Review

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New Insights into the Structure and Function of the Thyroid Hormone Receptor

Key Words

Thyroid hormones · Nuclear receptors · Transcription · Gene regulation · DNA binding protein

Introduction

The importance of the thyroid gland in body functions was recognized as early as 1827 [84]. In the late 19th century, it was found that thyroidectomy led to myxedema which was relieved by the injection of a glycerin extract of thyroid [48]. Loss of thyroid function was also associated with cretinism [57]. The active components of the gland were subsequently identified as thyroxine (T_4) by Harrington [26] and 3,3′,5-triiodo-*L*-thyronine (T_3) by Gross and Pitt-Rivers [24] (fig. 1). T_4 is more abundantly synthesized by the thyroid gland, but T_3 is more potent and more rapid in onset of action than T_4 [23–25]. The major source of T_3 is T_4 monodeiodination in peripheral tissues [9].

Thyroid hormone has diverse biological activities. In mammals, including humans, thyroid hormone is essential for normal growth, differentiation and development. Lack of thyroid hormone during neonatal stages leads to irrevocable defects in neuronal development [15]. Thyroid hormone also controls the metabolic activity of many tissues through actions in the metabolism of carbohydrates, proteins, lipids, vitamins, nucleic acids and ions [27]. Furthermore, thyroid hormone is also known to increase oxygen consumption and heat production in thissues except brain, spleen, gonadal tissue, lymph node, thymus and dermis [2].

The mechanisms by which thyroid hormone induces all these effects are not completely understood. The biological activities could result from direct and/or indirect effects and could be mediated by a change in the synthesis of proteins which control the T_3-induced cellular functions. Indeed, T_3 is known to positively and negatively regulate the synthesis of proteins. Notable examples of the former are pituitary growth hormone, Na^+,K^+-ATPase subunits, α-myosin heavy chain and proteins involved in lipogenesis such as malic enzyme and spot 14. Well-known examples of the latter group are thyrotropin and the β-myosin heavy chain. T_3 affects the synthesis of these proteins at the transcriptional level [34, 52] which had been shown to be mediated by the interaction of thyroid hormone nuclear receptor proteins (TRs) with thyroid hormone response elements (TREs) which are defined DNA sequences in the promoter regions of these genes. The characterization of the interaction of the TREs with TRs has recently been reviewed [17, 50].

T_3 and T_4 were also found to have effects which do not require protein synthesis. One notable example is the increase in the uptake of amino acids and carbohydrate in thymocytes, embryonic bone cells and brain tissues [20, 21, 71, 72]. In contrast to the effects mentioned above, which usually have a lag time, the response in the transport of amino acids and carbohydrate is rapid.

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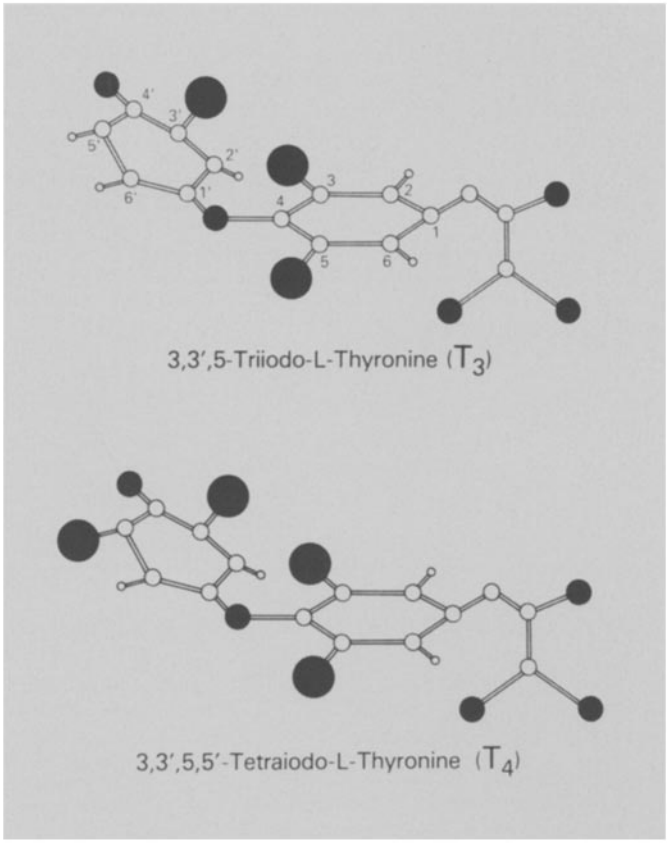

Fig. 1. The chemical structures of 3,3′,5-triiodo-*L*-thyronine (T_3) and 3,3′,5,5′-tetraiodo-*L*-thyronine (T_4). The positions of carbon atoms of the phenyl (inner) ring and phenoxy (outer) ring are marked.

This diversity in thyroid hormone action led investigators to propose that thyroid hormone action was initiated at multiple cellular sites. Indeed, in addition to the nuclear receptors, T_3-binding proteins have been detected in plasma membranes [45], mitochondria [76], cytosol [6] and endoplasmic reticulum [7]. However, the cloning of cDNAs encoding c-*erbA*s in 1986 has dramatically shifted the focus in the studies directed at understanding the mechanism of thyroid hormone action [64, 83]. Several articles have reviewed the biochemical and genetic evidence indicating that c-*erbA* proteins are indeed the thyroid hormone receptors [35, 54, 70]. This article will provide an up-to-date overview of the molecular diversity of the receptors, their multilevel regulation and their differential functions. The major focus, however, will be on the most recent developments regarding the structural and functional aspects of human TR subtype β1 (h-TRβ1).

Molecular Diversity of TRs

Using isotopic labeling, TRs were first identified in the nuclei of rat liver and kidney [53]. Subsequent studies have shown that TRs are present in many other mammalian tissues and cultured cells [55, 56, 63]. Rat pituitary tissue has the highest abundance of TR (10,000 molecules per cell) followed by liver (5,000 molecules per cell). It was also present in kidney and heart, but at much lower levels, and none was detected in adult rat testes [55]. Among cultured cells, pituitary growth hormone (GH)-producing cell lines have the most TR with about 15,000–20,000 molecules per cell [63]. Recently, high amounts of TR were found in several poorly differentiated human hepatoma cell lines [41]. However, the T_3-binding affinity is similar in all tissues and cultured cells and is in the range 10^{10}–10^{11} M^{-1}. Observed variations in the binding affinity are most likely due to the different conditions used in the in vitro binding assays. Using nuclear extracts of rat liver, the analog binding specificity has been extensively analyzed [32]. The affinity of these analogs for the TRs correlated well with their thyromimetic properties [32]. Before the genes for TRs were cloned, this tight correlation provided strong evidence that TRs mediated most, if not all, of the biological activity of T_3.

