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CONFORMATIONAL CHANGES OF HUMAN β1 THYROID HORMONE RECEPTOR INDUCED BY BINDING OF 3,3',5-TRIIODO-L-THYRONINE

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Summary - To understand the structural basis in the hormone-dependent transcriptional regulation of human β1 thyroid hormone receptor (h-TRβ1), we studied the conformational changes of h-TRβ1 induced by binding of 3,3',5-triiodo-L-thyronine (T_3). h-TRβ1 was treated with trypsin alone or in the presence of T_3, thyroid hormone response element (TRE) or T_3 together with TREs. Without T_3, h-TRβ1 was completely digested by trypsin. Binding of TREs had no effect on the tryptic digestion pattern. However, T_3-bound h-TRβ1 became resistant to tryptic digestion and yielded trypsin-resistant peptide fragments with molecular weight of 28,000 and 24,000. Chymotryptic digestion also yielded a T_3-protected 24 Kd peptide fragment. Using anti-h-TRβ1 antibodies and amino acid sequencing, the 28 Kd fragment was identified to be Ser^{202}-Asp^{456}. The 24 Kd tryptic fragments were found to be Lys^{239}-Asp^{456} and Phe^{240}-Asp^{456}. The 24 Kd chymotryptic fragment was identified to be Lys^{235}-Asp^{456}. The structural changes as a result of T_3 binding could serve as a transducing signal to modulate the gene regulating activity of h-TRβ1.

Introduction - The thyroid hormone, 3,3',5-triiodo-L-thyronine (T_3) promotes growth, induces differentiation and regulates metabolic effects. Recent studies have indicated that most, if not all of these effects are mediated through the interaction of T_3 nuclear receptors (TR) with the hormone response elements (TREs) of T_3 target genes (1). Two major TR isoforms have been isolated; TRα and TRβ, whose genes are located in chromosome 17 and 3, respectively. Recent studies have identified the consensus sequence of the half-site binding motif to be AGGT(C/G/A)A. The interaction of TRs with TREs leads to the alteration of the target gene activity. The interaction of TR with TREs is further modulated by other nuclear proteins including retinoid X receptor (2).

The TR-mediated activation or repression of the target genes is T_3-dependent. However, the structural basis for this T_3-dependency in the regulating activity of TR is not known. TRs are members of the steroid/retinoic acid receptor superfamily. By analogy with the steroid hormone

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Abbreviations: T_3, 3,3',5-triiodo-L-thyronine; h-TRβ1, human β1 thyroid hormone nuclear receptor; TRE, thyroid hormone response element; MAb, monoclonal antibody; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

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receptors, four domains could be assigned to human β1 TR, A/B (Met^{1}-Leu^{101}), C (Cys^{102}-Met^{169}), D (Ala^{170}-Lys^{237}) and E (Arg^{238}-Asp^{456}) (3). Domain E was previously thought to be the T_3 binding domain. However, we have recently shown that domain E alone does not bind T_3 and requires domain D to become a functional T_3 binder (4). Using circular dichroism and sequence analyses, we have recently proposed that TRβ1 is essentially composed of two functional domains; the DNA binding domain of known structure (5), linked by an α-helix (Ala^{170}-Gln^{200}) to a hormone binding domain (Lys^{201}-Asp^{456}) with an α/β barrel structure (6). It is possible that the hormonal signal for activation or repression of T_3 target genes is transmitted from the hormone binding domain via the α-helical hinge (Ala^{170}-Gln^{200}). This would mean that T_3 induces structure changes either in the hormone binding domain or the helical hinge or both. To understand the molecular basis of the hormone-dependent gene regulatory activity of h-TRβ1, the present studies used partial proteolysis and anti-TR antibodies as probes to assess whether binding of T_3 to the human TR, subtype β1 (h-TRβ1), leads to any conformational changes. We found that binding of T_3 renders h-TRβ1 less susceptible to proteolysis. Four T_3-protective sites are identified and are predicted to be on the surface of the globular T_3 binding domain.

