The Differential Hormone-dependent Transcriptional Activation of Thyroid Hormone Receptor Isoforms Is Mediated by Interplay of Their Domains*

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Human thyroid hormone nuclear receptor isoforms (TRα1 and TRβ1) express differentially in a tissue-specific and development-dependent manner. It is unclear whether these two isoforms have differential functions. We analyzed their interaction with a thyroid hormone response element with half-site binding motifs arranged in an everted repeat separated by six nucleotides (F2). Despite extensive sequence homologies, the two isoforms bound to F2 with different affinities and ratios of homodimer/monomer. Using F2-containing reporter gene, we found that the transcriptional activity of TRβ1 was 6-fold higher than that of TRα1. The lower activity of TRα1 was not due to differences in expression of the two isoforms because similar nuclear localization patterns were observed. To understand the structural determinants responsible for these differences, we constructed chimeric receptors in which hinge regions (domain D), hormone binding domains (domain E), and domains (D+E) were sequentially interchanged and their activities were compared. Chimeric TRs containing the domains D, E or (D+E) of TRβ1 showed increased propensities to form homodimers and mediated higher transactivation activities than TRα1. Thus, differential transactivation activities of TR isoforms are mediated by interplay of their domains and could serve as an important regulatory mechanism to achieve diversity and specificity of pleiotropic T3 effect.

Thyroid hormone receptors (TRs) are the products of two genes, TRα and TRβ, located on chromosomes 17 and 3, respectively. Alternate splicings of their primary transcripts produce isoforms of the protein (α1, α2, β1, and β2), which regulate the transcription of their target genes by binding to specific DNA sequences, known as thyroid hormone response elements (TREs). These contain repeats of a half-site binding motif with the sequence AGGTCA. Naturally occurring TREs can include sequences, known as thyroid hormone response elements (TREs). These contain repeats of a half-site binding motif with the sequence AGGTCA. Naturally occurring TREs can include adjacent palindromic repeats, as direct repeats separated by 4 nucleotides, and as everted repeats separated by 6 nucleotides (F2) (1, 2). The sequences of TRs have been divided into four separate domains, A/B, C, D, and E. Domain C contains two zinc fingers and is involved in binding of the receptors to TREs. Domains D and E are structurally linked, in so far as part of domain D is required for the biological function of domain E, which is to bind thyroid hormones (3). Domains D and E are also involved in binding to co-repressors and dimerization, respectively (4). The crystal structures of TRE-bound domains C of TRβ1 and the retinoid X receptor (5) and of domains D/E complexed with a thyroid hormone agonist (6) have recently been solved. These structures give important information on interaction within domains but reveal nothing about the modes and roles of the interaction between domains in intact receptors, which may have important biological significance.

Comparison of the sequences between the human TRα1 (w-TRα1) and human TRβ1 (w-TRβ1) indicates that except domain A/B, there is extensive sequence homology between the two isoforms, specifically 88% in domain C, 71% in domain D, and 86% in domain E. Despite this high sequence homology, biochemical evidence suggests that they could have isoform-specific roles in mediating the action of thyroid hormones. TRα and β genes are expressed at different stages during embryonic development (7, 8) and during amphibian metamorphosis (9). Moreover, these two isoforms are expressed differentially in different tissues (8, 10). More direct evidence to support the isoform-specific functional role of the TRα1 and TRβ1 was provided by using gene transfer experiments. Strait et al. (11) showed that the gene encoding PCP-2 is regulated by TRβ1 but not by TRα1. The 3,3',5-triiodo-L-thyronine (T3)-dependent negative regulation of thyroid tropin releasing hormone pro-moter was shown to be mediated by TRβ1 but not by TRα1 (12). Recently, using stably transfected neuronal cell line, Lebel et al. (13) showed that only cells that overexpress TRβ1, but not TRα1, can respond to T3 to exhibit morphological and functional characteristics indicative of neural differentiation.

