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Molecular Characterization of Thyroid Hormone Receptors from the Leopard Geckos, and Their Differential Expression in the Skin

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Thyroid hormones (THs) play crucial roles in various developmental and physiological processes in vertebrates, including squamate reptiles. The effect of THs on shedding frequency is interesting in Squamata, since the effects on lizards are quite the reverse of those in snakes: injection of thyroxine increases shedding frequency in lizards, but decreases it in snakes. However, the mechanism underlying this differential effect remains unclear. To facilitate the investigation of the molecular mechanism of the physiological functions of THs in Squamata, their two specific receptor (TRα and TRβ) cDNAs, which are members of the nuclear hormone receptor superfamily, were cloned from a lizard, the leopard gecko, Eublepharis macularius. This is the first molecular cloning of thyroid hormone receptors (TRs) from reptiles. The deduced amino acid sequences showed high identity with those of other species, especially in the C and E/F domains, which are characteristic domains in nuclear hormone receptors. Expression analysis revealed that TRs were widely expressed in many tissues and organs, as in other animals. To analyze their role in the skin, temporal expression analysis was performed by RT-PCR, revealing that the two TRs had opposing expression patterns: TRα was expressed more strongly after than before skin shedding, whereas TRβ was expressed more strongly before than after skin shedding. This provides good evidence that THs play important roles in the skin, and that the roles of their two receptor isoforms are distinct from each other.

Key words: thyroid hormone receptor, TR, skin shedding, reptile, Squamata, leopard gecko

INTRODUCTION

The thyroid hormones (THs), thyroxine (T4) and triiodothyronine (T3), are pleiotropic factors important for many developmental and physiological processes in vertebrates. There has been a lot of research into the physiological significance of THs in various vertebrates. For example, THs are known to be important for inner ear and retina development, liver metabolism in mice (Flamant and Samarut, 2003), metamorphosis in axolotl and Xenopus (Nakajima et al., 2005; Sachs et al., 2000; Safi et al., 2004), and embryogenesis and metamorphosis in many teleost fish (Power et al., 2001).

In reptiles, THs have been suggested to affect tail regeneration (Turner and Tipton, 1971), metabolic rate and metabolic enzyme activity (John-Alder, 1990; John-Alder and Joos, 1991), and shedding frequency (Chiu et al., 1967; Chiu and Lynn, 1970). Above all, their effect on shedding frequency is particularly interesting. In lizards, the injection of thyroxine increases shedding frequency, and thyroidectomy decreases it (Chiu and Lynn, 1970). The mechanisms underlying these completely opposite phenomena have not been clarified, partly due to the lack of investigation of the molecular mechanism of THs in reptiles.

THs can regulate target genes by interacting with thyroid hormone receptors (TRs), which are members of the nuclear receptor superfamily. Two isoforms, TRα and TRβ, have been isolated from species of four classes of vertebrate, but not from reptiles (Forrest et al., 1990; Kawakami et al., 2003; Murray et al., 1988; Yaoita et al., 1990). These isoforms share high homology and have similar biochemical properties. However, they have distinct spatial and temporal expression profiles in overlapping patterns, suggesting that two genes mediate both individual and common biological functions.

Unlike other squamate animals, the leopard gecko, Eublepharis macularius, is easily maintained and bred in the laboratory. The leopard gecko is therefore expected to become an experimental model. Indeed, several molecular studies of the endocrine system have already been conducted on this species (Endo and Park, 2004; Endo and Park, 2005; Ikemoto and Park, 2003; Ikemoto et al., 2004; Kato et al., 2005; Valleley et al., 2001).

In this study, we cloned TRα and TRβ from the leopard gecko to augment investigations on the molecular mechanisms of the physiological functions of THs in reptiles. In

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addition to identifying two isoforms of TR, we performed phylogenetic and expression analyses. We also demonstrated the differential expression of the TR isoforms, and herein discuss their possible roles in shedding.

MATERIALS AND METHODS

Animals
The leopard geckos (Eublepharis macularius) were treated according to the guidelines of the Bioscience Committee at the University of Tokyo. The animals were provided meal worms, crickets, water, and powdered calcium supplement ad libitum. Animals were anesthetized with sodium pentobarbital and killed by rapid decapitation, followed by complete bloodletting. Tissues and organs were immediately dissected, frozen in liquid nitrogen, and stored at –80°C until use.

