Role of Retinoid Receptor Coactivator Pockets in Cofactor Recruitment and Transcriptional Regulation*

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The nuclear receptor for retinoic acid (RAR) forms a heterodimeric complex with the retinoid X receptor (RXR). This RXR/RAR heterodimer binds to the promoter of retinoic acid target genes and recruits coactivators and corepressors to regulate gene expression. Currently, the relative role of each receptor monomer in regulating coactivator and corepressor recruitment remains unclear. Here we show that the receptor-associated coactivator 3 (RAC3) uses two separate LXXLL motifs to bind RAR and RXR. The mutation of the coactivator-binding pockets of RAR and RXR abolishes RAC3 binding. Although the coactivator pocket of RXR is essential for the function of the RXR homodimer, it has a minor role for the recruitment of RAC3 and trans-activation by the RXR/RAR heterodimer. Consistently, deletion of the activation helix of RXR enhances binding of RAC3 to the heterodimer, and mutation of the coactivator pocket of RXR had little effect on RXR/RAR activity. In contrast, the coactivator pocket and the activation helix of RAR are absolutely required. We also show that different residues of the RAR coactivator pocket are used differently for interactions with the corepressor silencing mediator for retinoid and thyroid hormone receptor (SMRT) and coactivator. These results indicate a differential role for each retinoid receptor to the overall binding of cofactors and regulation of transcription by the retinoid receptor heterodimer.

The steroid/nuclear hormone receptor superfamily is a large class of ligand-dependent transcription factors that plays critical roles in regulating genes involved in a wide array of biological processes including development and homeostasis (1). In the absence of a ligand, several receptors are able to repress basal transcription via functional interactions with the corepressors SMRT1 and N-CoR (nuclear receptor corepressor) (2, 3). Ligand binding triggers the release of corepressors and subsequent recruitment of coactivators, which enhance transcription by recruiting chromatin-modifying activities such as histone acetylation and methylation (4). Coactivators directly recruited by liganded receptors include members of the steroid receptor coactivator/p160 family such as SRC-1, transcriptional intermediary factor 2/gluocorticoid receptor interacting protein 1, and RAC3/activator of thyroid and retinoic acid receptors/amplified in breast cancer 1 (5). These coactivators contain highly conserved α-helical LXXLL motifs, where L is leucine and X is any amino acid (6, 7). Previous analyses of these motifs have indicated that motifs i, ii, and iii are critical to ligand-dependent interactions with nuclear receptors. In contrast, motifs iv, v, and vi are important for transcriptional activation likely via direct interaction with CREB-binding protein/p300 (6–8). Our laboratory and others have also uncovered a receptor-specific code of interaction, where different nuclear receptors prefer different LXXLL motifs of the coactivator (9–12).

Further insight into the biochemical basis of these interactions comes from crystal structures of the receptor ligand-binding domain complexed with the LXXLL peptide (13–16). These studies suggest a ligand-dependent formation of a hydrophobic pocket in the ligand-binding domain consisting of helices 3, 4, 5, and 12. The leucines of the α-helical LXXLL motif make direct contacts with this coactivator pocket, and a single LXXLL peptide interacts with each monomer of the receptor dimer. In addition, a “charge clamp,” consisting of a conserved glutamate in the AF-2 helix (helix 12/H12) and a conserved lysine in helix 3, positions the LXXLL motif into the coactivator pocket of the receptor (15). Interestingly, in the antagonist-bound structure, helix 12 mimics the LXXLL motif and occludes the coactivator site, consistent with the inability of coactivators to bind antagonist-bound receptors (16, 17). Recent studies also suggested a similar mechanism of interaction for the corepressors SMRT and N-CoR with unliganded receptors (18–20). Both proteins contain LXXLL-like motifs in their respective receptor-interacting domains (ID) (20). These motifs share a consensus sequence of LXXVH/XXXVL and form α-helices. Mutation of these motifs blocked the interaction with unliganded receptors and abolished transcriptional repression. Furthermore, using mutational analysis and molecular modeling, the coactivator motif seems to contact the receptor at the same surface that accommodates the LXXLL motif of the coactivator (18).