Using the TRs solubilized from the nuclei of GH_3 cells and various tissues, Samuels et al. [63] and others [1] estimated the molecular weight of TR to be the range of 47,000–57,000. The receptor has a sedimentation coefficient of 3.8 S and Stokes' radius of 3.3 nm. Its half-life is about 4.5 h and its synthetic rate is about 2,000 molecules/h/cell [63]. The indication that there could be multiple forms of TR came from photoaffinity labeling studies [59]. Two receptor forms with molecular weights of 57,000 and 47,000 were cross-linked to a T_3 analog (N-2-diazo-3,3,3-trifluoropropionyl-*L*-T_3) upon photoirradiation of the nuclear extracts of GH_1 cells. These two forms were not generated by indiscriminate proteolysis, UV peptide cleavage or protein-protein cross-linking. Furthermore, each form had a different half-life [5]. These observations were subsequently confirmed by cDNA cloning of TRs (see below).

Many attempts have been made to purify TR from tissues so that its structure could be studied in relationship to its complex biological functions. However, due to its low abundance and instability, the purification of TR has proven to be an extremely difficult task. The most highly purified preparation was only 4.2–4.9% TR with a yield of 6.4–14.7 μg from 4–5 kg of livers [29]. This difficulty impeded the progress in using the receptor protein se-

quence or anti-TR antibodies as a tool to clone the genes for TR. A breakthrough was achieved in 1986 when two groups simultaneously reported that the cellular homologs of v-*erbA* encode TRs [64, 83].

Molecular Cloning of Multiple Forms of TRs

The discovery that c-*erbA* proteins are TRs came from the realization that there is extensive sequence homology between v-*erbA* and steroid hormone receptors. v-*erbA* is an avian oncogene which by itself does not cause tumors, but enhances the transforming potential of the primary oncogene v-*erbB*. The finding that v-*erbA* and steroid hormone receptors shared significant sequence homology suggested that v-*erbA* and related molecules might exert their transcriptional regulatory effects in a manner similar to that of steroid hormone receptors.

Steroid hormone receptors are nuclear proteins which regulate target gene expression via DNA-receptor interaction. Extensive biochemical evidence had supported the hypothesis that the biological activity of T_3 was mediated by the gene-regulating activity of TRs. This similar mode of action led investigators to examine the possibility that v-*erbA* or a closely related protein could be a TR. Using v-*erbA* gene fragments as probes, a chicken-*erbA* (c-*erbA*) and a human-*erbA* (h-*erbA*) cDNA were isolated from a chicken embryonic [64] and human placental cDNA library [83], respectively. c-*erbA* has an open reading frame of 1,224 nucleotides and encodes a protein of 408 amino acids (M_r approximately 46,000). h-*erbA* has an open reading frame of 1,368 nucleotides and encodes a protein of 456 amino acids (M_r approximately 52,000). The proteins synthesized by in vitro transcription/translation of the two *erbA*s bound T_3 with K_d values of 0.05–0.2 n*M*. The K_d values as well as the rank order in the binding of T_3 and its analogs are similar to those determined for TR extracted from tissues and cultured cells [64, 83]. c-*erbA* is closer to v-*erbA* and is designated as the α form of TR. The TR form with a longer amino terminus, is designated TRβ. TRα and TRβ genes are localized on chromosomes 17 and 3, respectively [64, 83].

The finding that cellular homologs of v-*erbA* are TRs led to a flurry of activity in searching for TRs in other species. This resulted in the discovery of TRα and TRβ variants (fig. 2). By analogy with the steroid hormone receptors, four domains can be assigned to the TR isoforms: A/B, C, D and E. Sequence analysis indicates that there is a high degree of homology in domains C (86%), D (72%) and E (82%). However, very little or no sequence

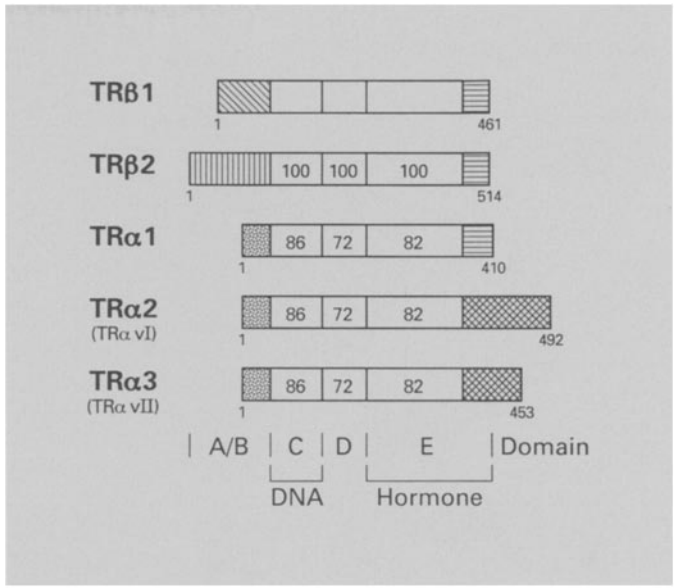

Fig. 2. TR isoforms and related proteins in the rat. The number of amino acids is deduced from the cDNA sequences. Different shadings represent totally dissimilar sequences. The extent (%) of sequences homology in domains C, D and E and the DNA- and hormone-binding domain are indicated.

similarity exists in domain A/B between the α and β form of *erbA*. In rat and human, the sequences of TRα2 (TRαvI) and TRα3 (TRαvII) are identical to that of TRα1 up to amino acid 370 [47, 49] and the remainder of the sequence bears no resemblance to the C-terminal sequence of TRα1. Variants TRα2 and TRα3 were derived from alternative splicing of the primary transcripts of the TRα gene. Unlike TRα1, however, these TRα variants do not bind T_3. A splicing variant of the TRβ gene was isolated from the pituitary of rat [28] and human [12]. In contrast to the alternative splicing event in TRα, TRβ2 differs from TRβ1 at the amino terminus (fig. 2). Because the hormone-binding domain in TRβ2 is identical to that of TRβ1, TRβ2 binds T_3 as well as TRβ1. In addition to human and rat, to date, the TRα and TRβ subtypes have also been found in chicken [74] and frog [85].

