Ligand dependent formation of retinoid receptors, receptor interaction protein 140 (RIP140) and histone deacetylase complex is mediated by a novel receptor interacting motif of RIP 140

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Running Title:

Ligand dependent formation of RAR/RXR, RIP140 and HDAC3 complexes
Summary

Receptor interacting protein 140 (RIP140) interacts with retinoic acid receptor (RAR) and retinoid receptor-X (RXR) in a ligand dependent manner and suppresses RA induction of its target genes. The receptor interacting motif is mapped to a C-terminal peptide sequence (LTKTNPILYYMLQK) of RIP140. The functional role of this motif in mediating the suppressive effects of RIP140 on RA induction is demonstrated in mutation studies. RA induces co-immunoprecipitation of histone deacetylase 3 with RAR/RXR in the presence of wild type RIP140, but not the C-terminal motif-deleted RIP140. A decrease in histone acetylation on the promoter region that carries an RA response element is associated with the expression of wild type RIP140, but not the mutant RIP140, in a dose-dependent manner. These data provide a molecular explanation for RIP140 acting as a novel ligand-dependent, negative modulator of RA regulated gene expression.
Nuclear receptors regulate target gene expression by binding to their cognate DNA response elements in the control region of its target genes and recruiting associate proteins to the transcription machinery (1-3). The associate proteins of nuclear receptors can be co-activators, co-repressors or co-regulators. A number of coactivators, mainly the p160 family, have been identified, including SRC-1/NCoA-1, TIF2/GRIP1/NCoA-2, and p/CIP/RAC3/ACTR/AIB1 (4-12), several of which have been shown to encode intrinsic histone acetyl transferase activities (4, 5, 12). On the contrary, corepressors are found to interact with histone deacetylases. For example, corepressors N-CoR/SMRT recruit histone deacetylases (HDACs) to remove specific acetyl groups from histone proteins of specific gene regulatory regions. As a result, chromatin is packed and gene activity is repressed (13, 14). Upon ligand binding to receptors, the AF-2 domain (helix 12) is repositioned, co-repressors are released and coactivators are recruited to activate target gene expression. One mechanism of gene activation is believed to be mediated by relaxation of chromatin due to the action of acetyl transferase encoded by the coactivator complexes.

It was first suggested that the molecular basis underlying nuclear receptor interaction with co-activators involved a signature motif "LXXLL", where L is a leucine and X is any amino acid, present in many coactivators (15-18). By studying the X-ray crystal structure of a ternary complex formed by PPAR-γ, the ligand and a 88 amino acids peptide of coactivator SRC-1, it was found that a charge clamp was formed on the ligand binding domain of PPAR which made a direct contact with the backbone atoms of the LXXLL helices of SRC-1 (19-21). Later, it was shown that aporeceptor interaction with corepressor involved a CoRNR box (L/I/XXI/VI) (where L is a leucine, I is an isoleucine, V is a valine and X is any amino acid) found in co-repressors such as N-CoR and SMRT. However, in competition experiments, CoRNR peptides were able to block both co-repressor and coactivator interaction with nuclear receptors (22), suggesting similar and probably overlapping receptor-interaction motif present in coactivators and corepressors. Furthermore, by studying mutant receptors and corepressors, it was found that mutations in nuclear receptor residues that directly participated in coactivator binding disrupted their interaction with corepressors (23, 24). It was then suggested that a consensus LXXI/HIXXXI/L sequence of corepressors is an extended helix compared to the LXXLL helix found in coactivators, and both helices were able to interact with nuclear receptors in the same receptor pocket (24).

The human receptor interacting protein 140 (RIP140) was first identified as a co-activator for a chimaeric estrogen receptor (25). However, the mouse RIP140 was characterized in this lab as a potent co-repressor for orphan nuclear receptor TR2 in the absence of putative ligands (26). Later, we demonstrated a strong ligand-dependent interaction of RIP140 with retinoic acid
receptor (RAR) and retinoid receptor X (RXR), mediated by a C-terminal segment of RIP140 (27). Two unique features of RIP140 are: a) In contrast to classical co-activators that interact with ligand bound hormone receptors to activate target gene expression, RIP140 suppressed gene activation by interacting with ligand-bound nuclear receptors in most reported studies (28-31), and b) The ligand-dependent receptor interacting motif of RIP140 does not involve any of its nine copies of LXXLL motif, rather it utilizes its C-terminal domain for holo-RAR/RXR interaction and a N-terminal amino acid (AA) #154-350 for Ah receptor interaction (26, 32).

