Serine 232 and Methionine 272 Define the Ligand Binding Pocket in Retinoic Acid Receptor Subtypes*

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The transcriptional response mediated by retinoic acid involves a complex series of events beginning with ligand recognition by a nuclear receptor. To dissect the amino acid contacts important for receptor-specific ligand recognition, a series of retinoic acid receptor (RAR) mutants were constructed. Transcriptional studies revealed that serine 232 (Ser232) in RARα and methionine 272 (Met272) in RARγ are critical residues for the recognition of their respective receptor-selective analogs.

The identification of these key amino acids in the ligand binding pocket is confirmed by the reported crystal structure of RARγ. Interestingly, the serine at position 232 in RARα gives an explanation for the observed differences in the affinity of the naturally occurring ligand, all-trans-retinoic acid (t-RA), in this receptor compared with that for the other receptors, since hydrogen bonding would not be permitted between the hydroyxyl of serine and the hydrophobic linker of t-RA. Using this model, a molecular mechanism for the transcriptional antagonism of a synthetic analog is suggested that involves an alteration in the structure of the receptor protein in the region around the AF2 domain in helix 12.

Retinoic acid and its analogs (retinoids) regulate cellular proliferation and differentiation in higher eukaryotes. The biological effects of these ligands are mediated by their binding to the retinoic acid receptors (RARs),† which are members of the superfamily of steroid/thyroid hormone nuclear receptors. These receptors act as transcriptional enhancers that bind in a sequence-specific manner to their response elements (retinoic acid response elements) located within the promoter region of distinct retinoid-responsive genes. RARs activate transcription of those genes after ligand bound to the receptor induces conformational changes leading to activation (1–4). A crystal structure of the ligand binding domain (LBD) of the closely related human retinoid-X receptor α revealed a novel protein fold, an antiparallel α-helical sandwich, common to the members of this superfamily. Examination of this structure for a potential binding pocket for its ligand, 9-cis-retinoic acid, unveiled two large hydrophobic cavities within the N-terminal portion of the LBD in the vicinity of the s1,s2 β-hairpin and α-helix H5 (5). These findings are supported by results from ligand-photoaffinity binding experiments, which identified important residues for receptor-ligand binding in the LDBs of the glucocorticoid receptor (6) and RARα (7, 8). The amino acid sequence alignment of the nuclear receptor LBDs indicates that most of the residues identified by photoaffinity labeling or site-directed mutagenesis correspond to the 9-cis-retinoic acid receptor residues surrounding the putative 9-cis-retinoic acid binding pocket (9).

Recently, the 2.0-Å structure of the LBD of human RARγ bound to all-trans-retinoic acid (t-RA) was reported showing details of ligand-receptor interactions (10). Several amino acids from α-helices H1, H3, H5, H11, H12, loops 6–7 and 11–12, and serine 289 (Ser289) from the β-sheet s1 were proposed as contact residues between the receptor and ligand. Amino acid sequence alignment of RARα, -β, and -γ revealed that among all of these residues, only three positions were variable: serine 222 (Ser222), alanine 225 (Ala225), and alanine 294 (Ala294) in H3, isoleucine 270 (Ile270), isoleucine 263 (Ile263), and methionine 272 (Met272) in H5 and valine 395 (Val395), valine 388 (Val388), and alanine 397 (Ala397) in H11. These amino acid residues therefore became obvious candidates as crucial amino acids responsible for determining RAR selectivity to certain synthetic analogs of t-RA.

Utilizing site-directed mutagenesis and RAR-selective retinoids, we previously identified serine 232 (Ser232) and threonine 239 (Thr239) from the N-terminal portion of the LBD of RARα and the corresponding alanine 225 (Ala225) and isoleucine 232 (Ile232) from RARβ to be essential for the recognition of retinoic acid and various analogs (11). In the present work, the amide linker region of Am-580 and the oxime linker region of BMS-185354 were used to precisely identify serine 232 (Ser232) in RARα and methionine 272 (Met272) in RARγ as critical for the specific interaction of the receptor and retinoid. Experiments with an RARα antagonist, BMS-185411, showed that Ser232 is also involved in the transcriptional antagonist activity of this compound. In RARα, an alanine (Ala225) at the position corresponding to Ser232 in RARα was shown to allow BMS-185411 to behave as an RARα-selective agonist. Analysis of several additional RARα- or RARγ-specific point mutants introduced along α-helix H3, H4/H5 and the Ω loop of RARγ detected a decrease in the transactivation activity of BMS-185411. The amino acids mutated in these experiments, according to the crystal model of RARγ LBD, do not directly interact with t-RA. This suggests

