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A nuclear hormone receptor-associated protein that inhibits transactivation by the thyroid hormone and retinoic acid receptors

(transcription/steroid receptor/two-hybrid system)

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ABSTRACT **Nuclear hormone receptors are transcription factors that require multiple protein–protein interactions to regulate the expression of their target genes. Using the yeast two-hybrid system, we identified a protein, *t*hyroid hormone *r*eceptor *u*ncoupling *p*rotein (TRUP), that specifically interacts with a region of the human thyroid hormone receptor (TR) consisting of the hinge region and the N-terminal portion of the ligand binding domain in a hormone-independent manner. Interestingly, TRUP inhibits transactivation by TR and the retinoic acid receptor but has no effect on the estrogen receptor or the retinoid X receptor in mammalian cells. We also demonstrate that TRUP exerts its action on TR and retinoic acid receptor by interfering with their abilities to interact with their DNA. TRUP represents a type of regulatory protein that modulates the transcriptional activity of a subclass of the nuclear hormone receptor superfamily by preventing interaction with their genomic response elements.**

Members of the nuclear hormone receptor superfamily are ligand-activated transcription factors (1–4). This superfamily includes the receptors for steroid hormones, thyroid hormones, lipophilic vitamins such as vitamins A and D, and the orphan receptors, which have structure consistent with other superfamily members but have no identified ligands. Nuclear receptors regulate gene expression by interacting with specific DNA sequences (response elements) in the promoters of target genes (5, 6). Receptors for the classical steroid hormones bind to their respective response elements in a ligand-dependent manner; however, some receptors such as the thyroid hormone receptor (TR) and retinoic acid receptor (RAR) bind to their response elements in a ligand-independent manner. The receptors modulate the rate of transcription of target genes by interacting with basal transcription machinery either directly (7–10) or indirectly via TATA binding protein-associated factors (11). The interaction of nuclear receptors with the basal transcriptional machinery may also be regulated by ligand binding. TR is an example of this level of regulation by ligand binding. In the absence of ligand, the ligand binding domain (LBD) of TR interacts with the basal transcription factor, TFIIB. In the presence of ligand this region of TR fails to interact with TFIIB (8). This complex ligand-dependent interaction of TR with TFIIB may play a role in the mechanism by which TR silences target gene transcription in the absence of ligand and activates target gene transcription in the presence of ligand (8, 12).

Recently, we demonstrated that TR must interact with a protein(s), termed a corepressor, to silence gene transcription (13). The corepressor interacts with a small region of TR consisting of the hinge region and the N-terminal portion of the LBD (amino acids 168–259). Using the yeast two-hybrid system, we attempted to identify the corepressor that interacts with this region of TR. In the course of these experiments, we serendipitously identified a protein that inhibits the ability of TR and the RAR to transactivate target genes.

MATERIALS AND METHODS

Plasmid Construction. The GAL4 DBD-TR168–259 yeast expression plasmid (pAS1cyh-TR168–259) (where DBD is DNA binding domain) was constructed by inserting the *Bam*HI/*Hin*dIII (filled) fragment of pABΔgal TR168–259 (13) into the *Bam*HI/*Sal* I (filled) sites of pAS1-cyh (14). pTRE2LacZ was constructed by inserting two copies of a DR4 oligonucleotide (15) into the *Bgl* II site of PC3 (16). p17merLacZ has been described previously (17). To construct pCBUB GAL TR168–456, *Hin*dIII-digested (and filled) pAB Gal TR168–456 was partially digested with *Bgl* II. The resulting *Bgl* II–*Hin*dIII (filled) fragment was inserted into the *Nco* I–*Pvu* II sites of pCBUB linker (17). YepTRβ was constructed by first inserting an *Eco*RI fragment containing the entire coding sequence of human TR β (hTRβ) into BCpV2 (18). The *Afl* II/*Kpn* I fragment of this vector was then subcloned into the corresponding sites of the yeast expression vector YepV1 (18). pCMV4-TRUP (where TRUP is TR uncoupling protein) was constructed from pBK-CMV-TRUP. pBK-CMV-TRUP was created by first inserting the *Xho* I fragment of pACT-TRUP (cor 1.3) into the *Xho* I site of pBK-CMV (Stratagene) followed by sequencing to determine the correct orientation. The *Hin*dIII–*Xba* I fragment of pBK-CMV-TRUP was then inserted into the *Hin*dIII–*Xba* I sites of pCMV4 (19) to create pCMV4-TRUP.

