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The TRAP220 component of a thyroid hormone receptor-associated protein (TRAP) coactivator complex interacts directly with nuclear receptors in a ligand-dependent fashion

CHAO-XING YUAN*, MITSUHIRO ITO*, JOSEPH D. FONDELL*†, ZHENG-YUAN FU, AND ROBERT G. ROEDER‡

Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, New York, NY 10021

Contributed by Robert G. Roeder, May 5, 1998

ABSTRACT **Cognate cDNAs are described for 2 of the 10 thyroid hormone receptor-associated proteins (TRAPs) that are immunopurified with thyroid hormone receptor α (TRα) from ligand-treated HeLa (α-2) cells. Both TRAP220 and TRAP100 contain LXXLL domains found in other nuclear receptor-interacting proteins and both appear to reside in a single complex with other TRAPs (in the absence of TR). However, only TRAP220 shows a direct ligand-dependent interaction with TRα, and these interactions are mediated through the C terminus of TRα and (at least in part) the LXXLL domains of TRAP220. TRAP220 also interacts with other nuclear receptors [vitamin D receptor, retinoic acid receptor α, retinoid X receptor α, peroxisome proliferation-activated receptor (PPAR) α, PPARγ and, to a lesser extent, estrogen receptor] in a ligand-dependent manner, whereas TRAP100 shows only marginal interactions with estrogen receptor, retinoid X receptor α, PPARα, and PPARγ. Consistent with these results, TRAP220 moderately stimulates human TRα-mediated transcription in transfected cells, whereas a fragment containing the LXXLL motifs acts as a dominant negative inhibitor of nuclear receptor-mediated transcription both in transfected cells (TRα) and in cell free transcription systems (TRα and vitamin D receptor). These studies indicate that TRAP220 plays a major role in anchoring other TRAPs to TRα during the function of the TRα–TRAP complex and, further, that TRAP220 (possibly along with other TRAPs) may be a global coactivator for the nuclear receptor superfamily.**

Nuclear hormone receptors comprise a superfamily whose individual members bind to and, in a ligand-dependent manner, activate transcription of specific genes that regulate biological processes such as cell growth, differentiation, and homeostasis (1). Despite the specificity intrinsic to individual receptors and cognate ligands and DNA binding sites, and the enormous complexity of the general transcriptional machinery (2) that serves as the ultimate target of nuclear receptors, other coactivators are also required for functional interactions between these components. Such cofactors (or intermediary factors) were first suggested by the demonstration of gene-specific interference between nuclear receptors (3), the presence of a conserved ligand-dependent activation domain (AF-2) (reviewed in refs. 4 and 5), cell- and promoter context-specific functions for some receptors (reviewed in ref. 6) and, more recently, by the inability of nuclear receptors to function optimally in cell free systems reconstituted with general initiation factors and coactivators that suffice for the function of other DNA-binding activators (7).

Most ligand-dependent nuclear receptor-interacting proteins that represent established or candidate cofactors have been identified and/or cloned by biochemical interaction or yeast two hybrid assays (reviewed in refs. 4 and 5). Coactivators thus identified include: (*i*) members of the SRC family (SRC-1/NCoA-1, TIF-2/GRIP-1/NCoA-2, and PCIP/ACTR/RAC-3/AIB-1; reviewed in refs. 4–6), (*ii*) the more general coactivators p300/CBP and the interacting coactivator PCAF (reviewed in refs. 4–6), and (*iii*) a group of unrelated factors that include RIP140, TIF-1, Trip-1, and ARA70 (reviewed in refs. 4–6), and the more recently described PBP (8), TRIP230 (9), and p120 (10). Most of these factors show ligand-dependent receptor interactions that depend on the integrity of the AF-2 domain, and demonstrations of AF-2-dependent coactivator functions have involved assays in living cells (yeast and mammalian) with ectopically expressed coactivators or microinjected antibodies (reviewed in refs. 4–6). Although mechanisms of action are unknown for most of these coactivators, the presence of histone acetyltransferase activities in CBP/p300 (11), PCAF (12), and some members of the SRC family (13, 14) suggest a role in chromatin structural modifications before the formation of a preinitiation complex.

In another approach, thyroid hormone receptor-associated proteins (TRAPs) were isolated by affinity purification of an epitope-tagged thyroid hormone receptor from cells grown in the presence of a cognate ligand (7). The coactivator function of the TRAPs was demonstrated in a cell free system reconstituted with purified DNA templates and components of the general transcription machinery, indicating that they act more directly to facilitate preinitiation complex formation or function (7). More recent studies have shown that the general coactivators PC4 and PC2, but apparently not the TAFs, are required for activation by the TR–TRAP complex (J.D.F., M. Guermah, S. Malik, and R.G.R., unpublished observations). This study reports the cloning and further characterization of individual TRAP components, which appear to play a broader role in nuclear receptor function.

MATERIALS AND METHODS

cDNA Cloning and Northern Blot Analysis. An immunopurified TR–TRAP complex from ligand-treated α-2 cells (7) was subjected to SDS/PAGE and peptides derived from TRAP220 and TRAP100 were subjected to microsequence analysis. An EST clone (EST33696) containing a TRAP220 peptide was used

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Abbreviations: ERα, estrogen receptor α; GST, glutathione *S*-transferase; PPAR, peroxisome proliferation-activated receptor; RARα, retinoic acid receptor α; RXRα, retinoid X receptor α; TRAP, thyroid hormone receptor-associated protein; TRα, thyroid hormone receptor α; hTRα, human TRα; VDR, vitamin D receptor.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AF055994 (TRAP220) and AF055995 (TRAP100)].

