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TIP27: a novel repressor of the nuclear orphan receptor TAK1/TR4

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ABSTRACT

The nuclear orphan receptor TAK1/TR4 functions as a positive as well as a negative regulator of transcription; however, little is known about the factors regulating or mediating its activity. Yeast two-hybrid analysis using the ligand-binding domain (LBD) of TAK1 as bait identified a novel TAK1-interacting protein, referred to as TIP27, which functions as a repressor of TAK1-mediated transactivation. TIP27 is a 27 kDa protein containing two zinc finger motifs. Mammalian two-hybrid analysis showed that TIP27 interacts specifically with TAK1 and not with several other nuclear receptors tested. The region between Asp^{39} and Lys^{79} of TIP27, referred to as TAK1-interaction domain (TID), is critical for its interaction with TAK1 while the TAK1-LBD from helix 3 until the C-terminus is required for the optimal interaction with TIP27. Pull-down assays demonstrated that the TIP27 physically interacts with TAK1 and supported the critical importance of the TID. Confocal microscopy showed that in the nucleus, TIP27 and TAK1 co-localize. TIP27 acts as a strong repressor of DR1-dependent transcriptional activation by TAK1. This repression does not involve the inhibition of TAK1 homodimerization or DR1 binding but may be due to an effect on co-activator recruitment by TAK1. Our results indicate that TIP27 functions as a TAK1-selective repressor.

INTRODUCTION

TAK1 (TR4 or NR2C2) forms, with the closely related receptor TR2 (NR2C1), a subclass of orphan nuclear receptors (1–4). TAK1 exhibits a typical nuclear receptor modular structure consisting of an N-terminal domain, a DNA-binding domain (DBD), a hinge domain and a ligand-binding domain (LBD) composed of a canonical structure of 12 helical regions (H1–H12) (5–7).

TAK1 is expressed in many tissues, including brain, thymus, testis and spleen (3,4,8). TAK1 is differentially regulated during spermatogenesis and in particular highly expressed in pachytene spermatocytes. In the brain, its expression is limited to specific regions, including hippocampus, thalamus, dentate gyrus and the granule cells of the cerebellum. TAK1 is also highly expressed in hematopoietic cells (9). Ectopic expression of TAK1 induces proliferation of promyelocytes thereby implicating TAK1 in the regulation of differentiation and proliferation of myeloid progenitor cells. This action, however, appears to be independent of the DBD of TAK1.

TAK1 binds as a homodimer to DNA response elements consisting of direct repeats of the consensus sequence AGGTCA spanned by 1–5 nt (DR1–5) (10,11). The homodimers bind with highest affinity to DNA elements containing a DR1. Although TAK1 does not form heterodimers with retinoid X receptor (RXR), it is able to form a dimeric complex with TR2 and the androgen receptor (AR) (1,10,12). The transcriptional activity of TAK1 is cell type dependent; in certain cell types TAK1 can induce DR1-dependent transcription while in other cell types it represses transcription (1,10,11). In addition, TAK1 is able to repress the transcriptional activation induced by several other nuclear receptors, including the peroxisome proliferator-activator receptor (PPAR), vitamin D receptor (VDR), RXR and retinoic acid receptor (RAR) (1,10,12,13). This repression appears to be mediated at least in part by competition for the same response element. Steroid 21-hydrolase, the erythroid ε- and γ-globin genes, and several viral promoters have been identified as potential targets for TAK1 regulation (14–17).

Recruitment of co-repressors and co-activators is a critical step in nuclear receptor-mediated repression and activation of gene expression, respectively (18–21). Through their histone deacetylase or acetylase activities, respectively, such complexes induce changes in chromatin structure. The insight obtained into the action of other nuclear receptors suggests that the transcriptional regulation by TAK1 also involves recruitment of various transcription intermediary factors. This is supported by a recent study showing that TAK1 and TR2 are part of a 540 kDa repressor complex bound to a DR1 element in the erythroid ε- and γ-globin genes (17). However, very little is known about the identity of the proteins associated with this complex.

To identify proteins that modulate or mediate the regulation of transcription by TAK1, we performed yeast two-hybrid analysis using the hinge-LBD region of TAK1 as bait. In this study, we describe the identification of a protein that specifically interacts with TAK1. This protein, referred to as TAK1-interacting protein 27 (TIP27), is a 27 kDa nuclear protein containing two putative zinc finger motifs. TIP27 was

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found to be identical to the recently described JAZF1, a protein with unknown function (22). TIP27 mRNA is found in several tissues and is most highly expressed in testis. Pull-down analysis demonstrated that TAK1 and TIP27 form a protein complex. Deletion mutation analyses identified a 40 amino acid TAK1-interaction domain (TID), i.e. critical for the interaction of TIP27 with TAK1 while the LBD of TAK1 is important in its interaction with TIP27. Reporter gene analyses showed that TIP27 is a strong repressor of the induction of DR1-dependent transactivation by TAK1 while TIP27 has little effect on the transcriptional activation by the nuclear receptors PPARα and RORγ. This repression does not involve an inhibition of TAK1 homodimerization or DR1-binding but may be due to an effect on co-activator recruitment by TAK1. Our results suggest that TIP27 functions as a TAK1-selective co-factor that may play an important role in mediating transcriptional repression by TAK1.

MATERIALS AND METHODS

Plasmids

The reporter plasmid pFR-LUC was obtained from Stratagene (La Jolla, CA). The vectors, pM and pVP16, and the β-galactosidase reporter plasmid pCMV-βgal were purchased from BD Biosciences (Palo Alto, CA). pSG5-TAK1, pcDNA3-RTR, pcDNA3-TAK1, $(DR1)_3$-CAT, pVP16-RTR (GCNF or NR6A1), pVP16-RORγ and pM-RORγ(Δ41) were described previously (1,23–25). pVP16-AR, pVP16-ERα, pVP16-LXRα, pVP16-PR-A, pVP16-PPARγ and pVP16-RXRα were provided by Donald P. McDonnell (Duke University) (26). Full-length and deletion mutants of pVP16-TAK1, pM-TAK1 or pM-TIP27 were created by placing each fragment in-frame with VP16(AD) or Gal4(DBD), respectively. These fragments were generated by PCR using TAK1- or TIP27-specific 5′- and 3′-primers to allow the PCR fragments to be subcloned into the multiple cloning site of each vector. Details on the length of each deletion are described in the text and figure legends. Point mutations in VP16-TAK1 and pM-TIP27 were introduced using a QuickChange site directed mutagenesis kit (Stratagene). pcDNA4-TIP27 was generated by inserting a full-length TIP27 cDNA that was obtained by PCR using the expressed sequence tag clone BG699065 as a template, into the BamHI/EcoRI sites of pcDNA4HisMaxA. pCMV-Myc-TIP27 and pCMV-Myc-TIP27(ΔTID) were generated by subcloning PCR-amplified TIP27 full-length and TIP27(ΔTID) into EcoRI/NotI and BglII/SalI sites of pCMV-Myc (BD Biosciences), respectively. pEGFP-TIP27 was generated by cloning full-length TIP27 in-frame into the EcoRI and SalI sites of pEGFP-N2 (BD Biosciences). pET41-TIP27 encoding the GST-TIP27 fusion protein was created by PCR amplification of the full-length coding region and insertion into the BamHI/NotI sites of pET41c (Novagen, Madison, WI). pGEX-TIP27(ΔC101) containing the N-terminus up to Pro^{101}, pGEX-TIP27(39-101) encoding TIP27 from Asp^{39} to Pro^{101} and pGEX-TIP27(ΔN79) containing the C-terminus from Lys^{79} were created by PCR amplification and PCR products inserted in-frame into the BamHI/EcoRI sites of pGEX-5X-3 (Amersham). All constructs were verified by restriction analysis and DNA sequencing.

