Effects of Retinoid Ligands on RIP140: Molecular Interaction with Retinoid Receptors and Biological Activity†

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ABSTRACT: Receptor interacting protein 140 (RIP140) interacts with retinoic acid receptor (RAR) and retinoid X receptor (RXR) constitutively, but hormone binding enhances this interaction. The ligand-independent interaction is mediated by the amino and central regions of RIP140 which contain a total of nine copies of the LXXLL motif, whereas the agonist-induced interaction is mediated by its carboxyl terminus which contains a novel motif (1063−1076, LTKTNPILYYMLQK). The ligand-independent interaction could be enhanced slightly by agonists, whereas the ligand-dependent interaction was strictly agonist dependent for both RAR and RXR. In the context of heterodimers, ligand occupancy of RXR played a more dominant role for both molecular interaction and biological activity of RIP140. Competition and mutation studies demonstrated an essential role for ^{1067}Asn and ^{1073}Met for a ligand-dependent interaction. A model was proposed to address the constitutive and agonist-dependent interaction of RIP140 with RAR/RXR.

The nuclear receptor superfamily consists of a large group of ligand-inducible transcription factors that are involved in a variety of biological processes. This superfamily includes receptors for various small hydrophobic molecules such as steroids, thyroid hormones, and retinoids. Also included in the superfamily are a large group of receptors of which the ligands remain to be identified, named orphan receptors (*1*−*4*). Nuclear receptors function by binding to their cognate DNA sequences (hormone response elements) to either suppress or activate transcription of target genes. The transcriptional regulatory properties of these receptors are mediated, in part, by their ability to recruit coregulatory proteins called coactivators or corepressors (*5*, *6*). Aporeceptors and antagonist-bound receptors are found to be associated with corepressor complexes such as NCoR/SMRT, which are known to recruit histone deacetylases (HDACs)[1] (*7*). Binding of agonists induces a conformational change in the receptors, resulting in the displacement of corepressor complexes followed by the recruitment of coactivators such as the p160 family, p300/CBP and P/CAF (*8*−*11*). The histone acetyltransferase activity of coactivator complexes modifies lysine residues in amino-terminal tails of histone proteins, resulting in an open chromatin structure for activated transcription. The ligand-dependent exchange of coactivators and corepressors appears to be the basis of nuclear receptor action in gene regulation.

The molecular basis of the interaction between corepressors and nuclear receptors was shown to involve a consensus sequence of L/IXXI/VI (the CoRNR motif), where L is a leucine, V is a valine, and X can be any amino acid. Interestingly, the interaction of holonuclear receptors with coactivators was shown to involve a similar motif, LXXLL, found in most coactivators (*9*, *12*). In competition studies, CoRNR peptides were able to block receptor interaction with either coactivators or corepressors, suggesting that receptors interact with coactivators and corepressors through an adjacent, and perhaps overlapping, domain on the receptor surface which recognizes these very similar motifs of the coregulators. It was also shown that the corepressor interacting motif could also be a more extended helix, LXXI/HIXXXI/L (*13*, *14*).

Receptor interacting protein 140 (RIP140, also named NRIP1) is the most enigmatic member among the known receptor coregulators. The human RIP140 was first identified as a coactivator for a chimeric estrogen receptor (*15*, *16*). The mouse RIP140 was cloned and characterized as a corepressor for orphan receptor TR2 (*17*). Later it was shown to be associated with many other hormone receptors in a ligand-dependent manner, including retinoic acid receptor (RAR), retinoid X receptor (RXR), and peroxisome proliferative-activator receptor (PPAR) (*16*). However, most of

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[1] Abbreviations: RIP140, receptor interacting protein 140; RAR, retinoic acid receptor; RXR, retinoid X receptor; HDAC, histone deacetylase; PPAR, peroxisome proliferative-activating receptor; DR5, direct repeat with five nucleotides in the spacer; TNT, in vitro transcription and translation; GRIP1, glucocorticoid receptor interacting protein 1.

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these studies, including our own studies of the RAR/RXR system, demonstrated a ligand-dependent suppressive role for RIP140 in these hormone signaling pathways (*18*−*20*). More recently, a direct association of RIP140 with both class I and class II HDACs (*21*) as well as a ligand-enhanced formation of the RAR/RXR/RIP140 complex both in vitro and in vivo (*22*) was demonstrated in our laboratory. The ligand-enhanced interaction with holoreceptors would categorize RIP140 as a coactivator, whereas its ability to directly recruit HDACs and suppress hormone-induced gene expression would suggest a corepressor role for RIP140. Interestingly, while RIP140 contains nine copies of the LXXLL interaction motif, its ligand-enhanced interaction with holoreceptor appeared to be attributed to a novel peptide (LTKTNPILYYMLQK) found in its carboxyl-terminal region (amino acids 1063−1076) (*22*). This region was shown to be essential for RIP140's biological function as a corepressor for the RAR/RXR heterodimer, because a mutant deleted in this motif failed to suppress RA induction of an RA-responsive reporter gene (*22*). The presence of nine LXXLL motifs in the amino and central portions and the novel peptide in the carboxyl terminus of this molecule prompted us to carefully examine and compare ligand-dependent and ligand-independent interactions of RIP140 with nuclear receptors, as well as the effects of specific ligands.

RAR and RXR mediate the pleiotropic effects of retinoids on morphogenesis, homeostasis, cell growth and differentiation. By using different retinoid ligands in the RAR/RXR system, we determined the molecular basis of the ligand-dependent and ligand-independent interactions between RIP140 and the retinoid receptors RAR and RXR and the effects of specific agonists/antagonists on this interaction. Second, by introducing point mutations, we identified the essential amino acids in the novel carboxyl peptide of RIP140 that are required for its specific agonist-dependent interaction with holo-RAR/RXR. This domain was found to be competitive to RIP140 in terms of receptor interaction and the suppressive biological activity. Finally, the effects of various retinoids on the suppressive activity of RIP140 were examined in the RAR/RXR-mediated gene activation system.

