Characterization of Receptor-interacting Protein 140 in Retinoid Receptor Activities

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Receptor-interacting protein 140 (RIP140) contains multiple receptor interaction domains and interacts with retinoic acid receptors in a ligand-dependent manner. Nine LXXLL receptor-interacting motifs are organized into two clusters within this molecule, each differentially interacting with retinoic acid receptor (RAR) and retinoid X receptor (RXR). RAR interacts with the 5′ cluster, whereas RXR interacts with both clusters. Additionally, a third ligand-dependent receptor-interacting domain is assigned to the very C terminus of this molecule, which contains no LXXLL motif. In mammalian cells, receptor heterodimerization is required for efficient interaction of RAR/RXR with RIP140. Furthermore, the heterodimeric, holoreceptor cooperatively interact with RIP140, which requires the activation function 2 domains of both receptors. By using different retinoic acid reporter systems, it is demonstrated that RIP140 strongly suppresses retinoic acid induction of reporter activities, but coactivator SRC-1 enhances it. Furthermore, an intrinsic repressive activity of RIP140 is demonstrated in a GAL4 fusion system. Unlike receptor corepressor, which interacts with antagonist-bound RAR/RXRs, RIP140 does not interact with antagonist-occupied RAR/RXR dimers. These data suggest that RIP140 represents a third coregulator category that is able to suppress the activation of certain agonist-bound hormone receptors.

Nuclear receptors are transcription factors that modulate the promoter activities of target genes by binding to specific hormone response elements. Members of this superfamily have been expanded to include the conventional steroid and nonsteroid hormone receptors and a large number of orphan nuclear receptors (1, 2). In most cases, the functional receptors require dimer formation of receptors. The steroid hormone receptors, such as androgen receptor, estrogen receptor (ER), and orphan receptors TR2, TR4, and chicken or albumin upstream promoter-transcription factors form homodimers, whereas nonsteroid hormone receptors, such as retinoic acid (RAR) thyroid, and vitamin D receptors, and oxysterol and fatty acid receptors (PPARs) heterodimerize with a common partner, retinoid X receptor (RXR) (2, 3). In addition to these common receptor dimerization pathways, heterodimerization can also occur for orphan receptors TR2/TR4 and ERα/ERβ (4, 5).

The modular structure of nuclear receptors can be divided, from the 5′- to the 3′-ends, into the N-terminal region containing activation function 1 (AF-1) domain, the DNA binding domain, the hinge region, and the ligand binding domain (LBD). A ligand-dependent AF-2 domain is located in LBD. Recent studies have revealed that transrepression by apo receptors is mediated by histone deacetyltransferase complexes, which are recruited by corepressors N-CoR/silencing mediator of retinoid and thyroid hormone receptor through interaction with the hinge region of apo receptors (6, 7). Upon ligand binding, a conformational change in the LBD releases corepressors and recruits coactivators by re-positioning helix 12 (AF-2 domain). Several putative coactivators, mainly the p160 family, have been identified, including SRC-1/NCoA-1, TIF2/GRIP1/NCoA-2, and p300 and CBP-interacting protein/receptor-associated coactivator 3/activator for thyroid retinoic acid receptor/amplified in breast cancer-1 (8–14). The SRC-1 and activator for thyroid and retinoic acid receptor, together with integrator CBP/p300, have been shown to encode histone acetylase activities (12, 15, 16). It is suggested that nuclear receptor/coregulator (coactivator or corepressor) complexes alter the chromatin structure by modifying the acetylation status of histone, thereby regulating the expression of target genes.

It has been demonstrated that the LXXLL motifs in SRC-1 and TIF2 are essential for these coactivators to interact with holoreceptors (17, 18). The human receptor-interacting protein (human RIP140), originally cloned as a strong ER-interacting protein, has been proposed as a coactivator based upon the presence of nine LXXLL motifs in this molecule and its ligand-dependent interaction with nuclear hormone receptors (19, 20). However, its role in nuclear receptor-mediated transcriptional regulation has been controversial (21–23). We have recently isolated the mouse homologue of human RIP140 and demonstrated that the mouse RIP140 functions as a coactivator for orphan receptor TR2 in a ligand-independent manner (21). Intriguingly, although RIP140 interacts with RAR in its holo form, it also interacts with apo-TR2. In addition, this ligand-independent interaction of RIP140 with TR2 is also mediated by the putative AF-2 of TR2 and the LXXLL motifs of RIP140.