Two-Hybrid System. The yeast strain y190 (*MAT***a**, *leu2-3, 112, ura3-52, trp1-901, his2-D200, ade2-101, gal4Δgal180Δ URA3 GAL-lacZ, LYS GAL-HIS3, cyh*^{r}) containing pAS1cyh TR168–259 was transformed with a human B-lymphocyte cDNA library in pACT (14) and plated on SC medium lacking tryptophan, leucine, and histidine (containing 25 mM 3-aminotriazole) as described by Durfee *et al.* (14). His$^+$ colonies exhibiting β-galactosidase activity using the filter lift assay were further characterized. β-Galactosidase activity was determined using chlorophenyl red β-D-galactopyranoside (CPRG) as described (14). To recover the library plasmids, total DNA from yeast was isolated and used to transform *Escherichia coli*

Abbreviations: TRUP, thyroid hormone receptor uncoupling protein; TR, thyroid hormone receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; ER, estrogen receptor; EMSA, electrophoretic mobility shift assay; DBD, DNA binding domain; hTRβ, human TR β; LBD, ligand binding domain; CAT, chloramphenicol acetyltransferase; tk, thymidine kinase.

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(HB101). Transformants were identified on minimal medium lacking leucine and containing ampicillin. To ensure that the correct cDNAs were identified, library plasmids isolated were retransformed into y190 containing pAS1cyhTR168–259 and β-galactosidase activity was determined. The specificity of the interaction of TRUP with TR was determined by mating y190 containing pACT-TRUP with the strain y187 (*MATα, gal4, gal80, his3, trp1-901, ade2-101, ura3-52, leu2-3,-112, URA3 GAL-lacZ met-*) containing either pAS1-SNF, pAS1-CDK2, pAS1-p53, or pAS1-lamin. The β-galactosidase activity of these diploids was examined using the filter lift and CPRG methods. The interaction of TRUP with GAL TR 168–456 and full-length TR was examined using a modified two-hybrid system. The yeast strain BJ2168 (genotype *MATa, prc1-407, Prb1-1122, pep4-3, leu2, trp1, ura 3-52*) was transformed with either pCBUB GAL TR 168–456 or YepTR and their corresponding reporters (p17mer LacZ and pTRE2 LacZ, respectively). Also included was empty pACT for controls or pACT-TRUP. β-Galactosidase activity was quantitated as described (17).

Transfections. Transfections of the Lmtk$^-$ cells and chloramphenicol acetyltransferase (CAT) assays were performed as described (20). Equivalent amounts of empty expression vector (pCMV4) were included in cells cotransfected with only a receptor and reporter and not pCMV4-TRUP. The expression and reporter plasmids pRSV-TR, pRSV-RAR, pRSV-RXR, pRSV-ER, DR4-tkCAT, DR5-tkCAT, DR1-tkCAT, and ERE2-tkCAT have been described (15, 21–23).

Electrophoretic Mobility Shift Assay (EMSA). All proteins used in the EMSA were *in vitro* translated using the TNT system (Promega) according to the manufacturer's protocol. The plasmids used for *in vitro* translation of the receptors, pT7-TR, pT7-RAR, pT7-RXR, and pT7-ER, have been described (15, 21–23). pBK-CMV-TRUP was used to *in vitro* translate TRUP. The EMSA was performed using unlabeled translated receptors that were preincubated for 15 min at room temperature with unlabeled *in vitro* translated TRUP in a total volume of 8 μl [containing 2 μl of 5× bandshift buffer (1× bandshift buffer = 60 mM KCl/5 mM $MgCl_2$/20 mM Hepes, pH 7.5/2 mM dithiothreitol/10% glycerol)]. Approximately equal amounts of receptor and TRUP were incubated, as determined by SDS/PAGE analysis of ^{35}S-labeled protein made in parallel with the unlabeled protein used in the EMSA. Two microliters of a solution containing 0.1 ng of ^{32}P-labeled DNA response element and 300 ng of pGEM (digested with *Hpa* II) was added to the reaction mixture and incubated an additional 15 min at room temperature. The response elements (DR1, DR4, DR5, and estrogen receptor response element) and the methods for their labeling have been described (15, 23). Protein–DNA complexes were resolved on a 5% polyacrylamide gel (15).

