Fig. 1B, and data not shown). In common with both RARα and RARβ, addition of ATRA further increased reporter gene activation by RARγ (Fig. 1B, *open bars*). We conclude that under the conditions used here, neither RARβ nor RARγ is able to repress reporter gene expression; instead they mediate a hormone-independent activation of target gene expression. It should be noted that identical expression and reporter vectors were used for all three isotypes, that all three receptor isotypes induced approximately equal reporter activation in the presence of hormone (Fig. 1B), and that all three receptors were expressed at comparable levels in the transfected cells in the absence of hormone (although the levels of RARβ were slightly increased in the presence of ATRA; Fig. 2).

RARβ Functioned as a Constitutive Transcriptional Activator in Multiple Cell Type and Promoter Contexts

Transient transfections of HeLa or COS-1 cells or use of a different reporter construct (M-pTK-Luc $RARE_2$) produced essentially the same results as did the CV-1 and pTK-Luc-$RARE_3$ system: in all cases, RARβ failed to repress and instead mediated an activation of reporter gene expression in the absence of hormone ligand (data not shown). Notably, most vertebrate cells express RARs endogenously (2), and this includes the CV-1, COS-1, and HeLa cells used here. The presence of these endogenous RARs could be detected as an ATRA-dependent activation of the pTK-Luc-$RARE_3$ construct that occurred in these cells even in the absence of an ectopically introduced RAR construct (note the effects of ATRA, *open bars*, on the pTK-Luc-$RARE_3$ reporter when tested with the pSG5 vector-only control in Fig. 1B, *right*). Conversely, in the absence of ATRA, the level of endogenous RARα in many of these cell lines appeared sufficient to repress the pTK-Luc-$RARE_3$ reporter, with the consequence that the introduction of ectopic RARα did not necessarily result in a further reduction of pTK-Luc-$RARE_3$ reporter expression (compare the effects of pSG5-RARα *vs.* pSG5 vector-only, Fig. 1B, *filled bars*). In support of this interpretation, when introduced without an ectopic RAR, the pTK-Luc empty reporter, which lacks response elements, displayed substantially higher levels of expression in the absence of hormone than did the otherwise identical pTK-Luc reporter containing RAR

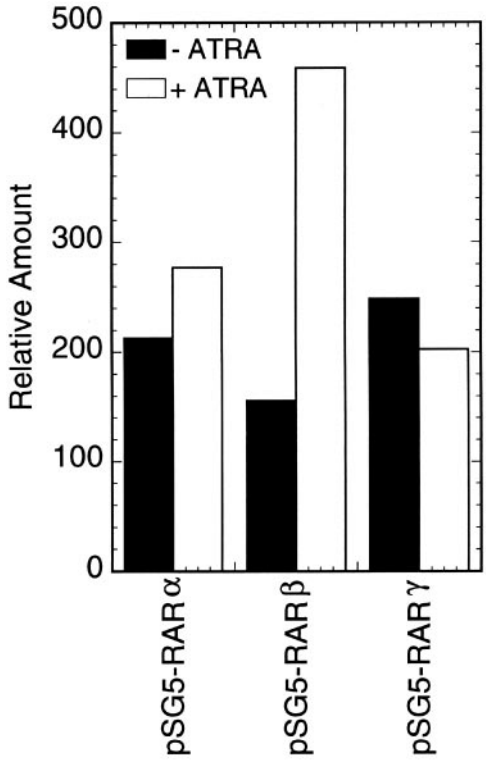

Fig. 2. The different RAR isotypes are expressed at comparable levels in transfected cells in the absence of hormone. CV-1 cells were transfected with the various pSG5-RAR isotype constructs using the same protocol as in Fig. 1B. After 24 h, the cells were either maintained in hormone-free media (*filled bars*) or transferred to media containing 400 nM ATRA. After an additional 24 h, the cells were harvested and lysed, and the lysates were analyzed by SDS-PAGE and by immunoblotting using RAR-directed antisera. The resulting RAR bands were quantified by using an ECL plus detection system and Storm System image analysis. The relative expression levels of the different RAR isotypes, normalized to correct for differences in the immunoreactivity of the antisera toward the different isotypes (see *Materials and Methods*), are presented.

response elements (pTK-Luc-RARE$_3$; Fig. 1B, *right panel*, *closed bars*). We used two approaches to circumvent this problem. We first used the L929 cell line that has previously been reported to be permissive for RARα-mediated repression (33). We found that, in agreement with this previous report, the ectopic expression of RARα caused a detectable repression of reporter gene transcription in the L929 cell line that was not seen when using an empty pSG5 vector control (Fig. 3A). Notably, RARβ retained the same phenotype in the L929 cells as it displayed in our other cells lines: a lack of repression, and the converse ability to activate reporter gene expression even in the absence of ATRA (Fig. 3A). To more fully preclude the contribution of endogenous RARs (or other mammalian transcription factors that might promiscuously recognize our RARE reporter elements), we next repeated our transfection studies using a GAL4-DNA binding domain (DBD)-RAR fusion system. By replacing the RAREs in the reporter with a GAL-17mer recognition site and by substituting the native DNA binding domain of the RAR with that of the *Saccharomyces cerevisae*-derived GAL4DBD, this system produces a reporter that responds exclusively to the ectopic GAL4-RAR fusion proteins and is not subject to regulation by endogenous RARs. Using this method, a GAL4DBD-RARα fusion mediated an unambiguous repression in the absence of hormone and a strong activation on addition of ATRA when compared with the pGAL4DBD vector control (Fig. 3B). In contrast, the GAL4DBD-RARβ fusion failed to repress and instead activated the pTK-GAL-17mer reporter in the absence of hormone, and this activation was further stimulated by addition of ATRA (Fig. 3B). These results support our results with the native RARs and indicate that the transcriptional properties of RARα and RARβ diverge in the absence of hormone, with the former mediating target gene repression and the latter mediating activation. Furthermore, given the nature of the GAL4-DBD constructs, our results demonstrate that the receptor A/B domain, a region previously shown to contribute to transcriptional activation by many nuclear hormone receptors, is not required for the hormone-independent transcriptional activation properties of RARβ.

