

Isotype-Restricted Corepressor Recruitment: a Constitutively Closed Helix 12 Conformation in Retinoic Acid Receptors β and γ Interferes with Corepressor Recruitment and Prevents Transcriptional Repression

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Received 8 October 2002/Returned for modification 21 November 2002/Accepted 21 January 2003

Retinoic acid receptors (RARs) are ligand-regulated transcription factors that play multiple roles in vertebrate development and differentiation. RARs as a class are capable of both repressing and activating target gene expression. Transcriptional repression is mediated through the recruitment of corepressor proteins such as SMRT. Notably, vertebrates encode three major forms of RARs, α , β , and γ , and these distinct RAR isotypes differ in the ability to recruit a corepressor. RAR α strongly interacts with SMRT and can repress target gene transcription, whereas RAR β and γ interact with SMRT only weakly and fail to repress. We report here the use of a genetic suppressor approach, based on a yeast two-hybrid interaction assay using *Saccharomyces cerevisiae*, for the isolation of RAR β mutants that have gained the RAR α -like corepressor phenotype, i.e., a strong interaction with SMRT and the ability to repress gene expression in vertebrate cells. Analysis of these gain-of-function mutants indicates that the different corepressor interaction properties of RAR α , β and γ are determined by a gating mechanism through which amino acid differences in the helix 3 region of these receptors influence the position of the receptor C-terminal helix 12 domain. As a consequence, the RAR β and RAR γ receptors appear to adopt a constitutively closed helix 12 conformation in the absence of hormone that may approximate the conformation of RAR α when bound to hormone agonist. This closed helix 12 conformation in RAR β and RAR γ blocks corepressor binding, prevents repression, and permits significant levels of target gene activation even in the absence of hormone. We refer to this phenomenon as a “gate-latch” model of corepressor regulation.

Retinoic acid receptors (RARs) are hormone-regulated transcription factors that regulate multiple aspects of vertebrate development and differentiation (9, 44, 45, 47, 59, 68). RARs exert these effects principally by binding to specific DNA sequences and regulating the expression of adjacent target genes (9, 44, 45, 47, 59, 68). In common with many other members of the nuclear receptor family, RARs possess bimodal transcriptional properties and can function either by repressing or by activating transcription of their target genes (19, 47, 57). This dual mode of transcriptional regulation is dependent on the ability of the receptors to associate with coregulator proteins denoted coactivators and corepressors (reviewed in references 13, 19, 30, 35, 39, 47, 48, 53, 57, 65, 71, and 80). Corepressors such as SMRT and its paralog, N-CoR, bind the nuclear receptor directly and can tether additional components of a larger corepressor complex, including TBL-1 and an array of histone deacetylases (HDACs) (13, 19, 30, 35, 39, 47, 48, 53, 57, 65, 71, 80). Conversely, the known coactivators that can function with the RARs include SRC-1, GRIP-1, ACTR, p300/CBP, pCAF, and the DRIP/TRAP/Mediator complexes (13, 30, 35, 39, 47, 48, 65, 71, 80). In turn, corepressors and coactivators modulate transcription by modifying the chromatin template and by interacting with the general tran-

scriptional machinery to promote or inhibit formation of a preinitiation complex (2, 4, 12, 23, 34, 49, 55, 60, 67, 72, 75, 77, 78).

Nuclear receptors typically recruit the SMRT/N-CoR corepressor complex and repress transcription in the absence of hormone or when bound to hormone antagonists (19, 57). Alternatively, the binding of an agonist by the receptor induces dissociation of the corepressor complex, the binding of coactivators, and transcriptional activation (19, 57). Corepressors are tethered to the unliganded nuclear receptor through the interaction of L/V-X-X-I/V-I amino acid motifs within SMRT and N-CoR with a docking surface on the receptor composed primarily of portions of helix 3 and helix 4/5 of the hormone binding domain (31, 32, 46, 51, 56, 79). In the absence of hormone, the C-terminal receptor helix 12 is thought to assume an extended conformation that allows corepressor access to its docking site (6, 19, 31, 32, 51, 56, 79). Conversely, the binding of a hormone agonist appears to induce a conformational change in the receptor, leading to the assumption by helix 12 of a more sequestered position lying across the receptor surface (7, 58). This sequestered helix 12 position occludes the corepressor docking site, resulting in release of corepressor, and simultaneously contributes to the formation of a new interaction surface that allows recruitment of the L-X-X-L-L amino acid motifs found in many coactivators (19, 31, 32, 42, 51, 56, 57, 79). Hormone antagonists confer additional conformations of helix 12 that destabilize coactivator binding and/or further favor corepressor binding (for examples, see references 8, 33, 54, 64, 66, 73, 79, and 85). Helix 12 therefore represents

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a hormone-operated, multiposition toggle switch that plays a key role in gating access of both corepressors and coactivators to their docking sites on the nuclear receptors.

Three distinct genetic loci encode RARs in vertebrates, resulting in the synthesis of three major RAR isotypes: α , β , and γ (9, 45, 68). Alternative mRNA splicing and promoter utilization result in further diversification within each isotype. The three RAR loci are strongly conserved in evolution and mediate distinctive biological functions, yet they encode receptors that share substantial amino acid sequence identity and possess similar biochemical properties (9, 45, 68). One intriguing difference among the different RAR isoforms that might contribute to their distinct functions in vertebrate development and physiology has been previously reported: RAR α recruits the SMRT corepressor and represses target gene transcription in the absence of hormone, whereas RAR β (and RAR γ) does neither (76; H. Hauksdottir, B. Farhoud, and M. Privalsky, submitted for publication). Instead, RAR β and RAR γ mediate significant levels of transcriptional activation even in the absence of hormone, and this activation is further augmented in response to the all-*trans* retinoic acid (ATRA) ligand (Hauksdottir, Farhoud, and Privalsky, submitted). Unexpectedly, prior studies by Wong and Privalsky indicated that these different corepressor interaction properties of RAR α , β , and γ map outside of the known corepressor docking site, suggesting that the ability of different isoforms of RAR to recruit a corepressor (and to repress transcription) is mediated by a region of the receptor distinct from the actual site of corepressor contact (76).

To better understand this phenomenon, we have now employed a genetic suppression-mutagenesis scheme to identify alterations in RAR β that are able to confer the RAR α -like corepressor interaction phenotype. Using this method, we have determined that RAR β (and RAR γ) has a fully functional SMRT docking site but that access of SMRT to this docking site is blocked by helix 12, which in RAR β and RAR γ assumes a sequestered position in the absence of hormone that approximates the conformation of the agonist-bound RAR α . This constitutively closed helix 12 conformation of RAR β and RAR γ appears to be stabilized by hydrophobic interactions between amino acids in receptor helix 3 and helix 12. Disruption of this helix 3-helix 12 interaction by the amino acid substitutions found naturally in helix 3 of RAR α , or by artificial mutagenesis of RAR β and RAR γ , confers a more open helix 12 conformation, enhanced corepressor binding, and transcriptional repression in the absence of hormone. Consistent with this "gate-latch" model for helix 12 of RAR β and RAR γ , these two isotypes also display an enhanced ability to recruit p160 coactivators and to activate transcription in the absence of hormone compared to that of the unliganded RAR α . We conclude that tertiary interactions confer inherently different conformations on helix 12 of the unliganded RAR α , β , and γ and that this evolutionarily programmed, differential gating of helix 12 contributes to the distinct cofactor recruitment and transcriptional properties of these different receptor isotypes.

MATERIALS AND METHODS

Molecular clones. The molecular clones of human RAR α , RAR β , and RAR γ cDNAs were previously described, as were the constructs for pGBT9-SMRT

(representing codons 71 to 769 in the TRAC1/SMRT clone), pGEX-KG-SMRT (representing codons 406 to 769 in the TRAC1/SMRT clone), pGEX-KG-SRC1 (representing codons 560 to 1136 of SRC1), and pGEX-KG-ACTR (representing codons 621 to 821 of ACTR) (11, 24, 28, 62, 76, 81). The RAR β isolate, originally obtained from a human hepatocellular carcinoma and designated HAP, encodes a Met at position 407 whereas most other RAR β isolates have been reported to encode a Leu in this position (16). This possible genetic polymorphism did not result in a functional difference in our assays, and comparable results were obtained with constructs in which Met 407 was replaced by a Leu (data not shown). Chimeras between the different RAR isotypes were constructed by ligation of suitable restriction fragments or by overlap extension PCR methodology (1), whereas point mutations were generated by a QuikChange oligonucleotide-mediated mutagenesis procedure (Stratagene, La Jolla, Calif.). Using standard restriction enzyme, PCR, and ligation procedures (1), the constructs of pACT2-RAR α (representing codons 86 to 462 for RAR α), pACT2-RAR β (representing codons 79 to 448 for RAR β), and pSG5-GAL4DBD-SMRT (representing codons 71 to 769 in the TRAC1/SMRT clone) were created. The pGEX-KG-SMRT(RID1) construct was created by using a PCR procedure to flank the SMRT receptor interaction domain 1(RID-1) CoNR box motif (RVVTLAQHISEVITQDYTR) with *Bam*HI and *Sma*I restriction sites (1); the resulting PCR fragment was excised and then joined in frame to the glutathione *S*-transferase (GST) sequence within pGEX-KG (21). All clones were confirmed by DNA sequence analysis.

