A Unique Role of the β-2 Thyroid Hormone Receptor Isoform in Negative Regulation by Thyroid Hormone

MAPPING OF A NOVEL AMINO-TERMINAL DOMAIN IMPORTANT FOR LIGAND-INDEPENDENT ACTIVATION

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Negative regulation by thyroid hormone is mediated by nuclear thyroid hormone receptors (TRs) acting on thyroid hormone response elements (TREs). We examine here the role of human TR-β2, a TR isoform with central nervous system-restricted expression, in the regulation of target genes whose expression are decreased by triiodothyronine (T3). Using transient transfection studies, we found that TR-β2 achieved significantly greater ligand-independent activation on the thyrotropin-releasing hormone (TRH) and common glycoprotein α-subunit genes than either TR-β1 or TR-α1. A chimeric TR-β isoform containing the TR-β2 amino terminus linked to the TR-α1 DNA- and ligand-binding domains functioned like the TR-β2 isoform on these promoters, confirming that the amino terminus of TR-β2 was both necessary and sufficient to mediate this effect. By constructing deletion mutants of the TR-β2 amino terminus, we demonstrate that amino acids 89–116 mediate this function. This domain, important in ligand-independent activation on negative TREs, is discrete from a previously described activation domain in the amino-terminal portion of TR-β2. We conclude that the central nervous system-restricted TR-β2 isoform has a unique effect on negative regulation by T3 that can be mapped to amino acids 89–116 of the amino terminus of the human TR-β2.

Thyroid hormone receptors (TRs) are both ligand-independent and -dependent transcription factors and are part of the nuclear receptor superfamily containing discrete functional domains (Ref. 1, and for review, see Refs. 2–5). In vertebrates, TR isoforms derive from two genes: c-erbA-α and c-erbA-β. By alternative RNA splicing, the c-erbA-α locus gives rise to TR-α1 and c-erbA-α2-β (6); the latter does not bind T3 and may inhibit the function of other TRs (7–9). Using alternative 5‘ exons and presumably separate promoters, TR-β1 and TR-β2 are derived from the c-erbA-β locus (10, 11). Thus, TR-β isoforms differ only in their amino-terminal domains (see Fig. 1A). Although the α1 and β1 TR isoforms are expressed ubiquitously (12), TR-β2 mRNA is found almost exclusively in the pituitary (10), hypothalamus (13), and at very low levels in some other parts of the central nervous system and periphery (14–16).

The transcription of many genes is affected by TRs. On positively regulated genes, like the growth hormone and myosin heavy chain genes, TR causes ligand-independent silencing and T3 activates transcription (17–19). Other genes are negatively regulated by thyroid hormone, such as the thyrotropin releasing hormone (TRH) (20, 21), TSH-α (22, 23) and β-subunit (24–27), epidermal growth factor (28), β-myosin heavy chain (29), Rous sarcoma virusLTR (30), and keratin (31) genes. Although they have been studied less extensively than positively regulated genes, they exhibit ligand-independent activation and ligand-dependent repression by TRs (30–32). Transcriptional regulation by TRs occurs via binding to TREs; these are composed of the half-site consensus sequences AG-GTCA, which binds TR (33). Positive response elements (pTREs) consist of either palindromic, direct-repeat, or inverted-repeat configurations (34–36). Some negative thyroid hormone response elements (nTREs) have also been mapped. A monomeric site can be found in the TRH (20, 21) and TSH-β genes (24–26), and another response element in the TRH gene consists of two sites separated by 11 base pairs (20). For these response elements, the direction and spacing of the half-sites is important for transcriptional modulation (32).

Considering the restricted localization of its expression, TR-β2 may play a unique role in negative regulation by thyroid hormone of centrally located genes, but its specific role in negative regulation has not been extensively studied. We focused this study on the amino termini of TR-β1 and TR-β2 since this is the only difference between these isoforms. Our results show that TR-β2 is the only TR isoform capable of significant ligand-independent activation of the TRH and TSH-α promoters, resulting in increased repression in the presence of T3. The area responsible for this unique pathway of negative regulation by TR-β2 is located in the area between the 89th and the 116th amino acids of its amino terminus.