EXPERIMENTAL PROCEDURES

Ligands and Peptides. The ligands used in this study include RAR agonist Am80 (8×10^{-9} M), RAR antagonist AGN193109 (1×10^{-6} M), TD550 (1×10^{-6} M), RXR agonist AGN194204 (1×10^{-6} M), and RXR antagonist HX531 (2×10^{-7} M). Am80, TD550, and HX531 were dissolved in DMSO as described (*23*, *24*). AGN193109 and AGN194204 were dissolved in ethanol. Peptides were synthesized in the Microchemical Facility, University of Minnesota.

Plasmid Constructs. RIP140 and its various deletions cloned in the GST expression vector pGEX-2T (Promega) were as described (*17*, *22*). The coding regions for each construct were as follows: the full-length (RIP-f), amino acids 1−1161; the N-terminal domain (RIP-N′), amino acids 1−496; the central domain (RIP-cent), amino acids 333−1007; the C-terminal domain (RIP-C′), amino acids 977−1161; and a small C-terminal peptide (R36), sequence LTKTNPILYYMLQK. Point mutations were generated in RIP-C′ by a two-step PCR. The full-length RIP140, RAR, and RXR mammalian expression vectors, as well as the reporter plasmid carrying a direct repeat 5 (DR5)-tk-luciferase, have been described previously (*17*).

GST Pull-Down Assays. GST-fused RIP140 proteins were expressed in *Escherichia coli* and partially purified using glutathione−agarose beads (Sigma). ^{35}S-Labeled or unlabeled full-length RAR or RXR were produced using an in vitro transcription−translation system (Promega). After binding of GST fusion protein to glutathione−agarose beads and extensive washing with 1× PBS and 0.1% NP-40, the beads were resuspended in a binding buffer (20 mM HEPES, pH 7.5, 100 mM NaCl, 0.5 mM EDTA, 0.1% Triton X-100, 10% glycerol). Receptors were preincubated with ligands in the binding buffer for 15 min on ice and added to the beads. Protein interaction was allowed to occur for 1 h at 4 °C. After washing, bound protein was eluted in SDS electrophoresis loading buffer, separated on a 10% SDS−PAGE, and visualized by autoradiography.

Competition pull-down assays using wild-type (wt) or mutant peptides (M1, M2, M3, and M4) were performed by incubating receptors with bound ligand and peptides (50 μM) in binding buffer for 1 h at 4 °C and then adding to bead-bound GST−RIP140 protein.

Analysis of the Biological Activity of RIP140. COS-1 cells were maintained in DMEM supplemented with 10% FBS at 37 °C in 5% CO_2. Cells were plated at a density of 5×10^4 cells/well in 24 well plates. Cells were transiently transfected by the calcium phosphate precipitation method with a mixture of RAR (0.1 μg), RXR (0.1 μg), RIP140 (0.1 μg), DR5-tk-luciferase (0.6 μg), and a CMV-Lac-Z internal control (0.05 μg). Transfected cells were treated with various combinations of ligands (Am80, AGN193109, AGN194204, and HX531) for 24 h. Forty-eight hours after transfection, cells were harvested and the total cell lysate was analyzed for luciferase and Lac-Z activity (*18*). The fold induction by ligands in the presence or absence of RIP140 was calculated and plotted. Reported values are the average of three independent experiments done in duplicate. For the competitive nature of C′ to the biological activity of RIP140, the same transient transfection system was used, in the absence or presence of C′ expression vector.

Modeling Methods. A prediction of the docking conformation of the RIP140 amino acid sequence LTKTNPILYYMLQKGS to RXRα was completed with the program INSIGHT II (Biosym Technologies, San Diego, CA). Most of the main chain coordinates for the RIP140 peptide were taken from that of the coactivator SRC-1 bound to RXRα as found in the heterodimer structures of PPARγ/RXRα (PDB code 1DKF). However, the crystal structures of the nuclear receptor box 2 regions of the glucocorticoid receptor interacting protein 1 (GRIP1; PDB code 3ERD) and nuclear receptor interacting domain (MTIF2; PDB code 1BSX) were also used as templates to extend the carboxyl-terminal end of this model polypeptide by two residues. The structurally conserved side chains were maintained. Side chains with additional and/or different atoms were given extended rotamer conformations from the point where continuity ended. Both lengths and torsional angles were then regularized.

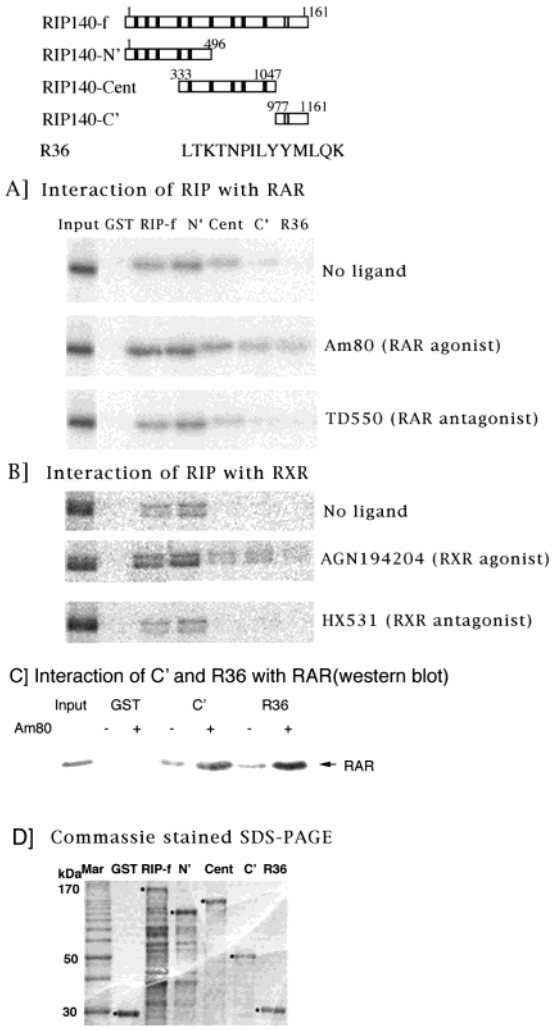

FIGURE 1: GST pull-down tests of the interaction of various GST−RIP140 fusions with RAR or RXR. The RIP140 domains used in each fusion are shown at the top of this figure. The LXXLL (solid bar) and LYYML (hatched bar) motifs are depicted. (A) RIP140 interaction with RAR. (B) RIP140 interaction with RXR. Ligands used are indicated at the right of each panel. (C) Western blot analysis of the interaction of C′ and R36 with RAR in the presence and absence of ligand Am80. (D) A Commassie-stained gel with GST and various RIP140 domains immobilized on GST beads, each marked by an asterisk.