Cotransfection of RXR as Heterodimer Partner Further Stimulates the Hormone-Independent Transcriptional Activation Properties of RARβ

Many nuclear hormone receptors can heterodimerize with other members of the family, and these heterodimeric species are believed to play important roles in mediating the hormone response (3, 4, 9). Retinoid X receptor (RXR) is a particularly important heterodimer partner for many nuclear hormone receptors, and RXR/RAR heterodimers are known to bind DNA more strongly and display enhanced transcriptional regulatory properties, compared with homodimers of either parental receptor (3, 4, 9, 34). We therefore conducted additional transient transfection studies to determine whether RXR might alter the transcriptional properties of RARβ. Transfecting an RXR expression construct alone had little or no effect on the pTK-Luc-RARE$_3$ reporter in the absence of hormone (Fig. 4, *top*). When cotransfected, ectopic RXR slightly attenuated the ability of RARα to repress in the absence of hormone, but greatly enhanced the ability of RARβ to activate reporter gene expression under the same conditions (Fig. 4, *top*). As anticipated from the results of previous studies (3, 4, 9), cotransfecting the RXR heterodimer partner in the presence of ATRA enhanced transcriptional activation by both RARα and RARβ, with this effect being more pronounced for the RARα isotype (Fig. 4, *bottom*). It should be noted that introduction of ectopic RXR also enhanced ATRA-mediated reporter gene activation by the endogenous RARs present in these CV-1 cells (Fig. 4, *bottom*; note

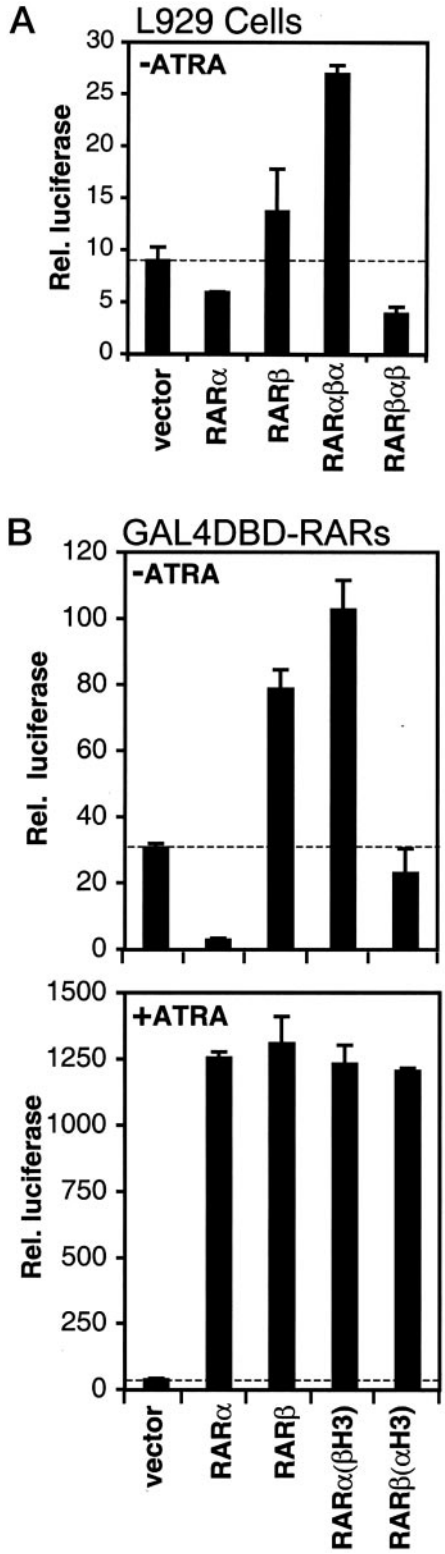

Fig. 3. RARα represses transcription in L929 cells, and in a GAL4DBD fusion assay system. A, In L929 cells, RARα (but not RARβ) represses transcription in the absence of hormone. A nonrecombinant pSG5 vector (vector) or a pSG5 vector encoding wild-type RARα, wild-type RARβ, the RAR$\alpha\beta\alpha$ chimera, or the RAR$\beta\alpha\beta$ chimera was introduced into L929 cells together with the pTK-Luc-βRARE$_3$ luciferase reporter. A pCH110 lacZ reporter was included as an internal normalization control. The cells were subsequently maintained in the absence of hormone and harvested, and the relative luciferase levels were determined as in Fig. 1B. The average of duplicate experiments and the SD are shown. B, When expressed as a GAL4-DBD-fusion, the unliganded RARα (but not RARβ) represses reporter gene transcription in CV-1 cells. CV-1 cells were transiently transfected with an expression vector either encoding no receptor, or encoding GAL4-DBD fusions of the hormone binding domains of RARα, RARβ, RARα(βH3), or RARβ(αH3), together with a pGL3-GAL4–17mer luciferase reporter vector. A pCH110 lacZ reporter was included as a internal control for transfection efficiency. Cells were subsequently incubated in the absence (*top*) or presence (*bottom*) of 400 nM ATRA, were harvested, and were analyzed for relative luciferase activity as in Fig. 1B. The average of duplicate experiments and the SD are shown.

the RXR-only lane). We conclude that ectopic RXR expression does not confer on RARβ the ability to repress, but instead further enhances the hormone-independent transcriptional activation properties of the RARβ isotype.