Genetic suppression-yeast two-hybrid strategy. The RAR β hormone binding domain was subjected to random mutagenesis by propagating our pACT2-RAR β clone in XL1-Red cells according to the instructions of the manufacturer (Stratagene). The mutated plasmids were recovered as a mixed population, and the sequences corresponding to the RAR β hormone binding domain were excised and reintroduced into a nonmutated pACT2 background (to exclude mutations generated within the vector backbone itself). This reconstituted pACT2-mutated RAR β plasmid population was then amplified and cotransformed together with pGBT9-SMRT into the *Saccharomyces cerevisiae* strain SFY526, thus selecting for growth in the absence of Leu and Trp (62). Separate yeast transformants (10,000) were selected, replica patched on fresh, appropriately supplemented synthetic dextrose agar plates, and tested for the ability to express β -galactosidase by use of a 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) overlay technique (37). Briefly, the patched yeast colonies were permitted to grow for 24 h at 30°C; the plates were then overlaid with a molten 0.5% agarose-0.5 M NaPO₄ (pH 7.0)-0.1% sodium dodecyl sulfate (SDS)-0.02% *N,N*-dimethyl formamide-0.2% X-Gal solution and were incubated for an additional hour at 37°C for colorimetric development. Yeast colonies that displayed a more intense blue color in the X-Gal assay than did control transformants containing the wild-type pACT2-RAR β (i.e., indicative of an enhanced RAR β -SMRT interaction) were identified visually, and the corresponding pACT2-mutant RAR β plasmid DNAs were recovered by transformation of *Escherichia coli* DH5 α . Once recovered, the pACT2-mutant RAR β plasmid DNAs were amplified and reintroduced into fresh yeast SFY526 cells together with the pGBT9-SMRT vector (62). pACT2-mutant RAR β plasmids that retained the ability to produce intensified β -galactosidase expression in these secondary yeast transformants (i.e., retained the enhanced RAR β -SMRT interaction) were analyzed further by sequence determination, by a liquid-based β -galactosidase assay, and by the procedures described below.

Mammalian two-hybrid assay. Approximately 5×10^4 CV-1b cells were aliquoted per well in 24-well culture plates containing Dulbecco modified Eagle's medium (DMEM) supplemented with hormone-depleted 10% fetal bovine serum. The cells were permitted to attach overnight at 37°C. Immediately prior to transfection, the cells were rinsed with phosphate-buffered saline and placed in DMEM supplemented with 10% hormone-depleted fetal bovine serum. Transfections were performed using the Effectene protocol as recommended by the manufacturer (Qiagen, Valencia, Calif.); 12.5 ng of pSG5-GAL4DBD-SMRT, 50 ng of pSG5-GAL4AD receptor, 50 ng of pGL2-GAL-17mer luciferase reporter, 25 ng of pCMV-lacZ (used as an internal transfection control), and 112.5 ng of pUC18 were employed per well (29, 62, 76). The culture medium was replaced 24 h later with fresh medium containing either ATRA or an equivalent amount of ethanol carrier alone. After an additional 24 h at 37°C, the cells were harvested and lysed and the luciferase and β -galactosidase activities were determined as previously described (29, 76).

Transient transfection assay. CV-1b cells were plated in DMEM supplemented with hormone-depleted 10% fetal bovine serum as described above. The cells were rinsed with phosphate-buffered saline, placed in DMEM supplemented with 10% hormone-depleted fetal bovine serum, and then transfected using the Effectene protocol (Qiagen) and 50 ng of the pTK-Luc retinoic acid response element (RARE) reporter vector, 10 ng of the appropriate pSG5-RAR

expression vector, 5 ng of pCMV-lacZ, and 185 ng of pUC18 (24). The culture medium was replaced 24 h later with fresh medium containing either ATRA or an equivalent amount of ethanol carrier alone. The cells were harvested 24 h later, and the luciferase and β -galactosidase activities were determined as previously described (24).

Protein-protein interaction assay in vitro. Glutathione *S*-transferase (GST) corepressor and GST coactivator proteins were synthesized in *E. coli* BL-21 cells containing the corresponding pGEX-KG vector (pGEX-KG-SMRT, pGEX-KG-SRC1, or pGEX-KG-ACTR), the bacteria were lysed, and the GST fusion proteins were bound to a glutathione-agarose matrix (21). 35 S-radiolabeled RARs were synthesized in vitro by a coupled transcription-translation (TnT) system (Promega, Madison, Wis.). Each radiolabeled receptor (typically 2 to 5 μ l of TnT product per reaction) was then incubated with the immobilized GST fusion protein of interest (approximately 50 ng of GST fusion protein immobilized to 10 μ l of agarose matrix per reaction) in a total volume of 120 μ l of HEMG buffer (4 mM HEPES [pH 7.8], 100 mM KCl, 0.2 mM EDTA, 5 mM MgCl₂, 0.1% NP-40, 10% glycerol, 1.5 mM dithiothreitol) at 4°C (62). The binding reactions were performed with 96-well multiscreen filter plates (Millipore, Bedford, Mass.) placed on a roller drum to ensure constant mixing. After a 3-h incubation, the filter wells were washed by centrifugation four times with 200 μ l of ice-cold HEMG buffer each, and any radiolabeled RAR proteins remaining bound to the immobilized GST fusion proteins were subsequently eluted with 50 μ l of 10 mM glutathione in 50 mM Tris-HCl, pH 7.8. The eluted proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (62) and were visualized and quantified using a PhosphorImager/STORM system (Molecular Dynamics, Sunnyvale, Calif.).

Protease resistance assay. Using the coupled TnT system (Promega), 35 S-radiolabeled RAR proteins were synthesized in vitro. For each time point, 1 μ l of the TnT reaction products was diluted to 16 μ l in 50 mM Tris-HCl (pH 6.8) containing either 1 μ M ATRA or an equivalent amount of ethanol carrier. After 10 min on ice, 4 μ l of carboxypeptidase Y (CPY) (1 mg/ml; Sigma) was added to each sample and the tubes were transferred to 10°C to initiate the digestion (28, 42). At each time point indicated, the proteolysis was terminated by the addition of 20 μ l of SDS-PAGE buffer and the samples were rapidly frozen and stored on dry ice. The samples were subsequently quickly denatured by heating to 95°C for 10 min and were resolved by SDS-PAGE, and the proteolytic degradation products were visualized and quantified by PhosphorImager analysis.

RESULTS

RAR α interacts with SMRT corepressor strongly and represses transcription in the absence of hormone, whereas RAR β and γ interact with SMRT poorly and fail to repress. Consistent with prior reports (76), we determined that RAR α bound to a GST-SMRT construct with high affinity in the absence of hormone, whereas RAR β and γ displayed only a very weak interaction with GST-SMRT under the same conditions (Fig. 1A). Addition of ATRA disrupted both the strong binding seen for RAR α and the residual binding observed for the RAR β and γ isotypes, resulting in loss of all three receptor isotypes from the immobilized GST-SMRT (Fig. 1A). The impaired (relative to RAR α) ability of RAR β and γ to recruit SMRT in vitro was also observed in mammalian two-hybrid assays in vivo (Fig. 1B) when N-CoR was substituted for SMRT (76) and in the presence of a retinoid X receptor (RXR) heterodimer partner (data not shown). Paralleling these isotype-specific differences in corepressor binding, RAR α repressed the expression of an RARE-thiamine kinase (TK) promoter luciferase reporter in transfected CV-1b cells in the absence of hormone, whereas RAR β and γ failed to repress reporter gene expression under these conditions (Fig. 1C and 2B). Instead, both RAR β and γ induced significant levels of reporter gene activation in the absence of hormone, and this activation was further increased by the addition of ATRA; RAR α , in contrast, induced reporter gene activation only in the presence of the ATRA agonist (Fig. 1C and 2B).

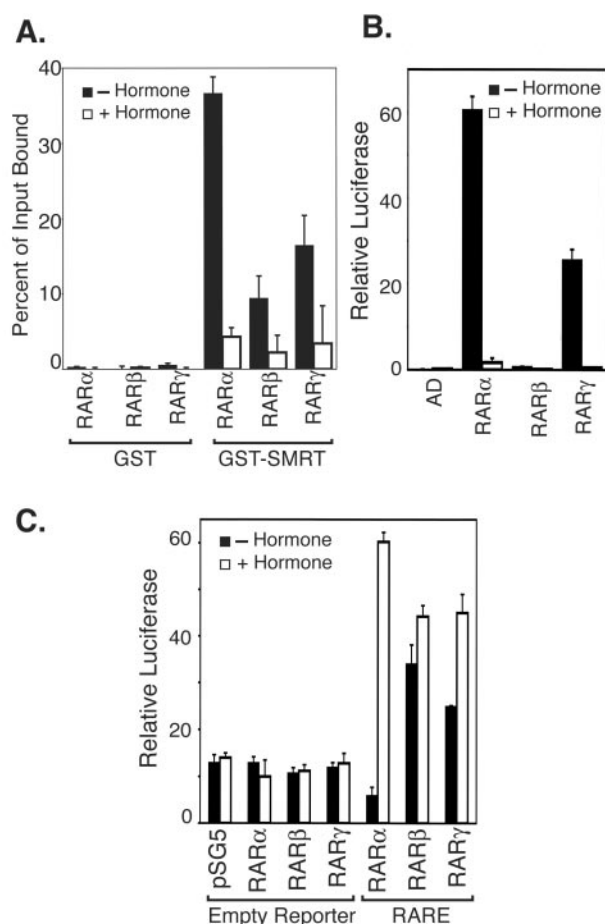


FIG. 1. RAR α , but not RAR β or RAR γ , interacts with SMRT corepressor in vitro and in vivo and represses reporter gene expression in transfected cells. (A) RAR α efficiently binds to SMRT corepressor in a GST pull-down assay, but RAR β and RAR γ do not. Radiolabeled RAR α , β , or γ , as indicated below the panel, was synthesized in vitro by a coupled TnT reaction and incubated with a GST or a GST-SMRT construct previously immobilized to a glutathione-agarose matrix. Incubations were performed in the absence (filled bars) or presence (open bars) of 1 μ M ATRA. The radiolabeled receptors remaining bound to the GST-SMRT matrix after repeated washing were eluted, resolved by SDS-PAGE, and quantified by PhosphorImager analysis. The percentage of each input protein bound to the GST or GST-SMRT matrix is shown; the averages and standard deviations from duplicate experiments are noted. (B) RAR α , but not RAR β or RAR γ , efficiently interacts with SMRT corepressor in a mammalian two-hybrid assay. pSG5 clones expressing GAL4AD alone or fused to the hormone binding domain of RAR α , β , or γ (as noted below the panel) were cotransfected by transient transfection into CV-1 cells together with a GAL4DBD-SMRT construct and a GAL4-(17mer)-luciferase reporter vector. A pCMV-lacZ reporter was included as an internal normalization control. The cells were incubated in the absence or presence of 500 nM ATRA for 24 h prior to harvest and lysis. Relative luciferase activity, indicative of an interaction between receptor and corepressor, was determined. (C) The different RAR isoforms differ in their abilities to regulate reporter gene expression in the absence of hormone. An empty pTK-Luc reporter (Empty Reporter), or the same reporter containing three RAREs, was introduced into CV-1b cells by transfection together with a pSG5 vector expressing the native RAR α , RAR β , or RAR γ . A pCMV-lacZ reporter was included as an internal normalization control. The cells were incubated in the absence or presence of 500 nM ATRA for 24 h prior to lysis and analysis. Relative luciferase activity was calculated as described for panel B; two or more independent experiments, each in duplicate, were performed, and the averages and standard deviations are shown.