RESULTS

Interaction of RIP140 with RAR and RXR. It is known that RAR and RXR can potentially exist as homodimers or heterodimers. To examine the effects of ligands on RIP140 interaction with either receptor alone, GST pull-down assays were performed by using a panel of RAR and RXR agonists and antagonists. The previously dissected RIP140 domains fused to GST (top of Figure 1) include the N-terminal domain which contains five copies of the LXXLL motif (N′), the central region which also contains five copies of the LXXLL motif (Cent, overlapping with N′ at one LXXLL motif), the C-terminal domain that contains a novel LYYML motif (C′), the dissected novel motif named R36, and the full-length RIP140 fusion (RIP-f). These GST fusion proteins were each purified and incubated with RAR or RXR expressed as ^{35}S-labeled proteins. The receptors were first incubated with various ligands, followed by incubation with different GST−RIP140 fusions. Figure 1A shows the pull-down results of RIP140 interaction with RAR, Figure 1B shows interaction with RXR, Figure 1C shows a more sensitive, Western blot, analysis of RAR interaction with C′ and R36, and Figure 1D shows a Commassie blue stained gel, revealing each RIP140 fusion protein eluted from the GST beads.

As shown in Figure 1A, a constitutive interaction of RIP140 with RAR was observed for the RIP140-f, N′ and Cent fusions, whereas specific agonist-dependent interaction was observed for both the C′ and R36 fusions (panel b). Therefore, for RAR, the multiple LXXLL motifs (as seen in the full-length, the N′, and the Cent fusions) in the context of RIP140 contributed to its constitutive interaction with the nuclear receptors, whereas its novel C-terminal motif (C′ or R36) indeed was responsible for a specific agonist-dependent interaction. In the case of RXR (Figure 1B), the RIP-f and N′ fusions showed positive interaction with receptors in the presence or absence of ligands, whereas the Cent, C′, and R36 were positive only in the presence of agonist. Therefore, for RXR, the five copies of LXXLL in the RIP140 N-terminal domain were responsible for a constitutive interaction, whereas the LXXLL motifs in its central portion, as well as the novel C-terminal motif, were responsible for its specific agonist-dependent interaction. For both RAR and RXR homodimers, agonist binding also enhanced the interaction of RIP-f and N′ with the receptor. The binding of antagonist to either RAR (Figure 1A, panel c) or RXR (Figure 1B, panel c) did not affect the constitutive interaction mediated by the RIP140-f, N′, and cent fusions in the case of RAR and RIP140-f and the N′-terminal region in the case of RXR, but it did abolish the agonist-dependent interaction of C′ and R36 with the receptors. The ligand dependency of receptor interaction with C′ or R36 was more obvious for RXR as compared to RAR, as a faint band was detected for RAR interaction even in the absence of ligand (Figure 1A, panel a). In an attempt to further examine this weak ligand-independent interaction of C′ and R36 with RAR, a Western blot analysis was conducted to carefully monitor this interaction as shown in Figure 1C. GST-fused protein was incubated with a nuclear extract of RAR-transfected COS-1 cells in either the presence or absence of ligand (Am80). After extensive washing, protein was separated on a SDS−PAGE, transferred to a PVDF membrane, and probed with an RAR antibody (Affinity Bioreagents). Consistently, a weak band was detected for C′ and R36 even in the absence of ligand, which was significantly enhanced in the presence of ligand, confirming the weak constitutive interaction of RAR with C′.

These results suggest the presence of two independent types of molecular interaction of RIP140 with nuclear receptors. The first involves an agonist-dependent interaction that is mediated by the novel C-terminal motif (for both RAR and RXR) and/or the LXXLL motifs present in the central portion of this molecule (for RXR). The second type involves a constitutive interaction mediated by the multiple LXXLL motifs present in the N-terminal portion (for both RAR and RXR), and/or those present in the central and carboxyl portions of RIP140 (for RAR). It can be concluded that, for both RAR and RXR, the novel motif in the C-terminus of RIP140 is indeed responsible for its specific agonist-dependent/enhanced interaction with receptors.

Interaction of RIP with RAR/RXR Heterodimers. The interaction of RIP140 with RAR/RXR heterodimers was also

