Structure of the Carboxy-Terminal Region of Thyroid Hormone Nuclear Receptors and Its Possible Role in Hormone-Dependent Intermolecular Interactions

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ABSTRACT: The thyroid hormone nuclear receptors (TRs) are ligand-dependent transcription factors. To understand the molecular basis of ligand-dependent transactivation, we studied the structure of their carboxy-terminal activation domain. We analyzed the structures of the peptides derived from the C-terminal sequences of human TR subtypes β1 (h-TRβ1) and α1 (h-TRα1) and a human TR mutant, PV, by circular dichroism (CD). Mutant PV has a C-terminal frameshift mutation and does not bind to the thyroid hormone, 3,3′,5-triiodo-L-thyronine (T_3). Analyses of the secondary structures of the peptides by CD indicate that five amino acids, EVFED, are part of an amphipathic α-helix which is required to maintain the structural integrity of the hormone binding domain. A monoclonal antibody, C4 (mAb C4), which recognizes both h-TRβ1 and h-TRα1 was developed. Using a series of truncated mutants and synthetic peptides, we mapped the epitope of mAb C4 to the conserved C-terminal amino acids, EVFED. Analysis of the binding data indicates that binding of T_3 to either h-TRβ1 or h-TRα1 was competitively inhibited by mAb C4. Deletion of C-terminal amino acids including EVFED led to a total loss of T_3 binding activity. Thus, part of the T_3 binding site is located in this five amino acid segment. T_3 may transduce its hormonal signal to the transcriptional machinery via interaction with EVFED at the C-terminus of TRs.

Thyroid hormone receptors (TRs),[1] members of the steroid hormone/retinoic acid nuclear receptor superfamily, are hormone-dependent transcription factors. By amino acid sequence analysis, these receptor proteins can be divided into domains A−E, with distinct functions. Domain C, which binds to specific thyroid hormone response elements (TREs) of target genes, is expected to have a structure similar to those determined for the glucocorticoid (Luisi et al., 1991) and estrogen receptors (Schwabe et al., 1993). Domains D and E have recently been shown to be structurally and functionally linked, in that part of domain D is essential for the hormone binding activity of domain E (Lin et al., 1991; McPhie et al., 1993). Alternative splicing of the two TR genes, *TR*β and *TR*α, found on chromosomes 3 and 17, respectively, gives rise to three receptors, TRβ1, TRβ2, and TRα1. These proteins are highly homologous, except in their A/B domains.

Unliganded TRs repress basal levels of transcription, but binding of hormone can cause either positive or negative regulation of target gene expression (Lazar, 1993; Cheng, 1995). The molecular mechanisms of these hormone-dependent gene regulatory activities are still unknown. Analysis of naturally occurring h-TRβ1 mutants showed that this activity is mapped to the extreme carboxy-terminal sequence of the ligand binding domain E (Tone et al., 1994). Lin et al. showed that deletion of the last eight amino acids completely abolished the binding activity of human TR subtype β1 (h-TRβ1) to the thyroid hormone, 3,3′,5-triiodo-L-thyronine (T_3) (Lin et al., 1991). Chatterjee et al. further showed that removal of the last nine amino acids from h-TRβ1 produced a molecule which was devoid of transcriptional regulatory activity and could not bind T_3 (Chatterjee et al., 1991). We showed that this deletion resulted in incomplete folding of the hormone binding domain (McPhie et al., 1993), indicating the importance of this region in the structure of the entire receptor.

The sequence in question is highly conserved among nuclear receptors. It contains hydrophobic and acidic residues and has been postulated to form an amphipathic α-helix (Zenke et al., 1990). Schmitz et al. showed that acidic sequences, which are capable of forming helices, are found in a number of activation domains, even in proteins unrelated to the nuclear receptor family (Schmitz et al., 1994). They proposed that these are initially unstructured but that secondary structure is induced by association with their target molecules. Here we report studies on the structure of the activation domain regions from normal TRs and a mutant receptor (PV), in which a frameshift mutation has changed the sequence of this region of the molecule. We studied their interaction with a monoclonal antibody, as a simple model for the as yet unknown natural target molecule for TRs in the transcriptional activation pathway.

MATERIALS AND METHODS

Tran ^{35}S-label (1093 Ci/mmol) was purchased from ICN (Irvine, CA). [3′-^{125}I]T_3 (2206 Ci/mmol) was purchased from

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[1] Abbreviations: T_3, 3,3′,5-triiodo-L-thyronine; TR, thyroid hormone nuclear receptor; h-TRβ1, human TR subtype β1; h-TRα1, human TR subtype α1; h-PR, human progesterone receptor; mAb, monoclonal antibody; CD, circular dichroism; SDS; sodium dodecyl sulfate.

DuPont New England Nuclear (Boston, MA). h-TRα1, h-TRβ1, and truncated mutants of h-TRβ1 (ED41, MD32, KD29, KD25, and KP28) were synthesized using TNT system according to the manufacturer's instructions (Promega, Madison, WI). Peptides were synthesized by Peptide Technologies Co. (Gaithersburg, MD) by solid-phase synthesis. The purity was confirmed by high-pressure liquid chromatography, and the identity was verified by amino acid analysis and mass spectrometry.

Circular Dichroism Measurements. In aqueous solution, short peptides rarely adopt the same conformation as in a globular protein (Brown & Klee, 1971). However, by the addition of detergents, it is possible to vary hydrophobicity of the solution and to mimic the environments experienced by a peptide in proteins (Wu et al., 1981; Zhong & Johnson, 1992). Thus, using circular dichroism, it is possible to map which conformations are possible for a given sequence and conversely to exclude forbidden conformations. We have used this technique to explore the behavior of C-terminal peptides of h-TRβ1 (peptide 2358), h-TRα1 (peptide 2359), mutant PV (peptide 2228), and a hybrid (peptide 2453).

Circular dichroism (CD) spectra of peptides and proteins and thermal melting curves were measured as described earlier, although spectra of proteins KD29 and PV06 were recorded in 0.2 M sodium phosphate buffer, pH 8.0. The high ionic strength and pH were necessary to ensure the solubility of PV06, which was found to aggregate under conditions used in earlier experiments (McPhie et al., 1993). Initial concentrations were measured by amino acid analysis of stock solutions and then routinely measured by absorbance measurements at 280 and 215 nm (Prasad et al., 1986). The spectra were analyzed in terms of secondary structures using the CONTIN program (Provencher & Glockner, 1981).

Preparation of mAb C4. The immunogens for producing antibodies in mice were obtained by expression of h-TRα1 in *Escherichia coli* using the plasmid pCLC13, which contains the coding region of h-TRα1 and a T7 promoter (Lin et al., 1994). h-TRα1 was expressed and partially purified from *E. coli* as described (Lin et al., 1991; Lin & Cheng 1991).