At present, the molecular basis of isoform-specific gene regulation is not understood. It was suggested that the different homodimerization potentials of the two isoforms may underlie the functional differences. TRβ1 is known to bind to F2 and the TRE site on cardiac β-myosin heavy chain mainly as a homodimer, whereas TRα1 forms homodimer poorly (14, 15). These differences, however, are not eliminated by removal of A/B domains from the molecules (16) and consequently must arise from the remainder of the receptors. However, it is not clear that they are a consequence only of differences in sequence. They may also be caused by changes in interactions between domains in the intact receptors. Because of their marked effects on the properties of the receptors as transcrip-

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‡ The abbreviations used are: TR, thyroid hormone receptor; T3, 3,3',5-triiodo-L-thyronine; TRβ1, human TR subtype β1; TRα1, human TR subtype α1; RXRβ, rat retinoid X receptor, subtype β; TRE, thyroid hormone response element; EMSA, electromophoretic mobility gel shift assay; F2, chicken lysozyme TRE; CAT, chloramphenicol acetyltransferase.
tion factors, we have investigated their origins by construction of a series of six chimeric receptors, in which domains A/B/C, D, and E from the two isoforms are joined in all possible combinations. We have measured their affinities for TR under identical conditions and their binding to an F2 TRE. We also determined the TR-dependent transcripitional activity of the wild type and chimeric receptors. We found that the domains C, D, and E are functionally linked, and the differential transcriptional activity of the two isoforms is mediated by interplay of their domains.

EXPERIMENTAL PROCEDURES

Construction of Plasmids Encoding Chimeric TRα1 and TRβ1—The T7-expression plasmids of the six chimeric receptors (see Fig. 2) were derived from the T7 expression plasmids of w-TRα1 (pCLC13) (17) and w-TRβ1 (pCJS3) (18). For cloning purpose, two restriction enzyme sites, NsiI and BamHI, were introduced into the boundaries between domains C and D and domains D and E of w-TRα1 (nucleotide positions 412–414 (AAG/Lys to AAA/Lys) and 616–621 (GGCCAGC/Gly-Ser to GGATCC/Gly-Ser)) to yield a new T7 expression plasmid of w-TRα1, pCH1. Only one restriction site, BamHI, was required to introduce into the boundary between domains D and E of w-TRβ1 (nucleotide positions 991–996 (GGCCAGC/Gly-Ser to GGATCC/Gly-Ser)) because in pCJS3 (w-TRβ1) the NsiI already existed which yielded a new T7 expression plasmid of w-TRβ1, pCH3. The introduction of these restriction sites was carried out by in vitro mutagenesis kit according to the manufacturer’s instructions (Bio-Rad). The introduction of these two new restriction sites into TRα1 and the BamHI site into TRβ1 did not change the amino acid sequences of TR isoform proteins. The six chimeric receptors were constructed by exchanging the domains between w-TRα1 and w-TRβ1 using NsiI, BamHI, and the 3′ EcoRI site immediately downstream of the termination codons of w-TRα1 (nucleotide position 1306 for TRα1 and 1672 for TRβ1 (pCJS3)) to yield T7 expression plasmids pCH1, pCH2, pCH3, pCH4, pCH5, and pCH6 for βαβ, ββα, βαα, ααβ, αββ, and αβββ, respectively (see Fig. 2). The coding sequences for the six chimeric receptors were verified by restriction map analyses and direct DNA sequencing.

The mammalian expression plasmids of the TRα1 and TRβ1 chimeric receptors were derived from the corresponding w-TRα1 and w-TRβ1 expression plasmids, pCLC61 and pCLC51 (19), respectively. The expression of w-TRα1 and w-TRβ1 is driven by cytomegalovirus promoter. To prepare the mammalian expression plasmids of chimeric receptor of TRβ1, pCLC51 was restricted by NsiI followed by ligation with pCDMCH3 in the presence of deoxynucleotides. The coding sequence of the w-TRβ1 in pCLC51 was then released by treating the linearized and blunt-ended pCLC51 with HindIII, thereby providing the vector for ligation to the proper chimeric TR coding fragments. The wild type and chimeric TRβ1 coding fragments were derived from the above T7 expression plasmids (pCHβ, pCH1, pCH2, and pCH3) by treating the plasmids with EcoRI. After ligation, the fragments were released by treating with NdeI. An adaptor (HindIII/NdeI) was used in the final ligation of TR coding fragments to the vectors to yield plasmids pCDMCH3, pCDMCH1, pCDMCH2, and pCDMCH3 for w-TRβ1, βαβ, ββα, and βαα, respectively. The mammalian expression plasmids of w-TRα1 and its chimeric receptors were prepared similarly except that the vector was derived from pCLC61. The resulting mammalian plasmids were pCDMCHA, pCDMCH4, pCDMCH5, and pCDMCH6 for w-TRα1, ααβ, αβα, and αβββ, respectively.