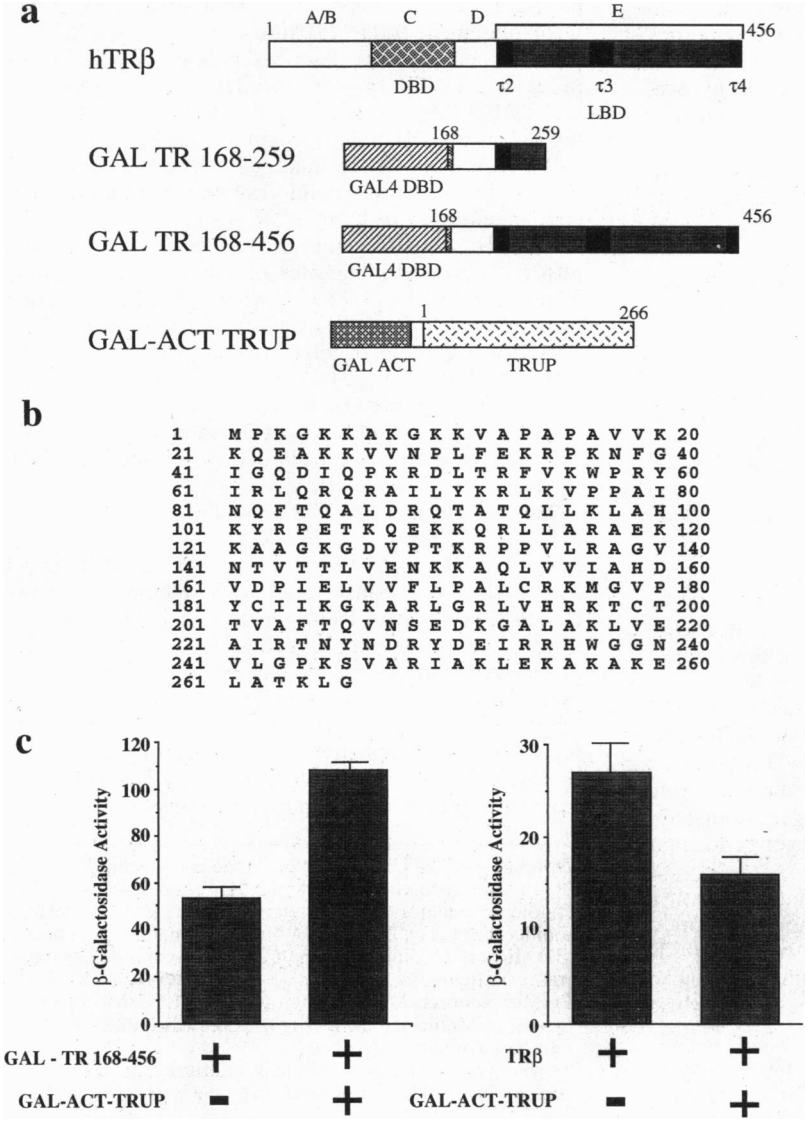

FIG. 1. Identification of a protein that interacts with the hTRβ using the yeast two-hybrid system. (*a*) Schematic of full-length TR, the GAL4 DBD TR (amino acids 168–259) fusion protein used for screening a cDNA library fused to the GAL4 activation domain (GAL-ACT), the GAL4 DBD-TR (amino acids 168–456) fusion protein, and the GAL4-ACT fusion protein identified, which specifically interacts with the 168- to 259-amino acid fragment of TR. τ2, τ3, and τ4 represent previously characterized transactivation domains (6). (*b*) The identified cDNA encodes a previously identified 266-amino acid protein, surf-3 or L7a ribosomal protein (GenBank accession no. M36072), which is in frame with the N-terminal GAL4-ACT. After functional characterization, we termed the identified protein TRUP. (*c*) TRUP interacts with the entire LBD of TR; however, it decreases the transcriptional activity of full-length TR.