Differential Tissue Expression of TRα and TRβ Genes

The discovery that there are multiple forms of TRs poses a challenging question. Do the α and β subtypes of TR each mediate distinctive T_3 functions? This possibility could provide a viable molecular explanation for the diverse actions of T_3. To address this question, the tissue

Table 1. Tissue levels of TR mRNAs and T_3 binding capacity in whole nuclei (B_{max})

	α_1 fmol/mg DNA	α_2 fmol/mg DNA	β_1 fmol/mg DNA	B_{max} ng T_3/mg DNA	$B_{max}/(\alpha 1+\beta 1)$
Liver	0.23±0.05	0.25±0.06	1.06±0.4	0.66±0.05	0.51
Cerebrum	3.4±0.47	31.0±2.1	4.2±0.28	0.43±0.02	0.06
Cerebellum	2.43±0.15	15.7±1.9	0.17±0.04	0.12±0.03	0.05
Kidney	0.52±0.11	2.4±0.8	2.8±0.87	0.16±0.02	0.05
Heart	0.56±0.12	1.29±0.16	1.53±0.39	0.38±0.04	0.18
Spleen	0.035±0.013	0.054±0.015	0.034±0.01	0.018	0.26
Testis	0	1.3±0.26	0	0.0023	–

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differential and developmental expression of TRs were studied by several laboratories. Earlier examination of TR tissue expression was mainly at the mRNA level. Most tissues examined expressed α1, α2 and β1 mRNA but in variable proportions [77, 78]. Table 1 shows that expression of TRβ1 mRNA is highest in the cerebrum followed by kidney, heart and liver, whereas the highest expression of TRα1 mRNA is in the cerebrum and cerebellum, while it is low in liver. In contrast, none of the TR mRNAs are abundantly expressed in the spleen. No TRα or TRβ was detected in the testis. Only a low amount of α2 mRNA was seen in the testis. This could explain the results of earlier work in which no T_3-binding activity was detected in testis [55].

However, the mRNA expression level may not reflect the amounts of functional TRs at the protein level. Additional posttranscriptional regulation could occur which may be subtype- and/or tissue-specific. The need for assessing the expression level of TR proteins led to the development of subtype-specific antibodies which made it possible to evaluate the tissue distribution of TR proteins [39, 62, 69, 77]. Various ratios of TRα1- and TRβ1-specific T_3-binding activity was found in tissue extracts (table 1), and marked variations in specific T_3-binding activity/mRNA ratios were detected. The ratio in cerebrum is only 1/10 of that in liver in spite of the fact that the combined mRNA value of TRα1 and TRβ1 in cerebrum is nearly 6-fold higher than that of liver. This nonlinearity is also prominent in cerebellum, heart, kidney and spleen to various degrees. If T_3-binding capacity truly reflects the level of the expressed receptors, this indicates that there are translational and/or posttranscriptional processes which are tissue dependent.

TRβ2 was first reported to be expressed only in pituitary [28]. Subsequently, however, using the polymerase chain reaction, TRβ2 mRNA was found to be widely distributed throughout the brain [11]. This was also confirmed by immunohistochemical localization [37]. Recently, T_3-binding activity which is immunoreactive to TRβ2 was also found to be present in the nuclear extracts of liver, kidney, brain and heart. Even though TRβ2-associated binding activity only accounts for 10–20% of total T_3-binding capacity, the protein/mRNA ratio is unexpectedly high as compared to those found for TRα1, TRα2 and TRβ1 [69]. The possibility exists that TRβ2 protein is very effectively transcribed or the protein is more stable than other TRs. These recent studies indicate that all four isoforms are expressed in tissues in variable amounts. The mechanism and functional consequences of the differential expression will require further study.

Isoform-Specific Functional Roles of TRs

The differential expression suggests that TR isoforms may have distinctive functional roles. To test this hypothesis, the temporal expression of TR isoforms was examined during embryonic development of rat brain [13, 78] and liver [13, 62, 78] and during amphibian metamorphosis [73]. The TRα gene is expressed substantially earlier during embryonic development than TRβ. In the fetal rat brain, the expression of TRα1 was clearly detectable on the 14th day of gestation and continued to be expressed during gestation and after birth. In contrast, TRβ1 expression was virtually nondetectable until day 17, after which a surge (a 40-fold increase) occurred between the 17-day fetus and the 10-day-old neonate. Similar subtype-specific

developmental expression was also seen in the liver in that TRα1 was predominantly expressed in the early fetus and TRβ1 rose only later in gestation and then remained constant.

The stage of development at which a sharp rise in TRβ1 expression in the rat brain occurs corresponds to the time when the T_3 level is known to rise. T_3 is known to affect the development of the central nervous system. This suggests that TRβ1 could mediate specialized functions in brain development during this critical period. On the other hand, the constant presence of TRα1 during the entire period of fetal gestation suggests that this isoform may be needed for overall fetal development. This hypothesis was further supported by a similar finding in developing tadpoles. TRα genes are highly expressed throughout tadpole development, whereas TRβ mRNA levels remain low until the beginning of prometamorphosis. A surge of TRβ gene expression coincides with the increase of endogenous thyroid hormonel level. TRβ gene expression declines toward the end of metamorphosis [73].

A more definitive role for the TRβ gene in neural differentiation was demonstrated by using a stable transfected neuronal cell line (Neuro-2a) which overexpressed TRβ1 [36]. When these cells are treated with T_3, cells arrest in G_0/G_1 and exhibit morphological and functional characteristics indicative of neural differentiation. In contrast, T_3 had no effect on the differentiation of Neuro-2a cells that overexpress TRα1. These results strongly support a role for TRβ1 in neural differentiation [36]. Recently, this distinctive role was further supported by studies using human hepatoma cells. Lin et al. [41] reported that TRβ1 is overexpressed in the least differentiated human hepatoma cell lines, whereas TRα1 is expressed at a much lower level irrespective of the differentiation state. The expression of h-TRβ1 was probably turned off when cells were well differentiated.