Materials and Methods

Production of monoclonal antibody C3: Mice were immunized with purified truncated fragment of h-TRβ1 (KD29; Lys^{201}-Asp^{456}) which contains the hormone binding domain of h-TRβ1 (4). Spleen cells from the immune mice were fused with P3x63Ag8653 myeloma cells by the method described previously (7). Initially, the hybridomas were screened by enzyme-linked immunosorbent assay using purified KD29. The positive hybridomas were rescreened by immunoprecipitation with [^{35}S]methionine labeled h-TRβ1 prepared by *in vitro* transcription/translation. The positive hybridomas were isolated and cloned by a limiting dilution method as described previously (7). The monoclonal antibody C3 was prepared in large quantity in mouse ascites fluids and was affinity purified using protein-G-Sepharose CL-4B (Pharmacia, Piscataway, NJ) as described (7).

Localization of the antigenic site of monoclonal antibody C3: To map the antigenic site of MAb C3, the [^{35}S]methionine labeled fragments of h-TRβ1, DD28 (Asp^{211}-Asp^{456}), KD25 (Lys^{235}-Asp^{456}), KP28 (Lys^{201}-Pro^{448}), KH27 (Lys^{201}-His^{436}) and KP24 (Lys^{201}-Pro^{414}) were prepared by *in vitro* transcription/translation according to Lin et al. (4). These [^{35}S]-methionine labeled proteins were immunoprecipitated with MAb C3 or a control MAb MOPC. The immunoprecipitates were analyzed by a 10% SDS-PAGE. The radioactive bands were detected by autoradiography.

Partial proteolysis of the *in vitro* translated h-TRβ1: Three μl of the [^{35}S]-methionine-labeled *in vitro* translated h-TRβ1 prepared as described in (4) was digested with 5-50 ng/μl of TPCK-treated trypsin or chymotrypsin (Sigma Co., St. Louis, MO) for 15 min at 23°C. h-TRβ1 was digested alone or after preincubation with 100 nM T_3 or thyroid hormone response element (TRE; 1 μg) for 1 hr at 23°C. The TREs used were the idealized palindrome (TREpal), DR + 4 which is a direct repeat with four gaps and F2, the chicken lysozyme gene TRE. These three TREs were synthesized as described in (8). After proteolysis, the digests were analyzed by 15% SDS-PAGE. The peptide fragments were detected by autoradiography.

Determination of amino terminal sequence of the proteolytic fragments: h-TRβ1 was expressed and purified by a modified procedure described by Lin et al. (9). Briefly, the inclusion bodies obtained as described in (9) were resuspended in 6 M guanidine hydrochloride in 0.1 M Tris/pH 8.0. The solution was diluted so that the protein concentration was 8 mg/ml and dithioerythritol was added to give a final concentration of 0.3 M. After incubation for 2 hr at 23°C, a 100-fold dilution was made with a buffer containing 0.5 M L-arginine hydrochloride, 8 mM oxidized glutathione, 10 μM $ZnCl_2$ and 0.1 M Tris•HCl/pH 8.0. After incubation for 45 min at 4°C, EDTA was added to give a final concentration of 2 mM. The solution was kept at 4°C for 40 hrs. The solution was dialyzed against 0.1 M urea, 0.02 M Tris•HCl/pH 7.4 (3x changes

of dialyzate) and loaded onto an 8 ml Q Sepharose column equilibrated with 0.05 M Tris•HCl/pH 8.0. After washing the column with 50 mM Tris•HCl/8.0 containing 0.15 M NaCl, h-TRβ1 was eluted with 0.4 M NaCl. The fractions were assayed for T_3 binding and for purity by SDS-PAGE.

The purified h-TRβ1 (20-25 μg) was treated with increasing concentrations of trypsin in the presence of 1 μM of T_3. The trypsin resistant peptides were blotted onto Problot (ABI, CA) for amino acid sequencing.