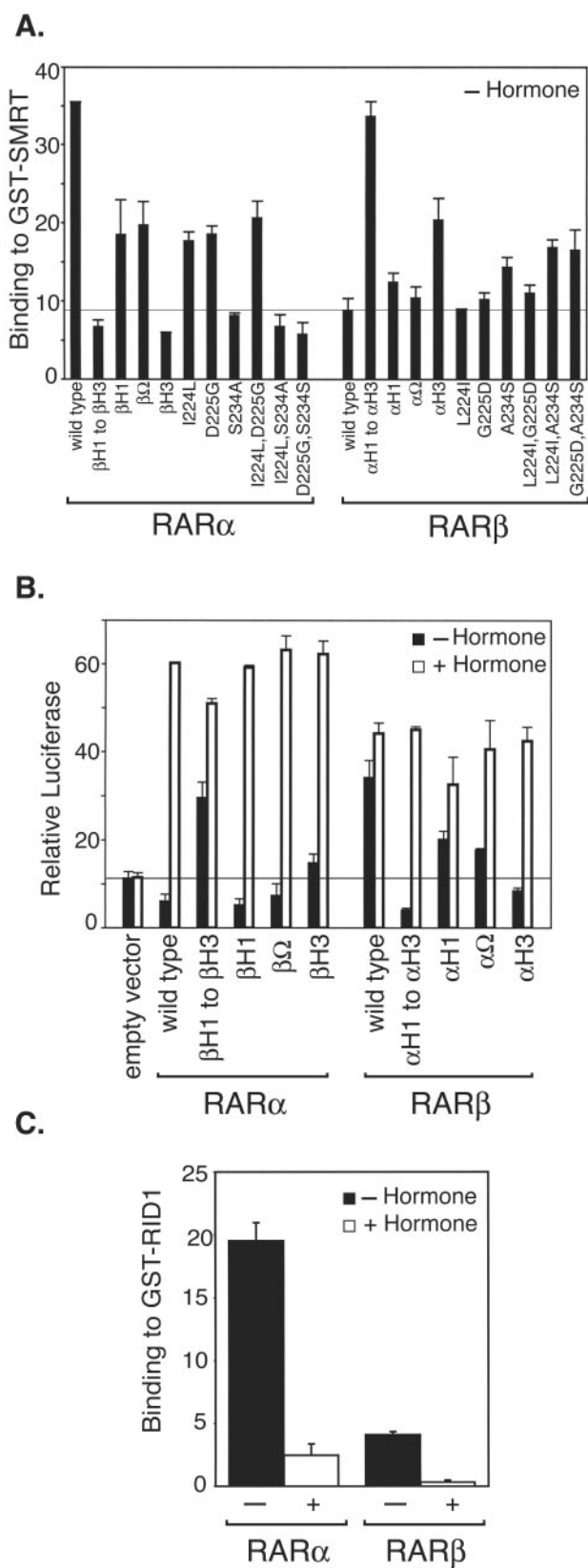


FIG. 2. The differing corepressor and transcriptional regulatory properties of RAR α and - β map primarily to helix 3 within the hormone binding domains of these receptors. (A) Chimeras of RAR α that contain the helix 3 domain of RAR β display poor corepressor binding,

These differences in the abilities of RAR α , - β , and - γ to induce repression versus activation of reporter expression in the absence of hormone were also observed in the presence of an RXR α expression vector, in L929 cells, and in the employment of GAL4-DNA binding domain (GAL4DBD) fusions of the corresponding RAR isotypes (Hauksdottir, Farboud, and Privalsky, submitted). Overexpression of SMRT in the transfected CV-1 cells did not convert either RAR β or RAR γ into transcriptional repressors (data not shown).

The differences in corepressor recruitment by RAR α and - β map primarily to differences in amino acid sequence in helix 3 of the ligand binding domain. To map the receptor determinants responsible for the different corepressor recruitment properties of the different RAR isotypes, we created a series of chimeric constructs between RAR α and - β and tested the chimeric receptors for the ability to bind to SMRT. Wong and Privalsky previously demonstrated that constructs of RAR β that contained the ligand binding domain derived from RAR α displayed the RAR α phenotype and interacted with SMRT with high affinity both in vitro and in vivo (76). Reciprocally, constructs of RAR α that possessed the ligand binding domain from RAR β exhibited the RAR β phenotype and failed to efficiently interact with SMRT by either GST pull-down or by mammalian two-hybrid assay (76). Analysis of additional chimeras further mapped the relevant corepressor recruitment determinants to the N-terminal portion of the ligand binding domain, encompassing helices 1 through 3 (Fig. 2A and 3A). More detailed dissection demonstrated that the identity of the RAR helix 3 region played a dominant role in determining the SMRT binding properties of the two different RAR isoforms. For example, RAR chimeras that possessed an N-terminal half of helix 3 derived from the RAR α sequence interacted with SMRT strongly, whereas RAR chimeras that possessed the corresponding N-terminal half of helix 3 derived from the RAR β sequence displayed little or no corepressor interaction (Fig. 2A). Nonetheless, it should be noted that the helix 1 and Ω loop subdomain of the RAR hormone binding domain did

whereas chimeras of RAR β that contain the helix 3 domain of RAR α display efficient corepressor binding. Wild-type RAR α , wild-type RAR β , or different chimeras and point mutants of the two isotypes, as indicated below the panel, were assayed as described for Fig. 1A for the ability to bind to a GST-SMRT construct in vitro. All assays were performed in the absence of hormone; binding of each RAR to a nonrecombinant GST construct was less than 1% of input. (B) Chimeras of RAR α bearing the RAR β helix 3 domain activate reporter gene expression in the absence of hormone, whereas chimeras of RAR β possessing the RAR α helix 3 domain repress expression. The different RAR chimeras and mutants described for panel A were tested for the ability to activate or repress the pTK-Luc-RARE reporter gene in transient transfections of CV-1b cells, as described for Fig. 1C. The cells were incubated in the absence (filled bars) or presence (open bars) of 500 nM ATRA and harvested 24 h later, and relative luciferase activity was determined. The average and standard deviation of two or more experiments are shown. (C) The different affinities of RAR α and - β for SMRT are maintained when a corepressor domain restricted to the RID-1 region is employed. A GST protein interaction assay was performed as described for Fig. 1A but employed a recombinant GST-SMRT construct limited to the RID-1 domain (i.e., the more N terminal of the two RID domains in SMRT). The averages and standard deviations for two or more independent experiments, performed in duplicate, are shown.

