TRAM-1, A Novel 160-kDa Thyroid Hormone Receptor Activator Molecule, Exhibits Distinct Properties from Steroid Receptor Coactivator-1*

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Nuclear hormone receptors (NRs) are ligand-dependent transcription factors that regulate target gene transcription. We report the molecular cloning and characterization of a novel human cDNA encoding TRAM-1, a thyroid hormone receptor activator molecule, a ~160-kDa protein homologous with SRC-1/TIF2, by far-Western-based expression screening. TRAM-1 binds to thyroid hormone receptor (TR) and other NRs in a ligand-dependent manner and enhances ligand-induced transcriptional activity of TR. The AF-2 region in NRs has been thought to play a critical role in mediating ligand-dependent transactivation by the interaction with coactivators. Surprisingly, TRAM-1 retains strong ligand-dependent interaction with an AF-2 mutant of TR (E457A), while SRC-1 fails to interact with this mutant. Furthermore, we identified a critical TRAM-1 binding site in rat TRb1 outside of AF-2, as TRAM-1 shows weak ligand-dependent interaction with a helix 3 ligand binding domain TR mutant (K288A), compared with SRC-1. These results suggest that TRAM-1 is a coactivator that may exhibit its activity by interacting with subdomains of NRs other than the AF-2 region, in contrast to SRC-1/TIF2.

The nuclear hormone receptors (NRs) regulate target gene transcription in response to various ligands including steroids, thyroid hormone (T₃), retinoids, and vitamin D. The liganded NRs bind to their cognate response elements, located in the promoter regions of target genes, and stimulate transcription by the interaction with coactivators. Functional analysis of NRs has shown that there are two major activation domains.

The N-terminal domain (AF-1) contains a ligand-independent activation function, whereas the extreme C-terminal region of the ligand binding domain (AF-2) exhibits ligand-dependent transactivation (1). The AF-2 region is conserved among NRs, and deletion or point mutations in this region impair transcriptional activation without changing ligand and DNA binding affinities (2–4). Recent x-ray crystallographic studies of the ligand binding domain of NRs revealed that the ligand induces a major conformational change in the AF-2 region (5–7), suggesting that this region may play a critical role in mediating transactivation by a ligand-dependent interaction with coactivators. Several putative coactivators of NRs have been identified, using either far-Western or yeast two-hybrid techniques, including SRC-1, RPI40, TIF1, TIF2, and CREB binding protein (CBP)/p300 (8–14). As expected, these proteins fail to interact with AF-2 mutants of NRs (10–14).

Using bacterially expressed thyroid hormone receptor (TR) as a probe, a cDNA expression library was screened, and a novel cDNA encoding TRAM-1, a putative thyroid hormone receptor activator molecule, was isolated. TRAM-1 belongs to a nuclear receptor coactivator (NCoA) gene family that includes SRC-1 and TIF2. TRAM-1 exhibits ligand-dependent, and unexpectedly, AF-2-independent interaction with TR, a biochemical feature distinct from SRC-1/TIF2. Our findings suggest that TRAM-1 may serve a novel pathway for transcriptional activation of NRs by interacting with subdomains other than the AF-2 region.

EXPERIMENTAL PROCEDURES

Plasmid Preparation—GST-rat TRb1 (wild type), GST-rat TRb1 mutant (E457A), GST-mouse retinoid X receptor β (RXRβ), GST-human retinoid acid receptor β (RARβ), and GST-human estrogen receptor (ER) plasmids were described previously (10). Human pCAF in pCX (Dr. Y. Nakatani, National Institutes of Health, Bethesda, MD) was subcloned into pBK-CMV (Stratagene), and mouse CBP (residues 1628–2260) was subcloned in pSKII (Dr. M. Montminy, Joslin Diabetes Center, Boston, MA). Two fragments of TRAM-1, residues 577–800 or 800–1215, were fused to a GST plasmid (15) by inserting BamHI/XhoI- and XhoI/SacI-digested fragments of TRAM-1(PIT) cDNA in pBluescript (Stratagene) to generate GST-TRAM-1A (residues 595–780) (10) into the GST plasmid. Expression vectors for rat TRb1 and its mutants (E457A and K288A) and mouse RXRβ were based in pcDNA/AMP (Invitrogen). E457A was created by PCR amplification using primers containing the codon 457 mutation and restriction enzyme sites at both ends. K288A was created using an in vitro mutagenesis kit (Promega). F-SRC-1 in pBluescript was subcloned into pcDNA/AMP. N-TRAM-1 in pBK-CMV was created by restriction digestion of full-length TRAM-1 in pBK-CMV by XhoI, followed by self-ligation. C-TRAM-1 in pBK-CMV was created by subcloning of the XhoI-digested fragment into pBK-CMV in the proper orientation. The luciferase (LUC) reporter constructs, the chick lysozyme thyroid hormone response element (TRE), F2-thymidine kinase; LUC, luciferase; kb, kilobase(s); CBP, CREB binding protein; RSV, Rous sarcoma virus.
nase (TK-LUC in PT109, and artificial direct repeat TRE, DR4-TK-LUC in PT109, were described previously (16).