Results

Binding of T_3 renders h-TRβ1 resistant to tryptic digestion: To understand the molecular basis of the hormone-dependent transcriptional activity, we examined the possible structural changes induced by binding of T_3 to h-TRβ1. We used partial proteolysis and anti-h-TRβ1 antibodies as probes. Figure 1 shows the autoradiogram of partial tryptic digestion of the *in vitro* translated h-TRβ1 under different conditions. In the absence of T_3 and DNA, h-TRβ1 was completely digested when the trypsin concentration was greater than 25 ng/μl (lanes 4 and 5). However, when T_3 was present, the digestion pattern was different. Two peptides with molecular weight of 28 Kd and 24 Kd became resistant to trypsin digestion (lanes 8-10). Binding of h-TRβ1 to F2 had no effect on the digestion pattern of h-TRβ1 (lanes 13-15 vs. lanes 3-5). Simultaneous addition of F2 and T_3 did not change the protective effect of T_3 from trypsin digestion (lanes 8-10 vs. 18-20). DR+4 and TREpal were also evaluated and no affect on the tryptic digestion pattern was found (data not shown).

The resistance to tryptic digestion is intrinsic to the ability of the T_3 receptor to bind T_3. A mutant receptor which was derived from the generalized thyroid hormone resistance kindred PV has lost totally its T_3 binding activity due to an insertion and frame shift mutation at codon 443 (Fig. 2) (10). As shown in Fig. 2, the mutant receptor was digested by trypsin similarly whether T_3 was present or absent.

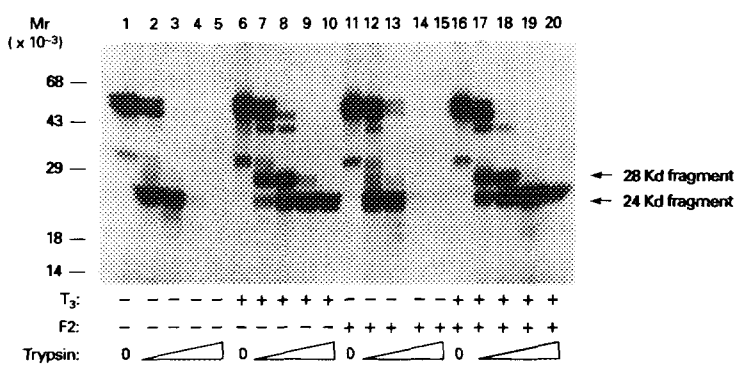

Figure 1. Autoradiograms of the peptide fragments derived from partial tryptic digestion of the *in vitro* translated [^{35}S]methionine-labeled h-TRβ1. Three μl of the [^{35}S]methionine labeled h-TRβ1 prepared as described in Methods were digested with 5 ng/μl (lanes 2, 7, 12 and 17), 10 ng/μl (lanes 3, 8, 13 and 18), 25 ng/μl (lanes 4, 9, 14 and 19) or 50 ng/μl (lanes 5, 10, 15 and 20) of TPCK-treated trypsin for 15 min at 23°C. Lanes 1-10 are without F2 and lanes 11-20 are with 1 μg of F2. T_3 (100 nM) was present in lanes 6-10 and 16-20. The digestion was terminated by the addition of 9 μl of 2X sample buffer (3% SDS, 0.07 M Tris, 11% glycerol and 5% mercaptoethanol). The peptide fragments were analyzed by 15% SDS-PAGE and autoradiographed.

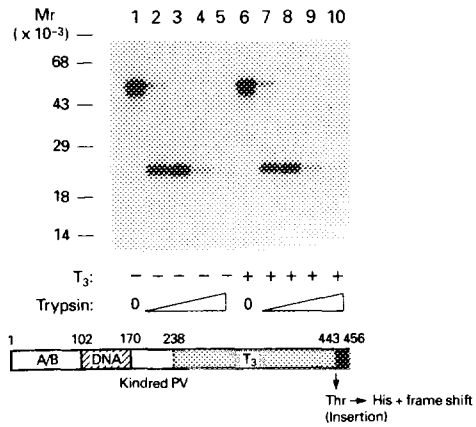

Figure 2. Autoradiograms of the peptide fragments derived from partial tryptic digestion of [^{35}S]methionine-labeled mutant receptor PV. Three µl of [^{35}S]methionine labeled mutant receptor PV was digested with 5 ng/µl (lanes 2 and 7), 10 ng/µl (lanes 3 and 8), 25 ng/µl (lanes 4 and 9) or 50 ng/µl (lanes 5 and 10) of TPCK-treated trypsin for 15 min at 23°C. The mutant receptor PV was digested in the absence (lanes 2-5) or presence of 100 nM T_3 (lanes 7-10). After digestion, the samples were processed similarly as described in Fig. 1.

