Identification of Sulphydryl-modified Cysteine Residues in the Ligand Binding Pocket of Retinoic Acid Receptor \( \beta \)

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The diverse biological functions of retinoic acid (RA) are mediated through retinoic acid receptors (RARs) and retinoid X receptors. RARs contain a high affinity binding site for RA which is sensitive to treatment with sulphydryl modification reagents. In an attempt to identify which Cys residues are important for this loss of binding, we created three site-specific RAR\( \beta \) mutants: C228A, C258A, and C267A. The affinity for RA of all three mutant receptors was in the range of that of the wild type protein, suggesting that none of these Cys residues are critical for RA binding. Rather, these modified Cys residue(s) function to sterically hinder RA binding; however, the modified Cys residues critical for the inhibition of binding differ depending on the reagent employed. Only modification of Cys\(^{228} \) is necessary to inhibit RA binding when RAR\( \beta \) is modified by reagents which transfer large bulky groups while both Cys\(^{228} \) and Cys\(^{267} \) must be modified when a small functional group is transferred. These data suggest that both Cys\(^{228} \) and Cys\(^{267} \) lie in the ligand binding pocket of RAR\( \beta \). However, Cys\(^{228} \) lies closer to the opening of the RAR\( \beta \) ligand binding pocket whereas Cys\(^{267} \) lies more deeply buried.

Retinoic acid is the potent mediator of the biological effects of vitamin A that include growth, differentiation, and morphogenesis (for review, see Ref 1). These actions of retinoic acid are mediated by two evolutionarily distinct groups of nuclear receptors that belong to the multigene family of steroid/thyroid hormone receptors called retinoic acid receptors (RARs)\(^{1} \) and retinoid X receptors (RXRs) (for review, see Ref. 2). The RARs and RXRs are each made up of three receptor types, designated \( \alpha, \beta \), and \( \gamma \) (2, 5–11). In dimeric form, these proteins function as ligand-dependent transregulatory factors by binding to DNA sequences located in the promoter of target genes called retinoic acid-responsive elements (RAREs) or retinoid X-responsive elements (RXREs). In vitro binding studies have demonstrated that both all-trans-RA (RA) and 9-cis-RA are ligands for the RARs, whereas only 9-cis-RA has been shown to be a ligand for the RXRs (12, 13).

Like other members of the steroid/thyroid hormone superfamily, RARs and RXRs consist of six functionally distinct domains designated A–F (2). Unique functions have been described for several of these domains. The A and B domains are important for the ligand-independent transactivation function (AF-1). The C domain, which contains two zinc fingers, is important for both DNA binding and receptor dimerization. The E domain is functionally complex. In addition to containing all the information necessary for high affinity ligand binding, it also contains a ligand-dependent transactivation function (AF-2) and accessory dimerization sequences.

Recently there have been major advances in understanding the nature of the ligand binding domain of RARs and RXRs. High resolution crystal structures of the ligand binding domains of apo-RXR\( \alpha \) and holo-RAR\( \gamma \) have demonstrated that these receptors share a similar overall fold (14, 15). Furthermore, several amino acids within the ligand binding domains of RARs have been reported to be functionally important for high affinity binding of RA. Previous studies from this laboratory have demonstrated that the positively charged amino acid residues, Arg\(^{209} \) and Lys\(^{220} \), are critical for the high affinity binding of RA and retinoid specificity of RAR\( \beta \) (16, 17). Ostrowski et al. (18) have reported that Ala\(^{225} \)/Ile\(^{228} \) of RAR\( \beta \) and the homologous amino acid residues Ser\(^{129} \)/Thr\(^{132} \) of RAR\( \alpha \) are important for discrimination of subtype-specific synthetic ligands, and Lupisella et al. (19) have demonstrated using fluorescence quenching techniques that Trp\(^{237} \) is within the ligand binding site of RAR\( \gamma \). Finally, Dallery et al. (20) and Sani et al. (21) have demonstrated a loss of RA binding after treatment of the EF domain of RARs and chicken skin RXRs, respectively, with several sulphydryl-modifying reagents suggesting that Cys residue(s) may be located in the ligand binding pocket of these receptors. The goal of this work was to identify which Cys residue(s) are important for this loss of RA binding and to address the role that this Cys residue(s) plays in the binding of RA.

