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# Cloning and Characterization of a New Subtype of Thyrotropin-Releasing Hormone Receptors

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**A new subfamily member of thyrotropin releasing hormone (TRH) receptor gene, *TRHR2*, was isolated from rat brain cDNAs. The deduced amino acid sequence of *TRHR2* is 51 % identical to that of rat TRH receptor gene which was reported previously. Northern blot analysis with *TRHR2* probe revealed brain-specific expression of a 9.5 kb mRNA. In a binding experiment using the TRHR2-expressing COS cells, specific binding of TRH to TRHR2 was observed with Kd value of 9 nM which was equivalent to the Kd value (= 13 nM) of TRH binding to the TRH receptor previously reported. The active metabolite of TRH, histidyl-proline diketopiperazine, or cyclo(His-Pro), showed no specific binding activity. These results suggest that TRHR2 is a novel subtype of TRH receptor.** 

Thyrotropin releasing hormone (TRH, thyroliberin) is a tripeptide hormone, which is distributed mainly in hypothalamus, as well as extrahypothalamic brain and peripheral tissues (1, 2). TRH is originally thought as a hormone whose primary effect is the enhancement of secretion of thyrotropin (thyroid stimulation hormone, TSH) and prolactin from anterior pituitary gland (3, 4). TRH might be also involved in release of growth hormone, $\alpha$-melanocyte stimulating hormone, somatostatin and vasopressin (5). Prepro TRH gene-disrupted mouse showed a phenotype of mild hypothyroidism and significant glucose intolerance (6). In addition to these endocrinotropic effects, TRH have been shown to possess various neurostimulatory effects by acting on the central nervous system (CNS) as neurotransmitter or neuromodulator (5, 7). Additional evidences suggesting the CNS effects of TRH are the findings of changes in TRH levels in specific areas of brain in Huntington's chorea, schizophrenia, Alzheimer's disease and depression (8, 9).

A specific receptor gene for TRH, *TRHR*, was first cloned in 1990 (10), and its mRNA is distributed in the anterior lobe of pituitary gland, hypothalamus, and other peripheral tissues (11, 12, 13, 14). This distribution pattern correlate with the intrabrain distribution of TRH. However, the presence of more than one subtype(s) of TRH receptors is suggested by the discoveries of several synthetic TRH analogues with preferential CNS effects with weak thyrotropic effects (15, 16). Thus, presence of another subtype of TRH receptor is suggested especially in the extra hypothalamus-pituitary region in brain.

Here, we report the molecular cloning of a novel subtype of TRH receptor, which might explain pleiotropic effects of TRH, especially the CNS effects.

## MATERIALS AND METHODS

*Cloning, sequencing and northern blot analysis of rat TRHR2 cDNA.* Rat thalamus and hypothalamus poly $(A)^+$ RNA was reverse transcribed into cDNA using RNA-PCR kit (Takara). The cDNAs were subjected to polymerase chain reaction (PCR) with degenerate primers designed from NPY receptors Y1, Y2, Y4, Y5 and Y6 (17, 18, 19, 20, 21): a sense primer, 5′-TNATGGACCACTGG(G/A)TCTTTG-3′ (N stands for G, A, T or C), corresponding to the extracellular region between transmembrane regions 2 and 3, and an antisense primer, 5′-CCATAAAAGNNGGGGTTGAC-3′, corresponding to transmembrane region 7. The PCR condition was 94 °C 2 min, followed by 35 cycles of 94 °C 30 sec, 48 °C 1 min, and 72 °C 1 min 30 sec, and finally 8 min incubation at 72 °C. The amplified products were purified by Wizard PCR Preps DNA Purification System (Promega) and cloned into pCR2.1 vector using TOPO TA Cloning Kit (Invitrogen). The obtained clones were subjected to single path sequencing and BLAST search by Wisconsin Package (GCG). One of the sequences showed significant similarity to known G protein-coupled receptors. An oligonucleotide designed from the sequence, 5′-GACGTGCTGGCTGCTTGGGCATCAC-3′, was then used as a probe to screen a SuperScript rat brain cDNA library (Life Technologies) by Gene Trapper cDNA Positive Selection System (Life Technologies). One positive clone, designated as pBG1-1, was sequenced completely. Rat multiple tissue northern blot (Clontech) was hybridized with a pBG1-1 cDNA probe in QuikHyb solution (Stratagene) at 68 °C for

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1 hr and washed in 0.1 x SSC containing 0.1 % SDS at 60 °C for 30 min.