Mice were immunized with highly enriched h-TRα1 according to the method of Obata et al. (Obata et al., 1988, 1989). Spleen cells from the immune mice were fused with P3X63Ag8653 myeloma cells by a method described previously (Obata et al., 1989). Initially, the hybridomas were screened by enzyme-linked immunosorbent assay using purified h-TRα1. The positive hybridomas were rescreened by immunoprecipitation with [^{35}S]methionine-labeled h-TRα1 prepared by *in vitro* transcription/translation products of pCLC13. The positive hybridomas were isolated and cloned by a limiting dilution method as described previously (Obata et al., 1989).

Preparation of Polyclonal Antibody T1 against PV Mutant. An 18-mer peptide (2228) corresponding to the deduced amino acid residues 445−463 of the carboxy-terminal region of the PV mutant was synthesized. PV mutant was derived from a kindred (PV) of thyroid hormone resistant patient (Parilla et al., 1991). The peptide was conjugated to keyhole limpet hemocyanin (KLH) via disulfide bond formation. KLH (16 mg) was reacted with 5 mg of Traut's reagent (Pierce) at 22 °C for 2 h in 0.5 mL of 200 mM Na_2HPO_4, pH 8.0. The derivatized KLH was separated from the reaction mixture on a PD 10 column, previously equilibrated with 200 mM Na_2HPO_4, pH 8.0. The derivatized KLH was further reacted with 25 mM 5,5′-dithiobis(2-nitrobenzoic acid) (Pierce, Rockford, IL) for 15 min at 22 °C and passed through a PD 10 column. Peptide 2228 (12 mg) was incubated with the derivatized KLH for 15 min at 22 °C to complete the disulfide exchange reaction. The KLH−peptide conjugate was used to immunize two rabbits as described (Fukuda et al., 1988). The positive serum was identified by enzyme-linked immunosorbent assay as described previously (Hasumura et al., 1986) and further confirmed by immunoprecipitation with [^{35}S]methionine-labeled PV mutant protein, which was synthesized by *in vitro* transcription/translation. This polyclonal antibody was designated as T1.

Immunoprecipitation. The ^{35}S-labeled h-TRα1, h-TRβ1, or truncated proteins (1−2 μL) synthesized by using the TNT system were incubated with 1 μg of mAb C4 for 30 min at 4 °C. After incubation, the ^{35}S-labeled antigen−mAb C4 complex was absorbed by Staph A, which were preincubated with rabbit anti-mouse heavy and light chains (Fukuda et al., 1988). The immunoprecipitates were analyzed by SDS−polyacrylamide gel electrophoresis and autoradiographed.

Construction of Truncated PV Mutant Expression Plasmid (pZJPV06). The preparation of truncated PV (Leu^{203}−D^{463}; PV06) expression vector (pZJPV06) was completed in two steps. The first step was to introduce six histidine codons immediately followed by a new *Pst*I site 5′ to the ATG site of pITPV. pITPV is a PV mutant *E. coli* expression vector whose expression is under the control of the T7 promoter (Mixson et al., 1993). The histidine tag/*Pst*I site containing plasmid, pZJPVH6, was constructed by phagemid mutagenesis according to the procedure described in the Bio-Rad Muta-Gene phagemid mutagenesis kit. In order to prepare a uracil-containing single-strand phagemid DNA, pITPV which contains mutant PV was transformed in *E. coli* CJ236. An isolated colony was picked and placed in 2 mL of LB medium containing 50 μg/mL ampicillin at 37 °C for 16 h. On the next day, 2 mL of overnight culture was used to inoculate 50 mL of LB medium and incubated at 37 °C until OD_{600} was 0.3. Seventy microliters (50 mg/mL) of kanamycin was added. Incubation was continued for an additional 6 h. The phage in the supernatant was precipitated by 3.5 M ammonium acetate and 20% PEG 6000 and then dissolved in 200 mL of a high-salt buffer (300 mM NaCl, 100 mM Tris·HCl, and 1 mM EDTA). The uracil phagemid single-strand DNA was purified by phenol extraction. The sequence of the mutagenesis primer containing the His tag/*Pst*I site was TGTAAGGCCATTTTCTGTCTGCAGGT-GATGGTGATGGTGATGTCTCATATGTAT-ATCTCCTTCTTAAAGTTA. The phosphorylated primer was annealed to the single-strand DNA. The second-strand DNA was synthesized by T4 polymerase and subsequently ligated. The clone was selected in *E. coli* MV1190. The histidine tag containing clone pZJPVH6 was confirmed by DNA sequencing. pZJPVH6 was further digested with *Pst*I to remove the codons that were 5′ upstream of codon 203 and religated to produce pZJPV06. The sequence of the resulting plasmid was verified by DNA sequencing.

Purification of Truncated PV Mutant Protein PV06. BL21(DE3)pLysS cells (from Novagen) were transformed with plasmid pZJPV06. The transformed cells were plated onto LB agar plates containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. The plates were incubated at 37 °C overnight. The next day, colonies were transferred

into 1 L of LB medium containing 100 μg/mL ampicillin and 34 μg/mL chloramphenicol. Cells were grown until OD_{600} reached 0.6−0.65 and then induced with 1 mM isopropyl β-D-thiogalactopyranoside for an additional 2.5 h. Cells were harvested by centrifugation at 3500*g* for 20 min. The supernatant was decanted, and cells were suspended in 200 mL of phosphate-buffered saline containing 10 μg/mL aprotinin, 1 μg/mL leupeptin, and 0.1 mM phenylmethanesulfonyl fluoride. Cells were then thawed and lysed by sonication for 3−5 cycles. Each cycle consisted of 30 s of sonication and 30 s of cooling.

The pellets which contain the inclusion bodies were obtained by centrifugation at 1000*g* for 20 min. The inclusion bodies were washed once with 50 mM Tris-HCl, pH 7.4, 20 mM EDTA, 0.5 M NaCl, and 2.5% Triton X-100 and resuspended using tissuemizer/homogenizer until no lumps were visible. The pellets were obtained by centrifugation at 15000*g* for 20−30 min followed by four washes with 50 mM Tris·HCl, pH 7.4, and 20 mM EDTA with centrifugation at 15000*g* for 10−15 min between each wash. The final pellets were stored overnight at −70 °C.

The pellets were thawed, resuspended in 6 M guanidinium-HCl and 100 mM Tris·HCl, pH 8.0, and incubated at 25 °C for 1 h. The protein concentrations was determined by the method of Lowry and were diluted to 8 mg/mL. Dithioerythritol was added to a final concentration of 0.3 M. The samples were then kept at 25 °C for an additional 2 h. After 2 h, the samples were diluted 100-fold with the refolding buffer (100 mM Tris·HCl, pH 8.0, 0.5 M L-arginine, 8 mM oxidized glutathione, and 0.01 mM $ZnCl_2$) by adding each sample to the rapidly stirring refolding buffer at a rate of 0.5 mL/s. After incubation for 30−45 min at 4 °C, EDTA was added to a final concentration of 2 mM. This solution was further incubated for 40 h at 4 °C. The solution was dialyzed against 20 mM Tris-HCl, pH 7.4, and 100 mM urea. Dialysis buffer was changed 3−4 times with a 12-h period between each change.