In the current report we have shown that, similar to RAR\( \alpha \), RA binding to both RAR\( \beta \) and RAR\( \gamma \) is inhibited by the sulphydryl-specific modifying reagents DTNB and MMTS and that the sulphydryl group of the reactive Cys residue(s) of RAR\( \beta \) lies in or near the retinoid binding pocket. These Cys residue(s) alone do not play a critical role in RA binding. Rather, these modified Cys residue(s) of RAR\( \beta \) function to sterically hinder the binding of RA. The modified Cys residues critical for the inhibition of RA binding differ depending on the reagent em-

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\(^{1}\) The abbreviations used are: RAR, retinoic acid receptor; RA, all-trans-retinoic acid; RXR, retinoid X receptor; RARE, retinoic acid response element; RXRE, retinoid X response element; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MMTS, methyl methanethiosulfonate; PCR, polymerase chain reaction; BE, β-mercaptoethanol; DTT, dithiothreitol; NEM, N-ethylmaleimide; p-HMB, p-hydroxymercuribenzoate; TBP, tri-n-butyl-phosphate, NaN3, sodium cyanide; GR, glucocorticoid receptor.
played. Only modification of Cys"228 is necessary when RARβ is treated with reagents that transfer large bulky groups to reactive Cys residues, whereas both Cys"228 and Cys"367 must be modified when a small functional group is transferred such as thiomethyl or thioacrylate to fully inhibit RA binding. Taken together these data suggest that Cys"228 most likely lies closer to the opening of the ligand binding pocket of RARβ, whereas Cys"367 lies more deeply buried within the ligand binding pocket.

MATERIALS AND METHODS

Plasmid Constructs and Site-directed Mutagenesis—Mutants were created according to the PCR site-directed mutagenesis technique described by Higuchi et al. (22). pSG5-RARβ, a gift from Professor Pierre Chambon, Strasbourg, France, was linearized with XbaI and used as a template for the preparation of each mutant. Both sense (s) and antisense (as) oligonucleotide primers were purchased from Ransom Hill BioScience (La Jolla, CA). The GCT codon was as used to encode the mutant Ala residue indicated in bold and underlined in the mutagenic primers.

For the preparation of C228A, two separate PCR fragments were prepared using the primer pairs RARβ 5'-s (5'GGAGGAGGATCCTCGAGAAGCTGCTGATGATGG3') and RARβ 3'-as (5'GAAGAGGACCTTACGCACGGTGGTA3') plus C228A-as (5'CTTAAAGCCCTTGGTAG3') and RARβ 3'-as (5'GAAGAGGACCTTACGCACGGTGGTA3'), respectively. The two PCR fragments were purified, annealed, and amplified in a second PCR reaction using the RARβ 5'-s and RARβ 3'-as primers. Likewise, the C258A and the C267A mutants were constructed using the RARβ 5'-s and the RARβ 3'-as primers and the following mutagenic primers: C258A-s (5'AAAGCGGCGTTCCGATATC3'), C258A-as (5'GATACTTACGCGGGCCGTG3'), C267A-s (5'TCTCAGATCTGGACGATGATTAC3') and C267A-as (5'ATACCTGATGGAACTCTGG3'). The MscI-Stul restriction fragment containing each Ala mutation was exchanged with that of full-length wild type RARβ previously cloned in frame in the NoviI restriction site of pET29 (Novegcn). In all cases, the presence of the specific mutation and the lack of random mutations were verified by DNA sequence analysis (23).

The entire coding sequences of mouse RARα and RARγ (gift from Professor Pierre Chambon) were cloned in frame into the BamHI-HindIII (RARα) or NovI (RARγ) restriction sites of pET29a (+).

Expression of RARβ and Preparation of Receptor Extracts—Each RAR expression construct was transformed into Escherichia coli K12 strain BL21(DE3) cells (Novagen) (24). Ten ml from a frozen glycerol stock was used to inoculate 5 ml of LB medium containing 30 μg/ml kanamycin and shaken at 37 °C until the A₆₀₀ was 0.6–1.0. This culture was stored overnight at 4 °C, and the following morning 1 ml of cells was used to inoculate 50 ml of LB medium containing 30 μg/ml kanamycin. This culture was incubated with shaking at 37 °C until the A₆₀₀ reached 0.6 to 1.0. At this time the cells were induced to express the recombinant S-Tag RAR fusion proteins by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM. Ninety minutes later the cells were harvested by centrifugation at 5000 × g for 15 min.