Electrophoresis Gel Mobility Assay (EMSA)—The probe, F2, was 32P-labeled similarly as described (20). Briefly, two complementary oligonucleotides containing the F2 sequences as shown in Sequence 1 below, were annealed and the recess 3′-end filled with DNA polymerase (Klenow fragment) in the presence of [α-32P]dCTP. The labeled oligonucleotides were separated on a 12% polyacrylamide gel and purified by electrophoresis.

For EMSA, unlabelled TRs synthesized by in vitro transcription/translation were used. The synthesized receptor proteins were quantified by measuring the intensity of the 32P-labeled protein bands after SDS-polyacrylamide gel electrophoresis using PhosphorImager (Molecular Dynamics, CA). The 32P-labeled protein was synthesized concomitantly by using amino acid mixture minus methionine but with [35S]methionine (4 μl; 1190 Ci/mmol). Based on the quantitation of the labeled receptors, the amounts of the unlabeled receptors were calculated. For the determination of the binding constants of TRs to F2, equal amounts of the in vitro translated unlabelled receptors were incubated with increasing concentrations of the labeled probes (0.2–120 fmol) in the binding buffer (25 mM Heps, pH 7.5, 5 mM MgC12, 4 mM EDTA, 10 mM dithiothreitol, 0.11 μ NaCl, and 0.4 μg of single-stranded DNA). In some experiments, XRβR prepared as described by Meier et al. (20) was added. After incubation for 30 min at 25 °C, the reaction mixture was loaded onto a 5% polyacrylamide gel and electrophoresed at 4 °C for 2–3 h at a constant voltage of 250 V. The gel was dried and autoradiographed. The intensities of retarded bands and free probes were quantified by PhosphorImager. The binding data were analyzed based on the equations and considerations as described below.

Analysis of Binding Data from EMSA—Binding of glucocorticoid nuclear receptors to response elements with adjacent identical half-sites has been successfully analyzed using a simple two-site cooperative model, which ignored dimerization of free receptors in solution (21). Since dimerization of bound TR’s has never been detected, we interpret our results in a similar way (Equation 1). Receptor (R) can bind to either TRE half-site (D) to give monomeric complexes (DR and RD) or to both yielding a dimeric complex (RDR).

\[
\begin{align*}
K_1 & \quad K_2 \\
2R + D & \quad \text{DR} \quad \text{RDR} \quad \text{RD} \\
K_1 & \quad K_2
\end{align*}
\]

We assume that a receptor molecule can bind to either half-site on an empty TRE (D) with a binding constant \( K_1 \) and on a monomeric complex (DR or RD) with a binding constant \( K_2 \). If \( K_2 = sK_1 \), then \( s \) is the cooperativity parameter. Positive cooperativity implies \( s > 1 \), i.e. stronger binding of the second TR than the first to the TRE. The concentration of monomeric complexes

\[
[D]_0 = [D] + [DR] + [RD] + [RDR]
\]

\[
[D^\alpha] = K_1 \cdot [D] + K_1 \cdot [R] \cdot [D] \quad \text{Eq. (2)}
\]

The total concentration of TRE

\[
[D^\alpha] = K_1 \cdot [D] + K_2 \cdot [R] \cdot [D] \quad \text{Eq. (3)}
\]

Consequently

\[
[monomer] = \frac{2 \cdot [D] \cdot [R]}{1 + 2 \cdot [D] \cdot [R] + K_1 \cdot [R]^2 + K_2 \cdot [R]^2} \quad \text{Eq. (4)}
\]

\[
[dimer] = \frac{K_1 \cdot [D] \cdot [R]}{1 + 2 \cdot [D] \cdot [R] + K_1 \cdot [R]^2} \quad \text{Eq. (5)}
\]