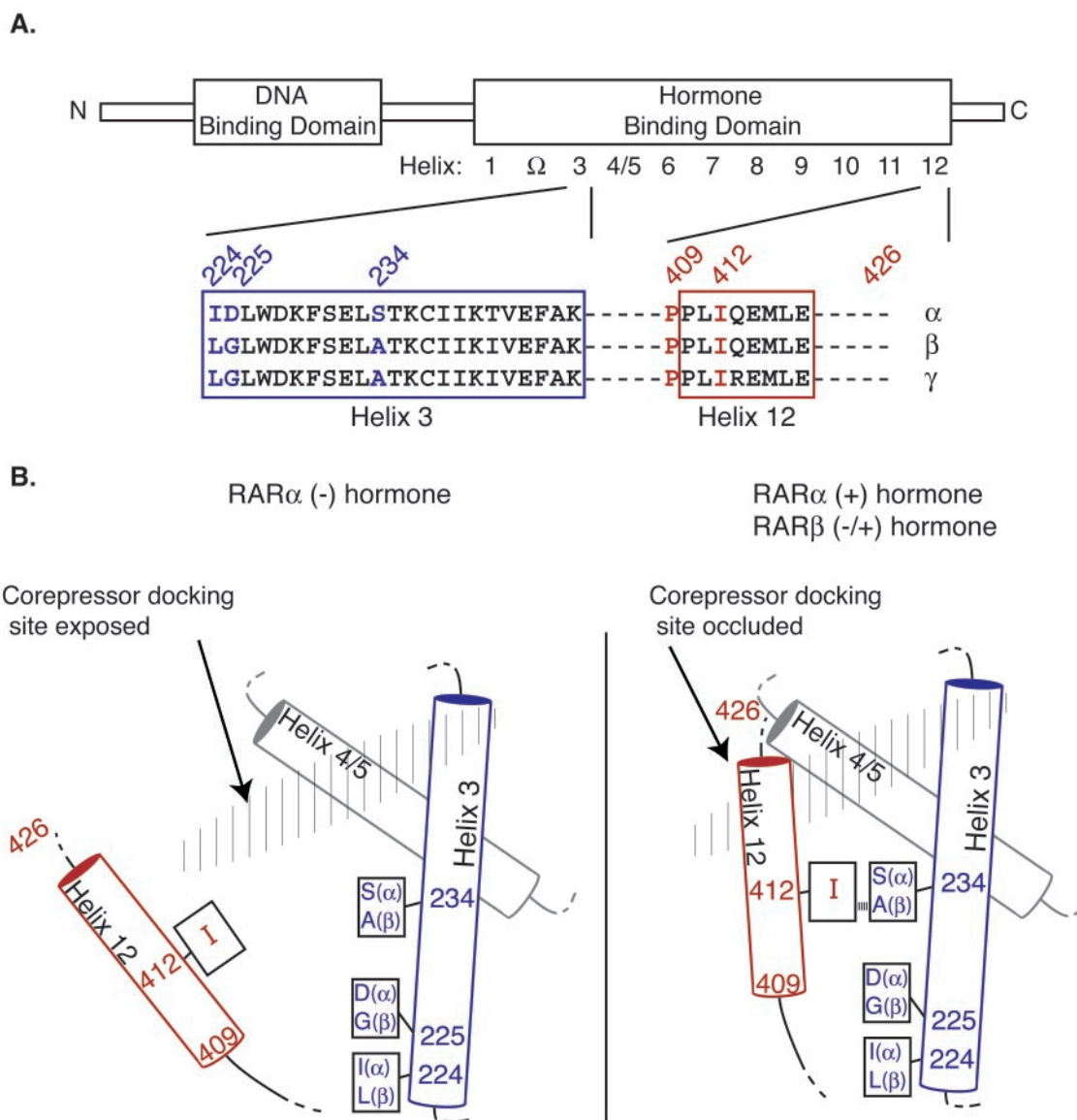


FIG. 3. Schematics of the different RAR isotypes are presented. (A) A schematic representation of a generic RAR is depicted. The locations of the DNA and hormone binding domains, and the various structural features noted in the text, are indicated. The amino acid sequences of the different RAR isotypes are compared for helix 3 and helix 12. Amino acids described in the text are highlighted. (B) A model for the structures of the RAR α and - β isotypes in the absence and presence of hormone is presented. The proposed orientations of helix 3, helix 4/5, and helix 12 in the absence or presence of a hormone agonist are shown for RAR α and RAR β . The proposed docking surface for the corepressor (representing portions of helix 3 and helix 5/6) is depicted by hatched shading. The locations of amino acids 224, 225, 234, 409, 412, and 426 are indicated.

contribute, if more modestly, to the overall strength of the corepressor interaction (note that an RAR β construct containing the entire helix 1- Ω loop-helix 3 region of RAR α interacted more strongly with SMRT than did an RAR β construct containing only the RAR α helix 3; Fig. 2A).

The ability to bind to SMRT in these interaction assays was also paralleled by transcriptional properties of these RAR constructs; RAR β chimeras that contained the relevant RAR α helix 3 sequences lost the ability to activate reporter gene expression in the absence of hormone, whereas RAR α chimeras that contained the equivalent helix 3 RAR β sequences failed to repress and displayed measurable hormone-indepen-

dent activation instead (Fig. 2B). Here again, although the source of the helix 3 sequences played the dominant role in defining the transcriptional properties of the different isotypes in the absence of hormone, the identity of the helix 1 and the Ω loop also contributed (compare the effects of exchanging only helix 3 to the effects of exchanging the entire helix 1- Ω loop-helix 3 region; Fig. 2B).

There are three amino acid differences within the relevant regions of helix 3 of RAR α and of RAR β , located at positions 224, 225, and 234 (to simplify discussion, we utilize a common numbering system for all three isotypes, as described in reference 58) (Fig. 3A). We therefore employed site-specific mu-

tagenesis to exchange the RAR α and - β sequences at these three helix 3 amino acids either individually or in sets of two. The majority of the isotype-specific corepressor interaction correlated with the identity of amino acid 234: an Ala 234-to-Ser substitution in RAR β significantly elevated the ability of this isotype to bind to SMRT, whereas the reciprocal Ser 234-to-Ala substitution in RAR α resulted in a substantial decrease in SMRT binding (Fig. 2A). Nonetheless, individual or double mutations at codons 224 or 225 also affected, if more modestly, the RAR/SMRT interaction (Fig. 2A). We conclude that all three of these amino acid differences between helix 3 of RAR α and helix 3 of RAR β contribute to the different corepressor interaction properties of these different isotypes but that codon 234 plays a dominant role in this regard.

The different corepressor interaction properties of RAR α and - β reflect their abilities to interact with the previously identified (L/V)-X-X-I/V-I motif within the SMRT corepressor. The SMRT and N-CoR corepressors are postulated to bind to RAR α by the interaction of the nuclear receptor with L/V-X-X-I/V-I motifs within the corepressor (31, 32, 51, 56). Are the isotype-specific effects of helix 3 noted above manifested by altering the ability of RAR α and - β to interact with these previously identified L/V-X-X-I/V-I corepressor motifs, or does helix 3 influence corepressor binding by contacting a previously unrecognized domain within the SMRT/N-CoR corepressor? To answer this question, we engineered a GST fusion protein that was restricted to the 19 amino acids immediately encompassing the L/I-X-X-I/V-I motif within the RID-1 of SMRT (i.e., the more N terminal of the two interaction domains in SMRT [76]). This abstracted SMRT subdomain retained the ability to bind to the radiolabeled RAR α isoform but, in common with the native SMRT protein, exhibited little or no binding to the RAR β isoform (Fig. 2C). We conclude that the different helix 3 sequences of RAR α and - β function by altering, directly or indirectly, the ability of these receptors to interact with the L/I-X-X-I/V-I motifs previously identified as the principal receptor contact surface on the SMRT/N-CoR corepressor.

Use of a yeast-based genetic suppression screen demonstrates that an intact receptor helix 12 is required for manifestation of the RAR β phenotype. To better understand the molecular mechanism behind the different corepressor interaction properties displayed by RAR α and - β , we next employed an unbiased genetic screen to identify any additional receptor domains involved in this phenomenon. We first devised a yeast two-hybrid assay in which a GAL4DBD-SMRT fusion construct and a GAL4 activation domain (GALAD)-RAR fusion construct were introduced together into *S. cerevisiae* cells bearing a GAL-17-mer-lacZ reporter (62). Consequently, an interaction between SMRT and RAR should manifest as enhanced β -galactosidase expression. The strong SMRT interaction properties of RAR α , and the weak SMRT interaction properties of RAR β , were accurately mimicked in this system, with the RAR α allele generating high levels of β -galactosidase expression and resulting in deep blue staining of the resulting recombinant yeast colonies in an X-Gal overlay assay, whereas the RAR β allele produced much lower levels of β -galactosidase expression and pale blue yeast colonies in the same assay (Fig. 4A and data not shown). Investigations with negative controls (devised by omitting either of the GAL4

fusion constructs, by using empty vectors, or by using irrelevant protein fusions in these assays) resulted in virtually undetectable levels of β -galactosidase expression and little or no X-Gal reactivity (Fig. 4 and data not shown).

We next introduced random mutations into the RAR β molecular clone by its propagation in a *mutSDT* strain of *E. coli*, recovered the mutated RAR β cDNA population, excised the RAR β open reading frame, and introduced it back into a wild-type GAL4AD-plasmid vector backbone. The resulting GAL4AD-RAR β plasmid population was transformed into yeast possessing the wild-type GAL4DBD-SMRT, and the resulting yeast recombinants were screened visually for alterations in β -galactosidase expression. Of 10,000 colonies analyzed, 9 exhibited a significantly elevated level of β -galactosidase expression indicative of an enhanced interaction between RAR β and the SMRT corepressor (data not shown). The same increase in β -galactosidase expression was observed when the corresponding yeast recombinants were reisolated and reexamined in a liquid β -galactosidase assay and when the GAL4AD-RAR β mutant plasmids from these primary yeast transformants were recovered by transformation of *E. coli* and reintroduced into secondary yeast transformants bearing a native GAL4DBD-SMRT construct (Fig. 4A and data not shown). These controls confirmed that the enhanced two-hybrid interaction phenomenon was inherent to the mutated RAR β alleles and was not due to either an epigenetic phenomenon or an unrecognized mutation in either the yeast host or in some other component of the two-hybrid system. An additional 287 mutant RAR β colonies displayed a nearly complete loss of β -galactosidase expression (i.e., white colonies instead of light blue); these "down" mutations, presumably receptor mutations that further disrupted the weak SMRT interaction characteristic of the wild-type RAR β , were not analyzed further.

The GAL4-RAR β plasmids that exhibited an enhanced SMRT interaction phenotype were recovered from the yeast and were subjected to DNA sequence analysis. The 9 plasmids represented three distinct mutations; all three mutant alleles retained the wild-type RAR β sequence throughout the helix 1 to helix 11 region but possessed substitution or frameshift mutations within or adjacent to receptor helix 12 (P409S, I412T, and a frameshift at 426 [426fs]; Fig. 3A). These RAR β "SMRT-up" mutants also displayed a detectably enhanced SMRT interaction in a mammalian two-hybrid analysis and (with the exception of 426fs) conferred strong transcriptional repression in transfected mammalian cells (Fig. 4B and D). The 426fs RAR β mutant paradoxically activates the reporter in our transfection experiments; although we do not understand the basis of this phenotype, it is possible that by appending novel amino acids onto the RAR C terminus, this frameshift has altered other properties of the receptor, such as coactivator recruitment, that might account for this effect.