*C.-X.Y., M.I., and J.D.F. contributed equally to this work.
†Present address: Department of Physiology, University of Maryland, School of Medicine, 660 West Redwood Street, Baltimore, MD 21201.
‡To whom reprint requests should be addressed. e-mail: roeder@rockvax.rockefeller.edu.

to screen a Jurkat cDNA library (Stratagene). Positive clones were sequenced and ligated to construct the full-length TRAP220. The N-terminal sequence of TRAP100 matched that of a protein specified by human KIAA0130 gene cDNA (15), and gene-specific primers were used to PCR amplify the N- and C-terminal halves of TRAP100 from α-2 cell-derived cDNA. The resulting fragments were ligated to create a full-length TRAP100 cDNA, which was verified by DNA sequencing. A 0.7 kb cDNA encoding residues 405–644 of TRAP220 and a 1.5 kb cDNA encoding residues 1–489 of TRAP100 were used to probe a human multiple tissue Northern blot (CLONTECH).

Plasmid Constructions. All primer sequences and details of subcloning (not shown here) are available on request. A TATA box and 130 bp G-less region (−53 to +130) from Δ53ML (7) were inserted in pGL3-Basic (Promega) to generate pTATA/130. Four TRE (one site sequence, AGGTCAcagcAGGTCA) (16) sites, all with the same head-to-tail orientation, were cloned into pTATA/130 to create pTRE4/130. Similarly, two TRE sites were inserted into an alkaline phosphatase minimum-promoter luciferase reporter (LBK-Basic; S. Stevens, unpublished data) to generate pTRE2-LBK-luc. Four human vitamin D receptor (VDR) binding sites (17) were ligated into pTATA/130 to create pVDRE4/130. TRAP220 was subcloned into pGEM5Z for *in vitro* translation (Promega). Point mutations were introduced into TRAP220 cDNA by using the Altered Sites II *in vitro* Mutagenesis System (Promega). Intact and mutated TRAP220 cDNA segments were then subcloned into pCIN4 (18) to create pCIN4-TRAP220 constructs, and human (h) thyroid hormone receptor α (TRα) was subcloned into pNT7-SB (19) to generate pNT7-TRα, for expression in transfected cells. For expression of glutathione *S*-transferase (GST) fusion proteins, cDNAs encoding TRAP220/1 (residues 405–644), TRAP220/2 (residues 622–701), TRAP100(1–253), hTRα, human peroxisome proliferation-activated receptor α (PPARα) (obtained by reverse transcription–PCR), mouse PPARγ_2 (20), hVDR (21), and human estrogen receptor α (ERα) (302–595) were subcloned into pGEX vectors (Pharmacia). hVDR was FLAG-tagged and subcloned further to create pVL1392-FLAG-hVDR.

Antibody Production and Western Blot. GST-TRAP220/2 and GST-TRAP100(1–253) were expressed in and purified from *Escherichia coli* strain BL21(DE3), and 10 mg of each were gel-isolated for antibody production in rabbits (Covance, Denver, PA). Mouse mAbs against hTRα-1 (Santa Cruz Biotechnology) were used as specified by the manufacturer. Western blots were visualized by enhanced chemiluminescence (Amersham).

***In Vitro* Protein–Protein Interaction and Far Western Analyses.** Proteins were synthesized and labeled with [^{35}S]methionine in a coupled transcription–translation system (TNT kit; Promega). Immobilized GST-fusion protein (1.5 μg) and 2 μl of ^{35}S-labeled input proteins were used in GST pull-down assays. For coimmunoprecipitation (7), 2 μl of anti-FLAG M2 affinity gel (Kodak) and 3 μl of ^{35}S-labeled input proteins were used. For Far Western analyses, 500 ng of purified TR–TRAP complex was resolved by SDS/PAGE and transferred onto a nitrocellulose membrane. The blots were probed with *in vitro* translated, ^{35}S-labeled hTRα-1 or hTRα-1 C terminus (residues 122–410) in the presence and absence of 10^{-7} M T_3.

Gel Filtration. Purified TR–TRAP complex (1 μg) was fractionated on Superose 6 (SMART, Pharmacia) in buffer BC300 (7) containing 0.1% Nonidet P-40 in the absence of T_3. Fractionated proteins were resolved by SDS/PAGE and visualized either by silver staining or by Western blot.

Transient Transfection. NIH 3T3 cells were maintained in DMEM with 10% calf serum. Twelve hours before transfection, confluent cells were split (1:5) into 6-well plates with DMEM plus 10% dialyzed calf serum (Sigma). Calcium phosphate precipitation plus CalPhos Maximizer (CLONTECH) was used in transfections (19). After 36-hr incubation with DNA precipitates, the cells were harvested and luciferase activities were measured with luciferase assay system with reporter lysis buffer (Promega). pRSV-β-gal was employed as an internal control for normalization of transfection efficiency.

Baculovirus-Mediated Expression of VDR, TR, and Retinoid X Receptor (RXR), and *in Vitro* Transcription. pVL1392-FLAG-VDR, -TRα and -RXRα were cotransfected into Sf9 cells with BacVector-3000 (Novagen). Expressed human VDR, TRα, and RXRα were purified (to 99% homogeneity) on anti-FLAG M2 agarose. *In vitro* transcription assays were performed as described (7).

