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Novel human ZAKI-4 isoforms: hormonal and tissue-specific regulation and function as calcineurin inhibitors

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We identified a thyroid hormone [3,5,3′-tri-iodothyronine (T_3)]-responsive gene, ZAKI-4, in cultured human skin fibroblasts. It belongs to a family of genes that encode proteins containing a conserved motif. The motif binds to calcineurin and inhibits its phosphatase activity. In the present study, we have demonstrated three different ZAKI-4 transcripts, α, β1 and β2, in human brain by 5′- and 3′-RACE (rapid amplification of cDNA ends). The α transcript was identical with the one that we originally cloned from human fibroblasts and the other two are novel. The three transcripts are generated by alternative initiation and splicing from a single gene on the short arm of chromosome 6. It is predicted that β1 and β2 encode an identical protein product, β, which differs from α in its N-terminus. Since α and β contain an identical C-terminal region harbouring the conserved motif, both isoforms are suggested to inhibit calcineurin activity. Indeed, each isoform associates with calcineurin A and inhibits its activity in a similar manner, suggesting that the difference in N-terminus of each isoform does not affect the inhibitory function on calcineurin. An examination of the expression profile of the three transcripts in 12 human tissues revealed that the α transcript is expressed exclusively in the brain, whereas β transcripts are expressed ubiquitously, most abundantly in brain, heart, skeletal muscle and kidney. It was also demonstrated that human skin fibroblasts express both α and β transcripts, raising the question of which transcript is up-regulated by T_3. It was revealed that T_3 markedly induced the expression of α isoform but not of β. This T_3-mediated increase in the α isoform was associated with a significant decrease in endogenous calcineurin activity. These results suggest that the expression of ZAKI-4 isoforms is subjected to distinct hormonal as well as tissue-specific regulation, constituting a complex signalling network through inhibition of calcineurin.

Key words: chromosome 6, DSCR1L1, RNA-ligase-mediated 5′-RACE, thyroid hormone.

INTRODUCTION

Using a differential display of mRNA by PCR, we cloned ZAKI-4 cDNA as a thyroid-hormone-responsive gene from cultured human skin fibroblasts [1]. Recently, it has been demonstrated that ZAKI-4 (assigned as DSCR1L1 by Human Nomenclature Committee) belongs to a family of genes that encode proteins containing a conserved motif of ISPPXSPP among different species [2]. The family in humans includes ZAKI-4 [1], DSCR1 [3] and DSCR1L2 [4], and they share 61–68% identity in amino acid sequences. ZAKI-4 and DSCR1 genes are abundantly expressed in human brain, heart and muscles [1,5], whereas DSCR1L2 is expressed ubiquitously [2]. The products of ZAKI-4 gene family bind to calcineurin A (CnA), the catalytic subunit of calcineurin, through their conserved C-terminal region and inhibit its activity [6–8].

Calcineurin is a serine/threonine protein phosphatase activated by the Ca^{2+}/calmodulin signalling [9,10]. It consists of the catalytic subunit (CnA) and calcium-binding regulatory subunit (calcineurin B). The discovery that immunophilins, cyclophilin and FKBP12 (FK506-binding protein), form inhibitory complexes with calcineurin in the presence of immunophilin-binding drugs such as cyclosporin A (CsA) and FK506 [11] established calcineurin as a mediator of T-cell activation [12–14]. Activation of calcineurin results in the dephosphorylation of a transcription factor known as the nuclear factor of activated T-cells (NF-AT) and its nuclear translocation [15–17]. NF-AT then binds to DNA co-operatively with other transcription factors such as AP-1 to activate transcription of the genes encoding cytokines such as interleukin 2 [17]. By inhibiting calcineurin activity, CsA and FK506 prevent the nuclear translocation of NF-AT secondary to dephosphorylation, thereby suppressing T-cell activation [15].

Apart from T-cell activation, calcineurin also plays a pleiotropic role in regulating neuronal plasticity [13,18] and apoptosis [19,20] and hypertrophy of cardiac [21,22] and skeletal muscles [23,24].

To elucidate the function of ZAKI-4 on calcineurin, we first examined the expression of ZAKI-4 transcript in human brain tissues, where calcineurin is abundant [25]. Three transcripts were identified. One transcript, termed α, is identical with the ZAKI-4 sequence which we originally reported [1], and the other two are novel and termed β1 and β2. Tissue distribution of each transcript, their differential regulation by thyroid hormone and their function on calcineurin will be described for the first time.

MATERIALS AND METHODS

Cloning of human ZAKI-4 cDNAs

Human brain tissue was obtained from a patient with malignant glioma. Normal tissues adjacent to the glioma were separated and immediately frozen in liquid nitrogen and kept at −80 °C

Abbreviations used: CHO, Chinese-hamster ovary; CnA, calcineurin A; CsA, cyclosporin A; DTT, dithiothreitol; FBS, foetal bovine serum; FISH, fluorescence *in situ* hybridization; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NF-AT, nuclear factor of activated T-cells; RACE, rapid amplification of cDNA ends; RLM-5′-RACE, RNA-ligase-mediated 5′-RACE; T_3, 3,5,3′-tri-iodothyronine.

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The nucleotide sequences for ZAKI-4β1 and β2 have been deposited in the GenBank* database under accession nos. AY034085 and AY034086 respectively.

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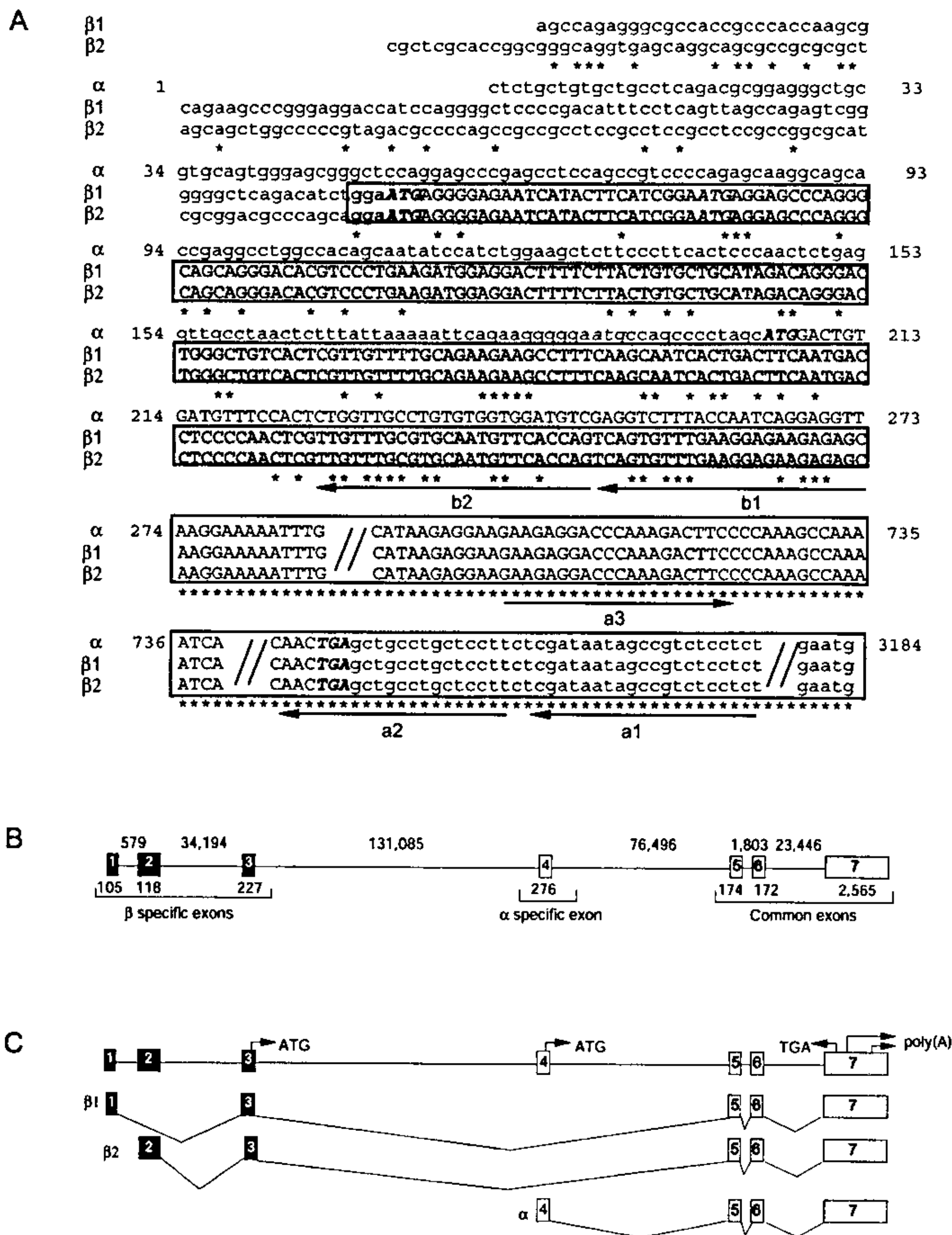