This study was aimed to first dissect the LXXLL-less motif of RIP140 that interacts with ligand bound RAR/RXR. This motif was determined by using different molecular approaches, including two-hybrid interaction, coimmunoprecipitation and GST-pull down assays. The functional role of this motif in the suppressive effects of RIP140 on RA induction of target gene expression was demonstrated in mutation studies. Finally, association of HDAC3, RIP140 and RAR/RXR was demonstrated to be ligand dependent in coimmunoprecipitation experiments, and histone acetylation decreased on the promoter region carrying an RA response element in the presence of RIP140. These data provided a molecular explanation for RIP140 acting as a novel ligand dependent negative modulator of RA regulated gene expression.
**Experimental Procedures**

**Construction of expression vectors for mammalian two hybrid interaction tests**

RIP140 as well as its deletions was each fused to the Gal4 DNA binding domain of pM vector (Clontech, Palo Alto, CA) for dissecting the interacting domain by either restriction digestion or polymerase chain reaction (PCR) of RIP140 cDNA. The full length RIP (RIP-F), N-terminal domain AA #1-496 (RIP-N), central portion AA #496-1006 (RIP-cent) and C-terminal domain AA #977-1118 (RIP-C) were described previously (26). R-18 was made by Hind III digestion of RIP-C, R-19 was made by Sma I digestion of R-18, and R-20 to R-32 were made by PCR cloning. The ligand binding domain of RAR and RXR was each fused to the pVP16 vector (Clontech, Palo Alto, CA) for two-hybrid test, the reporter (Gal4-luc) and the techniques for culturing COS-1 cells, transfection, and luciferase and lacZ assays were as described (26). Cultures were maintained in DMEM medium containing dextran charcoal-treated serum. All-trans RA (at-RA) and 9-cis RA (9c-RA) was each added at a final concentration of 5 x 10^{-7} M. Each experiment was carried out in triplicate. At least three independent experiments were conducted to obtain the mean and the standard error of the mean.

**Coimmunoprecipitation tests**

COS-1 cells were co-transfected with RIP140 (wild type, mutant L-384 as described later, or an empty vector), RAR\(\alpha\), RXR\(\beta\) and flag-tagged HDAC3 (33). Cells were treated with vehicle or at-RA (1\(\mu\)M) 24 hours following transfection, and harvested 24 hours later for resuspension in lysis buffer (50 mM Tris-Cl [pH 8.0], 150 mM Na Cl, 10% glycerol, 0.5% triton X-100, 1 mM PMSF and a protease inhibitor cocktail). Cells were sonicated twice in 20 second pulses on ice and lysates were clarified by centrifugation at 10,000 xg for 10 minutes. For immunoprecipitation, 150-200 \(\mu\)l of total lysate was incubated with anti-flag antibody (Sigma, St Louis, MO) at 4 C for three hours, followed by the addition of 20 \(\mu\)l of protein G agarose resin (Sigma, St. Louis, MO), continued incubation at 4 C for 1 hours and washing with 0.1% NP-40 in PBS. Beads were resuspended in loading buffer for separation on a 10% SDS-PAGE. Proteins were transferred to PVDF membranes, incubated with anti-RIP140 (Santa Cruz Inc., Santa Cruz, CA), anti-RAR\(\alpha\) or anti-RXR\(\beta\) (Affinity Bioreagents, Golden, CO). After washing, blots were incubated with a secondary antibody against the species from which the primary antibody was derived from, followed by extensive washing, and then detection with ECL (Amersham Pharmacia Biotech, Piscataway, NJ).
In vitro protein interaction test