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‡ The abbreviation used are: RAR, retinoic acid receptor; t-RA, all-trans-retinoic acid (t-RA); RARα, RARα-selective retinoic acid receptor; RARβ, RARβ-selective retinoic acid receptor; RARγ, RARγ-selective retinoic acid receptor; BMS-185354, [5,5,8,8-tetramethyl-2-naphthalenyl]hydroxyiminomethyl]-2-naphthalene-carboxylic acid.
that the effect of these residues on the potency and selectivity of BMS-185411 could be due to intramolecular interactions within the receptor itself. These findings support the conclusion that the transcriptionally active ligand-receptor complex is a result of a series of direct and indirect interactions between the receptor and its selective ligand, which reflect the dynamic nature of both components.

MATERIALS AND METHODS

Plasmids—Plasmids pRARα0 and pRARβ0 (12), which contained full-length human RARα and β, respectively, were gifts from Dr. P. Chambon (IGBMC, Strasbourg, France). The chloramphenicol acetyltransferase reporter plasmid containing a 46-base pair retinoic acid response elements of the laminin B1 gene was provided by Dr. L. Gudas (13). All-trans-retinoic acid was purchased from Sigma. Am-580 (14), BMS-185411, and BMS-185354 (15) were synthesized by Bristol-Myers Squibb Central Chemistry, Wallingford, CT. [3H]RA was purchased from NEN Life Science Products.

The polymerase chain reaction protocol described by Cotecchia et al. (16) was used to construct several chimeric retinoic acid receptors. DNA fragments synthesized by polymerase chain reaction were cloned directly into pRARα0 using a combination of the BstEII, BsgI, and BclI cloning sites found in RARα. The corresponding cloning sites, which are not present in RARβ cDNA, were introduced into DNA amplified from this receptor using synthetic amplification primers. Amplification and recombinant primers were made using the Pharmacia Biotech Inc. Gene Assembler-4. To construct the chimera RARα(E)β, the BsgI site at position 1194 in RARβ was removed using a similar protocol.

Retinoid Transactivation and Competition Analysis—Transfection of HeLa cells with DNA encoding wild type RARα, RARβ, and chimeric receptors was performed as described (11, 12). Retinoid efficacy was measured by the concentration of induced chloramphenicol acetyltransferase gene product obtained from transfected cells utilizing t-RA and the receptor-selective synthetic retinoids. The levels of stimulation are expressed as EC50 values, which reflect the concentration of the test compound giving 50% of the stimulation for t-RA at 10−6 M. Numbers represent the mean of a minimum of three independent experiments.

![Chemical structures of the ligands used in transactivation and competition assays.](http://www.jbc.org/)

To evaluate antagonist activity of test retinoids, a transactivation competition assay was used. Transfected HeLa cells were treated 16 h with 10−7 M t-RA, with or without BMS-185411 at concentrations of 10−8 to 10−5 M. These cells were then harvested and crude extracts prepared. Chloramphenicol acetyltransferase protein level was assayed as described above. The antagonist activity of BMS-185411 is measured as an IC50 for the inhibition of transactivation produced by t-RA.

Binding of synthetic ligands to the receptors was accomplished by a competition binding assay described in Ref. 11.

DNA and Protein Analysis—DNA sequence analysis and protein structure predictions were performed using GeneWorks 2.3 DNA-protein analysis software from IntelliGenetics, Inc.

RESULTS

Characterization of Retinoic Acid Receptor α-, β-, and γ-Specific Activities Using Receptor-selective Retinoids—Wild type RARα, β, and γ transiently transfected into HeLa cells were used to establish the profile of receptor-specific transactivation responses utilizing t-RA and the receptor-selective synthetic retinoids Am-580, BMS-185411, and BMS-185354 (Fig. 1). In these experiments, Am-580 was found to be an RARα-selective agonist, and BMS-
185411 showed specific agonist activity for RARβ with an EC₅₀ of 34 nM, and BMS-185354 selectively activated RARγ with an EC₅₀ of 28 nM (Table I).

Effects of Serine 232 in RARα on Specific Interactions with Receptor-selective Ligands—Utilizing the chimeric RAR, RARα(nE)β, where the N-terminal portion of RARβ domain E was subcloned into an RARα background by polymerase chain reaction-assisted site-directed mutagenesis, two sets of residues, serine 232 (Ser232)/alanine 225 (Ala225) and threonine 239 (Thr239)/isoleucine 232 (Ile232) in RARα and RARβ were found to be essential for receptor-ligand-specific interactions (11).