Figure 1 ZAKI-4 gene organization and its transcripts

(A) Three transcripts encoded by ZAKI-4 gene. RACE revealed three different transcripts, α, β1 and β2, encoded by ZAKI-4 gene. The primer positions for the conventional 5′-RACE were illustrated as a1 and a2, and that for 3′-RACE as a3 [1]. For the RLM-5′-RACE, nested primers were designed at b1 and b2. The α transcript is identical with that reported previously [1] as ZAKI-4 (GenBank™ accession no. D83407). The other two, β1 and β2, are novel. Non-coding sequences are depicted in lower-case and coding in upper-case letters. Translation initiation and stop codons are shown in bold italic. *, identical sequence among all the transcripts. Note that β1 and β2 transcripts share an identical sequence as shown in shaded boxes. All the transcripts share the sequence at the 3′-end, boxed in white where several parts of the sequence were omitted. (B) Gene organization of ZAKI-4. All the information was obtained from the following sequences (clone names are given in parentheses): AL359633.15 (RP11-795J1), AL390741 (RP11-38F19) and AC015547 (RP11-195J23). Numbers of nucleotides for introns are shown on the top and for exons at the bottom. Although the length of exons 3, 5, 6 and 7 could be precisely determined from a comparison of the gene and cDNA structure, the length of exons 1, 2 and 4 is putative. (C) Generation of three transcripts by alternative initiation and splicing. β1 and β2 mRNAs are generated by alternative initiation from exon 1 and exon 2 respectively. They are joined to exon 3 shared by β1 and β2 and to exons 5–7 shared by all the transcripts. The α mRNA starts from exon 4, joining to exons 5–7. A translation-initiation site (ATG) for β1 and β2 is present in exon 3 and that for α in exon 4. The stop codon (TGA) for all the transcripts is located in exon 7. Note that two polyadenylation sites are present in the 3′-non-coding region of exon 7.

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until use. Informed consent was obtained from the patient for the use of the surgical specimen. Total RNA was extracted by the method reported by Chomczynski and Sacchi [26].

For conventional 5′- and 3′-RACE (rapid amplification of cDNA ends), a kit from Life Technologies (Grand Island, NY, U.S.A.) was used [27]. cDNAs were prepared by reverse transcription using the brain RNA as a template. The primers were identical with those in our previous report [1] and are depicted as a1, a2 and a3 in Figure 1(A). The sequencing of the cloned 5′-RACE products revealed the existence of additional transcript(s) other than the one we previously reported as ZAKI-4. Thus the original ZAKI-4 sequence is termed α.

To characterize further the transcripts other than α, RNA-ligase-mediated 5′-RACE (RLM-5′-RACE) [28] was performed using a kit (FirstChoice RLM-RACE; Ambion, Austin, TX, U.S.A.). In this method, the total RNA sample was first treated with calf intestinal phosphatase to dephosphorylate the 5′-phosphate from all RNA species except the mRNAs with the cap structure. It was then treated with tobacco acid pyrophosphatase to remove the cap, leaving a 5′-phosphate. It was thus possible to ligate an RNA adapter oligonucleotide to mRNA species with an intact cap structure. Nested PCR using gene- and adapter-specific primers should amplify the 5′-end of the mRNA of interest. Based on the information obtained from the conventional 5′-RACE, we used the following primers: 5′-GCTCTCTTCTCCTTCAAACACTGA-3′ (outer primer, the position indicated by b1 in Figure 1A) and 5′-CTGGTGAACATTGCACGCAAACAA-3′ (inner primer, indicated by b2 in Figure 1A). The PCR-amplified fragments from RLM-5′-RACE were then cloned into pGEM-T Easy vector (Promega, Madison, WI, U.S.A.). The clones having inserts with the RNA adapter-specific sequence were identified by treatment with *Bam*HI, because the restriction site was present only in the adapter but not in the vector. The clones containing only cDNA inserts with the adapter *Bam*HI site were sequenced in both directions by the fluorescent dideoxy chain-termination method using a sequencer (ABI 373A; PerkinElmer, Norwalk, CT, U.S.A.).

Database search and sequence alignment

The non-redundant nucleotide sequence databases, including GenBank® [29], dbEST (expressed sequence tags database) [30], dbGss [31], High Throughput Genome [32], dbSTS [31], EMBL, GENES and GENOME databases, were searched with BLAST 2 served by GenomeNet (http://blast.genome.ad.jp). CLUSTALW (version 1.8) provided by DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html) [33] was used for the sequence alignment. The searches were made between March and May 2002.

Fluorescence *in situ* hybridization (FISH) analysis

The full-length human ZAKI-4α cDNA harbouring the sequence common to α and β was used for the FISH analysis. Human metaphase chromosomes were prepared from normal male blood lymphocytes, cultured in α-minimal essential medium supplemented with 10% foetal calf serum and phytohaemagglutinin (M form; Life Technologies) at 37 °C for 72 h. The lymphocyte cultures were synchronized by treatment with bromodeoxyuridine (0.18 μg/ml; Sigma). After washing three times with serum-free medium to release the block, the cells were recultured for 6 h in α-minimal essential medium with thymidine (2.5 μg/ml; Sigma). The chromosomal spread from lymphocytes was hybridized with the probe labelled with biotinylated dATP using the BRL (Grand Island, NY, U.S.A.) BioNick labelling kit [34,35]. FISH signals and the 4′,6-diamidino-2-phenylindole band pattern were recorded separately by taking photographs. Chromosomal location was assigned by superimposing the FISH signals with 4′,6-diamidino-2-phenylindole banded chromosome [36].

