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TNIP1 is a corepressor of agonist-bound PPARs

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ABSTRACT

Nuclear receptor (NR) coregulators include coactivators, contributing to holoreceptor transcriptional activity, and corepressors, mediating NR target gene silencing in the absence of hormone. We identified an atypical NR coregulator, TNFα-induced protein 3-interacting protein 1 (TNIP1), from a peroxisome proliferator activated receptor (PPAR) α screen of a human keratinocyte cDNA library. TNIP1's complex nomenclature parallels its additional function as an NF-κB inhibitor. Here we show TNIP1 is an atypical NR corepressor using two-hybrid systems, biochemical studies, and receptor activity assays. The requirements for TNIP1–PPAR interaction are characteristic for coactivators; however, TNIP1 partially decreases PPAR activity. TNIP1 has separable transcriptional activation and repression domains suggesting a modular nature to its overall effect. It may provide a means of lowering receptor activity in the presence of ligand without total loss of receptor function. TNIP1's multiple roles and expression in several cell types suggest its regulatory effect depends on its expression level and the expression of other regulators in NR and/or NF-κB signaling pathways. As a NR coregulator, TNIP1 targeting agonist-bound PPAR and reducing transcriptional activity offers control of receptor signaling not available from typical corepressors and may contribute to combinatorial regulation of transcription.

Introduction

The majority of ~300 nuclear receptor (NR)[2] coregulators identified to date are coactivators; far fewer corepressors have been isolated. Coactivators such as the p160/steroid receptor coactivator (SRC) and thyroid hormone receptor-associated proteins (TRAP) [1] associate with agonist-bound receptors via the coactivator's NR box, a short, amphipathic, leucine-rich motif (LXXLL, L = leucine, X = any amino acid). Physical association of unliganded NRs with corepressors e.g., nuclear receptor corepressor (NCoR) or silencing mediator for retinoid and thyroid hormone receptor (SMRT) is mediated by amphipathic leucine-rich helices (L/I-XX-I/V-I or L-XXX-I/L-XXX-L; I = isoleucine, V = valine, X = any amino acid) in the coregulator referred to as corepressor/nuclear receptor (CoRNR) boxes [2]. For most NR-coregulator interactions, the aporeceptor associates with corepressors and upon agonist binding undergoes a conformational change, shedding the corepressor and associating with a coactivator. However, the discoveries of ligand dependent corepressor (LCoR), receptor interaction protein (RIP) 140, and in this report TNIP1 have recognized a distinct group of coregulators. These proteins represent a novel, functionally allied group of coregulators using NR boxes, targeting the agonist-bound receptor's activating function-(AF) 2 region but reducing receptor-mediated gene transcription. This distinguishes them from typical NR corepressors such as SMRT and NCoR ([3] for review) and provides additional means of reducing NR activity without the more-extreme loss of activity conferred in the absence of ligand by SMRT or NCoR.

Three PPAR subtypes, PPARα, PPARδ, and PPARγ have been identified (NR1C1, NR1C2 and NR1C3) and each has been implicated in multiple aspects of keratinocyte response to natural and synthetic PPAR ligands [4–8]. As PPAR coregulators ([9] for review) would participate in these responses, we report here on a novel PPAR coregulator isolated via a two-hybrid screen of keratinocyte cDNA. Intriguingly, this PPAR-interacting clone matched coding sequences previously isolated via protein–protein interaction with (i) the HIV protein Nef, (ii) the HIV protein matrix, and (iii) the human zinc finger protein A20 [10–12]. In each case, different names were

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[2] *Abbreviations used:* aa, amino acid; ABIN, A20-binding inhibitor of NF-kB; AF-2, activating function 2; CoRNRB, corepressor NR box; DBD, DNA binding domain; NR, nuclear receptor; PPAR, peroxisome proliferator activated receptor; TNIP1, TNFα-induced protein 3-interacting protein 1.

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given to the isolate (Naf, VAN and ABIN, respectively). As found in the National Center for Biotechnology Information database, the sequence is named TNIP1 for TNFAIP3-interacting protein 1, and we use that designation in this report. Beyond the roles described for TNIP1 under its aliases of Naf, VAN, and ABIN are its recent gene-disease associations in lung cancer and psoriasis [13,14].

Given the diversity of previous TNIP1 investigations and having isolated TNIP1 as a PPARα-interacting protein the objectives of this study became (i) to examine the expression range of TNIP1, (ii) to determine the ligand requirements and amino acid motifs within NR and TNIP1 necessary for their interaction, and (iii) to establish the consequences to PPAR activity from TNIP1 expression. Here we provide evidence for TNIP1 as an atypical NR corepressor. Despite association with PPARs in the presence of their respective ligands, TNIP1 partially represses their activity. TNIP1 does not interact with the PPAR heterodimer partner retinoid X receptor (RXR) even in the presence of that receptor's 9-*cis* retinoic acid (RA) ligand. TNIP1's wide tissue distribution suggests it may play an important regulatory role in multiple cell types. Corepressors such as TNIP1 and LCoR [15], using NR boxes, targeting the agonist-bound receptor's AF-2 region, and yet reducing receptor-mediated gene transcription, may provide finer levels of control by lessening rather than completely silencing NR activity.