Both the 28 Kd and 24 Kd peptide fragments contain the C-terminus and the hormone binding domain: To identify the location of the 28 Kd and 24 Kd fragments in h-TRβ1 molecules, we used anti-h-TRβ1 antibodies. C-91 is an antipeptide antibody whose antigenic site is located in the C-terminus in the region of amino acids 445-456 (11). We further developed a MAb C3 which specifically recognizes the hormone binding domain of h-TRβ1. Using a series of truncated h-TRβ1 and immunoprecipitation (see Methods), we mapped its antigenic site to be located in the region of Lys^{235}-Pro^{414} (data not shown). Figure 3 shows that both 28 Kd and 24 Kd peptide fragments are recognized by antibodies C3 (lanes 12-15) and C91 (lanes 17-20) indicating that both peptides contain the C-terminus and the hormone binding domain. These results further indicate that 24 Kd fragment is derived from the 28 Kd fragment.

Binding of T_3 also renders h-TRβ1 resistant to chymotryptic digestion: To evaluate the extent of structural changes induced by T_3, we used chymotrypsin to explore other sites which were affected. Lane 17 in Fig. 4 indicates that when the chymotrypsin concentration was 5 ng/µl, two peptides with the molecular weight of 43 Kd and 27 Kd became resistant to chymotryptic digestion in the presence of T_3. However, these two peptides were digested when the chymotrypsin was increased to 10 ng/µl (lane 18, Fig. 4). However, a 24 Kd fragment remained when the concentration was increased to 25 ng/µl (lane 19, Fig. 4); whereas in the absence of T_3, h-TRβ1 was completely digested (lane 14, Fig. 4). This 24 Kd fragment has the same molecular weight as the peptide which is resistant to tryptic digestion (lanes 7-10, Fig. 4).

Identification of the sites with altered protease sensitivity induced by T_3 binding: To identify the proteolytic sites whose sensitivity to proteases have been altered as a result of T_3 binding, we used the h-TRβ1 expressed in *E. coli* and purified so that the peptide fragments could be isolated and sequenced. The purified h-TRβ1 had been characterized and shown to bind T_3 with affinity and analog specificity similar to those of the *in vitro* translated

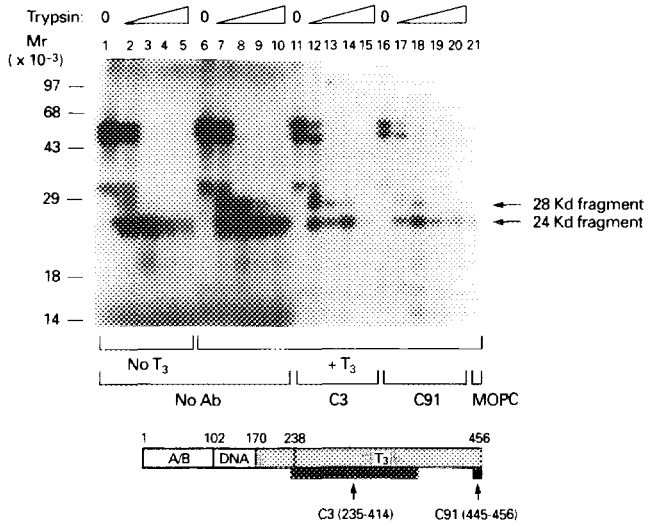

Figure 3. Autoradiograms of the immunoprecipitates of the tryptic peptide fragments of [^{35}S]methionine labeled h-TRβ1 prepared by *in vitro* translation. Three μl of [^{35}S]methionine labeled h-TRβ1 was treated with trypsin similarly as described in Fig. 1. The tryptic digests were immunoprecipitated with 1 μg MAb C3 (lanes 11-15), C91 (lanes 16-20) or a control antibody MOPC (lane 21). For comparison, lanes 2-5 and 7-10 were tryptic digests which were not treated with antibodies.