Retinoids are important physiological molecules that regulate a variety of cellular processes (24, 25). Two major types of retinoid receptors are present, each containing three subtypes. RARα, RARβ, and RARγ bind to both all-trans-RA (at-RA) and 9cis-RA (9c-RA), whereas RXRα, RXRβ, and RXRγ bind only to 9c-RA. For therapeutic applications in cancer management, a number of specific agonists and antagonists have been developed recently, such as the RAR-specific agonist TPNPB and antagonist AGN193109 (26). In this study, we determined the
domain requirements for the ligand-dependent interaction of RIP140 with RAR/RXR and compared this interaction with its ligand-independent interaction with certain orphan receptors as demonstrated in our previous studies (21) in order to gain insights into the role of RIP140 in RA signaling pathways. We report here that the LXXLLS of RIP140 have different affinities for RAR/RXR, and a C-terminal region that contains no LXXLL motif also interacts with RAR/RXR. We further demonstrate that heterodimerization of RAR/RXR is required for their interaction with RIP140, and ligand occupancy of both receptors cooperatively enhances this interaction. By using different reporter systems, it is demonstrated that RIP140 consistently suppresses RA induction of reporter activities, possibly through its intrinsic repressive activity, which is transferable, as demonstrated in a GAL4 fusion system. RIP140 may be categorized as a coregulator that utilizes a novel mechanism to suppress the activation of certain agonist-bound hormone receptors.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Vectors and Reporters**—The DEF domains (the ligand binding domains) of both mouse RARs (primers 5′-GAATTTCATGTCGACGTC-3′ and 5′-CTCGAGTCCTGTTGCGT-3′) and RXR (primers 5′-GGGAATTCAAAAGGGAGGGATGCGT-3′ and 5′-CCCTCAAGGTTGAGAGTTG-3′) were generated in polymerase chain reactions and cloned into the EcoRI and SalI sites of the pGEX-2T vector (Amersham Pharmacia Biotech) for the production of glutathione S-transferase (GST) fusion proteins in *Escherichia coli*. For expression in yeast, the same DNA fragments were inserted into the pBD-GAL4 Cam vector (Stratagene, La Jolla, CA) to generate GAL4BD-RARLD and GAL4BD-RXRLD. The AD fusions of the N-terminal, central, and C-terminal portions of RIP140 in the pAD-GAL4 vector (Stratagene) were as described previously (21).

For the mammalian two-hybrid interaction tests, the LBDs of RAR and RXR and their AF-2 deletion mutants (primers for RARBDΔAF-2: 5′-CTCGAGTCATGCAGACGA-3′ and 5′-CTCGAGTCATGCAGACGA-3′; and for RXRBDΔAF-2: 5′-GGGAATTCAAAAGGGAGGGATGCGT-3′ and 5′-CCCTCAAGGTTGAGAGTTG-3′) were also generated in polymerase chain reactions, were cloned into the pVP16 vectors (CLONTECH, Palo Alto, CA) at EcoRI and SalI sites to generate the VP16 fusions. In the VP16-RARBDΔAF-2 construct, the C-terminal 52 amino acids of RARLBD were deleted. The C-terminal 19 amino acids of RXRLBD were deleted in the VP16-RXRBDΔAF-2 construct. The GAL4BD fusions of all the RIP140 expression vectors constructed in pvector (CLONTECH) were as described previously (21), and the BD-N-CoR containing the C-terminal portion of mouse N-CoR (residues 1843–2453) was cloned into the EcoRI and SalI site of the pVT vector as described (3). The SRC-1 expression vector (under the control of a cytomegalovirus promoter) was kindly provided by Dr. M. Evans (27). The expression of the full-length RIP140, RARs, and RXRs for the transient transfection assays was under the control of a cytomegalovirus promoter in the pDNA3.1 (Invitrogen, Carlsbad, CA) expression vector.