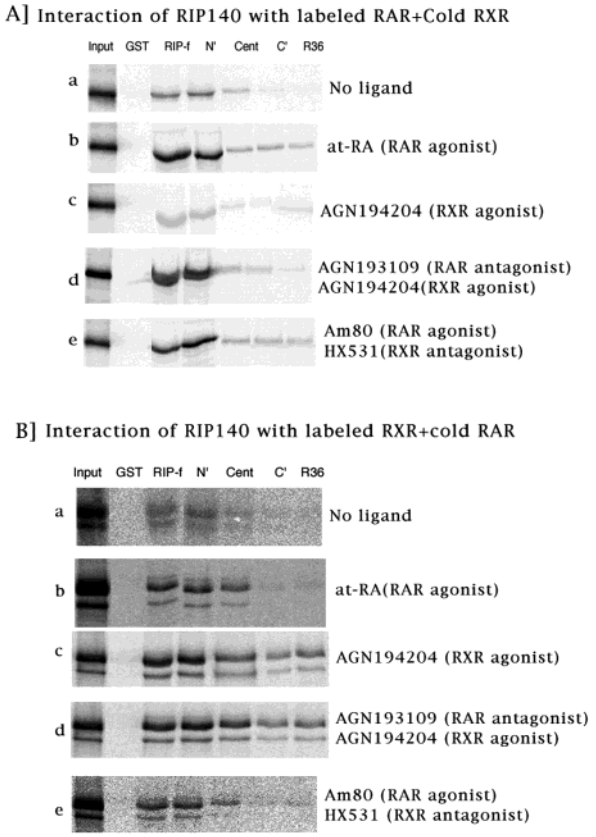

FIGURE 2: GST pull-down tests of the GST−RIP140 interaction with the RAR/RXR heterodimer. (A) Interaction using ^{35}S-labeled RAR and cold RXR. (B) Interaction using ^{35}S-labeled RXR and cold RAR. Ligands used are indicated at the right of each panel.

examined in GST pull-down assays. In these experiments, only one of the two receptor partners was labeled with ^{35}S-Met. Figure 2A shows the binding pattern obtained with labeled RAR and cold RXR, and Figure 2B shows the results of using labeled RXR and cold RAR. The results of using RIP-f or N′ fusions with either RAR or RXR labeled were similar to that with receptor homodimers in terms of ligand-independent interaction or antagonist-bound receptors (data not shown). However, agonist-dependent interaction mediated by the C-terminus of RIP140 appeared more obvious with labeled RXR in the heterodimer (Figure 2B), because only in the presence of RXR agonists (panels c and d) could the interaction be detected for both the C′ and R36 fusions. This interaction was not affected even when RAR was occupied by an antagonist (Figure 2B, panel d), and agonist-bound RAR was not effective in recruiting C′ or R36 to the RAR/RXR heterodimer (Figure 2B, panel b), further suggesting a dominant role of RXR in mediating the heterodimeric receptor's interaction with RIP140. When RAR was labeled in the heterodimer (Figure 2A), either RAR or RXR agonists could facilitate the ligand-dependent interaction (panels b−e). Thus, RXR seemed to play a more dominant role in this ligand-dependent interaction mediated by C′. In the case of the RIP140 Cent fusion with RAR labeled (Figure 2A), interaction was observed for either apo-RAR (panel a) or agonist-occupied RAR or RXR (panels b−e). However, with labeled RXR (Figure 2B), the Cent fusion required at least one agonist-occupied receptor (panels b−e), consistent with the results of interaction with homodimeric RXR (Figure 1B) in that the central fusion could be involved in both ligand-independent and agonist-dependent interactions. As compared to RAR or RXR homodimers, heterodimers behaved slightly different in terms of interacting with N′ fusion. There was a ligand-enhanced interaction of this RIP fusion protein with both receptors.

Therefore, it can be concluded that both the RIP-f and N′ fusions can interact with heterodimeric receptors ligand-independently and the interaction is enhanced by ligands. The C′ fusion interacts with receptors in a more agonist-dependent fashion. In terms of the central portion of RIP140, a general rule seems to require at least one agonist for a significant interaction with RXR, although a weak interaction is also seen without any ligands (panel a). Of particular interest is the observation that the RAR antagonist (AGN193109) failed to block the interaction of RIP140, triggered by RXR agonist AGN194204 (panel d), with either RAR (Figure 2A) or RXR labeled (Figure 2B), suggesting that the nature of ligand occupancy on the RXR moiety in the context of RAR/RXR heterodimer seems to play a more important role for the heterodimeric receptor's interaction with RIP140.

Amino Acid Residues Essential for Ligand-Dependent Interaction. The ligand-dependent interaction of RIP140 was mediated by its C-terminus, as shown in the results of the C′ and the dissected R36 peptide fusions. It was interesting that the C′ peptide lacks any LXXLL motif but contains a LYYML sequence in the region of amino acids 1063−1076 (LTKTNPILYYMLQK). A computer model was created to identify essential amino acids required for the interaction between this peptide and receptors (see Experimental Procedures). From this analysis, two amino acids, ^{1067}Asn and ^{1073}Met, were predicted to be important. The Met residue of this motif offers a larger buried surface area that is more hydrophobic in character than Leu, and therefore a higher binding affinity to receptors is expected for this novel RIP140 motif. The Asn residue may be important due to a prediction of hydrogen bonds formed with the receptor and as one residue at the interface that is polar rather than nonpolar in character. An Asn residue at this location is also conserved in the corepressors NCoR and SMRT. On the basis of the modeling four mutant peptides were made as shown at the top of Figure 3. The ^{1073}Met residue was changed to Lys (M1), to Leu (M2, as in a typical LXXLL motif), or to Ile (M3). The ^{1067}Asn was altered to Gly (M4).

Competition assays by the GST pull-down method were completed with wild-type (wt) and the mutant peptides (Figure 3). An excess (50 μM) of wild-type or mutant peptide was incubated with the receptors for 1 h and then added to R36 immobilized on GST beads. The results of the association of the R36 with RAR (panel A), RXR (panel B), and RAR/RXR in which RAR was labeled (panel C) are shown in Figure 3. It appeared that a similar pattern of competition was observed for either homo- or heterodimers in the presence of specific agonist. As predicted, either no interaction with RXR or very weak interaction with RAR was detected in the absence of ligand (top panels of Figure 3A−C). The wt peptide was fully competitive to R36 interaction with RAR, RXR, or heterodimers. The M1 peptide where Met was substituted with Lys failed to compete for RAR, RXR, and heterodimer. As expected, the M2 peptide where Met was changed to Leu, like a typical LXXLL motif, was also competitive but not as efficient as the wild-type peptide. The M3 peptide with the Met mutated to Ile