EXPERIMENTAL PROCEDURES

The negative response element reporter constructs included the 5’-flanking sequences for the TRH (20) and the common glycoprotein α-subunit gene (23) fused upstream of the luciferase reporter gene. The construct TRETK contains two copies of idealized pTREs arranged as a palindrome upstream of a minimal thymidine kinase promoter fused to the luciferase gene (37). A thymidine kinase control plasmid contains 198 base pairs of the 5’-flanking region fused to the luciferase gene (TK198) (27). All promoter constructs fused to the luciferase reporter gene contained two transcriptional stop sequences upstream of the promoter to prevent read-through transcription (pSV0-ALΔ5‘ vector; Ref. 38).

The cDNAs encoding human TR-α1, TR-β1, TR-β2, and chimeric or
mutated TRs were inserted into the expression vector pSG5, which employs the SV40 early promoter (39). The human TR-\(\beta\)2 amino terminus was obtained by polymerase chain reaction (PCR) amplification of genomic DNA and ligated to the \(\text{Sac}I\) site of TR-\(\beta\) common sequences forming the TR-\(\beta\)2 cDNA. The DNA sequence and location of the methionine start codon for translation were identical to a previous report.2

Chimeric TR-\(\beta\)2aa was constructed by introducing a \(\text{Sac}I\) site into TR-\(\alpha\) at amino acid 52 to allow for exchange with the TR-\(\beta\)2 amino terminus. The amino-terminal deletion TR-\(\Delta\beta\)b was constructed using PCR to introduce a Kozak initiation sequence at the beginning of the DNA binding domain (41). The TR-\(\beta\)2 amino-terminal deletion constructs were made using PCR to introduce an \(\text{Xba}I\) site at either amino acid 87 or 116 (to ligate with TR-\(\beta\) common region). An identical Kozak initiation sequence preceded by an \(\text{Eco}RI\) site was present at the 5' end of each amino-terminal deletion construct.

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2. K. Damm, National Center for Biotechnology Information, 1993, GenBankTM Data Bank accession number G437814.
The reporter used for heterologous expression system was UAS-TK fused upstream of the luciferase gene (42). The amino-terminal cDNAs from the TR-β2 deletion constructs were cloned in-frame with the GAL4-DNA as an EcoRI-SacI insert in the pBXGI vector (43). The integrity of all constructs was confirmed by restriction endonuclease digestion and dideoxy sequencing.

The CV-1 cell line was used for all experiments. Transient transfections were performed in 6-well plates on subconfluent cells using the calcium-phosphate technique without glycerol shock. Except where otherwise noted, 1.6 mg of reporter construct and 80 ng of receptor expression vector (or equivalent of vector alone) were introduced into each well. 16 h after transfection, culture medium was replaced with culture medium containing fetal bovine serum treated with anion exchange and activated charcoal; 10 nM T3 was added when indicated. 36–40 h after transfection, cells were harvested and assayed for luciferase activity. Data were from at least three independent experiments, performed in triplicate, and are displayed as mean ± S.E.

A probe representing the -78 to -36 sequence of the human TRH promoter (20) (5′-GGGACCCCTCCGCTGACCTCACCACGGAGC-CGCCTGG-3′) was radiolabeled using PCR and [α-32P]dCTP (400 μCi/mmol, NEN Life Science Products). Proteins were made by in vitro translation in rabbit reticulocyte lysate (TNT kit, Promega) using TR isoforms or RXRα cDNAs in pSG5 and T7 polymerase. Three μl of each protein was used in each reaction with radiolabeled probe (100,000 cpm). Gel-mobility shifts were performed as described previously (20).