The Difference in Transcriptional Regulation by the Different Isotypes Maps Principally to Receptor Helix 3 Located within the Hormone Binding Domain

The different corepressor binding properties of RARα and RARβ map within a region encompassing helix 1, the Ω loop, and helix 3 in the hormone binding domain of these receptors (Fig. 5A and *middle panel* of B; Ref. 31). We next tested whether the same receptor region was also responsible for the differing transcriptional properties of these isotypes in our reporter gene assays. An RAR$\alpha\beta\alpha$ chimera that contained the helix 1-Ω-helix 3 region of RARβ displayed the transcriptional properties of RARβ in CV-1 transfection assays, whereas an RAR$\beta\alpha\beta$ chimera that contains the helix 1-Ω-helix 3 region of RARα displayed the RARα transcriptional phenotype (Fig. 5B, *right*, *filled bars*). Analogous results were obtained from transfections of L929 cells in the GAL4DBD-RAR fusion assay system and in the presence of ectopic RXR (Figs. 3 and 4, and data not shown). Therefore, at this level of resolution, the failure of RARβ to repress correlated with the failure of RARβ to interact with corepressor. To more precisely identify the amino acid sequences that are responsible for the difference in repression by RARα and RARβ, we analyzed an additional series of chimeras in which helix 1, the Ω loop, or the relevant portions of helix 3 were individually swapped between the RARα and RARβ archetypes. These chimeras were first tested for their ability to activate or repress the pTK-Luc-RARE reporter. The RARα(βH1) and RARα($\beta\Omega$) chimeras displayed the transcriptional properties of the RARα receptor, whereas the RARα(βH3) construct failed to repress and displayed a

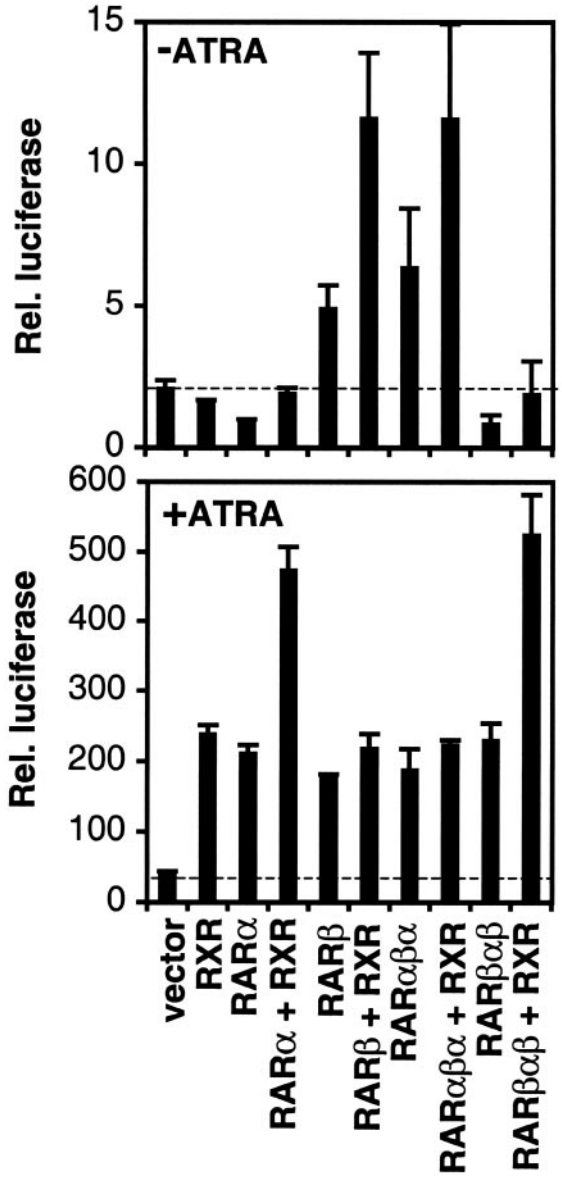

Fig. 4. Cotransfection of RXR enhances the hormone-independent transcriptional activity of RARβ. A pSG5 expression vector encoding no receptor, wild-type RARα, wild-type RARβ, the RAR$\alpha\beta\alpha$ chimera, or the RAR$\beta\alpha\beta$ chimera was introduced into CV-1 cells in the presence or absence of a pSG5-RXRα expression vector, as indicated. The pTK-Luc-βRARE$_3$ luciferase and pCH100 lacZ reporters were also included in each transfection. The cells were subsequently incubated in the absence (*top*) or presence (*bottom*) of 400 nM ATRA, and the relative luciferase levels were determined as in Fig. 1B. The average of duplicate experiments and the SD are shown.

hormone-independent reporter gene activation resembling that of the RARβ receptor (Fig. 6A, *left*). Reciprocally, the RARβ(αH1) and RARβ($\alpha\Omega$) constructs retained most (but not all) of the hormone-independent transactivation properties of wild-type RARβ, whereas the RARβ(αH3) chimera was greatly impaired in its ability to activate transcription in the absence of hormone (Fig. 6A, *right*). All constructs retained the ability to activate the reporter gene in the presence of ATRA (Fig. 6B). Therefore, we conclude that the divergent transcriptional properties of RARα and RARβ in the absence of hormone map primarily, but not exclusively, to differences in their helix 3 sequences, with the helix 1 and Ω-loop regions making a more minor, but still detectable, contribution. Notably, the divergent transcriptional properties of these different chimeras largely paralleled their ability to interact with SMRT corepressor: constructs possessing the RARβ helix 3 interacted only weakly with SMRT *in vitro*, whereas constructs containing the RARα helix 3 strongly interacted with SMRT in the absence of hormone (Fig. 6C, *filled bars*). The sequence of the helix 1 and the Ω-loop region also contributed, although in a minor fashion, to corepressor binding in that the RAR(αH1) and RAR($\alpha\Omega$) chimeras displayed slightly elevated abilities to bind to SMRT when compared with RARβ wild-type (Fig. 6C, and data not shown). Addition of hormone agonist (ATRA), as expected, caused dissociation of SMRT from all the RAR constructs tested (Fig. 6C, *open bars*). We extended these results to the GAL4DBD-RAR fusion/GAL-17mer reporter system (Fig. 3B). A GAL4DBD-RARβ (αH3) chimera that contains the relevant portions of helix 3 of RARα displayed the RARα property of repressing reporter gene expression in the absence of hormone; the reciprocal GAL4DBD-RARα(βH3) chimera that contains the relevant helix 3 sequences derived from RARβ exhibited the RARβ property of activating reporter gene expression in the absence of hormone (Fig. 3B, *top*). Again, all constructs mediated strong transcriptional activation in the presence of ATRA (Fig. 3B, *bottom*). We conclude that the inability of RARβ to interact efficiently with SMRT corepressor predominantly reflects isotype-specific amino acid differences in helix 3 and helps account for the inability of the RARβ isotype to repress transcription in transfected cells.