GST pull-down assay was conducted as described (24). Various RIP140 segments as shown in Fig. 2A were cloned by transferring each corresponding fragment of RIP140 dissected from the pM fusions to a GST vector (26). R-33, R-34, R-35, R-36 and R-37 was each derived from R-18, R-20, R-21, R-28 and R-31, respectively. The full length RAR and RXR was each expressed from T7 promoter and labeled with 35S-Met where indicated using a TNT kit (Promega, Madison, WI). E. coli BL21 transformed with the GST-fusion vectors was induced with 0.1 mM IPTG for 4 hours, and fusion proteins were purified from glutathione-Sepharose columns. The partially purified GST-RIP fusion protein was incubated with 35S-Met labeled RAR or RXR in the presence of unlabeled receptor partner. RA was added at the concentration of 10^{-6}M. Peptide LTKTNPILYYMLQK (RIP140 amino acid #1063 - 1076) of either L- or D-amino acids was synthesized and purified by the microchemical facility of U. Minnesota.

Determination of biological activities of RIP140 and its mutants on RA induction of target genes

The expression vectors for RAR, RXR and full length RIP140 were as described (26). The mutant RIP140 (L-384) deleted only in the receptor interacting motif (AA #1063-1076) was made by ligating AA #1077-1118 fragment to AA #1- 1063 of RIP140. Reporter carrying a direct repeat-5 (DR5)-tk-Luc and tests of RA induction in COS-1 system were as described (26).

Chromatin immunoprecipitation (ChIP) assay

COS-1 cells were transfected with the DR5-tk reporter, RAR, and RXR expression vectors, and either a CMV-driven wild type RIP140 expression vector, the L-384 mutant, or an empty vector. ChIP assay (33) was performed according to the manufacturer's recommendation (Upstate Biotechnology, Lake Placid, NY). Following transfection for 24 hours, cellular histone was cross-linked to DNA by adding formaldehyde to a final concentration of 1%. Precipitated chromatin was incubated with an anti-acetylated Histone 3 antibody (Upstate Biotechnology) overnight at 4C, treated with proteinase K and purified by phenol extraction. For PCR detection of precipitated chromatin DNA, primers for the tk promoter region (130 bp) following the DR5 site are 5'AGCGTCTTTGTCATTGGCG3' and 5'TTAAGCGGGTGCATGCAG3'. Control PCRs to amplify the CMV promoter were conducted by using primers 5'CTGACCGCCCAACGAC3' and 5'GACTAATACGTAGATG3' which allowed CMV promoter region to be amplified in the size of 255 bps.
Results

In vivo ligand dependent RAR/RXR-interaction of RIP140 detected by two hybrid interaction tests

Previously, we have confirmed that RIP140 interaction with RAR and RXR depends upon the presence of ligands and utilizes a small C-terminal segment of RIP140 which lacks a typical LXXLL motif (27). To determine the molecular basis of this ligand-dependent RIP140 interaction with RAR and RXR, we first utilized mammalian two-hybrid interaction tests. As shown in Fig. 1, pM-RIP140 interaction with pVP-RAR or pVP-RXR + RAR is mediated by the C-terminal segment between amino acids AA #977 and #1161, and this interaction is dependent upon the presence of ligand, at-RA for pM-RAR, and at-RA + 9c-RA for pM-RXR + RAR (columns 1-3). The C-terminal segment was further deleted from AA #1118 to #1161, resulting in R-18 which maintained a very similar pattern of interaction (column 4). R-18 was further deleted to retain only AA #977 - 1006 (R-19), #977 - 1033 (R-21) and #977 - 1076 (R-20). Among these deletions, only R-20 was able to interact with RAR/RXR (column 6), indicating that the interacting motif was located between AA #1033 - 1076. To confirm this result, 5’-deletions were made from R-18 to generate R-22 and R-23, each retaining AA #1023 - 1118 and AA #1084 - 1118, respectively. As predicted, R-22 (column 8) but not R-23 was able to interact with RAR/RXR, suggesting that interacting motif resides in the central portion of R-18. This was supported by the positive result of construct R-24 which contained only AA #1023 - 1076 (column 10). Further deletions from the 3’-end to generate R-25 (AA #1023 - 1063) and R-27 (AA #977 - 1063) abolished interaction. However, 5’-deletions of R-24 to generate R-26 (AA #1047 - 1076) and R-28 (AA #1063 - 1076) did not affect the interaction (columns 12 and 14). R-28 was the smallest clone that remained fully functional to interact with RAR/RXR. This was confirmed by the positive results of two constructs containing this region, R-29 and R-30, and the negative results of further deletions, R-31 (AA #1069 - 1076) (column 17) and R-32 (AA #1069 - 1076). The pattern of interaction with pM-RXR + RAR in the presence of 9c-RA alone was very similar to that of at-RA + 9c-RA (data not shown).

