A Regulatory Role for RIP140 in Nuclear Receptor Activation

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Transcriptional regulation of gene expression by nuclear receptors requires negatively and positively acting cofactors. Recent models for receptor activation propose that certain receptors in the absence of ligands can recruit corepressors while ligand binding results in conformational changes leading to the recruitment of coactivators. Previous work has established a coactivator role for the SRC-1 family members as well as an involvement of the coactivators CBP/p300 in nuclear receptor signaling. However, in addition to coactivators, ligand-activated nuclear receptors bind a number of different proteins that possibly serve other functions. Using peroxisome proliferator-activated receptor-α (PPARα) as bait in a yeast two-hybrid screening, we have isolated nuclear factor RIP140 whose function in receptor activation is unclear. We now report a detailed characterization of RIP140 action with a focus on the retinoid X receptor (RXR) heterodimeric receptors PPAR and thyroid hormone receptor (TR). We show that putative PPAR ligands enhance the interaction of RIP140 with the rat PPAR subtypes α and γ in solution but not with PPAR/RXR heterodimers on DNA. However, RIP140 forms ternary complexes in the presence of RXR ligands. Similar experiments with TR support the high affinity of RIP140 to the RXR subunit and also suggest that either partner in the TR/RXR heterodimer can independently respond to ligand. Coactivation experiments in yeast and mammalian cells confirm the coactivator role for SRC-1, but not for RIP140. We provide important evidence that the *in vitro* binding of RIP140 and SRC-1 to nuclear receptors is competitive. Since RIP140 generally down-regulates receptor activity in mammalian cells and specifically down-regulates coactivation mediated by SRC-1, we propose a model in which RIP140 indirectly regulates nuclear receptor AF-2 activity by competition for coactivators such as SRC-1. (Molecular Endocrinology 12: 864–881, 1998)

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INTRODUCTION

Transcriptional regulation mediated by members of the nuclear hormone and orphan receptor superfamily is a physiological process essential for development and maintenance of normal cellular functions. Receptors for nonsteroid ligands such as thyroid hormone (TR), retinoids [retinoic acid (RAR) and retinoid X (RXR)], vitamin D_3 (VDR), eicosanoids, and fatty acids [peroxisome proliferator-activated receptor (PPAR)], and many orphan receptors, for which ligands have not yet been identified, bind mainly as heterodimers with RXRs to direct repeat response elements in their target genes (1). The ability of RXR to bind and respond to its ligand 9-*cis*-retinoic acid (9-*cis*-RA) is differentially influenced by its heterodimerization partner. While permissive heterodimers (*i.e.* PPAR/RXR, LXR/RXR) allow signaling by RXR (2–6), nonpermissive heterodimers (*i.e.* TR/RXR, RAR/RXR) seem to inhibit RXR signaling (7–9).

Within the nuclear receptor superfamily, PPARs have acquired unique ligand-binding properties since they can bind to a broad range of structurally diverse compounds including peroxisome proliferators (*i.e.* clofibrates, WY-14, 643), antidiabetic thiazolidinediones (*i.e.* BRL49653), eicosanoid metabolites (*i.e.* prostaglandin PGJ_2, leukotriene LTB_4), and naturally occurring fatty acids (*i.e.* linoleic acid, oleic acid) (10–15). In addition, since the PPAR/RXR heterodimer is permissive also to RXR signaling, PPARs may be able to integrate diverse ligand signals and to participate in the control of such different biological events as lipid homeostasis, adipocyte differentiation, inflammation, and carcinogenesis (16, 17). PPARs comprise a subfamily with three different subtypes: PPARα and γ exert major functions in tissues important for fatty acid metabolism and lipid homeostasis such as liver or adipose tissue, whereas the function of the ubiquitously expressed PPAR β/δ is unclear.

Most nuclear receptors, including PPARs, share a typical domain structure: almost identical Zn-finger-type DNA-binding domains flanked by nonconserved N-terminal regions usually exhibiting a constitutive transcriptional activation function (AF-1) and con-

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served C termini possessing a multifunctional ligand-binding domain (LBD) necessary for ligand binding, heterodimerization, and ligand-dependent transcriptional activation (AF-2). Central to AF-2 function appears to be a highly conserved amphipathic α-helix located at the C-terminal end of the LBD (E region), termed AF-2 AD, τc, or Tau-4 (18–21). The contribution of both AF-1 and AF-2 activation domains to the transcriptional activity of the entire receptor varies extremely between receptors. In addition, many transcriptionally active nuclear receptors do not possess a potent AF-1 function. Further, the nonconservation of the N-terminal regions between different receptor subtypes (compare, for example, PPARα, β, and γ) argues against a conserved mechanism for AF-1 function. For these reasons, the recruitment of AF-2 coactivators is likely to represent a critical and conserved step in ligand-dependent transcriptional activation by nuclear receptors.

Although the AF-2 of nuclear receptors has been reported to interact directly with components of the basal transcriptional machinery, further experimental evidence has argued strongly for the existence of additional proteins acting as corepressors or coactivators (1, 22, 23). Receptors such as TR, RAR, or the orphan receptors RevErb and COUP, known to act as potent repressors in the absence of ligand, have been demonstrated to recruit the corepressors N-CoR/RIP13 and SMRT/TRAC (9, 24–30). Further, recent data suggest an additional involvement of corepressors in the regulation of antagonist-bound steroid receptors (31). Upon ligand binding, nuclear receptors undergo substantial conformational changes in their LBD, including a rearrangement of the activation domain helix that leads to the dissociation of corepressors and allows the association of coactivators as well as cofactors serving different functions. Protein-protein interaction screenings have provided several candidate proteins acting as cofactors for ligand-activated receptors (22, 23). Among these proteins, only the promiscuous coactivators, CBP/p300 (32–34) and members of the SRC-1 family of cofactors (SRC-1/N-CoA1, TIF-2/GRIP1/N-CoA2, p/CIP/ACTR), have been convincingly demonstrated to act as coactivators for many nuclear receptors (33, 35–41). The recent discovery of intrinsic histone acetyltransferase (HAT) activity in these coactivators functionally links nuclear receptor activation to histone acetylation and chromatin derepression (42–44).

In addition to SRC-1 coactivators (p160), biochemical studies using ligand-bound ER, RAR, and PPAR revealed the existence of a second group of predominant AF-2 cofactors with a molecular mass of approximately 140 kDa (9, 45, 46). As a major component of p140, nuclear factor RIP140 was originally identified in breast cancer cell lines and subsequently isolated by expression cloning using the ER AF-2 in the presence of estradiol (46, 47). Its ubiquitous expression and its ability to interact with the AF-2 of various nuclear receptors (48, 49) suggested that RIP140 might represent a common nuclear receptor cofactor. However, the coactivation effect of RIP140 on nuclear receptors in transient transfections was minimal and increasing amounts of RIP140 resulted in repression or down-regulation of receptor activity (32, 47). Also, microinjection experiments suggest that RIP140 cannot functionally substitute for coactivators of the SRC-1 family *in vivo* (50). These findings indicate alternative functions of RIP140 in nuclear receptor signaling.

To address this issue, we now report the characterization of RIP140 action with a focus on PPAR for several reasons. First, we identified RIP140 multiple times in a yeast two-hybrid screen for liver proteins interacting with the AF-2 domain of the rat PPARα. Second, recent reports support the coactivator function of SRC-1 for PPAR (4, 41). Although biochemical studies reveal predominant binding of p140 (RIP140) to PPAR in solution and as RXR heterodimer under certain conditions (4), the relevance of these findings has not yet been specifically addressed. Third, previous studies on RIP140 have mainly focused on the estrogen receptor (47, 48, 51). Therefore, analyzing RIP140 function in a different receptor context is of a certain interest, considering the structural and regulatory differences between steroid hormone receptor homodimers and RXR heterodimers with regard to ligand action and cofactor binding. In addition to PPAR and RIP140, in our study we included TR, a RXR heterodimer partner characteristic for nonpermissive heterodimers, and SRC-1, an AF-2 cofactor with established coactivator functions. Our results indicate that RIP140 has properties of a negative coregulator of ligand-activated nuclear receptor complexes that antagonizes coactivation mediated by SRC-1.

RESULTS

Cloning of Nuclear Factor RIP140 by Its Interaction with GAL-PPARα in the Yeast Two-Hybrid System

We used a GAL4-based yeast two-hybrid system (52) to identify proteins interacting with the rat PPARα. We first constructed several GAL4 DNA-binding domain [GAL4, amino acids (aa) 1–147] fusion proteins containing either the wild-type PPARα aa 1–468 (WT), aa 83–468 (ΔN), or aa 166–468 (LBD) and tested them for transcriptional activity in the yeast screening strain HF7c. Since only the GAL-PPARα LBD did not cause any background growth on medium lacking histidine, this bait was used to screen an activation domain-tagged human liver-specific cDNA library. A summary of the identified clones encoding putative PPAR-interacting proteins (PIPs) is given in Table 1. One clone (PIP32) was isolated multiple times and repetitively in independent screenings. The cDNA insert of PIP32 consists of 2492 bp encoding a single open reading frame of 728 aa. A database search revealed that PIP32 is identical to nuclear factor RIP140 (aa 431-

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1158) (47). In addition to RIP140, we isolated clones encoding two other known nuclear receptor cofactors, namely the corepressor N-CoR (26) and the putative androgen receptor coactivator ARA70 (53), as well as partial clones encoding two unknown proteins (hPIP13, hPIP67), the characterization of which is not the subject of this study. Furthermore, in an independent screening of a rat liver library, we isolated two different clones, both of which encode the LBD of RXRβ. Whereas all PIPs interacted well with PPARα in terms of *HIS3*-mediated growth induction, only human (h)PIP32 (RIP140), hPIP13, hPIP67, and rat (r)RXRβ, but not hPIP78 (N-CoR) or hPIP82 (ARA70), activated the *lacZ* reporter efficiently (Table 1), suggesting different affinities of these interacting proteins to the receptor.