The receptors for retinoic acid (RAR), thyroid hormone (TR), vitamin D3 (VDR), and peroxisome proliferators (PPAR) form heterodimeric complexes with the retinoid X receptor (RXR). These RXR heterodimers bind DNA and regulate gene expres-

benzoic acid; m1, leucine 276/valine 280 from helix 3; m2, valine 298/leucine 301 from helix 5.
sion. However, the role of each receptor monomer in the context of a receptor dimer in regulating coactivator and corepressor recruitment remains largely unknown. In this study, we have characterized the mechanism of recruitment of the coactivator RAC3 and corepressor SMRT to the RXR/RAR heterodimer. We demonstrate that the multiple LXXLL motifs of RAC3 are differentially required for interactions with RAR and RXR. The coactivator and corepressor binding pockets of RAR overlap extensively. However, differences in contribution from each helix of the pocket are evident. We find that although the coactivator pocket of RXR is essential to coactivator binding and transcriptional activation by the RXR homodimer, this pocket is not sufficient for the RXR/RAR heterodimer. In contrast, the coactivator pocket of RAR is absolutely required and sufficient for the function of the RXR/RAR heterodimer. Consistently, deletion of the AF-2 helix from RXR enhances RAC3 binding to the RXR/RAR heterodimer, whereas deletion of the AF-2 helix from RAR abolishes RAC3 binding and transcriptional activation by the receptor heterodimer.

MATERIALS AND METHODS

GST Pull-down Assay—GST fusion proteins were expressed in Escherichia coli BL21 cells and purified with glutathione-Sepharose beads. Approximately 5 μg of purified GST fusion protein was incubated with 5 μl of 32P-labeled protein with moderate shaking at 4 °C overnight in binding buffer (20 mM HEPES, pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl2, 0.05% Nonidet P-40, 1 μM dihydrotestosterone, and 1 μg/ml bovine serum albumin). The bound protein was washed three times with binding buffer, and the beads were collected by centrifugation. The bound protein was eluted in SDS sample buffer, subjected to SDS-polyacrylamide gel electrophoresis, and detected by autoradiography.

Site-directed Mutagenesis—Site-directed mutagenesis was conducted by the QuickChange site-directed mutagenesis system (Stratagene). All mutations were confirmed by DNA sequencing.

Cell Culture and Transient Transfection—HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in 5% CO2. Cells were plated for transfection in Dulbecco's modified Eagle's medium supplemented with 10% resin charcoal-stripped fetal bovine serum in 12-well plates 1 day prior to transfection. HEK293 cells were transfected using the standard calcium-phosphate method. Twelve hours after transfection, the cells were washed with phosphate-buffered saline and re-fed fresh medium containing indicated concentrations of ligand. After 24 h, the cells were harvested for β-galactosidase and luciferase activities as described previously (22).

RESULTS

LXXLL Motif Preferences for RARs and RXRs—To determine the LXXLL motif preferences for RAR and RXR, we used the above stable complex to re-examine the LXXLL motif requirement for RAC3 binding to the RXR/RAR heterodimer (Fig. 1E). Strikingly, when wild-type RAR was heterodimerized with the AF-2 helix-deleted mutant RXR443, this complex interacted with RAC3 much more strongly than did the wild-type heterodimer in the presence of atRA (Fig. 1, compare C, lane 3 with E, lane 2), suggesting that the AF-2 helix of RXR may inhibit the binding of RAC3 to the RXR/RAR heterodimer on DNA. Similarly, the RXR homodimer bound specifically to the DR1 probe, which was supershifted after the addition of wild-type RAC3-ID in a 9-cis-RA-dependent manner (Fig. 1D). Consistent with the GST pull-down data, the LXXLL motif i or ii mutation of RAC3 binding to VDR, whereas motifs i and ii are equally important for interaction with RXR, contrasting with the motif iii preference for VDR.
is most critical to RAC3 interaction with the RXR/RAR heterodimer, with motif i also contributing but to a lesser extent.