After dialysis, the solution was centrifuged at 7500*g* for 20−30 min at 4 °C. The supernatant was loaded onto a Q-Sepharose column (bed volume, 15 mL) preequilibrated with 50 mM Tris·HCl, pH 8.0, and 50 mM NaCl. Sample was loaded at a rate of 1−2 mL/min and washed with the equilibration buffer followed by equilibration buffer with 0.15 M NaCl. The protein was eluted with 0.4 M NaCl and 50 mM Tris·HCl, pH 8.0. The purity of the proteins was evaluated by using a 12% SDS−polyacrylamide gel. For CD studies, the protein eluted from the column was dialyzed against 0.2 M phosphate buffer, pH 8.0.

The truncated protein KD29 was prepared similarly as described above except that the expression plasmid used was pJL06 (Lin et al., 1991).

Binding of T_3 to h-TRα1 and h-TRβ1 in the Absence or Presence of mAb C4. h-TRβ1 or h-TRα1 (3 μL) prepared by the *in vitro* transcription/translation system was incubated with 0.2 nM [^{125}I]T_3 in the absence or presence of increasing concentrations of unlabeled T_3 in buffer B (50 mM Tris·HCl, pH 8.0, 0.2 M NaCl, 10% glycerol, 0.01% lubrol, and 1 mM dithiothreitol). In some experiments, increasing concentrations of mAb C4 or IgG fractions from normal mouse were present. After incubation of the mixture (0.266 mL) at 20 °C for 1 h, the [^{125}I]T_3-bound TRs were separated from the free [^{125}I]T_3 by passing the mixture through a Sephadex G-25 column (bed volume, 3 mL) as described previously (Lin et al., 1991).

Table 1: Conformation of C-terminal Peptides of TRs. Predicted Structures and Best Fits to Observed CD Spectra, Selected by the CONTIN Program[a]

I. Predicted Structures of Peptides

									e	e	e	e	e	e	e	e	e					
2453 (β1/PV hybrid)	C	P	T	E	L	F	P	P	L	F	L	G	S	V	R	G						
2228 (mutant PV)	C	P	H	R	T	L	P	P	F	V	L	G	S	V	R	G	L	D				
	|	|	•	•		•	|	|	•	•	|			•	•			•		
2358 (h-TRβ1)	C	P	T	E	L	F	P	P	L	F	L	E	V	F	E	D						
	446						452									461						
2359 (h-TRα1)	C	P	T	E	L	F	P	P	L	F	L	E	V	F	E	D	Q	E	V			
							e	e	e	h	h	h	h	h	h	h	h	h	h	h	h	h

II. Measured Structures of Peptides

	% in peptide			
			1%SDS	
	buffer[b]	0.1% SDS[c]	pH 7.5[b]	pH 2.0[d]
2453 (β1/PV hybrid)	h, 0	h, 0	h, 0	h, 0
	e, 59	e, 58	e, 61	e, 51
2228 (mutant PV)	h, 0	h, 5	h, 4	h, 4
	e, 52	e, 44	e, 52	e, 50
2358 (h-TRβ1)	h, 5	h, 12	h, 15	h, 29
	e, 62	e, 50	e, 51	e, 34
2359 (h-TRα1)	h, 3	h, 6	h, 10	h, 22
	e, 55	e, 50	e, 45	e, 42

[a] Analyses were carried out as described in McPhie et al. (1993). h, α-helical residues; e, β-strand residues. [b] 0.1 M sodium phosphate buffer, pH 7.5. [c] 5 mM sodium phosphate buffer, pH 7.5. [d] Adjusted to pH 2.0 with phosphoric acid.

RESULTS

Essential Role of the Five Amino Acids EVFED in Determining the Structure of the Hormone Binding Domain at the C-Terminus. By deletion analysis, we have previously demonstrated that elimination of the last C-terminal eight amino acids led to the loss of structural integrity of the hormone binding domain (Lin et al., 1991). The last C-terminal eight amino acids were suggested to form the last helix of the eight-stranded α/β barrel which was proposed to be the structure of the hormone binding domain (McPhie et al., 1993). The finding that this region is also critical for transcriptional activation (Chatterjee et al., 1991) prompted us to further study the structure of the C-terminal region of the TRs.

We synthesized peptides 2358 and 2359, which correspond to the last C-terminal amino acids of h-TRβ1 and h-TRα1, respectively (see Table 1). We investigated their secondary structures by CD. We also took advantage of the naturally occurring mutant PV derived from a thyroid hormone resistant patient. It has a frameshift mutation beginning at codon 448 as a result of a base insertion at nucleotide 1642 (Parrilla et al., 1991; Table 1). In mutant PV, three out of the last five amino acids as compared to the wild type h-TRβ1 undergo nonconservative mutation (Table 1). This mutant receptor has completely lost the T_3 binding activity (Meier et al., 1992, 1993). We therefore synthesized peptide 2228 (Table 1) with the altered C-terminal sequence of mutant PV and peptide 2453, a hybrid, in which the first

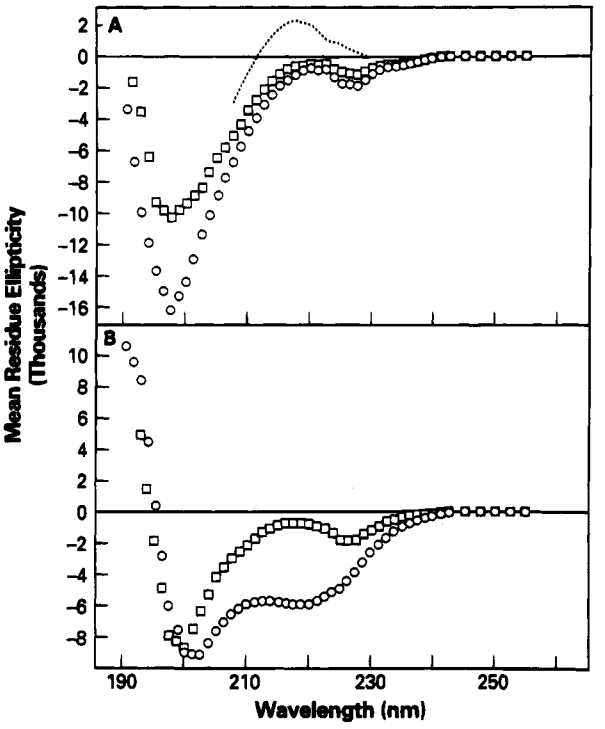


FIGURE 1: Ultraviolet CD spectra of peptide 2358. (A) CD spectra of peptide 2358 (100 μg/mL) in 0.1 M sodium phosphate, pH 7.5 (□), 6 M guanidinium-Cl (•••), or 0.1% SDS (○). (B) CD spectra of peptide 2358 in 1% SDS, pH 7.5 (○), or 1% SDS, pH 2 (□).