Cell culture

CV-1 and COS-1 cells were cultured in DMEM supplemented with 10% fetal bovine serum.

Yeast two-hybrid screening

The Gal4 yeast two-hybrid system was purchased from BD Biosciences. Library screening was conducted according to the manufacturer's instructions. Briefly, the bait construct pGBKT7-TAK1 was generated by cloning the region encoding the hinge and LBD of human TAK1 into the EcoRI and blunt-ended BamHI sites of the vector pGBKT7. The pGBKT7-TAK1 was transformed into *Saccharomyces cerevisiae* strain AH109(MATα). Yeast two-hybrid library screening was carried out by the yeast-mating procedure using *S.cerevisiae* Y187(MATα) pre-transformed with a pACT2 human testis MATCHMAKER cDNA library (BD Biosciences). After mating, positive clones were selected on minimal Synthetic Dropout medium (-Trp/-Leu/-His) containing 5 mM 3-amino-1,2,4-triazole. One of the positive in-frame cDNA clones, referred to as TIP27, was identified and characterized in this study.

DNA sequencing

Plasmids were purified using Wizard miniprep or midiprep kits from Promega. Automatic sequencing was carried out using a BigDye Terminator Cycle Sequencing Ready reaction kit (Applied Biosystems) and an ABI Prism 377 automatic sequencer. DNA and deduced protein sequences were analyzed by the SeqWEB sequence analysis software packages.

Northern-blot analysis

A multi-tissue blot containing poly(A)$^+$ RNA (2 μg) from 10 human tissues were purchased from Ambion (Austin, TX). Northern-blot analysis was performed as described previously (27) using a ^{32}P-labeled probe containing the full-length coding region of TIP27.

In vitro pull-down assay

Escherichia coli Tuner(DE3) cells (Novagen) transformed with pET41-TIP27, pGEX-TIP27(ΔC101), pGEX-TIP27 (ΔN79) or pGEX-TIP27(39-101) were grown at 37°C to mid-log phase, the synthesis of glutathione *S*-transferase (GST) proteins was then induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; 0.1 mM final concentration) at 30°C. After 3 h of incubation, cells were collected, resuspended in BugBuster protein extraction reagent (Novagen) and incubated as described in the manufacturer's protocol. Cellular extracts were then centrifuged at 15 000 *g* and the supernatants containing the soluble GST proteins were collected. Equal amounts of GST-TIP27 proteins or GST protein (Sigma, St Louis, MO) were incubated with glutathione–Sepharose 4B beads and washed in phosphate-buffered saline. [^{35}S]methionine full-length TAK1 and RTR were obtained by *in vitro* translation using the TNT-coupled reticulocyte lysate system from Promega and the vectors pSG5-TAK1 and pcDNA3-RTR. The GST- and GST-TIP27-bound beads were then incubated with the [^{35}S]methionine receptor in 0.2 ml binding buffer (20 mM Tris–HCl, pH 7.6, 100 mM KCl, 0.05% NP-40, 0.1 mM EDTA, 10% glycerol and 1 mM

PMSF). After 1 h incubation at 4°C, beads were washed five times in binding buffer and then boiled in 15 µl of 2× SDS–PAGE loading buffer. Solubilized proteins were separated by 10% SDS–PAGE and the radiolabeled proteins visualized by autoradiography.

Immuno-pull-down (IP) assay

p3XFlag-CMV-TAK1 or p3XFlag-CMV-RORγ was co-transfected with pCMV-myc-TIP27 in COS-1 cells. After 48 h, cells were harvested and lysed in binding buffer. Subsequently, 90% of the cell lysates were then incubated with anti-Flag M2 agarose resin (Sigma) for 1 h at 4°C with agitation. The resin was then washed five times with binding buffer. The pulled-down protein complexes were then examined by western-blot analysis using an anti-myc (Invitrogen, Carlsbad, CA). Proteins in the remaining cell lysates were mixed with sample buffer and examined by western-blot analysis using either an anti-c-myc or anti-Flag M2 antibody (Sigma).

Subcellular localization

pEGFP-TIP27 or pEGFP-C1 plasmid DNA were transfected into COS-1 cells using FuGENE 6. In certain instances, pEGFP-TIP27 was co-transfected into COS-1 together with p3XFlag-CMV-TAK1 or p3XFlag-RORγ. After 24 h, cells were fixed in 4% paraformaldehyde. For cells co-transfected with p3XFlag-CMV-TAK1 or p3XFlag-CMV-RORγ, immunofluorescent staining was performed by incubating cells with anti-FLAG M2 monoclonal antibody (Sigma) followed by incubation with Alexa Fluor® 594 goat anti-mouse antibody (Molecular Probes). Fluorescence was examined in a Zeiss confocal microscope LSM 510 NLO (Zeiss, Thornwood, NY).

Reporter gene assays

Cells were plated in 12-well dishes at 2×10^5 cells/well and 20 h later co-transfected (1 µg of total DNA in 2 ml) with 0.3 µg of $(UAS)_5$-LUC or $(DR1)_3$-CAT, 0–0.3 µg of various expression plasmids as indicated and 0.003 µg pRL-SV40 (Promega) which served as an internal control to monitor transfection efficiency. Total amount of DNA was kept constant by adding pZeo and/or pcDNA4HisMaxA. Cells were transfected in Opti-MEM (Life Technologies) and 3 µl FuGENE 6 transfection reagent (Roche). Cells were incubated for 48 h and then assayed for luciferase activity with a dual luciferase kit (Promega). CAT activity was determined by using a CAT enzyme-linked immunosorbent assay kit (Roche), and the level of β-galactosidase activity was determined using a luminescent β-galactosidase detection kit (Clontech) according to the manufacturer's instructions. The firefly LUC or CAT reporter activity (relative to the Renilla LUC activity) was calculated and plotted. Transfections were performed in duplicate and each experiment was repeated at least twice.

RESULTS

Identification of TIP27

As reported for other nuclear receptors (20,28), transcriptional regulation by TAK1 involves interactions with and recruitment of other nuclear proteins (17). Since a number of well-established co-modulators of nuclear receptors did not interact with TAK1 (T. Nakajima and A.M. Jetten, unpublished data), we began to identify proteins that influence or mediate the transcriptional regulation by TAK1. To identify TAK1-interacting proteins, we performed yeast two-hybrid analysis using the hinge-LBD region of TAK1 as bait. A total of unique yeast clones were obtained that induced high β-galactosidase reporter activity in yeast two-hybrid analysis. One of these clones, encoding a novel TAK1-interacting protein referred to as TIP27 (Figure 1A), was selected for further study.

