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Liganded RARα and RARγ interact with but are repressed by TNIP1

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ABSTRACT

Nuclear receptor (NR) transcriptional activity is controlled by agonist binding and concomitant exchange of receptor-associating corepressor proteins for NR box-containing, receptor AF-2-targeting coactivator proteins. We report here that TNIP1 is an atypical NR coregulator. Requirements for TNIP1-RAR interaction—its NR boxes, ligand, and the receptor's AF-2 domain—are characteristic of coactivators. However, TNIP1 reduces RAR activity. Repression is partially relieved by SRC1, suggesting interference with coactivator recruitment as a mechanism of TNIP1 repression. TNIP1 does not bind RXRα and RARα AF-2 domain, necessary for that receptor's association with TNIP1, is insufficient to confer upon RXRα interaction with TNIP1. Preferential interaction of RARα over RARγ with TNIP1 can be mapped to RARα ligand binding domain helices 5–9 and suggests regions outside the receptor helix 12 modulate interaction of NRs and NR box-containing corepressors. TNIP1 repression of RARs in the presence of RA places it in a small category of corepressors of agonist-bound NRs.

Introduction

Transcriptional activity of nuclear receptors (NRs) is modulated not only by ligands but also by coregulator proteins that act either as coactivators or corepressors of receptor function. Coregulators facilitate or inhibit transcription of NR target genes through chromatin modification or interaction with other components of cellular transcriptional machinery [1]. Typically, in the absence of agonist, the receptor interacts with a corepressor protein such as nuclear receptor corepressor (NCoR) or silencing mediator of retinoid and thyroid receptors (SMRT). Through interaction with histone deacetylase (HDAC) or other repressor proteins, corepressors help maintain the chromatin in a compact state preventing target gene transcription [2]. Agonist binding the receptor causes a conformational change in the activation function 2 (AF-2) domain within helix 12 of the ligand binding domain (LBD) causing release of the corepressor, recruitment of a coactivator, and transcriptional activation. Characteristic of coactivator proteins is the NR box, a leucine rich amino acid motif, LXXLL (L = leucine, X = any amino acid), which is used to bind to the receptor AF-2 domain [3].

The model of the apo-receptor being bound by a corepressor and the holo-receptor by coactivator was challenged with the discovery that receptor interacting protein 140 (RIP140) acts as a corepressor in the presence of bound agonist [4]. Indeed several corepressors of agonist-bound NRs have since been characterized [5]. Their interaction requirements with NRs are those of coactivators: they bind the receptor in the presence of ligand and usually rely on NR boxes to do so. Their method of receptor repression has been shown to involve competition with coactivators for receptor binding, recruitment of HDACs or other transcriptional repressors or a combination of these mechanisms [5,6].

In search for new coregulators we previously carried out a yeast two-hybrid screen with peroxisome proliferator activated receptor (PPAR) for NR-interacting proteins from a human keratinocyte cDNA library. We identified TNFα-induced protein 3 (TNFAIP3)-interacting-protein 1 (TNIP1), a protein previously identified as Naf, VAN, and ABIN-1 and shown to associate with HIV proteins nef and matrix [7,8] and to repress the activity of nuclear factor κB (NF-κB) [9], as a PPAR coregulator capable of repressing that receptor's activity in the presence of ligand (Flores et al., submitted). To improve our understanding of TNIP1's function as NR corepressor we examined its interaction with other group 1 NRs [10] characteristic of keratinocytes [11], retinoic acid receptors α and γ (RARα and RARγ), and a group 2 receptor retinoid X receptor (RXRα), the heterodimer partner [12] for both RARs and PPARs Here we report that TNIP1 does not interact with RXRα but exhibits ligand-dependent association with RARα and γ and acts as RAR corepressor in presence of all-*trans*-RA (ATRA). The repression is partially relieved by steroid receptor coactivator 1 (SRC1), suggesting interference with coactivator binding as a possible repression mechanism. The AF-2 domain of RARs is necessary but not sufficient for interaction with TNIP1. Among RARs, RARα is preferred over RARγ as TNIP1 interaction partner. In this regard, we

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identified a region within the LBD of RARα responsible for this subtype difference—region not previously shown to play any role in NR-coregulator association.

Materials and methods

Plasmids. The βRARE-tk-CAT and RXRE-tk-CAT constructs have been previously described [13]. TRE-tk-CAT reporter was made by inserting three copies of TRE palindrome [14] into pBLCAT2. The βRARE-tk-Luc was prepared by subcloning the βRARE repeats and the tk promoter from βRARE-tk-CAT into pGL4.10 (Promega, Madison, WI). The pG5-luc reporter was prepared by subcloning the GAL4 binding sites and the E1B promoter from pG5-CAT into pGL4.10. TNIP1 in pOTB7 backbone was obtained from ATCC (Manassas, VA) and subcloned into pVP16 vector for mammalian two-hybrid assays or pCDNA3.1-HA for receptor transactivation and immunoprecipitation assays. The pCDNA3.1-HDAC1-Flag construct was obtained from Dr. Eric Verdin via Addgene (www.addgene.org, Addgene plasmid 13820). RARα/RARγ chimera constructs were generated by subcloning fragments of RARα LBD into existing restriction sites within RARγ using standard techniques. For αγ1 and αγ2 constructs, the restriction sites were engineered into the RARα inserts by PCR using primers shown in Table 1. The αγ3 construct was prepared by cloning a PciI-BamHI fragment of RARα into the corresponding sites in RARγ. RXRα-R construct was prepared by site directed mutagenesis of the RXRα AF-2 domain using primers shown in Table 1 for conversion of the RXRα AF-2 domain (TFLMEMLE) to RARα AF-2 (PLIQEMLE). In all cases, the mammalian two-hybrid constructs were prepared in the pM vector expressing appropriate receptor DEF domains and the constructs for receptor transactivation assays were prepared in the pSG5 vector expressing full-length receptors. All site directed mutants were generated using QuikChange XL (Stratagene, LaJolla, CA) kit according to manufacturer instructions. All constructs were verified by sequencing.