Three Amino Acids that Differ in Helix 3 of RARα, RARβ, and RARγ Contribute to the Different Transcriptional Properties of These Three Different Isotypes

There are three amino acids that differ between RARα and RARβ within the relevant region of helix 3: Ile 224, Asp 225, and Ser 234 in RARα are Leu, Gly, and Ala in RARβ, respectively (Fig. 5A). We therefore mutated these amino acids, both individually and in pairs, to the corresponding sequence in the opposite isotype and tested the resulting receptors for their regulatory properties in transfection assays. Replacing the RARβ amino acid at any one position with that of RARα decreased the ability of RARβ to induce reporter gene expression in the absence of hormone, with the G225D mutation having the most severe effect (Fig. 7, *top*). Certain double substitutions, such as the L224I/A234S mutation, conferred

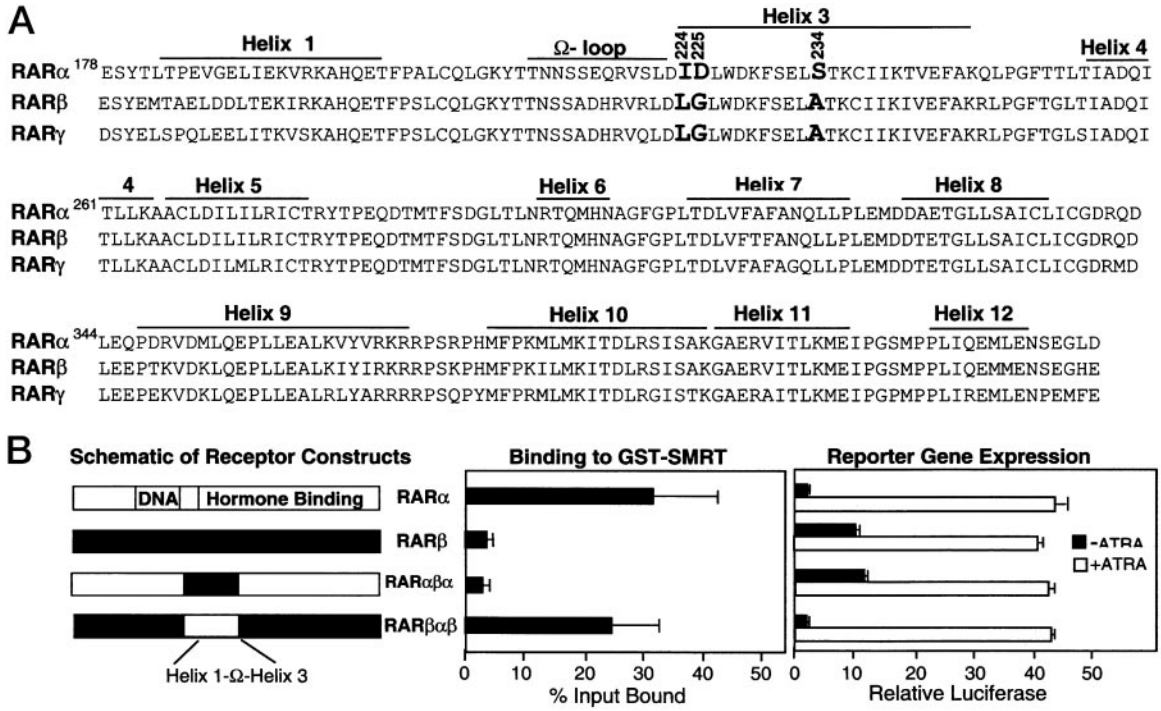

Fig. 5. The different transcriptional regulatory and SMRT corepressor binding properties of RARα and RARβ map to sequence differences within the hormone binding domain of these receptors. A, A comparison of the amino acid sequences of the hormone binding domains of RARα, β, and γ is presented. A common numbering system, based on the sequence of RARγ, is used for ease of comparison. The regions corresponding to helix 1–12 in the crystallographic structure of the liganded RAR are indicated *above* the amino acid sequence, as is the position of amino acids 224, 225, and 234 that contribute to the differing corepressor and transcriptional regulatory properties of the different isotypes (see the text). B, Chimeric constructs of RARα and RARβ demonstrate that the corepressor binding and transcriptional regulatory properties of these isotypes map within the helix 1-Ω loop-helix 3 region of these receptors. *Left*, A schematic of the different receptor constructs used in our experiments. *Middle*, The ability of these different RAR constructs to bind to GST-SMRT in the absence of hormone is shown (expressed as percentage input bound). *Right*, The ability of these different RAR constructs to regulate transcription of the pTK-Luc-RARE3 reporter in the absence (*filled bars*) or presence (*open bars*) of 400 nM ATRA is shown; transfections were performed as in Fig. 1B. The average of duplicate experiments and the SD are shown.

a more inhibitory effect on hormone-independent reporter gene activation by RARβ than did either individual mutation (Fig. 7, *top*). Reciprocally, changing the amino acids in helix 3 of RARα to the corresponding RARβ sequence generated a more RARβ-like phenotype; this was more evident for the I224L/D225G double and the complete-triple amino acid substitution than for any single mutation (Fig. 7, *top*). In contrast, point mutations in the helix 1 region (*e.g*. RARα double mutant I190T, V193I or RARβ double mutant T190I, I193V) and in the Ω loop region (RARβ R221S) did not result in any significant change in the ability of the receptor to interact with corepressor *in vitro* or repress transcription *in vivo* (data not shown). All mutant clones were able to strongly activate reporter gene expression in the presence of ATRA (Fig. 7, *bottom*). We conclude that all three helix 3 amino acids that differ between RARα and RARβ, located at positions 224, 225, and 234 in the protein sequence, contribute to the lack of repression and to the constitutive activation properties of RARβ. Notably, RARγ, which exhibits a constitutive transcriptional activation phenotype similar to that of RARβ, also possesses an RARβ amino acid sequence at these three helix 3 positions (Fig. 5A); changing these amino acids in RARγ to the RARα sequence converted the former from a hormone-independent activator to a transcriptional repressor (data not shown), indicating that this same region of helix 3 operates to define the distinctive transcriptional regulatory properties of all three isotypes.

