Functional Analysis of a Transactivation Domain in the Thyroid Hormone β Receptor*

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Hormone-dependent transcriptional activation (AF-2) by the thyroid hormone β receptor (TRβ) localizes to its carboxy-terminal domain. A putative transcriptional sequence within this domain was analyzed by mutating individual residues to alanine. Mutant receptor carboxy-terminal domains were tested coupled to the heterologous DNA binding domain of Gal4. A single mutant receptor (E400A) showed normal hormone binding and activation, whereas several others (P453A, F455A, L456A, F458A) exhibited impaired transactivation which correlated with their reduced ligand binding. Two mutations (L454A, E457A) were able to dissociate these properties, generating transcriptionally defective mutant proteins with preserved hormone binding. A further conservative substitution (E457D) was also non-functional, and these three mutations were equally deleterious when tested in the context of full-length TRβ with a natural thyroid hormone response element containing promoter. This loss of activity was not due to altered DNA binding or expression of mutant receptors in cultured cells. They also retained the ability to recruit VP16-tagged retinoid X receptor in vivo as well as bind the basal transcription factors TFII B and TBP in vitro. Our observations indicate that conserved hydrophobic (Leu454) and charged (Glu457) residues mediate AF-2 activity of TRβ, possibly via a co-activator that has yet to be identified.

The thyroid hormone receptor (TR) is a hormone-inducible transcription factor which is a member of the nuclear receptor superfamily. On the basis of amino acid sequence homology, these proteins can be divided into domains (A to F) which mediate distinct functions (1). A central zinc finger (C) domain mediates binding to regulatory DNA sequences or thyroid hormone response elements (TREs), usually located in the promoter region of target genes. The receptor can interact with TREs either as a homodimer or as a heterodimer with the retinoid X receptor (RXR) (2, 3). The carboxy-terminal (D/E/F) region of the receptor encompasses its ligand binding function as well as a series of heptad repeats of hydrophobic residues that are important for dimerization (4, 5). The unliganded receptor represses basal levels of transcription and hormone binding mediates both positive and negative regulation of target gene expression (6–8).

Functional analyses of nuclear receptors have shown that they contain two types of transcriptional activation function. The amino-terminal A/B domain contains a constitutive activation function (AF-1), whereas transcriptional activation mediated by the carboxy-terminal D/E/F domain (AF-2) is strictly hormone-dependent. Alternative splicing of two thyroid hormone receptor genes generates three receptors, TRα1, TRβ1 and TRβ2, that exhibit high homology in their DNA and hormone binding domains, but divergent amino acid sequences in the A/B region lead to differences in AF-1 activity between receptors (10). Thus, chick TRα exhibits constitutive activation of the rat growth hormone gene and the Rous sarcoma virus promoters (11, 12). In the latter case, AF-1 activity has been mapped to the amino-terminal region of TRα (12), whereas the A/B region of TRβ1 has not been found to contain significant autonomous activation function (7). However, the carboxy-terminal domains of both α and β thyroid hormone receptors contain a strong ligand-inducible activation function which can be transferred to heterologous DNA binding domains (6, 7). The viral oncoprotein v-erbA, a highly mutated version of its cellular counterpart c-erbA, or chick TRα, exhibits sequence-specific DNA binding, but has lost its ability to regulate transcription in a hormone-dependent manner. Previous analyses of chimeric c-erbA and v-erbA proteins have shown that this function maps to 9 amino acids at the extreme carboxyl terminus of c-erbA (13). This sequence motif is highly conserved among many nuclear receptors (Fig. 1) and consists of hydrophobic and negatively charged residues which may form an amphipathic α-helix (13). We have shown that deletion of the homologous region in the human thyroid hormone β receptor (hTRβ1) results in a transcriptionally inactive receptor which is also unable to bind thyroid hormone (T3) (14). Further evidence to support the importance of this motif is provided by functional studies of naturally occurring hTRβ mutants in thyroid hormone resistance syndrome; Zavacki et al. (15) have shown that a proline to histidine mutant at codon 453 (P453H), immediately preceding the putative amphipathic helix, is transcriptionally impaired. We have shown that other naturally occurring mutations (P453A, P455S, P457T) at this position impair activation of a reporter gene containing a direct repeat TRE despite the presence of supramaximal T3 concentrations (16).