RNA preparation and cDNA synthesis
Total RNA was extracted using ISOGEN (NIPPON GENE, Tokyo, Japan). The cDNAs used as templates for RT-PCR were synthesized from 3 µg denatured total RNA using 5 µM oligo(dT) primer and 100 units of M-MLV Reverse Transcriptase (Promega, Tokyo, Japan). The cDNAs used as templates for RT-PCR were immediately dissected, frozen in liquid nitrogen, and stored at –80°C until use.

Comparative study
Comparison of the amino acid sequences of various TRs
The nucleotide sequences of the entire ORFs of the TRs from the leopard gecko and from several species representing all other vertebrate classes were aligned using CLUSTAL X with default settings. The alignment of the nucleotide sequences was used to generate a phylogenetic tree, using the neighbor-joining method (Saitou and Nei, 1987). Bootstrap values were calculated with 1000 replications to estimate the robustness of internal nodes. The GenBank accession numbers of TRs used in the phylogenetic analysis are as follows: Homo sapiens (human) TRα, M24748; Homo sapiens TRβ1, X04707; Mus musculus (mouse) TRα, MMCREBA1; Mus musculus TRβ1, S62756; Gallus gallus (chicken) TRα, Y00987; Gallus gallus (chicken) TRβ1, X51555; Oryctolagus cuniculus (rabbit) TRα, X51556; Oryctolagus cuniculus (rabbit) TRβ1, X51557; Canis lupus familiaris (dog) TRα, X51558; Canis lupus familiaris (dog) TRβ1, X51559; Mus musculus (mouse) TRγ1, X51560.

Molecular phylogenetic analysis
The nucleotide sequences of the entire ORFs of the TRs from the leopard gecko and from several species representing all other vertebrate classes were aligned using CLUSTAL X with default settings. The alignment of the nucleotide sequences was used to generate a phylogenetic tree, using the neighbor-joining method (Saitou and Nei, 1987). Bootstrap values were calculated with 1000 replications to estimate the robustness of internal nodes. The GenBank accession numbers of TRs used in the phylogenetic analysis are as follows: Homo sapiens (human) TRα, M24748; Homo sapiens TRβ1, X04707; Mus musculus (mouse) TRα, MMCREBA1; Mus musculus TRβ1, S62756; Gallus gallus (chicken) TRα, Y00987; Gallus gallus (chicken) TRβ1, X51555; Oryctolagus cuniculus (rabbit) TRα, X51556; Oryctolagus cuniculus (rabbit) TRβ1, X51557; Canis lupus familiaris (dog) TRα, X51558; Canis lupus familiaris (dog) TRβ1, X51559; Mus musculus (mouse) TRγ1, X51560.

Table 1. Oligonucleotide primers used for RACE, RT-PCR, and sequencing.

<table>
<thead>
<tr>
<th>Name</th>
<th>Nucleotide sequence</th>
<th>Usage</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRα</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SE01</td>
<td>GCCGCTGGAGGATCCCATTTCCGTG</td>
<td>Seqencing</td>
</tr>
<tr>
<td>SE02</td>
<td>ACCGNAAYCAGTYCAGYTS</td>
<td>Degenerate PCR</td>
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<tr>
<td>SE03</td>
<td>ATGCTGAAATCTTCTGACTGCG</td>
<td>Seqencing</td>
</tr>
<tr>
<td>SE04</td>
<td>CCTAGCAGCCGATGGGCTGATGC</td>
<td>3' -RACE</td>
</tr>
<tr>
<td>SE05</td>
<td>CCGACCTGCGATTTGGGCTTG</td>
<td>Seqencing</td>
</tr>
<tr>
<td>SE06</td>
<td>CCCACCTCATACACCTCGGACACAC</td>
<td>Seqencing</td>
</tr>
<tr>
<td>AS01</td>
<td>TTSGGCGCAGAAGTGGGAAAT</td>
<td>Degenerate PCR</td>
</tr>
<tr>
<td>AS02</td>
<td>CTTTGCTCCCACGCGGATGCGG</td>
<td>Seqencing</td>
</tr>
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</tr>
<tr>
<td>AS06</td>
<td>GGTGCCCAACCCCTATACACACGC</td>
<td>Seqencing</td>
</tr>
<tr>
<td>TRβ1</td>
<td></td>
<td></td>
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<tr>
<td>SE03</td>
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<td>3' -RACE</td>
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<td>Seqencing</td>
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<td>SE05</td>
<td>TGGGAGATGGCAGTCAGAGGGA</td>
<td>3' -RACE</td>
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<tr>
<td>SE06</td>
<td>GCCAACGACACTCCCTCCTGTG</td>
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</tr>
<tr>
<td>AS06</td>
<td>GATTTCTCAACGTCCAAATTTTCCA</td>
<td>Seqencing</td>
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</tbody>
</table>