The RIP140 construct for the *in vitro* translation reaction was as described previously (21).

The reporter for the mammalian two-hybrid system (GALA4-kt-luciferase) was as described previously (4, 21). The DR5-kt-luciferase reporter was a gift from Dr. R. M. Evans (28). The IR0-kt-luciferase was constructed by placing six copies of a IR0 element (GTCGGGTCACG-erase) was as described previously (4, 21). A GAL4-tk-luciferase reporter (400 ng), and an SV40-lacZ internal control (25 ng) were cotransfected into COS cells. Luciferase and lacZ activities were determined as described (21).

**RESULTS**

**Identification of a C-terminal Receptor Interaction Domain Lacking LXXLL Motifs**—In our previous study, we identified the mouse homologue of human RIP140 as a co-repressor of orphan receptor TR2, named mRIP140 (21). The mRIP140 interacted strongly with apo-TR2, but its interaction with RAR required the addition of RA in a yeast two-hybrid interaction test (21). In our study, experiments were conducted to define the role of RIP140 in RA signaling pathways and to examine the domain requirement and ligand dependence of its interaction with RAR and RXR. We first performed GST pull-down assays to examine the interaction between RIP140 and RAR/RXR. The LBD of both RAR and RXR was each fused to the C terminus of GST protein (constructs GST-RARLBD and GST-RXRLBD) and expressed in *E. coli*. The purified GST fusion proteins and the control GST alone were then bound to glutathione-conjugated Sepharose beads and incubated with *in vitro* translated, radioactive-labeled RIP140 protein. The ligand, at-RA, 9c-RA, or vehicle was added to the reactions. After extensive washes, the reactions were resolved by SDS-polyacrylamide gel electrophoresis and subsequently detected in a PhosphorImager. As shown in Fig. 1, the RARLBD interacts with full-length RIP140 in the presence of ligand, at-RA, or 9c-RA (lanes 4–6), consistent with the previous results of yeast two-hybrid tests. Furthermore, the RXR ligand 9c-RA, but not the RAR ligand at-RA, potentiates the RXR/RIP140 interaction (lanes 7–9), indicating that RIP140 is a potential transcriptional modulator for both holore-AR and RXR. In the control experiments (Fig. 1, lanes 1–3), GST protein alone cannot interact with RIP140 with or without the addition of RA. The **bottom panel** of Fig. 1 shows the Coomassie Blue staining of the gel, confirming equal amount of protein loading in each lane.

We next utilized the yeast two-hybrid interaction tests to dissect the RAR- and RXR-interacting domains of RIP140. It is known that the receptor-interacting motif, LXXLL, is responsible for the ligand-dependent interaction of coactivator with nuclear receptors. To examine whether the nine LXXLL motifs of RIP140 were responsible for its interaction with RAR/RXR, the coding region of mRIP140 was divided into three parts, each fused to the GAL4 activation domain (GALA4AD) to generate constructs mRIP1/1–495 containing the 5′ LXXLL cluster: mRIP/623–951, containing the 3′ LXXLL cluster, and mRIP/977–1161, containing the very C terminus, which lacked any LXXLL motif (Fig. 2, *top panel*). Yeast cells containing a lacZ reporter with GAL4 binding sites (Fig. 2, *top panel*) were cotransfected with one of these constructs and the GAL4BD
fused to a final concentration of 5 nM. Interactions with BD-RARLBD and BD-RXRLBD in yeast. RA was less region (5–10 KDa) of GAL4 binding site. The fold induction of each RIP140/RAR or RXR pair in the presence of RA is indicated above the bars. The relative luciferase activity unit level was normalized with the lacZ activity. The fold induction of reporter activity was determined by comparing the relative luciferase activity unit with RA to that of without RA.