Materials and methods

Yeast and mammalian expression constructs, one- and two-hybrid assays

Selection stringency, ligand concentrations, vectors for yeast two-hybrid screen of a human keratinocyte cDNA library with human PPARα ligand-binding domain (LBD), mutagenesis methods, and receptor mutants were previously described in detail [16]. Briefly, the mutations are consensus amino acids (italicized) replaced with conservative substitutions (underlined) e.g., second TNIP1 NR box LKK*LL* to LKKAA and PPARα AF-2 *LL*QEIY to AAQEIY [16]. In this report, a quantitative β-galactosidase assay was used to assess interaction between pGAD10-coregulator proteins and pGBKT7-nuclear receptor DEF domains in the yeast two-hybrid system. pGBKT7-lamin C (Clontech, Mountain View, CA) was used as the negative control. Yeast strains Y187 and AH109 were lithium acetate transformed with pGAD10-coregulator (TNIP1) and pGBKT7-nuclear receptor (PPAR) DEF constructs, respectively, and selected on media lacking leucine for pGAD10 constructs or lacking tryptophan for pGBKT7 constructs. Colonies from each transformation were mated and selected on double drop-out media (-leucine/-tryptophan). Colonies expressing both constructs were picked from solid selective media, grown in triplicate in liquid selective media (with vehicle or ligand) overnight, and used as an inoculum for 4 h cultures with vehicle or ligand. β-galactosidase activity was determined from chlorophenol red-β-D-galactopyranoside (Roche Applied Science, Indianapolis, IN) conversion measured at OD578 by the following equation: $(1000 \times \mathrm{OD578})/(v \times \mathrm{time} \times \mathrm{OD600})$ where v is a concentration factor for the pelleted yeast volume (0.5 ml in these assays), time is reaction duration (in minutes) and OD600 provides a normalization to yeast cell number. NR constructs had no autonomous activation of reporter genes [16]. Mammalian two-hybrid assays in COS-7 cells were performed with pM vector expressing receptor LBDs and pVP16 vector expressing 43a or full-length TNIP1.

The mammalian one-hybrid assay employed here utilized the TNIP1 cDNA cloned in-frame after the Gal4 DNA binding domain (DBD) cDNA in the SV40 promoter-driven pGal4DBD (pM vector, Clontech) and the reporter construct, pGal4UAS-tk-CAT [17], which has basal expression from the thymidine kinase (TK) minimal promoter regulated by the upstream activating sequence (UAS). One day after plating, COS-7 cells were transfected with a standard calcium phosphate protocol and 72 h later assayed for CAT expression via ELISA (Roche Applied Science) as described [18].

Mammalian cell culture and transfections

HaCaT keratinocytes [19] or COS-7 (ATCC, Manassas, VA) were cultured in a 3:1 mix of DMEM and F12 (Invitrogen, Carlsbad, CA) with 10% FBS (HyClone, Thermo Scientific, Rockford, IL) and 100 U/ml penicillin and 100 μg/ml streptomycin. Receptor assays were from cells plated in triplicate wells 24 h before transfection in the above media but with 10% charcoal-stripped FBS. HaCaT keratinocytes were transfected via Effectene (Qiagen, Valencia, CA) according to the supplier's protocols and COS-7 cells were transfected via the calcium phosphate coprecipitation method as detailed previously [20]. Mouse 3T3-L1 preadipocyte cells (ATCC) were maintained and differentiated as previously described [21]. Preadipocytes were cultured in DMEM with 10% calf serum (Hyclone). Differentiation induction media was DMEM containing 10% FBS, 1 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine (Sigma, St. Louis, MO). Neonatal human primary keratinocytes were cultured in serum-free KGM-Gold media with Bullet-kit supplements (cells and reagents from Lonza, Walkersville, MD) at final calcium concentrations indicated in the results. Ligand treatments, cell collection, and reporter assays have been described [16,18]. Empty vector addition to standardize plasmid copy number and controls for NR reporter transfections were as described [18]. Reporter expression was normalized and tested for repeatability of results from independent transfections as described [22].

In vitro pull-down assays

^{35}S-methionine-labeled PPARγ LBD from *in vitro* transcription/translation (Promega; Madison, WI) and glutathione Sepharose column-purified GST or GST-TNIP1 expressed in BL21 RIPL (Stratagene; La Jolla, CA) from pGEX5 were incubated at 4 °C, washed three times (100 mM NaCl, 1 mM EDTA, 0.02% Igepal, 20 mM Tris–Cl, pH 8.0) and eluted with SDS–PAGE sample buffer.

Multiple tissue cDNA PCR

PCR amplification of human cDNA (Clontech) was done with Titanium Taq (Clontech) and 5 ml template; cDNA template amounts from different tissues were adjusted by the manufacturer to normalize target mRNA abundance of different house-keeping genes. TNIP1 primers were forward 5′-CCA CCA CCT TCT CCC TCC TT-3′ and reverse 5′-GCA TCT TCA CCT TCT TCT CG-3′. Ubinuclein primers [23] were forward 5′-AGA AGC CAT GCA GTG ACA C-3′ and reverse 5′-AGC TCT GGG TAG AAG AAC-3′. Primers crossed intron–exon boundaries. For both the TNIP1 and ubinuclein amplification, conditions were as follows: initial denaturation, 2 min at 94 °C, followed by 38 cycles at 94 °C for 20 s, 59 °C for 50 s, and 68 °C for 1 min with a final extension of 68 °C for 5 min.

TNIP1 plasmids

A human lung cDNA (ATCC) in pOTB7 was sequenced on both strands and determined to contain full-length, 636 amino acids, TNIP1. pVP16-TNIP1m1&m2 was used as template to generate carboxyl terminus truncations by PCR. The common forward primer for truncations was 5′ AAG G*GG ATC* CTC ATG GAA GGG AGA-3′ (Bam HI site, *italics*; TNIP1 start codon, underlined). The reverse primers were: TNIP1 1–206, TTG AT*A AGC TT*T TAG GAG GTG CGC TG; TNIP1 1–221, TGA GC*A AGC TT*T TAA GCC TCG TTC TC; TNIP1