Examination of the tissue-specific expression of ZAKI-4 transcripts

To investigate the expression profile of each ZAKI-4 transcript, duplicate multiple tissue Northern-blot membranes (ClonTech, Palo Alto, CA, U.S.A.) were hybridized with the probe specific to either α (exon 4) or β (exon 3). See Figure 1(B) for exons 3 and 4. Each probe was prepared by reverse transcriptase–PCR using the following primers: 5′-CTCTGCTGTGCTGCCTCAAACGCG-3′ (sense) and 5′-CTCCTGATTGGTAAAGACCTCGAC-3′ (antisense) for α; 5′-ATGAGGGGAGAATCATACTTCATC-3′ (sense) and 5′-GCTCTCTTCTCCTTCAAACACTGA-3′ (antisense) for β. After cloning into pGEM-T Easy vector (Promega), the authenticity of the sequences was confirmed before use. The cDNA inserts purified from agarose gel were labelled with [α-^{32}P]dCTP (3000 Ci/mmol; PerkinElmer Life Science, Boston, MA, U.S.A.) using a random-primed DNA labelling kit (Boehringer Mannheim, Mannheim, Germany).

To assess whether β1 or β2 is preferentially expressed in some tissues, the same membranes were stripped of the probes by heating in 0.5% SDS at 100 °C for 10 min. Each membrane was then hybridized with oligonucleotide probe specific to each transcript. β1-Specific 5′-CCCCCCGACTCTGGCTAACTGAGGAAATGT-3′ probes and β2-specific 5′-CTGCTGGGCGTCCGCGATGCGCCGGCGGAG-3′ probes were labelled at their 5′-ends with [γ-^{32}P]ATP (6000 Ci/mmol; PerkinElmer Life Science) by T4 polynucleotide kinase using a kit (Megalabel; Takara, Tokyo, Japan). All the hybridized membranes were exposed to Kodak X-AR films (Eastman Kodak, Rochester, NY, U.S.A.) at −80 °C.

Regulation of ZAKI-4α and ZAKI-4β expressions by thyroid hormone in cultured human skin fibroblasts

Human skin fibroblasts were cultured in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical, Tokyo, Japan), supplemented with 10% foetal bovine serum (FBS). The origin of the fibroblast was reported previously [1]. After the cells were grown to confluence, the medium was replaced with that containing FBS in which thyroid hormone was depleted by treatment with activated charcoal [37]. After 24 h incubation in this medium, the cells were treated with 3,5,3′-tri-iodothyronine (T_3; 10^{-8} M) for 12 h, and total RNA was extracted and subjected to Northern-blot analysis. The membrane was hybridized with α- or β-specific probe. The construction of the probes was described above. The same membranes were rehybridized with human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe [1]. Radioactivity of each mRNA was determined by BAS2000 bioimage analysing system (Fuji Photo Film Co., Tokyo, Japan). The mRNA levels for human ZAKI-4α and -4β were normalized to that for GAPDH. The experiments were performed in duplicate dishes and repeated three times.

In vitro protein–protein binding assay of ZAKI-4 isoforms with calcineurin

To investigate possible difference in the association of each ZAKI-4 isoform with CnA, it was co-synthesized with each isoform by *in vitro* transcription-coupled translation using TNT®-coupled rabbit reticulocyte lysate system (Promega). The same amount of plasmid (1 μg), expressing either ZAKI-4α (Zα), ZAKI-4β (Zβ) or the common region (Zc) shared by Zα and Zβ,

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and CnA was incubated in the presence of [^{35}S]methionine (1175 Ci/mmol; PerkinElmer Life Science). The synthesized products were then diluted in the binding buffer [50 mM Tris/HCl (pH 7.4)/120 mM NaCl/0.5 % Nonidet P40/1 mM dithiothreitol (DTT)/proteinase inhibitor cocktail (Boehringer Mannheim)] and immunoprecipitated with an antibody raised against ZAKI-4α synthesized in *Escherichia coli* [38]. The antibody-bound products were separated with Protein G–agarose beads (Amersham Biosciences, Piscataway, NJ, U.S.A.). After washing, the products were eluted in 20 μl of a dissociation buffer (50 mM Tris/HCl/100 mM DTT/2 % SDS/0.1 % Bromophenol Blue/10 % glycerol) at 100 °C for 10 min and analysed by SDS/PAGE.

All the cDNAs coding Zα, Zβ and Zc were amplified by PCR from human brain cDNAs. To construct the cDNA for Zc, the sense primer was designed to contain an ATG start codon preceded by Kozak's sequence [39]. The sense primers for each construct were: 5′-GTCAGCATGGACTGTGATGTTTCCA-CTCTG-3′ (sense) for Zα; 5′-GTCAGCATGAGGGGAGAC-GCCTACTTCATCGGA-3′ (sense) for Zβ; 5′-GCTAGCCA-CCATGGAAAAATTTGGGGGACTGTTTCGGACTTAT-3′ (sense) for Zc (Kozak's sequence with initiation codon in boldface was underlined). The antisense primer was common to all the constructs and its sequence was 5′-TCTAGATCAGTTGGACA-CGGAGGGTGGCAGGCC-3′. All the cDNAs were inserted into the pGEM-T Easy vector (Promega), where the transcription was driven by SP6 promoter. A cDNA coding human CnA was also amplified using the primers 5′-GCTAGCATGTCCGAGC-CCAAGGCAATTGAT-3′ (sense) and 5′-TCTAGATCACTG-AATATTGCTGCTATTACTGCC-3′ (antisense) based on the sequence information available at National Center for Biotechnology Information (accession no. NM-000944) and subcloned into the same vector.

Determination of calcineurin activity

Calcineurin activity in the human skin fibroblasts was determined using ^{32}P-labelled casein as substrate [40]. The fibroblasts were cultured as described above and harvested at intervals after addition of T_3 (10^{-8} M). They were washed twice with PBS and the cell pellets were kept frozen at −80 °C until the assay. The fibroblasts (2×10^6) were disrupted by sonication on ice in 50 μl of a solution containing 50 mM Tris/HCl (pH 7.5), 0.1 mM EGTA, 1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF and 5 μg/ml each of the protease inhibitors leupeptin, aprotinin and soyabean trypsin inhibitor. The resulting lysates were centrifuged at 10000 *g* for 10 min at 4 °C, and the supernatants were collected. For preparation of the substrate, 10 mg of dephosphorylated casein was incubated for 3 h at 30 °C in 1 ml of a reaction mixture containing 50 mM Tris/HCl (pH 7.0), 0.5 mM DTT, 2 mM $MgSO_4$, the catalytic subunit of cAMP-dependent protein kinase (2500 units/ml) and 1.67 μM [γ-^{32}P]ATP (3000 Ci/mmol; PerkinElmer Life Science). The reaction was terminated by the addition of 0.33 ml of 100 % (w/v) trichloroacetic acid, followed by incubation for 1 h on ice. The precipitated protein was separated by centrifugation at 10000 *g* for 10 min at 4 °C and washed twice with cold acetone (−20 °C). The pellet was dissolved in 4 ml of a solution containing 50 mM Tris/HCl (pH 7.0) and 0.5 mM DTT, and then dialysed overnight twice against the same buffer [41]. The calcineurin assay was initiated by the addition of 3 μl of the cell lysate supernatant to 25 μl of the phosphorylated casein. After incubation at 30 °C for 10 min, the reaction was terminated by the addition of 100 μl of 20 % (w/v) trichloroacetic acid and 25 μl of BSA (6 mg/ml). The samples were centrifuged at 10000 *g* for 10 min at 4 °C, and the radioactivity in the supernatants was measured in a liquid-scintillation counter.