Nuclear extracts were made from CV-1 cells transfected as described above with 10 μg of expression plasmid/100-mm plate. Cells were harvested, and nuclei were purified as described by Surks et al. (44). After the final centrifugation, the nuclear pellet was resuspended in 30 mM Tris, pH 8.0, 0.4 mM NaCl, 5 mM MgCl2, 2 mM EDTA, 10% glycerol, dithiothreitol, and protease inhibitors. 60 μg (Bradford assay) of nuclear extracts and 1 μl of in vitro translated TR-β2 (as a control) were used for Western blotting using standard techniques and a C4 antibody (Dr. Cheng, National Institutes of Health, Bethesda, MD), which is directed against the carboxyl terminus of TR-β. Detection was accomplished using the BM Chemiluminescence Western blotting kit (Boehringer Mannheim, Germany).

RESULTS

Shown in Fig. 1A is a schematic representation of the different TR constructs used in this study. In Fig. 1B, amino acid sequences of the human, rat, mouse, and chick amino terminus of TR-β2 are compared. To map the specific effect of TR-β2, six deletion constructs were made. These are shown in Fig. 1C, and the deleted (Δ) region is underlined in Fig. 1B. All receptor constructs were inserted into a viral expression vector (pSG5) for use in transient transfection studies.

We first wanted to determine if TR-β2 was different from other TR isoforms with respect to negative regulation of the TRH and common α-subunit genes. In the absence of ligand (T3), TR activates transcription of negatively regulated genes, termed ligand-independent activation. It is clear that TR-β2 has an increased capacity to activate in the absence of ligand compared with the other TR isoforms (4.0-fold versus 1.1–1.4-fold) on the TRH promoter (Fig. 2A) and α-subunit promoter...
Western blot analysis of nuclear extract obtained from CV-1 cells transfected with different TR isoforms on the TRH reporter construct. Again, transactivation when T3 is present (ligand-dependent repression) versus ligand (ligand-independent activation); and 2) repression of components: 1) activation of transcription in the absence of increased ligand-independent activation of TR-β2, however, seems to be due to the effect is preserved in different species and may have physiological relevance.

However, the TR isoforms could not ligand independently activate a control promoter (TK199, Fig. 2C), indicating the specificity of the effect for the promoters and reporter constructs used in this study. Moreover, we find no significant ligand-dependent repression of this construct in CV-1 cells under the conditions used in this study (data not shown). Thus in contrast to a previous report, our results cannot be explained by a nonspecific effect of TR on the luciferase reporter gene (45).

To determine if the amino-terminal domain of TR-β2 was responsible for its enhanced ligand-independent activation in comparison with the other TR isoforms, we transfected the TR-β2 amino terminus to the TR-α DNA- and ligand-binding domains forming the chimeric receptor, TR-β2αα. As expected, the TR-β2αα construct had properties similar to TR-β2, and not to TR-α, both on TRH- and α-subunit reporter constructs (Fig. 2, A and B, respectively). This confirms that the amino terminus of TR-β2 mediates the unique ligand-independent activation capacity of TR-β2.

The effect of TRs on negatively regulated genes has two components: 1) activation of transcription in the absence of ligand (ligand-independent activation); and 2) repression of transcription when T3 is present (ligand-dependent repression). Shown in Fig. 2D is ligand-dependent repression of the different TR isoforms on the TRH reporter construct. Again, TR-β2 yielded greater ligand-dependent repression versus the other isoforms (2.3-fold versus 1.15–1.3-fold). The increased -fold ligand-dependent repression, however, seems to be due to increased ligand-independent activation of TR-β2 since the actual levels of repressed transcription are similar for all reporter constructs (data not shown, and Fig. 3).