RESULTS

TRAP220 and TRAP100 cDNA Clones. cDNAs encoding TRAP220 and TRAP100 were isolated (see *Materials and Methods*) on the basis of amino acid sequences (underlined in Fig. 1*A*) derived from cognate polypeptides in the immunopurified TR–TRAP complex. The ORF of TRAP220 (Fig. 1*A*) encodes a novel human protein containing 1,581 residues, including a region (residues 635–703) nearly identical to that specified by a short cDNA (Trip 2) isolated in a yeast two hybrid screen with rat TRβ1 as bait (22). Although the cDNA-encoded protein has a calculated mass of only 168 kDa, the *in vitro* translated product has an electrophoretic mobility equivalent to that of TRAP220 (detected by immunoblot) in the natural TR–TRAP complex (Fig. 1*B*) and thus appears to be a full-length protein with an aberrant mobility (possibly due to the high serine content). TRAP220 contains two of the LXXLL domains previously implicated in nuclear receptor interactions (reviewed in ref. 6) and one is found in the Trip 2 fragment (boxed residues in Fig. 1*A*). cDNA sequence analyses also revealed a spliced mRNA variant containing a frame shift after glycine-216 and a stop codon at position 263 (data not shown), suggesting the possibility of a truncated TRAP220 (lacking a receptor interaction domain) that might have a distinct regulatory function.

The TRAP100 cDNA encodes a 989 aa residue protein (Fig. 1*A*) with a calculated mass of 110 kDa and an electrophoretic mobility similar to that of the TRAP100 in the natural TR–TRAP complex (Fig. 1*B*). TRAP100 has a presumptive zinc finger in the N-terminal region (residues 83–117), a putative ATP/GTP binding site in the central region (residues 436–448), and six dispersed LXXLL motifs (boxed residues in Fig. 1*A*).

Northern blot analysis of multiple human tissues showed that TRAP220 and TRAP100 are expressed ubiquitously, although at varying levels (Fig. 1*C*). Both transcripts are relatively abundant in skeletal muscle, heart, and placenta.

TRAPs Remain in a Multiprotein Complex in the Absence of TR. To investigate further the nature of the TR–TRAP complex, including the possibility of heterogeneity and the existence of a TRAP complex in the absence of TR and ligand, an immunopurified TR–TRAP complex isolated from ligand-treated cells (7) was subjected to gel filtration (Superose 6) in the absence of ligand. Analysis by silver stain (Fig. 2*A*) and by immunoblot (Fig. 2*B*) with antibodies to TRα and selected recombinant TRAPs (220, 150, and 100) showed a peak in the 1.5 MDa range that contained essentially all TRAPs, but no TRα, and a peak in the 660 kDa range that contained all detectable TRα and a small fraction (<5%) of TRAPs. These results, demonstrating a persistent association of TRAPs *in vitro* in the absence of TRα and ligand, suggest the possibility that the formation of the TR–TRAP complex *in vivo* may result from a ligand-induced association of TRα with a preformed (ligand-independent) TRAP complex.

Interactions of TRAP220 and TRAP100 with Nuclear Receptors. To test which TRAPs interact with TRα we first probed the entire complement of TRAPs (in the natural TR–TRAP complex) by a Far Western analysis. Intact TRα and a C-terminal fragment (TRα-C, residues 122–410) containing the ligand-binding and AF-2 domains interacted with

A.

TRAP220

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MKAQGETEESEKLSKMSSLLERLHAKFNQNRPWSETIKLVRQVMEKRVVMSSGGHQHLVS 60
CLETLQKALKVTSLPAMTDRLESIARQNGLGSHLSASGTECYITSDMFYVEVQLDPAGQL 120
CDVKVAHHGENPVSCPELVQQLREKNFDEFSKHLKGLVNLYNLPGDNKLKTKMYLALQSL 180
EQDLSKMAIMYWKATNAGPLDKILHGSVGYLTPRSGGHLMNLKYYVSPSDLLDDKTASPI 240
ILHENNVSRSLGMNASVTIEGTSAVYKLPIAPLIMGSHPVDNKWTPSFSSITSANSVDLP 300
ACFFLKFPQPIPVSRAFVQKLQNCTGIPLFETQPTYAPLYELITQFELSKDPDPIPLNHN 360
MRFYAALPGQQHCYFLNKDAPLPDGRSLQGTLVSKITFQHPGRVPLILNLIRHQVAYNTL 420
IGSCVKRTILKEDSPGLLQFEVCPLSESRFSVSFQHPVNDSLVCVVMDVQDSTHVSCKLY 480
KGLSDALICTDDFIAKVVQRCMSIPVTMRAIRRKAETIQADTPALSLIAETVEDMVKKNL 540
PPLAGPGYGMTTGNNPMSGTTTPTNTFPGGPITTLFNMSMSIKDRHESVGHGEDFSKVSQ 600
NPILTSLLQITGNGGSTIGSSPTPPHHTPPPVSSMAGNTKNHPMLMNLLKDNPAQDFSTL 660
YGSSPLERQNSSSGSPRMEICSGSNKTKKKKSSRLPPEKPKHQTEDDFQRELFSMDVDSQ 720
KPIFDVNMTADTLDTPHITPAPSQCSTPPTTYPQPVPHPQPSIQRMVRLSSSDSIGPDVT 780
DILSDIAEEASKLPSTSDDCPAIGTPLRDSSSSGHSQSTLFDSDVFQTNNNENPYTDPAD 840
LIADAAGSPSSDSPTNHFFHDGVDFNPDLLNSQSQSGFGEEYFDESSQSGDNDDFKGFAS 900
QALNTLGVPMLGGDNGETKFKGNNQADTVDFSIISVAGKALAPADLMEHHSGSQGPLLTT 960
GDLGKEKTQKRVKEGNGTSNSTLSGPGLDSKPGKRSRTPSNDGKSKDKPPKRKKADTEGK 1020
SPSHSSSNRPFTPPTSTGGSKSPGSAGRSQTPPGVATPPIPKITIQIPKGTVMVGKPSSH 1080
SQYTSSGSVSSSGSKSHHSHSSSSSSASTSGKMKSSKSEGSSSSKLSSSMYSSQGSSGS 1140
SQSKNSSQSGGKPGSSPITKHGLSSGSSSTKMKPQGKPSSLMNPSLSKPNISPSHSRPPG 1200
GSDKLASPMKPVPGTPPSSKAKSPISSGSGGSHMSGTSSSSGMKSSSGLGSSGSLSQKTP 1260
PSSNSCTASSSSFSSSGSSMSSSQNQHGSSKGKSPSRNKKPSLTAVIDKLKHGVVTSGPG 1320
GEDPLDGQMGVSTNSSSHPMSSKHNMSGGEFQGKREKSDKDKSKVSTSGSSVDSSKKTSE 1380
SKNVGSTSVAKIIISKHDGGSPSIKAKVTLQKPGESSGEGLRPQMASSKNYGSPLISGST 1440
PKHERGSPSHSKSPAYTPQNLDSESESGSSIAEKSYQNSPSSDDGIRPLPEYSTEKHKKH 1500
KKEKKKVKDKDRDRDRDKDRDKKKSHSIKPESWSKSPISSDQSLSMTSNTILSADRPSRL 1560
SPDFMIGEEDDDLMDVALIGN
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TRAP100