Ligand-Independent Interaction of RIP140 with PPAR in the Yeast Two-Hybrid Sytem

RIP140 has been reported previously to associate *in vitro* directly with nuclear receptors in the presence of ligands (47–49). Unexpectedly, in the yeast two-hybrid system, the RIP140 C terminus (PIP32) interacted strongly with PPARα in the absence of any added activator or ligand. To explore the interaction of PPARα with the wild-type RIP140 *in vivo*, we cloned the full-length cDNA encompassing aa 1–1158 into a GAL4-activation domain (GAD)-plasmid creating GAD-RIP140 and tested for interaction in a yeast two-hybrid mating assay. First, all mated two-hybrid strains were analyzed on selective plates lacking histidine for growth. Second, to assess the interaction efficacy quantitatively, the strains were grown in nonselective media and analyzed for β-galactosidase activity in a liquid assay. As Fig. 1A shows, RIP140 interacts strongly and in a ligand-independent manner with GAL-PPARα in yeast. The presence of potent PPARα activators, such as the peroxisome proliferator WY-14,643, does not further enhance the interaction; neither do added fatty acids such as oleic acid or linoleic acid. Similarly, although the PPARγ subtype interacts strongly with RIP140, the potent synthetic

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Table 1. Human Liver cDNA Clones Isolated in a Yeast Two-Hybrid Screening for PPAR-Interacting Proteins (PIPs)

Clone	Isolates[a]	ORF[b]	Identity	Similarity	Colony Color[c]
PIP32	3	728	hRIP140[d]		Blue
PIP78	2	764		mN-CoR[d] (>90%)	White
PIP82	1	433	hARA70/RFG[d]		White
PIP67	1	439			Blue
PIP13	1	44			Blue

[a] Number of clones isolated in a screening of $>10^6$ transformants.
[b] Amino acids in frame with the GAL4 activation domain (GAD).
[c] X-gal filter assay (2 h) yeast screening strain HF7c.
[d] Accession numbers: RIP140 (X84373), ARA70/RFG (L49399/X77548), N-CoR (U35312).

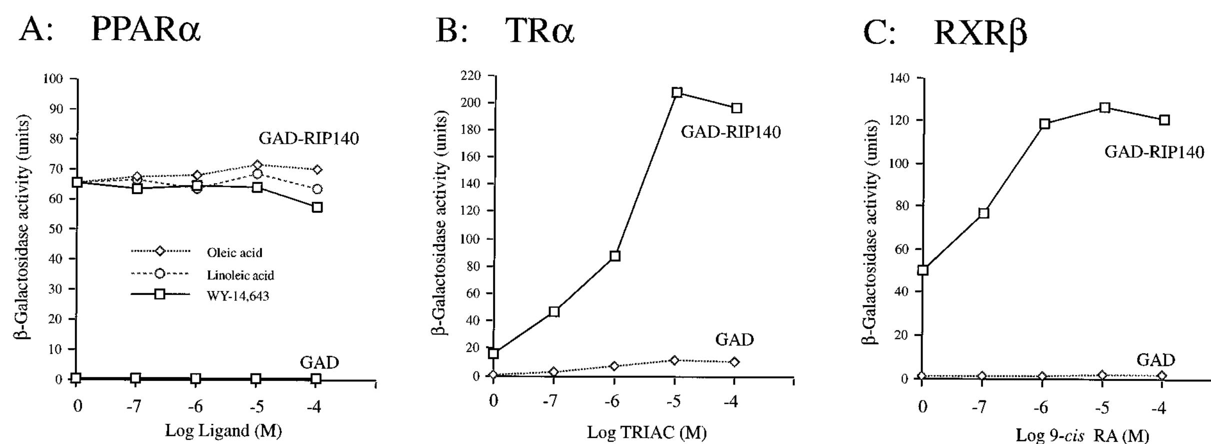

Fig. 1. Ligand-Independent Yeast Two-Hybrid Interaction of PPARα with RIP140

Yeast strains HF7c (*MATa*) expressing GAL4 fusion proteins to PPARα (aa 166–468), TRα (aa 122–410), or RXRβ (aa 153–451) and Y187 (*MATα*) expressing the GAD fusion to RIP140 WT (aa 1–1158) were selected after mating for the presence of both two-hybrid plasmids. Diploids were grown in liquid culture with the indicated amounts of ligands. For TRα and RXRβ, ligands were TRIAC and 9-*cis*-RA, respectively. The GAD control monitors the transcriptional activity of the GAL4 fusion proteins (background). Relative β-galactosidase activities were determined from each cell-free extract and expressed in relative units.

ligand BRL 49653 did not enhance the two-hybrid interaction (data not shown). Ligands clearly enhanced the interaction of RIP140 with thyroid hormone receptor (TR) or RXR (Fig. 1, B and C), in agreement with previous *in vitro* studies on those receptors (48, 49). Note that RXR still shows substantial ligand-independent interaction with RIP140 in the two-hybrid system. The ligand-independent two-hybrid interaction of PPARα is not restricted to RIP140, since we and others (41) observed similar strong ligand-independent interactions with SRC-1 and TIF-2 (data not shown).

Ligand-Enhanced Interaction of RIP140 and SRC-1 with PPAR *in Vitro*

To provide biochemical evidence for the ligand-dependent binding of RIP140 to PPAR, we prepared fusion proteins of the rat PPAR subtypes α and γ with glutathione *S*-transferase (GST) and tested their ability to bind *in vitro* translated [^{35}S]methionine-labeled RIP140 in pull-down assays. As shown in Fig. 2, RIP140 binds weakly in the absence of any ligand to both subtypes (Fig. 2, A and B, lane 2). However, addition of the putative PPARα ligand WY-14,643 further stimulated the interaction in a dose-dependent manner (Fig. 2A, lanes 3 and 4). In contrast, ETYA, a synthetic arachidonic acid analog and ligand for the *Xenopus* PPARα subtype (13), did not affect the interaction of the rat subtype with RIP140 (Fig. 2A, lanes 5–7). Next, using the PPARγ subtype, we demonstrated clearly that increasing concentrations of BRL49653, a synthetic thiazolidinedione ligand (14), enhanced the binding of RIP140 (Fig. 2B, lanes 2–6), whereas the putative natural ligand prostaglandin 15-deoxy-$\Delta^{12,14}$ PGJ_2 (11, 15) failed to affect the interaction under our assay conditions. The binding of RIP140 was specific to PPARα/γ, because GST alone or GST-TFIIB did not retain any labeled protein under our assay conditions (Fig. 2A, lanes 8 and 9). For comparison, we also analyzed the binding of RIP140 to TR and RXR (Fig. 2C). Apparently, both receptors already enhance the RIP140 interaction at lower ligand concentrations (100 nM, lanes 3 and 7). However, quantitative analysis reveals that the maximal amount of retained RIP140 at 100 μM ligand concentration is comparable between PPAR (17.4% of the input, Fig. 2B lane 6), TR (17.2% of the input, Fig. 2C, lane 5) and RXR (14% of the input, Fig. 2C, lane 9). The slightly different ligand enhancement (PPAR, 5-fold; TR, 6-fold; RXR, 8.7-fold) might be due to minor differences in the ligand-independent interaction. Finally, we were interested in

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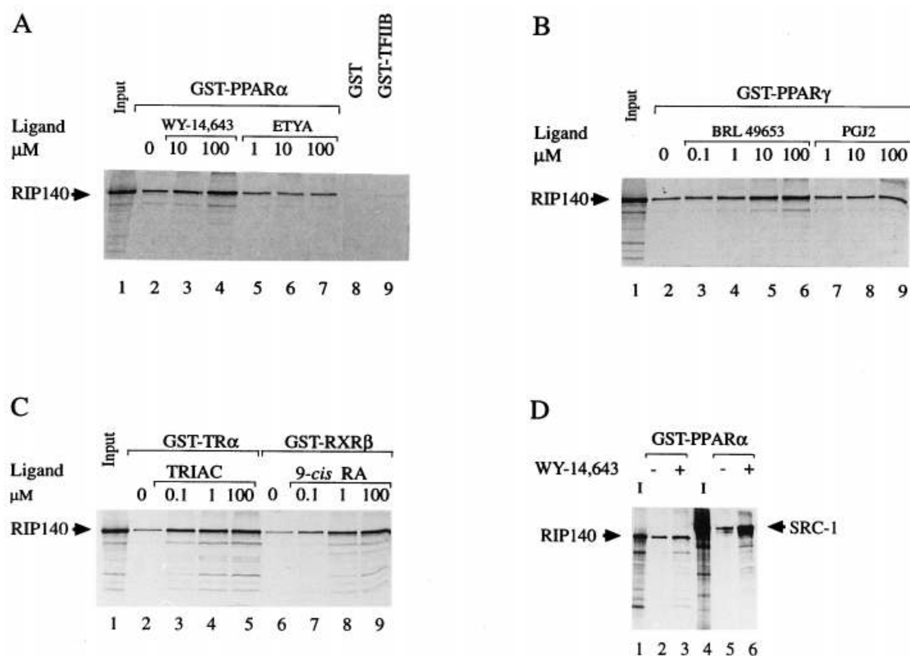


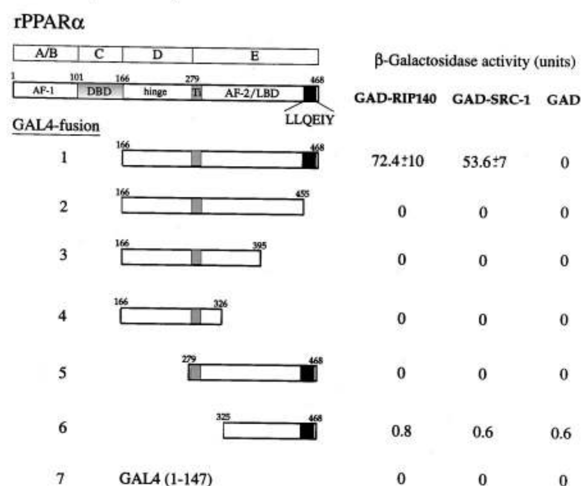
Fig. 2. Ligand-Enhanced *in Vitro* Interaction of RIP140 with PPARα and γ

RIP140 (aa 431-1158) or SRC-1 (aa 1–1061) were synthesized in the presence of [^{35}S]methionine by *in vitro* translation and analyzed with the indicated GST-fusion proteins bound to glutathione-Sepharose beads in a pull-down assay. The input always represents 20% of the amount of labeled protein used in the pull-down assay. A and B, The LBDs of PPARα (aa 166–468) or PPARγ (aa 175–475) were fused to GST and analyzed for RIP140 binding in the absence (lanes 2, DMSO) or presence of the indicated ligands. PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2. GST alone or GST-TFIIB (aa 1–316) do not bind RIP140. C, RIP140 interaction with GST-TRα (aa 122–410) or GST-RXRβ (aa 153–451) in the absence or presence of their respective ligands, TRIAC and 9-*cis*-RA. D, PPARα interacts ligand-dependently with RIP140 or SRC-1. Both cofactors were synthesized and analyzed separately for PPARα binding in the absence (DMSO) or presence of WY-14,643 (100 μM). The approximate sizes of RIP140 and SRC-1 are 80 and 114 kDa, respectively.

comparing the interaction of PPARα with RIP140 to that with the nuclear receptor coactivator SRC-1 (35). We *in vitro*-translated RIP140 and SRC-1 separately and performed the pull-down experiment with GST-PPARα in the absence or presence of ligand (Fig. 2D). Clearly, the WY-14,643 compound enhances the interaction of both cofactors with PPARα (RIP140, 3.4-fold; SRC-1, 7.7-fold). It is important to note that under different pull-down conditions using less GST fusion protein (100 ng instead of 1 μg), RIP140 no longer interacts with the unliganded PPARγ LBD, resulting in stronger ligand dependency of the interaction (see below, Fig. 9A).