The receptor extracts were prepared from a 50-ml culture by resuspending the cell pellet in lysis buffer (50 mM Tris, pH 8.0, 0.1% Triton X-100, and 200 μg/ml lysozyme) at a final concentration of 1 ml of lysis buffer/10 μl of induced culture, freezing at −70 °C followed by rapid thawing at 37 °C, sonication until the solution lost its viscosity, and centrifugation at 17,000 × g for 30 min. The supernatant was aliquot and stored frozen at −80 °C. Total protein concentration of the receptor extracts was determined with the Bio-Rad protein assay kit using the entire coding sequences of mouse RARα, RARβ, and RARγ on RA Binding—Fig. 1, A–F, depicts the results of the treatment of the full-length recombinant RARα, RARβ, and RARγ fusion proteins with the Cu, the modifying reagents DTNB and MMTS. Similar to what has been previously reported (20, 21), full-length RARα exhibits a dramatic reduction in RA binding upon treatment with both of the hydrophobic-modifying reagents (Fig. 1, A and B). Furthermore, this loss of RA binding is highly specific and is not due to the disruption of structural disulfide bonds in the protein since it is reversible to a level comparable with that of the untreated RARα upon the addition of the reducing agents BME or DTT. Likewise, RA binding to RARβ and RARγ was similarly affected by both DTNB (Fig. 1, C and E) and MMTS (Fig. 1, D and F). In all cases this inhibition of RA binding was also highly specific since it was reversed by reducing agents. Note that RA binding by RARγ following MMTS treatment was reversible to approximately 50% of that of untreated RARγ using DTT. However, treatment of the MMTS-modified RARγ with the more hydrophobic reducing reagent TBP (27) restored RA binding to a level comparable with that of the untreated RARγ.

We next wished to determine if any of these reagents contain reactive vicinal diithiols which are responsible for this loss of RA binding. For these experiments we used sodium arsenite which had been demonstrated to cross-link two thiol groups that are spatially close (vicinal diithiols) (28, 29). Treatment of the recombinant RARα, RARγ, and RARγ fusion proteins with 10 mM sodium arsenite did not result in any significant inhibition of RA binding (data not shown).

In order to determine if the modified Cys residues following DTNB and MMTS treatment are located within the RA binding
FIG. 1. Inhibition of RA binding activity after treatment of RARs with sulphydryl-modifying reagents. Receptor extracts containing RARα (A and B), RARβ (C and D), and RARγ (E and F) were treated with the indicated concentrations of either DTNB (A, C, and E) or MMTS (B, D) or TBP (F).
Dryl modification, we have created three site-specific mutants of RA binding in the absence of the chemical modifying reagent for each protein (% of control binding). Values are mean ± S.E. for at least three independent experiments performed in duplicate. Inset, samples of receptor extract containing RARβ were preincubated with 1 μM unlabeled RA for 3 h at 27°C. Following the 3-h incubation the receptor samples were treated for 5 min on ice with 1 mM NEM (+NEM) or ethanol (−NEM) followed by addition of 10 mM BME and further incubation on ice for 10 min. Unbound RA was removed by charcoal/dextran extraction followed by incubation of the samples at 4°C for 30 min to allow the dissociation of the bound RA. RA binding was then determined for samples in each of the treatment groups using 15 nM [3H]RA as described in the legend to Fig. 1. Values are mean ± S.E. of eight experiments. Western blot analysis of receptor extracts containing the wild type and mutant RARβ fusion proteins demonstrated a major band that migrated at the same position (approximate molecular mass of 55 kDa) along with several smaller molecular weight degradation products also of similar size. In addition, the wild type and mutant RARβ fusion proteins displayed a similar level of expression (data not shown).