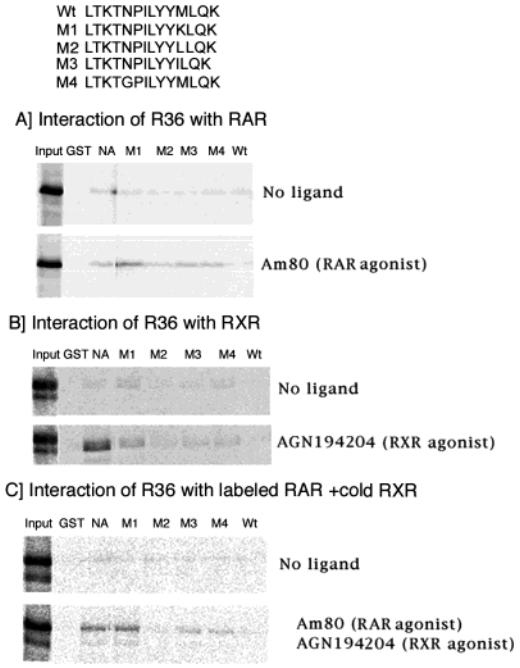

FIGURE 3: Peptide competition in GST pull-down assays. The sequences of the wild-type (wt) and various mutant peptides, with several mutations underlined, are shown at the top of this figure. (A) The results of using labeled RAR alone. (B) The results of using labeled RXR alone. (C) The results of using the RAR/RXR heterodimer with RAR labeled. Ligands used are indicated at the right of each panel.

competed to a lesser degree, further supporting the hypothesis that the Met residue is essential for the ligand-dependent interaction. The M4 peptide containing the Asn to Gly mutant was not very efficient in competition, particularly for RAR, indicating that the amino-terminal flanking sequence to the helical segment is also important in stabilizing the ligand-dependent interaction of this novel motif.

To further confirm these observations, mutations were introduced into the wild-type C-terminal domain of RIP140. This was done by a two-step polymerase chain reaction to generate ^{1068}Pro/Ala or ^{1073}Met/Lys mutations. The mutated protein, expressed as GST fusion protein, was tested in pull-down assays as shown in Figure 4. The wild-type C′ was able to interact with either RAR (Figure 4A) or RXR (Figure 4B) in a ligand-dependent manner whereas the mutation completely abolished this interaction. This C′ mutant was examined for interaction with receptors in the presence of different ligands as shown in Figure 1, and it was found that it also failed to interact with receptors in the presence of either RAR or RXR ligands (data not shown), further confirming that Met was absolutely required for ligand-dependent interaction of this novel RIP140 motif. Furthermore, as the mutation affected interaction with both RAR and RXR, it is likely that both RAR and RXR could interact with a similar or overlapping site of this novel RIP140 motif.

To gain an insight into the relative receptor-binding affinity of this novel peptide as compared to RIP-f or its N′ portion, a competitive GST pull-down assay was conducted using either GST−RIP-f or N′ immobilized on GST beads. The ^{35}S-labeled RXR/RAR heterodimer was incubated with beads in the presence of 50 μM peptide (LTKTNPILYYMLQK), followed by extensive washing. The results are shown in Figure 5A. The comparable expression of these proteins

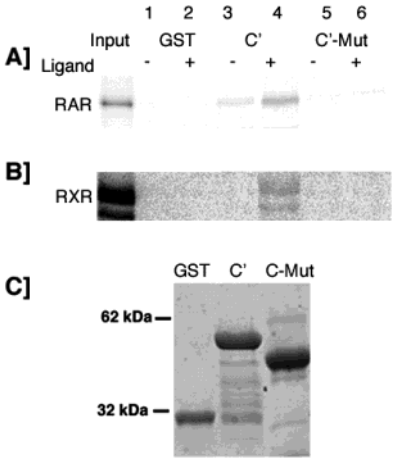

FIGURE 4: GST pull-down assay of the mutated C′ interaction with receptors. (A) The results using ^{35}S-labeled RAR. (B) The results using ^{35}S-labeled RXR. Lanes: 1 and 2, GST control; 3 and 4, wild-type C′; 5 and 6, mutant−C′. Lanes 1, 3, and 5 show the results in the absence of ligands and lanes 2, 4, and 6 show the results in the presence of ligands (Am80 for RAR in panel A and AGN194204 for RXR in panel B). (C) The Commassie-stained gel showing the fusion protein bound to GST beads.

revealed by PAGE is shown in Figure 5B. The peptide indeed effectively competed with the N′ portion of RIP140, but not RIP-f which contains this high-affinity motif, suggesting a relatively stronger binding of receptors with this carboxyl-terminal motif of RIP140 than its N′ portion which contains only typical LXXLL motifs.

The competitive nature of C′ was also confirmed in an assay for biological activities of RIP140 as shown in Figure 5C. As expected, in transient transfection assays, RIP140 suppressed RA induction of the reporters (compare columns 1 and 2). However, the suppressive activity of RIP140 could be blocked by the expression of C′ in a dose-dependent manner (Figure 5C). Thus, the C′ is not only able to compete with RIP140 for receptors binding but also block RIP140 activity. To further confirm that RIP140/RAR/RXR indeed formed complexes on target DNA DR5, a modified pull-down assay (*25*) was performed where DNA/RIP140/RAR/RXR could be simultaneously pulled out in the same complex (data not shown).