h-TRβ1 (9). The partial tryptic digestion of h-TRβ1 was carried out similarly as the *in vitro* translated [^{35}S]-labeled h-TRβ1. In the presence of T_3, a 28 Kd fragment was detected (lanes 6-9 vs. lanes 2-5, Fig. 5A). Furthermore, the 24 Kd fragment was more resistant to tryptic digestion in the presence of T_3 (lanes 7-9 vs. lanes 3-5, Fig. 5A). To ensure that the 28 Kd and 24 Kd

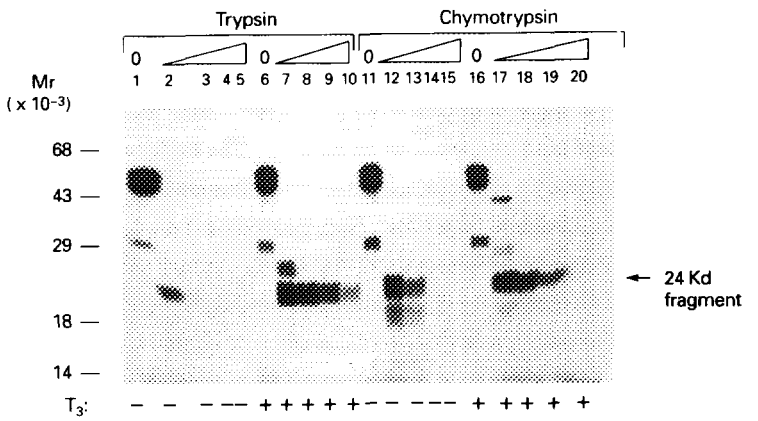

Figure 4. Comparison of tryptic and chymotryptic resistant peptides induced by T_3-binding. Three μl of the [^{35}S]methionine labeled h-TRβ1 was treated with trypsin (lanes 2-5 and 7-10) or chymotrypsin (lanes 12-15 and 17-20) in the absence (lanes 2-5 and 12-15) or presence of 100 nM T_3 (lanes 7-10 and 17-20). The concentrations of trypsin for lanes 2-5 and 7-10 were the same as in Fig. 1. The concentrations of chymotrypsin were 5 ng/μl (lanes 12 and 17), 10 ng/μl (lanes 13 and 18), 25 ng/μl (lanes 14 and 19) and 50 ng/μl (lanes 15 and 20). After tryptic or chymotryptic digestion, the peptides were analyzed by 15% SDS-PAGE and autoradiographed.

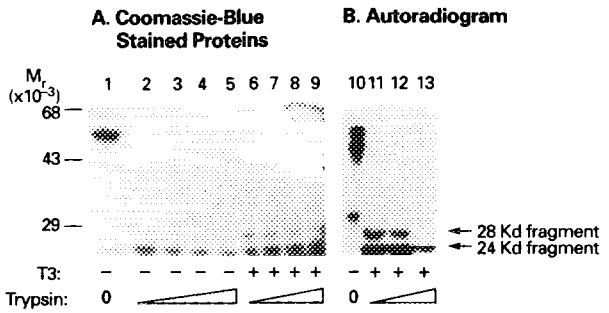

Figure 5. Partial tryptic digestion of h-TRβ1 purified from *E. coli*. (A) Purified h-TRβ1 (6 μg) in 50 mM Tris HCl/8.0 and 0.1M NaCl was digested with trypsin (0.5 ng/μl for lanes 2 and 6; 1 ng/μl for lanes 3 and 7; 2 ng/μl for lanes 4 and 8; 5 ng/μl for lanes 5 and 9) in the absence (lanes 2-5) or presence of 1 μM T_3 (lanes 6-9) for 15 min at 23°C. The digestion was terminated by the addition of 16 μl of 5X sample buffer. The peptides were analyzed by 10% SDS-PAGE and stained with Coomassie Blue. (B) Purified h-TRβ1 treated similarly as in (A) was coelectrophoresed with [^{35}S]methionine labeled h-TRβ1 which was partially digested with trypsin as described in Fig. 1. After 10% SDS-PAGE, the gel was dried and autoradiographed.

fragments are the same as those derived from the *in vitro* translated h-TRβ1, we coelectrophoresed the unlabeled h-TRβ1 purified from *E. coli* and the [^{35}S]-labeled h-TRβ1 prepared by *in vitro* translation. Lanes 11-13 of Fig. 5B indicate that the 28 Kd and 24 Kd derived from h-TRβ1 purified from *E. coli* and [^{35}S]-labeled prepared by *in vitro* translation comigrated.