In this study we have mutated individual residues within this extreme carboxy-terminal sequence of hTRβ1 and have separated the transcription activation and ligand binding functions of the D/E/F domains.

EXPERIMENTAL PROCEDURES

Expression Vectors and Reporter Constructs—Mutagenesis of hTRβ and construction of expression vectors and reporter constructs was as described previously (16). The Gal4-TR expression vectors contained wild type or mutant hTRβ (residues 174–461) in pSG424. Full-length wild type and mutant hTRβ cDNAs were expressed using a vector containing the Rous sarcoma virus (RSV) enhancer and promoter. VP16-RXRs contains the full-length hRXRa cDNA introduced into......
Assays-The cDNAs encoding hTBP and hTFIIB were cloned into mobility shift assays-The Gal4 DBD and Gal4-TR fusions were synthesized (34) and used to normalize luciferase values for transfection efficiency.

AASV. The reporter plasmid UAS-TKLUC contains Gal4 recognition sites in the promoter of the human elongation factor la (hEFT la) gene-driving expression of β-galactosidase (17). Cell Culture and Transfection Assays: JEG-3 cells were grown in OptiMEM containing 10% fetal calf serum, and 1% (v:v) antibiotic-glycoside mix (Life Technologies, Inc.). 18 h prior to transfection the cells were transferred to OptiMEM containing 1% penicillin/streptomycin/fungizone. Triplicate plates of cells were transfected by a 4-h exposure to calcium phosphate. Following a 36-h incubation, cells were lysed, and luciferase activity was measured as described previously (16). β-Galactosidase activity was also determined and used to normalize luciferase values for transfection efficiency.

Synthesis of Mutant Proteins, Ligand Binding, and Electrophoretic Mobility Shift Assays: The Gal4 DBD and Gal4-TR fusions were synthesized in vitro as either unlabeled or [35S]methionine-labeled proteins using the TNT reticulocyte lysate system (Promega, Southampton, United Kingdom). For use in protein-protein interaction assays, the relative translation efficiency of [35S]labeled products was determined as described previously (16). The TβR binding affinity of unlabeled in vitro translated fusions was measured using a modification of a filter binding assay (16). Data are the mean of at least two separate determinations, each performed in duplicate.

For electrophoretic mobility shift assay, the sequence of the oligonucleotide containing the 17-mer UASGal4 binding site was: 5′-agcttCGAGGACTGTCCTCAGGct-3′ (flanking sequences shown in lowercase). Reactions containing 2% of each unlabeled in vitro translated protein and 0.1 pmol of 32P-labeled DNA were performed as described elsewhere (16).

Expression of GST Fusion Proteins and Protein-Protein Interaction Assays: The cDNAs encoding hTBP and hTFIIB were cloned into pGEX4T1 and expressed in Escherichia coli as GST fusion proteins (GST-TBP and GST-TFIIB), whereas GST alone was generated similarly from pGEX2T. Each protein was purified using glutathione-Sepharose 4B affinity resin (Pharmacia, Milton Keynes, United Kingdom) according to the manufacturer's instructions but was not removed from the gel matrix. After purification, these proteins were equilibrated in PD buffer (50 mM Tris-HCl, 0.1 M KCl, 0.14 M NaCl, 0.5% nonidet P-40, 10% glycerol, pH 8.0) and adjusted to a protein concentration of 1 mg ml⁻¹.

For protein-protein interaction experiments, aliquots of the GST, GST-TBP, and GST-TFIIB glutathione-Sepharose complexes (~1 µg of protein) were made up to 10 µl in PD buffer, 2.5 µg of bovine serum albumin added, and then incubated for 5 min at room temperature. After the addition of a further 190 µl of PD buffer, equal counts (1-5 µl of 32P-labeled in vitro translated protein were added and the mixture incubated with continuous agitation at room temperature for 1 h in the presence or absence of 5 µM TβR. Sepharose-bound GST or GST fusion proteins and associated labeled Gal4-TR were then pelleted by centrifugation at 1500 g for 10 min. The supernatant was then added to 1 ml of NCS buffer (20 mM Tris-HCl, 0.1 M NaCl, 1 mM EDTA, 0.5% Nonidet P-40, pH 8.0), suspended in SDS sample buffer, and analyzed by denaturing polyacrylamide gel electrophoresis. Gels were Coomassie-stained to check that equal amounts of GST fusion protein were present and then autoradiographed.