Effect of Site-specific Mutation on RA Binding—Fig. 3 shows representative saturation curves and corresponding Scatchard plots for wild type and each mutant RARβ fusion protein. Average apparent Kd values were determined for each receptor using at least three separately prepared receptor extracts (Table I). The Kd value of 0.6 nM RA for wild type RARβ fusion protein is in good agreement with previous reports (16, 17). Both C258A and C267A displayed a similar affinity for RA comparable with that of wild type RARβ. Interestingly C228A, which has a Kd value of 4.4 nM, displayed a small decrease (approximately 7-fold) in affinity for RA when compared with that of wild type RARβ.

Effect of Treatment of C228A, C258A, and C267A Mutants with DTNB and p-HMB—Since the three mutant RARβs have an affinity for RA in the range of that of the wild type RARβ, it is unlikely that sulfhydryl modification of RARβ inhibits RA binding by obliterating a critical direct interaction between the ligand and any one of these Cys residues in the ligand binding domain of the receptor. Therefore, the alternative mechanism of inhibition involving steric blocking of RA entry into the binding pocket upon sulfhydryl modification of RARβ was explored. We tested this hypothesis by subjecting each of the Cys mutants to sulfhydryl modification with the assumption that if one or more chemically modified Cys residues mediate the inhibition of RA binding, then replacement of the reactive Cys with an unreactive Ala residue should result in a receptor with RA binding activity comparable with that of the unmodified receptor.

Fig. 4A shows the results of DTNB treatment of each of the mutants, plotted along with wild type RARβ for comparison. The profiles for inactivation of RA binding by C258A and C267A after DTNB treatment are similar to that of wild type RARβ suggesting that Cys258 and Cys267 are either not accessible to modification by DTNB or that modification of either of these residues by DTNB is not sufficient to interfere with RA binding. On the other hand, RA binding to C228A is not affected by DTNB modification. This strongly suggests that modification of Cys228 alone with DTNB is responsible for the inhibition of RA binding following DTNB treatment.

In order to confirm this conclusion we have also used the sulfhydryl-specific modifying reagent p-HMB to test each mutant in a similar fashion. Modification of a sulfhydryl by p-HMB involves the transfer of a substituted benzyl ring which is similar in size and structure to the thiobis(2-nitrobenzoic acid)
moiety which is transferred during DTNB modification. As can be seen in Fig. 4B, the profiles of inactivation of RA binding by the wild type and mutant RARβs treated with p-HMB are essentially identical to that of their respective DTNB-modified receptor. These results further demonstrate that modification of Cys\textsuperscript{228} alone with either DTNB or p-HMB is sufficient to inhibit RA binding.

Effect of Treatment of C228A, C258A, and C267A Mutants with MMTS—In order to determine if the size of the modifying group is important, we performed similar experiments using MMTS. Unlike DTNB and p-HMB, MMTS treatment of a protein results in the transfer of the small thiomethyl-blocking group to reactive sulfhydryl groups (27). Fig. 5A shows the RA binding activity of the wild type and mutant fusion proteins after modification with MMTS. C258A demonstrated a loss of RA binding upon MMTS treatment which was identical to that of wild type RARβ further demonstrating that Cys\textsuperscript{258} is not the Cys residue whose modification inhibits the binding of RA. C228A modified with MMTS displayed RA binding which was approximately 85% of that of the unmodified C228A. This is consistent with the findings obtained with DTNB and p-HMB which demonstrate that Cys\textsuperscript{228} is an important reactive Cys. Interestingly the MMTS-treated C267A RARβ displayed approximately 60% of the RA binding activity of the unmodified C267A (Fig. 5A). Furthermore, this inhibition of RA binding by the unmodified C267A was not reversible with DTT but was reversible with the hydrophobic reducing agent TBP (Fig. 5B).

Since both C228A and C267A retained a considerable amount of RA binding following treatment with MMTS, we
next measured the apparent $K_d$ of both the MMTS unmodified and modified forms (treated with 0.25 nM MMTS) of these two receptors along with C258A (Table I). No specific RA binding was observed up to a concentration of 20 nM by the MMTS-modified C258A. This further demonstrates that either Cys 228, Cys267, or both are the modified sulfhydryls in RARβ responsible for the loss of RA binding upon MMTS modification. Interestingly the $K_d$ values of the MMTS-modified forms of both C267A and C228A were very similar to that of their unmodified form (Table I). This suggests that the inactivation of RA binding of RARβ by MMTS, which involves the transfer of the small thiomethyl-blocking group, requires the modification of both Cys228 and Cys267.