Based upon these results, it is concluded that RIP140 interacts with RAR and RXR in a ligand dependent manner, and the interaction with liganded RAR and RXR is mediated by a small C-terminal peptide sequence (LTKTNPIYYMLQK), from AA #1063 to A. A. #1076.

In vitro ligand dependent interaction of RIP140 detected by GST pull down assays

To confirm the ligand-dependent RAR/RXR-interacting motif of RIP140, in vitro interaction tests based upon GST pull down assays were performed. In these tests, RIP140
portions were expressed as GST fusions and RAR/RXR was each expressed in TNT, with either one labeled with $^{35}$S-Met. Fig. 2A shows the maps of the representative clones, Fig. 2B shows the GST pull-down experiments that utilized labeled RAR and Fig. 2C shows the Coomassie blue stained gel separating partially purified RIP fragments (labeled with "*$\cdot$" signs on the right). As shown in Fig. 2B, all the clones that contain AA #1063-1076, i.e., R-33, R-34 and R-36, interacted with RAR/RXR in the presence of at-RA, while clones deleted in this motif, i.e., R-35 and R-37, failed in the test. This result further supports the notion that RIP140 interacts with holo RAR/RXR through the C-terminal AA sequence #1063-1076, and the interaction requires ligand binding to either one molecule of the receptor dimer.

To confirm the specificity of this peptide sequence in mediating RIP140 interaction with RAR, peptide competition was conducted in GST pull-down assays using the smallest RIP140 clone R-36 as shown in Fig 3A. Peptide was added at the concentration range of 2 $\mu$M - 20 $\mu$M and either RAR (top panel) or RXR (bottom panel) was labeled in the receptor input. The specific bands pulled-down by R-36 for either RAR-labeled or RXR-labeled receptor dimer was effectively competed out by the addition of 2 $\mu$M peptide. At the concentration of 20 $\mu$M, this peptide was able to compete with R-36 for more than 90%. To further substantiate the specificity in competition experiment, a peptide with the same sequence of D-amino acids was tested in parallel, as shown in Fig. 3B. The L-peptide successfully competed in this experiment (lane 3), whereas the D-peptide failed to compete even at a concentration as high as 100 $\mu$M (lane 4). Therefore, it is concluded that the C-terminal AA #1063-1076 of RIP140 is a ligand-dependent, specific RAR/RXR interacting motif.

**Functional role of receptor interacting motif of RIP140 in suppression of RA induction**

The biological activity of RIP140 in hormone receptor actions has been controversial. In the RAR/RXR system, we have consistently observed a ligand-dependent interaction of RIP140 with RAR and RXR heterodimers, which resulted in strongly suppressed RA induction of reporter activities (27). To determine if the interaction of RAR/RXR is required for the biological activity of RIP140, represented as suppression of RA-induced reporter activity, a mutant RIP140 (L-384) was constructed that was deleted specifically in the region AA#1063-1076. Transfection experiments were conducted to determine RA induction of reporter activities in the presence of RIP140 or this mutant as shown in Fig. 4. Consistent with our previous observations, RA induced reporter activities for greater than 50 folds (columns 1 and 2). In the presence of wild type RIP140, the basal level reporter activity remained the same in the absence of RA (column 3), but RA-induced reporter activity decreased for more than 20 folds (column 4). Very differently, the mutant, L-384, failed to effectively suppress RA induction (columns 5 and 6). This result confirmed that the suppressive effect of RIP140 on RA reporters was mediated by its
direct interaction with RAR/RXR through the C-terminal AA #1063 - 1076 sequence in a ligand-dependent manner.