RESULTS AND DISCUSSION

To identify proteins that interact with and mediate and/or modulate the activity of TR we employed the yeast two-hybrid system (24). A fragment of TR consisting of the hinge region and the N-terminal portion of the LBD (amino acids 168–259) was fused to the GAL4 DBD (Fig. 1*a*). Previously, we demonstrated that this region of TR interacts with proteins that are required for its function (13). The GAL4 DBD-TR 168–259 construct was used to screen a human B-lymphocyte cDNA library (fused to the GAL4 transactivation domain) (14). Approximately 500,000 recombinants were screened, of which 20 were classified as positive. One positive clone, cor 1.3, interacted with TR but not with other unrelated proteins such as lamin, p53, cyclin, and SNF (data not shown). cor 1.3 cDNA (≈900 bp) was recovered from the yeast, sequenced, and shown to contain an open reading frame (ORF) of 798 bp (266 amino acids) (Fig. 1 *a* and *b*). The initiation codon in the identified ORF was in frame with the sequence of the GAL4 activation domain. Based on its interaction with TR and subsequent functional characterization, we named the protein encoded by cor 1.3 TRUP. Sequence comparison indicated that TRUP is identical to the previously identified surf-3, PLA-X, and the L7a ribosomal protein cDNAs (25–27). Although this protein has been recovered previously from ribosomal preparations, and has been shown to be contained within the nucleus/nucleolus by histochemistry, it has no proven function (26).

To initially characterize the interaction of TRUP with the intact LBD of TR, we fused the hinge and entire LBD (amino acids 168–456) of TR to the GAL4 DBD (GAL TR 168–456) (Fig. 1*a*) and examined the ability of TRUP to interact with this protein in the two-hybrid system. As shown in Fig. 1*c*, coexpression of GAL-ACT TRUP increased the transcriptional activity of GAL TR 168–456. More importantly, we examined the ability of TRUP to interact with full-length TR utilizing its own DBD and not the GAL4 DBD. Intriguingly, coexpression of GAL-ACT TRUP significantly decreased the transcriptional activity of full-length TR (Fig. 1*c Right*).

To investigate the inhibitory activity of TRUP on the function of TR, we subcloned the TRUP coding sequence (without the GAL4 activation domain) into a mammalian expression vector. Lmtk$^-$ cells were cotransfected with an expression vector for the full-length TR and a reporter containing a TR response element (DR4) upstream of the thymidine kinase promoter-CAT gene reporter (tkCAT) (15) in the presence or absence of an expression vector for TRUP. In cells transfected with TR and the reporter, normal hormone-dependent activation of the reporter was observed (Fig. 2*a*, lanes 3 and 4). However, in cells cotransfected with TRUP, TR was no longer able to activate reporter gene transcription (Fig. 2*a*, lane 6). The inhibitory effect of TRUP on TR transactivation was dose dependent (data not shown). Furthermore, in the presence of TRUP, TR was less effective in silencing the basal transcription of a reporter gene in the absence of ligand (data not shown).

To examine whether the inhibitory effect of TRUP is specific for TR, we also examined the effects of TRUP on RAR, a nuclear hormone receptor closely related to TR. Lmtk$^-$ cells were cotransfected with an expression vector for