Effects of RAR/RXR Agonists and Antagonists on the Biological Activity of RIP140. To determine the effects of ligands on the ability of RIP140 to suppress receptor-mediated induction of target genes, transient transfection assays were conducted. COS-1 cells were transfected with RAR and RXR in the presence or absence of RIP140. The transfected cells were treated with ligands for 24 h, followed by assays for luciferase and Lac-Z activity. As expected, higher induction was observed when both receptors were bound by agonists [Figure 6, column 5, compared to either RAR (column 1) or RXR (column 3) bound by agonist]. As expected, RIP140 always suppressed the specific reporter activity (Figure 6, compare filled to open columns), and the fold of suppression was higher (approximately 3-fold) when agonist was applied as compared to antagonist (approximately 1.5−2-fold). Furthermore, an even stronger suppression was observed in the case where both RAR and RXR agonists were used (3.8-fold), suggesting that a cooperative binding of the holoreceptors promotes the recruitment of RIP140 and, hence, a stronger suppression. The suppression

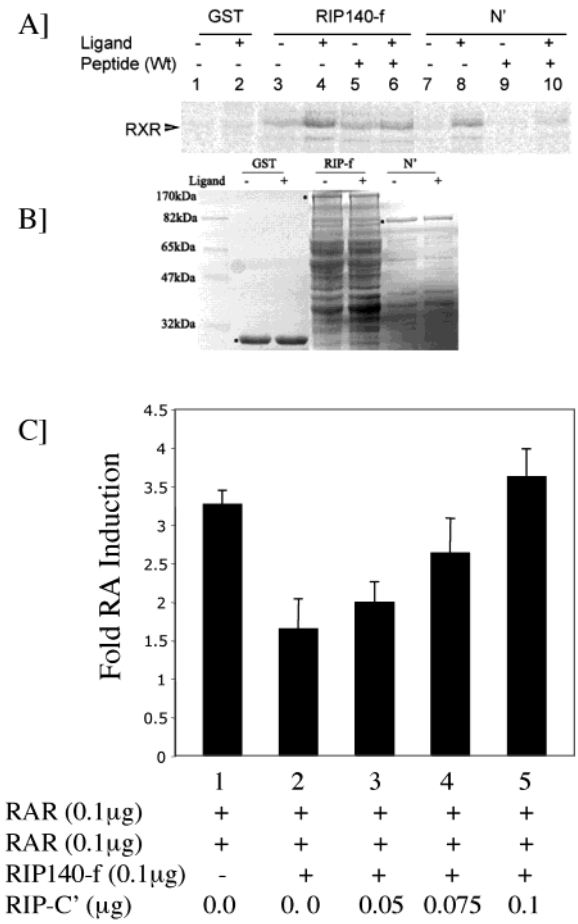

FIGURE 5: Competition of LXXML and LXXLL motifs for binding to RAR/RXR heterodimers with labeled ^{35}S-RXR. (A) The ^{35}S-labeled RXR associated with GST (lanes 1 and 2), RIP-f (lanes 3−6), and N′ (lanes 7−10) in the absence (lanes 1, 3, 5, 7, and 9) or presence (lanes 2, 4, 6, 8, and 10) of ligands (Am80 and AGN194204). Competition was done by adding 50 μM peptide (lanes 5, 6, 9, and 10). (B) A Commassie-stained gel where the bands of synthesized RIP140 protein are indicated with asterisks. (C) A blockage of RIP140-f activity by C′ in transfection experiments. Suppression of RA induction by RIP140-f (comparing columns 1 and 2) was blocked by the C′ expression in a dose-dependent manner (columns 3−5).

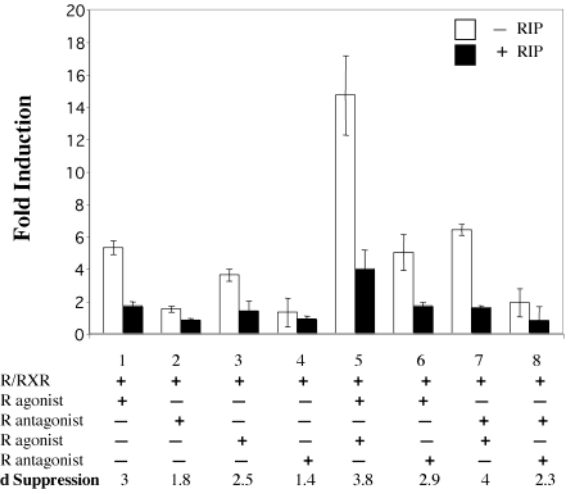

FIGURE 6: Effect of retinoid ligands on the suppressive activity of RIP140 in RAR/RXR-mediated gene activation. COS-1 cells were cotransfected with RAR and RXR in the presence (black bars) or absence (open bars) of RIP-f. Retinoids were added 24 h before the determination of reporter activity. Fold of induction of DR5-tk-luc obtained is plotted.

was also observed when either RAR (bar 2), RXR (bar 4), or both (bar 8) was bound with antagonists, consistent with the observation that RIP140 also interacted with antagonist-occupied RAR and RXR through its N-terminal and/or central regions (Figures 1 and 2).

It is concluded that RIP140 indeed acts as a negative regulator of RAR and RXR in the induction of RA target genes. The suppression is much more obvious for agonist-occupied receptors. In the presence of antagonists, the reporter activity is also suppressed by RIP140 because RIP140 can be recruited to the receptors under this condition (Figures 1 and 2). Either RAR or RXR ligand can enhance the recruitment of RIP140, but the strongest suppressive activity of RIP140 is seen when the agonists of both receptors are present.

DISCUSSION

The notion that ligand (agonist) dependent activation of nuclear receptors is mediated by coactivator recruitment was based on several mutational analyses and mapping of the AF-2 domains of nuclear receptors (*26*−*30*). Subsequent identification of corepressor molecules such as NCoR (*31*) and SMRT (*32*) further revealed the complexity of ligand modulation of these transcription factors. Corepressor interaction was reported for several receptors and transcription factors. From these reports it was demonstrated that corepressor association with nuclear receptors was either antagonist-dependent (*33*, *34*) or triggered by aporeceptors (*35*, *36*).