DISCUSSION

In Contrast to RARα, the RARβ and RARγ Isotypes Do Not Repress, But Can Instead Activate Transcription in the Absence of Hormone

Although the different RAR isotypes have a significant degree of functional redundancy, each of the isotypes also mediates unique functions in the organism (2, 3,

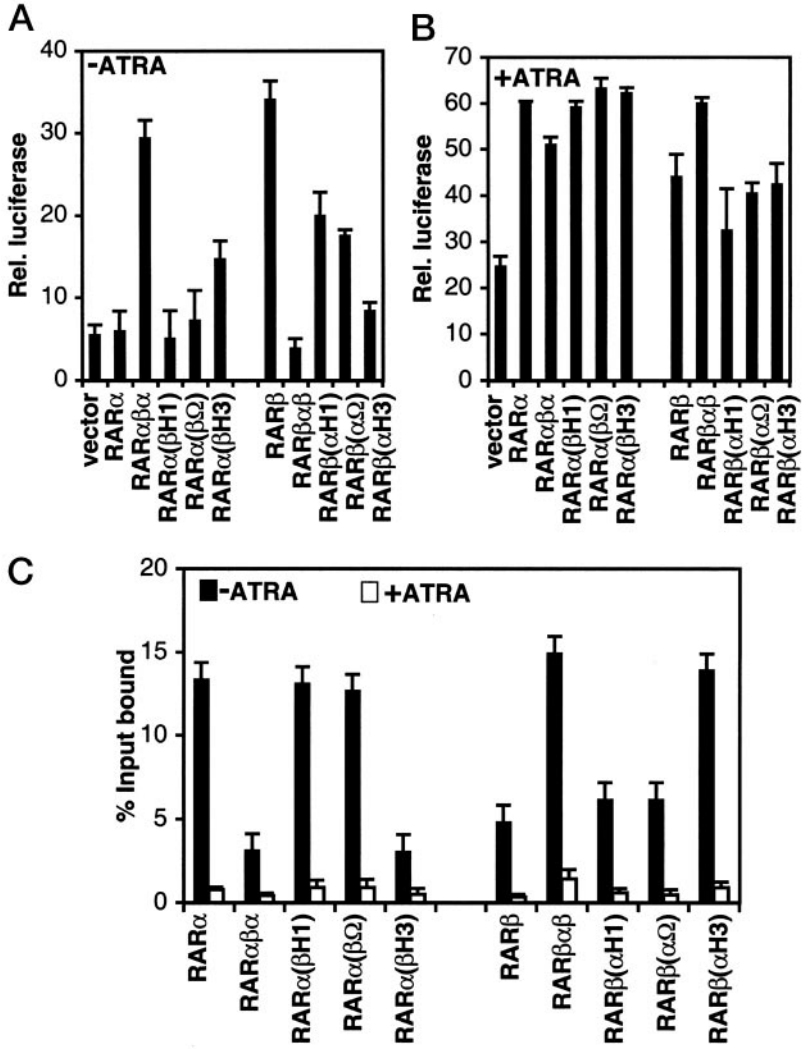

Fig. 6. The different transcriptional regulatory and SMRT corepressor binding properties of RARα and RARβ map to sequence differences within helix 3 of the hormone binding domain of these receptors. Different RARα constructs bearing the helix 1 (H1), omega loop (Ω-loop), or N-terminal portion of helix 3 of RARβ were created, as were the reciprocal constructs of RARβ. A and B, The distinct transcriptional regulatory properties of RARα and RARβ map to helix 3. The various RAR chimeras were tested as pSG5 constructs for the ability to activate or repress transcription of the pTK-Luc-RARE$_3$ reporter gene in transient transfections of CV-1 cells, as in Fig. 1B. The cells were subsequently incubated in the absence (A) or presence (B) of 400 nM ATRA, and the relative luciferase activity was determined as before. C, The distinct corepressor interaction properties of RARα and RARβ also map primarily to helix 3. The same chimeras were tested for the ability to bind to SMRT *in vitro* using a GST-pulldown assay. The amount of each RAR protein bound to the GST-SMRT construct in the absence (*filled bars*) or presence (*open bars*) of 400 nM ATRA is shown, expressed as a percentage of input. The average of duplicate experiments and the SD are shown in all cases.

5, 28–30, 35). The different RAR isotypes display very similar DNA and hormone binding properties *in vitro* (3). However, in a prior study, we reported that the abilities of RARα, RARβ, and RARγ to interact with SMRT corepressor are very different (31). In the current study, we examined whether these differences in corepressor interaction *in vitro* manifest as functional consequences *in vivo*. We report that there is a marked difference in the transcriptional properties of RARα, RARβ, and RARγ in the absence of hormone: RARα appears unique in its ability to repress reporter gene expression, whereas both RARβ and RARγ fail to repress and instead mediate a substantial level of transcriptional activation in the absence of ectopic hormone agonist. Moreover, there is a close correlation between the ability of the three RAR isotypes to repress *in vivo* and their ability to bind efficiently to SMRT corepressor *in vitro*. Nonetheless, all three RAR isotypes fully dissociate from SMRT, bind to coactivators, and mediate similar, strong reporter gene activation in the presence of an RAR hormone agonist, such as ATRA. These differences, seen in the absence of hormone, are likely to contribute to the physiological and developmental nonredundancy of the three differ-