To isolate the regions of the TR-β2 amino terminus important for ligand-independent activation, we constructed a number of deletions that are shown in Fig. 1C. Constructs can be viewed as pairs, with progressive deletions of the 5’ end of the amino terminus (21, 51, 89 amino acids) with or without a deletion of the area between amino acids 87–116 (the Δ constructs). Fig. 3 illustrates both ligand-independent activation (−T3) and ligand-dependent repression (+T3) for these deletion constructs on the TRH reporter construct. Clearly, whenever amino acids 87–116 are deleted, TR-β2 loses its ligand-independent activation and is not significantly different from data obtained from transfection of either TR-β1, TR-Δββ (a construct without any amino terminus), or empty vector (pSG5, −) alone. However, deletions of the first 20, 50, or 89 amino acids does not reduce ligand-independent activation of TR-β2. This indicates that amino acids 89–116 of TR-β2 are responsible for its unique properties in negative regulation of the TRH gene by T3. Furthermore, as shown in Fig. 1B, this area is completely conserved in the chicken and less so in the mouse and rat, supporting a potential physiological significance.

At the bottom of Fig. 3 is a Western blot analysis of wt and mutant TR-β2 expression in nuclear extracts obtained from transfected CV-1 cells. A band of approximately 60 kDa (arrow) was detected after a wt TR-β2 transfection but not after transfection of pSG5 alone (−), indicating detection of TR-β2 protein in transfected cells. Progressively smaller bands of similar intensity were detected in CV-1 cells transfected with TR-β2 deletion constructs. Thus, these functional data on the TRH promoter cannot be explained by differences in expression levels of the TR-β2 deletion constructs.

Similar results were obtained on the α-subunit reporter construct (Fig. 4). Interestingly, deletion of either the first 50 or 88 amino acids actually enhanced ligand-independent activation on the α-subunit gene, suggesting that the first 50 amino acids of TR-β2 may mask ligand-independent activation of the 89–116 region on certain promoters.

A transactivation domain specific to the TR-β2 isoform (chicken) was recently ascribed, using a GAL4 heterologous expression system, to a region between the 29th and 76th amino acids of the TR-β2 amino terminus (46). We used this model system to prove that the region responsible for a unique effect in negative T3 regulation was discrete from this previously described domain. Results from these mapping experiments are shown in Fig. 5. This figure demonstrates that the human TR-β2, like the chicken TR-β2, has a transactivation
domain (15.3-fold versus 0.8-fold activation for TR-β1) mapping primarily to a region between amino acids 21 and 50 in this study and overlapping the previously described chicken TR-β2 transactivation domain. This graph also denotes that the deletion of the area from amino acids 89 to 116 does not significantly affect activation in this heterologous system (no difference between TR-β2 wt and 1–120Δ or 21–120 and 21–120Δ). Thus, the domain important for ligand-independent activation on negative TREs is different from the previously described transactivation domain.

We next tested the TR-β2 deletion constructs on a reporter construct containing two copies of an idealized palindromic pTRE (TRETK). Fig. 6 demonstrates that transfection of TR-β1 yields greater -fold T3 activation than wt TR-β2. Note when you examine TR-β2 constructs as pairs (1–120 with 1–120Δ, 21–120 with 21–120Δ, etc.), the Δ construct always displays greater T3 activation than its full-length counterpart. In particular, the 1–120Δ construct relative to the full-length 1–120 TR-β2, achieves activity similar to wt TR-β1. This suggests that the nTRE domain we describe here might antagonize the positive T3 regulatory properties of TR-β2. It also strengthens our conclusions on the nTRE reporters since the Δ constructs that yielded very little activity on negatively regulated genes now show increased T3 activation on TRETK compared with their pair constructs with an intact 89–116 domain. Thus, there appears to be an opposite effect of the 89–116 domain in positive versus negative regulation by T3.

It can also be noted that the constructs with no or little activation in the heterologous expression system (51–120Δ, 89–120, Fig. 5) show significantly lower ligand-dependent activation of this positively regulated TRE than the other constructs containing the amino acids 21–50, such as wt TR-β2, 1–120Δ, 21–120, and 21–120Δ. This implies that the transactivation domain located between amino acids 21 and 50 has its importance primarily for positive regulation by T3.