very N and C termini of RIP140 (mRIP/1–495 and mRIP/977-1161, respectively), addition of ligands dramatically enhances the reporter activities, indicating a stronger interaction as a result of ligand binding to the receptors. Whereas both the 5’ and 3’ LXLLL clusters interacted well with holo-RXR (lanes 7–10), only the 5’ cluster had a high affinity for RAR (lanes 1 and 2). Surprisingly, the C terminus of RIP140 (mRIP/977-1161), a region containing no LXLLL motifs, also interacts strongly with both holo-RAR and RXR (lanes 5, 6, 11, and 12). Similar results were obtained in the mammalian two-hybrid tests, in which the three portions of RIP140 were fused to GAL4BD and RAR and RXR were fused to VP16. However, the interactions were strictly ligand-dependent (data not shown). These data suggest that the LXLLL clusters of RIP140 have different affinities for holo-RAR and RXR. Furthermore, a C-terminal fragment containing no LXLLL motif is also able to interact strongly with holo-RAR and RXR.

RAR/RXR Dimerizations Are Required for an Efficient Interaction with RIP140 in Mammalian Cells—It is believed that most nuclear receptors bind DNA as dimers. For instance, coregulators SRC-1 directly contacts the AF-2 of dimeric receptors through the LXLLL motifs, thereby connecting the receptors to the basal transcription machinery (31). Although the three partial RIP140 fragments interact well with both holo-RAR and RXR in the yeast (Fig. 2), only RXR, and not RAR, interacts strongly with RIP140 in mammalian cells (see below). Because RXR, but not RAR, could form homodimers of its own, it was speculated that the full-length RIP140 interacted with receptors only in their dimeric forms in mammalian cells. To test this possibility and to examine whether AF-2 of RAR and/or RXR was also essential for RAR/RXR interaction with RIP140, the mammalian two-hybrid tests were conducted. In this experiment, the full-length RIP140 was fused to GAL4BD (BD-RIP140, Fig. 3, top panel), and RARLBD, RXRLBD, and their AF-2 deletion mutants (RARLBDΔAF-2 and RXRLBDΔAF-2) were each fused to VP16 protein. COS-1 cells

Fig. 1. Ligand-dependent RIP140 interaction with RA receptors demonstrated in GST pull-down assays. Six μg of purified GST, GST-RARLBD, or GST-RXRLBD protein was bound to glutathione-Sepharose beads and incubated with radioactive labeled, in vitro translated RIP140. at-RA or 9c-RA was added to the reactions at a final concentration of 5 × 10^{-7} M. The reactions were resolved by SDS-polyacrylamide gel electrophoresis and subsequently detected in a PhosphorImager. The positions of RIP140, GST-RARLBD, GST-RXR-LBD, and GST are indicated on the left, and the positions of a protein ladder are indicated on the right. Lane 10 shows a 50% input of the radioactive labeled RIP140 protein. The lower panel shows the Coomassie Blue staining of the gel, indicating equal amounts of protein loading.

Fig. 2. Dissecting the receptor interaction domains of RIP140 using yeast two-hybrid interaction tests. RIP140 was dissected into three parts including the 5’ LXLDs, 3’ LXLDs, and C-terminal LXLDless region (top panel); each was fused to the GALAD and tested for the interactions with BD-RARLBD and BD-RXRLBD in yeast. RA was added to a final concentration of 5 × 10^{-7} M when indicated. The relative interaction strength was determined by the liquid lacZ assays, which measured the activity of a lacZ reporter containing three copies of GAL4 binding site. The fold induction of each RIP140/RAR or RIP140/RXR pair in the presence of RA is indicated above the bars.
were cotransfected with BD-RIP140, different combinations of VP16 fusions (Fig. 3, lower panel), a luciferase reporter containing GAL4 binding sites (GAL4-tk-luciferase), and a lacZ internal control construct. As shown in Fig. 3, ligand-bound RARLBD interacts very weakly with RIP140 (lanes 4–6), which is mediated by the AF-2 of RAR because deletion of AF-2 completely abolishes this interaction (lanes 7–9). In contrast, ligand-bound RXRLBD interacts strongly with the full-length RIP140 (10-fold induction of the reporter activity; Fig. 3, compare lane 10 to lane 12), which is also AF-2-dependent (lanes 13–15). Interestingly, in the presence of both receptors and at-RA, interaction of receptors with RIP140 results in a 6-fold reporter activity (Fig. 3, lane 17), a 3-fold stronger interaction than that obtained using RAR alone (Fig. 3, lane 5). Moreover, when 9c-RA, the ligand for RAR and RXR, was present, a synergistic induction of reporter activity (25-fold) was observed (Fig. 3, compare lane 18 to 17 and lane 12 to 6). These data indicate that dimerizations are required for an optimal interaction between full-length RIP140 and RAR/RXR in mammalian cells, and the AF-2 of the receptors is indispensable for their interaction with RIP140.