The concentrations of monomer, dimer, and TRE are measured on the gel from the known specific activities of the DNA probes. However, the recombinant TRs are produced in cell lysates. It was not possible to determine how much of the protein in each lysate was intact, competent TR, i.e. [R] is unknown. In each experiment, in each lane, we can measure a ratio,

\[
r = \frac{[dimer]}{[monomer] = \frac{K_1 \cdot [D] \cdot [R]^2}{2 \cdot [D] \cdot [R] + K_2 \cdot [R]^2} \quad \text{Eq. (6)}
\]

\[
r = \frac{2}{K_2} \quad \text{Eq. (7)}
\]

\[
[r = \frac{2 \cdot r}{K_2} \quad \text{Eq. (8)}
\]

\[
5' \quad \text{AAGGGATCTTCTTGGCCCGCTGACCGTCAATGTCCTAGAGGA} \quad 3'
\]

\[
3' \quad \text{ATATTGCGACGCTCAATGGCTTACGTCAG} \quad 5'
\]

SEQUENCE 1
Substituting Equation 8 into 5 and 6 gives

\[ [\text{monomer}] = \frac{4 \cdot K_d \cdot [D] \cdot r}{(K_2 + 4 \cdot K_1 \cdot r + 4 \cdot K_1 \cdot r^2)} \] (Eq. 9)

\[ [\text{dimer}] = \frac{4 \cdot K_d \cdot [D] \cdot r^2}{(K_2 + 4 \cdot K_1 \cdot r + 4 \cdot K_1 \cdot r^2)} \] (Eq. 10)

For each combination of recombinant TR and TRE, values of $K_1$ and $K_2$ were estimated by fitting the measured concentrations [monomer] and [dimer] simultaneously to Equations 9 and 10 as functions of $[D]_0$ and $r$, with the constraints $K_1 > 0$, $K_2 > 0$. Analyses were performed using the PC-MLAB program (Civlized Software, Bethesda, MD). It must be pointed out that this procedure violates one basic assumption of least squares curve fitting, i.e. that experimental uncertainties in plotted data parallel the y axis. Here we have uncertainties along both axes. Together with the problems of the gel retardation method, which requires separation of reactants and products, perturbing the system from equilibrium, as discussed previously (16), could result in some uncertainty in $K_1$ and $K_2$. Consequently, the values given in Table I may be only approximate.

Binding of TR to the Wild Type and Chimeric Receptors—The binding was carried out by incubating the in vitro translated TR proteins with 0.4 nM [125I]T3 in the presence or absence of increasing concentrations of unlabeled T3 (0.1 to 10 nM) or in 0.25 ml of buffer B (50 mM Tris, 8.0, 0.2% sodium chloride, 0.01% Lubrol, and 20% glycerol) for 90 min at 25 °C. TR-bound [125I]T3 was separated from the unbound radioligand in a Sephadex G-25 (fine) column (5.5 x 1 cm), as described (3).

The binding data were analyzed by using Equation 11 based on direct competition between [125I]T3 and the unlabeled T3 for a single site on the receptor. The concentration of radioactive complex is given by Equation 11:

\[ [R_b] = [R_I] + [h] \frac{[c]}{K_0 + [h] + [c]} \] (Eq. 11)

where $[R_I]$ is the total concentration of receptor, [h] and [c] are the concentrations of [125I]T3 and the unlabeled T3, respectively, and $K_0$ is the dissociation constant of the hormone-receptor complex. The data were fitted directly to Equation 11 using the PC-MLAB program (Civlized Software, Bethesda, MD), to evaluate $K_0$ and $[R_I]$.

**RESULTS**

**Differential Interaction of TRα1 and TRβ1 with F2**—Previously it has been shown that TRβ1 binds to F2 mainly as a homodimer, whereas TRα1 binds to F2 both as a homodimer and as a monomer (14, 15). However, there was no quantitative comparison in the differential binding of F2 to the two isoforms. We therefore compared the binding affinities of F2 to the two isoforms. Fig. 1 shows the binding of TRα1 and TRβ1 to F2 in a concentration-dependent manner. Consistent with previous observations (14, 16), TRβ1 bound to F2 predominantly as a homodimer. Interestingly, when F2 concentration was higher than 15 fmol, weak binding of TRβ1 to F2 as a monomer was clearly detected (lanes 13–16). However, TRα1 bound to F2 differently from TRβ1. As shown in Fig. 1, at all corresponding F2 concentrations, TRα1 bound both as a homodimer and as a monomer. It clearly had a higher propensity to form monomer than TRβ1 (lanes 1 versus 9; lane 2 versus 10; lane 3 versus 11; lane 4 versus 12; lane 5 versus 13; lane 6 versus 14; lane 7 versus 15; and lane 8 versus 16).