**Ligand-dependent complex formation of RAR/RXR, RIP140 and histone deacetylase (HDAC) detected by coimmunoprecipitation**

Previously, we have demonstrated a direct interaction of RIP140 with HDAC3 and that the immunoprecipitates of anti-RIP140 encodes HDAC activity (33). We have therefore hypothesized that RIP140 could function as a novel ligand-dependent co-repressor for nuclear receptor actions by recruiting HDAC to nuclear receptors in a ligand dependent manner. To test this hypothesis, we then examined whether HDAC3 can form immunocomplexes with RIP140 and RAR/RXR in vivo, and if the formation of these complexes is ligand dependent. COS-1 cells were transfected with expression vectors for RIP140 (wild type, mutant L-384 or an empty vector), flag-HDAC3, RAR$\alpha$ and RXR$\beta$. Anti-flag antibody was used to precipitate proteins complexed with HDAC3. As shown in Fig. 5, RAR$\alpha$ (lane 4, panel A), RXR$\beta$ (lane 4, panel B) and RIP140 (lane 4 panel C) were all detected in immunocomplexes in the presence of at-RA. In the absence of RA, these proteins were either absent or detected at a negligible level (lane 6 of each panel). To confirm the specificity of RIP140 action in facilitating the formation of these immunocomplexes, two control experiments were conducted by using either the L-384 mutant RIP140 which was deleted in its C-terminal motif (lane 5) or an empty vector (no RIP140, lane 8). Under either condition, immunocomplex formation was much less efficient as shown in the much reduced level of RAR (panel A), RXR (panel B) and RIP140 (panel C) in the precipitates. To monitor the efficiency of protein expression in these cultures, total lysates were examined on the same blots for the expression of each component as shown on lanes 1-3 and 7. This result strongly supports our hypothesis that RIP140 facilitates immunocomplex formation of HDAC3 with RAR/RXR in the presence of RA and that the C-terminal receptor-interacting motif of RIP140 is required for this activity.

**Deacetylation of RA responsive promoter by RA in the presence of RIP140**

Our demonstrations of HDAC activity in the immunoprecipitates of anti-RIP140 (33) and RA induced complex formation of RIP140, HDAC3 and holo-RAR/RXR (Fig. 5) would predict decreased acetylation of chromatin histones around the promoter region of RAR/RXR target genes in the presence of RIP140 and RA. To test this possibility, we employed ChIP assays by using a classical DR5-tk reporter as a model, which was also used to examine the biological activity of RIP140 on RA regulated target gene expression (Fig. 4). In this assay, acetylated chromatin can be precipitated with anti-acetyl H3 and the precipitated DNA fragments detected by PCR. On the contrary, hypoacetylated chromatin is precipitated less efficiently, therefore, less
DNA is amplified. As shown in the top panel of Fig. 6A, an expected 130 bp fragment can be amplified efficiently from cells transfected with the control vector (lane 1) but not from cells co-transfected with RIP140 (lane 2), indicating a decrease in acetylation on tk promoter region regulated by the DR5 element. The negative controls where a nonspecific rabbit antiserum was used are shown in lanes 3 and 4. Two positive controls of input DNA are shown in lanes 5 and 6. Lane 7 shows a negative control of water, and lane 8 shows a positive control of plasmid DNA.

For an internal control of this assay, the acetylation status of the CMV promoter used in the expression vector was monitored in parallel experiments as shown in the lower panel. Since no DR5 is present in the CMV promoter-driven expression vector, this promoter is highly acetylated, and therefore amplified efficiently, regardless the presence or absence of RIP140 (lanes 1 and 2).

To confirm the specificity of RIP140 effects, ChIP assay was conducted by using various concentrations of RIP140 expression vector and the L-384 mutant as shown in Fig. 6B. Deacetylation of the promoter (top panel) occurs in the presence of wild type RIP140 in a dose dependent manner (lanes 1-4), whereas the promoter remains acetylated at the same level in the presence of mutant RIP140 (lane 5) as that of control (lane 1). Second panel shows input DNA, third panel shows non-specific IgG control, and the bottom panel shows a Western blot of transfected RIP140 expression in these cultures. These results clearly show the specificity of hypoacetylation on the promoter containing the DR5 element as a result of expressing wild type RIP140/RAR/RXR in the presence of RA, but not in the presence of mutant RIP140 deleted in the C-terminal receptor-interacting motif. It is concluded that the co-immunoprecipitated complex of RIP140, holo-RAR/RXR and HDAC3 is correlated with decreased histone acetylation on the promoter driven by the RAR/RXR target element DR5, and that the C-terminal receptor interacting motif of RIP140 is required for this activity.
Discussion