Isolation of TRAM-1—

The expression cDNA library screen by far-Western blotting was performed as described previously (10). Human pituitary (Stratagene) and 293 kidney embryonic cell libraries (Dr. J. A. DeCaprio, Dana-Farber Cancer Institute, Boston, MA) in ZAPII were screened using 10^5 cpm/ml of 32P-labeled ligand binding domain (LBD) of rat TRβ1 in the presence of 1 μM T3. The remaining 5′-end sequence was cloned by PCR amplification from the human pituitary library with the pBS-specific sense primer, 5′-GGAAACAGCTATGACCATGAT-TACG-3′, and the cDNA-specific antisense primer of the original clone obtained by far-Western screening, 5′-AAACACTTGTGTTAACCAG-GTCCTCTTGCT-3′. A full-length cDNA (TRAM-1) was reconstructed in pBK-CMV from overlapping TRAM-1 clones. Nucleotide sequences of positive clones were determined using an Applied Biosystems 377 DNA sequencer.

Northern Blot Analysis—

Northern blot analysis was performed using human multiple tissue poly(A)^+ RNAs (2 μg per lane; CLONTECH), according to the protocol of the manufacturer. The random primed 32P-labeled DNA probes used were nucleotides 1153–2213 of TRAM-1 and nucleotides 1274–1896 of F-SRC-1.

Preparation of In Vitro Translated Proteins and GST Pull-down Assays—

Full-length and fragments of TRAM-1 in pBK-CMV, and TR and RXR in pcDNA/AMP, were transcribed and translated in rabbit reticulocyte lysate (Promega) according to the instructions of the manufacturer. The GST-fusion protein pull-down assay was performed as described previously (10).

Electrophoretic Mobility Shift Assay (EMSA)—Deoxyribonucleotides

Fig. 1. Features of TRAM-1. A, amino acid sequence comparison of TRAM-1 and F-SRC-1. Amino acids are denoted by the single letter amino acid code. An alignment of TRAM-1 and F-SRC-1 (10) was performed using Genestream Search (http://genome.eerie.fr). Sequence alignment is shown with amino acid identity ( ), and conservative substitution ( ) is indicated in the line between the two sequences. The two primary cDNAs encoding TRAM-1 (293) and TRAM-1 (PIT) start at amino acid residues 497 and 577, respectively (boldface). The alternative splicing region of TRAM-1 (aa 904–918) is underlined. Interaction consensus motifs (LXXLL) in TRAM-1 (aa 621–625, 685–689, 738–742, and 1057–1061) and F-SRC-1 (aa 633–637, 690–694, 749–753, and 1434–1437) are indicated by bold letters.

B, multiple human tissue Northern blot analysis using TRAM-1 (top panel) and SRC-1 (bottom panel). Top panel, TRAM-1 shows a major 9-kb transcript with a minor 5.5-kb mRNA (arrows). Bottom panel, SRC-1 shows a major 8.5-kb transcript with a minor 7-kb mRNA (arrows). C, schematic diagram of comparison of TRAM-1 with p/CIP. The poly Q region of TRAM-1 is located at the C terminus, compared with that of p/CIP. The C-terminal tail sequence of TRAM-1 bears no homology with that of p/CIP. D, nucleotide and deduced amino acid comparison between the C-terminal tails of TRAM-1 and p/CIP. TRAM-1 is highly conserved with p/CIP until amino acid residue 1321. One nucleotide deletion of p/CIP at codons 1296–1297 (TAG) results in a protein sequence again homologous with TRAM-1, shown by shaded box.