Further investigation of the role of each residue alone was accomplished by a second series of mutants, RARα(nE-S225)β, RARα(nE-T232)β, RARα(nE-S232)β, and RARβ(S225). In the RARα(nE-S225)β mutant, alanine 225 in RARα(nE)β was substituted by a serine as found in RARα (Fig. 2). In transactivation experiments, the RARα-selective agonist, Am-580, activated RARα(nE-S225)β with a profile similar to wild type RARα, with an EC₅₀ of 2.5 nM. Both the RARβ-selective agonist, BMS-185411, and the RARγ-selective agonist, BMS-185354, showed little or no transactivation activity with this mutant (Table I). These results suggested that Ser²³² in RARα is the residue that is primarily responsible for this receptor’s characteristic activities with selective retinoids like Am-580, which contains an amide linker. This conclusion was further supported by results of transactivation experiments with Am-580 and RARα(nE-T232)β, where isoleucine 232 (Ile²³²) in RARα(nE)β was replaced by the threonine found in RARα (Fig. 2). This substitution did not alter the transactivation profile observed for the double mutant, RARα(nE-S225/T232)β, or the single mutant, RARα(nE-S225)β, indicating that only the serine residue at position 225 is responsible for interaction with the amide linker of Am-580 (Table I). In addition, substitution of isoleucine 232 (Ile²³²) by serine instead of threonine (mutant RARα(nE-S232)β) showed an activity profile identical to that of the wild type RARα, indicating that addition of a second serine at this position will not alter the transactivation selectivity for Am-580. Finally, analysis of a single point mutant of RARβ, RARβ(S225), showed that the single amino acid substitution of alanine 225 (Ala²²⁵) by serine resulted in a mutant receptor that responded to both t-RA and Am-580 with an identical profile to wild type RARα and showed only weak transactivation with the RARγ-selective, BMS-185354 (Table I). The EC₅₀ of 1.8 nM for Am-580 with RARβ(S225) verified that Ser²³² in RARα is the only amino acid which selectively interacts with the amide linker of the compound.

Methionine 272 Is Responsible for RARγ-specific Transactivation Activity in Response to RARγ-selective Retinoids—Examination of amino acid differences among RARα, RARβ, and RARγ within the first 100 residues of the N-terminal portion of domain E revealed only a limited number of amino acid differences (Fig. 2). These suggested that the receptor chimera, RARα(nE)β, could be used as a host to create five constructs targeted to identify the amino acids involved in specific RARγ-ligand interactions.

In a series of transactivation experiments, neither the RARβ arginine 212 substitution with RARγ glutamine (Gln²¹²) (RARα(nE-Q212)β) nor the RARβ isoleucine 246 substitution with RARγ serine (Ser²⁴⁶) alone (RARα(nE-S246)β) or in combination (RARα(nE-Q212/S246)β) was sufficient to convert a ligand-selective response from the RARβ type into the RARγ type using the RARγ-selective BMS-185354. This suggests that both Gln²¹² and Ser²⁴⁶ are not contact amino acids between
RARγ and its selective ligand (Table I). The replacement of the RARβ isoleucine 263 (Ile263) by methionine, which corresponds to the RARγ methionine 272 (Met272) (Fig. 2), resulted in chimera RARα(nE-Q212/S246/M263). In transactivation experiments, all three selective retinoids showed an EC50 for this mutant receptor similar to that for the wild type RARγ (Table I). This suggests that the methionine may be responsible for the RARγ-specific interactions observed in this class of receptor-selective ligand. The results of experiments with RARα(nE-M263) showed an EC50 for the single mutant similar to that for wild type RARγ (Table I). Taken together, these results strongly suggest that a single isoleucine-methionine substitution is sufficient to convert the chimeric receptor, RARα(nE), which shows an RARβ-specific type of transactivation, into a receptor with an RARγ-specific type of transactivation.

**Fig. 3. Model of molecular interactions between the retinoic acid receptor and receptor-selective retinoids.** In this model (A), serine 232 (Ser232) from α-helix H3 in RARα interacts with the amide linker region of AM-580 (double arrow). Similarly, in C a hydrogen bond between Ser232 and the carbonyl group from the amide linker of BMS-185411 (double arrow) may stabilize this compound within the ligand binding site of RARα and force the hydrophobic moiety of BMS-185411 toward the α-helix H12 affecting its alignment (solid arrow) and interfering thus AF2 transactivation activity. B, methionine 272 (Met272) from α-helix H5 in RARγ interacts with the oxime linker region of BMS-185411 (double arrow).