Using a gel-shift assay, we evaluated DNA-binding of the different deletion constructs. In data not shown, TR-β2 deletion constructs were in vitro translated to a similar extent as demonstrated in [35S]methionine labeling of translation products. TR-β2 deletion constructs show similar heterodimeric binding to the site 4 DNA probe, which is an important nTRE present in the TRH promoter (Fig. 7). This confirms the integrity and similar translation efficiency of TR-β2 deletion construct in vitro and indicates that differences in DNA-binding of the TR-β2 deletion constructs are unlikely to cause the observed effects in negative T3 regulation we describe.
DISCUSSION

This paper is the first report mapping a unique domain of the TR-β2 isoform in negative regulation by T₃. We demonstrate that TR-β2 is a better ligand-independent activator of TRH and α-subunit gene expression than either TR-α1 or TR-β1. As a consequence of increased ligand-independent activation, -fold T₃ repression is also significantly greater with the TR-β2 isoform. The area responsible for this enhanced ligand-independent activation of TR-β2 was mapped to amino acids 89 to 116 of the amino terminus. This enhanced ligand-independent activation of TR-β2 in CV-1 cells was not due to differential protein expression or DNA binding, suggesting that the mechanism of ligand-independent activation involves direct interaction of the TR-β2 amino terminus with either transcriptional cofactors or the basal transcription machinery itself.

Maintenance of TR-α and TR-β genes through evolution of vertebrates suggests that TR isoforms might have different roles in thyroid hormone regulation of gene expression. TR isoform specificity has not been extensively studied although there has been recent interest in this subject. For example, both TR-α1 and TR-β1 readily form heterodimers, but TR-β1 tends to form a stronger homodimer (41, 47). Also, on a palindromic positive TRE, Ng et al. (48) have described that TR-β2 forms a much stronger homodimer than TR-β1. Some functional differences have also been noted on positive TREs. For instance, Hollenberg et al. (41) have shown that TR-β2 has increased ligand-independent repression relative to TR-β1. This was recently confirmed by another group (49).

Less well known is whether TR isoform-specific T₃ regulation extends to negatively regulated genes (TRH, and TSH subunit genes). Recent insight into the role of TR-β isoforms in control of TSH expression has been obtained from studies where the TR-β locus was disrupted in mice by homologous recombination. These animals lack both TR-β isoforms and clearly have increased thyroid hormone levels and inappropriate TSH secretion, indicating central thyroid hormone resistance (50). These data prove the importance of the TR-β isoforms in negative T₃ regulation of the pituitary and hypothalamus since the remaining TR-α1 expression was not sufficient to maintain normal thyroid hormone levels. It is unclear from this study, however, if loss of one or both TR-β isoforms is required to observe the resistant phenotype or if loss of ligand-independent function by TR-β isoforms has any physiological significance in these animals. Based on the results reported here, we suggest that TR-β2 may be more important than TR-β1 in negative regulation by thyroid hormone and speculate that the loss of ligand-independent activation on target genes in the pituitary and hypothalamus in TR-β knock-out animals may tend to minimize their resistant phenotype.

The molecular mechanisms responsible for differences in TR-β1 versus TR-β2 function have been studied by several groups. While differences in DNA-binding and function of these isoforms have been suggested (41, 51), recent studies from this laboratory have begun to provide a rationale for a unique role of TR-β2 in thyroid hormone action. Our laboratory (52) recently demonstrated that ligand-independent activation by TR-β2 was unaffected by the nuclear co-pressors, N-CoR, while ligand-independent activation by either TR-α1 or TR-β1 was masked. This finding suggests that TR-β2 is the only TR isoform able to mediate significant negative T₃ regulation in the presence of N-CoR. In addition, we have recently suggested that mutant TR-β2 may mediate the syndrome of pituitary resistance to thyroid hormone (53). PRTH mutants, for example, had no significant dominant negative activity as TR-β1 isoforms on positive or negative TREs. However, when PRTH mutants were expressed as TR-β2 isoforms, they had strong dominant negative activity on the negative TREs.