RIP140 and SRC-1 Require the Entire PPARα LBD for Interaction

The two-hybrid clone PIP32 (RIP140 aa 431-1158) was isolated with GAL-PPARα fusion proteins containing regions D/E (LBD, aa 166–468; Fig. 3A). Neither RIP140 nor SRC-1 interact with N-terminal parts of the receptor (AF-1, DNA binding domain, data not shown). To further delineate the minimal region of the LBD required for interaction with both cofactors, we created a series of N- and C-terminal deletions of the rat PPARα LBD and analyzed them in the yeast two-hybrid system. As summarized in Fig. 3A, only the intact LBD was able to interact with either cofactor. Already a small C-terminal deletion of 13 amino acid residues encompassing the AF-2 AD core motif abolished the interaction. Surprisingly, similar results were obtained by successive deletions from the N-terminal end of the LBD, indicating that regions located outside the conserved C-terminal AF-2 core are indispensible for interaction. Note that the weak activity seen with GAL-PPARα (aa 325–468) is independent of the presence of RIP140 or SRC-1. This might indicate the derepression of the weak transcriptional activation function (AF-2 AD core) caused by deletion of N-terminal LBD regions. The results from the liquid β-galactosidase assay were confirmed using the very sensitive growth assay. Only GAL-PPARα (aa 166–468; Fig. 3A, fusion 1) interacted with GAD-RIP140 or GAD-SRC-1 and allowed growth on -HIS medium (data not shown). The nonfunctionality of the shorter PPARα fragments is not due to protein instability or major expression differences in yeast, since all GAL-PPARα fusions were detected at similar levels in Western blots (Fig. 3B).



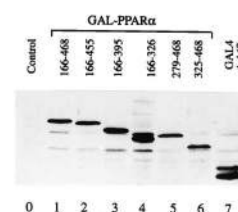


Fig. 3. The Interaction of RIP140 or SRC-1 with PPARα Requires the Integrity of the LBD

A, Schematic illustration of the functional domains of rPPARα. On *top*, the general nomenclature for the different domains A–E of nuclear receptors is given. *Numbers* indicate the aa position defining the borders of the individual domains: DBD (DNA-binding domain), LBD, AF-1/-2 (N- or C-terminal activation function), Ti (conserved part defining the N terminus of the LBD). The yeast strains HF7c (*MATa*) expressing different PPAR fragments (numbers 1–6) fused to GAL4 (aa 1–147) and Y187 (*MAT*α) expressing wild-type RIP140 (aa 1–1158) or wild-type SRC-1 (aa 1–1061) fused to GAD, or GAD alone were mated, diploids were grown in liquid culture in the presence of histidine, and the β-galactosidase activity was determined. "0" indicates no activity above background (yeast extracts); these matings did not induce the *HIS3* reporter when selected for growth in the absence of histidine. B, Western analysis for expression of the GAL-PPARα fusion proteins. A monoclonal antibody directed against GAL4 (aa 1–147) detects specifically the GAL4-PPARα fusion proteins in whole-cell extracts prepared from yeast expressing the two-hybrid combinations with GAD-RIP140 used for the β-galactosidase activity assay. The antibody did not detect any GAL4 protein from yeast extracts alone (control).

PPARα AF-2 Mutations Affect the Interaction with RIP140 and SRC-1

Most ligand-activated nuclear receptors contain a conserved short sequence motif ΦΦXEΦΦ (Φ stands for large hydrophobic aa) located at the very C-terminal end of the AF-2/LBD, referred to as AF-2 AD, AF-2 core, τ4, or τc (18–21). This motif seems to posses key regulatory functions for several reasons. First, it constitutes an autonomous (although weak) activation domain and is likely to be a target for transcriptional cofactors. Second, mutations of conserved hydrophobic residues as well as the conserved glutamic acid residue affect both the transcriptional activity as well as the interaction with cofactors. Third, from the crystal structures of the retinoid acid and thyroid hormone receptors, we learned that the AF-2 core forms a part of an α-helix and undergoes a ligand-dependent structural rearrangement (54).

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Based on analogy to mutations in the AF-2 core described for several other nuclear receptors, we substituted aa L459/L460 (mutation M3) and E462 (mutation M2) of the rat PPARα with alanine (Fig. 4A). In addition, to create a mutation outside the conserved AF-2 core region, we substituted L434/V438 (mutation M1) with alanine. Both residues are part of the putative heterodimerization helix and are believed to be important for the structural stability of the LBD. To analyze the interaction of RIP140 and SRC-1 with the PPARα mutations, we constructed the corresponding GAL-PPAR LBD fusions and tested them in the two-hybrid system. From the data presented in Fig. 4B, we conclude that hydrophobic aa of the AF-2 core are the most important determinants for the interaction with both RIP140 and SRC-1. We observed different effects of the E462→A mutation (M2): whereas RIP140 still interacts to 30% compared with the WT LBD, the mutation completely abolished the interaction with SRC-1. Surprisingly, the mutation M1 uncovered further differences between the two cofactors: the interaction with RIP140 is only slightly decreased (consider the apparently lower expression of M1, Fig. 4C), whereas the SRC-1 interaction is definitely affected. These data imply overlapping, but nonidentical, interaction surfaces of RIP140 and SRC-1 on PPARα. Alternatively, RIP140 and SRC-1 might bind with different affinities to similar parts of the LBD.

Multiple NR Box-Containing RIP140 Domains Interact with PPAR

The original fragment of RIP140 isolated in the yeast two-hybrid screen (PIP32) contained the C-terminal 728 aa, suggesting that this part was responsible for the interaction with PPARα. To further determine which region(s) was required for receptor interaction, we constructed a series of RIP140 deletions and as-

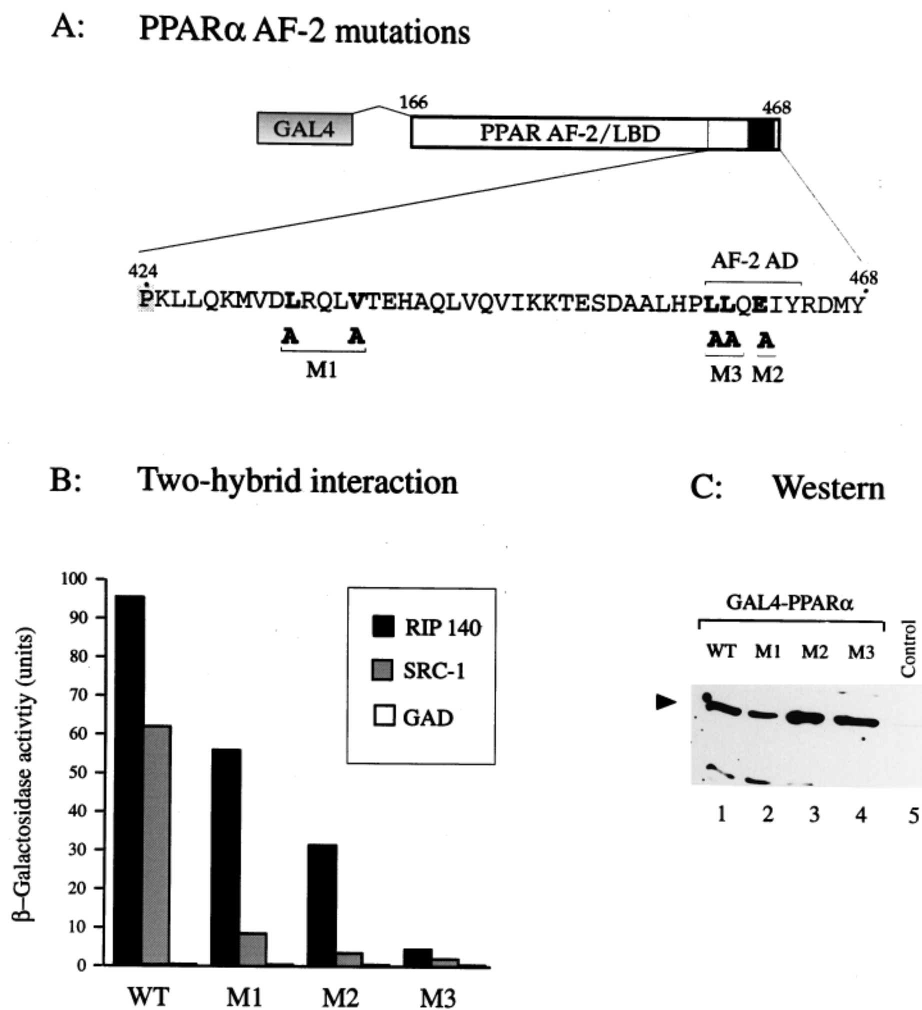

Fig. 4. Influence of PPARα AF-2 Mutations on the Interaction with RIP140 or SRC-1

Yeast two-hybrid analysis was performed similarly to the experiment shown in Fig. 3. A, Localization of the AF-2 mutations at the C-terminal end of PPARα. B, Yeast strains expressing the different mutated GAL4-PPARα fusion proteins and wild-type RIP140 or SRC-1 fused to GAD were mated and grown in liquid culture in the presence of histidine, and the β-galactosidase activity was determined. C, Western analysis for expression of the mutated GAL-PPARα fusion proteins.