The effects of these SMRT-up mutations were most pronounced when tested in the RAR β background; the same substitutions, when engineered into RAR α , displayed much less of an effect on the already strong corepressor interaction properties of the latter isotype (Fig. 4B). The I412T RAR β mutant also displayed an enhanced SMRT interaction in a GST pulldown assay, whereas P409S and 426fs had relatively little or no effect in this *in vitro* assay (Fig. 4C); we presume that differences in the ionic environment or the presence of

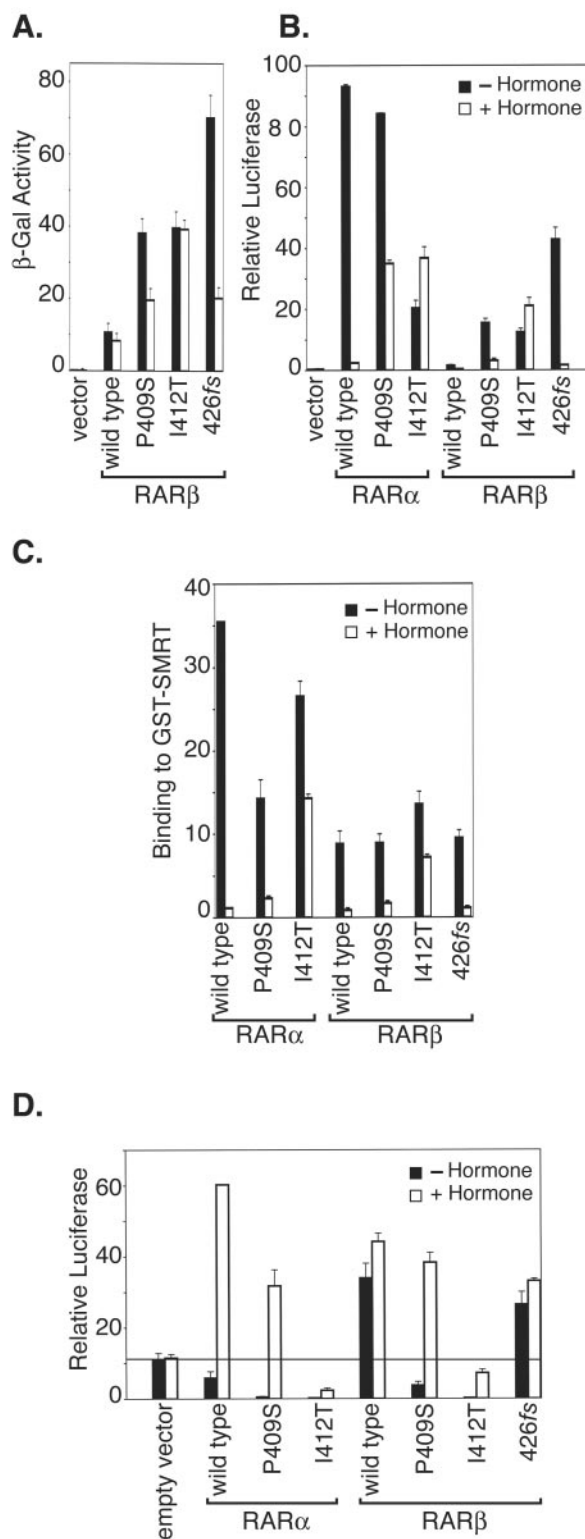


FIG. 4. Gain-of-function mutations in the helix 12 domain of RAR β confer an elevated interaction with corepressor and the ability to repress reporter gene expression. (A) Mutations in helix 12 confer an elevated SMRT interaction on RAR β in a yeast two-hybrid analysis. Yeast cells were cotransformed with pACTII-GALAD-RAR β and pGBT9-GAL4DBD-SMRT constructs. Transformants were grown to stationary phase, diluted 1:5, and grown for an additional 12 h at 30°C in the absence (open bars) or presence (filled bars) of 1 μ M ATRA

additional factors in intact cells stabilizes the interaction of the P409S and 426fs RAR β mutants with SMRT in two-hybrid and transcription assays in a manner that is not fully recapitulated in the GST pull-down. Overall, our results indicate that helix 12 interferes with the ability of the unliganded RAR β (but not RAR α) to bind to SMRT.

RAR β possesses a corepressor binding site, but access to this site appears to be blocked by a constitutively closed position of helix 12. In most unliganded nuclear receptors, helix 12 is thought to be in an extended position that permits access of a corepressor to a docking surface composed primarily of portions of receptor helix 3 and helix 4/5 (presented schematically in Fig. 3B). Binding of ligand by RAR α is proposed to reorient helix 12 into a more sequestered position that occludes the corepressor binding site and generates a novel docking surface for coactivators (19, 57) (Fig. 3B). The location and nature of our intragenic suppressor mutations suggested that an isoform-specific interaction between RAR β helix 3 and helix 12 operates to gate helix 12 closed even in the absence of hormone, blocking access of SMRT to its docking site. Specifically, the Ala 234 in helix 3 of RAR β is in a position to interact with Ile 412 in helix 12 when helix 12 is gated closed (58); this hydrophobic pairing between helix 3 and helix 12 may be sufficient to favor the closed helix 12 conformation even in the absence of a ligand (Fig. 3B). Notably, this hydrophobic pairing is disrupted by the more-polar amino acids present in RAR α (e.g., Ser at 234) or in our (I412T) RAR β mutant, both of which bind SMRT strongly (Fig. 3B). Our other RAR β mutants that confer an increase in the SMRT interaction are also likely to function by disrupting this helix 3-helix 12 interaction: the P409S substitution interrupts a hinge on which helix 12 rotates, and the 426 frameshift adds extraneous sequence to helix 12 that is likely to prevent proper packing of the folded helix 12 against the receptor surface (Fig. 3B). Significantly, simply deleting helix 12 also conferred strong corepressor binding and repression on RAR β (Fig. 5). Taken as a whole, our results indicate that the RAR β isoform possesses a fully functional corepressor docking site but that this site is occluded by a constitutively closed helix 12 position.

A series of site-directed mutations that disrupt the hydrophobic interaction between amino acids 234 and 412 in RAR β convert RAR β into a transcriptional repressor in vivo and

prior to assay. Data are presented as β -galactosidase activity relative to the corresponding optical density of the culture at 600 nm. The results from four different transformants were combined for each mutant; the averages and standard deviations for three independent experiments are shown. (B) Mutations in helix 12 confer an elevated SMRT interaction on RAR β , but not RAR α , in a mammalian two-hybrid analysis. The different helix 12 mutations assayed as described for panel A were tested for their effect on the ability of RAR α and RAR β to interact with SMRT in a mammalian two-hybrid assay, as described for Fig. 1B; relative luciferase is plotted. (C) The I412T mutant enhances SMRT binding by RAR β , but not RAR α , in a GST-protein interaction assay in vitro. The overall protocol was as described for Fig. 1A. (D) Mutations in helix 12 of RAR β confer the ability to repress target gene expression. The various helix 12 mutations were introduced into either the RAR α or RAR β background, as indicated below the panel, and the resulting receptors were tested for the ability to repress or to activate the pTK-Luc-RARE reporter in the absence (filled bars) or presence (open bars) of 500 nM ATRA as described for Fig. 1C.

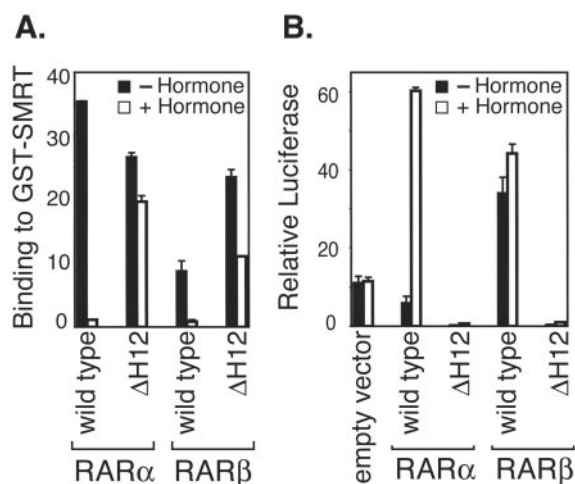


FIG. 5. Deleting helix 12 greatly enhances the ability of RAR β to bind to SMRT corepressor in vitro and to repress target gene expression in transfected cells. (A) Deletion of helix 12 increases corepressor binding. Wild-type and C-terminal-deleted RAR α (amino acids 1 to 403) and RAR β (amino acids 1 to 396) were tested for the ability to bind to GST-SMRT by the protocol described for Fig. 1A. (B) Deletion of helix 12 converts RAR β from a hormone-independent activator to a constitutive repressor of reporter gene expression in transiently transfected CV-1b cells. The protocol described for Fig. 1C was used.

elevate corepressor binding in vitro. We next created and characterized a series of amino acid substitutions in helix 3 and helix 12 to further test our hypothesis that a hydrophobic interaction between these two domains was involved in gating access of the corepressor to the receptor. Replacement of Ala 234 in RAR β with Cys (approximately the same size and hydrophobicity as Ala) retained the RAR β phenotype of activating, rather than repressing, target gene expression in transfected cells (Fig. 6A). The A234T mutation in RAR β also retained the RAR β phenotype; by several criteria, threonine is considered to be more hydrophobic than serine, and this property may account for the RAR β -like ability of the A234T mutant to activate transcription in the absence of hormone.

In contrast, replacement of Ala 234 with amino acids expected to disrupt the interaction of helix 3 with helix 12 (replacements involving charged or bulky residues, such as A234D, A234K, or A234I), converted RAR β into the RAR α -like phenotype, resulting in repression in the absence of hormone and activation in the presence of hormone (Fig. 6A). An A234V substitution, representing a hydrophobic amino acid of intermediate size, generated an intermediate phenotype (Fig. 6A). Replacing the helix 12 amino acid in RAR β with amino acids smaller or more polar than the wild-type Ile 412 (e.g., I412A, I412C, I412D, I412K, or I412S) also converted RAR β into an RAR α -like repressor, whereas a more conservative replacement, I412V, generated an intermediate effect on reporter expression (Fig. 6A). Although the I412A and I412C mutants remained hormone responsive, the more highly polar or charged RAR β I412D, I412K, and I412S mutants failed to activate on addition of ATRA and behaved as constitutive repressors (Fig. 6A); possibly these latter mutations are sufficiently disruptive to the proper folding of helix 12, or to hormone binding, so as to prevent corepressor release-coactivator