Abbreviations for degenerate nucleotides: K, G or T; M, A or C; R, A or G; S, C or T; V, A or G; Y, C or T. N represents all four nucleotides.
Fig. 1. Nucleotide and deduced amino acid sequence of the cDNA encoding (A) TRα and (B) TRβ of the leopard gecko. Nucleotides (upper row) are numbered from 5' to 3', beginning with the initiator codon (ATG) in the coding region. Amino acid residues (lower row) are numbered beginning with the first Met residue in the ORF. The C and E/F domains are indicated by solid and dashed underlining, respectively. The D domain is between these two domains.
Fig. 2. Alignment of the predicted amino acid sequence of (A) TRα and (B) TRβ of the leopard gecko with homologs from other species. Dots indicate identity of amino acids with those of the TRs of the leopard gecko. Dashes indicate gaps inserted during alignment. The domains are indicated.
Expression analysis of TRs

To identify the target organs of THs, the spatial expression pattern of the TRs was examined by RT-PCR. Twenty-five nanograms of cDNA from the whole brain, heart, liver, small intestine, large intestine, testis, ovary, and thymus, and 5 ng from the pituitary, were amplified using primers specific for TRs. The primer sets used were TRαSE03 and TRαAS02 for TRα, and TRβSE03 and TRβAS03 for TRβ (Table 1). The PCR products were visualized by electrophoresis on a 1.2% TAE agarose gel and stained with ethidium bromide. Each DNA fragment was extracted from the gel and directly sequenced to confirm its identity.

Temporal expression analysis of TRs in skin

Total RNA was extracted from the skin of three animals within 24 hours before or after shedding. cDNA from the skin (7.5 ng) was amplified using the specific primers described above. The PCR conditions were as follows: 94°C for 3 min; 30 cycles of 94°C for 40 s, 64°C for 25 s, and 72°C for 30 s; and 72°C for 2 min. The PCR products were analyzed by electrophoresis on a 1.2% TAE agarose gel.
Molecular phylogenetic analysis

A phylogenetic tree of the TRs was constructed using the entire ORF nucleotide sequences of selected species representing all classes of vertebrate (Fig. 3). The TRs formed two groups, TRα and TRβ, in accordance with these two isoforms being derived from distinct genes. As expected, both the leopard gecko TRs clustered with their chicken homologs.

Expression analysis

As expected, a wide TR distribution was observed. RT-PCR products of the expected size were obtained from all tissues and organs examined (Fig. 4). The authenticity of the RT-PCR products was confirmed by direct sequencing. A control without RT was also used for 40 or 45 cycles of PCR, and no signal was detected (data not shown).

![Fig. 4. Expression of the TR mRNAs in the leopard gecko. Five nanograms of cDNA from the pituitary, and 25 ng of cDNA from the whole brain, heart, liver, small intestine, large intestine, testis, ovary, and thymus, were subjected to PCR for TRs and β-actin of the leopard gecko.](image)

![Fig. 5. Expression of TR mRNAs in the skin of the leopard gecko. The samples subjected to RT-PCR were taken from three animals before (indicated by "B") or after (indicated by "A") skin shedding. RT-PCR products beneath each horizontal bar were from mRNA taken from the skin of the same animal.](image)

Temporal expression analysis in skin

The expression of the TRs in the skin was investigated by RT-PCR. TRα was expressed more strongly in the skin after shedding than before. In contrast, TRβ was expressed more strongly before shedding than after.