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sayed their binding to PPARα *in vivo* (two-hybrid assay) and to PPARγ *in vitro* (GST pull-down). The results are shown in Fig. 5. Constructs 2–6 and 9 represent smaller fragments of the C-terminal interaction clone PIP32, whereas constructs 7 and 8 are parts of the RIP140 N terminus (not present in PIP32). Using the sensitive *HIS3* growth reporter in the two-hybrid assay, we were surprised to find that all deletions were able to interact with GAL-PPARα. However, the quantitative liquid β-galactosidase assay revealed different interaction intensities. To verify the two-hybrid results *in vitro*, we performed pull-down assays with GST-PPARγ in the presence of ligand (Fig. 5B). We focused on RIP140 fragments 1–7 covering the entire protein and containing both N- and C-terminal interaction domains. Again, all different parts of RIP140 interact efficiently with GST-PPARγ. Quantification of input and pull down supports the notion that, with the exception of the N-terminal fragment 7 (2.2% input), comparable amounts (15–25% input) of the different RIP140 fragments were retained on GST-PPAR. Subsequent pull-down experiments with RIP140 N- or C-terminal fragments fused to GST and *in vitro*-translated full-length PPAR, TR, and RXR support the notion that ligand-dependent receptor interaction *in vitro* is mediated predominantly by the RIP140 C terminus (Fig. 5C). Important differences between the *in vivo* two-hybrid assay and the *in vitro* GST pull-down assay, in particular the ratio between the interacting partners, the sensitivity of both assays, the influence of the GAD tag on the structure of the RIP140 interaction surface, and the folding and ligand status of the PPAR LBD expressed in yeast or bacteria, might acount for the apparent discrepancy between the two systems in some interactions (compare, for example, fragment 5 with fragment 7).

During the course of this work, several groups independently reported the identification of a short conserved peptide motif LxxLL (refered to as NR box) in RIP140 and other AF-2 cofactors (50, 55, 56). RIP140 possesses as many as nine such motifs distributed over the entire sequence and present in all fragments except the C-terminal fragment 5 (Fig. 5A, aa 951-1158). This fragment contains, however, a NR box-like motif, in which one of the conserved leucine residues is replaced by methionine (LxxML).

RIP140 Forms a Ternary Complex with RXR Heterodimers Bound to DNA

To address the possibility that RIP140 might form a ternary complex with the PPAR/RXR heterodimer on DNA, we performed electrophoretic mobility shift assays (EMSA). We preincubated *in vitro*-translated full-length receptor proteins with the ^{32}P-labeled DR1 binding site derived from the rat acyl-CoA oxidase (ACO) gene promoter as probe in the absence or presence of the appropriate ligands and added purified recombinant HIS-tagged RIP140 protein (aa 747-1158). To achieve a greater mobility of the heterodimer complex and to be able to detect ternary complexes, both RXRα and RXRαΔC (see below) lack the first 102 aa of the N terminus. Consistent with previous results, PPARγ and RXRα form a DNA-bound complex in the absence of added ligand (Fig. 6A, lane 1), and neither receptor alone or in combination with RIP140 can bind to the ACO-peroxisome proliferator response element (PPRE) (data not shown). Addition of either PPAR or RXR ligands did not affect the DNA binding under our conditions (lanes 2–4). When purified RIP140 C terminus was included in the binding reaction, the complex was supershifted in the presence of the RXR ligand 9-*cis*-RA, indicating the formation of a ternary complex. Surprisingly, we failed to detect any RIP140 ternary complex in the presence of the potent PPARγ ligand BRL49635. To exclude subtype- or ligand-specific differences between PPARs, we repeated the experiment with PPARα and observed basically identical results (data not shown): 9-*cis*-RA, but not the PPAR-ligand (WY-14,643), induced the RIP140 ternary complex. Note that the *in vitro*-translated PPARs interacted in a ligand-dependent manner with GST-RIP140C in the pull-down assay (Fig. 5C and data not shown). Although we can not exclude differences between the sensitivity of the two *in vitro* approaches, it is very likely that both DNA binding and heterodimerization induce a different PPAR conformation affecting the interaction with RIP140.

Since RIP140 ternary complex formation was induced by 9-*cis*-RA, it was reasonable to propose that RIP140 binding depends entirely on the RXR subunit of the PPAR/RXR heterodimer. To investigate whether a functional RXR AF-2 is required for RIP140 binding, we deleted the C-terminal half of the AF-2 AD core/helix 12 (RXRΔC) necessary for ligand-dependent transcriptional activity and interaction with cofactors (30, 57, 58). As seen in Fig. 6A (lane 9–14), RXRΔC forms stable heterodimers with PPAR. However, addition of RIP140 and 9-*cis*-RA (lanes 13 and 14) or PPAR ligands (lanes 12 and 14) does not result in the formation of the ternary complex. This result strongly suggests that RIP140 ternary complex formation with PPAR/RXR heterodimers depends on the functional AF-2 of the RXR subunit.

The recruitment of AF-2 cofactors such as RIP140 to the PPAR/RXR heterodimer in response to RXR ligands is in good agreement with the active role of RXR as permissive partner *in vivo* (2–4, 6). In contrast, in the TR/RXR heterodimer, RXR is believed to act as a nonpermissive partner, unable to bind its ligand *in vitro* and to activate transcription *in vivo* (7). Thus, we repeated the EMSA experiment with the TR/RXR heterodimer bound to a labeled synthetic DR4 binding site as probe. In contrast to the situation with the PPAR/RXR heterodimer, the mobility of the TR/RXR heterodimer containing the wild-type RXR (including the N terminus) was sufficient to distinguish the heterodimer from the RIP140 ternary complex. As demonstrated in Fig. 6B, addition of RIP140 and either 3,3,5-triiodothyroacetic acid (TRIAC), 9-*cis*-RA, or

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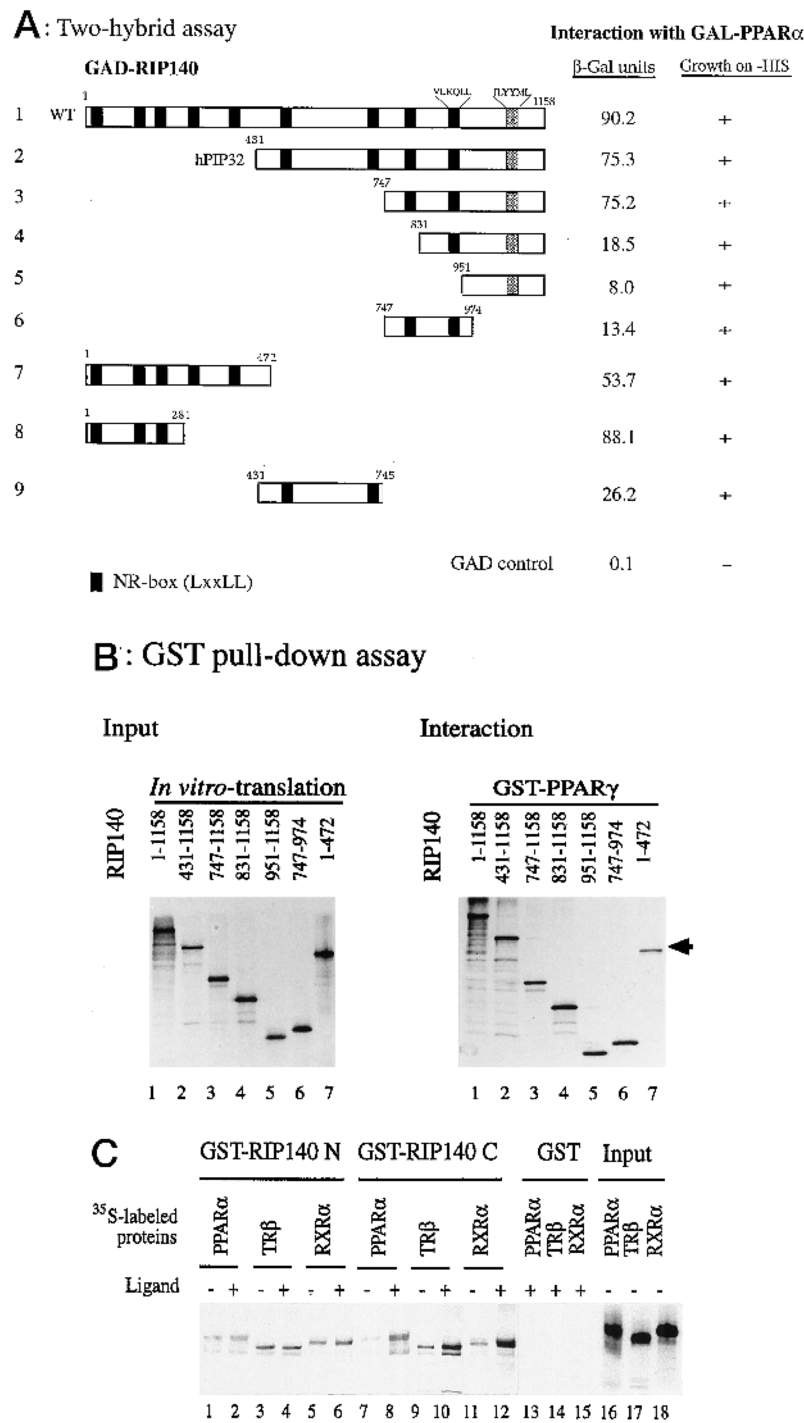

Fig. 5. Multiple RIP140 Domains Mediate the Interaction with PPAR

A, *In vivo* interaction of RIP140 fragments with PPARα in yeast. A *schematic illustration* for the RIP140 protein highlights the NR box interaction motifs (55). RIP140 fragments fused to GAD were analyzed for interaction with GAL4-PPARα (aa 166–468) in a two-hybrid liquid β-galactosidase assay and for induction of the *HIS3* growth reporter. Note, that fragment 2 represents the partial clone PIP32 isolated in the initial two-hybrid screen. *Numbers* indicate the aa defining the N- or C-terminal borders of the individual fragments. B, *In vitro* interaction of RIP140 fragments with GST-PPARγ in a pull-down assay. The same RIP140 fragments 1–7 as for the yeast two-hybrid interaction in panel A were synthesized by *in vitro* translation (*left panel*, input ~20%) and analyzed for binding to GST-PPARγ in the presence of 100 μM BRL 49653 (*right panel*). The approximate sizes of the RIP140 fragments 1–7 are 127, 80, 46, 37, 23, 26, and 51 kDa, respectively. The *arrowhead* indicates the position of the weaker interacting N-terminal fragment 7. C, *In vitro* interaction of translated wild-type receptors with GST-RIP140N (aa 1–281) and GST-RIP140C (aa 747-1158) in the absence (DMSO) and presence of the appropriate ligands WY-14,643 (100 μM), TRIAC (1 μM), and 9-*cis*-RA (1 μM).