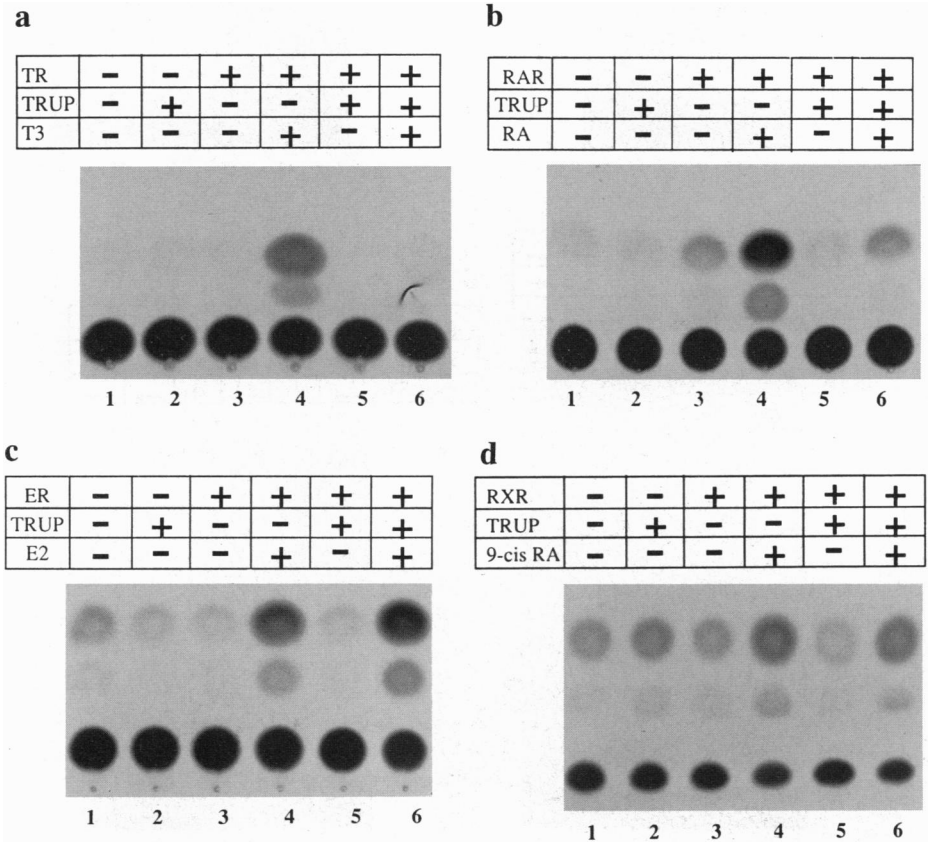

FIG. 2. Inhibition of TR- and RAR-mediated transactivation by TRUP in mammalian cells. (*a*) Representative data from CAT assays from Lmtk$^-$ cells transiently transfected with pRSV-TR (1 μg) and DR4-tkCAT (5 μg) in the presence or absence of pCMV4-TRUP (5 μg). (*b*) pRSV-RAR (1 μg) and DR5-tkCAT (5 μg) were cotransfected into Lmtk$^-$ cells in the presence or absence of pCMV4-TRUP (5 μg). (*c*) CAT assay data from cells transfected with pRSV-ER (1 μg) and ERE2-tkCAT (5 μg) in the presence or absence of pCMV4-TRUP (5 μg). (*d*) pRSV-RXR (1 μg) and DR1-tkCAT (5 μg) were cotransfected into Lmtk$^-$ cells in the presence or absence of pCMV4-TRUP (5 μg). T3, addition of 1 μM triiodothyronine; RA, addition of 1 μM *all-trans*-retinoic acid; 9-cis, addition of 1 μM 9-*cis*-retinoic acid; E2, addition of 1 μM estradiol. RXR, retinoid X receptor; ER, estrogen receptor.

full-length RAR and a reporter containing a RAR response element (DR5) upstream of tkCAT (15) in the presence or absence of an expression vector for TRUP. In cells transfected with RAR and the reporter, normal hormone-dependent activation of the reporter was observed (Fig. 2*b*, lanes 3 and 4) and, in a manner similar to TR, overexpression of TRUP antagonized the ability of RAR to transactivate (Fig. 2*b*, lane 6). To examine the specificity of the inhibitory effects of TRUP, we overexpressed TRUP along with ER and RXR and their respective reporters. TRUP had no effect on the abilities of these nuclear receptors to transactivate their target genes (Fig. 2 *c* and *d*). Overexpression of TRUP alone did not affect any of the reporters examined.