Isolation of TRAM-1—The expression cDNA library screen by far-Western blotting was performed as described previously (10). Human pituitary (Stratagene) and 293 kidney embryonic cell libraries (Dr. J. A. DeCaprio, Dana-Farber Cancer Institute, Boston, MA) in ZAPII were screened using 10^5 cpm/ml of 32P-labeled ligand binding domain (LBD) of rat TRβ1 in the presence of 1 μM T3. The remaining 5′-end sequence was cloned by PCR amplification from the human pituitary library with the pBS-specific sense primer, 5′-GGAAACAGCTATGACCATGAT-TACG-3′, and the cDNA-specific antisense primer of the original clone obtained by far-Western screening, 5′-AAACACTTGTGTTAACCAG-GTCCTCTTGCT-3′. A full-length cDNA (TRAM-1) was reconstructed in pBK-CMV from overlapping TRAM-1 clones. Nucleotide sequences of positive clones were determined using an Applied Biosystems 377 DNA sequencer.

Northern Blot Analysis—Northern blot analysis was performed using human multiple tissue poly(A)^+ RNAs (2 μg per lane; CLONTECH), according to the protocol of the manufacturer. The random primed 32P-labeled DNA probes used were nucleotides 1153–2213 of TRAM-1 and nucleotides 1274–1896 of F-SRC-1. Preparation of In Vitro Translated Proteins and GST Pull-down Assays—Full-length and fragments of TRAM-1 in pBK-CMV, and TR and RXR in pcDNA/AMP, were transcribed and translated in rabbit reticulocyte lysate (Promega) with 35S-methionine according to the instructions of the manufacturer. The GST-fusion protein pull-down assay was performed as described previously (10).
containing F2 or DR4 TRE (16) were end-labeled with \[^{32}P\]ATP by T4 polynucleotide kinase. Unlabeled in vitro translated receptor proteins, 20 ng of GST-fusion proteins, and a 50,000-cpm oligonucleotide probe were mixed and incubated together before being subjected to electrophoresis and autoradiography.

Transient Transfection Experiments—CV-1 cells were transiently transfected using the calcium phosphate coprecipitation method in 6-well plates with 1.7 μg of reporter plasmid containing the F2 or DR4 TRE, fused to TK-LUC cDNA; 20 ng of Rous sarcoma virus (RSV)-b-galactosidase plasmid as a internal control; 100 ng of TRb1; and various amounts of TRAM-1 or F-SRC-1 expression plasmids as detailed in the legend of Fig. 2. Empty expression vectors were added to equalize total transfected plasmid DNA concentrations. Cells were grown for 24 h in the absence or presence of \(10^{-7} \text{M T}_3\) in serum-free media and harvested. Cell extracts were then analyzed for both luciferase and β-galactosidase activities to correct for transfection efficiency. The corrected luciferase activities of untreated samples were normalized to the luciferase activities of samples containing the vector alone in the absence of the ligand (1-fold basal). The results shown are the mean ± S.D. (\(n = 3\)).

RESULTS AND DISCUSSION

Isolation, Expression and Function of TRAM-1—To identify TRAMs, we employed a far-Western approach previously used to clone a full-length version of human SRC-1 (F-SRC-1) cDNA (10). We screened cDNA expression libraries with a \[^{32}P\]labeled probe containing the LBD of rat TRb1 in the presence of T3. Two positive clones, termed TRAM-1(293) and TRAM-1(PIT), from a 293 cell line and a human pituitary library, respectively, were identified. The nucleotide sequences of the coding regions and 3'-ends of these two clones were determined and found to be overlapping. TRAM-1(293) cDNA is ~6 kb in length, contains a poly(A) signal, and encodes an open reading frame (ORF) of 913 amino acids (aa), whereas TRAM-1(PIT) cDNA contains an ORF of 848 aa. The remaining 5'-sequence of TRAM-1 mRNA was cloned using 5'-rapid amplification of cDNA ends. A full-length version of the cDNA, designated as TRAM-1 cDNA, consists of a protein coding region of 4272 base pairs flanked by 191 base pairs of 5'-untranslated and 3.5 kb of 3'-untranslated regions with a poly(A) tail. The ORF of TRAM-1 cDNA encodes 1424 aa with a calculated molecular size of TRAM-1 of 155.3 kDa. In TRAM-1(293) cDNA, the region corresponding to aa 904–918 was missing, probably due to alternative splicing. Comparison of these sequences with those in the GenBank showed that TRAM-1 shares homology with SRC-1 (Fig. 1A) and TIF2 sequences (33.2 and 42.2% identity, respectively). Thus, TRAM-1 belongs to the 160-kDa protein subset of the NCoA gene family, which has a basic helix-loop-helix (bHLH)/PAS domain in the N-terminal, a nuclear receptor binding domain in the central, and a glutamine (Q) rich sequence in the C-terminal regions (Fig. 1C).