Inhibitory action of each ZAKI-4 isoform on calcineurin activity was studied in Chinese-hamster ovary (CHO) cells from A.T.C.C. (Manassas, VA, U.S.A.; accession no. CCL-6) by transfecting a plasmid expressing constitutively the active form of CnA (ΔCnA) [13] together with that expressing Zα, Zβ or Zc. For the transient transfection assay, all the cDNA inserts in the pGEM-T Easy plasmids described above were subcloned into pShuttle (ClonTech). Calcineurin activity was determined 48 h after the transfection. The activity was corrected by the amount of protein determined by a micro-assay kit (Bio-Rad Laboratories) using BSA as a standard. Transfection efficiency was monitored by co-transfecting a plasmid expressing β-galactosidase (pCMV β-gal; Promega). The enzyme activity was expressed as a percentage of the mean value of the control [42].

Assessment of the inhibitory activity of ZAKI-4 isoforms on calcineurin-mediated activation of NF-AT

A human T cell line (Jurkat) was maintained at a cell density less than 8×10^5 cells/ml in RPMI 1640 supplemented with 10 % FBS in an atmosphere of 5 % CO_2 and 100 % relative humidity. The cells were electroporated to transfect with a reporter plasmid (pIL2-luciferase) containing human IL-2 promoter upstream of the luciferase gene, those expressing ZAKI-4 isoforms and pCMV β-gal to monitor the transfection efficiency. The electroporation was performed at settings of 350 V, 960 μF in a 0.4 cm cuvette with Bio-Rad Gene Pulser System (Nippon Bio-Rad Laboratories, Tokyo, Japan). After electroporation, the cells were diluted and aliquoted into a 6-well tissue culture plate. Twenty four hours after the transfection, stimulation of calcineurin and subsequent activation of NF-AT were initiated by adding 2 μM ionomycin and 20 ng/ml PMA [13]. Luciferase and β-galactosidase activities were determined 24 h after the addition of ionomycin/PMA. When indicated, a calcineurin inhibitor CsA at a final concentration of 1 μM was added 15 min before the addition of ionomycin/PMA.

The pIL2-luciferase, human IL-2 promoter sequence from −326 to +47 [17], was PCR-amplified and cloned into the upstream of luciferase gene of the pGL3 basic vector (Promega).

Statistical analysis

Data were compared among three or more groups by one-way analysis of variance and Fisher's *post hoc* test.

RESULTS

Three different transcripts generated from ZAKI-4 gene

Conventional 3′- and 5′-RACE and RLM-5′-RACE revealed three different transcripts encoded by ZAKI-4 gene (Figure 1A). The α transcript was identical with that we reported previously [1] as ZAKI-4 (GenBank® accession no. D83407). The other two were novel and named as β1 and β2 according to the location of the corresponding exons in the genome (see Figure 1B). As shown in Figure 1(A), both β1 and β2 shared an identical sequence harbouring two possible translation initiation codons (italic in the shaded box). Since the two codons were not followed by the optimal 'G' [39], we assigned the first ATG as translation initiation sites for β transcripts. In the α transcript, two in-frame ATG codons were located at nucleotide positions 190 and 205. The second ATG was concordant with the Kozak rule, and was assigned as the translation start site [39,43].

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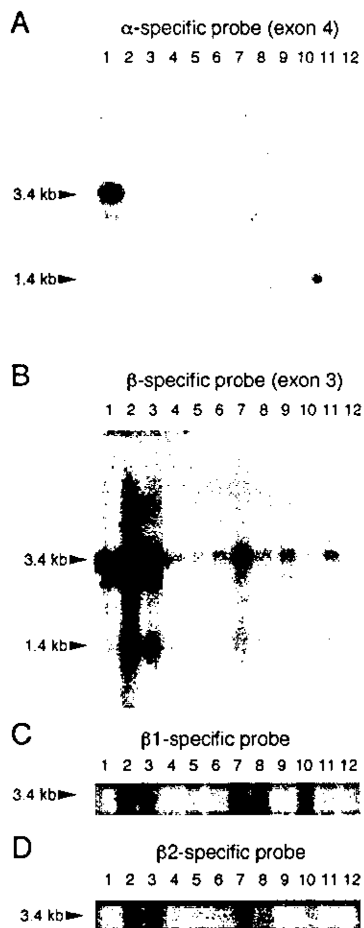

Figure 2 Expression of ZAKI-4α and β mRNAs in various human tissues

Northern-blot analysis of polyadenylated [poly(A)+] RNA from 12 human tissues: brain (lane 1), heart (2), skeletal muscle (3), colon (4), thymus (5), spleen (6), kidney (7), liver (8), small intestine (9), placenta (10), lung (11) and peripheral blood leucocytes (12). First, the duplicate membranes were hybridized with the α-specific region encoded by exon 4 (**A**) or with the β sequence common to β1 and β2 coded by exon 3 (**B**). The same membranes were used to study the expression profile of β1 (**C**) and β2 (**D**), using specific oligonucleotide labelled at 5'-end with [γ-^{32}P]ATP. The positions of marker bands (kb) are indicated to the left of the blots.

BLAST search with α, β1 and β2 cDNA sequences identified five overlapping genomic clones, enabling us to construct the complete organization of ZAKI-4 as a single gene. As shown in Figure 1(B), the gene consists of seven exons. Exon 1 corresponds to the non-coding region specific for β1, whereas exon 2 corresponds to the non-coding region specific for β2. Exon 3 encodes the region common to β1 and β2. Exon 4 corresponds to the sequence specific to α. The last three exons are shared by all the transcripts. It is thus speculated that the transcripts are generated by alternative initiation and splicing (Figure 1C). Figure 1(C) also depicts the location of translation start codons for β transcripts in exon 3 and for α in exon 4. The site of stop codon is located in exon 7. Note that two polyadenylation sites are located in the 3'-non-coding region of exon 7 [1]. FISH localized the ZAKI-4 gene on the short arm of chromosome 6p12 (results not shown).

Expression of ZAKI-4α and β mRNAs in various human tissues

We previously demonstrated that ZAKI-4 mRNA is expressed abundantly in brain, heart, skeletal muscle and liver [1]. Identification of α, β1 and β2 transcripts led us to examine the expression profile of each ZAKI-4 transcript. First, duplicate multiple tissue Northern-blot membranes were hybridized either with the α-specific region encoded by exon 4 or with the β sequence common to β1 and β2 encoded by exon 3. As shown in Figure 2(A), the α transcript was detected only in the brain, whereas the β transcript was expressed ubiquitously, with abundant expressions in brain, heart, skeletal muscle and kidney (Figure 2B). Note that two mRNA species of size 3.4 and 1.4 kb were detected with α and β probes. These mRNAs are likely to be generated by alternative polyadenylation as described above (Figure 1C) [1]. To explore possible differences in the tissue-specific expression profiles of β1 and β2, oligonucleotide probes were used to rehybridize the membranes. As shown in Figures 2(C) and 2(D), both transcripts were distributed in a similar manner, as demonstrated by the β-specific probe (exon 3).