Identification of Critical Coactivator-binding Residues of RAR and RXR—A coactivator-binding pocket, consisting of residues from helices 3, 4, 5, and 12, that accommodates the LXXLL motif of coactivators has been identified (13–16). In light of these findings, we decided to characterize the coactivator-binding pockets of RAR and RXR, by creating site-directed mutations, and determine the relative contribution of each coactivator-binding pocket of the RXR/RAR heterodimer to the recruitment of RAC3. We aligned several receptor sequences and identified conserved residues that form direct contacts with the LXXLL motif in the crystal structures of estrogen receptor α, TRβ, and PPARγ (Fig. 2A). These residues were mutated in the context of full-length RARα and RXRα. These mutations are homologous to those made in the TRβ, which retained their ability to bind hormone (13). Each RAR mutant was expressed in vitro (Fig. 2B) and tested for interactions with RAC3 in a GST pull-down assay (Fig. 2C). As expected, GST-RAC3-ID pulled down a significant amount of wild-type RAR in the presence of 1 μM atRA, compared with only minimal binding to GST alone. However, mutation of any of the conserved pocket residues drastically reduced this interaction. In particular, the V240R, L261R, and E412K mutations each abolished RAC3 binding completely, whereas F249R retained slight interaction with RAC3. Therefore, these residues in the coactivator-binding pocket of RAR are critical for RAC3 binding, suggesting that the coactivator pocket is conserved among different nu-
clear receptors and for interactions with different SRC coactivators.

Similarly, we analyzed the conserved residues in the coactivator pocket of RAR or RXR were mutated based on homologous amino acids (in bold) of estrogen receptor α, TRβ, and PPARγ to contact the LXW motif in the respective crystal structures. A, autoradiograph of in vitro translated 35S-labeled RAR probes demonstrates equal expression of wild-type (wt) and mutant proteins. B, GST pull-down assay of the indicated RAR probe with GST-RAC3-ID in the presence of 1 μM atRA. D, the autoradiograph of in vitro translated 35S-labeled RXR probes. E, GST pull-down assay of the indicated RXR probe with GST-RAC3-ID in the presence of 1 μM 9-cis-RA.

Contribution of Each of the RAR and RXR Coactivator Pockets to the Recruitment of RAC3—To determine the relative contribution of each of the two coactivator-binding pockets of the RXR/RAR heterodimer to the interaction with RAC3, we conducted a gel mobility shift assay with coactivator pocket-mutated receptors. Consistent with the above experiment, the wild-type RXR/RAR heterodimer bound to the DR5 element was significantly shifted by the wild-type RAC3-ID in the presence of atRA (Fig. 3A, lane 1). Interestingly, each of the four coactivator pocket mutations in RARα eliminated the RAC3-dependent supershift despite the presence of an intact coactivator pocket in RXRα (lanes 3–10). It seemed that the F249R mutant retained weak binding to RAC3-ID. We found that the addition of 9-cis-RA to the reactions had no effect on the binding (data not shown), suggesting that the RXR coactivator pocket alone is not sufficient to recruit RAC3 to the RXR/RAR heterodimer in the absence of a functional RAR coactivator pocket.

To further investigate the role of the RXR coactivator pocket in the recruitment of RAC3, the effect of each of the two RXR coactivator pocket mutations on binding of RAC3 to the RXR homodimer on DNA was tested (Fig. 3B). As demonstrated above, the wild-type RXR homodimer strongly bound the DR1 probe, and this complex was shifted by RAC3-ID in the presence of 9-cis-RA (Fig. 3B, lanes 1 and 2). The RXR coactivator pocket mutants retained the ability to form homodimers and bind DNA, suggesting that these mutations did not disrupt the structure of the receptor. However, we found that the m1 mutation had a weaker DNA binding activity, and the m2 mutation seemed to have an enhanced DNA binding (lanes 3–6). Nonetheless, both RXR mutants showed no evidence of binding RAC3-ID (lanes 4 and 6), indicating that the coactivator pocket of RXR is required for the recruitment of RAC3 to the RXR homodimer on DNA.