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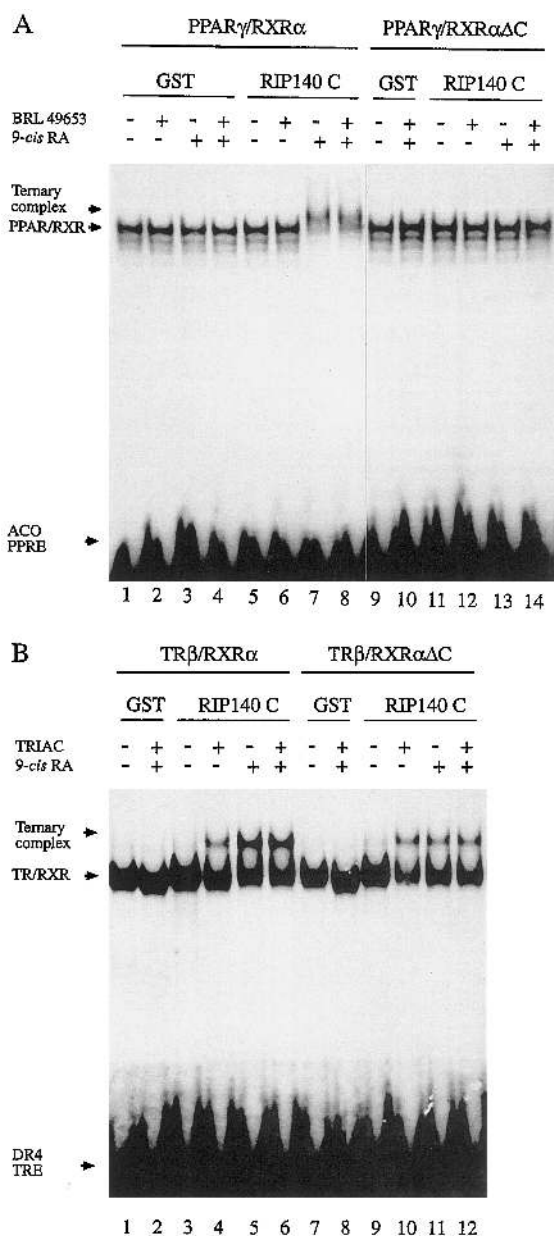

Fig. 6. RIP140 Forms Ternary Complexes with Nuclear Receptor Heterodimers on DNA

Electrophoretic mobility shift assays were performed using *in vitro* translated nuclear receptors and bacterially expressed HIS-tagged RIP140 (aa 747-1158) or GST control proteins (~500 ng). The receptors were incubated with ^{32}P-labeled double-strand-oligonucleotide containing the direct repeat response element and purified proteins under different ligand conditions as indicated at the *top* of each panel. A, 9-*cis*-RA-dependent interaction of RIP140 with the PPARγ/RXRα heterodimer bound to a DR1-type response element (ACO-PPRE). RXRαΔC deletes conserved hydrophobic residues of the AF-2 helix 12. Note that for mobility reasons both RXRα and RXRαΔC lack the first 102 aa of the N terminus. Ligand concentrations were 100 μM for BRL49653 and 1 μM for 9-*cis*-RA. B, Ligand-dependent interaction of RIP140 with the TRβ/RXRα heterodimer bound to a DR4-type response element [synthetic thyroid hormone response element (TRE)]. Both ligands (TRIAC and 9-*cis*-RA) were used at concentrations of 1 μM.

both ligands in combination, induced the formation of the ternary complex (lanes 4–6). The putative ternary complex is not formed after addition of GST control protein (lanes 1 and 2). Note that the RXR-ligand 9-*cis*-RA appears to be more effective than the TR-ligand TRIAC under the conditions of our bandshift assay (1 μM ligands), supporting the idea that RIP140 might have a higher affinity to the ligand-activated RXR subunit in different heterodimeric receptor complexes. It is also interesting to note that the ternary complex with TR/RXR bound to both ligands exhibited the same mobility as the ternary complexes observed with only one ligand-bound receptor, indicating that the number of RIP140 molecules recruited to the heterodimer was identical. Although the stoichiometry of the ternary complex is unknown, our results strongly suggest that heterodimeric receptors bind RIP140 as a functional unit and not as independent subunits. This view is further supported by the experiment shown in Fig. 6B (lanes 7–12) in which we used the C-terminal truncated RXR. Surprisingly, the RXR ligand 9-*cis*-RA still induced the RIP140 ternary complex, although the differential recruitment of RIP140 in response to the individual ligands appears to be lost with the mutated RXR (compare lanes 4/5 and 10/11). These results suggest strongly different modes of interaction of RIP140 with PPAR/RXR or TR/RXR heterodimers, respectively.

SRC-1, But Not RIP140, Functions as Potent Nuclear Receptor Coactivator in Yeast

Having established that RIP140 interacts with the ligand-activated AF-2/LBD of nuclear receptors in solution and with heterodimers bound to DNA, we attempted to clarify the role of RIP140 in transcriptional activation. Recent studies in yeast suggested that RIP140 activates transcription when tethered to DNA and serves as a nuclear receptor coactivator in yeast (48, 51). However, when fusing the RIP140 WT protein to the GAL4 DNA-binding domain, we observed only negligible transcriptional activity in our yeast system (data not shown). Next, to monitor the ability of RIP140 to serve as a coactivator for PPAR or TR in yeast, we established an *in vivo* transactivation assay. In contrast to the two-hybrid situation, RIP140 was now fused only to the SV40 nuclear localization signal. As shown in Fig. 7, RIP140 WT (aa 1–1158) or C-terminus (aa 431-1158), and for comparison, the nuclear receptor coactivator SRC-1 WT (aa 1–1061), were individually coexpressed with PPAR or TR fused to the GAL4 DNA-binding domain and assayed for activation of the *lacZ* reporter. Importantly, none of the RIP140 fragments functions as potent coactivators in our yeast system, in contrast to the strong coactivation seen with SRC-1. Basically similar results were observed on plates (2 days growth) as revealed from X-gal filter assays (data not shown).

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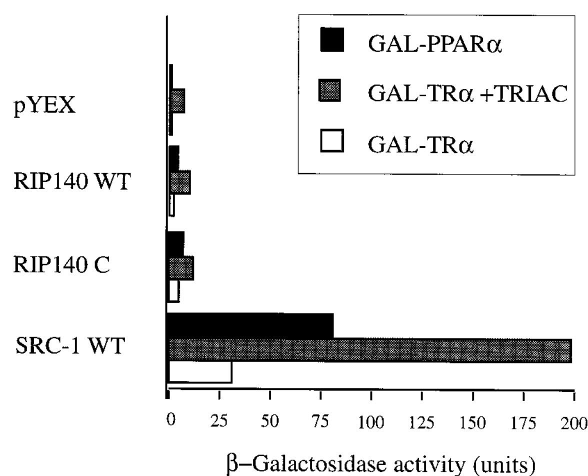

Fig. 7. SRC-1, but Not RIP140, Coactivates PPARα and TRα in Yeast

Liquid β-galactosidase assay using mated yeast strains (HF7c::Y187) containing GAL4-receptor fusions and RIP140 or SRC-1 proteins fused to a nuclear localization signal peptide in pYEX: RIP140 WT (aa 1–1158), RIP140 C (aa 431-1158), SRC-1 WT (aa 1–1061). GAL4-TRα was analyzed in the absence or presence of 1 μM TRIAC.

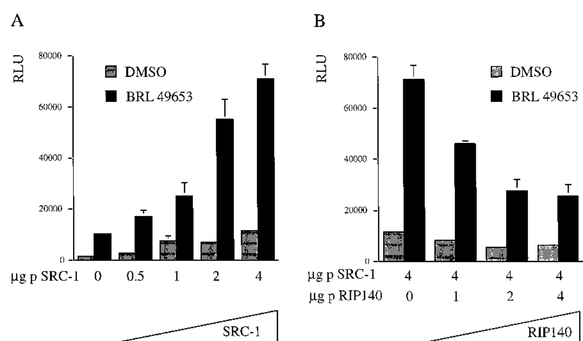

Fig. 8. RIP140 Antagonizes SRC-1 Coactivation of a PPAR-Responsive Reporter Gene

A, CV-1 cells were cotransfected with 1 μg PPRE-tk-luciferase reporter plasmid and increasing amounts of pSG5-based expression plasmids for SRC-1 in the absence (DMSO) or presence of 5 μM BRL 49653. B, CV-1 cells were cotransfected with reporter plasmid (1 μg), SRC-1 expression plasmid (4 μg), and increasing amounts of RIP140 expression plasmid in the absence (DMSO) or presence of 5 μM BRL 49653. Similar results were obtained in three independent experiments.

RIP140 Antagonizes SRC-1 Coactivation in Mammalian Cells Possibly through Competition for Binding to Nuclear Receptors

Previous transient transfection studies in mammalian cell lines have established that SRC-1 can serve as a coactivator for PPARγ (4, 41). To examine whether SRC-1 coactivates a PPAR-responsive luciferase reporter, we cotransfected CV-1 cells with the reporter plasmid and increasing amounts of SRC-1 expression vector in the absence or presence of the PPARγ specific ligand BRL 49653 (Fig. 8A). SRC-1 coexpression potently activated the reporter up to 4 μg dose of the expression vector. In contrast, coexpression of similar amounts of RIP140 decreased the reporter activity in response to ligand (data not shown), consistent with results obtained for ER (47). We conclude that SRC-1, but not RIP140, may serve as a PPAR coactivator in mammalian cells. Considering the possibility that RIP140 may counteract PPAR coactivation mediated by SRC-1, we cotransfected constant amounts of SRC-1 expression vector with increasing amounts of RIP140 expression vector. The result (Fig. 8B) shows clearly that RIP140 exerts a dominant negative effect on SRC-1-mediated coactivation.

Since both RIP140 and SRC-1 seem to exhibit similar interaction characteristics, we now asked whether the dominant negative effect of RIP140 seen *in vivo* might be due to competitive binding of both cofactors to receptors. An *in vitro* GST pull-down assay was performed with GST-PPARγ protein bound to gluthathione-Sepharose and [^{35}S]methionine-labeled cofactors in the absence or presence of purified histidine-tagged RIP140 (aa 747-1158) protein (Fig. 9A). First, the purified RIP140 C terminus almost completely eliminated binding of the *in vitro*-translated wild-type RIP140 (lanes 3 and 4), confirming our assertion that the C terminus is the predominant ligand-dependent interaction site. Second, binding of the purified RIP140 protein to PPARγ completely inhibited the binding of *in vitro*-translated SRC-1 (lanes 7 and 8), indicating that binding of both cofactors to the receptor is mutually exclusive. Third, the binding of *in vitro*-translated RXRα to PPARγ (heterodimerization) was not affected by adding purified RIP140 protein (lanes 11 and 12), indicating that the competition seen between RIP140 and SRC-1 was not due to nonspecific protein effects. It should be noted that the use of limiting amounts of GST protein (100 ng) for the competition pull-down assay apparently abolished the ligand-independent RIP140 interaction observed with higher amounts (1 μg) of GST-PPARγ (Fig. 2). Control Western blots (Fig. 9B) using antibodies recognizing PPARγ demonstrate that relatively equal amounts of GST-PPARγ protein were used for the pull-down experiment. Furthermore, using an anti-(HIS$_6$) antibody we could detect the purified HIS-RIP140C protein bound to purified GST-PPARγ, supporting the notion that the interaction between RIP140 and PPARγ is direct and not mediated by other proteins.