DISCUSSION

The THs are pleiotropic factors important for many functions in vertebrates. In reptiles, THs are suggested to affect tail regeneration, metabolic rate, metabolic enzyme activity, and shedding frequency. To augment the investigation of the molecular mechanism of THs in reptiles, we characterized their receptors at the molecular level.

In this study, we cloned two isoforms of TR from the leopard gecko, *Eublepharis macularius*. This is the first molecular identification of full-length TRs from reptiles. The deduced amino acid sequences of the cDNAs demonstrated the classic modular structure of members of the nuclear receptor superfamily, and exhibited high identity with their homologs in other species. The highest identities were shown with their chicken homologs (96 and 98%). There are eleven conserved Cys residues in the C domain of TRα. Although the seventh Cys in IgTRα, which does not contribute to the formation of a disulfide bond or zinc finger (Zhao et al., 1998), is substituted by Ser, other Cys residues are completely conserved in all TRαs. It is therefore conceivable that cloned IgTRα does not lose the ability to bind DNA by this substitution. In the future, ligand binding studies will be helpful. In the phylogenetic tree, TRs formed groups, TRα and TRβ. Both IgTRs clustered with their chicken counterparts, as expected.

mRNA expression of the IgTRs was detected in all the tissues and organs examined, indicating that thyroid hormones are pleiotropic factors important for many functions in the leopard gecko as well as other animals. In lizards, it has been reported that THs can regulate cardiac function (Venditti et al., 1996), enzyme activity in the liver (John-Alder, 1990), and testis activity (Cardone et al., 2000; Plowman and Lynn, 1973). It is therefore conceivable that TRs mediate such effects in these organs. In other species, the expression of TRs has been also demonstrated in various tissues, but significant isoform-specific functions are poorly understood. For instance, although the expression of TRs in the adult brain has been demonstrated in mammals, their specific roles have not yet been clarified (Schwartz et al., 1992). It is known that THs can regulate steroidogenesis; however, although the expression of TRs in the human ovary has been confirmed, their specific roles remain unknown (Zhang et al., 1997). There is less information for the testis, and the type of TR expressed there remains controversial (Maran, 2003).

THs are known to regulate the shedding frequency in Squamata. Intriguingly, the effect of thyroxine appears to be reversed between lizards and snakes. To obtain a better understanding of the potential role of THs in skin shedding, we analyzed the temporal expression of TRs in the skin. The expression of TRα was stronger after skin shedding than before, whereas the result was the opposite for TRβ. Although Chiu et al. (1967) have discussed the indirect effect of THs on shedding frequency, our results strongly suggest that THs can directly affect the skin, and that the two isoforms of TR play distinct roles in skin shedding.

The shedding cycle can be divided into two phases: resting and proliferation. As the skin is in the resting phase after shedding (Maderson and Licht, 1967), our result suggests that TRα plays a role in the resting phase, such as maintaining this condition so as not to enter the proliferation phase. Furthermore, we suspect that TRβ mediates the effect of THs in the proliferation phase, since TRβ was...
strongly expressed in the skin before shedding. It is conceivable that the condition of the skin taken before shedding in this experiment was at around the last stage of the proliferation phase (Maderson and Licht, 1967). This is supported by an in vitro study demonstrating that the epidermis can differentiate by itself but cannot shed (Flexman et al., 1968). This indicates that the capacity for the complex changing pattern of cell differentiation is intrinsic to the epidermis, but that shedding is not. Extrinsic factor(s) is/are necessary for shedding, and THs may be one such factor. It has also been reported that THs have no effect on the skin in the proliferation phase, either directly or indirectly, as even thyrotoxicomized animals shed (Chiu et al., 1967). This discrepancy may be because it is always difficult to completely remove the thyroid surgically (Chiu et al., 1967). The interpretation of the physiological significance of up- or down-regulation of mRNA expression of TRs needs further study, such as in situ hybridization to analyze where and when during the shedding cycle TRs are expressed. The differential expression of TR isoforms has also been reported for other species, such as frogs during development (Sachs et al., 2000). Although both isoforms appear to be involved in regulating metamorphosis, their functional differences are not yet clear.

In this report, we characterized leopard gecko TRs and demonstrated the possibility of their direct involvement in skin shedding. These results will facilitate investigation of the physiological significance of THs and of the molecular mechanisms by which they regulate shedding frequency in Squamata.

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