The *TIP27* gene encodes an mRNA of ~3.1 kb and a basic protein with a molecular mass of 27.1 kDa (Figure 1A) and a pI of 8.83. MotifScan and ScanProsite identified two putative zinc finger motifs (Figure 1A) with the consensus sequence $CX_4CX_{12}HX_4H$, one at the N-terminus between Cys^{14} and His^{37} (ZF1), and one at the C-terminus between Cys^{175} and His^{198} (ZF1). A third putative zinc finger motif (ZF3; $CXCX_{12}HX_4H$) was observed between Cys^{210} and His^{230}. In addition, TIP27 contains a Glu/Asp-rich region between Glu^{131} and Glu^{146}. TIP27 was found to be identical to the recently described JAZF1, a protein with unknown function (22). The human TIP27 sequence exhibited high homology (98% identity at the amino acid level) with an unpublished mouse sequence (GenBank accession no. AK050474) representing the mouse homolog of TIP27. In addition, the N- and C-termini of TIP27 containing, respectively, the first and second zinc finger motifs of TIP27 exhibit high homology with a hypothetical *Drosophila* protein (GenBank accession no. NP_651853), which suggests that it might be the *Drosophila* homolog of TIP27 (Figure 1B). The zinc finger motifs of TIP27 did not show homology with other zinc finger proteins.

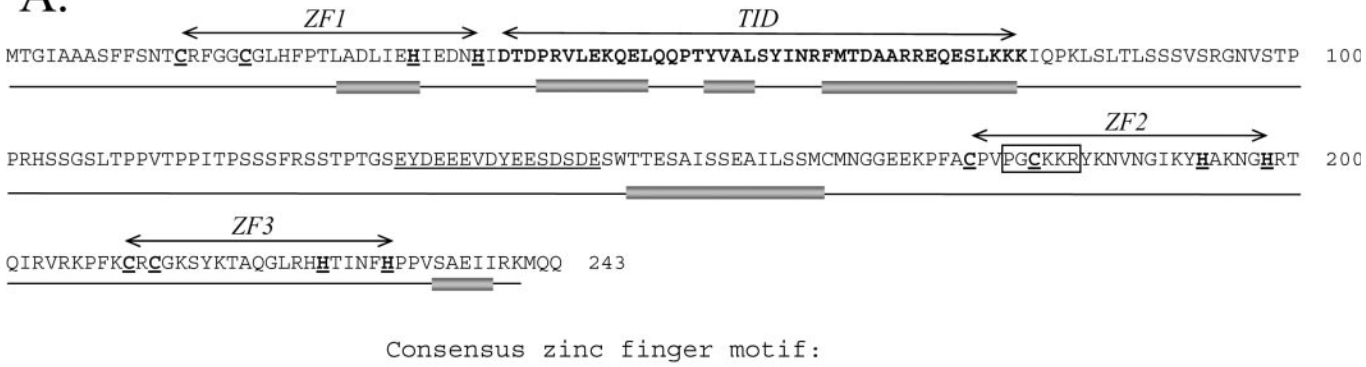
Tissue-specific expression of TIP27

To examine the pattern of tissue-specific expression of TIP27, northern-blot analysis was performed using RNA from several human tissues (Figure 2). TIP27 mRNA was most highly expressed in adult testis, at moderate levels in colon, placenta, prostate and ovary, and at low levels in several other tissues. In testis, the radiolabeled TIP27 probe hybridized to two major transcripts 3.2 and 0.9 kb in size while other tissues contained mainly the 3.2 kb transcript. The 3.2 kb TIP27 transcript (GenBank accession no. AY372319) contains a 2.1 kb 3′-untranslated region (3′-UTR). The 115 bp of 5′-UTR sequence contains a stop codon at 64 nt in agreement with our conclusion that 'MTGI . . .' is the N-terminus of TIP27 and that the 3.1 kb transcript encodes the full-length TIP27 protein.

TIP27 interacts selectively with TAK1

Since TIP27 was identified by yeast two-hybrid analysis, we examined its interaction with TAK1 by mammalian two-hybrid analysis. As shown in Figure 3, co-transfection of pM-TIP27 and VP16-TAK1 into CV-1 cells greatly enhanced $(UAS)_5$-driven LUC reporter activity confirming the interaction between these two proteins observed in yeast two-hybrid analysis. To examine the specificity of this interaction, we determined whether TIP27 was able to interact with the LBD of several other nuclear receptors, including AR, estrogen receptor α (ERα), progesterone receptor (PR), liver X receptor α (LXRα), PPARγ, RXRα, retinoid-related

A.



B.

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TIP27: ASFFSNTCRFGGCGLHFPTLADLIEHIEDNHIDTDPRVLEKQELQQPTYVALSYINRFMT 66
 DHP: -V-LI-V-KYN---IT--S-S---S----T---Y--K-V-QK-QA--ACLP---VL--I- 61

TIP27 : DAARREQESL..KKKIQPKLSLTLSSSVSRGNVSTPPRHSSGSLTPPVTPPITPSSSFRS 124
 DHP : EEN-K-AG-FLASNTTA-PTTNSG-NAAGH--GNVELKR...K-AIKHHSYSMS--NRST 118

TIP27 : STPTGSEYDEEEVDYEESDSDESWTTESAISSEAILSSMCMNG........GEEKPFACP 176
 DHP : .------.M--D-MVVT--EDSNDS----EF---F-MRYGSRHS-GGSNGTP-N------- 177

TIP27 : VPGCKKRYKNVNGIKYHAKNGHRTQIRVRKPFKCRCGKSYKTAQGLRHHTINFH 230
 DHP : -----------------S----KKDG----GY--H-----------KN-ALHT- 231
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Figure 1. (**A**) The amino acid sequence of human TIP27. The two zinc finger motifs (ZF1 and ZF2) and TID are indicated. The Cys and His residues participating in the tetrahedral configuration of the zinc fingers are indicated in boldface and underlined. The nuclear localization signal is boxed. The Glu/Asp-rich region is underlined. The TIP27 sequence was submitted to GenBank under accession no. AY372319. Bars indicate putative α-helical regions within TIP27. The consensus sequence of the two zinc finger motifs is shown at the bottom. (**B**) Partial sequence homology between TIP27 and a hypothetical *Drosophila* protein (DHP), the putative *Drosophila* homolog of TIP27. Zinc finger motifs are underlined.

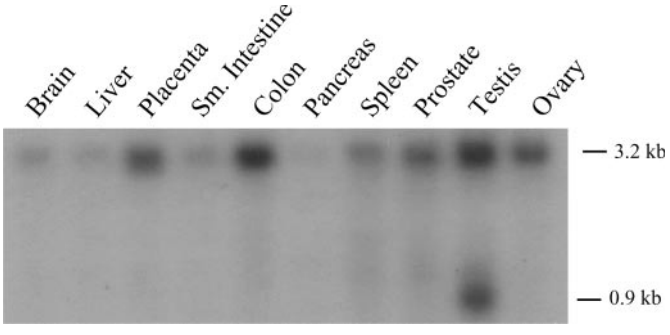

Figure 2. Tissue-specific expression of TIP27. Poly(A)$^+$ RNA (1.5 μg) from 10 different human tissues was examined by northern-blot analysis. Membranes were hybridized to a ^{32}P-labeled probe for TIP27 as described in Materials and Methods.