DISCUSSION

RIP140 and SRC-1 as AF-2 Cofactors for Ligand-Activated PPARs

RIP140 and SRC-1 serve as cofactors for the ligand-dependent AF-2 activation domain of most ligand-

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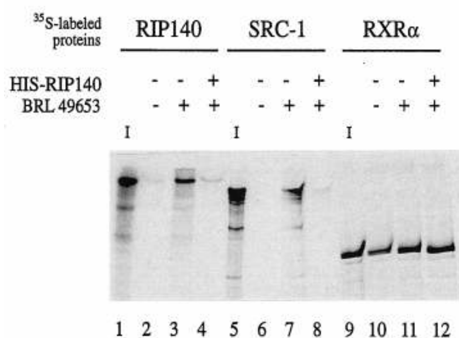

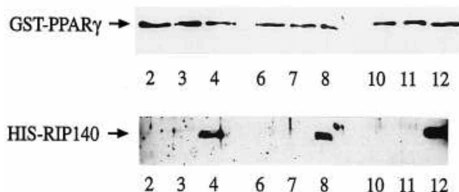

Fig. 9. RIP140 and SRC-1 Competitively Bind to PPARγ *in Vitro*

A, The *in vitro* interactions of GST-PPARγ with RIP140 (aa 1–1158), SRC-1 (aa 1–1061), and RXRα (aa 103–467) were analyzed in a GST pull-down assay in the absence (DMSO) or presence of 100 μM BRL 49653. The experimental conditions were otherwise similar to those described in Fig. 2, except that the amounts of GST-PPAR protein was reduced (~100 ng) and ~500 ng histidine-tagged RIP140 (aa 747-1158) were added if indicated (lanes 4, 8, and 12). The size of the major translation products is 127 (RIP140), 114 (SRC-1), and 43 (RXR) kDa, respectively. 'I' represents 20% input. B, Control Western blots detect both GST-PPARγ and the retained HIS-RIP140 protein.

activated nuclear receptors. Therefore, it was not surprising that yeast two-hybrid screenings described here and by others (41) identified both proteins as cofactors for members of the PPAR subfamily. The strong constitutive interaction of PPARs with RIP140 and SRC-1 (41) in yeast might have several explanations. Two-hybrid systems sense protein-protein interaction indirectly through the transcriptional activation of reporter genes. Due to the high sensitivity of certain reporters, ligand-independent interactions between overexpressed receptor and cofactor proteins could perhaps be sufficient to induce maximum transcription activation. Note that strong ligand-independent yeast two-hybrid interactions in combination with nonresponsiveness to ligands have also been reported for other nuclear receptors with AF-2 cofactors (59, 60). Furthermore, while we cannot exclude the possibility that our yeast strains are somehow defective in uptake of certain PPAR activators, the strong interaction of PPAR in the absence of exogenous ligands could be explained, in part, by the presence of endogenous ligands such as fatty acids in yeast, leading to an active receptor conformation sufficient for interaction with cofactors. This view is supported by previous yeast reconstitution studies (61, 62) demonstrating 1) the constitutive activity of the PPAR/RXR heterodimer, 2) the nonresponsiveness to peroxisome proliferators, but responsiveness to RXR ligands, and 3) the possible involvement of the yeast peroxisomal β-oxidation cascade in the generation of endogenous PPAR ligands. Alternatively, the unliganded PPAR LBD might perhaps differ structurally from receptors such as TR and RXR with regard to the position of helices 3/4 and 12. Consistent with that, our attempts to define the minimal interaction domain of PPARα with RIP140 and SRC-1 revealed that the interaction was dependent on the integrity of the entire LBD. Deletion or mutation of the AF-2 AD helix in PPARα decreased the interaction with both cofactors, indicating that this conserved domain is necessary for interaction. However, LBD regions other than the AF-2 AD contribute to the interaction with RIP140 and SRC-1 as well, confirming the current model that multiple regions serve important structural roles in establishing interactions between helix 12 and the LBD core, allowing the formation of a complex interaction surface for cofactors (54).

We and others have previously demonstrated that peroxisome proliferators, such as WY-14,643, and natural fatty acids activate the PPARα subtype in mammalian cells (2, 63). Here we show that WY-14,643 enhances the interaction of the rat PPARα with RIP140 and SRC-1 *in vitro*, consistent with similar results recently reported by Wahli and co-workers (13) using the *Xenopus* PPARs and SRC-1 in pull-down assays. This suggests that at least some peroxisome proliferators and endogenous fatty acids may act as PPARα ligands through direct binding to the receptor. In addition, binding of the synthetic ligand BRL 49635 to PPARγ efficiently enhances the interaction with both cofactors *in vitro*. However, several PPAR activators and putative endogenous ligands (for instance, PGJ_2, LTB_4, and ETYA) did not enhance the RIP140 interaction in our assays. Such discrepancies may be partially due to limitations of the pull-down approach using GST fusion proteins, since Li *et al.* found that bacterially expressed receptor LBDs could in some way be defective in proper folding and ligand binding (64). This is important considering the difficulties observed by us and others in obtaining soluble and functional PPAR protein of mammalian origin (13). Further, it is not unlikely that species-specific differences between the PPAR subtypes may complicate the interpretation of these *in vitro* results.

With respect to cofactors relevant for ligand activation by PPARs, the question remains whether, in addition to RIP140/SRC-1 proteins, other perhaps specific cofactors contribute to the transcriptional regulation in response to ligands. For example, the rat enzyme deoxyuridine triphosphatase (dUTPase) was isolated as a PPAR-interacting protein (65). However, the interaction domain of dUTPase seems to be spe-

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cific for rodents, arguing against the importance of dUTPase as a general PPAR cofactor. One should also consider that p140/p160 proteins identified in biochemical studies (4, 9, 45, 46) were purified mostly from a limited number of cell lines (CV-1, HeLa, and breast cancer cell lines), leaving the possibility open that other cell lines or tissues might contain a different set of cofactors. For example, nothing is known about relative expression levels of AF-2 cofactors in adipose tissue, one of the major target tissues of PPARγ action.

A Coactivator Function for RIP140?

Summarizing the results presented here and in previous studies, RIP140 shares important features with coactivators of the SRC-1 family (40, 46–49). Biochemically, these proteins have been identified from several cell extracts as the predominant nuclear receptor AF-2 cofactors in the presence, but not in the absence, of ligands. Using different experimental approaches, RIP140 and SRC-1 have been demonstrated to interact directly and ligand dependently with the AF-2. Nuclear receptor modifications that abolish the AF-2 activity have been shown also to abolish the interaction with both cofactors. Furthermore, RIP140 and SRC-1 form ternary complexes with ligand-activated receptor dimers bound to DNA. Also, RIP140 and SRC-1 family members seem to be ubiquitously expressed and have been demonstrated individually to colocalize with receptors to the nucleus (37, 47). In summary, both cofactor classes fulfil important criteria crucial for coactivator function.

However, a critical evaluation of the experimental data presented here and elsewhere might question such a role for RIP140, especially when compared with the evidence provided for SRC-1 and related coactivators. In fact, SRC-1/TIF-2 (31, 35, 37, 40, 41) and CBP/p300 (32–34, 36), but not RIP140, have been shown to significantly enhance the AF-2 activity of several nuclear receptors in mammalian cells, clearly indicating a role as nuclear receptor coactivators. The requirement for SRC-1 and CBP in ligand-regulated receptor activation has been further evaluated in inhibition experiments using microinjected antibodies (32, 33, 50). Coinjection of expression vectors for SRC-1 and TIF-2, but not for RIP140, could reverse the inhibitory effects of anti-SRC-1 IgG on ligand-dependent expression of a *lacZ* reporter gene, clearly demonstrating that RIP140 functionally cannot replace coactivators of the SRC-1 family (50). Further, far-Western analysis of cellular proteins binding mainly to ligand-activated ER suggested that different cell lines primarily contain relatively large amounts of p160, but apparently variable amounts of p140 (4, 9, 33, 45, 46). SRC-1 coactivators are now considered to act as histone acetyltransferases (HAT) and to function in concert with several other proteins exhibiting acetyltransferase activity, *e.g*. CBP/p300 and P/CAF (42–44, 66–68). Intriguingly, CBP/p300 as part of the RNA polymerase II holoenzyme might perhaps link nuclear receptors directly to the basal transcription machinery (69). Although RIP140 has been proposed to act as a bridging protein to the basal transcription machinery, there is no evidence for the involvement of RIP140 in such complexes in mammalian cells, consistent with the notion that RIP140 does not interact with TATA box binding protein (TBP), TFIIB, or CBP/p300 (33, 47). It should be noted that RIP140 coexpression in yeast only resulted in a minimal coactivation effect under nonsaturating ligand concentrations (51), whereas the coexpression of SRC-1 family members could efficiently restore the AF-2 activity of nuclear receptors (70, 71).