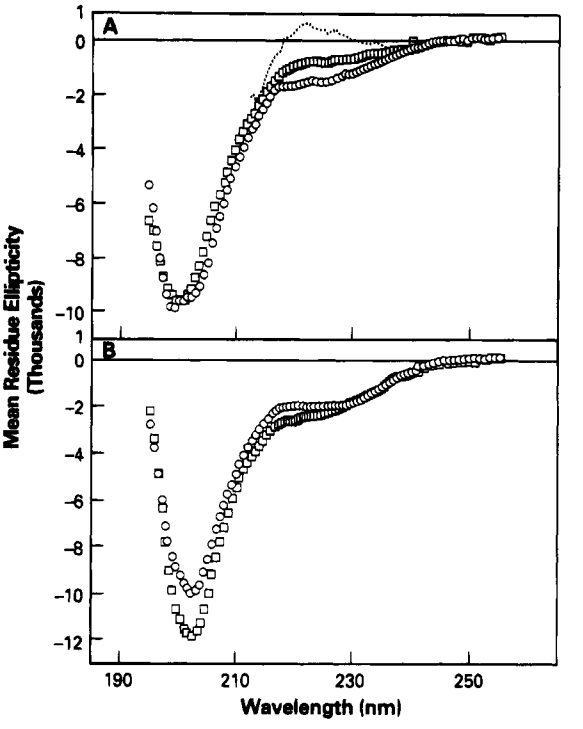


FIGURE 2: Ultraviolet CD spectra of peptide 2228. (A) CD spectra of peptide 2228 (100 μg/mL) in 0.1 M sodium phosphate, pH 7.5 (□), 6 M guanidinium-Cl (•••), or 0.1% SDS (○). (B) CD spectra of peptide in 1% SDS, pH 7.5 (□), or 1% SDS, pH 2 (○).

eleven residues were derived from h-TRβ1 and the last five were from mutant PV and compared their secondary structures with those of the corresponding wild-type h-TRβ1 and h-TRα1, peptides 2358 and 2359, respectively.

Figure 1 shows CD spectra of peptide 2358 from h-TRβ1. The isolated peptide shows a deep minimum at 198 nm and a shoulder at 225 nm, a spectrum which is usually taken to indicate an unfolded conformation. However, the spectrum is drastically altered by 6 M guanidinium chloride, a solvent in which most peptides are true random coils. High concentrations of SDS intensified the band at 225 nm, especially at low pH, reflecting formation of α-helix in the acidic region of the peptide. Analysis of the spectra by the CONTIN program reinforced this conclusion (Table 1). Similar changes in CD spectra were produced by high concentrations of the helix-permitting solvents methanol and trifluoroethanol (not shown). High concentrations of detergent resulted in similar changes in the CD spectrum of peptide 2359, derived from the carboxy terminus of h-TRα1 (Table 1), showing that both of these peptides have an inherent potential to form an α-helix conformation in a suitable environment.

In contrast, the CD spectra of peptide 2228, from mutant PV protein, showed little change under conditions favoring the formation of the α-helix, such as 1% SDS (Figure 2 and Table 1) or high concentrations of alcohol (not shown). Thus, changes in the sequence of this peptide resulting from the frameshift mutation have greatly reduced its potential for forming any helical structure. Identical behavior was shown by peptide 2453 (Table 1). These results indicate that the helix-forming potential is localized in the carboxy-terminal region of its sequence, EVFED.

Critical Role of the Last C-Terminal Helix in the Structural Stability of the Hormone Binding Domain of TRs. Analyses of the CD spectra of the peptides shown above indicate that the EVFED is an essential part of sequence to form a helical structure. To be certain that the same holds in the hormone binding domain, we analyzed the secondary structures of the hormone binding domains of the wild-type and mutant PVs. To simplify the secondary structural analyses, only the minimal T_3 binding domain was included. Previously we have shown that the minimal hormone binding domain of the h-TRβ1 includes part of domain D which starts at K^{206} and ends at D^{461}. The wild-type truncated protein KD29 binds T_3 with the same specificity as the intact wild-type receptor (Lin et al., 1991). To compare the secondary structures of the wild-type and mutant hormone binding domains, we constructed a T7 expression plasmid, pZJPV06, which encodes the coding region of the hormone binding domain of the PV mutant (L^{203}–D^{463}). We expressed the truncated protein, PV06, in *E. coli* and purified it to homogeneity as shown in lane 1 of Figure 3B. For comparison, we have also expressed the corresponding hormone binding domain of the wild-type truncated protein KD29 (see Figure 7C) in *E. coli* and purified it to homogeneity (Figure 3B, lane 2).

To be certain that the purified PV06 has the correct C-terminal PV mutant sequence, we developed polyclonal antibody T1 by using peptide 2228 as an immunogen. T1 recognized the intact mutant PV protein and PV06 protein synthesized by *in vitro* transcription/translation (Figure 3A, lanes 2 and 4, respectively), thus confirming the expected amino acids, at the C-terminus of mutant PV. In contrast, T1 does not recognize the wild-type h-TRβ1 (lane 5). Using Western blotting, we showed that PV06 purified from *E. coli* also has the correct protein sequence, as it is recognized by T1 (Figure 3C, lane 1). Consistent with the results in which T1 only reacted with mutant PV and not with wild-type TRβ1 (Figure 3A, lane 2 vs 5), T1 does not react with KD29 as