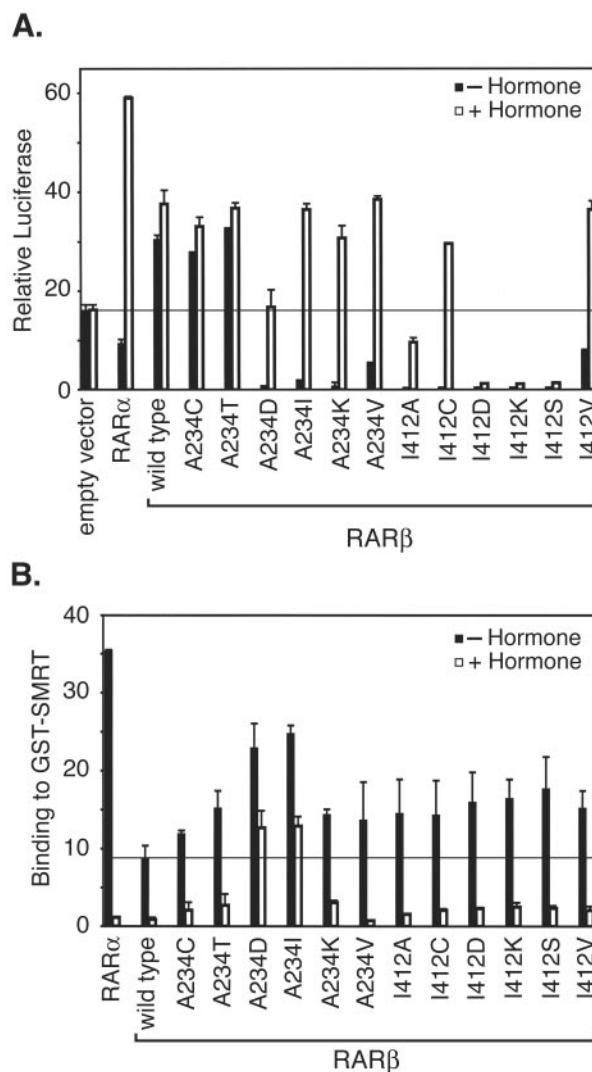


FIG. 6. Mutations in RAR β that disrupt the hydrophobic interaction surface between helix 3 and helix 12 increase corepressor binding and enhance repression of reporter expression. (A) Point mutations in helix 3 or 12 can convert RAR β into a repressor. The ability of wild-type RAR α or wild-type RAR β or specific amino acid mutants of the latter to repress or activate the pTK-Luc-RARE reporter in transiently transfected CV-1b cells was determined as described for Fig. 1C. (B) Point mutations in helix 3 or helix 12 can confer enhanced corepressor binding on RAR β . The ability of wild-type RAR α or RAR β or specific mutants of the latter to bind to SMRT in a GST protein interaction assay was determined as described for Fig. 1A.

recruitment even in the presence of an agonist. In general, the amino acid substitutions that conferred repression on RAR β in transfected cells also elevated corepressor recruitment in vitro, although the effects were not always proportionate in both assays (Fig. 6B). In conclusion, a variety of (otherwise diverse) amino acid substitutions that shared the ability to disrupt the hydrophobic character of the helix 3-helix 12 interaction surface also shared the ability to confer enhanced corepressor binding and enhanced transcriptional repression on RAR β .

Consistent with our model, the RAR α helix 12 is more accessible to protease in the absence than in the presence of

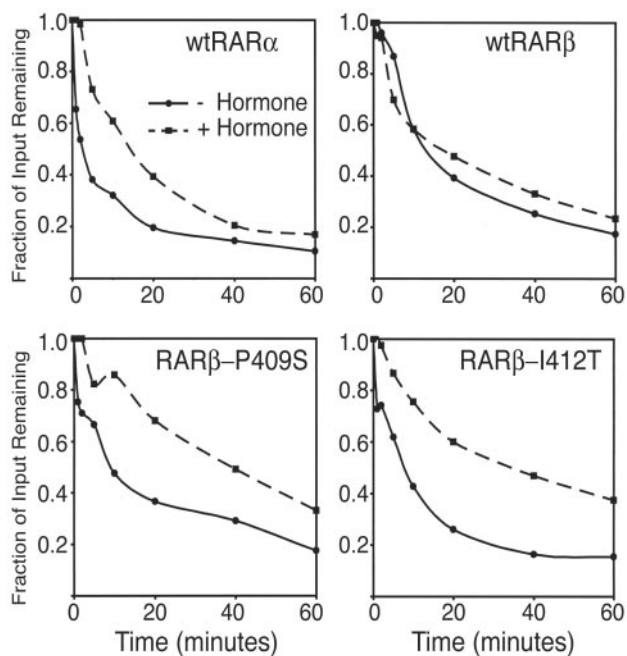


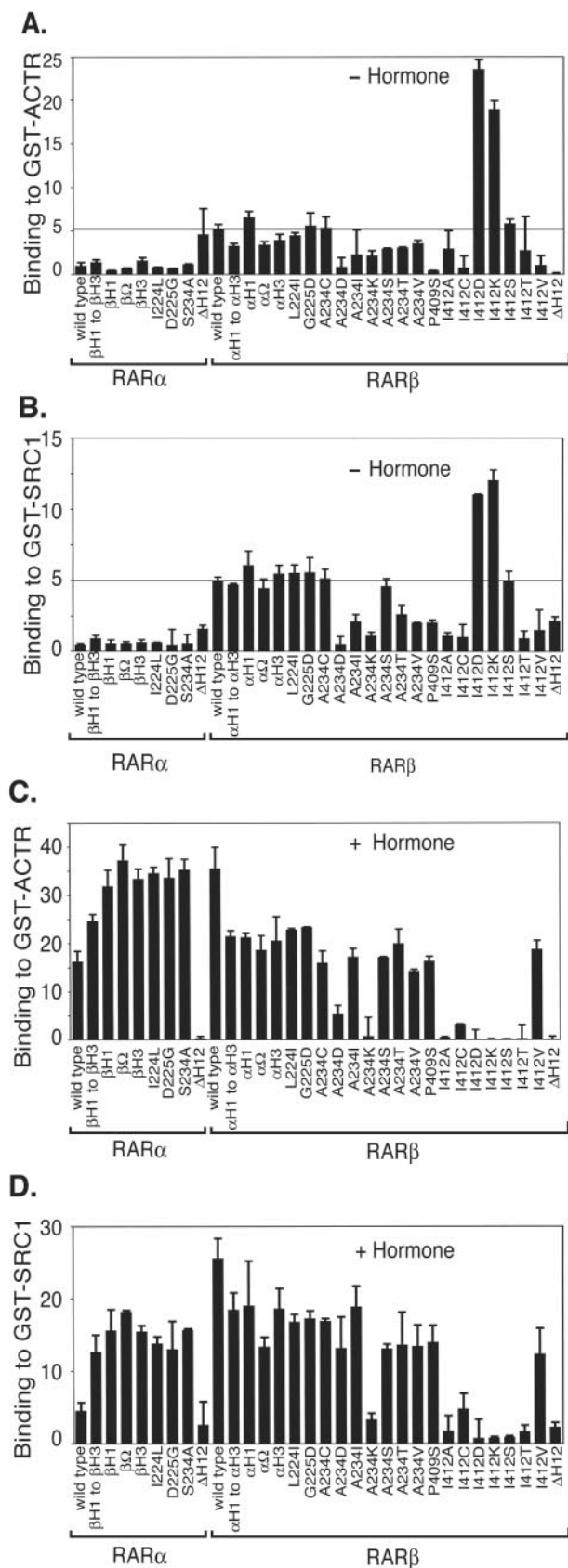
FIG. 7. RAR β is resistant to carboxypeptidase degradation in both the absence and presence of hormone, unlike RAR α and the RAR β -P409S or RAR β -I412T mutants. Radiolabeled receptor was synthesized *in vitro* by a coupled TnT reaction and incubated with CPY for the times indicated below the panel in the absence (solid line) or presence (dashed line) of 1 μ M ATRA. The resulting proteolytic products were resolved by SDS-PAGE and were visualized and quantified by PhosphorImager analysis. The fraction of full-length receptor remaining at each indicated time point is shown. A representative experiment is provided; analogous results were obtained in three independent experiments.

ATRA, whereas the RAR β helix 12 is in a sequestered position (resistant to proteolytic degradation) both in the absence and the presence of hormone. Unfortunately, no X-ray or nuclear magnetic resonance structure has been reported for the unliganded RAR α , - β , or - γ . The position of helix 12 in these receptors, however, can be approximated by the use of CPY, an exopeptidase that serves as a probe of the solvent accessibility of the protein C terminus (28, 42, 83). Consistent with the prevailing model of hormone-driven helix 12 gating, the wild-type RAR α was significantly more sensitive to CPY digestion in the absence of ATRA (indicative of an open helix 12 position) than in the presence of ATRA (suggestive of a more closed helix 12 position) (Fig. 7). In contrast, the wild-type RAR β was comparatively resistant to CPY digestion either in the absence or the presence of ATRA hormone, which is consistent with a constitutively sequestered helix 12 conformation (Fig. 7). Notably, our RAR β mutants that displayed an elevated (i.e., more RAR α -like) SMRT interaction also displayed a more RAR α -like pattern of CPY degradation: an increased sensitivity to carboxypeptidase digestion in the absence of hormone but protection in the presence of ATRA (Fig. 7 and data not shown). These results establish a correlation between the inability of the wild-type RAR β to recruit corepressor and the existence of a more sequestered helix 12 position in the unliganded conformation of this isotype.

RAR β is able to bind to p160 coactivators in the absence of

hormone. Our transfection studies established that RAR β not only fails to repress but also has a detectable ability to activate reporter gene expression even in the absence of ligand. To determine the basis for this property, we investigated whether RAR β possessed an elevated ability to bind to transcriptional coactivators in the absence of hormone compared to RAR α . Indeed, the unliganded RAR β displayed a three- to ninefold-greater interaction with ACTR and SRC-1 in a GST pulldown assay than did unliganded RAR α (Fig. 8A and B). This ability of RAR β to bind to coactivators in the absence of hormone was decreased by several of our mutations in helix 3 and helix 12 that increased corepressor binding; many of our substitutions at codon 234 and at codon 412, the P409S replacement, and the helix 12 deletion all disrupted coactivator binding by RAR β to a significant extent (Fig. 8B). Therefore, the enhanced coactivator binding by RAR β may be, in part, a consequence of the constitutively closed helix 12 conformation assumed by this isotype: the closed conformation in the unliganded RAR β may sufficiently mimic that of an agonist-bound RAR α to form a docking site for coactivator recruitment. Nonetheless, note that coactivator binding by RAR β was further substantially increased by the addition of ATRA, suggesting that the conformation of helix 12 in the unliganded RAR β is not identical to the agonist-induced conformation (Fig. 8C and D). Furthermore, analysis of our chimeric RAR α / β constructs indicated that regions of the RAR β receptor mapping outside of helix 3 were also critical contributors to the enhanced coactivator binding properties of this isotype relative to RAR α . Two of our highly charged substitutions into RAR β helix 12, I412D and I412K, paradoxically increased coactivator binding (Fig. 8A and B). We conclude that RAR β possesses an inherent ability to interact with p160 coactivators in the absence of hormone and that this property overlaps, but is distinguishable from, the inability of this receptor to bind the corepressor.