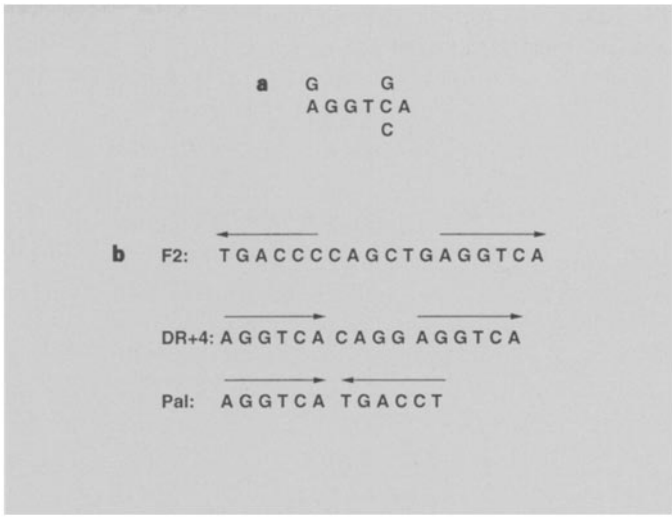

Fig. 3. The DNA sequence (**a**) and arrangement (**b**) of the TRE half-site binding motifs.

Structure and Transcriptional Activity

Interaction of the DNA-Binding Domain with DNA

The assignment of the domains in TRs was based on sequence homology to steroid hormone receptors and their structure has not yet been determined. However, the structures of the DNA-binding domain of the glucocorticoid and estrogen receptors have been determined by X-ray crystallography [42, 68]. Since the sequence homology between the DNA-binding domain of TRs and those of glucocorticoid and estrogen receptors is very high, it is reasonable to assume that their structures are basically identical. The DNA-binding domain is globular and contains two Zn-binding subdomains with distinct conformation and function. In the presence of DNA, the receptor dimerizes, placing the first finger of the DNA-binding domain of each monomer in direct contact with the DNA major groove. The dimer is stabilized by direct interaction of the second zinc fingers of each monomer [42, 68].

While the basic mode of interaction of the DNA-binding domain of TRs with DNA could be similar to that of steroid hormone receptors, the detailed molecular interaction is expected to be different. Glucocorticoid receptor and estrogen receptor form only homodimers and bind to DNA with a palindromic sequence of AGAACA and AGGTCA, respectively. TRs, on the other hand, bind to TREs which exhibit remarkable variability. The consensus sequence of the half-site binding motif, (A/G)GGT(G/C/A)A (fig. 3a) [33, 70], can be arranged as a direct repeat, as an inverted repeat or as an everted repeat (fig. 3b). The spacing between the two half-sites also varies. Furthermore, TRs not only form homodimers in binding to the TREs but also form heterodimers with the vitamin D receptor (VDR) [66, 67], all subtypes of the retinoic acid receptor (RAR-α, RAR-β and RAR-γ), retinoid X receptor (RXR-α, RXR-β and RXR-γ) [86], and also with other thyroid hormone receptor auxiliary proteins [80].

The ability of TRs to recognize the consensus sequence of the half-site binding motifs in different arrangements

provides a means to achieve diversity and selectivity in the recognition of T_3 target genes. Recent studies have indicated that the binding of TRs with TREs is TR subtype-, TRE- and T_3-dependent. TRβ1 binds to the TREs mainly as a homodimer in the following order: F2 (chicken lysozyme gene TRE, an everted repeat) >DR+4 (a direct repeat separated by 4 gaps) >Pal (an inverted repeat) (fig. 3b) [58]. TRα1 interacts with these TREs differently. TRα1 binds to DR+4, Pal and Lap (an everted repeat) weakly as a monomer but strongly in the homodimeric form [75]. Additional modulation of TR interaction with TREs is achieved by the binding of T_3. Binding of TRs to T_3 weakens the interaction of TRs to TREs. The extent of inhibition by T_3 is TRE dependent. For TRβ1, the inhibitory effect by T_3 is greater for F2 than for DR+4 [46].

The interaction of TRs with DNA is further modulated by the formation of heterodimers with thyroid hormone receptor auxiliary proteins. Heterodimerization has a dramatic effect on the interaction of TRs with TREs and increases the binding of TRs to TREs [61]. The enhancement is TRE and heterodimer partner dependent. So far, the best thyroid hormone receptor auxiliary proteins identified are the three RAR forms (α, β and γ), and the three RXR forms (α, β and γ). However, the RAR·TR heterodimer is less stable than RXR·TR [82, 86].

RXRβ·TRβ1 binds to TREs in the following order: F2 > DR+4 > Pal [58]. The molecular basis for this selectivity is not yet clear, but there are indications that the interaction of the DNA-binding domain of the RXR·TR heterodimer with TREs is modulated by other regions of the TR molecule [33, 60]. A TR has two other dimerization subdomains. One is a discrete, yet-to-be-defined region within the ligand-binding domain and the other overlaps with the DNA-binding domain [33]. RXR·TR binds to TREs which have the half binding motif in three different orientations with differential affinities. It is reasonable to assume that the dimerization contact sites would vary depending on the orientation of the half-site binding motif. Indeed, recent mutational analyses have indicated that binding of RXR·TR to DR+4 places RXR in the 5′ position of the direct repeat [33, 60]. This resultant polarity was postulated to be a consequence of a cooperative interaction of a loop (DR box) near the first finger of the TR DNA-binding domain with the second zinc finger of the RXR DNA-binding domain [60]. A short sequence (the A box) that is located carboxyl-terminal to the conserved DNA-binding domain could also be critically important for the resultant polarization [33]. Binding to a palindromic response element would require a rotation of approximately 180° of the DNA-binding domain with respect to its carboxyl-terminal dimerization interface. Therefore, the DR or A box of the TR would be aligned differently with the contact sites of the RXR as compared to a direct repeat. This flexibility in the DNA-binding properties of RXR·TR makes it possible to interact with an array of TREs. The differential affinity in the binding imparts selectivity and specificity in the binding of TR to the target genes.