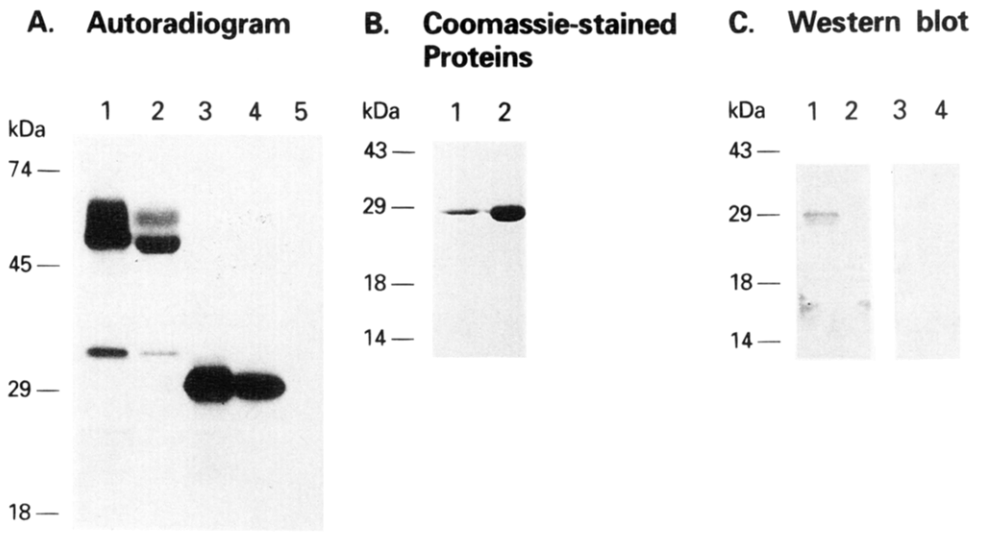


FIGURE 3: Immunoreactivity of intact mutant PV and its truncated PV06 with polyclonal Ab T1. (A) Three microliters of ^{35}S-labeled mutant PV (lanes 1 and 2), PV06 (lanes 3 and 4), or h-TRβ1 (lane 5) synthesized by *in vitro* transcription/translation were immunoprecipitated with 1 μg of T1 (lanes 2 and 4) as described in Materials and Methods. Lanes 1 and 3 are the *in vitro* translated mutants PV and PV06, respectively, as controls for comparison. (B) Purified PV06 (3.8 μg; lane 1) and KD29 (12 μg; lane 2) analyzed by 15% SDS−polyacrylamide gel electrophoresis. The purified proteins were visualized by Coomassie blue staining. (C) Western blotting. In a separate experiment, 1.5 μg each of PV06 (lanes 1 and 3) and KD29 (lanes 2 and 4) were analyzed by 15% SDS−polyacrylamide gel electrophoresis and transferred onto a nitrocellulose paper. The blots were incubated with Ab T1 (0.02 μg/mL) (lanes 1 and 2) or a control antibody, MOPC (lanes 3 and 4). After washing, the blots were incubated with goat anti-rabbit IgG conjugated with peroxidase. The color was developed using diaminobenzidine.

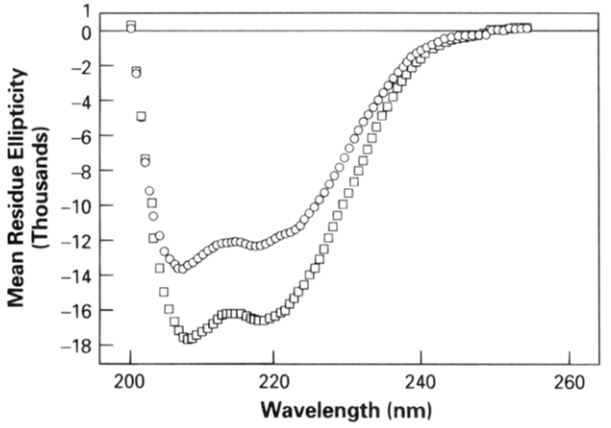


FIGURE 4: Ultraviolet CD spectra of proteins KD29 (□) and PV06 (○). Spectra were recorded on a Jasco J-500C spectropolarimeter, using a DP-500N data processor and 1-mm path length quartz cuvettes, in 0.2 M sodium phosphate buffer, pH 8.0, 25 °C. Protein concentrations were 100 μg/mL.

indicated by Western blotting (Figure 3C, lane 2). Lanes 3 and 4 of Figure 3C are controls to indicate the specificity of the Western blotting shown in lanes 1 and 2 of Figure 3C.

Analysis of CD spectra of the proteins (Figure 4) in terms of secondary structure gave selected best fits of 55% α-helix and 11% β-strand for KD29 and 30% α-helix and 45% β-strand for PV06. The estimates for KD29 are in reasonable agreement with those made using other methods from spectra measured under slightly different conditions (McPhie et al., 1993). Single-wavelength melting curves, following the change in ellipticity at 215 nm, showed that both proteins underwent cooperative unfolding at high temperatures (Figure 5). The thermal transitions were not fully reversible. The T_ms for KD29 and PV06 are 60 and 45 °C, respectively, indicating that the structure of KD29 is more stable than that of PV06.

The Epitope of mAb C4 Is Localized to the C-Terminal Pentapeptide EVFED of TRs. To develop a high-affinity monoclonal antibody to the C-terminal region of TRs, we used highly enriched h-TRα1 expressed in *E. coli* as an immunogen to obtain mAb C4. As shown in lane 4 of Figure 6, mAb C4 recognizes the intact h-TRα1 (MW 47 000) and the three smaller truncated h-TRα1s which were prepared by *in vitro* transcription/translation. These three smaller truncated proteins probably represent initiation at other

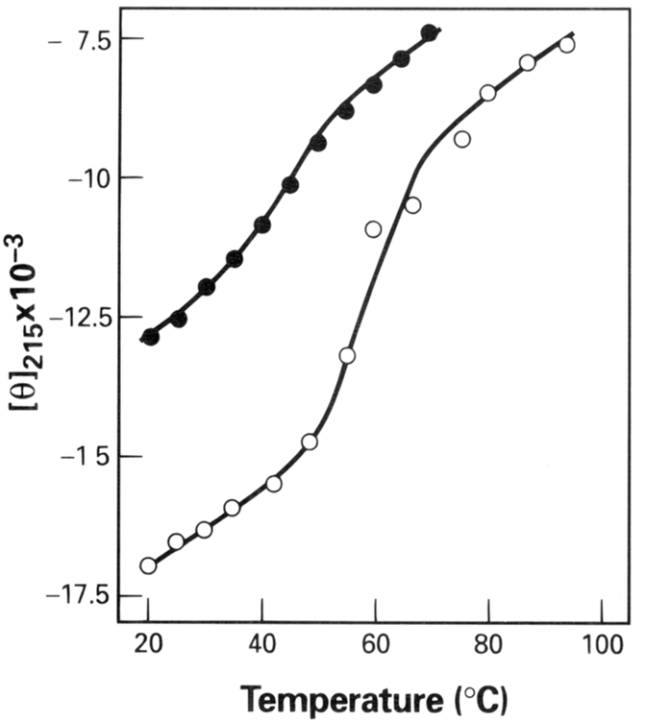


FIGURE 5: Thermal melting curves of proteins KD29 (○) and PV06 (●) measured by changes in CD at 215 nm. Changes were recorded on a Jasco J-500C spectropolarimeter. Protein solutions (100 μg/mL) were in 1-mm path length quartz cuvettes, held in a brass cell holder, which was thermostated by a Lauda circulating water bath. Temperatures in the cuvettes were measured with a Yellow Springs Instruments thermocouple.

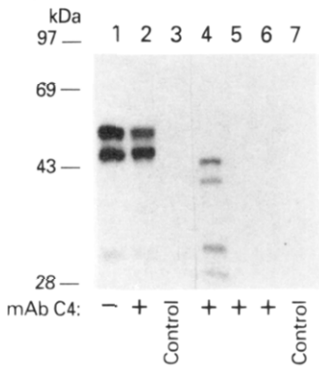


FIGURE 6: Autoradiogram of the immunoprecipitates of h-TRβ1 or h-TRα1 with mAb C4. Three microliters of ^{35}S-labeled h-TRβ1 (lanes 1−3 and lane 6) or h-TRα1 (lanes 4, 5, and 7) synthesized by *in vitro* transcription/translation were reacted with 1 μg of mAb C4 (lanes 2, 4, 5, and 6) or a control mAb, MOPC (lanes 3 and 7) as described in Materials and Methods. The competitor used in lanes 5 and 6 was h-TRα1 (5 μg) prepared in *E. coli*. The immunoprecipitates were analyzed by 10% SDS−polyacrylamide gel electrophoresis and autoradiographed.

downstream ATG sites (Nakai et al., 1988), suggesting that the epitope for mAb C4 is located toward the carboxyl end of the receptor. This recognition is specific because when a control antibody MOPC was used, no h-TRα1 was immunoprecipitated (Figure 6, lane 7). Furthermore, in the presence of 5 μg of highly enriched h-TRα1 prepared from *E. coli* extracts, no h-TRα1 was detected (Figure 6, lane 5). mAb C4 also recognizes the h-TRβ1. As shown in lane 2, three bands representing the intact (MW 55K) and two truncated h-TRβ1s (MW 52K and 35K), prepared by *in vitro* transcription/translation (Fukuda et al., 1988; Weinberger et al., 1986) were immunoprecipitated. Lane 1 shows the ^{35}S-labeled h-TRβ1 proteins for comparison. Again, this recognition is specific, as h-TRβ1 was not immunoprecipitated by the control antibody MOPC (lane 3). The cross reactivity was further confirmed by competition experiment. Lane 6 shows that none of the h-TRβ1 proteins were immunoprecipitated by mAb C4 in the presence of 5 μg of highly enriched h-TRα1 prepared from *E. coli* extracts. These results indicate that mAb C4 recognizes a common epitope present in both h-TRβ1 and h-TRα1.