From the above experiments, it is evident that the RAR coactivator pocket is absolutely required for recruiting RAC3 to the RXR/RAR heterodimer and that the RXR coactivator pocket is required for recruiting RAC3 to the RXR homodimer. However, it is not clear whether the RXR coactivator pocket contributes to RAC3 binding to the RXR/RAR heterodimer. Therefore, we compared the ability of RAC3 to bind the wild-type RXR/RAR heterodimer versus heterodimers in which the coactivator pocket of RXR or RAR was mutated (Fig. 3C).

Intriguingly, when wild-type RAR was dimerized with the mutant RXR, RAC3-ID was still capable of binding the complex significantly (lanes 5–8), contrasting to the lack of binding to the complex containing mutant RAR and wild-type RXR. This interaction was abolished when the RXR coactivator site was also mutated (lanes 9 and 10). These data suggest that the RAR coactivator pocket is the primary binding site to RAC3, whereas the RXR coactivator pocket is less critical.

To confirm the above observations, we repeated the experiment using RXR443, which dramatically enhances the interaction of the RXR/RAR heterodimer with RAC3-ID (Fig. 3D). When RXR443 harboring the m2 mutation was dimerized with RAR, a much more enhanced interaction with the RAC3-ID was still evident (lanes 5 and 6). In fact, nearly the entire RXR443/RAR complex was again shifted by RAC3-ID. A similar result was obtained with the RXR443-m1 double mutant (data not shown). These results suggest that the RAR coactivator pocket plays a minor role in recruiting RAC3 to the RXR/RAR heterodimer, in contrast to the essential role of the RXR coactivator pocket.

Contribution of Each of the RAR and RXR Coactivator Pockets to Transcriptional Activation—To assess the functional significance of mutating the coactivator pockets of RAR and RXR in vivo, we performed reporter gene assays to investigate the transcriptional activity of these coactivator pocket mutants. First, HEK293 cells were transfected with wild-type or mutant RAR along with a luciferase reporter containing DR5 response elements (Fig. 4A). In the absence of ligand, the wild-type and mutant RAR had little effect on reporter expression, except the
wild-type RAR and E412K had a slight repressive activity. In the presence of atRA, the reporter alone was stimulated about 2-fold, whereas transfection of wild-type RAR strongly stimulated the reporter expression about 10-fold. In contrast, all four RAR mutants failed to enhance reporter expression above the endogenous level. These observations correlate well with the above in vitro data in implicating a critical role of the RAR coactivator pocket in mediating transcriptional activation from responsive promoters.

We next sought to investigate the role of the RXR coactivator pocket in supporting transcriptional activation by the RXR homodimer or RXR/RAR heterodimer in vivo. HEK293 cells were transfected with wild-type or coactivator pocket-mutated RXR and a luciferase reporter driven by a DR1-containing promoter (Fig. 4B). The wild-type RXR displayed strong 9-cis-RA-dependent transcriptional activation on the DR1 promoter as expected. In contrast, the RXR m1 and m2 mutants were significantly impaired in their abilities to activate reporter gene expression. We noted that the m2 mutant retained some weak ligand-dependent activity; however, this may correlate with its residual binding to RAC3 in vitro (Fig. 2E) and/or enhanced homodimerization and DNA binding ability (Fig. 3B). Overall, these data suggest that the coactivator pocket of RXR is critical to transcriptional activation by the RXR homodimer, correlating to the binding of RAC3 in vitro.