It was recently shown that a more intricate regulation of TR action emerges when TR complexes with the VDR [67]. The polarity of the VDR·TR heterodimer is dictated by the sequence, spacing and arrangement of the half-site binding motifs. Thus, on the promoter of the rat 9K calbindin gene where the response element is a direct repeat separated by three gaps, (DR+3), the polarity is 5′-TR·VDR-3′, whereas in the promoter of the rat 28K calbindin gene which has a DR+4 as the response element, the polarity is reversed to 5′-VDR·TR-3′. The VDR·TR heterodimer can be activated by either ligand. Remarkably, ligand sensitivity is dependent on the polarity of the receptors. The receptor at the 3′ position is activated by a low concentration of its cognate ligand. A 10-fold higher concentration is required to activate the receptor at the 5′ position. Therefore, an additional regulation of the transcriptional activity is achieved via this polarity-dependent ligand sensitivity.

Structure and Function of the Hormone-Binding Domain

The hormonal signal must be transduced from the hormone-binding domain to the DNA-binding domain to activate or repress T_3-dependent target genes. To understand how the signal is transmitted, it is necessary to understand the structure of the hormone-binding domain.

Domain D Is Essential for Thyroid Hormone Binding of the TR

Unlike the DNA-binding domain for which the X-ray crystallographic structure is known for two members of the steroid hormone/RA receptor superfamily (glucocorticoid and estrogen receptors), the structure of none of the hormone-binding domains in the superfamily is known. As a first step to understanding the structure, Lin et al. [38] defined the hormone-binding-domain boundary of h-TRβ1 by deletion analysis. Surprisingly, the predicted hormone-binding domain (Lys^{235}–Asp^{456}, KD25) alone does not bind T_3 (fig. 4). An extension of the amino-termi-

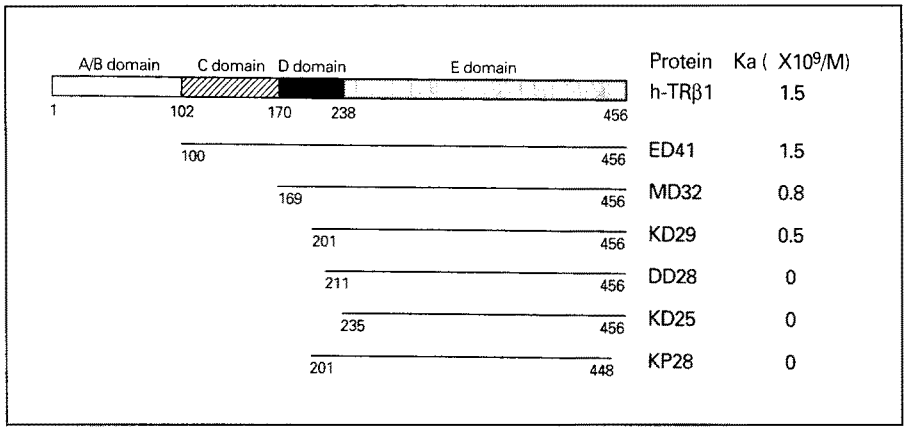

Fig. 4. Schematic representation of various deletion mutants of h-TRβ1. The full-length h-TRβ1 consists of 456 amino acids according to Weinberger et al. [83]. Domain assignment is based on Green and Chambon [22]. The K_as on the binding of T_3 to intact and truncated h-TRβ1 were determined by Lin et al. [38]. Adapted with permission from McPhie et al. [44] (copyright American Chemical Society, 1993).

nal sequence of domain E to include part of domain D (Lys^{201}–Asp^{235}) completes the structure of the hormone-binding domain and KD29 (Lys^{201}–Asp^{456}) can function as a T_3 binder. KD29 binds to T_3 analogs with the same order as intact TRβ1 (3,3′,5-triiodo-*L*-thyropropionic acid > *L*-T_3 > *D*-T_3 > *L*-T_4 > 3′,5′,3-triiodo-*L*-thyronine). However, even though KD29 has the required minimal structure to function as a T_3 binder, its T_3-binding affinity is one-third of that of the wild type. This indicates that additional structural elements in the D domain help stabilize the minimal T_3-binding domain. Indeed, a further extension of the amino-terminal sequence to include Met^{169}–Gln^{200} (MD32) led to an increase of T_3-binding affinity by nearly 2-fold (fig. 4). Inclusion of the DNA-binding domain (ED41) further stabilizes the minimal T_3-binding domain to the level of the intact h-TRβ1. This suggests that domains D and E are not independent but are structurally 'linked'. On the other hand, deletion of domain A/B has no effect on the T_3-binding activity of the hormone-binding domain [38].

The findings that the predicted hormone-binding domain requires domain D to function as a hormone binder prompted us to examine the structural requirement at the carboxyl terminus. Secondary-structure analysis predicted that the structure of the last eight amino acids is a helix. Deletion of this helix (KP28) resulted in the complete loss of T_3-binding activity. Therefore, the functional hormone-binding domain of h-TRβ1 starts at Lys^{201} and ends at Asp^{456} [38].

The Proposed Structure of the Hormone-Binding Domain of h-TRβ1 Is an α/β Barrel

The structures of the purified truncated h-TRβ1 fragments shown in figure 4 were analyzed by circular dichroism [44]. Figure 5 shows the far-ultraviolet CD spectra of

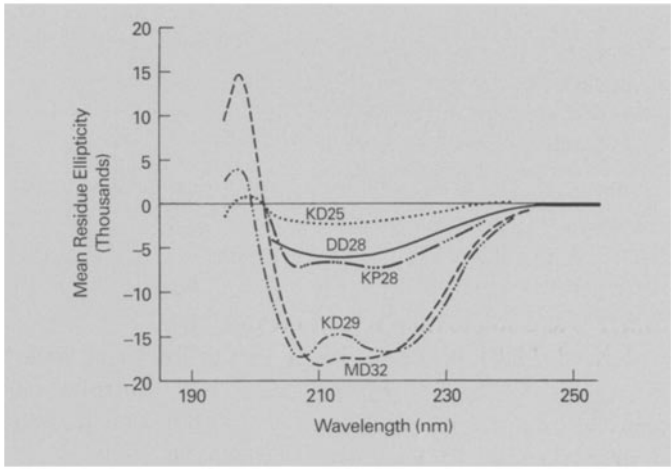

Fig. 5. Ultraviolet circular dichronism spectra of truncated fragments of h-TRβ1. Adapted with permission from McPhie et al. [44] (copyright American Chemical Society, 1993).

the active fragments MD32 and KD29 and the inactive fragments of KP28, DD28 and KD25. MD32 and KD29 both have intense spectra, with minima at 222 and 208–210 nm, respectively, and maxima around 190–195 nm, characteristic of α-helical structures. The spectra of KP28, DD 28 and KD25 were greatly reduced in intensity, with a minimum around 210–215 nm, a wavelength usually assigned to β-sheet structures. Their intrinsic spectra from circular dichroism were analyzed, and the results indicated that the fragment MD32 (all of domains D and E) contains extensive regions of α helix and β sheet.