RAR γ more closely resembles RAR β than RAR α in its transcriptional and corepressor interaction properties. We also investigated RAR γ , the third major RAR isotype. RAR γ possesses a helix 3 amino acid sequence identical to that of RAR β , as well as retaining the conserved Ile 412 in the helix 12 domain (Fig. 3A) (58). Consistent with these sequence identities, the transcriptional properties of RAR γ were very similar to those of RAR β ; RAR γ activated, rather than repressed, reporter gene expression in the absence of hormone, and RAR γ displayed a relatively weak interaction with the SMRT corepressor compared to RAR α (Fig. 1). Molecular determinants similar to those in RAR β appeared to be responsible for these phenomena in RAR γ : replacing helix 3 in RAR γ with that of RAR α , or introducing mutations in helix 12 that mimicked the enhanced SMRT mutations we isolated in RAR β , resulted in increased corepressor binding and transcriptional repression by RAR γ (Fig. 9A and B). As with RAR β , the RAR γ isotype displayed an elevated ability to interact *in vitro* with p160 coactivators in the absence of hormone (Fig. 9C and D). However, when examined in detail, RAR γ did diverge from both RAR β and RAR α in several of its properties. Most notably, the interaction RAR γ displayed with SMRT in either the GST pulldown or mammalian two-hybrid assay, although weak, was nonetheless stronger than that observed with RAR β (Fig. 1). Also the relative contributions of amino acids 224, 225, and 234



to the corepressor binding phenotype differed in details for RAR β and RAR γ , with the identity of amino acid 224 being more critical in the latter (Fig. 9B). It is possible that these differences in transcriptional regulation by RAR γ versus that by RAR α or RAR β reflect additional amino acid sequence differences either in helix 12 itself (most notably, amino acid 413 in RAR γ is an Arg, whereas both RAR β and RAR α have a Gln at this location; Fig. 3A) or in regions elsewhere in the coding domains of these different isoforms.

DISCUSSION

In the absence of hormone, RAR α binds SMRT/N-CoR corepressor strongly and is a potent transcriptional repressor; RAR β and - γ , in contrast, bind SMRT/N-CoR only weakly and fail to repress and can activate transcription even in the absence of hormone. In all vertebrates characterized to date, RARs are encoded by three distinct loci, denoted α , β , and γ . This coding scheme is evolutionarily tightly conserved, and the sequence conservation that a given isotype displays in comparison to its orthologs among different species is greater than that seen in comparison to its isotype paralogs within the same species. This evolutionary stability suggests that the different RAR isoforms perform distinct physiological functions. Consistent with this supposition, use of gene disruption techniques and isotype-specific ligands has confirmed that RAR α , - β , and - γ play unique as well as overlapping roles in gene regulation (9, 68). In the work presented here, we have identified at least one molecular basis for the divergent biological capabilities of the different RAR isoforms: RAR α can recruit the SMRT corepressor in the absence of a ligand and repress target gene expression, whereas the RAR β and RAR γ isoforms are unable to efficiently bind the SMRT corepressor and instead possess the ability to activate transcription in both the absence and the presence of hormone. The different cofactor and transcriptional properties of RAR α , - β , and - γ are likely to manifest in many aspects of retinoid signaling and help account for the incomplete biological redundancy previously noted for the different RAR isoforms. Note in this regard that the promoters for many of the RAR isoforms contain retinoid response elements and therefore respond to retinoic acid in a positive-feedback loop by which hormone induces additional receptor expression (26, 40, 41, 69); the constitutive activation properties of RAR β and RAR γ are likely to influence the dynamics of these feedback loops quite differently than would the repressive properties of the unliganded RAR α .

FIG. 8. RAR β displays an elevated ability to interact with p160 coactivators in the absence of hormone relative to RAR α . The ability of wild-type, chimeric, and point mutants of RAR α and RAR β to bind in vitro to GST-ACTR (amino acids 621 to 821) (A and C) or to GST-SRC1 (amino acids 560 to 1136) (B and D) was analyzed by the same general protocol employed for SMRT as described for Fig. 1A. The percentage of binding (relative to input) of each receptor to the coactivator matrix is shown. Receptor binding to a GST-only control was 0.3 to 0.7% of input. The averages and standard deviations of at least two independent experiments, each performed in duplicate, are provided. The assay was performed both in the absence (A and B) and presence (C and D) of 1 μ M ATRA.

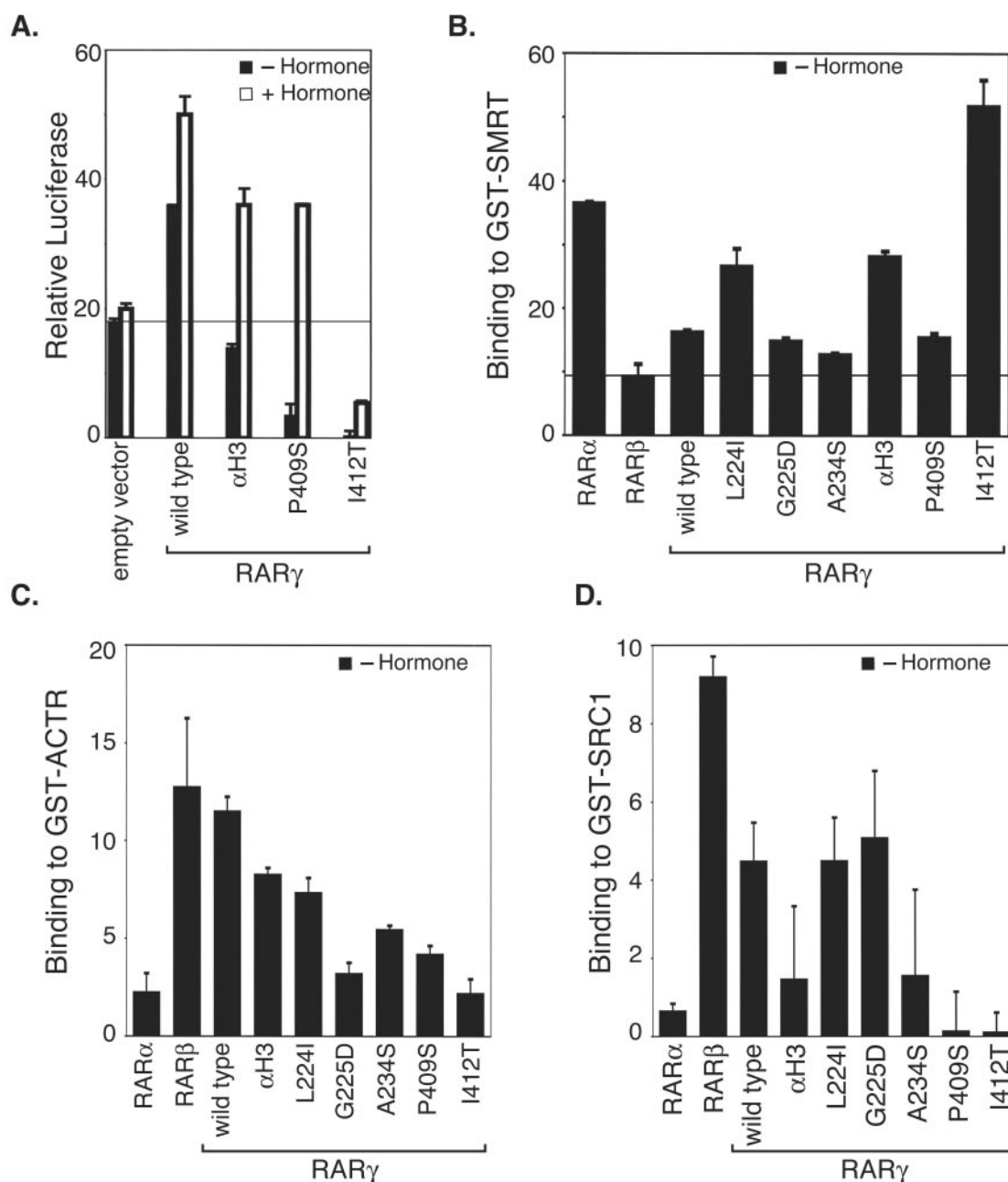


FIG. 9. RAR γ resembles RAR β in its corepressor binding, transcriptional regulation, and p160 coactivator binding properties. (A) RAR γ activates reporter gene expression in the absence of hormone, and this property maps to helix 3. Using the procedure described for Fig. 1A, the ability of wild-type RAR γ and the RAR γ chimeras and mutants (as indicated below the panel) to regulate the expression of the pTK-Luc-RARE reporter was tested by transient transfection of CV-1b cells. (B) RAR γ binds SMRT weakly, and this property maps primarily to helix 3 and helix 12. The ability of wild-type RAR α , RAR β , and RAR γ and of specific mutants of the last to bind to GST-SMRT in vitro was tested in the GST-protein interaction assay described for Fig. 1A. (C and D) RAR γ interacts with p160 coactivators in the absence of hormone more strongly than does RAR α . Using the GST protein interaction assay described for Fig. 7, the ability of wild-type RAR α , RAR β , and RAR γ to bind to ACTR (C) or to SRC-1 (D) was determined in vitro. The averages and standard deviations for at least two independent experiments are shown.

The different corepressor binding and transcriptional repression properties of RAR α , - β , and - γ map to amino acid differences within helix 3 of the ligand binding domains of these receptors. Use of chimeric constructs permitted us to demonstrate that the different corepressor interaction and transcriptional repression characteristics of RAR α , - β , and - γ

map predominantly to differences in the helix 3 sequences in the ligand binding domains of these different isotypes. RARs bearing the α isotype sequence within the N-terminal half of helix 3 bound to the SMRT corepressor strongly in vitro and repressed reporter gene expression in vivo, whereas RARs bearing the β or γ isotype sequence within the N-terminal half

of helix 3 interacted only weakly with the corepressor and failed to repress target gene transcription. There are three amino acid differences, at positions 224, 225, and 234, between the relevant region of helix 3 of RAR α and those of RAR β and γ , and all three contribute to some extent to the differences in corepressor recruitment and repression noted for the different isotypes. Intriguingly, although the C-terminal half of helix 3 comprises a portion of the corepressor docking site in the nuclear hormone receptors, amino acids 224 and 225 are distal to the actual sites of contact between corepressor and receptor, and amino acid 234 lies on the boundary of the known corepressor docking surface (31, 32, 46, 51, 56, 79). In fact, both RAR β and γ possess fully functional corepressor docking sites, but the accessibility of the corepressor to these docking sites appears to be restricted by the actions of helix 12, as discussed below.