This study demonstrates a novel holo-RAR/RXR interacting motif, LTKTNPIYYMLQK, of RIP140, which diverts from the reported LXXLL box found in coactivators or CoRNR box (L/IXXI/VI) found in corepressors. This motif mediates strong ligand dependent interaction of RIP140 with RAR/RXR as demonstrated in both in vitro and in vivo protein interaction tests. Interaction of RIP140 with RAR/RXR results in suppressed RA induction of target gene expression, and the presence of this motif in RIP140 is essential for this biological activity. RIP140 and RAR/RXR can each be efficiently co-immunoprecipitated with HDAC3 in the presence of RA, but less efficiently in its absence. RAR/RXR can not be co-precipitated efficiently with HDAC3 with the expression of the C-terminal motif-deleted RIP140, or without the expression of RIP140, suggesting a role of this C-terminal motif of RIP140 in enhancing RA-induced molecular interactions among these proteins. Finally, wild type RIP140 expression, but not the mutant RIP140, dose-dependently, renders hypoacetylation of promoter region of RA target gene in the presence of RA, suggesting recruitment of HDAC3 by RIP140/RAR/RXR complex to the RA responsive promoter in a ligand dependent manner. However, it remains to be determined whether the recruitment of HDAC3 to the RA-responsive promoter and the repression of gene expression as a result of RIP140 expression also occur for endogenous gene promoters, and if other HDACs can also be recruited by RIP140/RAR/RXR complexes.

RIP140 is able to interact with numerous nuclear receptors in a ligand dependent manner in the case of hormone receptors, and a ligand-independent manner in the case of orphan receptors. However, the receptor interacting domain of RIP140 vary among different receptor systems. For instance, its interaction with orphan receptor TR2 utilizes various portions of the molecule that contains LXXLL motif (26), whereas its interaction with holo-RAR/RXR utilizes the novel motif present in its C-terminus. While it has been demonstrated that its interaction with other hormone receptors can be mediated by its nine LXXLL motifs, the evidence for this type of interaction is less compelling. In our two-hybrid interaction tests we occasionally detected a very low level of interaction in the absence of ligand; however, this detection system can be complicated by nuclear environment of the cell types used and the activity of reporter. The ligand-dependent enhancement of RIP140 interaction with RAR/RXR through the LXXLL-less motif is significant and represents a novel example of holo-receptor interaction with its coregulator. Structural studies are required to resolve the molecular basis of this interaction.

The biological role of RIP140 has been debated as variable results were presented in different studies. While the initial study suggested a coactivator function of RIP140 in a chimaeric estrogen receptor system (25), many later studies from different labs have...
demonstrated RIP140 as a corepressor or negative coregulator (28-31). We first demonstrated that RIP140 suppressed TR2 target gene expression (26). Later, we found that RIP140 expression also suppressed RA induction of target gene despite its ligand-dependent interaction with RAR/RXR (27). More recently, we demonstrated a direct association of RIP140 with HDAC3 through its N-terminal domain, and the immunoprecipitated complexes pulled out with anti-RIP140 encodes HDAC activity (33). The current study extends from these previous studies and provides strong evidence for a suppressive role of RIP140 in RA mediated gene expression. A molecular explanation for the negative role of RIP140 in RA signaling pathways is presented, i.e., the recruitment of HDAC3 by RIP140/RAR/RXR complex to RA responsive promoters in an RA-dependent manner. This view contradicts the central dogma that ligand induces association of holo nuclear receptors with coactivators that encodes histone acetyl transferase activity. The fact that the novel receptor interacting motif of RIP140 diverts from the classical LXXLL box found in coactivators and the CoRNR box found in corepressors may explain the unique feature of RIP140. It will be interesting to examine how RIP140, as compared to other coactivators, interacts with holo-RAR/RXR. RIP140 represents the first negative cofactor for nuclear receptors that acts in a ligand dependent manner. How exactly this observation can be translated into specific biological event remains to be explored. It is noted that a number of studies have reported negative regulation of gene expression by a direct effect of RA on certain RA response elements found in the parathyroid hormone-related protein gene, the thyrotropin-beta gene, and the mouse Oct-3/4 gene (34-36), etc. It is tempting to speculate a role of RIP140 on specific gene suppression mediated by the direct action of RA-bound RAR/RXR in certain cell types or under specific conditions. It would be interesting to examine the difference in receptor conformation when complexed with a typical corepressor, coactivator or a novel coregulator like RIP140.
Acknowledgments