The effect of temperature was used to probe further the structure of the truncated proteins [44]. Increasing temperature produced cooperative sigmoidal thermal transitions in both MD32 and KD29 with extensive loss of sec-

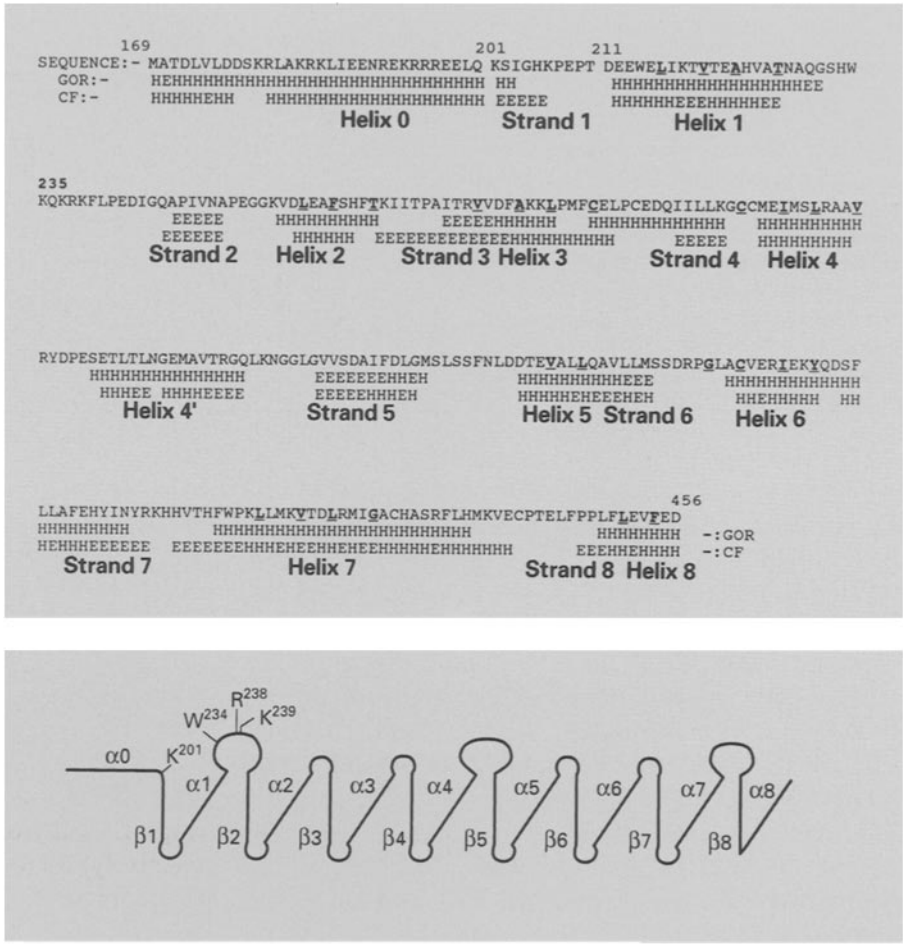

Fig. 6. a Secondary-structure predictions for domains D and E of h-TRβ1. The approximate locations of the predicted structural elements are shown in the bottom lines. Residues are predicted to be helical (H) or in an extended sheet (E). Secondary-structure predictions were made using the programs of Garnier et al. [16] and Chou and Fasman [10] which are abbreviated as GOR and CF, respectively. Adapted with permission from McPhie et al. [44] (copyright American Chemical Society, 1993). **b** The location of T_3-induced proteolytic-resistant sites. With permission from Bhat et al. [4].

ondary structure (T_m = 40 °C in each case). This shows the highly cooperative nature in the structure of MD32 and KD29, typical of a native globular protein. In contrast, neither DD28 nor KD25 showed sigmoidal thermal-unfolding curves, consistent with a lack of cooperative structural interactions in these proteins. Likewise, removal of the eight carboxyl-terminal amino acids led to a disruption of the globular structure [44].

Extensive analysis of sequences from the known TRs indicated that there are alternating stretches of α helix and β strand in domains D and E (fig. 6a). This is consistent with the structure of MD32 determined by circular dichroism. On the basis of these sequence and secondary-structure analyses, an α/β-barrel structure was proposed for the hormone-binding domain of h-TRβ1 (fig. 7). An α/β barrel has eight alternating α helix and β strands [14]. Each β strand is connected to an α helix. Thus, the eight β strands form the inner surface of the globular protein which is surrounded by α helices on the surface. This structural motif is called a barrel, and is found in a large family of enzymes. The cross section of the barrel is usually elliptical rather than circular. In addition, enzymes with an α/β-barrel domain frequently have other structural elements (α helices, β strands or an entire domain) which precede, interrupt or follow a barrel [14]. The structure proposed for the hormone-binding domain also contains a segment of extra helix (helix 4′; loop 4) which interrupts the barrel (fig. 6, 7). The T_3-binding site is proposed to be in the β barrel [44; and see below].