Northern blot analysis of poly(A)1 RNAs from human tissues (Fig. 1B) indicated that the major TRAM-1 mRNA is ~9 kb; a minor species (~5.5 kb) is also seen in several tissues. Although TRAM-1 is ubiquitously expressed, the expression pattern of TRAM-1 (top panel) is different from that of SRC-1 (bottom panel). TRAM-1 is highly expressed in placenta, whereas SRC-1 is most abundant in brain. This result suggests that the expression of TRAM-1 and SRC-1 may be differentially regulated and thus may play specific roles in NR-mediated gene expression in different target tissues.

To investigate the coactivator activity of TRAM-1, cotrans-
and 8 in the absence or presence of GST-TR and its GST-AF-2 mutant was analyzed by the pull-down assay. Of note, there are two major differences as protein. The amino acid sequence of TRAM-1 has 74.2% identity with that of p/CIP. Of note, there are two major differences as protein. The amino acid sequence of TRAM-1 has coactivator activity for TR function. TRAM-1 has such activity. Thus, the apparent functional differences between TRAM-1 and p/CIP may be due to different C-terminal tails, although a sequencing error of p/CIP cannot be ruled out.

In vitro Binding Assay—Binding of TRAM-1 to NRs was tested using GST-fusion proteins. As shown in Fig. 3A, in vitro translation of TRAM-1 mRNA generated a major full-length 35S-labeled product (160 kDa) (lane 1). In the presence of cognate ligands, 35S-labeled TRAM-1 protein showed increased binding with GST-TR (lanes 2 and 3), GST-ER (lanes 4 and 5), GST-RAR (lanes 6 and 7), and GST-RXR (lanes 8 and 9) fusion proteins. Thus, TRAM-1 can interact with several members of NRs in a ligand-dependent manner.

Recently, a consensus interaction motif, a helical leucine-charged residue-rich domain (LXXLL), was identified in a number of coactivators such as SRC-1, TIF2, RIP140, TIF1, and CBP/p300 (14, 17, 18). Since TRAM-1 contains three such motifs in the central region between aa 621–742 (region A), and another at aa 1052–1060 (region B), we generated N-terminal and C-terminal halves of TRAM-1 as in vitro translated proteins, termed N-TRAM-1 (aa1–780; containing region A) and C-TRAM-1 (aa 877–1424; containing region B), and studied their interaction with TR and its AF-2 mutant, E457A (Fig. 3B). Both N-TRAM-1 and C-TRAM-1 showed ligand-dependent interaction with GST-TR (lanes 2, 3, 7, and 8), indicating that there are multiple nuclear receptor interaction sites, probably via LXXLL motifs in these region. Surprisingly, while C-TRAM-1 failed to show ligand-dependent interaction with GST-E457A (lanes 9 and 10), N-TRAM-1 retains this function (lanes 4 and 5) similar to wild type. This result suggests that region A may mediate ligand-dependent, but AF-2 independent, interactions with TR.

Next, we generated GST-TRAM-1A (aa 577–800) and GST-TRAM-1B (aa 800–1215) fusion proteins, which contain regions A and B, respectively, and tested their interactions with in vitro translated 35S-labeled TR and RXR. As shown in Fig. 3C, TR showed ligand-dependent interaction with TRAM-1A (lanes 2 and 3) and little or no ligand-dependent interaction with TRAM-1B (lanes 4 and 5). RXR showed ligand-dependent interaction with TRAM-1A (lanes 7 and 8) but not TRAM-1B (lanes 9 and 10). This result confirms the recent reports that the function of an LXXLL motif also depends on other sequences (17, 18).

Region A, which contains three LXXLL motifs, is highly conserved among members of the NCoA (SRC-1, TIF2, p/CIP, and TRAM-1) gene family. On the other hand, the LXXLL motif in region B is not present in SRC-1, TIF2, and p/CIP. A transfection study using an SRC-1 mutated in the LXXLL motif in region A has shown that the mutant fails to enhance ligand-induced RAR- or ER-mediated transactivation (17, 18). Therefore, region A of TRAM-1 may play a more important role for ligand-dependent interaction with NRs than region B.