The data presented here extend these findings and demonstrate the importance of enhanced ligand-independent activation by TR-β2 for its effect on negative T₃ regulation. We found that ligand-independent activation was at least 2 to 3 three times greater with TR-β2 than with TR-α1 or TR-β2 on the TRH and common α-subunit genes. We could transfer this effect to TR-α1 by replacing its amino terminus with that of TR-β2 (Fig. 2, A and B), proving that the TR-β2 amino terminus mediated this effect. Absence of regulation of a control promoter (Fig. 2C) showed that this was not a nonspecific effect of TREs on the luciferase reporter gene or vector background as previously suggested by others (45). Feng et al. (40) and Satoh et al. (21) and have done similar studies on the human and mouse TRH gene, respectively, transfected into heterologous cell lines and reported absence of TR isoform specificity in T₃ inhibition. Differences between our results and their results could be explained by differences in cell lines or transfection conditions that were employed.

We then localized the region of the TR-β2 important for this effect to an area between amino acids 89 and 116 (Figs. 3 and 4). This region of the amino terminus was differentiated from a known transactivation domain located in the amino terminus of TR-β2 (46) using a heterologous GAL4 expression system (Fig. 5). When the TR-β2 amino terminus was fused to the GAL4 DNA-binding domain, activation of the UASTK reporter was more than 15-fold greater than when the TR-β1 amino terminus was utilized. Deletion of the first 50 but not the first 20 amino acids within the TR-β2 fusion construct resulted in a loss of the stimulatory effect on the UASTK reporter construct, suggesting a transactivation domain is located between amino acids 21 and 50. In contrast, no significant effect on activation was observed when the area important for negative regulation (amino acids 87–116) was deleted, supporting the conclusion that these two domains are distinct in location and function.

As a corollary, we studied positive regulation by T₃ on the palindromic element found in TRET3K (Fig. 6). These studies suggest that the domain we describe for negative regulation has an inhibitory role on positive regulation by T₃. For example, TR-β1 had a significantly greater T₃-dependent activation than TR-β2 on TRET3K, but deletion of amino acids 87–116 of TR-β2 (the Δ constructs) increased activation by TR-β2 to levels similar to TR-β1 (in the 1–120 Δ construct). These results suggest an opposite effect of the domain located between amino acids 89 and 116 of TR-β2 regarding negative and positive regulation by T₃. The exact mechanism of action of this domain remains to be elucidated but is not due to differences in DNA-binding, as suggested by electrophoretic gel-mobility shift assays (Fig. 7).

In this paper, we also confirm the existence of the transactivation domain of the amino terminus of human TR-β2, and we further map it to the area between amino acids 21 to 50. A previous report using a similar heterologous expression system had mapped this transactivation area to amino acids 29–76 of the chicken TR-β2 (46). Combining both reports, we suggest that the transactivation domain is located between the 29th and 50th amino acid of the TR-β2 amino terminus. Functional data on a palindromic element confirm and extend previous findings on the importance of this domain for positive regulation by T₃. TR-β2 constructs lacking this domain (constructs 51–120, 51–120Δ, 89–120) exhibit greatly reduced ligand-dependent activation compared with either wt TR-β2 or 21–120 construct.

We conclude TR-β2 plays a unique role in negative regulation by thyroid hormone by exhibiting greater ligand-independent activation and ligand-dependent repression of the TRH and
α-subunit of glycoprotein hormones genes. This effect is mediated by amino acid 89–116 of the amino terminus of TR-β2. Future studies will elucidate the interactions between this newly described domain and other cofactors in thyroid hormone action and help clarify the mechanism of negative regulation by thyroid hormone.

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