*Construction of plasmids for expression.* For binding assay, an expression vector pEF1x was constructed by modifying pcDNA3 (Invitrogen) as follows. pcDNA3 was digested by *Mlu*I, blunted by Klenow fragment, and self-ligated. The obtained plasmid was digested by *Afl*III and *Sma*I, blunted by Klenow fragment, and self-ligated to obtain the plasmid [A]. Human EF1$\alpha$ promotor (GenBank Accession number J04617) was amplified by PCR using oligonucleotide primers 5′-CGAGGATCCGTGAGGCTCCGGTGCCCGTC-3′ and 5′-CGGGTAAGCTTCACGACACCTGAAATGGAAGA-3′, digested by *Bam*HI and *Hin*dIII, and cloned into pUC19. This plasmid DNA was digested by *Xho*I, blunted by Klenow fragment, and self-ligated. The *Bam*HI and *Hin*dIII insert of this plasmid was subcloned into the *Bgl*II and *Hin*dIII sites of the plasmid [A].

The *Not*I/*Eco*RI insert fragment of pBG1-1 was subcloned into pEF1x vector (pEF1x-rTRHR2). Rat *TRHR1* cDNA was amplified from λZAP rat thalamus/hypothalamus cDNA library by PCR using primers 5′-GCGAATTCACAGCACAAAAGGTGGAGG-3′ and 5′-TAGCGGCCGCCGATGTTCTTACTGACTTC-3′, digested with *Not*I and *Eco*RI, and subcloned into pEF1x vector (pEF1x-rTRHR1).

*Cell culture.* COS−7 cells (8 x $10^6$ cells in 800 $\mu$l of serum−free Dulbecco's MEM) transfected with 40 $\mu$g of pEF1x−rTRHR and pEF1x−rTRHR2 by electroporation (220 V, 950 $\mu$F, using ECM−600 Electroporation System (BTX, San Diego, CA) with a 4 mm gap cuvette) were grown in Dulbecco's modified Eagle's medium supplemented with 10 % FBS, 100 IU/ml penicillin−G and 100 $\mu$g/ml streptomycin. These cells were grown in a 95 % air, 5 % $CO_2$ humidified atmosphere at 37°C for 48 hrs before membrane preparation.

*Binding experiments.* The cells were washed with 20 % sucrose solution containing154 mM NaCl, 10 mM KCl, 0.8 mM CaCl2 and 10 mM MOPS, then homogenized and centrifuged at 1,000 x g for 15 min at 4°C. The supernatant was centrifuged at 100,000 x g for 50 min at 4 °C. The pellets were resuspended in 5 mM HEPES buffer (pH 7.4) and centrifuged again. The membrane fraction was resuspended by a homogenizer in the same buffer and used for this study. Binding experiments were done according to the reported procedures with some modification (22). Binding of [$^3$H]TRH to the membrane preparations was performed in 0.2 ml 25 mM Tris buffer (pH7.4) containing 10 mM $MgCl_2$, 1 mM phenylmethylsulfonylfluoride, 0.1 % bacitracin and 0.5 % BSA. The membranes (600−700 ug/ml) were incubated at 4°C for 60 min. Bound and free peptides were separated by filtration using a GF/C glass filter (Packard). Specific binding was determined by subtracting non−specific binding activity from total binding activity in the presence of 10 $\mu$M TRH.

## RESULTS AND DISCUSSION

As a result of a study which was aimed at cloning of NPY receptor subtypes, we have unexpectedly isolated a GPCR-like clone, designated as *TRHR2*, from rat thalamus and hypothalamus cDNA. The *TRHR2* cDNA was 2,351 bp in length excluding poly(A) tail, encoding a 39 kDa protein of 352 amino acids. Because there is an in-frame stop codon 5′ upstream of the first ATG codon in the open reading frame, the cDNA should contain the entire coding region. The northern blot analysis by the *TRHR2* probe showed an apparent transcript of 9.5 kb, which was much larger than the size of the obtained cDNA (Figure 3), suggesting the presence of long 5′ and/or 3′ untranslated regions. The obtained cDNA lacked polyadenylation signal (AATAAA) in the 3′ untranslated region, which may be due to annealing