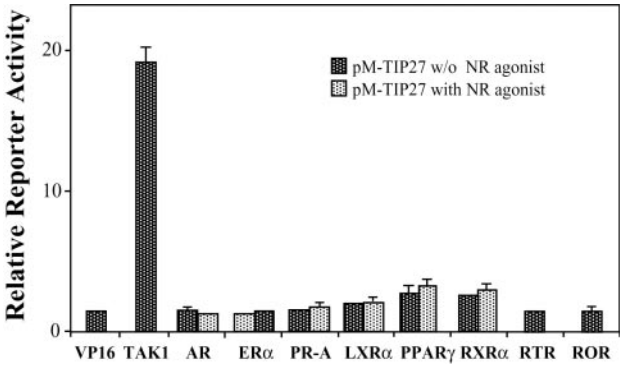

Figure 3. TIP27 interacts specifically with the nuclear orphan receptor TAK1. CV-1 cells grown in DMEM containing charcoal-stripped FBS were transfected with $(UAS)_5$-LUC, pRL-SV40, pM or pM-TIP27, and either VP16, VP16-TAK1, VP16-AR, VP16-ERα, VP16-PR-A, VP16-LXRα, VP16-PPARγ, VP16-RXRα, VP16-RTR or VP16-RORγ expression vectors as indicated. After 24 h, cells were treated with the respective ligand [1 μM 17β-estradiol (ERα), 22*R*-hydroxycholesterol (LXRα), GW347845 (PPARγ), SRI 11246 (RXRα), 5α-dihydrotestosterone (AR) or 100 nM progesterone (PR-A)] or vehicle and 48 h later assayed for reporter activities as described in Materials and Methods. The relative firefly LUC reporter activity was calculated and plotted as stated in Materials and Methods.

testis-specific receptor (RTR or GCNF) and retinoid-related orphan receptor γ (RORγ). AR, ERα, PR, PPARγ, LXRα and RXRα were analyzed in the presence and absence of their respective ligand. A small, ligand-independent increase in pM-TIP27-mediated reporter activity was observed when VP16-PPARγ(LBD) or VP16-RXRα(LBD) was co-transfected into the cells suggesting a weak interaction between TIP27 and these receptors (Figure 3). Increasing levels of VP16-PPARγ(LBD) did not further enhance reporter activity (data not shown). The LBD of the other receptors analyzed did not show any substantial interaction with TIP27, either in the presence or in the absence of ligand indicating that the interaction of TIP27 is rather selective for the LBD of TAK1. However, we cannot rule out that for some nuclear receptors TIP27 might interact with regions outside

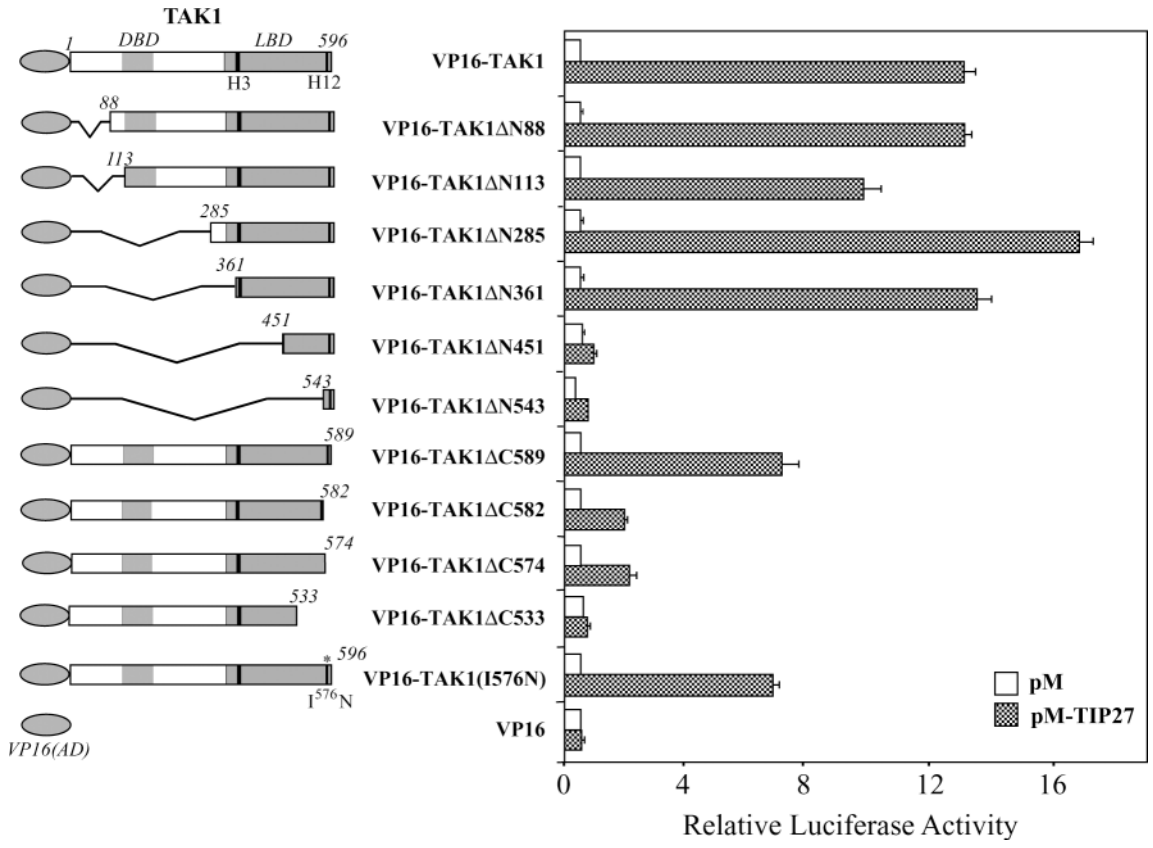

Figure 4. The TAK1 LBD is required for the interaction of TAK1 with TIP27. To determine the effects of various N- and C-terminal deletions and the $I^{576}N$ point mutation on the interaction of TAK1 with TIP27, CV-1 cells were transfected with $(UAS)_5$-LUC, pRL-SV40, pM or pM-TIP27, VP16 or various VP16-TAK1 mutants, as indicated. After 48 h, cells were collected and assayed for reporter activities as described in Materials and Methods. The relative reporter activity was calculated and plotted.

the LBD. In addition, the recruitment of TIP27 by nuclear receptors may depend on the promoter context of the target gene.

To determine what region in TAK1 is important for its interaction with TIP27, the effect of various TAK1 deletion and point mutations on this interaction was examined. N-terminal deletions up to Gly^{361} had little effect on this interaction; however, deletion up to Ile^{451} dramatically reduced reporter activity indicating that the H3–5 region within the LBD of TAK1 is required for its interaction with TIP27 (Figure 4). Deletion of 14 amino acids at the C-terminus, as in VP16-TAK1(ΔC582) removes part of the putative activation function 2 (AF2; ^{579}ILKMETA; helix 12), caused a 85% reduction in transactivation while transactivation is almost totally abolished in VP16-(TAK1(ΔC533). Mutating Ile^{576} in H12 to Asn decreased reporter activity by 50% (Figure 4). These results suggest that the TAK1 LBD from H3 until the C-terminus is required for optimal interaction with TIP27.

Identification of the TID

To identify the region within TIP27 required for its interaction with TAK1, we examined the ability of several TIP27 deletion mutants to interact with TAK1. Deletion of the N-terminus up to Asp^{39}, which contains the first zinc finger motif, rather increased than reduced reporter activity (Figure 5A). However, deletion up to Ser^{59} totally abolished the interaction between TIP27 and TAK1 suggesting that the region between Asp^{39} and Ser^{59} is required for this interaction. To further identify this region, the effect of C-terminal deletions on TIP27 interaction with TAK1 was investigated. C-terminal deletions up to Lys^{79} had little effect while deletion up to Ser^{59} greatly reduced the interaction with TAK1 (Figure 5A). These results indicate that the region between Asp^{39} and Lys^{79}, referred to as TAK1 interaction domain or TID, is critical for the interaction of TIP27 with TAK1.