The above data suggest a requirement of the RXR coactivator pocket for RAC3 binding and transcriptional activation by the RXR homodimer. However, our data also suggest that the RXR coactivator pocket is not as important in recruiting RAC3 to the RXR/RAR heterodimer as the RAR pocket or as the recruitment of RAC3 to the RXR homodimer. To investigate the functional significance of this differential requirement of the RXR coactivator pocket in different dimer configurations, we analyzed the effects of the RXR coactivator pocket mutations on transcriptional activation of the RXR/RAR heterodimer from a DR5-driven promoter (Fig. 4C). Coexpression of wild-type RXR and RAR enhanced reporter expression above the expression of RAR alone. Strikingly, both RXR coactivator pocket mutants were capable still of sustaining transcriptional activation from the DR5 promoter, in contrast to their severely impaired function at the DR1 promoter. Similarly, coexpression of RXR443 with RAR was also capable of sustaining transcriptional activation by RXR/RAR and coactivation by RAC3 (Fig. 4D). In fact, RXR443 slightly enhanced RAC3-mediated transcriptional coactivation (1.5-fold). Although, this effect is not as dramatic as the enhancement of binding to RAC3 in vitro, we have found that RXR443 also enhances recruitment of the SMRT corepressor to the RXR/RAR heterodimer. Furthermore, it has also been shown that RXR443 decreases ligand-dependent dissociation of SMRT (23). Therefore, the ability of RXR443 to support transcriptional activation in vivo may be compromised by enhanced corepressor association. These data suggest that the RXR coactivator pocket is critical to the activity of the RXR homodimer but is less important to the RXR/RAR heterodimer.

Involvement of the RXR Coactivator Pocket for Corepressor Interactions—Several recent studies have determined that SMRT and N-CoR contain LXXLL-like motifs that are required for interaction with unliganded TR and RAR (18–20). Therefore, we wished to determine whether the same residues within the RXR coactivator pocket that were required for RAC3 binding were also critical to binding of the corepressor SMRT (Fig. 5A). As expected, GST-SMRT-ID pulled down significant amounts of wild-type RAR in the absence of hormone. Interestingly, the V240R, F249R, and L261R mutations each inhibited the interaction substantially, with F249R and L261R more or less abolishing the binding; V240R had a more modest effect. In contrast, the E412K mutation in helix 12 did not alter the SMRT-RAR interaction, with was opposite of the strong effect on the RAC3-RAR interaction. These results suggest that the RAR coactivator pocket overlaps with a proposed corepressor pocket. However, distinct contributions of individual residues do exist, because V240R had a more modest effect on SMRT

2 C. Leo, X. Yang and J. D. Chen unpublished data.
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We have investigated the mechanisms by which the coactivator RAC3 and corepressor SMRT are recruited by retinoid receptors and how these interactions correlate with transcriptional activities of the receptors. We find that RAC3 preferentially utilizes the LXXLL motifs i and ii to bind RXR and RAR, with highest affinity of motif ii with RAR. We identify specific residues within the coactivator-binding pockets of RAR and RXR that are required for coactivator and corepressor bindings. We demonstrate that mutation of these coactivator pocket residues disrupts recruitment of RAC3 and transcriptional activities of the receptors. Interestingly, we also find that the integrity of the RXR coactivator pocket is not sufficient for the RXR/RAR heterodimer to recruit RAC3 and activate transcription, whereas this RXR pocket is absolutely required for the function of the RXR homodimer. Consistently, deletion of the AF-2 helix from RXR enhances rather than inhibits coactivator binding to the RXR/RAR heterodimer. Additionally, we demonstrate that several coactivator-binding residues in RAR are also involved in the binding of corepressor and regulation of transcriptional repression.