The 28 Kd and 24 Kd fragments were transferred to Problot and sequenced. The 28 Kd is a peptide beginning at Ser^{202}. The 24 Kd peptide is a mixture of two peptides beginning at Lys^{239} and Phe^{240}. Residues 201 and 239 are Lys and residue 238 is an Arg. The 24 Kd chymotryptic fragment begins at Lys^{235}. Residue 234 is a Trp. Therefore, the peptides which have become resistant to partial proteolysis are Ser^{202}-Asp^{456}, Lys^{239}-Asp^{456}, Phe^{240}-Asp^{456} and Lys^{235}-Asp^{456}.

Discussion

Using partial proteolysis and anti-h-TRβ1 antibodies as probes, we found that binding of T_3 induces conformational changes in h-TRβ1. Lys^{201}, Arg^{238}, Lys^{239} and Trp^{234} have become less susceptible to proteolytic digestion as a result of T_3 binding. The molecular basis of the T_3-induced changes in the sensitivity to proteolysis, however, is not entirely clear. The T_3-induced structural changes, however, could be better understood based on a model of the hormone binding domain of h-TRβ1 we have just recently proposed (6). Using circular dichroism measurements and sequence analyses, we have shown that the hormone binding domain of h-TRβ1 (Lys^{201}-Asp^{456}) consists of eight alternating α-helices and β-strands. Together with deletion analyses, we proposed that the hormone binding domain has a tertiary structure of an α/β barrel (6). According to this model, Lys^{201} is located at the end of helix 0 and the beginning of β-strand 1 (see Fig. 6). Trp^{234}, Arg^{238} and Lys^{239} are in the loop between helix 1 and β-strand 2 (Fig. 6). These four residues are exposed on the surface of the β-barrel. The T_3 binding site is located inside of the eight-stranded β-barrel, similar to that found for the major plasma thyroid hormone binding protein, transthyretin (12). Binding of T_3 could result in a more compact hormone binding domain

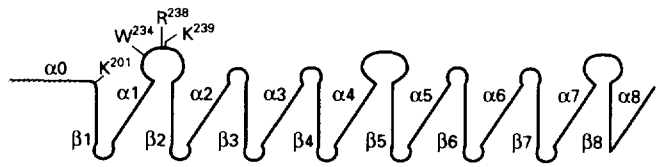

Figure 6. A diagrammatic representation of the secondary structural elements predicted in domains D and E (Met^{169}-Asp^{456}) of h-TRβ1 (6). The T_3-protected proteolysis sites mentioned in the text are indicated. α, helix; β, β-strands.

due to the possible interaction of T_3 with the hydrophobic residues inside the β-barrel. Alternatively, binding of T_3 could lead to an alteration of the local structure in the region of β-strand 1-helix 1-loop-strand 2, thereby leaving Lys^{201}, Trp^{234}, Arg^{238} or Lys^{239} less accessible to proteases.

Recently, it has been shown that binding of T_3 induces the dissociation of TR homodimer bound to DNA, thereby relieving the suppression effects of the unliganded TR (13). Furthermore, dissociation of the TR homodimers could also lead to the binding of functionally relevant TR/thyroid receptor accessory protein(s) to TRE which results in the activation or inhibition of transcription of the target genes. The T_3-induced conformational changes observed in the present studies could be the signal to affect the dissociation of TR homodimers from DNA. In our structure model, Lys^{201}, Trp^{234}, Arg^{238} and Lys^{239} are located in the amino terminal face of the barrel. They are on the same side of the DNA binding domain. Conceivably, the structural changes in the region between α0 and β-strand 1 and of the loop between helix 1 and β-strand 2 could directly or indirectly affect the interaction of DNA binding domain with DNA. The detailed molecular mechanism affecting the transcriptional activity of TR however, would require further analysis.

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