The binding data shown in Fig. 1 were analyzed, and the $K_a$ values of homodimeric (Kα) and monomeric (Kc) binding for TRβ1 were found to be 400 and 0.1 x 10^-10 M^-1, respectively, indicating an increase of 4000-fold in the binding affinity when TRβ1 was bound to F2 as a homodimer ($s = 4000$, Table I). Thus, binding of the first monomer of TRβ1 to F2 facilitated the binding of the second monomer. We designated "s" as the ratio of $K_a$ to measure the extent of positive cooperativity in the binding of TR to TREs. The $K_a$ values of homodimeric and monomeric binding of TRα1 to F2 were 300 and 3 x 10^-10 M^-1, respectively, which gave a substantially lower cooperativity ($s = 100$; see Table I) than that for TRβ1.

**Role of Domains in the DNA and TR Binding Activity of TR Isoforms**—To identify the molecular basis of the differential interaction of TRα1 and TRβ1 with F2, we interchanged the domains between the two isoforms and evaluated the effects of domain swapping on the F2 and TRβ binding activity. An examination of the sequences between the two isoforms indicates that there is no sequence homology in the A/B domain, whereas there is an 88, 71, and 86% homology in sequence in domains C, D, and E, respectively (Fig. 2f). We have previously shown that...
the removal of domain A/B has no effect on the interaction of TRβ1 with TREs (16). Therefore, we grouped domain A/B together with domain C as a unit and constructed the chimeric receptors by swapping domains A/B/C, D, and E (Fig. 2II). The sequences encoding the chimeric receptors in the constructs were confirmed by restriction map analyses and DNA sequencing.

To assess the T₃ binding activity, we prepared the receptors by in vitro transcription/translation and carried out competitive T₃ binding assays. The displacement curves for w-TRβ1 and its chimeric receptors are shown in Fig. 3A and for w-TRα1 and its chimeric receptors are shown in Fig. 3B. Binding data were analyzed, and the Kᵦ values are shown in Table II. The Kᵦ values for the binding of w-TRβ1 and w-TRα1 to T₃ were 0.36 ± 0.06 and 0.10 ± 0.037 nM, respectively, indicating that w-TRα1 bound to T₃ with an approximately 3-fold higher affinity than that of w-TRβ1. The 3-fold difference is very significant as indicated by the t test (p < 0.01). The difference in the binding affinity was not due to the different protein expression level by in vitro transcription/translation. As shown in Fig. 3C, lane 2 shows the two translation products of w-TRβ1 initiated from the ATGs (Met-5 and -32) with the molecular weights of −55,000 and −52,000 (26, 27) that have the combined intensity similar to that of w-TRα1 shown in lane 6 (Fig. 3C). Similar binding experiments were carried out for the chimeric receptors, and as shown in Fig. 3, A and B, no significant differences were observed in the binding curves within the same subtype. The Kᵦ values for the chimeric receptors are virtually identical to those of the wild type receptors (Table II), indicating that the domain swapping between the two isoforms had no effect on the T₃ binding activity.

In contrast to the T₃ binding activity, domain swapping had a dramatic effect on the interaction of chimeric receptors with F2. Lanes 2–5 of Fig. 4 compare the binding of w-TRβ1 (βββ, see Fig. 2) and its chimeric receptors to F2 by EMSA. Replacement of domains D or E of TRβ1 by that of TRα1 had no significant effect on the binding of βββ or ββα receptor to F2 as a homodimer, but an increase in the formation of monomer was seen (lanes 3 versus 2; lanes 4 versus 2). However, when both domains D and E were swapped, a dramatic increase in the monomer formation was detected. The extent of monomer formation was similar to that seen for w-TRα1 (ααα, lane 6 of Fig. 4A versus lane 5). We further measured an F2 concentration-dependent binding to each chimeric receptor (βββ, ββα, and ββα), similar to the experiments shown in Fig. 1, and determined their affinity constants. The binding data were analyzed, and the Kᵦ values are shown in Table I. Swapping of domain D or E of TRβ1 by that of TRα1 led to a 3- and 4-fold increase in the binding affinity of βββ or ββα to F2 as a monomer, respectively (K₁ = 0.3 and 0.4 x 10⁶ M⁻¹, respectively, versus 0.1 x 10⁶ M⁻¹ for w-TRβ1), but with little change in the binding affinity of these two chimeric receptors as a dimer (K₂ = 500 x 10⁶ M⁻²). On the other hand, when both domains D and E were swapped, a dramatic 20-fold increase in monomer binding affinity (K₁ = 2 x 10⁶ M⁻¹) was detected. Thus, inclusion of domain D or E of TRα1 facilitates the binding of TR as a monomer.