To further examine the effect of modification of only Cys228 with a small functional group compared with a large bulky group on RA binding, we have examined the effect of cyanalysis of DTNB-modified C258A and C267A. As demonstrated above in Fig. 4, inhibition of RA binding of DTNB-modified RARβ involves the modification of Cys228. Treatment of DTNB-modified Cys residues with NaCN results in the replacement of the bulky thiobis(2-nitrobenzoic acid) group with thiocyanide which is similar in size to the thiomethyl group transferred upon MMTS treatment (33, 34). It is important to note that MMTS-treated C267A and DTNB-modified C267A treated with...
Sulphydryl-modified Cys Residues in RARβ

The loss of RA binding following treatment with MMTS and DTNB cannot be explained by the elimination of critical interactions between any of the Cys residues examined and RA since each of the three Cys mutants have an apparent Kd for RA in the range of that of wild type RARβ. Interestingly, the C228A mutant did display a 7-fold decrease in affinity for RA when compared with wild type RARβ. This is not surprising since two amino acids of RARβ, close in the primary sequence of Cys228 (Ala225 and Ile232), have been demonstrated to be important for interaction with type-selective ligands (18). Furthermore, the crystal structure of holo-RARγ indicates that Cys237, the homologous residue to Cys228 of RARβ, lies within 4 Å of RA (15). It is possible that substitution of Cys228 with an Ala removes an interaction between this Cys residue and RA which contributes to the overall stability of holo-RARβ. On the other hand, it is also possible that this mutation has caused a small disruption of the local topography of the ligand binding pocket sufficient to result in this modest decrease in the affinity of C228A for RA.

Although inhibition of RA binding upon sulphydryl modification of RARβ is a result of steric blocking of RA binding, the Cys residues whose modification is critical for this inhibition of RA binding differ depending on the reagent employed. Wild type RARβ displays similar sensitivity to DTNB and MMTS treatment; however, the C228A and C267A mutants represent novel receptors with unique sensitivity to these two sulphydryl modifying reagents. Our finding of a differential response of these two mutants to reagents that transfer different size blocking groups is not unprecedented. It has been reported that specific sulphydryl modification of rabbit muscle creatine kinase with blocking groups of increasing size resulted in the incremental decrease in enzymatic activity (38). Similar results have been found for the GR. Modification of critical Cys residues in GR with MMTS results in a greater amount of residual binding of ligand than modification by iodoacetamide which transfers a relatively large blocking group (36).

Our data demonstrate that both Cys228 and Cys267 are located within the ligand binding pocket of RARβ such that when modified they inhibit the binding of RA. However Cys228 lies closer to the opening of the ligand binding pocket than Cys267 because Cys228 is accessible to modification by both large and small reagents and upon modification results in the inhibition of RA binding, whereas Cys267 is only sensitive to small reagents. We have estimated the size of DTNB and MMTS by measuring the carbon center to carbon center distances on molecular models. The dimensions of MMTS, when viewed as a molecular structure, are 3.0 Å in height and 4.0 Å in width, whereas those of DTNB are 6.0 and 7.4 Å, respectively. Based on our sulphydryl modification results, these measurements suggest that Cys267 is located in a region of the pocket not accessible to molecules or side chains of molecules much larger than 4.0 Å in diameter. Since RA is no greater than 4.0 Å at its carboxylate end and at least 5.0 Å at the β-ionone ring, we can conclude that Cys267 of RARβ is closest to the carboxylate end of RA. This conclusion is supported by the recently published crystal structure of the ligand binding domain of holo-RARγ which demonstrates that the homologous residue of Cys228 (Cys237) is within 4 Å of carbon 13 of RA (15). In addition, there is only one amino acid residue in the primary sequence between Cys267 and Arg276 of RARβ (Arg278 of RARγ). We have previously shown Arg276 of RARβ to be important for RA binding and retinoid specificity most likely by interaction with the carboxyl group of RA (16, 17), and the crystal structure of the ligand binding domain of RARγ places Arg278 within 4 Å of the carboxylate oxygen 22 of RA (15).
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