We were able to demonstrate in the two-hybrid system that TRUP interacts with and increases the transcriptional activity of GAL4 DBD-TR 168–456; however, TRUP decreases the transcriptional activity of full-length TR. The major difference between these two TR constructs is that the GAL4 DBD-TR 168–456 utilizes the GAL4 DBD and the full-length TR construct uses its natural DBD. Therefore, we hypothesized that TRUP may inhibit full-length TR function by interfering with the natural DNA binding function of TR, while having no

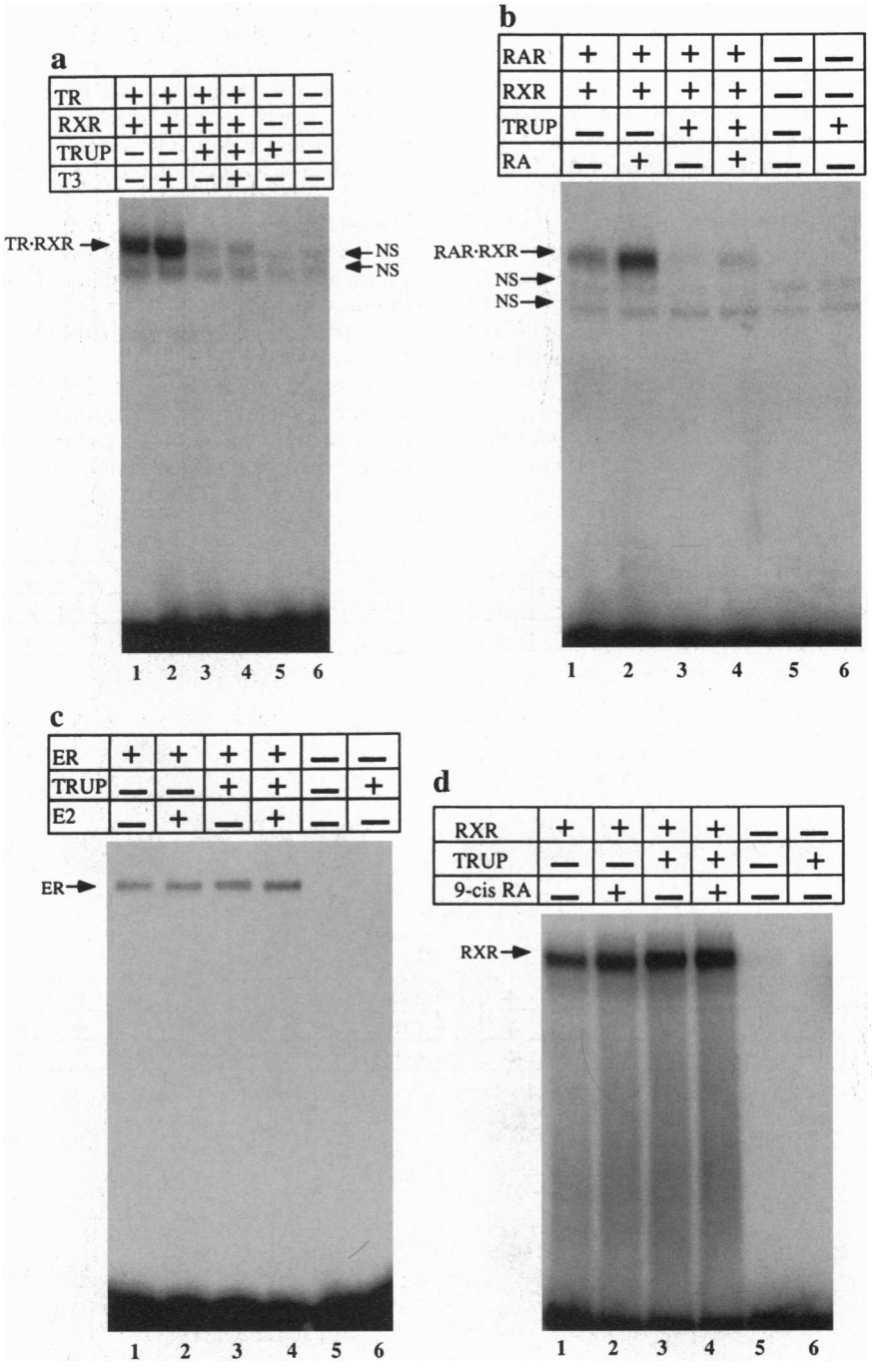

FIG. 3. TRUP prevents transactivation by TR and RAR by inhibiting their abilities to interact with DNA. (*a*) EMSA demonstrating the ability of TRUP to inhibit TR·RXR heterodimers binding to a ^{32}P-labeled DR4 element. (*b*) TRUP inhibits the ability of RAR·RXR heterodimers to bind to a DR5 element in an EMSA. (*c*) ER's ability to interact with an estrogen receptor response element is not affected by the presence of TRUP. (*d*) RXR's ability to bind to a DR1 element is also unaffected by the presence of TRUP. T3, addition of 1 μM triiodothyronine; RA, addition of 1 μM *all-trans*-retinoic acid; 9-cis, addition of 1 μM 9-*cis*-retinoic acid; E2, addition of 1 μM estradiol; NS, nonspecific complex that is present in lysate controls.

effect on the heterologous binding domain of the GAL4 DBD-TR 168–456 chimera. We addressed this possibility by examining the ability of *in vitro* translated TR, RAR, ER, and RXR to bind to their respective response elements in the presence and absence of *in vitro* translated TRUP in an EMSA. An equal amount of RXR was added to TR and RAR in the EMSA to facilitate the detection of retarded complexes. As shown in Fig. 3*a*, TR·RXR heterodimer bound to a ^{32}P-labeled DR4 element can be detected in the presence and absence of hormone (lanes 1 and 2). However, if the heterodimer is preincubated with an equal amount of TRUP (as determined by SDS/PAGE analysis) the ability of TR·RXR to bind the element is significantly decreased (Fig. 3*a*, lanes 3 and 4). Similar results were noted with the RAR·RXR heterodimer (Fig. 3*b*). A stoichiometric amount of TRUP is able to inhibit the ability of RAR·RXR heterodimers to bind to a DR5 element (Fig. 3*b*). The presence of hormone does not affect the ability of TRUP to interfere with DNA binding of TR·RXR or RAR·RXR heterodimers, indicating that the interaction of TRUP with TR and RAR is hormone independent (Fig. 3 *a* and *b*). Consistent with our transfection data, TRUP does not alter the DNA binding activity of either ER (Fig. 3*c*) or RXR (Fig. 3*d*).