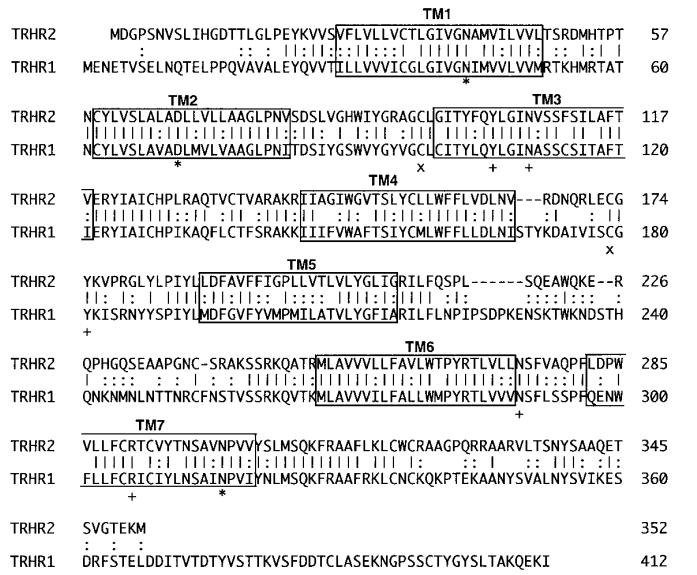


**FIG. 1.** Alignment of the deduced amino acid sequences of rat *TRHR1* and *TRHR2*. Identical and similar residues are shown by vertical lines and colons, respectively. The amino acid residue numbers are shown in the right. The postulated transmembrane regions are boxed. The Asn43, Asp71 and Asn316 whose interaction is critical for activation of TRHR1 are shown by asterisks. Cys98 and Cys179 forming disulfide bond are shown by x's. The amino acid residues, which interact with TRH, are shown by pluses. The nucleotide sequence of the rat *TRHR2* cDNA will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB015645.

of poly(T) primer to the A-rich sequence during the cDNA synthesis.

The deduced amino acid sequence of *TRHR2* showed 51 % overall identity with that of rat TRH receptor previously reported (Fig. 1). Thus, the classical TRH receptor, which has been referred to *TRHR*, are designated as *TRHR1*, in this report. Previous reports of binding and function studies as well as computer modeling showed that several residues highly conserved among rhodopsin/b-adrenergic receptor family GPCRs are also conserved in *TRHR1* and are important for the function of *TRHR1* (23 - 28). Interaction among these residues of TRHR1, Asn43 in transmembrane region (TM) 1, Asp71 in TM2 and Asn316 in TM7, which are highly conserved among the rhodopsin/b-adrenergic family, are important for TRH binding. Cysteine residues in the first and second extracellular loops of TRHR1, which form disulfide bond, are critical for high affinity biding of TRH (24). It was suggested that Asn110, Asn289, Tyr181, Tyr106 and Arg306 of TRHR1 interacts with TRH (25, 26, 27, 28). These residues were also conserved between *TRHR2* and *TRHR1* (Fig. 1.), raising a possibility that TRHR2 may be another subtype of TRH receptors.

To test the possibility that TRHR2 may be another subtype of TRH receptors, binding assay was per-

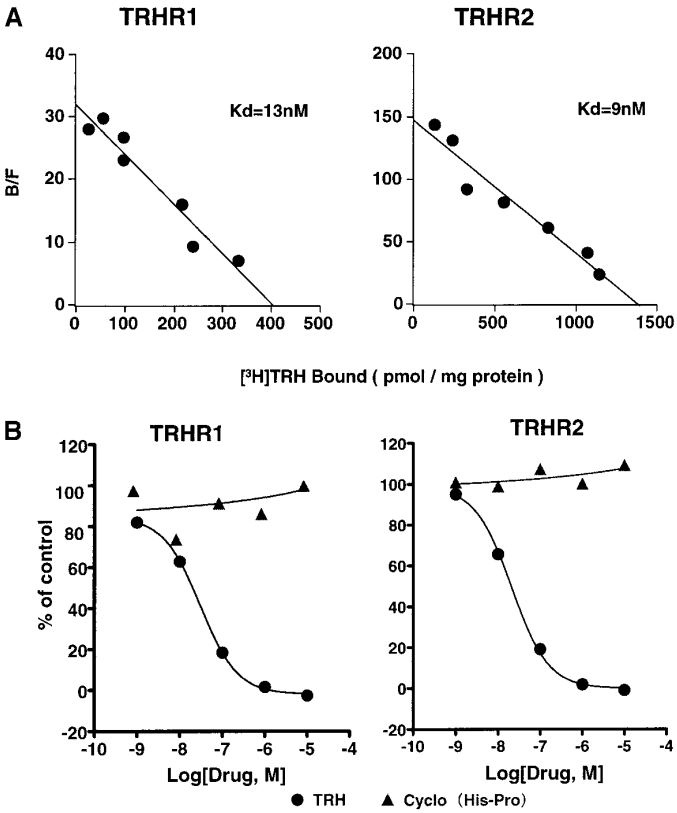


**FIG. 2.** (A) Scatchard plot analysis of [$^3$H]TRH specific binding to rat TRH receptors. The membranes were incubated with [$^3$H]TRH for 60 min at 4°C. Each point represents duplicate determinations from two independent experiments. (B) Inhibition of [$^3$H]TRH specific binding to rat TRH receptors by TRH and cyclo(His−Pro). The membranes were incubated with 2 nM [$^3$H]TRH for 60 min at 4°C. Data are expressed as percentages of total specific binding.