**Table II**

| Receptor                  | IC50 nM   |
|---------------------------|-----------|
| A/B | C | D | E | F | 400 ± 120 |
| A/B | C | D | E | F | NI         |
| A/B | C | D | E | F | NI         |
| A/B | C | D | E | F | 233 ± 58  |
| A/B | C | D | E | F | 200 ± 70  |
| A/B | C | D | E | F | 217 ± 41  |
| A/B | C | D | E | F | NI         |
| A/B | C | D | E | F | 433 ± 137 |

**Results of retinoic acid competition assay**

Comparison of transcriptional antagonism of BMS-185411 for wild type RARα, RARβ, RARγ, and chimeric receptors. Transactivation was stimulated by 100 nM t-RA and then competed with increasing concentrations of BMS-185411. The level of competition is expressed as IC50, which is the concentration of each compound which inhibits 50% of the transactivation induced by t-RA. Numbers represent the mean of a minimum of three independent experiments.
The retinoic acid receptors are ligand-dependent transcription factors that regulate the expression of genes involved in cell growth, differentiation, and development. Yet, despite the large number of biological activities mediated by the receptors, relatively little is known about the precise interaction of the receptor protein and its naturally occurring ligand, t-RA. This study was undertaken to clarify the role of some of the amino acid contacts that were described in a previous report (11) and identified in the x-ray crystal structure (10).

In our previous report, two amino acid residues from the N-terminal region of the LBD, Ser232 and Thr239 in RARα, and the corresponding Ala225 and Ile232 in RARβ, were shown to be critical for receptor-specific interactions with t-RA and various receptor-selective retinoids (11). Here, further analysis of these key amino acids using site-directed mutagenesis revealed that Ser232 in RARα and Met272 in RARγ (Fig. 2) are critical for receptor-selective ligand interactions. The serine residue interacts with the amide linker portion of Am-580 in RAR receptor-selective ligand interactions. The serine residue interacts with the amide linker of BMS-185411 in RARα and the methionine residue interacts with the oxime linker in an RARα-selective retinoid (Fig. 3). Defined in this way, these receptor-selective contact amino acids are located in the ligand binding pocket and must modulate the binding of naturally occurring retinoids as well.

Using the results generated here for synthetic ligands, Ser232 in RARα may participate in a hydrogen bond with the amide linker of Am-580 and its analogs, which links the compound to α-helix H3 (Fig. 3). In RARβ and RARγ, alanine residues at positions 225 and 234, respectively, correspond to Ser232 in RARα. The presence of a hydrophilic linkers, as seen in Am-580, in close proximity to a hydrophobic amino acid (Ala225 or Ala234) may result in negative interactions between the receptor and retinoid manifested as decreased affinity of the compound for both RARβ and γ (Fig. 3 and Table III). In contrast, mutant receptors containing Thr239 alone were found to have no effect on the activity of the RARβ-selective agonist, Am-580, and its analogs (Table I). This finding is supported by the results of experiments with the chimeric receptor RARα(NE-S232)β, where the Thr239 was substituted by serine.

The strategy outlined above was also used for the identification of RARγ-selective amino acid contacts with receptor-selective ligands. Several mutant receptors were made, particularly an isoleucine 263 substitution for methionine in RARα(NEβ), which produced RARα(NE-M263)β. This mutation was found to be completely sufficient to convert the RARβ type of response of RARα(NE)β with BMS-185354 into the RARγ type. Therefore, it is likely that the methionine residue at position 272 is exclusively responsible for the RARγ selectivity of BMS-185354 (Table I). Methionine is generally considered to be a hydrophobic amino acid. However, the presence of a sulfur atom in the side chain of this amino acid could account for weak hydrogen bonding (18) between this amino acid and the hydroxyl substituent of BMS-185354 (Fig. 1 and compound 2 in Ref. 11). On the other hand, the overall strong hydrophobic properties of methionine can also explain why there is only a moderate negative effect of RARγ-selective retinoids on RARβ, which contains an isoleucine residue at a position corresponding to Met272 (Figs. 2 and 3). These observations are supported by conclusions from the crystal structure of RARγ, where interactions between Met272 and t-RA were proposed (10). The Met272 is located on α-helix H5 (Fig. 2), which, according to the model from x-ray analysis, lies above the ligand (10). This arrangement also explains the results of experiments where the racemate (R,S)-1 of 6-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthyl) hydroxymethyl-2-naphthalene carboxylic acid and both its purified S-1 and R-1 enantiomers were used (19). All three compounds showed RARγ-selectivity with the S-1 enantiomer being 10-fold more potent than R-1. In this enantiomer, the hydroxyl group is more favorably positioned with respect to Met272 and, thus, Met272 appears to be the only amino acid required for receptor-specific recognition.