Differential Interaction of RIP140 with PPAR/RXR and TR/RXR Heterodimers

Ligand-dependent ternary complex formation of cofactors with receptor dimers on DNA is an important criterion for function. We demonstrated in EMSA experiments that RIP140 supershifts both TR/RXR and PPAR/RXR heterodimers under certain ligand conditions. Although PPAR ligands increased RIP140 binding to PPAR in solution, they failed to induce RIP140 ternary complexes with PPAR/RXR heterodimers on DNA. The reason for this is unclear, especially since the *in vitro* binding affinity of the BRL 49643 compound to PPARγ is comparable to that reported for TR or RXR ligands to their respective receptors (14). However, RIP140 binds strongly to the PPAR/RXR heterodimer in the presence of 9-*cis*-RA. Since this interaction was dependent on the functional RXR AF-2 helix 12, RXR might critically influence RIP140 binding to the PPAR/RXR heterodimer. Previous studies suggested that in nonpermissive RXR heterodimers, RAR and TR allosterically inhibit RXR from binding its ligand *in vitro* (7, 8). This is contradicted by recent observations that, at least in the case of RAR/RXR heterodimers, both partners can independently bind their ligands (64). Our studies support the subunit independency also for the DNA-bound TR/RXR heterodimer, since 9-*cis*-RA and TRIAC could independently induce the RIP140 ternary complex. These data do not conflict with the nonpermissivity seen *in vivo*, since RXR ligands alone may not be sufficient to dissociate dominant negative corepressors bound to the unliganded TR or RAR subunit. Surprisingly and in contrast to the situation with PPAR/RXR, we observed 9-*cis*-RA-dependent RIP140 ternary complexes with TR/RXR also with an RXR lacking the AF-2 helix 12. It remains to be shown whether this effect is due to a phantom ligand effect (58) of 9-*cis*-RA on TR, or whether RIP140 binding to RXR when heterodimerized with TR is not entirely dependent on helix 12. The latter possibility is not unlikely since the putative interaction surface for AF-2 cofactors is suggested to be complex and requires the contribution of other LBD parts than helix 12 (such as helix 3/4). Importantly, these differences between PPAR/RXR and TR/RXR heterodimers with re-

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spect to RIP140 binding support the concept that RXR heterodimers act as functional units with distinct specificities (30, 57, 58).

It is further interesting to note that in both the PPAR/RXR and the TR/RXR heterodimer, ligand-activated RXR appears to be very efficient in recruiting RIP140 to the complex. Independent far-Western studies aimed at detecting cofactors from CV-1 cell extracts using GST-RXR or the RAR/RXR heterodimer bound to DNA might support this notion since they revealed the predominant recruitment of p140 instead of p160, whereas in the absence of RXR ligands apparently either p140 or p160 can bind the heterodimer (4, 9). If true, the high affinity of RIP140 to the RXR subunit might have implications for the role of RIP140 *in vivo*, considering that RXR, as the ultimate heterodimerization partner for the majority of nuclear receptors, plays a central role in nuclear receptor activation. It remains to be determined how the recruitment of regulatory AF-2 cofactors, such as RIP140, through the activated RXR subunit contributes to the transcriptional activity of RXR heterodimers *in vivo*.

Competitive Binding of NR Box-Containing Cofactors to Nuclear Receptors

During the completion of our work, interaction domain mapping studies on TIF-1α, RIP140, and SRC-1 led to the identification of a short conserved peptide sequence LxxLL, serving as NR box or signature motif in a variety of coactivators, including SRC-1, TIF-2, ACTR, CBP, and p300, but also in other receptor cofactors, including RIP140, TIF-1, and several TRIPs (50, 55, 56). However, since this motif is found in many proteins not associated with receptor function, and since AF-2 interacting proteins such as ARA70 (53) obviously lack the LxxLL motif, it is likely that less-conserved motifs as well as additional structural features determine the interaction of AF-2 cofactors with receptors. For example, although the C-terminal RIP140 region (aa 981-1158) does not contain the consensus motif, we still detected an appreciable interaction with PPAR. In addition, we noticed that RIP140 N and C termini exhibit different interaction characteristics to nuclear receptors, although the interaction characteristics of NR box peptides derived from these domains do not allow any prediction for preferential binding (55). This suggests that the protein context and structural influences of outside regions are important interaction determinants of NR box-containing domains or proteins.

We have demonstrated that the C-terminal RIP140 interaction domain could not only compete for binding of wild-type RIP140 but, importantly, also for binding of wild-type SRC-1 to GST-PPARγ in pull-down assays. The competition observed in GST pull-down assays indicates similar interaction sites of RIP140 and SRC-1 on the LBD, but does not allow conclusions about the relative binding affinity of both cofactors. However, preliminary data (not shown) suggest that competition between RIP140 and SRC-1/TIF-2 also occurs in bandshift assays with receptor heterodimers using equal amounts of cofactor protein, suggesting that RIP140 and SRC-1/TIF-2 bind with similar affinities to receptors. The importance of competition in transcriptional activation may be illustrated, for example, by the recent demonstration that competitive interaction occurs between dTAF$_{II}$ 230 and the VP16 transcriptional activation domain on the TBP surface (72). Similarly, competitive binding between the adenovirus E1A protein and positive cofactors, such as the histone acetyltransferase P/CAF to the same domain of CBP/p300, might contribute to the negative effect of E1A on CBP/p300 signaling (67). Interestingly, competition between RIP140 and SRC-1 might act at a similar level considering the recent discovery of intrinsic HAT activity in SRC-1 coactivators and functional evidence for the existence of a complex between multiple HAT proteins. The capability of RIP140 to compete for binding of SRC-1 coactivators *in vitro* strongly suggests that the dominant negative *in vivo* effect of transiently expressed RIP140 on nuclear receptor activation or on AF-2-dependent synergism between nuclear receptors and transcription factors such as Pit1 (73) is primarily due to competition for binding and not to squelching or active repression mechanisms. Our finding concerning competition between RIP140 and SRC-1 has potentially important general implications for the mode of interaction of NR box-containing cofactors with the LBD. Considering the different interaction affinities of NR box peptides derived from several cofactors, one could assume that not all NR box-containing proteins bind with similar affinities to the LBD. For example, it remains to be seen whether cofactors such as CBP/p300 bind competitively with RIP140 or SRC-1 to receptors. Competitive binding will undoubtedly favor a limited number of cofactors with a relatively high binding affinity over the majority of low-affinity cofactors. In consequence, the number of biologically relevant cofactors might be smaller than anticipated from the abundancy of cofactors described today.

A Regulatory Role for RIP140

Our study supports the concept that cofactors such as RIP140 may function as regulatory proteins by critically influencing the stoichiometry of individual components of a transcriptional activation complex. Accordingly, a cell type expressing dominant levels of RIP140 would be predicted to exhibit a lower level of receptor activity compared with one with dominant levels of p160 coactivators. With the recent cloning of AIB1 (also known as p/CIP, ACTR), the third member of the SRC-1 coactivator class, from chromosomal regions amplified in certain breast and ovarian cancers (74), it became apparent how overexpression of one critical cofactor might determine the activity of the entire receptor-cofactor complex, resulting in deregulation of normal cellular functions. Current models

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suggest a role of RIP140 in regulating receptor activity directly as a coactivator and bridging factor to the basal transcription complex. However, consistent with previous suggestions for a role of RIP140 in receptor deactivation (46), our findings indicate that RIP140 may alternatively regulate nuclear receptor activity through competition with coactivators such as SRC-1.

MATERIALS AND METHODS

Plasmid Constructs

All described constructs were generated using standard cloning procedures (75) including PCR and verified by restriction enzyme analysis and DNA sequencing. Sequences of the oligonucleotides used for PCR and sequencing are available upon request.

Yeast Expression Plasmids All GAL4 DNA-binding domain (aa 1–147) fusion constructs were derived from the 2 μm plasmids pGBT9 or pAS2/2–1 (CLONTECH, Palo Alto, CA). GAL-PPARαLBD (aa 166–468) served as bait for the two-hybrid screening and was constructed by inserting a PCR fragment into *Eco*RI-*Sal*I linearized pGBT9. All GAL4-receptor LBD fusion proteins (for details see figure legends) were expressed from pAS2–1 after cloning the appropriate PCR fragments into the *Eco*RI-*Sal*I linearized vector. The GAD (aa 768–881) fusion constructs were derived from the 2-μm plasmids pGAD10, pGAD424, pGAD-GH, or pACT2 (CLONTECH). The GAD fusions to hRIP140 (aa 431-1158) and rRXRβ (aa 153–451) were isolated in two-hybrid screenings from pGAD10 cDNA libraries (CLONTECH) with GAL4-rPPARα as bait. GAD-SRC-1 was constructed by PCR amplification of the SRC-1 coding sequence (aa 1–1061) from pBK-CMV-SRC-1 (gift from B. W. O'Malley) and cloning into *Bam*HI-*Xho*I linearized pACT2. GAD-RIP140 constructs were generated as follows: GAD-RIP140 WT was constructed by PCR amplification of the RIP140 coding sequence (aa 1–1158) from pEF-RIP140 (gift from M. G. Parker) and cloning into *Bam*HI-*Xho*I linearized pACT2. The GAD-RIP140 fragments 3–6 were constructed by PCR amplification and cloning into *Eco*RI-*Sal*I linearized pGAD-GH. GAD-RIP140 (aa 431-1158, fragment 2) was created by transferring hPIP32 as a *Bgl*II fragment from the pGAD10-construct to pACT2 cleaved with the same enzymes. GAD-RIP140 (aa 1–472, fragment 7) was derived from GAD-RIP140 (aa 1–1158) using partial digestion with *Bam*HI and *Bgl*II and religation. GAD-RIP140 (aa 1–281, fragment 8) was derived from GAD-RIP140 (aa 1–1158) using digestion with *Xho*I and religation. To create GAD-RIP140 (aa 431–745, fragment 9), an *Eco*RI-*Sal*I fragment from GAD-hPIP32 (RIP140 aa 431-1158) was cloned into the corresponding site of pACT2. The yeast expression vector pYEX-RIP140 C (aa 431-1158) was constructed by internal deletion of an *Asp* 718-*Xho*I fragment (encoding the GAL4 activation domain of pGAD10) from pGAD10-hPIP32. pYEX was made subsequently from pYEX-RIP140 C by internal deletion of the RIP140 *Bgl*II fragment. pYEX RIP140WT and SRC-1WT fusions were made by inserting a *Bgl*II fragment from the corresponding pACT2-constructs into the *Bam*HI site of pYEX.

Mammalian Expression Plasmids Expression plasmids for RIP140 and SRC-1 were created by cloning of *Bgl*II fragments from the corresponding pACT2-constructs into pSG5 (Stratagene, La Jolla, CA). The PPRE luciferase reporter was generated by inserting a double-stranded oligonucleotide derived from the rat cytochrome P4504A6 gene (CYP4A6) (76) promoter 5′-AGCTTCTGAACTAGGGCAAAGTTGAG-3′ into a thymidine kinase (tk)-luciferase vector.