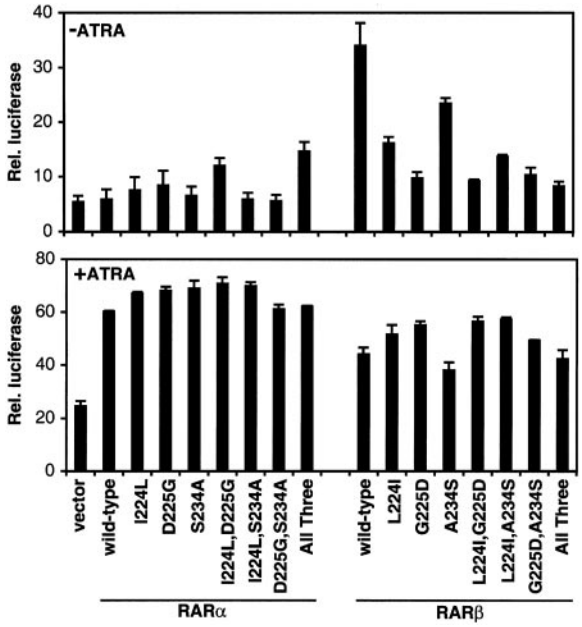

Fig. 7. All three amino acid differences between the relevant portions of helix 3 of RARα and RARβ contribute to the different transcriptional properties of these isotypes. Different RARα constructs bearing single, double, or triple amino acid substitutions characteristic of the RARβ sequence at codons 224, 225, or 234 were created by site-directed mutagenesis, as were the reciprocal substitution mutants of RARβ. These different RAR mutants were then tested as pSG5 constructs for the ability to activate or repress transcription of the pTK-Luc-RARE$_3$ reporter gene in transient transfections of CV-1 cells, as in Fig. 1B. The cells were incubated in the absence (*top*) or presence (*bottom*) of 400 nM ATRA, and the relative luciferase activity was determined as before. The average of duplicate experiments and the SD are shown in all cases.

ent RAR isotypes and may help to account for the conservation of these unique isotypes during vertebrate evolution. The different abilities of the three RAR isotypes to recruit SMRT may also influence the ability of the corresponding RXR/RAR heterodimers to activate transcription in response to RXR agonists (36), suggesting an additional biological consequence of the differing corepressor interaction properties of the three different RAR isotypes.

The Ability of RARα to Repress Transcription Is Obscured in Certain Cell Types, Apparently by the Presence of Endogenous Retinoid Receptors

Although RARα efficiently binds to SMRT corepressor both *in vitro* and *in vivo*, prior studies have noted that the ability of RARα to repress target genes in the absence of hormone varies in different cell types; in fact, the expression of ectopic RARα in many cell lines does not produce an overt repression of reporter gene expression (33). In agreement with these studies, our analysis demonstrated that ectopic expression of RARα repressed target gene expression most efficiently in L929 cells and had less of a repressive effect in several other cell lines. We noted that many of these same cell lines express high levels of endogenous RARs and exhibit an inherent ability to activate the pTK-Luc-RARE reporter in response to ATRA even in the absence of an experimentally introduced RAR. This suggested to us that the endogenous RARα present in these cells may cause an inherent repression of the reporter gene, precluding the detection of any further effects of an ectopically introduced RARα. Consistent with this hypothesis, the expression of a pTK-Luc reporter containing RARE sequences was suppressed in many cell lines compared with that of the same pTK-Luc reporter lacking RAREs, and this was observed even in the absence of an ectopically introduced RAR. To circumvent this experimental complexity, we created GAL4DBD fusions of the different RAR isotypes and tested their effects on a reporter gene construct engineered to be under the regulation of a GAL-17mer DNA site. The GAL-17mer reporter, unlike the pTK-Luc-RARE, displayed no retinoid responsiveness in the absence of an ectopic receptor construct, confirming that this reporter construct is not recognized by endogenous retinoid receptors. Using this experimental setup, we were able to detect unambiguous repression by GAL4DBD-RARα in the absence of hormone, whereas the comparable GAL4DBD-RARβ construct mediated significant reporter gene activation under the same conditions. As expected, addition of ATRA allowed both the GAL4DBD-RARα and GAL4DBD-RARβ fusions to activate the GAL-17mer reporter. We suggest that the prevalence of endogenous RARs in many cell lines has probably hampered prior experimental detection of the otherwise distinctive transcriptional properties of the different RAR isotypes.

We note that an alternative hypothesis might be advanced that certain of our cell lines may be able to produce retinoids intrinsically, and these intrinsic retinoids could serve to bind to, and obscure repression by, RARα in the absence of ectopic ATRA. Although we cannot completely discount this hypothesis, we observed no evidence for an involvement of intrinsic retinoids when using the GAL-17mer reporter system, in which the GAL4DBD-RARα construct functioned as a strong repressor in the absence of externally added ATRA.

The Different Transcriptional Properties of the Different RAR Isotypes Is Observed in Both the Absence and the Presence of an RXR Heterodimer Partner

RARs are believed to function primarily as RXR/RAR heterodimers *in vivo* (3, 4, 9, 34). The cell lines used in this study, as virtually all vertebrate cells, express significant levels of RXRs, and presumably these endogenous RXRs are available to form heterodimers with the ectopically introduced RARs studied here. It is difficult, however, to determine the extent of het-

erodimer formation in our transfections. We therefore investigated whether coexpressing RXR together with RARα or RARβ could alter the results of our transfection studies. Interestingly, cotransfection of RXRα did not confer transcriptional repression on RARβ, but rather further enhanced the hormone-independent activation properties of this isotype. We suggest that although RXRs have been shown to have a detectable, if weak, ability to bind to SMRT corepressor *in vitro*, the strength of this interaction is too modest *in vivo* to confer repression by either RXR homodimers or the RXR/RARβ heterodimers studied here.