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MKVVNLKQAILQAWKERWSDYQWAINMKKFFPKGATWDILNLADALLEQAMIGPSPNPLI 60
LSYLKYAISSQMVSYSSVLTAI(SKFDDFSRDLCVQALLDIMDMFCDRLSCHGKAEEC)IGL 120
CRALLSALHWLLRCTAASAERLREGLEAGTPAAGEKQLAMCLQRLEKTLSSTKNRALLHI 180
AKLEEASSWTAIEHSLLKLGEILANLSNPQLRSQAEQCGTLIRSIPTMLSVHAEQMHKTG 240
FPTVHAVILLEGTMNLTGETQSLVEQLTMVKRMQHIPTPLFVLEIWKACFVGLIESPEGT 300
EELKWTAFTFLKIPQVLVKLKKYSHGDKDFTEDVNCAFEFLLKLTPLLDKADQRCNCDCT 360
NFLLQECGKQGLLSEASVNNLMAKRKADREHAPQQKSGENANIQPNIQLILRAEPTVTNI 420
LKTMDADHSKSPEGLLGVLGHMLSGKSLDLLLAAAAATGKLKSFARKFINLNEFTTYGSE 480
ESTKPASVRALLFDISFLMLCHVAQTYGSEVILSESRTGAEVPFFETWMQTCMPEEGKIL 540
NPDHPCFRPDSTKVESLVALLNNSSEMKLVQMKWHEACLSISAAILEILNAWENGVLAFE 600
SIQKITDNIKGKVCSLAVCAVAWLVAHVRMLGLDEREKSLQMIRQLAGPLFSENTLQFYN 660
ERVVIMNSILERMCADVLQQTATQIKFPSTGVDTMPYWNLLPPKRPIKEVLTDIFAKVLE 720
KGWVDSRSIHIFDTLLHMGGVYWFCNNLIKELLKETRKEHTLRAVELLYSIFCLDMQQVT 780
LVLLGHILPGLLTDSSKWHSLMDPPGTALAKLAVWCALSSYSSHKGQASTRQKKRHREDI 840
EDYISLFPLDDVQPSKLMRLLSSNEDDANILSSPTDRSMSSSLSASQLHTVNMRDPLNRV 900
LANLFLLISSILGSRTAGPHTQFVQWFMEECVDCLEQGGRGSVLQFMPFTTVSELVKVSA 960
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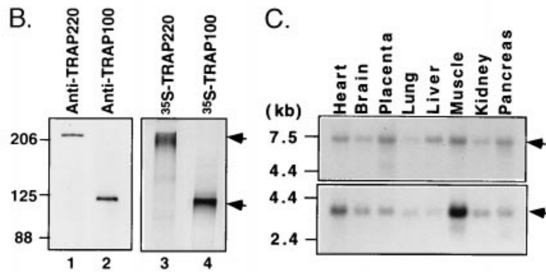
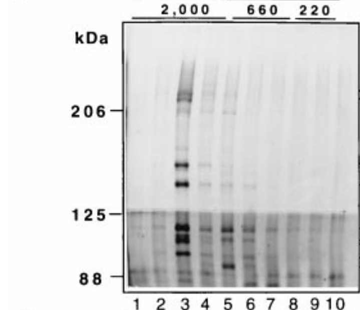


FIG. 1. Cloning and expression of TRAP220 and TRAP100. (*A*) Amino acid sequences of TRAP220 and TRAP100. Polypeptide microsequences are underlined. The circled glycine-216 indicates the point from which a truncated TRAP220 differs from full-length TRAP220. LXXLL motifs are boxed. A C4 type zinc finger region is bracketed. The putative ATP/GTP binding site is underlined with a broken line. (*B*) TRAP220 and TRAP100 expression. Lanes 1 and 2 show an immunoblot analysis of purified TR–TRAP complex probed with antibodies against recombinant TRAP220 and TRAP100. Lanes 3 and 4 show an SDS/PAGE autoradiographic analysis of ^{35}S-labeled TRAP220 and TRAP100 generated by *in vitro* translation. (*C*) Northern blot. (*Upper*) TRAP220 and (*Lower*) TRAP100.