Lanes 7–9 of Fig. 4 show that replacement of either domain D or E alone or both domains D and E of TRα1 by the corresponding regions of TRβ1 resulted in a similar reduction in the monomer formation (lane 6 versus lanes 7–9). The ratios of monomer to homodimer were clearly reduced in ααβ, αβα, and αββ. A more detailed analysis was carried out by determining the affinities in the binding of F2 to the chimeric TRαs. Their K₁, K₂, and s values are shown in Table I which indicate that there were only small changes in the values of positive cooperativity in ααβ, αβα, and αββ as compared with ααα (s = 50–100).

RXRs have been shown to heterodimerize with TRs and modulate the activity of TRs (1, 2). We therefore also examined the effect of domain swapping on the heterodimerization activity of the chimeric receptors. Similar to w-TRβ1 and w-TRα1, all chimeric TRs were capable of forming dimers with RXRβ on F2. No significant differences in the extent of formation of heterodimers were detected among the chimeric TRs (data not shown).

Role of Domains in the Differential Transactivation Activity of the Wild Type and Chimeric TRs—To assess the role of the domains in the transactivation activity of TRs, we constructed the mammalian expression vectors in which the expression of

### Table I

| TRs     | F₂, Kₐ(×10⁶ M⁻¹) | | | |
|---------|------------------|------------------|------------------|------------------|------------------|
| w-TRβ1 (βββ) | 0.1 | 400 | 4000 | | |
| βββ   | 0.3 | 500 | 1250 | | |
| ββα   | 0.4 | 500 | 1250 | | |
| βαα   | 2   | 400 | 200  | | |
| w-TRα1 (ααα) | 3   | 300 | 100  | | |
| ααβ   | 3   | 300 | 100  | | |
| αβα   | 3   | 300 | 100  | | |
| αββ   | 4   | 200 | 50   | | |

*Note: K₁ = 0.3 and 0.4 x 10⁶ M⁻¹, respectively, versus 0.1 x 10⁶ M⁻¹ for w-TRβ1,* but with little change in the binding affinity of these two chimeric receptors as a dimer (K₂ = 500 x 10⁶ M⁻²). On the other hand, when both domains D and E were swapped, a dramatic 20-fold increase in monomer binding affinity (K₁ = 2 x 10⁶ M⁻¹) was detected. Thus, inclusion of domain D or E of TRα1 facilitates the binding of TR as a monomer.

FIG. 2. Schematic representation of w-TRβ1, w-TRα1, and their chimeric receptors. I, the domain structure of the two isoforms and the extent of sequence homology. II, the chimeric receptors are designated by three-letter symbols, the first letter represents A/B and C domains; the second letter represents D domain, and the third letter represents E domain. The amino acid positions at the boundaries of domains C, D, and E are shown.
the wild type and chimeric TRs was driven by the cytomegalovirus promoter. We co-transfected the TR expression plasmids with F2-containing reporter into CV1 cells. Fig. 5 shows that w-TRβ1 (βββ) had a ~6-fold higher T₃-dependent transactivation activity than w-TRα1 (ααα; bars 2 versus 6 of Fig. 5). The lower transactivation of w-TRα1 was not due to the lower expression of w-TRα1 proteins in CV1 cells. Using high titer monoclonal antibody C4 (26), we had concurrently carried out immunocytochemical localization of TRs in CV1 cells and Western blotting for quantitation of the expressed TRs. w-TRs and chimeric receptors to TRE. On F2, swapping of domain D of w-TRα1 was replaced by that of w-TRβ1 expression plasmids into CV1 cells. Consistent with the previous findings (24), the T₃-dependent transactivation activity of w-TRβ1 mediated by F2