The Different Abilities of RARα, RARβ, and RARγ to Repress Transcription Map Predominantly to a Region within Helix 3 of the Hormone Binding Domain of the Receptor

Our analysis of chimeric constructs created between RARα and RARβ demonstrated that the identity of the helix 1-Ω loop-helix 3 region of the hormone binding domain determines the ability of the receptor to repress or to activate transcription in the absence of hormone. Although the helix 1 and Ω loop contributed to this phenotype, the preponderance of the different activation/repression functions of unliganded RARα and RARβ mapped to amino acid differences within the helix 3 region of these isotypes. Simply replacing portions of helix 3 of RARα with the corresponding sequence of RARβ resulted in a loss of repression, whereas the reciprocal substitution of portions of helix 3 of RARβ with the equivalent sequence of RARα conferred the ability to repress reporter gene expression. All three of the amino acids that differ between RARα and RARβ within the relevant helix 3 domain, at postions 224, 225, and 234, contribute to some extent to this phenomenon. The same region of helix 3 also appears to play the same role in defining transcriptional properties of RARγ: RARγ possesses a helix 3 sequence identical to that of RARβ, and in common with RARβ; RARγ does not repress transcription, but instead activates reporter gene expression in the absence of hormone ligand.

How might helix 3 be exerting these transcriptional effects? We observed a close correlation between the ability of the different RAR isotypes to repress reporter gene expression *in vivo* and their ability to bind SMRT corepressor *in vitro*: RARα binds corepressor strongly and represses, whereas RARβ and RARγ bind corepressor comparatively weakly and activate rather than repress, in the absence of hormone. Helix 3 forms a portion of the corepressor interaction/docking surface on the nuclear receptor (37–40). However, it is also well established that the conformation of receptor helix 12 also plays a major role in gating access of corepressors and coactivators to their docking surface on the nuclear hormone receptors (10, 11). A genetic analysis suggests that an interaction between helix 3 and helix 12 in RARβ and RARγ stabilizes a closed helix 12 position in these isotypes and blocks access of corepressor to its docking site in the absence of hormone ligand, whereas the RARα helix 3 sequence disrupts this helix 12 interaction, resulting in an exposed corepressor docking surface and corepressor binding (Farboud, B., H. Hauksdottir, Y. Wu, and M. L. Privalsky, submitted for publication). There is precedent for this proposal: for example, the helix 12 of Ultraspiracle (the *Drosophila* ortholog of RXR) and of the constitutively activated androstanol receptor (CAR) also assume a closed conformation in the absence of known ligand, although the structural basis for this phenomenon appears to differ for each receptor (41–43). We suggest that, unrestrained by corepressor binding, RARβ and RARγ are able to recruit coactivators and to confer modest target gene activation even in the absence of hormone. The RARα isotype, in contrast, is dependent on hormone agonist to release corepressor, recruit coactivator, and activate target gene expression.

MATERIALS AND METHODS

No experimental animals were used in this research.

Molecular Clones

The human RARα, RARβ, and RARγ cDNA clones were obtained from P. Chambon (Institute of Genetics and Molecular and Cellular Biology, College of France, Illkirch Cedex, France). This particular isolate of RARβ, originally obtained from a human hepatocellular carcinoma and denoted HAP, encodes a Met at position 416, whereas most other RARβ isolates have been reported to encode a Leu in this position (44). Notably, this possible genetic polymorphism did not result in a difference in function in our assays, and we obtained comparable results when the relevant experiments were repeated with a construct in which Met 416 was replaced by a Leu (data not shown).

The subcloning of pSG5-RAR$\alpha\beta\alpha$ and pSG5-RAR$\beta\alpha\beta$ was described elsewhere (31). To create the pSG5-RAR$\alpha\beta\alpha\alpha$ and pSG5-RAR$\beta\beta\alpha\beta$ chimeras, a *Sac*I restriction site was introduced between codons 198 and 199 in pSG5-RARβ and in pSG5-RAR$\alpha\beta\alpha$ by site-directed QuikChange mutagenesis (Stratagene, La Jolla, CA) to match a corresponding *Sac*I site that occurs naturally in pSG5-RARα. To avoid confusion arising from the different N-terminal lengths of the different RAR isotypes, we have adopted the convention of Ref. 45 and use in these descriptions an identical codon numbering system for all three isotypes based on that of RARγ. Subsequently, a *Sac*I-*Bcl*I fragment from RAR$\alpha\beta\alpha$ was swapped with the *Sac*I-*Bcl*I fragment from RARα to create RAR$\alpha\beta\alpha\alpha$, representing an RARα receptor with codons 187–233 derived from RARβ. Likewise, a *Kpn*I-*Sac*I fragment from RAR$\beta\alpha\beta$ was swapped with a *Kpn*I-*Sac*I fragment from RARβ to create RAR$\beta\beta\alpha\beta$, creating a RARβ receptor with codons 199–246 derived from the RARα sequence. The RAR helix 1, Ω loop, and helix 3 chimeras were created by PCR overlap extension (46). The RARα(βH1) chimera represents an RARα receptor with codons 183–200 derived from RARβ sequence. Likewise, the RARβ(αH1) chimera is a RARβ receptor wherein codons 183–200 have been replaced with the RARα sequence. The RARα($\beta\Omega$) chimera represents an RARα receptor with codons 201–223 replaced with RARβ sequence; the RARβ($\alpha\Omega$) chimera represents a receptor in which codons 201–223 in RARβ have been replaced with RARα sequence. In the RARα(βH3) chimera, codons 224–237 in RARα have been

replaced with RARβ sequence, whereas in the RARβ(αH3) chimera, codons 224–237 have been replaced with the RARα sequence. The pSG5-RARα(S232A), pSG5-RARβ(A225S), pSG5-RARβ(R221S), pSG5-RARα(IEKV→TEKI) and pSG5-RARβ(TEKI→IEKV) mutants were created from the wild-type pSG5-RARα and pSG5-RARβ clones by oligonucleotide-mediated, site-directed QuikChange mutagenesis.