Differential regulation of ZAKI-4α and β expressions by thyroid hormone in human skin fibroblasts

Although the α transcript was predominantly detected in the brain (Figure 2A), the cDNA was cloned as a thyroid-hormone-responsive gene in human skin fibroblasts [1]. Because of the

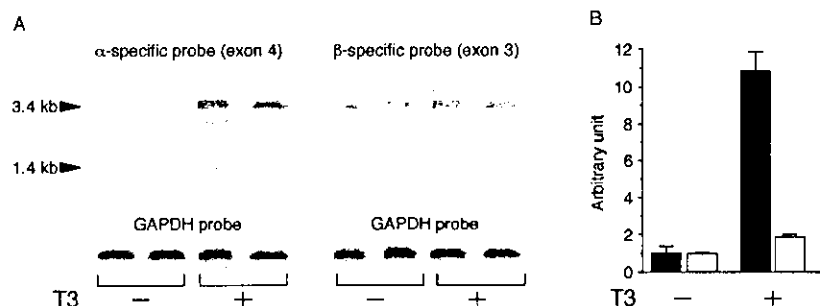

Figure 3 Differential regulation of ZAKI-4α and -4β expression by T_3 in human skin fibroblasts

Human skin fibroblasts were cultured in the presence or absence of T_3 (10^{-8} M) for 12 h. (**A**) Representative image of the Northern blot hybridized with either α- or β-specific probe. The experiments were performed in duplicate dishes for each group and repeated three times. (**B**) Relative amount of α or β mRNA corrected with that of GAPDH. Closed column, α mRNA; open column, β mRNA. The data are presented as means ± S.D. from three experiments.

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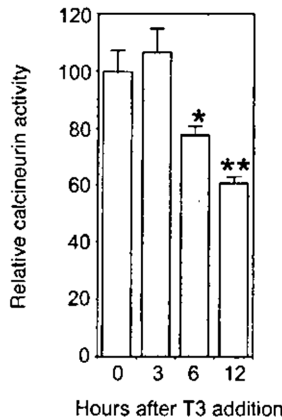

Figure 4 T_3 treatment results in a decrease in endogenous calcineurin activity

Human skin fibroblasts were incubated with T_3 (10^{-8} M). At intervals, the cells were harvested and endogenous calcineurin activity was determined. The calcineurin activity was corrected by the amount of protein. The enzyme activity was expressed as a percentage of the mean value of the control cells at 0 h. The data were expressed as means ± S.D. Statistical significances for 0–3 h and 0–6 h are $^{*}P < 0.05$ and $^{**}P < 0.001$ respectively.

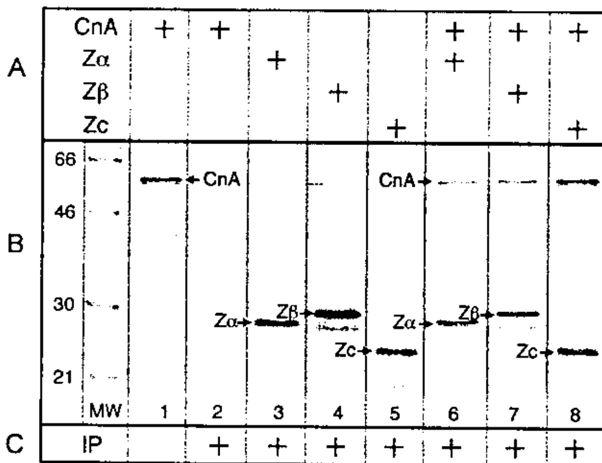

Figure 5 *In vitro* protein–protein binding assay of ZAKI-4 isoforms with calcineurin

CnA, Zα, Zβ and Zc were synthesized by *in vitro* transcription-coupled translation. (**A**) Plasmid used for each translation. (**B**) Image of SDS/PAGE. The position of each product was marked. MW indicates the molecular-mass marker (kDa). (**C**) The + sign indicates immunoprecipitation (IP) with an anti-ZAKI-4 antibody before SDS/PAGE. CnA was not immunoprecipitated by the antibody (lane 2), whereas Zα, Zβ and Zc were precipitated (lanes 3–5). Note that the antibody precipitated not only Zα, Zβ or Zc but also CnA, when they were co-translated (lanes 6–8).

ubiquitous distribution of the β transcript, we analysed its expression in the fibroblasts. As shown in Figure 3, both transcripts were detected in the fibroblasts, raising the question of which transcript responds to thyroid hormone. Northern-blot analysis revealed that T_3 treatment resulted in a marked increase (11-fold) in ZAKI-4α mRNA. However, only a slight increase was observed for ZAKI-4β mRNA. It is thus speculated that the major isoform responsive to thyroid hormone is ZAKI-4α, but not ZAKI-4β, in human skin fibroblasts.

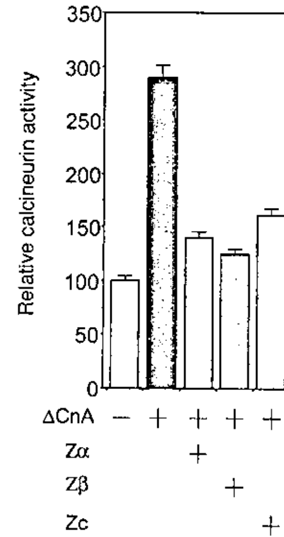

Figure 6 Inhibition of calcineurin activity by ZAKI-4 isoforms

CHO cells were transfected with a plasmid expressing ΔCnA together with the plasmid expressing Zα, Zβ or Zc. The cells were harvested 48 h after transfection for the determination of calcineurin activity. The transfection efficiency was monitored by pCMV β-gal. Transfection of the ΔCnA-expressing plasmid resulted in approx. 3-fold increase in the calcineurin activity. This increase was significantly decreased by the expression of Zα, Zβ or Zc. Note that no significant difference in calcineurin activity was observed by the expression of each isoform.

Inhibition of calcineurin activity by T_3-mediated increase in ZAKI-4α mRNA

Protein products of all the ZAKI-4 gene family share conserved C-terminal amino acid sequence, which has been shown to interact with CnA and inhibit its protein phosphatase activity [6–8]. It is thus possible that T_3-mediated increase in ZAKI-4α isoform inhibits endogenous calcineurin activity. As shown in Figure 4, T_3 treatment resulted in a significant decrease in calcineurin activity (22% of the basal level) at 6 h when ZAKI-4α mRNA started to increase (results not shown). The decrease was more evident (39% of the basal level) at 12 h when the mRNA reached the highest level. These results suggest that T_3-mediated increase in ZAKI-4α isoform is responsible for the decrease in calcineurin activity.

Association of ZAKI-4 isoforms with calcineurin

Although ZAKI-4α and -4β contain an identical C-terminal region harbouring the conserved motif interacting with CnA, it is possible that the N-terminus-specific sequence of ZAKI-4 isoform could modify the binding. We thus examined the interaction of Zα, Zβ and Zc with CnA. All the proteins were synthesized *in vitro* and subjected to immunoprecipitation with an anti-ZAKI-4 antibody. As shown in Figure 5, Zα, Zβ or Zc could be immunoprecipitated (lanes 3–5) by the antibody but CnA alone was not (lane 2). Co-translation of CnA with Zα, Zβ or Zc resulted in the pull-down of CnA with the antibody. It is thus demonstrated that both ZAKI-4α and -4β associate with CnA through their C-terminal common sequence.

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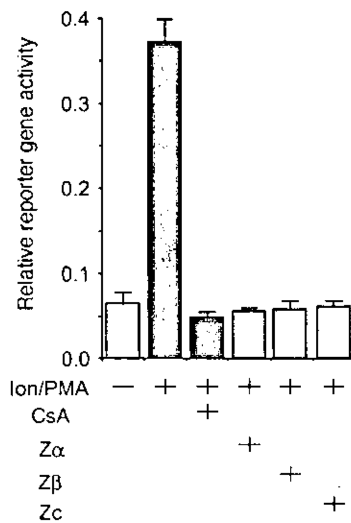

Figure 7 Inhibition of calcineurin-mediated activation of NF-AT by ZAKI-4 isoforms

Jurkat cells were electroporated to transfect pIL2-luciferase together with a plasmid expressing Zα, Zβ or Zc. Transfection efficiency was monitored by co-transfection of pCMV β-gal. Stimulation of calcineurin and subsequent activation of NF-AT were initiated by adding ionomycin (Ion; 2 μM) and PMA (20 ng/ml) 24 h after transfection. CsA (1 μM) was added 15 min before the addition of Ion/PMA. Luciferase activity was measured after 24 h and corrected by β-galactosidase activity. Experiments were performed in triplicate and repeated three times. Data are expressed as means ± S.D.