Recently, another group of synthetic retinoids were discovered that act to reverse the effect of retinoic acid in transactivation assays. BMS-185411 is an example of such a transcriptional antagonist, which is shown here to interact with Ser232 (Table II). We propose that the hydrogen bond between the hydroxyl group of BMS-185411 and the amide linker of BMS-185411 positions this compound adjacent to α-helix H3 and may also force the phenyl group of this compound into close proximity to α-helix H12, which is important for transactivation activity (Fig. 3) (21, 22). The crystal structure of RARγ suggests that when t-RA binds to the receptor, α-helix-H12 closes the entry opening by swinging upward and interacting with α-helix-H4 (10, 17, 20). The α-helix-H12 is then stabilized in this position by a salt bridge between glutamic acid 412 (Glu412) from H12 and lysine 264 (Lys264) from α-helix-H4 (10). Modeling studies of the receptor with this ligand suggest that the phenyl ring of BMS-185411 may interfere with the proper alignment of α-helix-H12 leading to the inactivation of this receptor, possibly by interfering with the function of the AF-2 transactivation domain. In RARβ, the lack of the hydrogen bond between alanine 225 and the amide linker of BMS-185411 may permit flexibility of the compound in the ligand binding pocket of this receptor thus avoiding interference with α-helix-H12 and leading to selective agonist activity (Fig. 3 and Table I).

A similar explanation could also be applied to RARγ, which contains an alanine residue in a position corresponding to RARβ-Ala225 and a methionine at position 272. Using the

**Table III**

_The apparent K_d for the binding of retinoids tested in this study using wild type RARα, RARβ, and RARγ_

| Receptor | Apparent K_d, nM |
|----------|-----------------|
| t-RA     | 1.7±0.2         |
| AM-580   | 11±5            |
| BMS-185411 | 2.5           |
| BMS-185354 | >5000         |

| Receptor | Apparent K_d, nM |
|----------|-----------------|
| RARα     | 0.5±0.1         |
| AM-580   | 41±94           |
| BMS-185411 | 40±10       |
| BMS-185354 | 60±17       |

**FIG. 4.** Model for the interaction of t-RA with RARα, -β, and -γ showing the position of the key amino acid contacts.
RAR\(_{α\text{(NE-M263)}}\)β mutant, BMS-185411 was approximately 10–15-fold less potent than the same compound with RAR\(_{α\text{ or wild type RARβ}}\) (Table I). This suggests that when BMS-185411 moves within the ligand binding pocket to avoid conflict with the α-helix-H12, the dimethyl groups from the tetramethyltetrahydroxynaphthalene portion of the compound interact with α-helix-H5 at position Met\(_{272}\) (Fig. 3). The corresponding Ile\(_{263}\) in RARβ is smaller and more hydrophobic than methionine; therefore, it can better accommodate hydrophobic dimethyl groups in its vicinity allowing for more potent agonist activity with RARβ (Table I).

The results obtained here for synthetic ligands can be used to explain the pattern of binding observed for the naturally occurring ligand of these receptors, t-RA. When bound to RARβ, t-RA encounters an entirely hydrophobic environment (Fig. 4), which is reflected in the low apparent \(K_d\) with Ile\(_{263}\) and Ala\(_{225}\) at the two key positions (Table III). In RARγ, t-RA encounters Met\(_{272}\), a partial hydrophobic residue, and Ala\(_{234}\) at these two positions giving an intermediate apparent \(K_d\). Finally, in RARα, an Ile\(_{270}\) and Ser\(_{232}\) generates hydrophilic character in the binding pocket and increases the apparent \(K_d\) accordingly.

In summary, these results suggest that ligands interact with the amino acids in the ligand binding pocket in a manner that involves subtle positioning between the top of the pocket, represented by the methionine at position 272, and the bottom of the pocket. Our studies with both receptor-selective agonists and antagonists suggest that the interaction between RARs and their selective retinoids has a dynamic nature where both protein as well as ligand affect the final conformation to form a transcriptionally active complex. The functional analysis detailed here provides experimental confirmation of the RARγ LBD x-ray crystal structure. In addition, the identification of specific amino acid contacts within the ligand binding pocket can be exploited for the design of compounds of pharmacologic importance aimed at increasing the biological response and eliminating unwanted side effects.

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