Although RXR was believed to be a silent partner in the RAR/RXR dimer, the synergistic induction of reporter activity (Fig. 3, lane 18) prompted us to investigate the possible cooperative binding of RAR and RXR receptor complexes to RIP140. COS-1 cells were cotransfected with either RARLBD/RXRLBDΔAF-2 or RARLBDΔAF-2/RXRLBD pair and tested their interaction with RIP140. As shown in Fig. 3, the holo-RARLBD/apo-RXRLBDΔAF-2/RIP140 interaction results in a reporter activity (Fig. 3, lane 20) similar to that of holo-RARLBD/apo-RXRLBD/RIP140 (lane 17), and the interaction is almost completely abolished in the holo-RARLBDΔAF-2/apo-RXRLBD/RIP140 pair (lane 23). This result indicates that an intact AF-2 of RAR in the holo-RAR/apo-RXR dimer is required for RIP140 interaction. Furthermore, the synergistic activation of reporter activity by 9c-RA (Fig. 3, lane 18) is abolished when either receptor is deleted in its AF-2 (Fig. 3, lanes 21 and 24). Over a wide range of RIP140 concentrations, this synergism is still observed (data not shown). Therefore, the AF-2 domains of both receptors are required for a cooperative interaction of holo-receptors with RIP140. Consistent with these results, the interaction of holo-RARLBD/hoaroRXRLBDΔAF-2/RIP140 (9c-RA as ligand, lane 21) is similar to that of holo-RARLBD/apo-RXRLBDΔAF-2/RIP140 (at-RA as ligand, lane 20), suggesting that once RXR is deleted in its AF-2, the ligand for RXR no longer has an effect on this interaction.

RIP140 Is a Negative Modulator of RA Signaling Pathway—Based upon the presence of LXXLL motifs and its ligand-dependent interaction with hormone receptors, human RIP140 was originally categorized as a coactivator (20). However, our previous studies showed that RA activation of a chimeric protein, GAL4BDRAR, was strongly suppressed by RIP140 in COS-1 cells (21). To confirm this observation and to examine this controversy, we utilized two RA reporter systems in this study. The first reporter contains a naturally derived R5-DR5 containing luciferase gene could be efficiently induced by RA in COS-1 cells (30), two sets of DR5 containing luciferase reporters were used in this experiment, including a heterologous reporter (DR5-tk-luciferase) and a reporter driven by the orphan receptor TR2–11 basal promoter (500 ng), expression vector for RAR, RXR, and RIP140 (50 ng each), and an SV40-lacZ internal control (25 ng). The control expression vectors were supplemented to assure an equal amount of DNA input in each reaction. These data confirm that in the same cellular environment, RA induction is enhanced by coactivator SRC-1 as expected but is abolished when RIP140 is expressed. Furthermore, RIP140 is able to suppress RA induction of two different types of RARE.

Our previous data showed that RIP140 was highly expressed in most of the adult mouse tissues (21). It has also been shown that this protein was moderately expressed in COS-1 cells (20). It is possible that suppression of RA induction by RIP140 in COS-1 cells is mediated by sequestering common components of the coactivator complex. To examine this possibility, the effect of RIP140 on RA induction was determined in the embryonal carcinoma cell line P19, a well characterized cell line model for RA-induced cellular differentiation and apoptosis (24, 25) that expressed very low level of RIP140. Because the DR5 containing RARb basal promoter containing the DR5 element (RARb-luciferase). P19 cells were cotransfected with either one of the reporters, different amounts of RIP140 expression vector, and an SV40-lacZ internal control. Consistent with the results obtained from studies conducted with COS-1 cells, the activity of the DR5-tk-luciferase is strongly induced by the addition of RA (Fig. 5A, lanes 1 and 2), and co-expression of RIP140 also suppresses RA induction in a dose-dependent manner (lanes 3–6). Similar results were obtained in experiments utilizing the RARb-luciferase reporter, as shown in Fig. 5B. These results further suggest that RIP140 is a negative modulator of RA signaling pathways.