Inhibition of calcineurin activity by ZAKI-4 isoforms through their common C-terminal region

To address further whether ZAKI-4 isoforms inhibit calcineurin activity *in vivo*, calcineurin activity was determined in CHO cells after transfection with a plasmid expressing ΔCnA together with a plasmid expressing Zα, Zβ or Zc. As shown in Figure 6, transfection of the plasmid expressing ΔCnA resulted in approx. 3-fold increase in calcineurin activity. This increase was markedly inhibited by co-transfection of the plasmid expressing Zα, Zβ or Zc. No significant difference in the degree of inhibition was observed by the expression of Zα or Zβ. These results together with the findings from *in vitro* protein–protein binding assay (Figure 5) suggest that ZAKI-4 isoforms inhibit calcineurin activity through their association with CnA. Furthermore, the difference in the amino acid sequence of ZAKI-4 isoforms at N-termini does not seem to affect the inhibitory action on calcineurin.

Inhibition of calcineurin-mediated NF-AT activation by ZAKI-4 isoforms

We further evaluated whether the inhibition of calcineurin activity by ZAKI-4 isoforms affects the calcineurin-mediated activation of NF-AT in Jurkat cells. When pIL2-luciferase was transfected, activation of calcineurin by ionomycin and PMA resulted in a marked increase in the luciferase activity (Figure 7), confirming the activation of NF-AT. This increase was completely inhibited by the addition of CsA or co-transfection of the plasmid expressing either Zα or Zβ. A similar result was also observed with co-transfection of the plasmid expressing Zc. It is thus speculated that calcineurin-mediated activation of NF-AT could be completely inhibited by overexpressing ZAKI-4α or -4β.

DISCUSSION

This is the first study to demonstrate the existence of three transcripts encoded by ZAKI-4 gene. The FISH analysis as well as BLAST search established that the transcripts are encoded by a single gene (approx. 271 kb) on chromosome 6 (p12). The genomic organization is quite similar to that of DSCR1 in human [5] and mouse [44], both harbouring seven exons. It was noted that the last three exons [5,6] in both ZAKI-4 and DSCR1 genes encode the conserved, C-terminal, CnA-interacting domain [6]. On the other hand, the deduced N-terminal amino acid sequences are divergent between α (25 amino acids) and β (75 amino acids) isoforms of ZAKI-4 gene products. The difference is generated by alternative exon usage, the former being encoded by exon 3 and the latter by exon 4 (Figures 1B and 1C). The sequence difference in N-termini does not seem to interfere with the calcineurin-inhibitory activity, as demonstrated by their similar association with the CnA (Figure 5) and by the inhibition of its enzyme activity (Figure 6). Calcineurin-mediated activation of NF-AT was also inhibited, as demonstrated by the IL-2 reporter gene assay (Figure 7). The result is compatible with a finding that 80-amino-acid deletion from N-terminus of mouse homologue of DSCR1 (corresponding to 80 N-terminal amino acids of α and 131 of β isoforms of human ZAKI-4 gene products) does not impair its calcineurin-inhibitory function [44].

Although not defined in the present study, it is tempting to speculate that the difference in N-termini of α and β isoforms might confer a function other than inhibition of calcineurin.

The Northern-blot analysis revealed that ZAKI-4α transcript is exclusively expressed in the brain, whereas the β transcripts are expressed in brain, heart, skeletal muscle, kidney and other organs (Figure 2). This result indicates that a unique transcription factor(s) is required for tissue-specific expression of each transcript. The regulation of the expression by hormones or other stimuli could be also different among the transcripts. Indeed, thyroid hormone markedly increased the expression of ZAKI-4α but not ZAKI-4β in human skin fibroblasts (Figure 3). This increase in α isoform by T_3 was associated with an inhibition of endogenous calcineurin activity (Figure 4). It is thus suggested that T_3 exerts pleiotropic effect on various cell types through inhibition of calcineurin.

In summary, the expression of ZAKI-4 isoforms is subject to distinct hormonal as well as tissue-specific regulation, constituting a complex signalling network through inhibition of calcineurin.

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Thyroid-hormone-dependent negative regulation of thyrotropin β gene by thyroid hormone receptors: study with a new experimental system using CV1 cells

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The molecular mechanism involved in the liganded thyroid hormone receptor suppression of the TSHβ (thyroid-stimulating hormone β, or thyrotropin β) gene transcription is undetermined. One of the main reasons is the limitation of useful cell lines for the experiments. We have developed an assay system using non-pituitary CV1 cells and studied the negative regulation of the TSHβ gene. In CV1 cells, the TSHβ–CAT (chloramphenicol acetyltransferase) reporter was stimulated by Pit1 and GATA2 and suppressed by T_3 (3,3′,5-tri-iodothyronine)-bound thyroid hormone receptor. The suppression was dependent on the amounts of T_3 and the receptor. Unliganded receptor did not stimulate TSHβ activity, suggesting that the receptor itself is not an activator. Analyses using various receptor mutants revealed that the intact DNA-binding domain is crucial to the TSHβ gene suppression. Co-activators and co-repressors are not necessarily essential, but are required for the full suppression of the TSHβ gene. Among the three receptor isoforms, β2 exhibited the strongest inhibition and its protein level was the most predominant in a thyrotroph cell line, TαT1, in Western blotting. The dominant-negative effects of various receptor mutants measured on the TSHβ–CAT reporter were not simple mirror images of those in the positive regulation under physiological T_3 concentration.

Key words: GATA2, negative-feedback loop, Pit1, thyroid hormone, thyroid hormone receptor, thyroid-stimulating hormone β gene (TSHβ gene).

INTRODUCTION

In the past 15 years, the mechanism of thyroid hormone receptor (TR) function in the positive transactivation of target genes has been studied extensively [1]. TR heterodimerizes with retinoid X receptor (RXR) and binds to a positive T_3 (3,3′,5-tri-iodothyronine)-responsive element (pTRE) on the promoter region of the genes. The typical pTRE is referred to as DR4 (direct repeat 4) in which two half sites (AGGTCA) are aligned in the same direction and separated by a space of four random nucleotides. In the absence of T_3, TR/RXR recruits co-repressors, such as NCoR (nuclear receptor co-repressor) and SMRT (silencing mediator for retinoid and thyroid hormone receptor), and associates with Sin3 and histone deacetylase (HDAC), inducing the suppression of target genes [2]. In the presence of T_3, TR/RXR dissociates from the co-repressor complex and recruits co-activators including p160 family proteins, CBP [CREB (cAMP-response-element-binding protein)-binding protein]/p300, PCAF (p300/CBP-associated factor) and others. The histone acetyltransferase activity of the co-activator complex relaxes the chromatin structure, resulting in transactivation.