TRAP220, but not with the other TRAPs, in a ligand (T_3)-dependent manner (Fig. 3*A*). This interaction was confirmed by coimmunoprecipitation of *in vitro*-translated, ^{35}S-labeled recombinant proteins. As shown in Fig. 3*B*, anti-FLAG mAbs that recognize FLAG-tagged TRα coimmunoprecipitate TRAP220, but not TRAP100, in a ligand-dependent fashion. To analyze domains in TRAP220 involved in receptor interactions, regions containing the first LXXLL motif (TRAP220/1, residues 405–644) and the second LXXLL motif (TRAP220/2, residues 622–701) were expressed as GST fusion proteins. As shown in Fig. 4*A*, intact TRα and TRα-C, but not an N-terminal fragment (TRα-N) containing the AF-1 and DNA-binding domains, were bound in a ligand-dependent manner to both GST-TRAP220/1 and GST-TRAP220/2, but not to GST alone. However, the binding affinity was much stronger for TRAP220/2. Thus, TRAP220/2 and the TRα C terminus containing the ligand-binding and AF-2 domains provide the major interface for the ligand-dependent TRα-TRAP220 interaction.



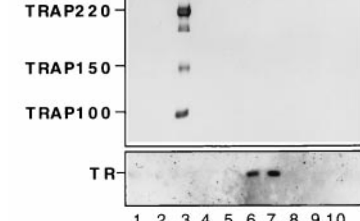


FIG. 2. Gel filtration of purified TR–TRAP complex in the absence of T_3. (*A*) Silver staining. One microgram of TR–TRAP complex was fractionated on Superose 6, resolved by SDS/PAGE with 6% (*Upper*) and 8% (*Lower*) acrylamide gels and detected by silver staining. The void volume was not collected. Bars on the right indicate positions of individual TRAPs. (*B*) Western blot analysis. Proteins (molecular weight <85 kDa) in the lower part of the SDS/PAGE analysis in *A* were transferred and probed with anti-TRα antibody. Proteins from an analysis equivalent to the SDS/PAGE analysis in *A* were probed with anti-TRAP220, TRAP150, and TRAP100 antibodies.

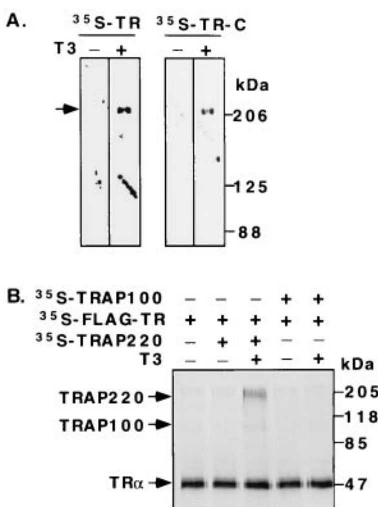


FIG. 3. Ligand-dependent interaction between TRα and TRAP220. (*A*) Far Western analysis. Five hundred nanograms of purified TR–TRAP complex was separated by SDS/PAGE (8% gel), blotted, and probed with ^{35}S-labeled full-length (TR) and C-terminal (TRα-C) hTRα in the presence and absence of 10^{-7} M 3,3′,5-triiodothyroacetic acid (T_3). Arrow indicates the position of the TRAP220 band. (*B*) Coimmunoprecipitation of TR-TRAP220 from *in vitro* translated products. The indicated combinations of ^{35}S-labeled TRAP220, TRAP100, and FLAG-TRα were incubated in the presence and absence of 3,3′,5-triiodothyroacetic acid (T_3), and subjected to immunoprecipitation with anti-FLAG antibodies (M2 agarose) that recognize only FLAG-tagged TRα.

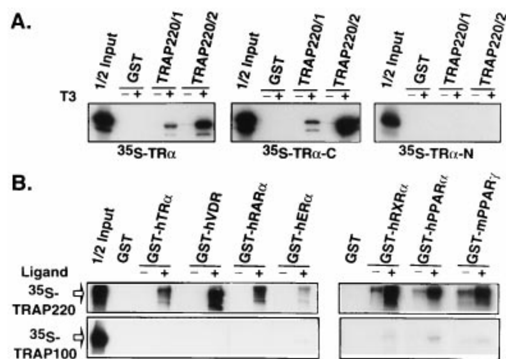


FIG. 4. Nuclear receptor interactions with TRAP220 and TRAP100. (*A*) Ligand-dependent interactions of GST-TRAP220/1 and -2 with ^{35}S-labeled TRα. GST or GST-TRAP220/1 and -2 fusion proteins (1.5 μg) were incubated with ^{35}S-labeled TRα, TRα-C, or TRα-N in the presence and absence of 10^{-7} M 3,3′,5-triiodothyroacetic acid. (*B*) Interactions between GST receptors and ^{35}S-labeled TRAP220 and TRAP100. GST and GST nuclear receptor fusion proteins (1.5 μg) were immobilized and incubated with [^{35}S]TRAP220 or [^{35}S]TRAP100 at either 300 mM KCl (TRα, VDR, RARα, and ERα) or at 100 mM KCl (RXR, PPARα, and mouse PPARγ). The cognate ligands were 10^{-7} M 3,3′,5-triiodothyroacetic acid (TRα), 10^{-6} M all-trans retinoic acid (RARα), 10^{-6} M 9-cis retinoic acid (RXRα), 10^{-7} M 1α,25(OH)$_2$D$_3$ (VDR), 10^{-4} M Wy14643 (PPARα), and 10^{-5} M 15-deoxy-D^{12,14}-prostaglandin J2 (mouse PPARγ).