This model awaits verification by X-ray crystallographic analysis. However, it is supported by the known X-ray crystallographic structure of a plasma thyroid hormone transport protein, transthyretin. The T_3-binding site of transthyretin is located in a β barrel [51]. Furthermore, the T_3-binding site is also modeled as a β barrel in thyroxine-binding globulin [81]. This model would lead to a simplified structure of h-TRβ1, in that h-TRβ1 consists of two functional domains: the DNA-binding domain of

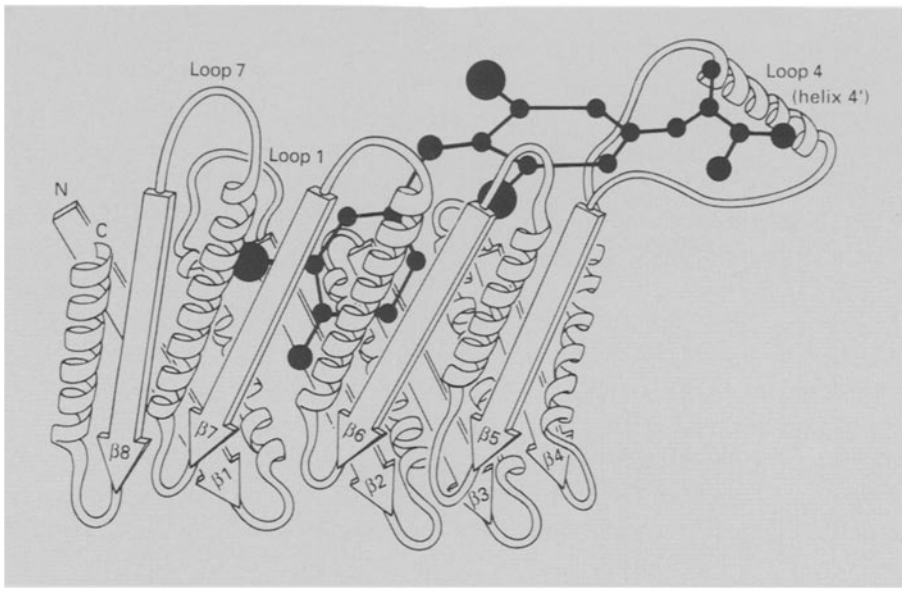

Fig. 7. Schematic representation of the suggested structure of the T_3-bound hormone-binding domain of h-TRβ1. The number of β strands is marked. The corresponding helices follow the β strands. The amino and carboxyl termini are denoted by N and C, respectively. The dimension of the β barrel is unknown at the present time, therefore, the relative size of T_3 and the β barrel are not necessarily in scale. Adapted with permission from Cheng et al. [8] (copyright American Chemical Society, 1994).

known structure (residues 102–169), linked by an α helix to a hormone-binding domain (residues 201–456) with an α/β-barrel structure.

Thyroid-Hormone-Binding Site of h-TRβ1

Elucidation of the nature of the T_3-binding site in the hormone-binding domain will certainly lead to an understanding of how T_3 modulates the transcriptional activity of TRs. To this end, attempts have been made to solve the structure of the hormone-binding domain by X-ray crystallography [43]. A T_3-binding fragment of rat TRα1 consisting of residues 122–410 was expressed and purified from *Escherichia coli.* This fragment encompasses the entire domain D and E. It binds to T_3 with an affinity similar to that of intact r-TRα1 (K_d = 0.06 n*M*) [43]. Crystals of this hormone-binding domain bound to T_3 were obtained. However, no information is as yet available on the structure due to the instability of the crystals and insufficient resolution of X-ray diffraction [43].

Biochemical methods have been used to map the T_3-binding site. Binding of T_3 and its analogs to the naturally occurring, mutated h-TRβ1 were characterized by Cheng et al. [8]. The mutant receptors were derived from patients with the syndrome of generalized thyroid hormone resistance and each had a point mutation in the hormone-binding domain (KT: R333W; TP: L445H; IR: D317H; NN: G342E; AH: P448H; OK: M437V; RL: F454C, and ED: A312T). The analogs were chosen so that the likely interaction sites of the hormone with the β barrel could be probed. A change of the steric orientation of the alanine side chain (*D*-T_3) as well as changes of location of iodine atoms on the phenyl and phenoxy rings of the thyronine rings (*L*-T_4 thyroxine and 3,3′,5′-triiodo-*L*-thyronine, respectively) led to a reduction in the binding to the mutant receptors (see fig. 1). The affinities were lower than those of wild-type h-TRβ1. On the basis of detailed analyses of the binding data and the structure of the hormone-binding domain as an eight-stranded α/β barrel, a model of the T_3-binding site was proposed [8]. The phenyl ring lies across the amino-terminal face of the hormone-binding domain with the phenoxy ring pointing downward into the barrel interacting with β strand 8 in the opposite side (fig. 7). The alanine side chain of the hormone forms an ionic bond with loop 4 of the α/β barrel. Loops 1 and 7 of the hormone-binding domain, which are located on the same side as the DNA-binding domain, fold over the top of the bound hormone [8].

Structural Changes Induced by the Binding of Thyroid Hormone

Before the cloning of the cDNA for TRs, Ichikawa and DeGroot [30] found that the binding capacity of the TR solubilized from rat liver nuclei was protected from tryptic digestion if T_3 is bound to the receptor. It was suggested that the protective effect was due to changes of structure induced by T_3. Recently, a more detailed analysis of the T_3-induced conformational changes was reported by Bhat et al. [4]. Using the purified recombinant TRβ1 and

partial proteolysis, T_3 was found to render h-TRβ1 less susceptible to tryptic and chymotryptic digestion. The proteolytic sites which are protected from proteolysis are Lys^{201}, Arg^{238}, Lys^{239} and Trp^{234} (fig. 6b). On the basis of the putative structure of the hormone-binding domain as an eight-stranded α/β barrel, Lys^{201} is located at the beginning of β strand 1 and Arg^{238}, Lys^{239} and Trp^{234} are located in loop 1 which is between helix 1 and β strand 2 (fig. 6, 7). These four sites are all on the amino-terminal face of the β barrel and on the same side of the DNA-binding domain. Loop 1 was proposed to fold over the bound hormone. Conceivably, the structural changes in the region between α0 and β strand 1 and loop 1 could directly or indirectly affect the interaction of the DNA-binding domain with DNA. This could in turn affect the transcriptional activity of h-TRβ1. However, the molecular details of T_3-induced changes in the interaction of DNA and the DNA-binding domain remain to be resolved.

Regulation of the Transcriptional Activity of TRs

To understand the regulation of the transcriptional activity of TRs, most studies have focused on analyses of the specificity and differential activity of various TREs. Since the discovery that RXRs are major heterodimerization partners, another level of transcriptional regulation was added to the complexity. These two areas were discussed above. Recently, a new dimension in the regulation of TR transcription was discovered. Phosphorylation was found to play a pivotal role in the transcriptional activity of TRs [3, 31, 40, 79].