Contrary to the positive regulation, little is known about the mechanism of T_3-dependent negative regulation of the genes. Negative regulation of target genes by T_3/TR is very important in terms of thyroid hormone homoeostasis. In fact, Feng et al. [3] have shown that approx. 50% of T_3-dependent genes in the liver are suppressed. Among the negatively regulated genes, TSH (thryoid-stimulating hormone, or thyrotropin) genes, which consist of an α-subunit (TSHα) and a TSH-specific β-subunit (TSHβ), are most important in the hypothalamus–pituitary–thyroid axis. It is clear that TR, especially TRβ, plays a critical role in the regulation of TSH, since patients with resistance to thyroid hormone (RTH) who possess mutations in the TRβ gene show impairment of TSH regulation [4].

Despite its importance, suppression of target genes by T_3/TR has not been investigated extensively. There are several difficulties in the study, and one of the main reasons has been the limitation of useful cell lines for the experiments. To study the positive regulation, the TR and reporter plasmid containing pTRE are introduced into cells such as CV1, and a T_3-induced increase in the transcriptional activity of the reporter gene can be easily observed. To study the negative regulation, however, the basal transcription activity should be high enough to detect the suppression. Unfortunately, it is very difficult to introduce the TSHβ gene into the non-pituitary cells commonly used for transfection experiments. Exceptionally, the transcriptional activity of the TSH gene promoter can be observed in some culture cells, such as GH3, JEG3 and TSA201, and there have been several reports analysing the negative regulation of TSHα and β using these cells [5–8]. Such culture cells do not themselves express the TSH gene, and we

Abbreviations used: CAT, chloramphenicol acetyltransferase; CBP, cAMP-response-element-binding protein-binding protein; CoR, co-repressor; DBD, DNA-binding domain; DMEM, Dulbecco's modified Eagle's medium; DR4, direct repeat 4. ER, oestrogen receptor; FCS, foetal calf serum; HDAC, histone deacetylase; Luc, luciferase; NCoR, nuclear receptor co-repressor; PPAR, peroxisome-proliferator-activated receptor; pTRE, positive T_3-response element; RAR, retinoic acid receptor; RTH, resistance to thyroid hormone; RXR, retinoid X receptor; SMRT, silencing mediator for retinoid and thyroid hormone receptor; SRC-1, steroid receptor co-activator-1; T_3, 3,3′,5-tri-iodothyronine; TR, thyroid hormone receptor; TRH, thyroid-stimulating-hormone-releasing hormone; TSH, thyroid-stimulating hormone (thyrotropin); VDR, vitamin D_3 receptor.

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do not know how transfected TSHα and β genes can be activated in these non-thyrotrophic cells. The fact that the mechanism of the TSH gene activation in these cells is completely unknown hinders the study of the T_3/TR-induced suppression of the TSHβ promoter activity.

The ideal cell line for the TSH study should have the following properties: (i) a reporter gene containing a TSH gene promoter is easily and efficiently expressed, and the basal activity of the expressed TSH in cells is sufficiently high; (ii) the cells do not contain endogenous nuclear receptors; and (iii) the cells are easily maintained under conventional culture conditions. In the present study, we established a new experimental system for the negative regulation of the TSHβ gene by T_3/TR using CV1 cells, the most frequently used cells in the study of T_3-dependent positive regulation. With this assay system, we provide data showing that unliganded TR itself may not be an activator of the TSHβ gene. The intact DNA-binding domain (DBD) of TRβ is crucial to the T_3/TR-dependent TSHβ gene suppression. Co-activators and co-repressors are not necessarily essential, but are required for the full suppression of the TSHβ gene. Among the three TR isoforms, β2 exhibited the most potent inhibitory function, and its protein level was predominant in a thyrotroph cell line, TαT1, in Western blotting. The dominant-negative effects of various TRβ mutants measured on the TSHβ–CAT (chloramphenicol acetyltransferase) reporter were not simple mirror images of those on the reporters for positive regulation under physiological T_3 concentration.

MATERIALS AND METHODS

Plasmid constructions

The TSHβ(−128/+37)–CAT reporter gene was constructed by fusing the human TSHβ promoter (−128 to +37) to the CAT gene [9]. Using partial digestion of TSHβ–CAT with *Eco*RI, the human genomic DNA fragment (−1193 to −129) was ligated into one of two *Eco*RI sites upstream of the TSHβ promoter region (−128 to +37) to generate TSHβ(−1193/+37)–CAT. The cDNA encoding mouse GATA2 was subcloned into the *Hin*dIII/*Xba*I site in pcDNA3 vector (Invitrogen, CA, U.S.A.). The expression vector pCMX containing wild-type human TRα1, human TRβ1 and rat TRβ2 were used. The human TRβ1-deletion mutants (pCI-C1, pCI-C2) [9], mutant TRβ1 in the zinc fingers [C127S, C145G and C164S (single letter amino acid codes, e.g. C127S is a $Cys^{127} \rightarrow$ Ser mutation)] [10] and in the CoR (co-repressor) box {AHT (mutations at positions 228, 229 and 233 in human TRβ1 to glycine, glycine and alanine respectively, as described in [2], according to the numbering system of Sakurai et al. [10a]) and P214R} [2,11] were previously described. The expression vectors pCMX containing the mutants TRβ1-F451X, E449X, K443E, R338W, G345R, F451I and F451L, all identified from patients with RTH, were described elsewhere [12–16]. Mutant TRβ1s (I280K, V284K, I302R and C309K) [17] and rat TRβ2-F504X and E502X, which have identical amino acid mutations with mutant TRβ1-F451X and E449X respectively, were artificially constructed by site-directed mutagenesis (Stratagene, La Jolla, CA, U.S.A.) and confirmed by sequencing.

Cell culture

CV1 and HEK-293T cells were grown in monolayer culture at 37 °C under CO_2/air (1:19) in Dulbecco's modified Eagle's medium (DMEM) containing 10 % (v/v) foetal calf serum (FCS), penicillin G (100 units/ml) and streptomycin (100 μg/ml). TαT1 cells, a thyrotroph cell line from the mouse pituitary, were seeded on Matrigel-coated plates (Becton Dickinson Labware, Bedford, MA, U.S.A.). The cells were maintained under the same conditions as CV1 and HEK-293T cells.

Transient transfection of TSHβ–CAT in CV1 and HEK-293T cells

CV1 and HEK-293T cells were trypsinized and plated in 60-mm diameter dishes for 24 h before the transient transfection using the calcium phosphate technique. The cells at a density of 10^6 cells/plate were transfected with 0.4 μg of wild-type and/or mutant TR expression vector along with 4.0 μg of the TSHβ–CAT reporter gene, 2.2 μg of β-galactosidase expression vector pCH111 (a modified version of pCH110; Pharmacia LKB Biotechnology, Piscataway, NJ, U.S.A.), 0.2 μg of human Pit1 expression vector pCB^{6+}-hPit1, 0.4 μg of mouse GATA2 expression vector pCDNA3-mGATA2 and pCMX empty vector as carrier DNA (a total of 7.2 μg of DNA per dish). After the cells were exposed to the calcium phosphate/DNA precipitates for 20 h, the medium was replaced with fresh DMEM containing 5 % (v/v) FCS depleted of thyroid hormones [18] or the medium was supplemented with 1 μM T_3. After incubation for an additional 24 h, the cells were harvested and the CAT activity was measured as described previously [19]. The transfection efficiency was normalized by the β-galactosidase assay. In each CAT reporter assay, we performed the transfection with CMV (cytomegalovirus)–CAT (10 or 25 ng/dish), the magnitude of which was adjusted to a value of 100 to standardize the activities of TSHβ–CAT.