![Image](https://example.com/image.png)

**FIG. 3.** Binding of the wild type and chimeric TRs to [³²P]TRE. Equal amounts of in vitro translated w-TRβ1 or its chimeric receptors (A), w-TRα1 or its chimeric receptor proteins (B) were incubated with 0.4 nM of [³²P]TRE in the absence or presence of increasing concentration of unlabeled T₃ (0.2, 0.5, 1, and 10 nM). The free and receptor bound [³²P]TRE were separated by Sephadex G-25 (fine) column and quantified. Data are expressed as % of [³²P]TRE bound in the absence of unlabeled T₃. The apparent affinity constants were calculated according to the binding Equation 11 shown under "Experimental Procedures." C, comparison of the size and expression level of the in vitro translated wild type and chimeric receptors by SDS-polyacrylamide gel electrophoresis. Three μl of the lysates containing the in vitro ³⁵S-labeled translated receptor proteins were loaded onto a 10% SDS-polyacrylamide gel. The gel was dried and autoradiographed.

| TRs          | Kₐ  |
|--------------|-----|
| w-TRβ1 (βββ) | 0.04 |
| βαβ          | 0.07 |
| ββα          | 0.08 |
| βαα          | 0.34 |
| w-TRα1 (ααα) | 0.10 |
| ααβ          | 0.14 |
| αβα          | 0.18 |
| αββ          | 0.14 |

**TABLE II**

Apparent affinity constants of the binding of w-TRβ1, w-TRα1, and chimeric receptors to F2

Increasing concentrations of the [³²P]-labeled F2 TRE were incubated with equal amounts of in vitro translated w-TRα1, w-TRβ1, or the chimeric receptor proteins. After EMSA, the intensities of the monomeric and homodimeric bands were quantified by PhosphoImager. k₁ and k₂ are binding constants for the association of a receptor molecule to a half-site on an empty TRE and on a monomeric complex, respectively. Cooperativity between the sites is measured by s=(k₂/k₁). Their values were calculated according to the equations described under "Experimental Procedures." The concentration of the [³²P]-labeled TRE was 12 × 10⁻² M, M, monomer; H, homodimer.
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between the half-site and upstream spacer sequence. The dimerization interface between RXR and TRβ1 lies across the minor groove of the spacer, involving mainly residues from the first zinc finger in TRβ1 and the second zinc finger in RXR. Of the many side chains identified as making DNA contacts, only one, K193R, is changed in TRα1. Mutagenesis experiments indicate that at least one other conserved region of TRs, Leu-367-Leu-374, located in domain E, is involved in dimerization of intact receptors. The structure of the separate ligand binding domains D + E of rat TRα1 has recently been determined (6).

The isolated protein is monomeric and gives no indication as to how this sequence, which forms “an extensive hydrophobic patch,” participates in dimerization. The analogous sequence in human RXR-α does form a dimer interface in crystals of its isolated ligand binding domain (29). It has been suggested that this dimerization sequence from domain E has no selective pressure on response element recognition but only serves to stabilize these homodimer complexes (30), being active in all dimerization interfaces. Biochemical data, on the polarity of binding and the specificity for particular spacings in DNA response elements shown by heterodimers formed by various members of the steroid/thyroid hormone receptor family, were readily explained using the crystal structure solved by Rastinejad et al. (5). This indicates the generality of the binding mode which they detected and predicts that homodimers formed by TRs on the three different types of TREs will have distinct dimerization interfaces. Homodimers formed on F2 elements will be symmetrical, with a dimerization interface including the first zinc fingers of domain C and the D domain α-helices of both proteins. The situation is further complicated by the spatial arrangements of the binding sites. For DR4, the centers of the two binding motifs are on the same face of the DNA, one turn of the DNA helix apart (5). We can predict that for F2 they will fall a little further apart, on opposite faces of the DNA. Under most conditions, binding of TRβ1 to an F2 response element occurs as a dimer complex. Since the two half-sites involved are basically identical, the observed low levels of half-sites involved in dimer formation (i.e., Kd ≫ Ka) should be considered. The binding of TRα1, TRβ1, and their chimeric receptors to F2 shows cooperative binding to the DNA with no interaction between occupied and unoccupied sites, or between two occupied sites. In general, less extreme situations, where both occupied-occupied and occupied-unoccupied interactions occur, must also be considered. The binding of TRα1, TRβ1, and their chimeric receptors to F2 shows cooperative binding (Kd ≫ Ka). Table I clearly shows that the enhanced positive cooperativity of binding shown by TRβ1 over TRα1 (i.e., its greater tendency to bind as a dimer) results mainly from the second of these causes. TRβ1 monomer complex is much less stable than the TRα1 form, and this lower stability is relieved by formation of the dimer complex. As noted above, the sequences involved in DNA binding are essentially identical in TRα1 and TRβ1. Consequently, the instability of the TRβ1 monomer complex must result from the overall structure of the receptor molecule and its effect on the binding interfaces. The data obtained with the chimeric receptors show that all proteins in which the DNA binding domain C is of the α form show monomeric binding to F2, like TRα1 (Table I, Fig. 4). However, exchange of only this domain to the β form (ααα ↔ βαα) is not sufficient to significantly enhance binding cooper-
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Receptors suggests that the higher transcriptional activity of receptors derived from domain C of TR resultant tertiary structure of domains D and E of TR basal transcriptional machinery (4). It is possible that the proposed to act as bridging factors between the TRs and the ing TRs have been reported (4, 32–35). Their function has been large conformational change on hormone binding (6, 26, 30). extreme carboxyl terminus of domain E, which undergoes a