The RIP140 coregulator has been characterized as both a coactivator and a corepressor. RIP140 is able to interact with a variety of nuclear hormone receptors including estrogen, and thyroid hormone receptors, RAR and RXR (*16*). In an earlier study, the LXXLL motifs scattered throughout the N-terminal and central portions of RIP140 were suggested to be responsible for ligand-induced interaction with holoreceptors (*37*). Our previous study showed that RIP140 interacted with the orphan receptor TR2 in a ligand-independent manner, resulting in further repression of transcription from TR2 target genes (*17*). We also provided evidence that the corepressive activity of RIP was due to its direct interaction with class I HDAC (*21*). The RIP140 domain that interacted with TR2 orphan receptor in a ligand-independent manner was mapped to the LXXLL-containing N-terminal and central portions of RIP140 (*17*), which is similar to what was seen in the case of RAR/RXR aporeceptors or antagonist-occupied receptors in this study (Figures 1 and 2). In contrast to the earlier study suggesting LXXLL motif-mediated ligand-dependent interaction of RIP140 with receptors, we consistently detected a ligand-dependent interaction of the C-terminal portion of RIP140, which lacks any LXXLL motif, with RAR/RXR. However, a slightly modified sequence (LYYML) was noticed in the C-terminal region of RIP140 (*18*). By extending this previous observation, this study attempted to carefully determine the molecular basis of RIP140 interaction with RAR and RXR. In particular, we have unambiguously confirmed a characteristic agonist-induced interaction of holoreceptors with RIP140 mediated by this novel C-terminal motif.

Several RAR/RXR-specific agonists and antagonists, as described in Experimental Procedures, were used to examine the effects on the interaction of RAR and RXR homo- and heterodimers with RIP140. Several domains of RIP140 were fused to GST and tested for interaction with RAR (Figure 1A) and RXR (Figure 1B) in pull-down assays. These interaction tests showed that the ligand-independent recruitment of RIP140 by RAR and RXR was mediated by the amino-terminal and central portions of the RIP140 protein, as seen in the case of interaction with orphan receptor TR2 (*17*). Presumably, the nine LXXLL motifs present in these regions of RIP140 were responsible for this ligand-independent interaction. In contrast, agonist-dependent recruitment of RIP140 involved its carboxyl-terminal domain and, in particular, the LYYML motif present in this domain. The ligand-dependent interaction of C′ and R36 with receptors was more obvious for RXR as compared to RAR. Although antagonist binding to nuclear receptors has been shown to alter the conformation of the AF2 domain in the native receptor (*38*, *39*), the constitutive interaction of RIP-f, RIP-N′, or the RIP-cent with either RAR or RXR receptors or with heterodimers (data not shown) was not affected by occupancy of receptors by antagonists, confirming the nature of constitutive interaction of RIP140 with RAR and RXR mediated by its N-terminal and central regions.

The interaction of receptor heterodimers with corepressor and coactivators has been extensively documented (*7*, *40*). While the heterodimer of RAR/RXR is predicted to be the predominant form of retinoid receptors acting on target genes, homodimers have also been suggested (*41*). RAR and RXR have two dimerization interfaces, the first being located in the DNA binding domain and the second in the ligand binding domain (*42*), although in vitro studies have shown that receptor dimerization and DNA binding can be ligand-independent processes and ligand-dependent homodimerization can occur for RXR (*43*−*45*). Most studies, in particular in vivo genetic knockout studies (*46*), argue for a heterodimeric receptor pair as the true physiological receptor unit and that ligand induces receptor heterodimerization which facilitates DNA binding of receptors (*41*, *47*). Other studies have shown that, in a heterodimer, RXR was "silenced" by apo-RAR (*48*, *49*) and that this silencing was relieved upon ligand binding to RAR (*50*). However, this was not the case for RIP140. In the present study for RAR/RXR heterodimers, agonist binding to either receptor was sufficient to recruit RIP140 to RAR/RXR dimer (Figure 2). A stronger recruitment of RIP140 was observed when both receptors were bound by agonists. The fact that occupancy of either receptor with its agonist in a heterodimer was sufficient to recruit RIP140 is consistent with other studies that suggested that both RAR and RXR subunits in a heterodimer not only autonomously bound to their cognate ligands but each was capable of recruiting coactivator upon binding by agonists (*51*). To this end, RIP140 behaved very much like a typical coactivator. However, unlike a typical coactivator, our data showed that the interaction of the RXR/RAR heterodimer with RIP140 (Figure 2B) was more prominent when RXR was bound by agonist, and this was not affected (or silenced) by occupancy of RAR with its antagonist. This would suggest that RXR may be the dominant receptor in the heterodimer in terms of recruiting RIP140. Thus, from our study and from others (*51*), it is tempting to speculate that, in the presence of RAR antagonist, RXR agonists can still regulate gene transcription under certain circumstances. The interaction of RIP-f and N′ was enhanced by ligand binding of receptor heterodimer, suggesting that the amino-terminal portion of RIP140 may also have a role in the ligand-enhanced recruitment of RIP140 to the receptors. The ligand-dependent interaction of C′ and R36 with RAR/RXR heterodimers was also seen in mammalian two-hybrid assays (*22*) where we have demonstrated that a C′-deleted RIP140 mutant drastically lost its ligand-enhanced interaction with receptors.