Two-hybrid and receptor transactivation assays. Yeast and mammalian two-hybrid assays were performed as previously described [15]. For receptor transactivation assays Cos-7 or HeLa cells were seeded in 12-well plates and 24 h later transfected using calcium phosphate for Cos-7 or FuGENE6 (Roche Applied Science, Indianapolis, IN) for HeLa with receptor constructs in the pSG5 backbone, pSG5-SRC1, pcDNA-HA-TNIP1, and the reporter construct as indicated in figures. RXRα was always cotransfected with RAR expression constructs. Appropriate empty vectors were used to keep the DNA amount constant. Cells were treated 16 h later with media containing vehicle (0.1% DMSO) or ligand indicated in the figures at 1 μM. After 24 h (for luciferase) or 48 h (for CAT) of ligand treatment the cells were collected and luciferase assays (Promega) or CAT ELISA (Roche) were performed according to manufacturer instructions.

Immunoprecipitations and HDAC assays. Cos-7 cells were transfected with pcDNA3.1-HA or pcDNA3.1-HA-TNIP for HDAC activity assays, or cotransfected with pcDNA3.1-HA-TNIP1 and pcDNA3.1-HDAC1-Flag for immunoblot detection of coimmunoprecipitation and 48 h later were washed with ice cold PBS and lysed with buffer containing 20 mM Tris pH8, 100 mM NaCl, 1 mM EDTA, 0.5% IGEPAL, and complete protease inhibitor (Roche). Lysates were diluted to 400 μg/mL protein concentration with lysis buffer and immunoprecipitated overnight with normal rat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) or HA rat monoclonal antibody (Roche) at 4 °C with rotation. The immunoprecipitates were incubated for 4 h with Protein A/G agarose beads (Santa Cruz) at 4 °C with rotation and washed four times with cold lysis buffer. The HDAC activity was determined using an HDAC fluorescent assay kit (Biomol Research Laboratories, Plymouth Meeting, PA) according to manufacturer instructions. For Western blots the immunocomplexes were eluted from the beads with Laemmli buffer and blotted with Flag antibody (Santa Cruz) using standard techniques.

Statistical analyses. Data was analyzed using Prism software (GraphPad, San Diego, CA). Student's *t*-test was used to compare two groups and one-way ANOVA with Newman-Keuls post hoc test was used for comparing more than two groups. *P*-values of less than 0.05 were considered significant.

Results and discussion

TNIP1 interaction with RARα and RARγ is dependent on ligand, NR boxes, and receptor AF-2 domain

We used the partial TNIP1 cDNA, clone 43a, as well as the full-length TNIP1 cDNA (Fig. 1A) to test for interaction with RARα and RARγ. Clone 43a contains one LXXLL NR box—amino acid sequence well known for its use by coregulators for interaction with liganded NRs [3,5]—while full-length TNIP1 contains two. Thus the partial cDNA provided us an initial tool to examine the role of one TNIP1 NR box versus two in interaction with retinoid receptors. In a yeast two-hybrid assay, clone 43a demonstrated strong interaction with RARα that was wholly dependent on ATRA and an intact NR box. There was no detectable association in the absence of ligand or when assayed in the presence of ligand but with the NR box mutated (m43a) from LKKLL to LKKAA (Fig. 1B). To assess AF-2 contribution to interaction between RARα and 43a, we introduced previously characterized [16] mutations (PLIQEMLE to PAAQEMLE) into this domain of RARα. This completely abolished the receptor's ability receptor to interact with clone 43a (Fig. 1B). Consistent with and extending these results for RARα, interaction between RARγ and clone 43a was also strictly dependent on ATRA, the receptor's AF-2 domain, and the clone's LKKLL NR box (not shown).

In mammalian two-hybrid assays, clone 43a demonstrated the same interaction requirements of ligand presence and receptor AF-2 integrity seen in yeast, but retained some association with RARα (Fig. 1B, inset) and RARγ (not shown) when expressed as an NR box mutant. This suggests that other non-canonical motifs

Table 1
Primers used to create the RARγ and RXR-R chimeric constructs with the engineered restriction sites or mutations underlined.

Construct		Primer sequence
αγ1	Forward	5'-TGCCAGCTGGGCAAGTATACTACGAA-3' (BstZ17I)
	Reverse	5'-CCGCAGGATCAGGATATCCAGGCA-3' (EcoRV)
αγ2	Forward	5'-GCCTGCCTGGATATCCTGATCCTG-3' (EcoRV)
	Reverse	5'-TTGGGGAACATGTGGGGGCGGCT-3' (PciI)
RXRα-R	Forward	5′-CTCTTCTTCTTCAAGCTCATCGGGGACACACC CATTGACCCTCTCATCCAGGAGATGCTGGAGGCGCCGCACCAAATGACTTAG-3′
	Reverse	5′-CTAAGTCATTTGGTGCGGCGCCTCCAGCATC TCCTGGATGAGAGGGTCAATGGGTGTGTCCCCGATGAGCTTGAAGAAGAAGAG-3′