Given the conservation of various motifs in the ligand-binding domains of nuclear receptors, and the common interaction with other coactivators through LXXLL domains (reviewed in ref. 6), we tested for TRAP220 and TRAP100 interactions with other receptors by GST pull-down assays. As shown in Fig. 3*A*, *in vitro*-translated TRAP220 showed significant ligand-dependent binding not only to TRα but also to VDR, retinoic acid receptor α (RARα), and ERα under moderately stringent binding conditions (300 mM KCl). Under less stringent conditions (100 mM KCl) TRAP220 showed interactions with RXRα, PPARα, and PPARγ that were enhanced significantly by cognate ligands. In contrast, and despite the presence of six LXXLL motifs, TRAP100 showed only marginal (ligand-dependent) interactions with ERα, RXRα, PPARα, and PPARγ.

***In Vivo* Function of TRAP220.** To test the functional role of TRAP220 *in vivo* the influence of TRAP220 mutants on a T$_3$/TRα-responsive, TRE-containing luciferase reporter was tested by cotransfection of NIH 3T3 and COS7 cells. Because similar results were obtained for both cell lines, only those for NIH 3T3 cells are shown. To help overcome limitations due to endogenous TRAPs, the TRα and TRAP220 expression vectors (Fig. 5*A*) were driven by the strong cytomegalovirus promoter and the dosages were optimized.

In the presence of T$_3$, intact (wild-type) TRAP220 moderately and reproducibly (in three experiments) enhanced TRα-mediated activation (Fig. 5*B*), consistent with results observed with a mouse homologue (PBP) of TRAP220 (8). The weak activation may reflect the normal function of TRAP220 within a larger TRAP complex, but also suggests that TRAP220 could be a limiting component. A mutation in the first LXXLL domain (mutant a) had no effect on the coactivator function of TRAP220 in the presence of ligand, whereas the same mutation in the second LXXLL domain (mutant b) completely abolished the observed coactivator function (Fig. 5*B*). This result indicates the primary importance of the second motif and is consistent with indications of a stronger receptor interaction of this motif in the direct binding assay (Fig. 4*A*). Given the likelihood that TRAP220 may function optimally only in conjunction with other TRAPs (7), we attempted to create a dominant negative mutant that would provide more compelling evidence for TRAP220 function in this context. As

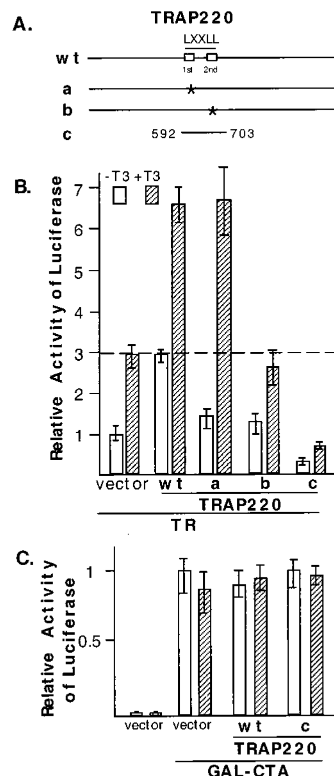


FIG. 5. *In vivo* function of TRAP220 in transfected NIH 3T3 cells. (*A*) Diagram of TRAP220 constructs. The two LXXLL motif-containing regions are represented by rectangles and corresponding point mutations (LXXLL to LXXAA) by asterisks. (*B*) Effects of TRAP220 on TR-mediated activation of the TRE-containing reporter. Cells were transfected with 2 μg of pTRE2-LBK-luc, 2 μg of pRSV-β-gal (internal control), 0.1 μg of pNT7-TRα, and either 3 μg of pCIN4-TRAP220 or an empty vector pCIN4 as indicated. Luciferase values have been normalized to β-galactosidase activity and corrected for the activity of the reporter in the absence of ectopic TRα expression. (*C*) Specificity of TRAP220 function. Cells were transfected with 2 μg of pG5-luc (19), 2 μg of pRSV-β-gal, 0.1 μg of pGAL-CTA (19), and 3 μg of pCIN4-TRAP220 or an empty pCIN4 vector. Open and solid bars indicate activities in the absence and presence, respectively, of 10^{-7} M 3,3′5-triodo-L-thyronine (T$_3$).

shown in Fig. 5*B*, and consistent with the results of the PBP study (8), a 112-residue TRAP220 fragment containing both LXXLL domains dramatically reduced the ligand-dependent TR-mediated activity.