To map out the epitope of mAb C4, we utilized a series of deletion mutants of h-TRβ1 which have been characterized previously (Figure 7C; Lin et al., 1991). We systematically analyzed their reactivities with mAb C4 by immunoprecipitation. As indicated in Figure 7A, mAb C4 recognizes the truncated mutants in which domain A/B (lane 4), a part of domain D (lanes 7 and 10, Figure 7A), or the entire domain D (lane 5 of Figure 7B) was deleted. However, when the last eight amino acids were deleted as in KP28 (lane 9 of Figure 7B), the recognition site was lost. In these experiments, we also used mAb C3 and the polyclonal antibody C91 as controls. The epitope of mAb C3 has previously been mapped to the region of K^{240}−P^{419} of domain E (Bhat et al., 1993). Ab C91 is a polyclonal antipeptide antibody whose antigen is the peptide C^{446}−D^{461} (Fukuda et al., 1988). As shown in lanes 1, 3, 6, and 9 of Figure 7A and lanes 4 and 8 of Figure 7B, mAb C3 immunoprecipitated the intact h-TRβ1, ED41, MD32, KD29, KD25, and KP28, respectively, as expected. Ab C91 reacted with KD25 (lane 3 of Figure 7B), as expected, but lost its reactivity when the last eight amino acids were deleted (lane 7 of Figure 7B). Taken

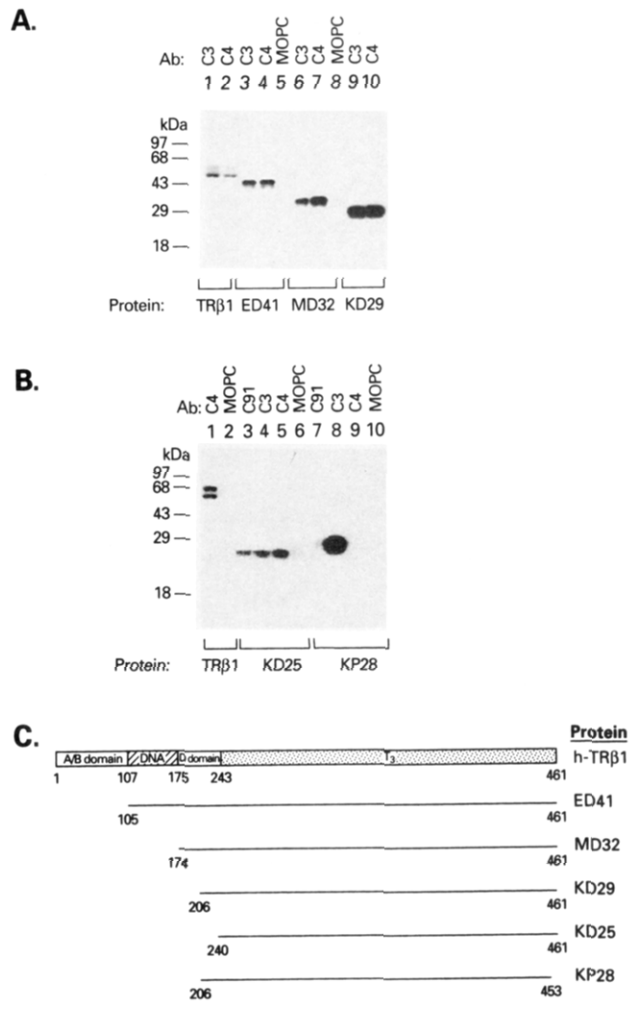
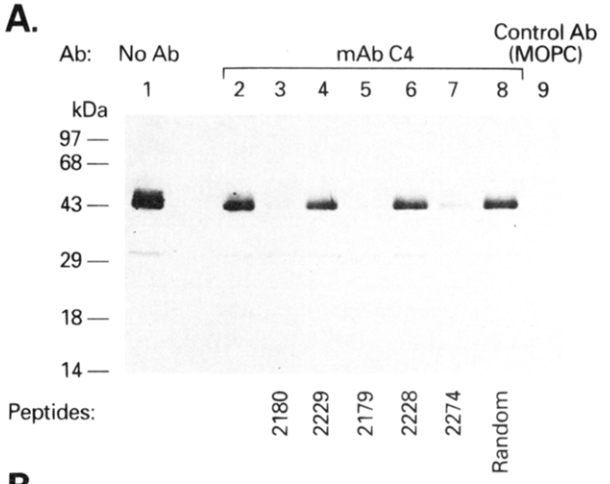


FIGURE 7: Mapping of the epitope of mAb C4 by immunoprecipitation of the amino-terminal (A) and C-terminal deletion mutants (B). Three microliters of ^{35}S-labeled h-TRβ1, ED41, MD32, KD29 (in A), KD25, and KP28 (in B) synthesized by *in vitro* transcription/translation were immunoprecipitated with 1 μg each of mAb C3, C4, the polyclonal Ab C91, or the control Ab, MOPC. The truncated proteins and antibodies used were as indicated. The immunoprecipitates were analyzed by 12% polyacrylamide gel electrophoresis and autoradiographed. (C) Schematic representation of the truncated proteins. The amino terminus and carboxy terminus are indicated. The numbering of the amino acids differs from those in Lin et al. (1991), McPhie et al. (1993), and Green and Chambon (1986), to reflect the consensus described in Beck-Peccoz et al. (1994a,b).

together, these results indicated that the epitope of mAb C4 lies in the region of L^{454}−D^{461}.

To further map out the precise epitope of mAb C4, we synthesized a series of peptides as shown in Figure 8B. We carried out immunoprecipitation to see whether these peptides can compete for the recognition with mAb C4 for intact h-TRβ1. Lane 1 of Figure 8A shows the h-TRβ1 synthesized by *in vitro* translation/transcription. In a 12% polyacrylamide gel, the 55K and 52K proteins were not very well separated. Lane 2 shows that the same three protein bands were immunoprecipitated by mAb C4. Lanes 3, 5, and 7 of Figure 8A show the effective blocking of the binding of h-TRβ1 to mAb C4 by peptides 2180, 2179, and 2274, respectively. In contrast, peptides 2229 and 2228 which have the sequence of mutant PV, failed to compete with h-TRβ1 for binding to mAb C4. Moreover, a peptide with random sequence also failed to compete for the binding of h-TRβ1 to mAb C4 (lane 8 of Figure 8A). These results indicate that the inhibition



B.