Distinct Properties of RIP140 and N-CoR—The GAL4BD fusion system has been widely used to identify the transferable, intrinsic activity of transcription factors and coregulators, such as the co-repressive activity of N-CoR and SMRT (32, 33). Our earlier study showed that when tethered to the promoter by GAL4BD fusion, the full-length RIP140 encoded an active repressive activity (21). To determine the domains of RIP140 that were responsible for this repressive activity, RIP140 protein
was dissected into three portions, the N terminus, containing amino acids residues 1–495 (mRIP1/1–495), the central part, containing residues 336–1006 (mRIP140/336–1006), and the C terminus, containing residues 977–1161 (mRIP/977-1161), and fused to the GAL4BD. Interestingly, in this GAL4 reporter system in COS-1 cells, all three parts of the RIP140 molecule strongly repressed the reporter activity (Fig. 6A, lanes 2–4). This repression was not caused by a sequestering effect because we did not observe any repressive activity in cells transfected with the counterparts fused to the VP16 expression vectors (data not shown).

With regard to the molecular mechanisms of co-repressor interaction, it is known that N-CoR interacts with apo-RAR or thyroid receptor and is released from the receptor as a result of agonist binding (32, 33). Furthermore, transcriptional silencing by antagonist-bound receptor is due to the inability of antagonist to release N-CoR from the receptor (34). In this study, we set up experiments to examine whether RIP140 could also employ a similar mechanism to suppress antagonist-bound receptors. We utilized an RAR-specific antagonist AGN193109 (26) in the GAL4-based mammalian interaction system and examined whether antagonist-occupied receptor could interact with RIP140. An interaction of RIP140 with antagonist bound receptor would suggest a possibility of this mechanism. For a comparison, the C-terminal receptor-interacting domain of N-CoR was fused to GAL4BD (BD-N-CoR), and the interactions of BD-N-CoR/VP16-RARLBD and BD-RIP140/VP16-RARLBD/VP16-RXRLBD were determined in COS-1 cells supplemented with agonist 9c-RA or antagonist AGN193109. As expected, N-CoR interacted strongly with apo-RAR, and addition of agonist, but not antagonist, released it from the receptor (Fig. 6B, lanes 1–3). In contrast, only agonist-bound receptors could interact with RIP140 (lanes 4–6). Furthermore, the addition of at-RA and 9c-RA enhanced the interaction between RIP140 and RAR/RXR in GST pull-down assays (Fig. 6C, lanes 2–4), but AGN193109 failed to potentiate this interaction (lane 5). Therefore, by using two types of interaction tests, it is demonstrated that RIP140 employs a very different mechanism to exert its suppressive activity on hormone receptors. Whereas both RIP140 and N-CoR encode an active, transferable, repressive activity as demonstrated in the GAL4BD fusion system, the mechanisms of their modulatory effects on receptor activities are distinct. N-CoR mediates transcriptional repression by antagonist-occupied receptors, whereas RIP140 interaction results in active suppression of the agonist-bound hormone receptors.