For h-TRβ1, phosphorylation was first reported to increase its binding to TREs by Lin et al. [40], whose findings were subsequently confirmed [3, 79]. Phosphorylation increases the binding of F2, DR+4, Pal and rat α-myosin heavy-chain gene TRE (a direct repeat) to h-TRβ1 as a homodimer [3, 79]. The degree of enhancement depends on the orientation of the half-site binding motifs. The degree of increase by phosphorylation is higher for F2 and DR+4 than for Pal [3]. However, consistent with the findings that binding of T_3 to h-TRβ1 is not affected by phosphorylation [40], the T_3-induced dissociation of homodimers is also not altered by phosphorylation [79].

The effect of phosphorylation on heterodimerization with RXR, however, is controversial. Sugawara et al. [79] found that the heterodimerization of h-TRβ1 with RXR or thyroid hormone receptor auxiliary proteins isolated from rat liver was not affected by phosphorylation. In contrast, Bhat et al. [3] observed a dramatic increase in the dimerization of h-TRβ1 with RXR. The increase was found to be about 10-, 7- and 6-fold for F2, DR+4 and Pal, respectively. The reasons for the discrepancy are not entirely clear. They could be due to different preparations of RXR and/or TRβ1 used by the two groups. The RXR used by Sugawara et al. [79] was prepared by the in vitro transcription/translation method, whereas the RXRβ used by Bhat et al. [3] was from nuclear extracts of Sf9 cells infected with RXRβ-containing virus. It is possible that posttranslation modification of the RXR could play a role in its interaction with TRβ1. The TRβ1 used by the two groups could also differ [3, 79]. Bhat et al. [3] purified h-TRβ1 from the inclusion bodies of *E. coli.* The method of preparation of h-TRβ1 used by Sugawara et al. [79] has not been published. This discrepancy should be resolved in future studies.

The functional significance of h-TRβ1 phosphorylation was further demonstrated by transient transfection experiments [3, 40]. The in vivo phosphorylation of h-TRβ1 is increased in a concentration-dependent manner by treating COS-1 or CV1 cells with a protein phosphatase inhibitor, okadaic acid [40]. Concomitant with the increase in h-TRβ1 phosphorylation, the transcriptional activity was also increased. Similar to in vitro DNA binding, the increase in the h-TRβ1-mediated transcriptional activity is TRE dependent. The increase was 1.7-, 2.3- and 3-fold for malic enzyme gene TRE (a direct repeat), Pal and F2, respectively [3]. Consistent with these results, Jones et al. [31] also reported a 6- to 10-fold and 11- to 45-fold increase of the transactivation activity mediated by F2 and DR+4, respectively, in the presence of okadaic acid. The differential increase probably reflects the different effects of phosphorylated residues on the dimerization interface due to the different orientation of domain C relative to the orientation of the half-site binding motifs. To evaluate whether heterodimerization of h-TRβ1 with RXRβ is modulated by phosphorylation, Bhat et al. [3] cotransfected h-TRβ1 and RXRβ into CV1 cells. They found that the RXRβ-modulated increase of h-TRβ1 transcriptional activity was further enhanced by okadaic acid, suggesting that heterodimerization is affected by phosphorylation. These in vivo results are consistent with the in vitro DNA binding observed by Bhat et al. [3].

The phosphorylation sites of h-TRβ1 remain to be identified. However, it is known that h-TRβ1 is phosphorylated at multiple sites [40, 79]. Serine is the major phosphorylated residue and threonine and tyrosine are minor participants [40]. It is important to point out that okadaic acid increases the level of phosphorylation of h-

TRβ1 by about 10-fold, whereas the increase in transactivation is only 1.7- to 3-fold. Therefore, phosphorylation of h-TRβ1 not only mediates the transactivation activity but is also involved in other yet-to-be-identified functions [3, 40].

Phosphorylation of TRs is not only limited to the β1 subtype: other isoforms were also found to be phosphorylated. c-TRα1 has been shown to be a phosphoprotein [18, 19]. Two of the phosphorylated sites have been identified. Ser^{12} was found to be phosphorylated by casein kinase II. It is uncertain which kinase(s) is responsible for phosphorylation of Ser^{28} as this was stimulated by activators of either protein kinase C or cAMP-dependent protein kinase. Since cAMP-dependent protein kinase phosphorylates c-TRα1 both in vitro and in vivo, it is possible that c-TRα1 is the direct substrate of cAMP-dependent protein kinase. These two phosphorylation sites are located in the A/B domain. Phosphorylation of these sites does not seem to change the affinity of DNA binding or affect nuclear localization. The functional consequences of phosphorylation of these sites remain to be elucidated.

Summary and Future Challenges

Following isolation of cDNA encoding TRs, much progress has been made in understanding their structure and transcriptional activity. Additional TR-mediated target genes are being identified and more variations in the consensus hexameric half-site sequences and the spacing are being discovered [65]. It has become increasingly clear that multilevel regulation modulates the transcriptional activity of TRs. The factors known so far to affect the transcriptional activities are the different types of TRE, thyroid hormone receptor auxiliary proteins, various heterodimer partners and phosphorylation.

However, there are many challenging problems to be solved. (1) So far, three subtypes of TRαs and two closely related receptor-like proteins (TRα2 and TRα3) are known. Does each TR subtype have a tissue-specific or differential function responsible for the diverse biological activity of T_3? If so, what is the molecular mechanism by which the specificity and selectivity are achieved? (2) Besides RXRs, are there other thyroid hormone receptor auxiliary proteins which function to modulate the activity of TRs? If so, what is the molecular nature of thyroid hormone receptor auxiliary proteins and by what mechanisms do they affect transcriptional activity? (3) What is the molecular basis of hormonal signal transduction? (4) How does TR produce negative regulation of its target gene? (5) Besides modulating the interaction of receptors with DNA, does phosphorylation of TRs play other functional roles? The answers to these questions will certainly lead us closer to understanding how T_3 orchestrates its pleiotropic effects on growth, differentiation and development. The information will ultimately help us understand the molecular basis of diseases due to abnormality of TRs.

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