In pull-down experiments, region A also displayed ligand-de-
Koenig (20) reported that TR is more dependent on SRC-1 than AF-2, in contrast to SRC-1. Previously, O’Donnell and colleagues (22) translated TRAM-1A and we used GST-TRAM-1A and GST-SRC-1A for EMSA. As shown in Fig. 4A, wild-type TR forms homodimers (lane 1) and heterodimers with RXR (lane 7) on an inverted palindrome TRE, F2. Addition of T₃ decreased homodimer formation (lane 2) as noted previously (19). Incubation of TR and TR/RXR with GST-SRC-1A produced a ligand-dependent TR/SRC-1A (lane 4) and TR/RXR/SRC-1A (lane 10) complex, respectively. Similarly, GST-TRAM-1A showed a ligand-dependent interaction with wild-type TR and TR/RXR. In addition, a RXR antibody could supershift TR/RXR/SRC-1A and TR/RXR/TRAM-1A complexes (data not shown), indicating that both SRC-1 and TRAM-1 can form a ligand-induced complex with TR/RXR heterodimer on F2 TRE. Interestingly, GST-TRAM-1B did not show any interaction with TR or TR/RXR on EMSA (data not shown), consistent with region A being more important for ligand-dependent interaction with NRs than region B.

Next, we compared the interaction of an AF-2 mutant of TR, E457A, with TRAM-1 and SRC-1. As shown in Fig. 4B, E457A showed similar properties of homo- and heterodimers formation as wild-type TR. While SRC-1A failed to show ligand-dependent interaction with E457A (lane 4) and E457A/RXR (lane 10), TRAM-1A still exhibited strong ligand-dependent interaction with E457A (lane 6) and E457A/RXR (lane 12). We speculated that TRAM-1 may interact with subdomains of NRs other than AF-2, in contrast to SRC-1. Previously, O’Donnell and Koenig (20) reported that TR61 point mutants of aa 288–300 (helix 3 region in the LBD of TR), which are conserved among NRs, have impaired transcriptional activation without altered ligand and DNA binding, similar to AF-2 mutants. X-ray crystallographic studies have shown that the AF-2 region comes into close contact with the helix 3 region after ligand binding (5–7). In EMSA (Fig. 4C), K288A also showed similar properties of homo- and heterodimers formation as wild-type TR. Interestingly, TRAM-1A shows negligible ligand-dependent interaction with K288A and K288A/RXR, compared with SRC-1. Similar results were obtained when we used the direct repeat TRE DR4 (data not shown). These EMSA studies indicate that although both TRAM-1 and SRC-1 interact with liganded TR, SRC-1 may preferentially bind to the AF-2 region; on the other hand, TRAM-1 may preferentially bind to helix 3 in the LBD of TR.

The C-terminal region of CBP has been reported to interact with SRC-1 (9) and P/CAF, which is a p300/CBP-interacting protein with histone acetylase activity (21). In addition, the progesterone receptor has been shown to interact directly with P/CAF (22). Thus, we tested whether CBP or P/CAF interacts with TRAM-1. As shown in Fig. 5, both CBP (aa 1626–2260) and P/CAF were significantly retained on GST-TRAM-1B complexes compared with GST-SRC-1A. This observation suggests that TR, TRAM-1, CBP/p300, and P/CAF can potentially form a ligand-dependent complex to mediate ligand-induced transactivation.

In summary, we have isolated cDNA, encoding a putative thyroid hormone receptor coactivator TRAM-1. TRAM-1 belongs to a 160-kDa NCOR family that includes SRC-1 and TIF2. However, TRAM-1 exhibits expression and interaction properties distinct from SRC-1. As such, TRAM-1 may be another component of a TR coactivator complex critical for TR action.

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Note Added in Proof: Alternate splice forms of human TRAM-1 cDNAs have recently been reported (Li, H., Gomes, P. J., and Chen, J. D. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8479–8484; Chen, H., Lin, R. J., Shilts, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) Cell 90, 569–580; and Anzick, S. L., Kononen, J., Walker, R. L., Azorsa, D. O., Tanner, M. M., Guan, X.-Y., Sauter, G., Kallioniemi, O. P., Trent, J. M., and Meltzer, P. S. (1997) Science 277, 965–968).

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