Peptides:		
(TRβ1)	2179:	P^{453} L F L E V F E D^{461}
(TRβ1)	2229:	P^{453} L F L E V^{458}
(TRβ1)	2180:	E^{457} V F E D^{461}
(TRα1)	2274:	E^{402} V F E D Q E V^{409}
(Mutant PV)	2228:	C^{446} P H R T L P P F V L G S V R G L D^{463}

FIGURE 8: Competition of binding h-TRβ1 to mAb C4 with C-terminal peptides of TRs. Four microliters of ^{35}S-labeled h-TRβ1 synthesized by *in vitro* transcription/translation were immunoprecipitated with 2 μg of mAb C4 in the absence (lane 2) or the presence of 150 μg of the peptides as described in Materials and Methods. The peptides used are indicated. The immunoprecipitates were analyzed by 12% SDS−polyacrylamide gel electrophoresis and autoradiographed. (B) Amino acid sequences of the C-terminal peptides.

in the binding of h-TRβ1 to mAb C4 by peptides 2180, 2179, and 2274 is specific. Since the common residues in these three peptides are EVFED, we concluded that the epitope for mAb C4 is EVFED. It is important to point out that EVFED is conserved in h-TRβ1 and h-TRα1.

mAb C4 Competes with T_3 for Binding to h-TRβ1 and h-TRα1. The identification of E^{457}VFED^{461} as the epitope for mAb C4 provided us with a tool to examine whether this region is directly involved in T_3 binding. Figure 9 indicates that T_3 binding to h-TRβ1 is inhibited by mAb C4 in a concentration-dependent manner. Nonimmune IgG failed to compete for T_3 binding. Furthermore, the binding of T_3 to h-TRα1 is also similarly inhibited in a concentration-dependent manner.

The inhibition in T_3 binding to h-TRβ1 or TRα1 by mAb C4 could be due to competitive binding for the same site or noncompetitive binding. To distinguish these two possibilities, we measured T_3 binding to h-TRβ1 in the presence of increasing concentrations of mAb C4. Binding of T_3 to receptor is measured by displacement of [^{125}I]T_3 from a hormone−receptor complex by increasing concentrations of unlabeled T_3. Assuming direct competition between hot and cold ligand for a single site on the receptor, then the concentration of radioactive complex is given by the equation

$$[\mathrm{Rh}] = \frac{[\mathrm{R}]_0{}^*[\mathrm{h}]}{K_d + [\mathrm{h}] + [\mathrm{c}]} \qquad (1)$$

where $[R]_0$ is the total concentration of receptor, [h] and [c] are the concentrations of hot and cold T_3, and K_d is the dissociation constant of the hormone−receptor reaction. We

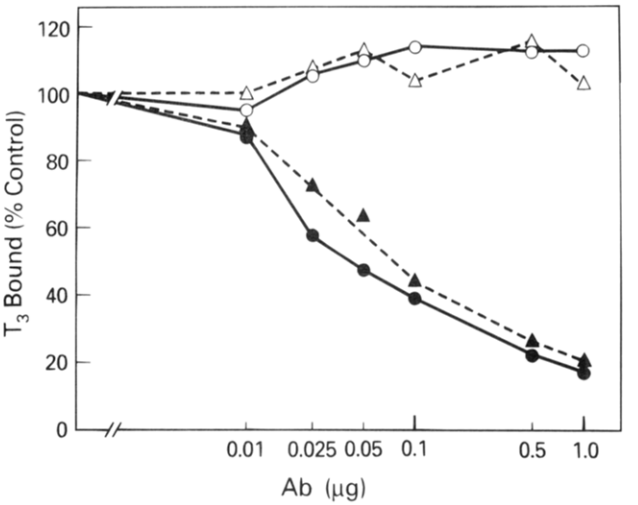


FIGURE 9: Competitive binding the T_3 and mAb C4 to h-TRβ1. Three microliters of h-TRβ1 (●, ○) or h-TRα1 (▲, △) synthesized by *in vitro* transcription/translation were incubated with 0.2 nM of [^{125}I]T_3 in the absence or with increasing concentrations of mAb C4 (●, ▲) or nonimmune mouse IgG (○, △) for 1 h at 25 °C. After incubation, the free and h-TRβ1 or h-TRα1-bound [^{125}I]T_3 were separated by Sephadex G-25 column chromatography as described in Materials and Methods.

can model the behavior of the system in the presence of mAb C4, by assuming a general case, where antibody can bind to both free receptor and receptor−hormone complex, with association constants K' and K'', respectively. Now, the measured radioactivity bound is given by

$$[\mathrm{Rh}] + [\mathrm{AbRh}] = \frac{[\mathrm{R}]_0{}^*[\mathrm{h}]}{\dfrac{K_d(1 + K'[\mathrm{Ab}])}{(1 + K''[\mathrm{Ab}])} + [\mathrm{h}] + [\mathrm{c}]} \qquad (2)$$

where [Ab] and [AbRh] are the concentrations of antibody and of antibody−receptor−hot T_3 complex, respectively. In particular, if the antibody binds only to free receptor ($K'' = 0$), then the apparent dissociation constant derived by fitting to eq 1 will increase linearly with antibody concentration, whereas binding of antibody to only the hormone−receptor complex ($K' = 0$) will facilitate hormone binding, causing K_d to decrease with antibody concentration. Binding of the antibody to both forms of the receptor will result in complex dependency of the apparent dissociation constant on antibody concentrations. Figure 10 shows that, over the concentration range measured, K_d increases linearly with mAb C4 concentration, clearly indicating that antibody binds only to free receptor; i.e., mAb C4 and T_3 are competing for the free receptor. Since the epitope for mAb C4 was mapped to the C-terminal EVFED, we concluded that part of the T_3 binding site of TR is located in this region.

DISCUSSION

TRs are ligand-dependent transcription factors which act both by binding to specific DNA sequences and by interaction with the components of the transcription complex directly or indirectly via bridging factor/mediator. Schmitz et al., have recently proposed a general model for such interaction to occur via a C-terminal transactivation domain which could assume the structure of an amphipathic α-helix (Schmitz et al., 1994). Barettino et al. presented mutational

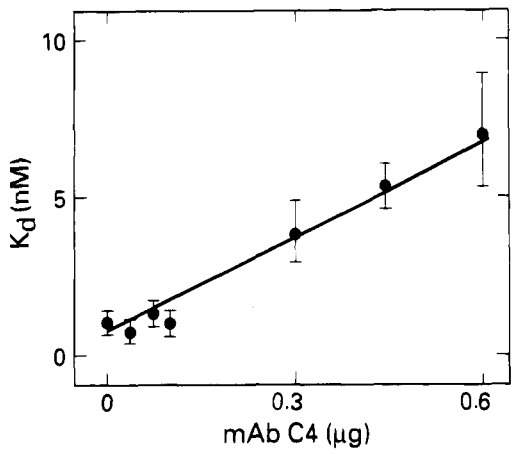


FIGURE 10: Effect of increasing concentrations of mAb C4 on the apparent dissociation constant (K_d) of T_3 from h-TRβ1. Competitive binding experiments were carried out as described in Lin et al. (1991) except that h-TRβ1 (200−300 ng) purified from *E. coli* was used in the competitive binding studies. The data are analyzed according to eqs 1 and 2. The data are averages of three experiments, each with duplicates. The bars represent means ± SDs (n = 3).

evidence for such a domain in the last carboxy 35 amino acids of chicken TRα1 (Barettino et al., 1994).