DISCUSSION

In this study, we have utilized molecular approaches to understand the domain requirement for RIP140 interaction with RAR/RXR, the biological effects of this interaction in terms of RA induction of target gene expression, and the mechanisms underlying this biological activity of RIP140. By using two-hybrid interaction tests, it was demonstrated that the 5’ and 3’ LXXLL clusters have differential affinities for receptors. Furthermore, only the 5′ LXD cluster interacts with holoRAR, but both LXXLL clusters interact strongly with holoRXR. Intriguingly, unlike other LXXLL-containing cofactors, RIP140 also utilizes a C-terminal region, which lacks LXXLL, to interact with RAR/RXR. The mammalian two-hybrid interacting test was used to study the interaction between RIP140 and RAR/ RXR dimer in mammalian cells. The LBD of either RAR or RXR interacts with the full-length RIP140 in a ligand-dependent manner as demonstrated in the GST pull-down assays; however, heterodimerizations of RAR/RXR are required for efficient interaction with the full-length RIP140 in mammalian cells. Furthermore, simultaneous binding of ligands to both receptors of the dimer results in a cooperative receptor interaction with RIP140. In transient transfection assays using different reporter systems in different cell lines, RIP140 consistently suppresses RA induction of reporter activities, sug-
gesting that it functions as a negative RA signaling modulator. Consistent with these observations, an active, transferable repressive activity is detected in RIP140 as demonstrated in the GAL4BD fusion system, and the active repressive activity is detectable even only a portion of this molecule is used, such as the N-terminal, central, and C-terminal portions. Unlike corepressor N-CoR, RIP140 does not interact with antagonist bound receptors. Rather, it actively suppressed the activation of agonist-bound receptors.

The receptor-interacting LXXLL motif, also called LXD or NR box, in the coactivators has been shown to interact with several helices of the ligand bound LBD including the AF-2 domain (35, 36). Unlike the well characterized coactivators, such as SRC-1/NCoA-1, TIF-2/GRIP-1, and activator for thyroid and retinoic acid receptor/p300 and CBP-interacting protein, each containing three LXXLs located in the central region of the proteins (37, 38), RIP140 has nine LXXLs scattering over the entire molecule (Fig. 2) (grouped into the 5’ and 3’ clusters). It has been shown that the three LXXLs in the p160/SRC-1 family have differential affinities toward different nuclear receptors and are essential for the interactions (37–39). We and others have also demonstrated that nuclear receptors preferentially interact with different portions of the RIP140 molecule (Refs. 21 and 22 and this study). For instance, in two-hybrid interaction tests in which RIP140 has been truncated into the different clusters, containing five and three LXXLs, re- spectively, RXR, PPAR, and TR2 all can utilize both the 5’ and the 3’ clusters, whereas RAR interacts only with the 5’ cluster. Because these biological tests can be complicated by other factors present inside the cells, it is not possible to compare these interactions directly using this type of assay. It has been proposed, based upon the PPARγ crystal structure study, that the three LXXLs in SRC-1 may mediate the cooperative binding of dimeric receptors to the coactivator (36). Whether the multiple LXXLLs in RIP140 can interact with receptors, also involving AF-2 in either an active or an inactive status. Alternatively, because the C-terminal LXD-less region can only interact with liganded RAR and RXR but not unliganded TR2 and PPAR, this region may account, to a certain extent, for this discrimination. It will be interesting to examine this LXD-less region of RIP140 in the future.

GST pull-down, yeast two-hybrid, and mammalian two-hybrid assays are three commonly used methods to examine protein-protein interactions. The advantage of the first two assays is their low backgrounds, which minimize the interference by other cellular factors often seen in the mammalian cells. This is evident when the interactions of BD-RIP140 and VP16 fusions of RA receptors are compared with that of BD-N-CoR and VP-RAR in COS-1 cells (Fig. 6). The reporter activities resulted from the interaction of RIP140 and liganded RA receptors (25-fold) is much lower than that of N-CoR and RAR (200-fold) in COS-1, whereas similar reporter activities for both interaction pairs are observed in yeast in our previous study (21). This is probably due to the presence of a repressive activity in the BD-RIP140 construct but not in the BD-N-CoR construct, which contains only the receptor-interacting domain. However, these two methods may suffer from the lack of other cellular factors that can be important for the target protein interaction. For instance, in pull-down assays, GST-RAR and GST-RXR can individually interact with the full-length RIP140 in the presence of ligand (Figs. 1 and 6C). However, in the