cule's affinity to that of the intact receptor, indicating regulatory interactions between these domains. The results shown in Fig. 3 indicate that the chimeric receptors derived from TRα1's domain C all have higher T3 binding affinity than the chimeric receptors derived from domain C of TRβ1, at least in the absence of DNA (see Table II). Thus this aspect of the behavior of a chimeric receptor is determined exclusively by the origin of its domain C, the DNA binding domain, reinforcing the importance of interdomain interactions.

The results on the effect of domain swapping on the transcriptional activity of the TR isoforms revealed that despite a higher T3 binding affinity, TRα1 and its chimeric receptors had lower transcriptional activity (Fig. 5). This was unexpected, suggesting that the mode of DNA binding to TRs overrides the advantage gained from higher T3 affinity. Inspection of the data further indicates that the extent of transcriptional activation by a receptor correlates better with the source of its DNA. It is clear that domains D and E of TRβ1 tend to impart higher transcriptional activity than those of TRα1. This in turn correlates well with the propensity of the chimeric receptors to form homodimers (see Fig. 4). The fact that w-TRα1 and its chimeric receptors formed heterodimers with the kXR as well as w-TRβ1 and its chimeric receptors suggests that the higher transcriptional activity of TRs which contain the domains D and E of TRβ1 was not mediated by the heterodimer pathway. This notion is further supported by the findings that the transfected kXR repressed the T3-dependent transactivation activity of the two isoforms with similar extent. Therefore, this higher transcriptional activity of TRβ1 lies most likely in the interactions of domains D and E with domain C in homodimers. Genetic experiments have shown that the hormone-dependent transactivation activity depends on a short amphipathic α-helix at the extreme carboxyl terminus of domain E, which undergoes a large conformational change on hormone binding (6, 26, 30). The sequence is conserved in both isoforms but is probably located in different sequence contexts in relation to the DNA binding domain or in the context of the entire molecule. Thus, it may function with differing efficacies in the two different environments. Recently, several co-repressors and one co-activator for several members of the receptor superfamily including TRs have been reported (4, 32–35). Their function has been proposed to act as bridging factors between the TRs and the basal transcriptional machinery (4). It is possible that the resultant tertiary structure of domains D and E of TRβ1 is less favorable to bind to a co-repressor. It is also possible that the structure of domains D and E of TRβ1 is such that the carboxyl-terminal α-helix is more easily accessible to a co-activator. Thus, domains D and E of TRβ1 may have a higher efficacy in transmitting the effects of conformational change of the carboxyl-terminal α-helix upon binding the hormone to regulate the interaction of the domain C with the target genes. These possibilities can only be distinguished when the x-ray crystallographic structures of ligand-bound intact TRα1 and TRβ1 are solved and compared. Our present studies indicate that domains C, D, and E are functionally linked and the interplay of these domains underlines the differential transcriptional activity of the two isoforms.

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