TRAP220 mutants a and b showed no effect on ligand-independent activity, whereas, unexpectedly, intact TRAP220 stimulated and TRAP220 mutant c inhibited this activity (Fig. 5*B*). These latter effects may reflect weak ligand-independent interactions between TRAP220 and TRα that are driven by high levels of TRAP220. Similar ligand-independent interactions and corresponding effects on transcription have been reported for other receptors and overexpressed coactivators in yeast (6, 8). One possible explanation for the enhanced ligand-independent basal activity is that overexpressed levels of intact TRAP220 result in the release of TR-associated corepressors such as NCoR and SMRT (for review, see ref. 5). However, the antirepression function of TRAP220 appears to require not only both LXXLL domains, but also the coactivator activity; the latter is indicated by the fact that TRAP220 mutant c failed to show antirepression and, instead, further inhibited the (ligand-independent) basal activity of the TR-driven promoter. In this case the dominant negative mutant may be blocking the activity of endogenous TRAP220 (or TRAP complex) on a distinct (activated) population of the transfected reporter genes.

Because both intact and mutant c forms of TRAP220 had effects on transcription of the TR-responsive promoter in the absence of ligand, it was essential to eliminate the possibility of the general effects of these factors on transcription. As shown in Fig. 5*C*, neither intact TRAP220 nor mutant c had any significant effect, in the presence or absence of T_3, on either the basal activity or the activator-enhanced (over 100-fold) activity of a reporter (pG5-luc) that contains five Gal4 sites upstream of the luciferase reporter. The activator (pGAL-CTA) in this case consisted of the Gal4 DNA binding domain fused to the C-terminal activation domain of human heat shock factor (19). These results provide further support for the significance and specificity of the coactivator activity of the dominant negative derivative (mutant c).

***In Vitro* Function of TRAP220.** Our previous study showed that the TR–TRAP complex could significantly activate a TRE-containing promoter both in nuclear extracts and in a system reconstituted with purified factors (7). As shown in Fig. 6*A*, the GST-TRAP220/2 fusion protein (containing the LXXLL domain that most strongly interacts with TRα) showed a significant (5-fold) inhibition of the TR-TRAP-enhanced activity on the TRE-containing promoter, with no effect on the control promoter, whereas GST alone had no effect on either promoter. These *in vitro* effects of TRAP220/2 parallel the effects of the dominant negative mutant c *in vivo* and further support the notion that TRAP220 is (or is part of) a *bona fide* coactivator.

In view of the suggestion from the receptor interaction data (Fig. 4*B*) that TRAP220 might be involved in VDR function, and given the ability of VDR to activate vitamin D receptor element (VDRE)-containing promoters in nuclear extracts (23), we tested the effect of TRAP220/2 on VDR-dependent transcription *in vitro*. As shown in Fig. 6*B*, a VDRE-containing promoter was modestly and selectively (relative to the control promoter) activated in response to VDR and RXR. In addition, GST-TRAP220/2 selectively abolished the VDR-mediated activity, with no effect on the control template, whereas GST alone had no effect. These functional data support the idea that TRAP220 is a more general coactivator for nuclear receptors.

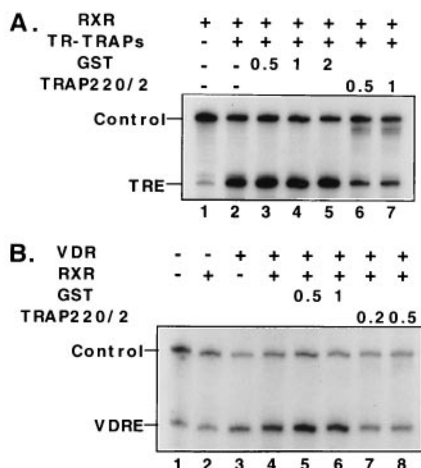


FIG. 6. TRAP220/2 inhibition of TR- and VDR-mediated transcription *in vitro*. (*A*) TRAP220/2 specifically inhibits TR-mediated transcriptional activation. Reactions (7) contained 5 μl of Namalwa nuclear extract (about 40 μg protein), 10^{-7} M 3,3′,5-triiodothyroacetic acid, 100 ng of RXRα, 100 ng of pTRE4/130, 25 ng of pWtML200 as an internal control template, 5 ng of TRAP-associated TR, and indicated amounts (in micrograms) of GST or GST-TRAP220/2. (*B*) GST-TRAP220/2 also inhibits VDR-mediated transcriptional activation. Reactions contained 5 μl of Namalwa nuclear extract, 25 ng of RXRα, 14 ng of baculovirus expressed hVDR, 10^{-7} M 1α,25$(OH)_2D_3$, 100 ng of pVDRE4/130, 15 ng of pWtML200, and the indicated amounts (in micrograms) of GST or GST fusion proteins.

DISCUSSION

Transcriptional coactivators, broadly defined as factors essential for the function of DNA-binding activators, but not for transcription initiation from core promoter elements by basal factors, provide an increasingly important level of regulation and fall into several groups: (*i*) those whose primary interactions are with specific DNA-bound activators, such as the early-described B cell-specific OCA-B (24) and ubiquitous CBP (25); (*ii*) those that are intimately associated with the general transcriptional machinery, including the TAF components of TFIID (reviewed in ref. 26) and the SRB/MED components associated with polymerase II (reviewed in ref. 27); and (*iii*) potentially more general factors, such as the human USA-derived positive cofactors (28) and nucleosome remoldeling factors (29). Such cofactors may function either to relieve the effects of negative constraints (chromatin structure, specific negative cofactors) or as adapters between activators and the general transcriptional machinery. In the case of nuclear receptors, many of the best studied coactivators fall into the first category and interact with receptors in a ligand-dependent fashion through the C-terminal AF-2 containing domain (see Introduction). This study shows that the human TRAPs represent a novel group of cofactors, provides insights into their mechanism of action, and suggests a more global role in the function or regulation of a variety of nuclear receptors.