Plasmids for *in Vitro* Transcription/Translation Proteins were synthesized *in vitro* using the T3 or T7 RNA polymerase-based, rabbit reticulocyte lysate-coupled transcription-translation kit (TNT, Promega, Madison, WI). pBK-CMVHA-RIP140WT was constructed by PCR amplification of the RIP140 coding sequence (aa 1–1158) from pEF-RIP140 (gift from M. G. Parker) and cloning into *Bgl*II-*Xho*I linearized pBK-CMV (Stratagene) containing a hemagglutinin epitope (77). pBK-CMVHA-RIP140 (aa 431-1158) was created by transferring hPIP32 as a *Bgl*II fragment from the pGAD10 construct to the pBK-CMVHA linearized with the same enzyme. The RIP140 fragments 3–6 (for details see Fig. 7B) were constructed by cloning *Eco*RI-*Sal*I fragments from the corresponding GAD yeast two-hybrid plasmids into pBK-CMVHA linearized with *Eco*RI-*Xho*I. pBK-CMVHA-RIP140 N (aa 1–472, fragment 7) was made by cloning a *Bam*HI-*Bgl*II fragment from pBK-CMVHA-RIP140 WT into pBK-CMVHA linearized with *Bgl*II.

rPPARγ1 (aa 1–475) was inserted after PCR amplification into a *Nde*I-linearized derivative of pET19B (Novagen, Madison, WI). pGEM3Z (Promega) containing rRXRα (aa 1–467) has been described previously. Plasmids expressing N- or C-terminal deletion variants of rRXRα (RXRΔN: aa 103–467, RXRΔC: aa 1–457, RXRΔN/C: aa 103–467) were created by PCR amplification and subcloning into pGEM vectors. pT7-hTRβ (aa 1–410) was a gift from Stefan Nilsson.

GST/HIS Fusion Constructs To create pGEX fusion constructs to rPPARα (aa 166–468), hTRα (aa 122–410), rRXRβ (aa 153–451), and hTFIIB (aa 1–316), *Eco*RI-*Sal*I fragments from the corresponding yeast two-hybrid plasmids or pBK-CMV-derivatives were inserted into pGEX4T-1 (Pharmacia, Piscataway, NJ) linearized with *Eco*RI-*Sal*I. pGEX-rPPARγ (aa 175–475) was constructed by PCR amplification and insertion into pGEX4T-3 linearized with *Bam*HI-*Not*I. Similarly, GST-RIP140 N (aa 1–281) and GST-RIP140 C (aa 747-1158) were derived from the corresponding yeast two-hybrid plasmids. $(His)_{10}$-tagged RIP140 (aa 747-1158) was constructed by cloning a *Bam*HI-*Not*I fragment from pGEX-RIP140 C into pET19 (Novagen).

Yeast Two-Hybrid Interaction Screening

To identify PPARα-interacting proteins, a human liver cDNA library (CLONTECH) in the activation domain vector pGAD10 was introduced into the yeast reporter strain HF7c (*MATa, ura-52, his 3–200, lys 2–801, ade 2–101. trp 1–901, leu 2–3, 112, gal4–542, gal80–538, LYS::GAL1-HIS3, URA3::(GAL4 17 mers)$_3$ -CYC1-lacZ*) bearing pGBT9-PPARα LBD. More than 3×10^6 transformants (as determined from plating on SD media lacking leucine and tryptophan) were plated onto selective synthetic medium (SD) lacking histidine, leucine, and tryptophan and grown for 3–5 days at 30 C. From 89 HIS^+ colonies, 69 remained positive after restreaking onto fresh selective plates. When assayed for β-galactosidase activity using an X-gal filter assay, yeast from eight colonies turned blue. Library plasmid DNA from all HIS^+ colonies was isolated after electroporation of total yeast DNA into *Escherichia coli* strain HB101 and selection on synthetic M9 media lacking leucine, followed by PCR analysis with GAD10-specific primers to detect insert-containing library plasmids. After classification using PCR and restriction analysis, 33 different cDNA inserts were sequenced using the GAD10 5′-primer.

Yeast Two-Hybrid Interaction Assay/Coactivation Assay

The mating approach was used for both yeast two-hybrid and coactivation assays. Briefly, pGAD or pYEX plasmids were introduced into the reporter strain Y187 (*MATα, ura3–52, his 3–200, ade 2–101. trp 1–901, leu 2–3, 112, gal4Δ, met-, gal80Δ, URA3::GAL1-lacZ*) and mated with HF7c (*MATa*) bearing various GAL4 constructs (pGBT9 or pAS2-derivatives) for 12–16 h in liquid YPD (yeast-peptone-dextrose)-rich medium. Diploid strains were selected for the presence of

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both Leu and Trp plasmids on plates lacking tryptophan and leucine. Qualitative yeast growth assays, quantitative liquid β-galactosidase assays, and all standard yeast manipulations were as essentially described.

Western Blotting

Yeast Two-Hybrid Assay Yeast whole-cell extracts were prepared as described (78) and fractionated by SDS/PAGE, and proteins were transferred onto a nitrocellulose filter (Amersham, Arlington Heights, IL). Filters were blocked with 5% milk powder in PBS-containing 0.5% Tween 80 and incubated with a 1:1000 dilution of a mouse monoclonal antibody raised against the GAL4 DNA binding domain (RK5C1, Santa Cruz Biotechnology, Santa Cruz, CA) in PBS/Tween 80 for 60 min at room temperature. After washing, the filters were incubated with horseradish peroxidase-conjugated anti-mouse IgG antibody (Amersham) at a dilution of 1:2000 in PBS/0.5% Tween 80 for 60 min. After washing, the GAL4 fusion proteins were visualized with x-ray film using an enhanced chemiluminescense system (ECL, Amersham).

GST Pull-Down Competition Assay Proteins in 1× SDS sample buffer were subjected to standard Western analysis. GST-PPARγ was detected with an rabbit polyclonal antibody (PA3–820, Affinity BioReagents, Golden, CO) recognizing the conserved C terminus of all PPAR subtypes, and HIS-RIP140C was detected with a mouse monoclonal antibody (dia 900, Dianova, Hamburg, Germany) recognizing the HIS tag.

Expression and Purification of GST- and HIS-Tagged Proteins

Log-phase cultures of *E. coli* BL218(DE3) carrying the appropriate fusion constructs were grown in LB medium containing 0.5% casamino acids and 0.5% glucose at 30 C and were induced with 0.2 mM isopropyl β-D-thiogalactoside for 2–3 h. Cells were recovered by centrifugation and lysed in resuspension buffer [1×PBS, 1 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml lysozyme, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 10 μg/ml deoxyribonuclease (DNase) I, 10 μg/ml ribonuclease (RNase) A] for 30 min with rotation at 4 C. The lysates were clarified by centrifugation at 10,000 × *g* for 30 min at 4 C and immediately used for the binding reaction (GST pull-down assay). HIS-tagged RIP140 for gel-shift assays was purified on a TALON affinity column after standard protocols (CLONTECH) and dialyzed against the bandshift buffer (20% glycerol, 5 mM dithiothreitol, 5 mM EDTA, 250 mM KCl, 100 mM HEPES, pH 7.5, 25 mM $MgCl_2$, 0.05% Triton X-100). Protein concentration was determined by the Bradford dye binding procedure (Bio-Rad Laboratories, Richmond, CA) The stability of the proteins and concentration were confirmed by SDS-PAGE analysis followed by Coomasie blue staining.

In Vitro Protein-Protein Interaction Assay (GST Pull-Down)

Approximately 1 μg GST fusion protein bound to glutathione-Sepharose-4B beads was incubated for 2 h with 4 μl *in vitro*-translated [^{35}S]methionine-labeled protein in the presence of 1 μl ligand in dimethylsulfoxide (DMSO) (final concentration between 1–100 μM) or DMSO alone in a total volume of 100 μl incubation buffer (50 mM KPi, pH 7.4; 100 mM NaCl; 1 mM $MgCl_2$; 10% glycerol; 0.1% Tween 20, 1.5% BSA) with rotation at 4 C. In the competition assay, purified HIS-tagged RIP140 was included into the binding reaction. Beads were collected by microfugation and washed three times for 15 min with incubation buffer without BSA. Washed beads were resuspended in 50 μl 1×SDS sample buffer, heated in boiling water for 5 min, and pelleted in a microfuge, and 10–15 μl of the supernatant were subjected to SDS-PAGE. To control the stability of the GST-fusion proteins and equal loading, gels were stained with Coomasie blue before autoradiography. For quantification, autoradiographs were analyzed using the GelPro Software (Media Cybernetics, Silver Spring, MD).

EMSAs

Receptor proteins were synthesized in rabbit reticulocyte lysate using the TNT coupled *in vitro* transcription/translation system (Promega). Double-stranded oligonucleotides (1 μg) containing either a PPRE corresponding to the rat ACO gene promoter (2) 5′-CTAGCGATATCATGACCTTTGTCCTAG-GCCTC-3′ or a synthetic DR4-TRE 5′-TCGATCAGGTCATT-TCAGGTCAGAG-3′ were end-labeled with [γ-^{32}P]ATP. Binding reactions (20 μl) included 1× reaction buffer (5% glycerol, 5 mM dithiothreitol, 5 mM EDTA, 250 mM KCl, 100 mM HEPES, pH 7.5, 1 μg poly(deoxyinosinic-deoxycytidylic)acid, 25 mM $MgCl_2$, 1 mg/ml BSA, 0.05% Triton X-100), protease inhibitors (′Complete′, Boehringer Mannheim, Indianapolis, IN), 0.5 ng labeled probe, 2 μl of each *in vitro* translated receptor proteins, and, where indicated, 1 μl ligands in DMSO. Purified GST or HIS-RIP140 protein (usually 200 ng/reaction) was added last and binding was allowed to proceed for 20 min on ice. Reactions were loaded on a 4% nondenaturing polyacrylamide gel containing 5% glycerol and electrophoresed for 2 h in 0.5×Tris-borate-EDTA at 4 C. Gels were dryed and autoradiographed.

Mammalian Cell Culture, DNA Transfections, and Luciferase Assays

CV-1 cells were maintained in DMEM supplemented by 10% FCS (GIBCO BRL, Gaithersburg, MD), 100 μl/ml penicillin, and 100 μl/ml streptomycin (GIBCO BRL). Cells were transiently transfected by the calcium phosphate method. Cotransfections were performed using 1 μg PPRE-tk-luc per plate and indicated amounts (1–6 μg) of pSG-derived expression vectors in the absence or presence of 5 μM BRL49653. Lysated cells were mixed with luciferin reagent and ATP reagent (Bio-Orbit) in the luminometer (Anthos Labtec Instruments, Salzburg, Austria) according to the protocol of GEN-Glow-1000 (Bio-Orbit, Turku, Finland). Cells were harvested 20 h after transfection. Diagnostic cotransfections with a control plasmid showed the reproducibility of the transfections. Therefore, the luciferase activities achieved did not have to be corrected; rather, the mean and SD from independent triplicate experiments are presented.

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