To further characterize this TID, the effects of additional deletion and point mutations within the TID on the interaction of TIP27 with TAK1 were examined. This analysis showed that TIP27(39-101) and TIP27(39-79) were able to induce reporter activity in mammalian two-hybrid analysis whereas TIP27(59-101) and TIP27(59-79) did not (Figure 5B). In addition, TIP27(ΔTID) lacking the TID did not induce reporter activity. These effects of these deletion mutations are in agreement with our conclusion that TID is localized between Asp^{39} and Lys^{79} and required for the interaction with TAK1. We next examined the effect of four point mutations within the TID on the interaction of TIP27 with TAK1. The point mutations $K^{47}P$, $L^{76}P$ and $A^{69}P$ potentially disrupt the two main α-helices contained within the TID while the point mutation $Y^{60}D$ might disrupt

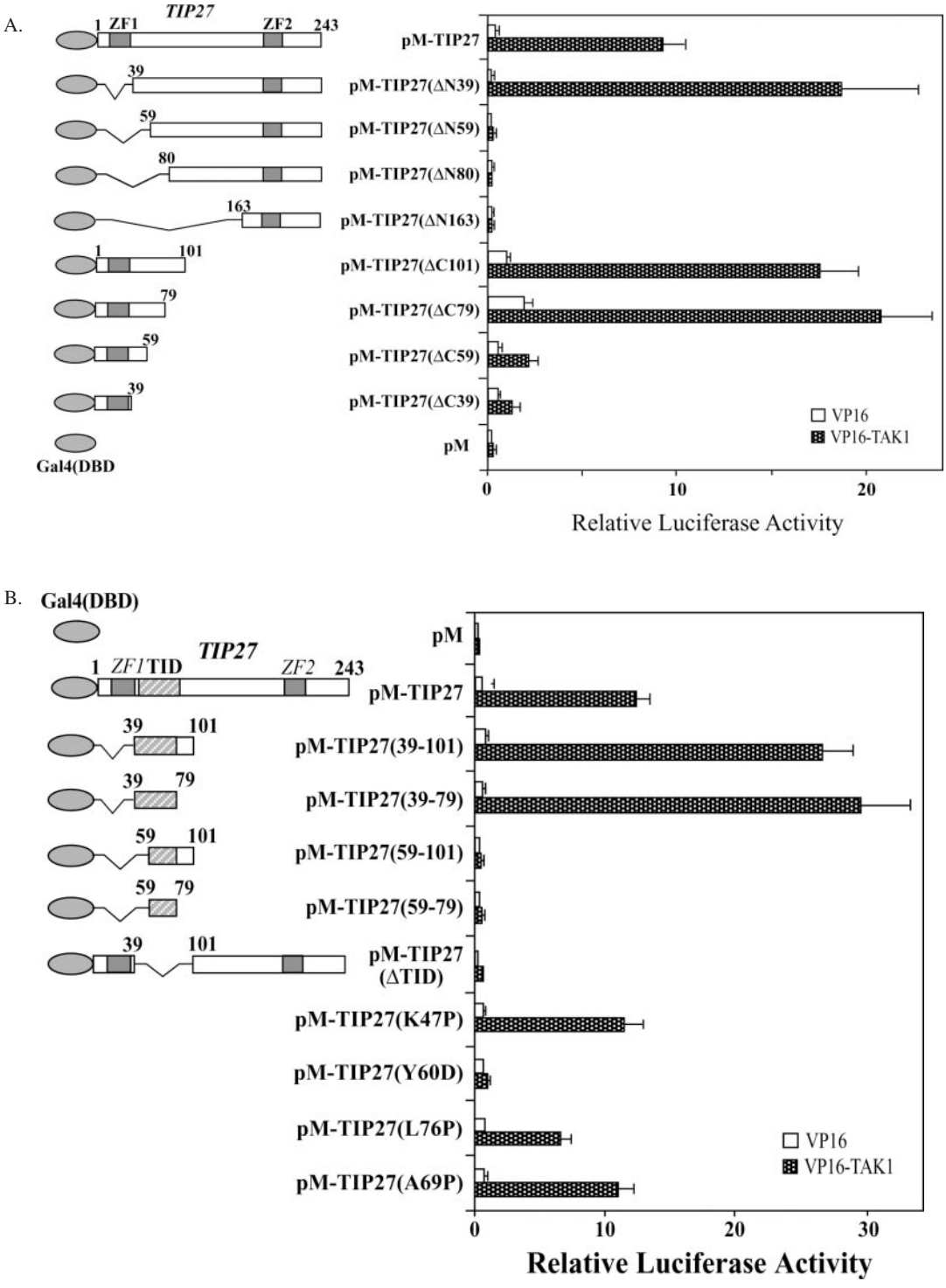

Figure 5. Identification of TID in TIP27. (**A**) Effect of several TIP27 N- and C-terminal deletions on the interaction of TIP27 with TAK1. (**B**) Mapping of the TID: effect of various deletion and point mutations within the TID on the interaction of TIP27 with TAK1. CV-1 cells were transfected with $(UAS)_5$-LUC, pRL-SV40, VP16 or VP16-TAK1, pM or various pM-TIP27 mutants, as indicated. After 48 h, the cells were collected and assayed for reporter activities as described in Materials and Methods. The relative reporter activity was calculated and plotted. The TID is localized between Asp^{39} and Lys^{79} of TIP27 (indicated by the hatched boxes). ZF, zinc finger domain.

potential phosphorylation of this tyrosine. The point mutation $Y^{60}D$ almost totally abolished the interaction between TIP27 and TAK1 while $L^{76}P$ reduced reporter activity ~40% (Figure 5B). This observation suggests that these amino acids are important in TIP27/TAK1 interactions and may function either as a structural determinant or as a target for phosphorylation. The point mutations $K^{47}P$ and $A^{69}P$ had little effect on the TIP27/TAK1 interaction.

TIP27 interacts with TAK1 *in vitro*

To determine whether TAK1 and TIP27 physically interact, we performed pull-down assays using GST-TIP27 and ^{32}S-labeled TAK1 (Figure 6). As shown in Figure 6B, GST-TIP27 was able to pull down TAK1. No TAK1 band was observed in the control (GST only). Pull-down assays with the TIP27 deletion mutants TIP27(ΔC101) and TIP27(39-101), which still contain the TID, showed that both deletion mutants were able to pull down TAK1 (Figure 6C). These GST-TIP27 proteins did not pull down the nuclear receptor RTR, which was shown in mammalian two-hybrid analysis not to interact with TIP27. GST-TIP279(ΔN79), lacking the TID, did not pull down TAK1 (Figure 6C). These results indicate that TAK1 and TIP27 physically interact and are in agreement with the conclusion that this interaction requires the TID of TIP27 (Figure 5).

To determine whether TAK1 and TIP27 form a complex in intact cells, we expressed Flag-TAK1 and myc-TIP27 fusion proteins in COS-1 cells and performed IP analysis with isolated protein lysates prepared from transfected cells. As shown in Figure 6D, Flag-TAK1 was able to pull down myc-TIP27 suggesting that in intact cells the two proteins are in a complex with each other. Flag-TAK1 did not pull down TIP27 lacking the TID (Figure 6D) while Flag-RORγ, a receptor unable to interact with TIP27, was unable to pull down TIP27 (data not shown). A similar result was obtained with Flag-PPARγ (data not shown). These results indicate the importance of the TID and the selectivity of the TIP27/TAK1 interaction.