Structure and Function of TRAPs. On the basis of cognate cDNA cloning and sequence analyses TRAP220 and TRAP100 are distinct from other nuclear receptor coactivators, with the exception of a recently reported mouse homologue of TRAP220 (8). Sequence analysis of most of the other TRAPs also confirms their novelty with respect to other coactivators (unpublished observations). In support of this finding and the idea of distinct functions, most or all of the TRAPs (including TRAP220 and 100) appear to reside in a single complex. TRAPs 220 and 100 show no obvious common motifs with other coactivators, except for the LXXLL motifs implicated in receptor-coactivator interactions (reviewed in ref. 6). In conjunction with this finding and in support of a major role for TRAP220 in anchoring other TRAPs to the receptor, TRAP220 showed strong ligand-dependent receptor interactions that were mediated by the C-terminal ligand-binding domain and dependent on an intact LXXLL domain. However, although no other TRAP showed comparably strong TR interactions, including TRAP100 with six LXXLL domains, the possibility of additional TRAP-receptor interactions in the context of the TR–TRAP complex is not excluded. That TRAP220 and associated TRAPs are involved in the function of the nuclear receptor AF-2 domain is suggested by the loss of receptor interactions in a mutated mouse homologue (PBP) of TRAP220 (8).

In further support of a nuclear receptor coactivator function for TRAP220, ectopically expressed TRAP220 moderately enhanced TRα function in transfected cells, whereas a truncated form of TRAP220 containing the LXXLL motifs acted as a dominant negative inhibitor of TRα function both in transfected cells and in a cell free system containing the TR–TRAP complex. The latter observation indicates that regions outside the receptor-interaction domain are also required for coactivator function. In addition, however, an apparent derepression effect of TRAP220 on ligand-independent TR function was dependent on both LXXLL-containing domains and the capability for coactivator function, suggesting that multiple TRAP220 domains may be required for dissociation of presumptive receptor-bound (co)repressors (see below).

Mechanism of Action of TRAPs in Relation to Other Nuclear Receptor-Interacting Cofactors. The fact that TRAPs enhance TRα function in cell free systems reconstituted with general transcription factors and naked DNA templates sug-

gests that they are involved directly in preinitiation complex assembly or function. This could involve interactions with general initiation factors or with general coactivators such as PC4 and PC2 (28) that enhance the intrinsic activity of these factors. At the same time, a possible role for TRAPs in reversing the action of specific negative cofactors (28) that might also be present in the partially purified reconstituted system is not excluded.

The currently available information on both TRAPs and other nuclear receptor-interacting cofactors suggests a general model for nuclear receptor function. Major features include (*i*) the ligand-independent binding of nuclear receptors (to target DNA sites within chromatin) along with corepressors (SMRT/NCoR, Sin3, and histone deacetylases) that help maintain a repressed state by deacetylation of nucleosomal histones (30, 31); (*ii*) ligand-mediated dissociation of (co)repressors plus concomitant binding of coactivators (SRC-1 related factors, p300/CBP, and PCAF) that contain, or interact with factors that contain, histone acetyltransferase activity (reviewed in refs. 4–6), with resulting acetylation of nucleosomal histones or possibly other factors (32); and (*iii*) binding of TRAPs (or a receptor–TRAP complex), perhaps with displacement of other coactivators (or receptor–coactivator complex), and subsequent interactions (see above) with general initiation factors or coactivators. This latter step could also involve direct (ligand-independent) interactions of nuclear receptors with general initiation factors (reviewed in ref. 6).

Complexity, Specificity, and Potential Regulation of TRAPs and Other Nuclear Receptor Coactivators. Although the receptor binding and coactivator functions for TRAPs were originally demonstrated for TRα, this study also shows ligand-dependent binding of TRAP220 to RARα, RXRα, VDR, PPARα, PPARγ, and ERα. This suggests a much broader receptor specificity for TRAPs, and functional studies showed that a dominant negative form of TRAP220 can inhibit both TRα and VDR function. Other nuclear receptor-interacting coactivators also function broadly with nuclear receptors (reviewed in refs. 4–6) and, in the case of p300/CBP, with many unrelated activators as well (reviewed in ref. 33).

Given the target gene DNA-binding and activation specificity intrinsic to the various nuclear receptors and their cognate ligands, as well as the enormous complexity of the general transcriptional machinery (containing the ultimate receptor targets), the question arises as to the reason for the unexpected complexity of the nuclear receptor coactivators. This may relate to the extreme diversity of physiological processes (in growth, differentiation, and homeostasis) that involve nuclear receptor function and the need for secondary regulatory events that are mediated through other signaling pathways that affect the abundance or activity of the cofactors. Cell- or cell-state specific variations in coactivators, as originally observed for the B cell-specific OCA-B (24), could help explain various cell- and promoter-specific effects of nuclear receptor/ligand function. Finally, the complexity of receptor interacting coactivators, most notably the 9–10 subunit TRAP complex, may also reflect a functional redundancy with other coactivators, such as those associated with the general transcriptional machinery. Indeed, recent studies suggest that the TAF components of TFIID may be dispensable for TR-TRAP function *in vitro* (J.D.F., M. Guermah, S. Malik, and R.G.R., unpublished observations).

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