RESULTS

Ligand Binding and Transcriptional Properties of Mutant Receptors—Individual amino acids from the proline at codon 453 to the extreme carboxyl terminus of hTβR1 were mutated to alanine. Chimeric fusion proteins, consisting of the D/E/F domain (residues 174-461) of wild type or mutant TβR coupled to a heterologous DNA binding domain from the yeast transcription factor Gal4 (residues 1-147), were assayed for their ability to activate the UAS-TK-LUC reporter gene which contains Gal4 binding sites upstream of the thymidine kinase promoter (Fig. 2a). The wild type receptor fusion stimulated UAS-TK-LUC activity in a hormone-dependent manner, with maximal activation occurring at 5 nM T3. In comparison, the mutant receptor fusions showed three types of activation profile; a glutamic acid to alanine substitution at codon 460 (E460A) did not alter TβR-dependent activation significantly; the majority of mutants (P453A, F455A, L456A, F459A) exhibited

FIG. 1. Sequence alignment of the potential amphipathic α-helical motif in TβR with the corresponding region in the thyroid/retinoic acid receptors (hTRα) (32), thyroid hormone α receptor (hTRα) (33), estrogen receptor (mER) (34), retinoic acid receptor (hRARα) (35); retinoid X receptor (hRXRα) (36), vitamin D receptor (hVDR) (37), peroxisome proliferator activated receptor (hPPARα) (38), and chicken ovalbumin upstream promoter (hOCUP-TF) (39).
a reduction in sensitivity to thyroid hormone such that higher  

$T_3$ concentrations were required to obtain a comparable stimu-

lation response to the wild type receptor, resulting in right-

shifted activation profiles; finally, two mutants (L454A, E457A)  
exhibited negligible or severely attenuated hormone respon-

siveness. Next we compared the transcription activation prop-

erieties of these Gal4-TR fusions with their ability to bind $T_3$ in vitro (Table I). In keeping with its preserved transcription function, the E460A mutant showed a $T_3$ binding affinity which was comparable with wild type receptor. A number of mutants exhibited reduced $T_3$ binding affinities which correlated with their functional impairment such that the rank order of their $T_3$ binding affinities (Table I) was mirrored by their relative Kd values (Fig. 2a; F455A = F453A > L456A > F459A). However, these properties were markedly discordant in the transcriptionally inactive mutants (L454A, E457A), in that their $T_3$ binding affinities were relatively normal.

Previous analyses of transcription factors such as Gal4 and VP16 had suggested that negatively charged residues were particularly important for transcription (18). Therefore we tested a further series of mutant receptor fusions to determine the importance of negatively charged residues within the putative transcription activator motif in the TRβ. A conservative substitution of the distal glutamic acid residue within this motif (E460D) was not functionally deleterious, whereas a similar substitution at the proximal position (E457D) impaired receptor function but not $T_3$ binding (Fig. 2a and Table I). A double mutant with conservative substitutions at both positions (E457D, E460D) also showed a markedly attenuated activation profile. Last, we tested a mutant incorporating 3 further amino acids at the carboxyl terminus which are present in TRα but not TRβ (Fig. 1). The presence of these residues (QEV) did not increase transcriptional potential to generate a “supraactive” receptor, despite the addition of a further negatively charged glutamic acid residue (Fig. 2a).

Three transcriptionally impaired mutants (L454A, E457D, E457A) with preserved ligand binding properties were studied further to determine whether the presence of amino-terminal A/B or DNA binding domain receptor sequences would modu-

late their deleterious effects. These mutations were introduced into full-length wild type TRβ and tested using the reporter gene MAL-TKLC. In comparison with wild type TRβ, all three mutant receptors were completely inactive, despite the presence of supramaximal $T_3$ concentrations (Fig. 2b). Notably, the E457D and E457A mutants, which retained a little AF-2 activity as Gal4-TR fusions with UAS-TKLC, were transcriptionally inert in their natural context. We have also observed that these three mutants are unable to activate a reporter gene containing a palindromic TRE configuration (TREP) and that the thyroid hormone analogue 3,5,3′-triiodothyronaic acid is not able to restore transcription (data not shown). Overall our results indicate that mutation of both hydrophobic and charged residues at the carboxyl terminus of TRβ markedly impaired AF-2 activation function without altering the hormone binding properties of the mutant receptors.