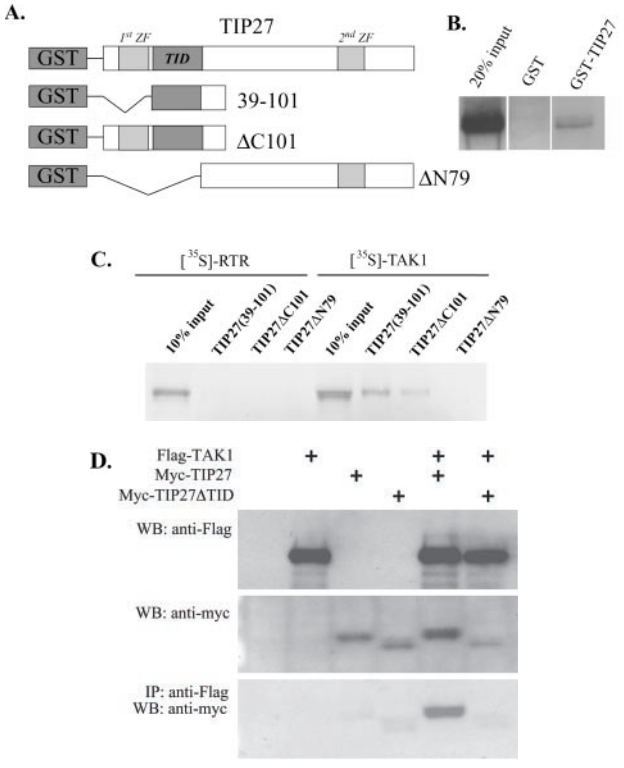

Figure 6. TIP27 interacts directly with TAK1. (**A**) Schematic representation of GST-TIP27 fusion proteins used in pull-down assays. (**B**) and (**C**) GST and different GST-TIP27 fusion proteins were bound to glutathione–Sepharose 4B beads and then incubated with [^{35}S]methionine TAK1 or the nuclear receptor RTR. After 1 h incubation, beads were washed extensively and bound proteins solubilized and analyzed by western-blot analysis. Radiolabeled proteins were visualized by autoradiography. (**D**) TAK1 pulls down TIP27, but not TIP27(ΔTID), from cellular extracts prepared from COS-1 cells co-transfected with p3XFlagCMV-TAK1 and pCMV-myc-TIP27 or pCMV-myc-TIP27(ΔTID). Protein lysates were prepared 48 h after transfection as described in Materials and Methods. One part was used in western-blot (WB) analysis using anti-Flag or anti-Myc antibodies, the remaining was used in IP assay using anti-Flag M2 agarose resin. Bound proteins were then examined by western-blot analysis using an anti-Myc antibody.

Subcellular localization

ScanProsite and PFSCAN analysis indicated the presence of a putative nuclear localization signal (NLS) at Pro^{178}-Tyr^{184} that is contained within the second zinc finger motif. To examine the subcellular localization of TIP27, COS-1 cells were transfected with pEGFP-TIP27 and 30 h later the subcellular distribution of EGFP-TIP27 was analyzed by confocal microscopy. Figure 7A shows that the full-length TIP27 protein localized to the nucleus. In more than 90% of COS-1 cells, TIP27 was detected predominantly in the nucleus and in most cells excluded from the nucleoli. Its punctated pattern suggests that it is associated with a larger nuclear complex.

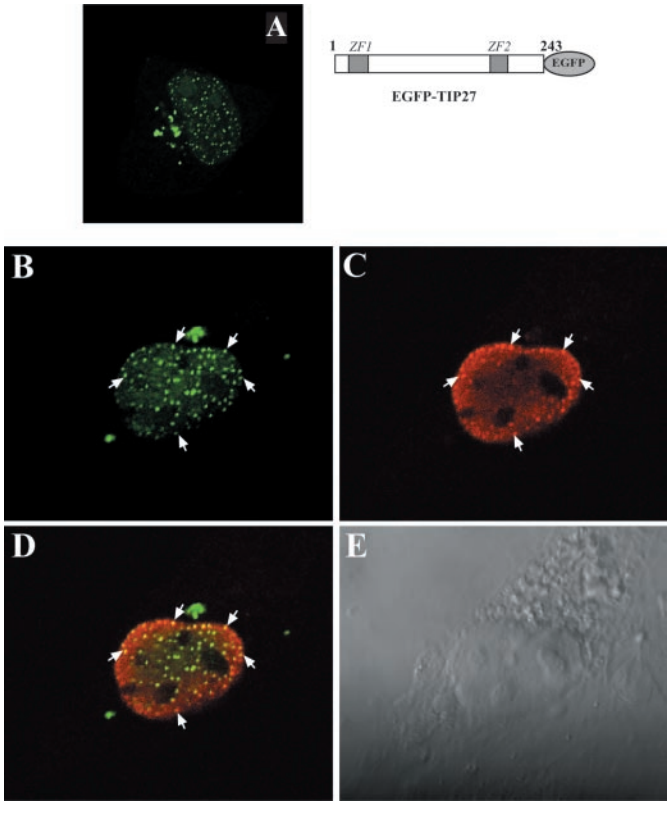

Figure 7. Localization of TIP27 and co-localization with TAK1. COS-1 cells were transfected with pEGFP-TIP27 (**A**) or co-transfected with pEGFP-TIP27 and p3XFlag-CMV-TAK1 (**B**–**E**) expression vectors as indicated. After 24 h, expression of EGFP-TIP27 and Flag-TAK1 fusion proteins was visualized using fluorescence confocal microscopy as described in Materials and Methods. Flag-TAK1 was detected by incubating cells with anti-FLAG M2 and subsequently with Alexa Fluor® 594 goat anti-mouse antibodies. (**A**) Localization of EGFP-TIP27 transfected alone into COS-1 cells; (**B**) localization of EGFP-TIP27 co-transfected with TAK1; (**C**) localization of TAK1 (Alexa Fluor® 594) co-transfected with TIP27; (**D**) overlap of the EGFP and Alexa Fluor® 594 images; and (**E**) DIC image.

Co-localization of TIP27 and TAK1

To examine the co-localization of TIP27 and TAK1, COS-1 cells were co-transfected with pEGFP-TIP27 and p3XFlag-CMV-TAK1. As shown in Figure 7B–E, both TIP27 and TAK1 were expressed in the nucleus in a very similar punctated pattern (see arrows). These results support the conclusion that in cells TIP27 and TAK1 are part of the same protein complex. No co-localization of TIP27 was observed with the nuclear orphan receptor Flag-RORγ (data not shown).

Inhibition of TAK1-induced transcription by TIP27

Transcriptional activation by TAK1 is mediated through binding to a DR1 response element (10). This transactivation has been reported to be cell type-dependent; TAK1 is able to induce transcriptional activation in COS-1 cells but not in CV-1 cells (29). To determine the function of TIP27, we examined its effect on the induction of TAK1-mediated transcription in COS-1 cells. For this purpose, cells were co-transfected with pSG5-TAK1 and pcDNA-TIP27 expression vectors and a $(DR1)_3$-CAT reporter. Transfection with pSG-TAK1 alone caused a 6.5-fold induction of DR1-dependent transcriptional activation while transfection with pcDNA-TIP27 alone had little effect on basal transcription (Figure 8A). However, co-transfection with increasing amounts of pcDNA-TIP27 effectively repressed TAK1-mediated induction of transcription (Figure 8A). In contrast, TIP27(ΔTID) had little effect on TAK1-mediated transactivation. In addition, expression of TIP27 had only a minor effect on the transcriptional activation induced by the nuclear receptors RORγ and PPARα suggesting that effect of TIP27 on transcription is highly selective for TAK1 and appears not to be mediated through a general effect on transcription (Figure 8B).