Our results demonstrate that TRUP interacts specifically with a subclass of nuclear hormone receptors and antagonizes their ability to transactivate by inhibiting their binding to specific DNA response elements. Another protein, calreticulin, has been demonstrated recently to have a similar effect on several nuclear receptors, including the androgen receptor, glucocorticoid receptor, and RAR (28, 29). Calreticulin inhibits the DNA binding of these receptors by binding directly to their DBD (28, 29), whereas TRUP likely exerts its effects by interacting primarily with a region of the receptor that is C-terminal of the DBD. The precise mechanism by which TRUP inhibits the DNA binding activities of TR and RAR is not clear and several possibilities exist. When TRUP binds to the receptor just C-terminal of the DBD, it may either allosterically alter the DBD rendering it inactive or it may sterically hinder the DBD from interacting with DNA. Alternatively, TRUP may interfere with the dimerization properties of TR and RAR preventing DNA binding. We prefer the former model based on the ability of TRUP to inhibit the DNA binding of TR monomers as determined in an EMSA (data not shown).

The unusual ability of TRUP to regulate the DNA binding abilities of only a subset of the steroid/thyroid/vitamin receptor superfamily may represent again another level of regulatory complexity for the nuclear hormone receptor superfamily at the level of the target gene. Ligand binding favors dimerization of these receptors and specific high-affinity interaction with DNA. Negative regulators such as calreticulin can specifically oppose this interaction, with respect to GR, AR, and RAR, while TRUP acts similarly for TR and RAR. Both negative regulators have been shown to function in transfection experiments in intact cells. Although the physiological relevance is not proven to date, such negative regulators may represent additional examples of a growing number of cellular factors and enzymes that oppose nuclear receptor function, among which are heat shock proteins, phosphatases, corepressors, and chromosomal histone structure. Variation in concentration of such regulators could determine the relative hormonal response of different tissues during development or in the differentiated state. Although much more complex than previously thought, this myriad of positive and negative factors appears to contribute to the highly controlled and graded functional response that results from ligand activation of receptors in animal cells.

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