The coactivator RAC3 contains three separate LXXLL motifs within its receptor-interacting domain. Based on previous studies of the crystal structure of liganded receptor with LXXLL peptide (15), it is likely that each receptor binds one LXXLL motif. Therefore, a receptor dimer may selectively utilize two LXXLL motifs to bind RXR and RAR, initially utilizes the LXXLL motif i and ii to bind RXR and RAR, with highest affinity of motif ii with RAR. We identify specific residues within the coactivator-binding pockets of RAR and RXR that are required for coactivator and corepressor bindings. We demonstrate that mutation of these coactivator pocket residues disrupts recruitment of RAC3 and transcriptional activities of the receptors. Interestingly, we also find that the integrity of the RXR coactivator pocket is not sufficient for the RXR/RAR heterodimer to recruit RAC3 and activate transcription, whereas this RXR pocket is absolutely required for the function of the RXR homodimer. Consistently, deletion of the AF-2 helix from RXR enhances rather than inhibits coactivator binding to the RXR/RAR heterodimer. Additionally, we demonstrate that several coactivator-binding residues in RAR are also involved in the binding of corepressor and regulation of transcriptional repression.

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part of the DNA-bound RXR/RAR heterodimer. In contrast, motifs i and ii are equally important to interactions with RXR in solution or when homodimerized and bound to DNA. Therefore, in the RXR/RAR heterodimer, motif ii may bind to RXR first. The subsequent interaction with RXR may be mediated by motif i. In the case of the RXR/VDR heterodimer, motif iii may bind to VDR first, followed by a secondary interaction between RXR and motif ii (9). Thus, the existence of three LXXLL motifs in RXC3-ID likely provides the coactivator with flexibility to adapt to different structural conformations that each receptor dimer assumes.

Recent crystallographic evidence has detailed the formation of a hydrophobic pocket induced by ligand binding to the receptor that serves as the docking surface for the LXXLL motif of coactivators (14–16). This pocket consists of helices 3, 4, 5, and 12 of the ligand-binding domain including a charge clamp formed by a conserved glutamate from helix 12 and a lysine from helix 3, which together precisely position the LXXLL motif within the pocket. Based on the interactions observed in the crystal structures of estrogen receptor α, TRβ, and PPARγ complexed with LXXLL peptides, highly conserved amino acids from RAR and RXR were selected for analysis in this study. We find that the mutation of valine 240, phenylalanine 249, or lysine 261, from helices 3, 4, and 5, respectively, each strikingly inhibits RAR interactions with RXC3 in vitro and transcriptional activation by the receptor in vivo. Mutation of the charge clamp glutamate, glutamate 412, shows the same effect. These mutations also abolish recruitment of RXC3 to a DNA-bound RXR/RAR heterodimer despite the presence of an intact coactivator-binding site in RXR. While it remains to be demonstrated, these single point mutations are unlikely to affect the overall structure of the receptor because they retain intact DNA-binding and RXR heterodimerization activities. These data suggest that an intact coactivator pocket is essential to RAR interactions with RXC3 and that coactivator interaction is required for the transcriptional activation function of RAR.

We also have investigated the ability of RXR, the common heterodimeric partner for nonsteroid receptors, to interact with RXC3 because it is not known what role RXR plays in recruiting coactivators to the RXR/RAR heterodimer on DNA. Although heterodimerization with RXR is essential for DNA binding, RXR has long been considered as a transcriptionally silent partner for partnering receptors (1, 24, 25). However, the RXR-specific ligand SR11237, when in combination with the RXR antagonist BMS453, can induce differentiation of NB4 acute promyelocytic leukemic cells and transcriptional activity of the receptor to control gene expression. In summary, our data suggest that each receptor in a receptor heterodimer is differentially required in the recruitment of coactivators and corepressors. The recruitment of coactivators and corepressors by the RXR/RAR heterodimer involves a complex series of interactions that in turn regulates the transcriptional activity of the receptor to control gene expression. Multiple RXC3 LXXLL motifs mediate the binding of coactivator to the liganded receptor, with different receptors preferring different motifs for interaction. Both coactivators and corepressors interact with a similar hydrophobic pocket of the ligand-binding domain, and the AF-2 helix of RXR seems to be an important regulatory motif in mediating cofactor recruitment. Overall, this study provides several new insights to the under-
standing of the mechanism by which individual coactivator pockets are utilized in a nuclear receptor dimer to recruit coactivators and corepressors.

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