Previous studies have shown that TAK1 binds as a homodimer to DR1 response elements (1,11). Therefore, the repression by TIP27 could be due to an inhibition of TAK1 homodimerization or the binding of TAK1 to DR1 response elements. To analyze the effect of TIP27 on homodimerization, we carried out mammalian two-hybrid analysis with Gal4(DBD)-TAK1 and VP16(AD)-TAK1. Homodimerization of TAK1 would lead to increased transactivation of the reporter through the activation domain (AD) of VP16. For this purpose, pM-TAK1 and VP16-TAK1 were co-transfected with $(UAS)_5$-LUC into CV-1 cells in the presence or absence of pcDNA-TIP27. As shown in Figure 8C, TIP27 did not inhibit Gal4(DBD)-TAK1 and VP16(AD)-TAK1 mediated transactivation. These results indicate that transcriptional activation through VP16 appeared not to be affected by TIP27 in agreement with the conclusion that repression by TIP27 is very selective for TAK1. In addition, these results suggest that TIP27 did not affect TAK1 homodimerization. Because VP16(AD)-mediated transactivation was not affected by TIP27, we were able to examine the effect of TIP27 on the binding of VP16-TAK1 to the TAK1 (DR1) response element. For this purpose, VP16-TAK1 and $(DR1)_3$-CAT were co-transfected into COS-1 cells with or without pcDNA-TIP27. As shown in Figure 8D, VP16-TAK1 caused a 10-fold increase in DR1-dependent transcriptional activation. TIP27 had little effect on this transactivation suggesting that it does not affect the binding of TAK1 to DR1.

DISCUSSION

In this study, we describe the identification of a novel TAK1-interacting protein, referred to as TIP27, which functions as a co-repressor for the nuclear orphan receptor TAK1. TIP27 is highly conserved (98%) between mouse and human and contains three putative C_2H_2-type zinc finger motifs. These motifs exhibit little homology with zinc fingers found in other mammalian zinc finger protein families; however, the regions at the N- and C-termini of TIP27 including the zinc finger motifs displayed high homology with two regions in an unpublished *Drosophila* protein identified in the GenBank database (Figure 1B). We hypothesize that this protein is the *Drosophila* homolog of TIP27. Examination of the subcellular localization of TIP27 showed that it localizes to the nucleus in a punctated pattern suggesting that it is part of a larger protein complex. These observations are in agreement with the hypothesis that TIP27 functions as co-modulator of TAK1.

TIP27 is expressed in several tissues, including placenta, colon, prostate and testis. As TAK1, TIP27 mRNA is most abundant in adult human testis where it is expressed as a 0.9 and 3.2 kb transcript. The smaller mRNA is restricted to the testis while all other tissues analyzed contain only the larger transcript. The generation of multiple and unique transcripts in the testis is not limited to TIP27 but has been reported for many genes, including TAK1 and RTR (8,30–32). These transcripts can be generated by alternative splicing, the testis-specific use of alternative promoter and transcription start sites, or the selection of alternative polyadenylation signals. Preliminary results have indicated that in testis the different TIP27 transcripts are generated by the use of different polyadenylation signals. Previous studies (4,8) have shown that TAK1 mRNA was most abundant in pachytene spermatocytes and expressed at lower levels in round spermatids. *In situ* hybridization analysis has shown that in testis TIP27 is also most highly expressed in pachytene spermatocytes and round spermatids (unpublished data). These observations suggest that in the testis the expression of TIP27 and TAK1 overlap. The observed co-expression is in agreement with the proposed functional relationship between these two proteins.

Recruitment of co-activators and co-repressors is a critical step in, respectively, nuclear receptor-mediated activation and repression of gene expression (20,28). This likely also applies to the activation and repressor functions of TAK1. However, several known co-activators and co-repressors, including NCoA1, NCoA2, PGC-1α, NCoR1 and NCoR2, that have been reported to interact with many nuclear receptors, do not appear to interact with TAK1 (T. Nakajima and A.M. Jetten, unpublished data). These observations suggest that in this regard, TAK1 behaves like an atypical receptor and, therefore, may recruit other co-activators and co-repressors that have specificity for TAK1. The identification of TIP27 as a selective modulator of TAK1, appears to support this conclusion. Both mammalian two-hybrid and pull-down analyses showed that the TIP27 gene encodes a protein that interacts selectively with the nuclear receptor TAK1. Confocal microscopy demonstrated that in the nucleus TAK1 and TIP27 co-localize suggesting that they are part of the same protein complex. Moreover, *in vitro* pull-down analysis indicated that TIP27 interacts directly with TAK1 while it did not bind the nuclear orphan receptor RTR. In many nuclear receptors