formed (Fig. 2a.). [3H]TRH specifically bound to TRHR1 with a high affinity (Kd =13 nM) as reported previously (29) (Fig.1). The membranes expressing rat TRHR2 also showed a specific binding of [3H]TRH with a similar affinity (Kd = 9 nM) (Fig. 2a). These results clearly suggest that the TRHR2 is a specific receptor of TRH.

Histidyl-proline diketopiperazine, often referred to cyclo(His-Pro), is known to be an active metabolite which decreases release of prolactin (30). A specific binding fraction for cyclo(His-Pro) has been demonstrated in adrenal cortical particles (31), suggesting the existence of a specific receptor for cyclo(His-Pro), although molecular identification of this receptor has not been reported. We have explored the possibility of TRHR2 being a receptor of cyclo(His-Pro), by testing whether cyclo(His-Pro) binds to TRHR2. But no specific binding was observed to TRHR1 or TRHR2, excluding the possibility that TRHR2 is a receptor of cyclo(His-Pro) (Fig. 2b.). Thus, we concluded that TRHR2 is a new subtype of TRH receptors.

The northern blot analysis showed predominant expression of *TRHR2* mRNA in brain, but not in heart, spleen, lung, liver, skeletal muscle, kidney and testis (Fig. 3). This expression pattern is distinct from that of *TRHR1*, in which no obvious expression was reported in brain by northern blot analysis (11). Thus, *TRHR2* may be involved in biological pathways which are distinct from those of *TRHR1*.

TRHR1 is a G protein-coupled receptor, which couples to $G_q$ and $G_{11}$ protein, leading to hydrolysis of phosphatidylinositol 4,5-diphosphate by phospholipase C, then to $Ca^{2+}$- and diacylglycerol-activated protein kinase action (32, 33). However, there have been reports that TRH possesses two distinct types of brain intracellular signaling systems, namely, a cyclic AMP- and inositol phosphate-based system, which vary with brain regions (34). It is possible that TRHR2 may be a trigger of such signaling pathways which are different from those of TRHR1. In support for this possibility is the findings in the amino acid alignment of *TRHR1* and *TRHR2*: all the putative transmembrane domoains were well conserved, while no significant homology was observed in the intracellular domain between the TM 5 and 6 as well as C-terminal intracellular domain, which are essential for the interaction with distinct G-proteins.

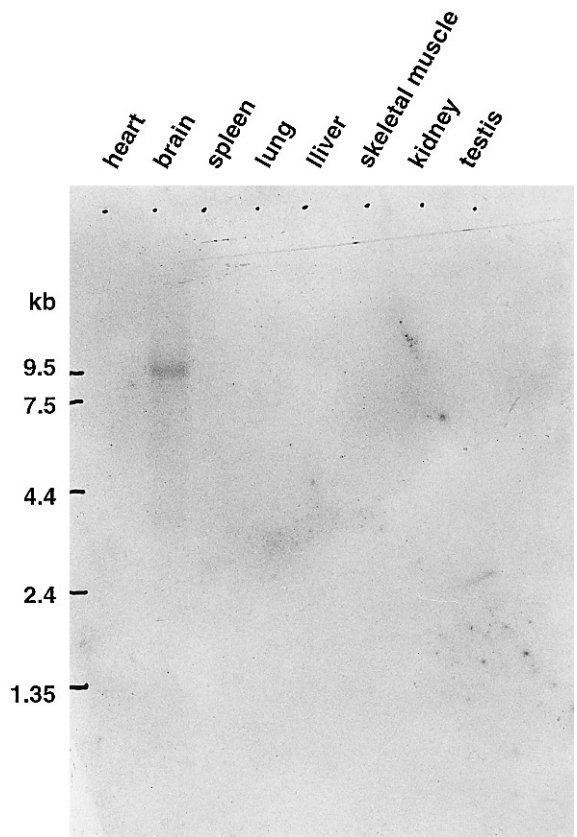


**FIG. 3.** Northern blot analysis of *TRHR2*. Rat multiple tissue northern blot (Clontech) was hybridized with rat *TRHR2* cDNA. Positions of size markers are indicated in the left.

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