DNA Binding, in Vivo Expression, and Interaction of Mutant Receptors—To determine whether these mutations alter the DNA binding properties of receptor fusions, electrophoretic mobility shift assays were performed using mutant Gal4-TR proteins and a 32P-labeled Gal4 binding site (UAS). All three mutants showed specific interactions with UAS that were comparable with wild type Gal4-TR. To exclude the possibility that the function of mutant receptors is impaired because their expression or stability in cultured cells is altered, we used an assay which has previously demonstrated in vivo interactions between RAR and RXR (19). Wild type or mutant Gal4-TR expression vectors were co-transfected with a second vector containing the activating domain of VP16 fused to hRXRα (VP16-RXRα). Interaction between these fusion proteins results in VP16-mediated transcriptional activation of a co-transfected UAS-TKLC reporter gene. UAS-TKLC activity was not stimulated upon co-transfection with wild type Gal4-TR nor VP16-RXRα expression vectors individually, nor with a combination of VP16-RXRα and Gal4 DBD (data not shown). However, co-transfection of VP16-RXRα with wild type Gal4-TR resulted in a marked induction of luciferase activity (Fig. 4), indicating a heterodimeric interaction between the carboxyl-

terminal domains of TR and RXR. Similarly, co-transfection of vectors encoding mutant Gal4-TRs with VP16-RXRα also in-

creased reporter gene activity, indicating that each mutant was expressed, bound UAS sequences, and retained the ability to interact with RXRα in cells. For comparison, we studied two artificial Gal4-TR fusions (L421R, L428R) in which conserved leucine residues at each end of the ninth heptad repeat known to be involved in dimerization had been changed to arginine (20). Although both heptad mutants bound UAS (Fig. 3), their ability to recruit VP16-RXRα was markedly impaired (Fig. 4), in keeping with their previously reported inability to het-

erodimerize with RXR in vitro (21, 22).

Mutant Receptor Interaction with Basal Transcription Factors—Previous studies have shown that the unliganded thyroid hormone receptor can bind the basal transcription factor TFIIB (23, 24) and that the addition of thyroid hormone decreases this interaction. Accordingly, we examined the binding of three mutant TRs (L454A, E457D, E457A) to TFIIB as well as TBP, to determine whether altered interactions with

![Figure 3](https://via.placeholder.com/150)

**Fig. 3.** DNA binding properties of Gal4-TR mutant chimeras. In vitro translated Gal4 DNA binding domain (Gal4) and wild type (WT) or mutant Gal4-TR fusions were incubated with 32P-labeled oligonucleotide containing the Gal4 recognition sequence (UAS) and analyzed by electrophoretic mobility shift assay as detailed under “Experimental Procedures.” RL, reticulocyte lysate.
basal transcription factors could account for their impaired transcriptional activity. In vitro synthesized [35S]methionine-labeled Gal4-TR fusions were incubated with GST or GST-TFIIB immobilized on glutathione-Sepharose beads. Although the wild type receptor fusion showed a low level of nonspecific binding (<10% of total) to the glutathione-Sepharose-GST complex, significant specific binding to TFIIB was demonstrated in the absence of hormone (Fig. 5b). This receptor-TFIIB interaction was markedly reduced following incubation with T3, as has been documented previously (24). In comparison, the mutant receptor fusions (L454A, E457D, E457A) showed comparable TFIIB binding which was also attenuated by ligand. Similar studies performed with GST-TBP also showed specific binding of wild type Gal4-TR which was markedly reduced by thyroid hormone (Fig. 5c). Again, the mutant fusions (L454A, E457D, E457A) which bind T3 with high affinity interacted in a comparable manner to wild type receptor.

**DISCUSSION**

We have shown that specific residues within a 9-amino acid sequence at the carboxyl terminus of TRβ are critical for the hormone-dependent activation function of the D/E/F domain of this receptor. Our studies indicate that a number of mutations within this sequence impair both hormone binding and transcriptional activation functions. However, mutation of two residues (Leu654, Glu657) which are highly conserved between members of the nuclear receptor superfamily (Fig. 1) enabled the two functions to be dissociated, resulting in selective impairment of transactivation while T3 binding was preserved. We have shown that this transcriptional impairment is not related to an inability of mutant proteins to bind DNA. Furthermore, these chimeric mutant Gal4-TR fusions retained the ability to recruit VP16-tagged RXR in cultured cells, as expected from their location outside the heptad repeat motif, suggesting that their expression, binding to UAS sequences, and dimerization in vitro is not altered.