We recently proposed a model for the structure of the ligand binding domain of h-TRβ1, in which the polypeptide chain is folded into an α/β barrel (McPhie et al., 1993). The extreme carboxy-terminal region of the protein was assigned an important role in this model, forming the last of the eight α-helices necessary for closure of the barrel. A truncated protein, KP28, in which the last eight amino acids (454−461) were deleted, was shown to be incompletely folded and unable to bind T_3. This region of the protein is also known to be important in direct protein−ligand interactions, since a number of point mutations isolated from patients with generalized resistance to thyroid hormone have been mapped in this area (Refetoff, 1992). Mutant receptor PV, in which the last 16 residues in the protein are changed by a frameshift mutation, is unable to bind T_3.

Structural arguments based solely on predictions may not be very convincing. Therefore, we examined the behavior of four peptides derived from the regions of interest in a series of solvents which have been shown to induce the formation of secondary structures compatible with a peptide's environment in a folded protein (Wu et al., 1981; Zhong & Johnson, 1992). In aqueous solution, all four peptides showed CD spectra generally regarded as characteristic of unfolded conformations (though formal analysis of the spectra using the CONTIN program indicates the presence of extensive amounts of β-strand). High concentrations of SDS or methanol induced the formation of extensive regions of α-helix in peptides 2358 and 2359 from h-TRβ1 and h-TRα1, respectively (Figure 1 and Table 1). However, the CD spectra of peptide 2228 from PV and hybrid peptide 2453 were essentially invariant with solvent conditions, showing that the helix-forming potential lies at the extreme carboxy-terminal region of the receptor proteins. Mapping these terminal sequences onto a helical wheel shows the highly amphipathic nature of this helix, all of the acidic side chains falling on one side (Zenke et al., 1992).

Monoclonal antibody mAb C4 recognizes a common epitope of h-TRβ1 and h-TRα1 and reduces binding of T_3 to both receptors similarly (Figure 9). The results shown in Figure 10 indicate that mAb C4 and T3 are competitive for wild-type h-TRβ1; i.e., binding one of these to the receptor excludes binding to the other. Figure 7 places the binding site for this antibody in the last eight amino acids of the h-TRβ1 sequence, since it does not recognize KP28 but binds to other truncated forms of the receptor. The location of the antigenic site can be mapped more closely by competition experiments, using a series of peptides derived from the carboxy-terminal region of the protein (Figure 8). Receptor−antibody binding is inhibited by peptides 2180, 2179, and 2274, which all contain the last five residues of the ligand binding domain (EVFED), but not by peptide 2229, which lacks the last three residues (FED). The epitope, EVFED, is conserved in TRβ1 and TRα1 receptors. It has long been known that the most antigenic side chains are those derived from aromatic residues (Sela & Arnon, 1960), suggesting that the antibody binding site will include F459. The naturally occurring point mutation F459C results in a 30-fold reduction in the affinity of h-TRβ1 for L-T_3 (Cheng et al., 1994), and more conservative changes produce smaller changes (Barettino et al., 1994), indicating the importance of this residue in hormone−receptor interactions.

The demonstration that five amino acids, EVFED, are part of the T_3 binding site provides a molecular basis to understand the critical role of the C-terminal region in the hormone binding and transactivation of TRs reported earlier (Lin et al., 1991; Chatterjee et al., 1991). Furthermore, the localization of T_3 binding determinants in this sequence raises the exciting possibility that the hormonal signal for transactivation may be transmitted by direct alteration of the structure of the C-terminal activation domain. Previously, we have found that T_3 induces conformational changes to make the hormone binding domain more "compact" and less susceptible to proteolytic degradation (Bhat et al., 1993). Our earlier CD studies showed no change in secondary structure of the hormone binding domain on binding T_3 (McPhie et al., 1993). Toney et al. (1993) showed that binding of Triac (3,3′,5-triiodo-L-thyroacetic acid) to intact h-TRα produced increases in ellipticity at 193 nm, which they interpreted to show "a subtle structural change...without a major perturbation of the secondary structure". These authors observed similar changes when h-TRα bound to an inverted repeat thyroid hormone response element (TRE), but not other TREs, which they took to indicate a specific environment for the carboxy-terminal domain in these complexes.

The CD spectra shown in Figure 4 reflect the secondary structure of hormone binding domains derived from the wild-type and mutant PV receptors. Both spectra are characteristic of proteins containing mixtures of α-helices and β-strands, but analysis of the spectra by the CONTIN program indicates that the structure of protein PV06 differs from that of protein KD29 derived from wild-type receptor. Thermal melting experiments indicate that the structure of PV06 is also less stable than that of KD29 (Figure 5). Thus, deletion of the carboxy-terminal sequence (McPhie et al., 1993) or its disruption by a frameshift mutation with loss of helix-forming potential results in incomplete folding of the receptor's ligand binding domain, with loss of biological function. These studies clearly demonstrate the importance of the structural integrity of the extreme carboxyl amphipathic α-helix for the structure of the thyroid hormone receptors. Contrary to the theory of Schmitz et al. (1994), the structure is not

induced solely in the presence of hormone.

Earlier studies on the behavior of the human progesterone receptor (h-PR) show close similarities to h-TRs. Deletion of the last 42 amino acids abolished hormone binding (Vegeto et al., 1992); hormone binding to intact h-PR increased resistance to proteolysis in the protein's carboxy-terminal 30−40 amino acids (Allan et al., 1992) and prevented binding of a monoclonal antibody raised against the receptor's last 14 amino acids (Weizel et al., 1992). It seems that ligand-induced conformational changes in the extreme carboxy-terminal region may perform an important role in intermolecular interactions throughout this receptor superfamily.

In the case of h-TRs, we have now located this important site in the last five (h-TRβ1) or eight (h-TRα1) amino acids, which form an α-helix in the intact protein. We have shown that binding of T_3 is competitive with that of mAb C4, a protein which binds specifically to this region of the receptor. In like manner, binding of T_3 could lead to the release of repressors which bind to the activation domain, providing a molecular switch for transcription. Alternatively, interaction of T_3 with the C-terminal region EVFED could induce a rearrangement of the activation domain to "fit" better or become more accessible to bridging factors, mediators, or other components of the transcriptional machinery. These possibilities require further investigation.

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