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References


Fig. 1. Mammalian two-hybrid interaction tests to dissect the receptor interacting motif of RIP140. A) RIP140 constructs generated in the pM vector. Numbers denote AA positions of RIP140. Restriction enzymes used are R: *Eco RI*, S: *Sma I*, H: *Hin dIII*. Results of the interactions tests are shown at the right hand column. B) Data of two-hybrid interaction tests from three independent experiments.

Fig. 2. GST pull-down assay to confirm RIP140 interaction with RAR and RXR in vitro. A) RIP140 constructs generated as GST fusions. Results from the pull down assays are shown at the right hand column. B) GST pull-down assay by using labeled RAR and unlabeled RXR. C) A protein gel demonstrating the presence of GST fusion protein in each construct, as marked with "*" signs.

Fig. 3. Peptide competition experiment to demonstrate specific interaction of the dissected receptor interacting motif carried on the smallest clone, R36, in GST pull-down assays. A) A dose-dependent competition of GST pull-down assay of R-36 interaction with RAR (top panel) and RXR (bottom panel) by the L-peptide. B) Competition with L-peptide (lane 3), but not D-peptide (lane 4). GST pull-down assay of R-36 interaction with RAR (top panel) and RXR (bottom panel) was conducted using 100 µM of peptide in the reactions.

Fig. 4. Demonstration of biological activity of RIP140 and its mutants. RA induction of the DR5-tk-luc was examined in the presence of a control expression vector (Cont, lanes 1 and 2), a wild type RIP140 (RIP-F, lanes 3 and 4) or a mutant RIP that is deleted in the receptor interacting motif (L-384, lanes 5 and 6).

Fig. 5. Co-immunoprecipitation experiments to demonstrate RA-dependent complex formation of HDAC3 with RIP140 and RAR/RXR. COS-1 cells were co-transfected with flag-HDAC3, RIP140 (wild type, lanes 1, 3, 4 and 6; mutant, lanes 2 and 5; or empty vector, lanes 7 and 8), RARα and RXRβ, in the presence (lanes 1, 2, 4, 5, 7 and 8) or absence (lanes 3 and 6) of RA. Total lysate was each monitored on Western blot (lanes 1-3 and 7) for the expression of these component. The lysate was precipitated with anti-flag and the immunocomplexes were resolved on PAGE, detected with anti-RARα (panel A), anti-RXRβ (panel B), and anti-RIP140 (panel C).

Fig. 6. ChIP assay to demonstrate specific changes in histone acetylation status on the tk promoter as a result of expressing RIP140 and RAR/RXR in the presence of RA. A) Deacetylation of tk promoter containing DR5 (top panel, lane 2), but not a non specific promoter CMV (bottom panel, lane 2) in the presence of RIP140. Positive controls are the input (lanes 5 and 6) and plasmid DNA (lane 8). Negative control reactions with a nonspecific rabbit antiserum are shown on lanes 3 and 4. Lane 7 shows a negative control of water. In the immunoprecipitated chromatin, histone acetylation decreases as a result of
expressing RIP140 (lane 2) as compared to expressing a control vector (lane 1). The top panel shows specific changes on tk promoter and the bottom panel shows the results of internal control, CMV promoter, where acetylation status remains the same regardless the presence of absence of RIP140. B) Specificity of RIP140-triggered deacetylation of tk promoter. ChIP was conducted as described in panel A, in the presence of indicated amounts of RIP140 (lanes 1-4), or the L-384 RIP140 mutant (lane 5). Top panel shows acetylation is gradually reduced as a result of expressing more wild type RIP140, second panel shows the input plasmid control, third panel shows nonspecific IgG control, and the bottom panel shows a Western blot of RIP140 expressed in these cultures.
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Ligand dependent formation of retinoid receptors, receptor interaction protein 140 (RIP140) and histone deacetylase complex is mediated by a novel receptor interacting motif of RIP 140

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