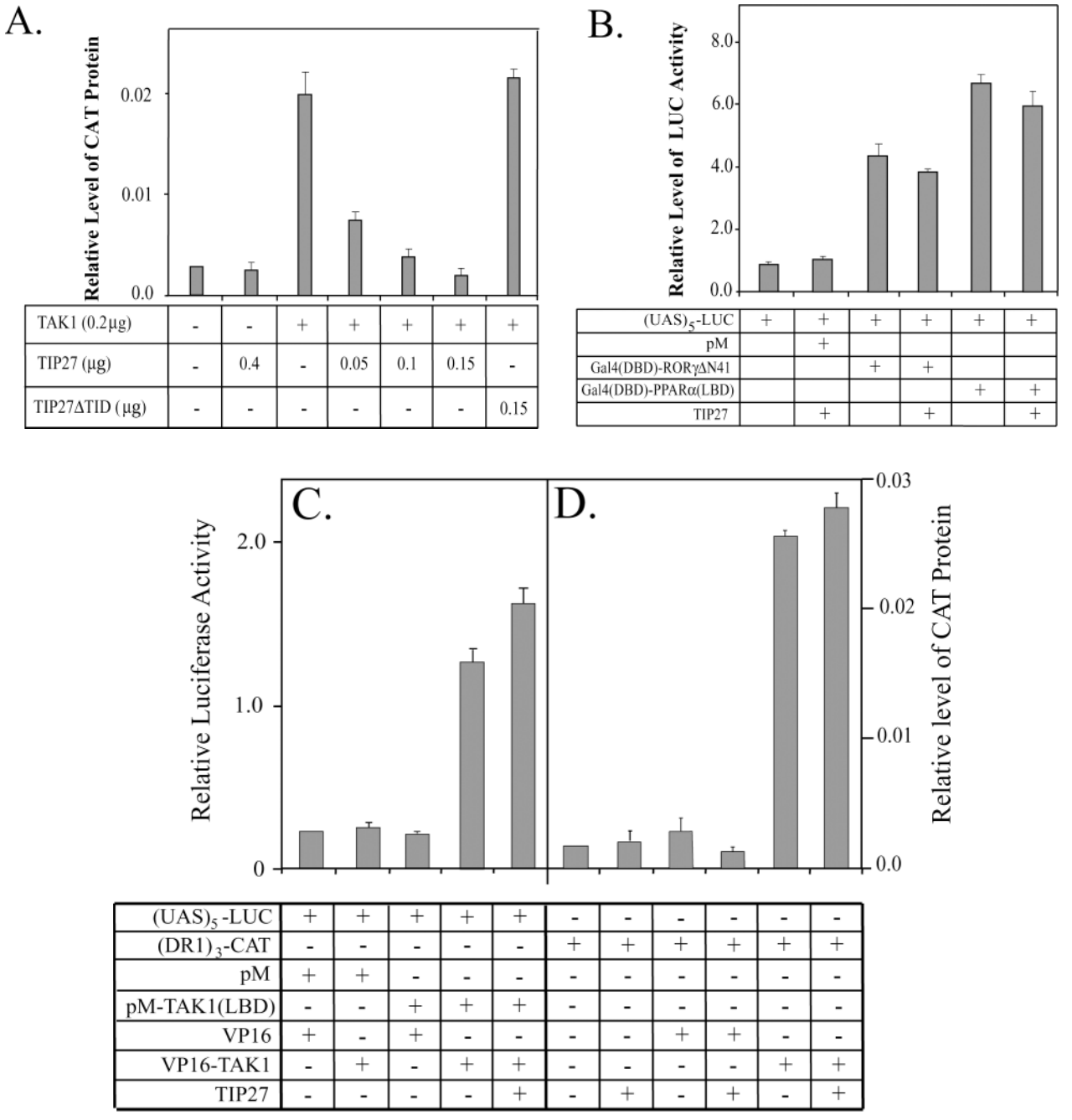

Figure 8. (**A**) and (**B**) TIP27, but not TIP27(ΔTID), functions as a repressor of DR1-dependent transcriptional activation by TAK1 and has little effect on RORγ- and PPARα-mediated transactivation. COS-1 cells were transfected with $(DR1)_3$-CAT, CMV-βGal, TAK1 and increasing amounts of pcDNA4-TIP27 expression vectors as indicated. Another series were transfected with $(UAS)_5$-LUC, pFR-LUC, Gal4(DBD)RORγ(ΔN41) or Gal4(DBD)PPARα(LBD) expression vectors in the presence or absence of pcDNA4-TIP27 (0.1 μg). The PPARα agonist GW327647 (100 nM) was added after 6 h to cells transfected with Gal4(DBD)PPARα(LBD). After 48 h, cells were collected and assayed for reporter activities as described in Materials and Methods. The relative reporter activity was calculated and plotted. (**C**) TIP27 does not affect VP16 transactivation function and TAK1 homodimerization. CV-1 cells were transfected with $(UAS)_5$-LUC, pM-TAK1 and VP16-TAK1 in the absence and presence of TIP27 expression vector (0.15 μg) as indicated. (**D**) TIP27 does not inhibit binding of TAK1 to DR1. COS-1 cells were transfected with $(DR1)_3$-CAT and VP16-TAK1 in the absence and presence of TIP27 expression vector (0.15 μg).

helices 3–5 together with helix 12 in the LBD form a hydrophobic cleft and patch clamp that serve as an interaction surface for co-repressors and co-activators. Deletion mutant analyses demonstrated that deletion of the helix 3–5 region or the putative AF2 domain (helix 12; ^{579}ILKMETA) of TAK1 dramatically reduces its interaction with TIP27 suggesting that TIP27 interacts with the LBD of TAK1 and that helix 3–5 and helix 12 are required for optimal interaction.

Deletion and point mutation analyses identified a 40 amino acid long region from Asp^{39} to Lys^{79}, referred to as the TID, as being critical for the interaction of TIP27 with TAK1. Deletion of this region and the point mutation Y^{60}D within the

TID abolished the interaction of TIP27 with TAK1. As demonstrated for the interaction of nuclear receptor with other co-repressors, the interaction of TIP27 is dependent on the LBD of the receptor. The binding of several co-activators and co-repressors are mediated by a α-helix containing an LXXLL or I/LXXII consensus motif (33–37). The co-repressor RIP140 interacts with nuclear receptors through LXXLL α-helix motifs, while other co-repressors, such as NCoR1 and NCoR2, bind receptors through a related motif with the consensus I/LXXII. Although the TID of TIP27 contains several α-helices (Figure 1A), it lacks the LXXLL or I/LXXII consensus sequence suggesting that the interaction TIP27 with TAK1 may involve a different interaction motif.

In addition to the TID, TIP27 contains three putative zinc finger motifs and a Glu/Asp-rich region. Zinc finger motifs have been involved in protein–protein as well as protein–DNA interactions. We speculate that the zinc finger domains and the Glu/Asp-rich region within TIP27 might play a role in the recruitment of other nuclear proteins to the TAK1–TIP27 complex. Alternatively, TIP27 might have additional roles, such as functioning as a transcription factor itself. Its zinc finger motifs could therefore be involved in the recognition of specific DNA motifs. Future studies are needed to establish the function of these domains.

Study of the function of TIP27 demonstrated that TIP27 almost totally abolished the induction of DR1-dependent transcription by TAK1 in COS-1 cells (Figure 8A). VP16-, RORγ- and PPARα-mediated transactivation were not or only slightly affected by TIP27 (Figure 8B and D). These results suggest that TIP27 is a rather specific repressor of TAK1-mediated transcriptional activation and not a general repressor of transcription. Deletion of the TID within TIP27 totally abrogates its repressor activity in agreement with our observations that this domain is required for TIP27/TAK1 interactions. Since the *in vitro* pull-down analysis showed that TIP27 interacts directly with TAK1, the repression of TAK1-mediated transactivation by TIP27 could be mediated through an inhibition of TAK1 homodimerization or the binding of TAK1 to its DNA response element DR1. However, the results presented in Figure 8C and D indicate that TIP27 does not inhibit TAK1 homodimerization and does not prevent the binding of TAK1 to DR1. Therefore, the mechanism by which TIP27 represses TAK1-mediated transactivation, differs significantly from that of the recently described TR4(TAK1)-associated protein TRA16, which exhibits no sequence similarity with TIP27 (29). The repression of TAK1-mediated transactivation by TRA16 involves an inhibition in TAK1 homodimerization and binding of TAK1 to its DNA response element DR1 (29). In addition, in contrast to TRA16, the interaction of TIP27 with TAK1 requires the LBD of TAK1 and is independent of its DBD.

Since TIP27 does not inhibit TAK1 homodimerization or DR1 binding, the repression of TAK1-mediated transactivation by TIP27 might involve inhibition of co-activator recruitment. Such a mechanism has been proposed for the inhibition of nuclear receptor-induced transcription by the co-repressor RIP140 (38,39). Binding of RIP140 to nuclear receptors competes with the recruitment of co-activators, such as NCoA1 and PGC-1α, and promotes the recruitment of histone deacetylases. However, it is not yet possible to test this hypothesis since several known co-activators, including NCoA1 and PGC-1α, are unable to interact with TAK1 (T. Nakajima and A.M. Jetten, unpublished data) and no other co-activators that interact with TAK1 have yet been identified. We are in the process of determining whether other TAK1-interacting proteins identified in our yeast two-hybrid screen function as co-activators of TAK1.

In summary, in this study we describe the identification of TIP27 as a novel nuclear modulator of the nuclear orphan receptor TAK1. Mammalian two-hybrid and pull-down analyses indicate that TIP27 interacts rather specifically with the LBD of the nuclear orphan receptor TAK1 and identify TID as a region within TIP27 that plays a critical role in the interaction of TIP27 with TAK1. Future studies have to determine the physiological function of TIP27 in the repression of gene transcription by TAK1.

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