The GAL4DBD-RAR-LBD fusions were created as follows. The hormone binding domains of wild-type RARα, RARβ, RARγ, or the appropriate mutants were amplified by PCR. Subsequently, these sequences were excised as *Sma*I-*Bam*HI restriction fragments and inserted into the corresponding sites in a pSG5-GAL4DBD mammalian expression vector (47). The M-pTK-Luc-RARE$_2$ reporter vector was created as follows: the M-pTK-Luc vector (48) was digested with *Xho*I and *Sal*I, and the direct-repeat-4 element was discarded. Two oligonucleotides were annealed (5′-TCGAGGGTAG GGTTCACCGA AAGTTCACTCT AGAGGGTTCA CCGAAAGTTC ACTCG-3′ and 5′-TCGACGAGTG AACTTCGGTG AACCCTCTAG AGTGAACTTT CGGTGAACCC TACCC-3′) to create a sequence containing three RARE elements with *Sal*I/*Xho*I compatible ends. Subsequently, the annealed oligonucleotides were cloned into the *Sal*I and *Xho*I sites on the vector. The pGL3-GAL-17mer reporter plasmid was created by annealing oligonucleotides containing two tandem GAL-17mer response elements with *Sal*I/*Xho*I compatible ends (5′-TCGACCGGAG GACAGTCCTC CGGCC GGAGGACAGT CCTCCGG-3′ and 5′-TCGACCGGAG GACTGTCCTC CGGCCGGAGG ACTGTCCTCCGG-3′). The annealed oligonucleotides were cloned into the *Sal*I and *Xho*I sites of the pGL3-luciferase reporter vector (Promega Corp., Madison, WI). The pTK-Luc-RARE$_3$ reporter, containing three copies of the RARE found within the human RARβ promoter, was obtained from A. Dejean (Pasteur Institute, Paris, France).

Transient Transfections

Transient transfections were performed as previously described (49). Briefly, CV-1, HeLa, or L929 cells were plated at a density of 9×10^4 cells per well in 12-well tissue culture plates, were permitted to attach, and were transfected using either a LipofectAMINE plus (Figs. 2–5; Life Technologies, Inc., Gaithersburg, MD) or Effectin (Figs. 1, 6, 7; QIAGEN, Valencia, CA) protocol. Ten nanograms of the pSG5 expression plasmid (either an empty vector or encoding the appropriate RAR isotype), 200 ng of luciferase reporter (pTK-Luc empty, pTK-Luc-RARE$_3$, M-pTK-Luc empty, M-pTK-Luc-RARE$_2$, or pGL3-GAL-17mer-Luc), and 100 ng of a pCH110-lacZ reporter plasmid (used as an internal transfection normalization control) were introduced per well, plus sufficient pUC18 plasmid to adjust each transfection to 500 ng total DNA. After a 3-h incubation at 37 C in the transfection media, either ATRA or an equivalent amount of hormone-free ethanol carrier was added to each well, and the cells were incubated for an additional 24 h at 37 C. Cells were then harvested and lysed, and the β-galactosidase and luciferase activities were detected as described previously (47).

RAR-Corepressor Binding Assays

Interactions between receptors and the SMRT corepressor were assayed *in vitro* by the use of a GST-pulldown protocol (50). GST or GST fused to TRAC-1 codons 406–769 (Ref. 47; corresponding to SMRTα codons 2109–25189 in Ref. 51) were produced in *Escherichia coli* and were purified by immobilization on a glutathione-agarose matrix (Sigma Chemicals, St. Louis MO). Radiolabeled RAR proteins were synthesized in a coupled transcription-translation system using the T7 promoter site within the pSG5 expression constructs (Promega Corp.). Protein binding reactions were performed as described previously (50). Briefly, radiolabeled receptor was incubated with the immobilized GST or GST-SMRT proteins for 1 h at 4 C, and unbound receptor was removed by four cycles of washing the glutathione agarose matrix with 1 ml each cycle of HEMG buffer. Any RARs remaining bound to the GST-SMRT matrix were then eluted by incubating the matrix with 10 mM glutathione in 50 mM Tris-Cl (pH 7.6), and the eluted protein was resolved by SDS-PAGE. Radiolabel was visualized and quantified by PhosphorImager analysis (Storm System, Molecular Dynamics, Inc., Sunnyvale CA).

Immunoblotting Analysis

CV-1 cells were transfected with pSG5 vectors expressing the RARα, RARβ, and RARγ isotypes using the transient transfection protocol detailed above. Two days after transfection, the cells were harvested by scraping and lysed in SDS-sample buffer, and the lysates were resolved by SDS-PAGE using a NuPage Tris-acetate buffer system (Invitrogen Inc., Carlsbad CA). The proteins were transferred by blotting to nitrocellulose membranes, and the membranes were blocked by incubation for 45 min in TBST buffer [10 mM Tris-Cl (pH 7.8), 150 mM NaCl, 0.1% Tween-20] plus 5% milk. The membranes were then washed in TBST buffer (2×5 min) and incubated for 1 h with rabbit polyclonal anti-RAR antisera diluted 1:1000 in 5 ml of TBST buffer plus 5% milk (antibody M454 from Santa Cruz Biotechnology, Inc., Santa Cruz CA). The membranes were then washed three times for 15 min in TBST buffer and incubated for 1 h with horseradish peroxidase-coupled, antirabbit IgG diluted 1:1000 in 5 ml of TBST buffer plus 5% milk. After additional washing in TBST, the RAR bands were visualized using ECL Plus (Amersham Pharmacia Biotech Biosciences, Piscataway, NJ) and quantified by scanning with the Storm System Imager using a chemiluminescence mode. Although capable of recognizing all three isotypes, the M454 antisera reacts preferentially with the RARγ isotype; we quantified these differences in M454 reactivity by using RAR isotypes synthesized to known levels by *in vitro* transcription and translation, and normalized the values obtained from immunoblots of transfected cells accordingly.

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