Analyses of transcription factors such as Gal4 and VP16 have led to the suggestion that one class of transactivation domain consists of acidic residues, either within amphipathic α helices or unstructured regions, which interacts with the basal transcriptional machinery via its high density of negative charge (18, 25). The putative amphipathic α-helix in TRβ is conserved among nuclear receptors (Fig. 1) and was first analyzed in the mouse estrogen receptor (mER) (26). Mutations of both negatively charged (Glu654, Asp657) or hydrophobic (Leu654, Leu657) residues in mER were associated with impaired transactivation. The authors also found that the A/B domain of mER could modulate AF-2 activity, such that some mutations were more deleterious in the context of an amino-terminally truncated versus full-length receptor. However, we found that the mutations in TRβ were also deleterious when tested as either Gal4-TR fusions or in the full-length receptor, suggesting that the A/B domain of TRβ either lacks significant autonomous (AF-1) activation function or that such activity only manifests in restricted cell-type or promoter specific contexts. Conversely, we have observed that some mutants retain more activation function as Gal4-TR chimeras compared with their full-length receptor contexts (E457D and E457A, Fig. 2, a and b; P453A, Fig. 2a; Ref. 16), suggesting that AF-2 activity may also be modulated by factors other than AF-1.

Another study with the chick TRα has shown that double mutations of acidic amino acids (Glu601, Glu605) to basic or hydrophobic residues were also associated with diminished transactivation (27). We have extended this observation by demonstrating the greater importance of the proximal charged residue (Glu637) at the homologous location in TRβ. Indeed, we have found that mutation of the distal acidic residue (Glu640) is functionally silent, in accordance with a similar observation in mER, and it is interesting to note that, although this distal residue is conserved in the TR, RAR, and RXR subfamilies, it is not conserved in other nuclear receptor groups (Fig. 1) (26). Our data have also delineated the critical role of the leucine residue at codon 454, signifying the importance of hydrophobic residues in mediating transactivation. In addition,
although we have found that the glutamic acid at position 457 is critical, this may not be related to the presence of an acidic residue in this position. The conservative mutation to aspartic acid is equally deleterious. Again, it is interesting to note that the orphan receptor COUP-TF, which has not been shown to exhibit ligand-
inducible transactivation, contains an acidic residue rather than glutamic acid residue in the position corresponding to Glu457 of TR (Fig. 1). Overall, our observations with TRβ are consistent with more recent studies of the activation domain of Gal4, which indicate that clusters of hydrophobic rather than negatively charged acidic residues are important for transcriptional activity (28, 29).

Studies with another prototypic transactivator, VP16, have shown that it interacts with the basal transcription factors TBP and TFIIIB. We therefore tested the hypothesis that the mutations which selectively abolished transactivation in TRβ also interfered with receptor interactions with TFIIIB or TBP. However, our data indicate that the mutant proteins bind both basal transcription factors with equal efficacy compared with wild type receptor. In the presence of T3, we have shown a marked attenuation of wild type receptor binding to TFIIIB, as documented previously (24), as well as to TBP. In keeping with their normal hormone binding affinities, the mutant proteins exhibited a similar T3-dependent reduction in TFIIB and TBP binding. The lack of quantitative differences in mutant TR interactions with TFIIIB or TBP, makes it less likely that these basal factors are involved in impaired transactivation. In conjunction with an earlier observation that the AF-2 activity in the estrogen receptor is not synergistic with the acidic activation function of VP16 (9), it suggests that transcriptional activation by ligand-inducible nuclear receptors and acidic activators may be mediated by distinct pathways.

Evidence that nuclear receptors may activate transcription via co-activators or adaptors was first provided by the finding that a protein with E1A-like activity was required in order to elicit maximal responsiveness to retinoic acid in embryonal carcinoma cells (30). More recently, Halachmi et al. (31) have demonstrated the existence of a 160-kDa protein (ERAP160) in MCF-7 cells which interacts with the carboxyl-terminal domain of the estrogen receptor in a strictly hormone-dependent manner. Furthermore, a carboxyl-terminal truncation mutant of the estrogen receptor (Δ554) lacking the previously characterized transactivation domain (26) was unable to interact with ERAP160. Whether the homologous region in TRβ which we have characterized also mediates hormone-dependent interactions with ERAP160 or a similar putative co-activator remains to be elucidated.

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