A hydrophobic interaction between helix 3 and helix 12 stabilizes the closed helix 12 position in RAR β and RAR γ in the absence of hormone, occluding corepressor binding and preventing repression. To determine the molecular basis behind the inability of RAR β and RAR γ to efficiently bind the SMRT corepressor, we utilized a yeast genetic screen to identify intragenic suppressors that can confer enhanced SMRT binding on the RAR β isoform. The enhanced SMRT-RAR β interaction mutations isolated by this approach all mapped within or proximal to the receptor helix 12 coding region. Simple deletion of helix 12 also conferred strong corepressor binding and strong transcriptional repression on both RAR β and RAR γ . We conclude that all three RAR isotypes possess functional docking sites for the SMRT corepressor but that these sites in RAR β and γ are rendered cryptic by the presence of the native helix 12 sequence. Notably, all three of the helix 3 amino acids that differ between RAR α , β , and γ are in a position to influence the position of helix 12. Amino acid 234 (an Ala in RAR β and γ but a Ser in RAR α) is in a position to interact with Ile 412 in helix 12 when the latter helix is in the agonist-bound conformation (58). Similarly, amino acid 224 (a Leu in RAR β and γ but an Ile in RAR α) and amino acid 225 (a Gly in RAR β and γ but an Asp in RAR α) are in close proximity to helix 11 and to the base of helix 12 (58).

Allosteric changes centered on this and adjacent regions of the nuclear receptors have been shown to play critical roles in modulating the repositioning of helix 12 in response to antagonists and to receptor mutations (18, 38). We propose that a hydrophobic interaction between complementary portions of helix 3 (centered on Ala 234) and helix 12 (centered on Ile 412), perhaps further facilitated by the allosteric contributions of the amino acids at positions 224 and 225, helps gate helix 12 closed in RAR β and γ in the absence of hormone and thus prevents corepressor binding by these isotypes. We further suggest that the helix 3 sequence of RAR α disrupts this helix 3-helix 12 interaction sufficiently so that helix 12 assumes an open conformation, allowing access of the corepressor to its docking surface in the absence of hormone but also permitting helix 12 to gate shut and to release the corepressor upon the binding of hormone. A similar concept, although operating through a structurally distinct mechanism, has been proposed for the constitutive androstane receptor; this receptor, in the absence of a physiological ligand, appears to assume an ago-

nist-like closed helix 12 conformation due, in part, to a stabilizing ionic interaction between the α -carboxylic acid of the receptor C terminus and a Lys located within helix 4 (17). Similarly, *Ultraspiracle*, the insect homolog of vertebrate RXR, also displays a closed helix 12 conformation in the absence of a ligand; this closed helix 12 conformation resembles that found in RXR and estrogen receptors when bound to certain mixed agonists-antagonists and is conferred on *Ultraspiracle* by an unusually positioned L1-3 loop that contacts and interlocks helix 3, helix 11, and helix 12 (5, 14).

Consistent with our hypothesis that RAR β and γ assume a constitutively closed helix 12 conformation, disruption of the contact surface between helix 3 and helix 12 by the introduction of polar, charged, or bulky amino acid substitutions in either helix results in increased corepressor binding and increased transcriptional repression by RAR β and RAR γ . The wide diversity of amino acid substitutions that confer corepressor binding on RAR β and on RAR γ supports our argument that these mutations do not generate a corepressor docking site de novo but rather, by disrupting the interaction of helix 3 with helix 12, expose a existing, but previously cryptic docking site. Notably, our P409S substitution and 426fs mutations that also enhance SMRT binding by RAR β are similarly positioned so as to interfere with the ability of helix 12 to pivot correctly into the sequestered position, either by disrupting the pivot itself or by appending irrelevant protein sequence onto helix 12.

Naturally occurring mutations in these same receptor domains have been implicated in other contexts in which corepressor interaction is favored. For example, the PML-RAR α oncoprotein plays a causal role in human acute promyelocytic leukemia, and the ability of this protein to recruit SMRT is closely associated with maintenance of the neoplastic phenotype (20, 22, 25, 27, 36, 43, 62). The native PML-RAR α protein releases from the corepressor in response to high levels of ATRA, inducing differentiation of the leukemic cell and clinical remission (20, 22, 25, 27, 36, 43, 62). Two mutants of PML-RAR α that are resistant to this ATRA-induced differentiation have been identified: one possesses a Leu-to-Pro substitution at position 411, whereas a second bears an Ile-to-Thr substitution equivalent to our RAR position 412 mutation (15, 63). Similarly, resistance to thyroid hormone syndrome, an inherited endocrine disease, is caused by the production of mutant thyroid hormone receptors (TRs) that fail to dissociate from the corepressor in response to physiological levels of hormone (10, 50, 61, 70, 84); the most abundant mutations identified in resistance to thyroid syndrome alter a Pro in the TR β , corresponding to codon 409 in RAR, to Ala, Ser, Thr, or Asp (10, 82). Analogously, a Val-to-Met mutation in the peroxisome-proliferator-activated receptor γ at a site equivalent to RAR position 234, or a Pro-to-Leu substitution at a site equivalent to position 410, prevents corepressor release by peroxisome-proliferator-activated receptor γ in response to a ligand and is associated with severe inherited type II diabetes (3). Taken together, these results suggest that interactions between helix 3 and helix 12 play a critical role in the gating of helix 12 in many nuclear receptors and that defects in these interactions can lead to a variety of endocrine and neoplastic disorders.

Structural probes employing exopeptidase support the hypothesis that RAR β and γ possess a constitutively closed helix 12 conformation. A crystallographic or nuclear magnetic resonance analysis of the unliganded RARs has not been reported. We therefore employed CPY as an enzymatic probe of the solvent accessibility of helix 12 in the different receptor isoforms. Consistent with the accepted model of a hormone-induced alteration from an open to a closed helix 12 position, the C terminus of RAR α is more resistant to CPY degradation in the presence, versus the absence, of the ATRA ligand. A similar ligand-induced alteration in the CPY susceptibility of helix 12 is also observed in the wild-type TR, which switches in a like manner from a transcriptional repressor to an activator in response to cognate hormone. In contrast, the C-terminal helix 12 of wild-type RAR β (and γ) displays a hormone-independent resistance to CPY consistent with a constitutively sequestered helix 12 position. Furthermore, mutations in either helix 3 or 12 that confer corepressor binding on RAR β and γ also confer CPY susceptibility on these isoforms, indicative of a close correlation between the exposed position of helix 12 and the ability to bind SMRT.

In addition to occluding corepressor binding, a closed conformation of helix 12 can also help form a docking surface for the L-X-X-L-L motifs that are found in many coactivators (19). In superficial agreement with this concept, both the RAR β and RAR γ isoforms exhibit higher levels of hormone-independent p160 coactivator binding in vitro and hormone-independent transcriptional activation in vivo than does RAR α . We note, however, that although both RAR β and γ bind coactivators in the absence of hormone, addition of hormone induces still higher levels of p160 binding, indicating that the conformation of these receptors in the unliganded state is not identical to that assumed by the same receptors on the binding agonist. Additionally, receptor sequences outside of helix 3 and helix 12 also contribute to the elevated, hormone-independent coactivator binding properties of RAR β and γ . Intriguingly, repression by unliganded RAR β generally proved to be dominant over activation; i.e., most of the RAR β mutants that recruit corepressor in vitro also repress reporter gene repression in transfected cells, even when these mutants retain the elevated SRC-1/ACTR binding characteristics of the unliganded wild-type RAR β . Although the specific coactivators responsible for the constitutive activation properties of RAR β and γ in cells are not known, our results suggest that RAR β and γ possess an innate ability to bind to p160 coactivators in the absence of hormone. However, for this innate coactivator binding to be manifested as target gene activation in vivo, it appears also necessary to prevent corepressor binding through the helix 3-helix 12 interaction elucidated in this work.

Helix 12 is a critical, multiposition toggle switch that controls corepressor and coactivator binding by nuclear hormone receptors and is programmed to operate differently in different receptor isoforms. Helix 12 in the ligand binding domain of the nuclear receptors is surprisingly malleable and can assume any of a spectrum of different conformations; these conformations differ in the unliganded receptor, in the presence of different agonists and antagonists, in response to posttranslational modifications of the receptor, and under the influence of the receptor dimer partner (for examples, see references 5–8, 14, 52, 58, 64, 74, and 79). The different helix 12 conformations, in

turn, play a key role in mediating the interactions of the receptor with auxiliary factors such as corepressors and coactivators (19, 57). Genetic defects that disrupt the ability of helix 12 to position properly in response to hormone lead to defects in cofactor recruitment and release that manifest as endocrine and neoplastic diseases (for examples, see references 3, 36, and 82). In the work reported here, we establish that different isoforms of the same receptor are evolutionarily programmed to display inherently different helix 12 conformations in the absence of hormone and therefore to manifest different cofactor recruitment and transcriptional regulatory properties. This observation helps explain the different roles that the three different RAR isoforms play in development and physiology and provides a rationale for the evolutionary conservation of these three distinct isoforms during the vertebrate divergence (9, 68). The closed position of helix 12 that we propose to be the null state of the unliganded RAR β and γ need not be immutable; however, posttranslational modifications, antagonists, or interactions with other proteins may, in theory, be able to open the helix 12 conformation in RAR β and γ and thereby confer corepressor binding and transcriptional repression. We are currently exploring this possibility.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant DK53528 from the National Institute of Diabetes, Digestive, and Kidney Diseases. B.F. was also funded, in part, by a Public Health Service predoctoral training award, T32GM07377, from the National Institute of General Medical Sciences.

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