| Interaction with RIP140 | Receptor | Activity in mammalian cells | Sequence of the AF-2 region | Ref. |
|------------------------|----------|-----------------------------|-----------------------------|------|
| Agonist-dependent      | ERα      | Suppression                 | LYDLLLLMLD                  | 20   |
|                        | RAα      | Suppression                 | MPQLPQLEKLE                 | This study |
|                        | RXRβ     | Suppression                 | IDFILMEKLE                  | This study |
|                        | TRα      | N/A*                        | FPPFLFLEVFE                 | 36   |
|                        | LXRX     | Suppression                 | LPLPILSE1WD                 | 23   |
| Agonist-independent    | PPARγ    | Suppression                 | LHPLDEQELY                 | 22, 23 |
|                        | TR2      | Suppression                 | PHILKMEPAD                  | 21   |
|                        | TR4      | Suppression                 | PYIILKMETAE                 | Unpublished |

* N/A, not applicable.

Footnote 2.

![Fig. 7. A model of physical and functional interactions between RIP140 and holo-RAR/RXR dimer.](image)

A. RAR requires RXR to form complex with RIP140 through its AF-2. However, when both receptors bind to their ligand, a conformational change induces a cooperative RIP140 interaction via the AF-2 of both receptors. B. RIP140 belongs to a third coregulator category that interacts with agonist bound hormone receptor and suppresses the activation of receptors by the agonist.
mammalian cells, strong interaction with RIP140 occurs only when both RAR and RXR are provided (Fig. 3). Similarly, the ligand-independent interaction of N-CoR and RAR is enhanced by adding VP16-RXR-LBD in COS-1, despite the lack of direct interaction between N-CoR and RXR (32, 33). Because RAR requires RXR to form a functional receptor dimer, the phenomenon observed in the mammalian two-hybrid system mimics more closely the in vivo events. Despite the observation that RXR may be a silent partner for some heterodimeric receptors, recent studies suggest that both receptors in the RAR/RXR heterodimer are capable of ligand binding and synergistically activating promoter activity, and this synergism requires intact AF-2 of both receptors (40, 41). Consistent with these findings, our data also show that an intact RAR in RAR/RXR dimer is sufficient to recruit RIP140 by a cooperative interaction; thus, a synergistic activation of reporter activity occurs between the holo-RAR/holo-RXR pair and RIP140. It has been demonstrated that two TIF2 molecules cooperatively bind to a holothylol/receptor-holo-RXR dimer in a gel shift assay (37). A model is proposed wherein RIP140 also binds to holo-RAR/ holo-RXR in a 2:1 ratio through the AF-2 of both receptors (Fig. 7A).

Regardless of the status of ligand occupancy, RIP140 consistently suppresses the reporter activities in transient transfection assays (Table I). In this study, we have utilized different reporter systems in different cell lines to examine the effects of RIP140 in RA signaling pathways. In P19 cells, which express a very low level of RIP140, RA induction, mediated by a DR5 element in the context of either a heterologous or a natural promoter (DR5-tk-luciferase and RAR

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Preparation of the mammalian two-hybrid system mimics more closely the in vivo events. Despite the observation that RXR may be a silent partner for some heterodimeric receptors, recent studies suggest that both receptors in the RAR/RXR heterodimer are capable of ligand binding and synergistically activating promoter activity, and this synergism requires intact AF-2 of both receptors (40, 41). Consistent with these findings, our data also show that an intact RAR in RAR/RXR dimer is sufficient to recruit RIP140 by a cooperative interaction; thus, a synergistic activation of reporter activity occurs between the holo-RAR/holo-RXR pair and RIP140. It has been demonstrated that two TIF2 molecules cooperatively bind to a holothylol/receptor-holo-RXR dimer in a gel shift assay (37). A model is proposed wherein RIP140 also binds to holo-RAR/ holo-RXR in a 2:1 ratio through the AF-2 of both receptors (Fig. 7A).

Regardless of the status of ligand occupancy, RIP140 consistently suppresses the reporter activities in transient transfection assays (Table I). In this study, we have utilized different reporter systems in different cell lines to examine the effects of RIP140 in RA signaling pathways. In P19 cells, which express a very low level of RIP140, RA induction, mediated by a DR5 element in the context of either a heterologous or a natural promoter (DR5-tk-luciferase and RAR