The effect of these ligands on the biological activity of RIP140 was tested in transfection studies. The suppressive activity of RIP140 was attributed to its direct association with HDACs through its amino-terminal domain, and therefore only RIP-f, but not C′ or dissected R36, was tested for the RIP140-mediated suppression of the RA-responsive promoter. The results paralleled the observations made in the GST pull-down assays in terms of the dominant role of RXR ligands. The combination of RXR agonist and RAR antagonist was as efficient as the combination of double agonists in recruiting the suppressive activity of RIP140 to an RA-responsive reporter (Figure 6, compare lanes 5 and 7). With sufficient evidence for a characteristic agonist-dependent interaction mediated by the novel C-terminal motif of RIP140, we then attempted to identify amino acids essential for this interaction. A computer model was generated to predict contact points between this motif and receptor surface. This model predicted two important residues, one at the ^{1073}Met which replaces Leu seen in a typical LXXLL motif and the second at ^{1067}Asn which was predicted to stabilize the contact. Pull-down assays using the motif-containing R36 construct with receptors were conducted in the presence of either wild-type or mutant peptides where ^{1067}Asn/Gly or ^{1073}Met/Lys, Leu, or Ile mutations were introduced (Figure 3). The results showed ^{1073}Met as the most important residue for ligand-dependent interaction of RIP140 with either homodimer or heterodimer because mutation at this residue indeed drastically affected this interaction, as revealed in both peptide competition (Figure 3) and mutation study in the context of the C-terminal domain of RIP140 (Figure 4). As predicted, the M2 mutant (^{1073}Met/Leu) competed well, but not as efficiently as the wt peptide, suggesting that the binding affinity of the wild-type peptide to receptors was perhaps stronger than the typical LXXLL peptide. The wild-type peptide competed well with N′, for binding to the heterodimer (Figure 5A), suggesting that this novel LYYML motif has a higher affinity toward receptors as compared to the typical LXXLL motif. This notion was further confirmed in the assay for biological activity (Figure 5C) where the suppressive activity of RIP140 could be effectively blocked by the expression of C′. Since these mutations disrupted C′ interaction with both RXR and RAR, it is possible that holo-RAR and holo-RXR could contact an overlapping or similar site on RIP140. For a heterodimer, the agonist-bound receptor could target this site, whereas the unliganded or antagonist-bound partner could be recruited by any of the LXXLL motifs. Presumably, one of the nine copies of LXXLL in the N-terminal and central portions of RIP140 would sufficiently accommodate the other partner, which requires little ligand stimulation. This would be similar to a recent study of the RAR/RXR heterodimer interaction

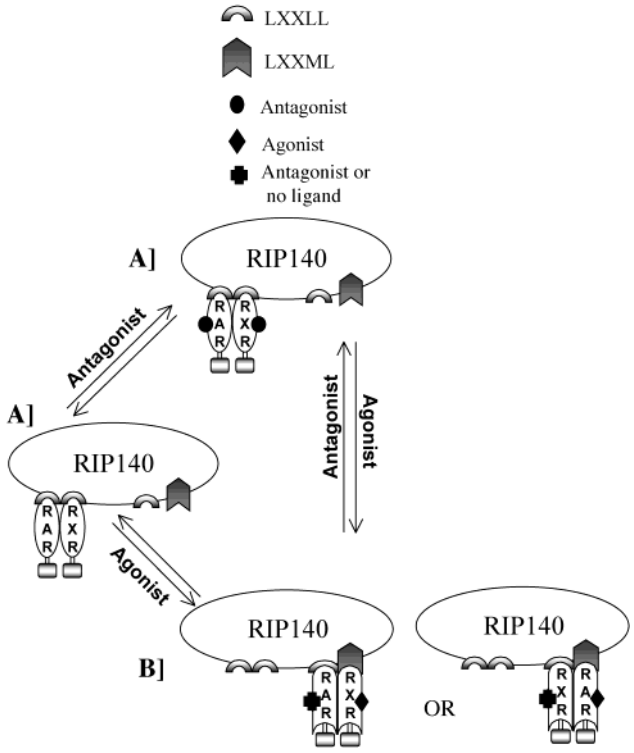

FIGURE 7: Diagrammatic representation of a model proposing the interaction of RIP140 with an agonist- or antagonist-bound RAR/RXR heterodimer. Form A represents the RIP140/apo-RAR/RXR complex and form B represents the RIP140/holo-RAR/RXR complex. While it is possible that receptors can form homodimers, heterodimers are believed to be the predominant forms of receptor units present in a physiological condition. This model is to address the more physiologically relevant condition where heterodimers are believed to be more abundant.

with TIF2, which revealed that either partner was able to interact with the coactivator, and both receptors bound to a single TIF2 molecule at distinct receptor interacting sites on the coactivator (*48*).

The ternary complex of RAR/RXR with either RIP-f or RIP-C′ could be detected on the DR5 oligonucleotide in a modified pull-down assay (*25*) by using ^{35}S-labeled RIP140 protein and ^{32}P-labeled DNA (data not shown). The role of DNA in the agonist-mediated recruitment of coactivator Src-1 or corepressor NCoR to RAR/RXR has also been documented (*33*). The fact that the RIP140/RAR/RXR complex can be tethered to the DNA would suggest that the suppressive activity of RIP140 in receptor-mediated gene activation is likely to be a direct suppression of target gene transcription. The effect of ligand in the interaction of RIP140 suggested a role of AF2 region of the receptor. Crystallographic studies on RAR and RXR ligand binding domains suggested a major repositioning of the "transactivation helix" H12 (that corresponds to the core of AF2) on ligand binding to receptor. With regard to the molecular interaction, we propose a working model as shown in Figure 7. In this model, RIP140 forms a complex with RAR/RXR in the absence of ligand or in the presence of antagonist (form A), mediated by the LXXLL motifs present in the N-terminal and central regions of RIP140. Upon the addition of one or two agonists, one of the receptors (the one bound by agonist) is recruited to the carboxyl-terminal LYYML motif of RIP140 with the other receptor remaining attached to any of the nine LXXLL motifs, forming a tighter ternary complex (form B). Therefore, it is possible that N′ and C′ regions of RIP140 act cooperatively to interact with the RAR/RXR receptor. At this point of our studies it is not possible to differentiate between the nine LXXLL motifs distributed in the N′ and central regions of RIP140. Future studies with targeted mutations in these LXXLL motifs are required to clarify this cooperative feature. Alternatively, a ternary complex could also form with only one holoreceptor binding to RIP140 and the second one retained by receptor heterodimerization.

Crystallographic studies of the RAR/RXR heterodimer with the RIP140 wild-type and mutant peptides would be helpful to unambiguously define the structural interface between these proteins. In terms of the RIP140-interacting surface of receptors, the involvement of ligands clearly suggested an essential role of the AF-2 region of receptors in mediating this interaction. The ligand-induced repositioning of the transactivation helix H12 may play a key role in docking or stabilizing the receptor−RIP140 complex upon the occupancy of ligands.

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