Antibodies and immunoblotting

The anti-FLAG antibody (M2; Sigma, St. Louis, MO, U.S.A.) was used to detect expression of the FLAG-tagged wild-type TRβ1 (pCMX-FLAG-hTRβ1), pCI-C1 and pCI-C2. The expression of wild-type TRβ1, and AHT, P214R, C309K, E457A, RT338W, G345R, K443E, C446X, E449X, F451X, F451I and F451L mutants were confirmed with the antibody against the C-terminal amino acid sequence of TRβ1 (MA1-215; Affinity Bioreagents, Golden, CO, U.S.A.). The antibody against the common C-terminal 40 amino acids among TRβ1, TRβ2 and TRα1, which was raised previously in our laboratory and referred to as 4BII [20], was utilized to confirm the comparable expression of pCMX-hTRβ1, pCMX-rTRβ2 and pCMX-hTRα1 in CV1 cells. To study the expression of TR isoforms in TαT1 cell, a Western blot was performed with specific antibodies against TRβ1 (J51; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), TRβ2 (06-540; Upstate Biotechnology, Lake Placid, NY, U.S.A.) and TRα1 (PA1-211A; Affinity Bioreagents). Each antibody was diluted with 0.5 % (w/v) non-fat dried milk in PBS. To confirm the expression of plasmids for wild-type and mutant TRs in CV1 cells, 3 μg/6 cm diameter dish of the expression plasmids were transfected and the whole-cell extracts were subjected to Western blotting with specific antibodies. Nuclear extracts from the TαT1 cells were prepared as described previously [9]. Briefly, the cells were suspended in one packed cell volume of buffer A (10 μM Hepes, pH 7.9, 1.5 μM $MgCl_2$ and 10 μM KCl) and sheared by passage through a 25 G needle five times on ice. They were centrifuged at 18000 g for 10 min and resuspended in buffer B [20 μM Hepes, pH 7.9, 25 % (v/v) glycerol, 420 μM NaCl, 1.5 μM $MgCl_2$ and 0.2 μM EDTA] for 30 min at 4 °C. The samples were dialysed against buffer C [20 μM Hepes, pH 7.9, 20 % (v/v) glycerol, 42 μM $(NH_4)_2SO_4$, 0.5 μM dithiothreitol and 0.2 μM EDTA] at 4 °C for 2 h, and stored at −80 °C. The protein concentration was determined by the method of Bradford (Bio-Rad, Hercules, CA, U.S.A.). As controls, we used whole-cell

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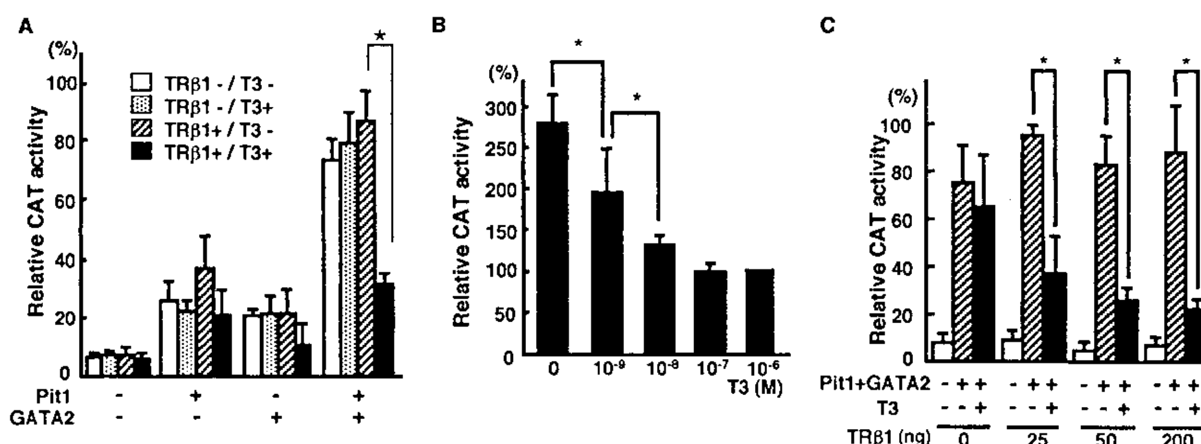

Figure 1 T_3-dependent negative regulation of the TSHβ promoter ($-128/+37$) by TRβ1 in CV1 cells

(**A**) TSHβ($-128/+37$)-CAT plasmid (4.0 μg) was transfected into CV1 cells with or without Pit1 (0.2 μg), GATA2 (0.4 μg) and TRβ1 (0.4 μg) plasmids. After incubation for 24 h with or without 1 μM T_3, the cells were harvested and the CAT activity was measured, with normalizing transfection efficiency by β-galactosidase activity. As an inter-assay control, 10 ng of CMV (cytomegalovirus)–CAT was transfected, the magnitude of which was adjusted to a value of 100. The experiment was repeated several times and the results are means $\pm$ S.D. *$P < 0.05$. (**B**) Dose dependency of T_3. TSHβ($-128/+37$)–CAT, Pit1, GATA2 and TRβ1 were expressed in CV1 cells and 0–1 μM of T_3 was supplemented. The CAT activity at each T_3 concentration was expressed relative to that at 1 μM T_3, which was adjusted to a value of 100. The results are means $\pm$ S.D. for three separate experiments. *$P < 0.05$. (**C**) Dose-dependency of the amount of TRβ1 expressed. pCMX-hTRβ1 (25–200 ng) was transfected in CV1 cells under the same conditions as described in **A**. CMV–CAT (10 ng) was used as the interassay control and the expression level of CMV–CAT was adjusted to a value of 100. The TSHβ($-128/+37$)–CAT activities without Pit1 or GATA2 (open bars), with Pit1 and GATA2 in the absence of T_3 (shaded bars), and with Pit1 and GATA2 in the presence of 0.1 μM T_3 (solid bars) are indicated. The results are means $\pm$ S.D. for four independent experiments. *$P < 0.05$.

extracts of CV1 cells transfected with pCMX plasmid expressing wild-type human TRα1 [21], TRβ1 and rat TRβ2.

Equal amounts (100 μg) of nuclear extract were separated by SDS/PAGE (10% gel) and transferred on to the Immobilon-P membrane (Millipore, Bedford, MA, U.S.A.). The membrane was incubated overnight at 4 °C in blocking solution [10% (w/v) non-fat dried milk in PBS]. After the membrane was washed in PBS with 0.05% (v/v) Tween 20 three times for 10 min, it was incubated for 1 h at room temperature (25 °C) with the TR-isoform-specific antibodies described above. After incubation with horseradish-peroxidase-linked donkey anti-mouse or anti-rabbit immunoglobulin (Amersham Biosciences, Little Chalfont, Bucks., U.K.) diluted 1:500 for 1 h at room temperature, the antibody–protein complexes were visualized using an ECL® (enhanced chemiluminescence) detection reagent (Amersham Biosciences).

Statistics

Each experiment was performed in duplicate more than three different times and each result is expressed as the mean $\pm$ S.D. The statistical significance was determined by ANOVA and Fisher's Protected Least Significant Difference (PLSD) test using StatView 4.0 software (Abacus Concepts, Berkeley, CA, U.S.A.). $P < 0.05$ was considered significant.

RESULTS

T_3-dependent suppression of the TSHβ gene by TR in CV1 cells

We have established an assay system to study the negative regulation of the TSHβ gene using CV1 cells. When simply transfected into CV1 cells, the TSHβ($-128/+37$)–CAT reporter gene was very low in its activity. Although the expression of Pit1 alone or GATA2 alone did not highly stimulate the activity, co-expression of both transcription factors dramatically enhanced the TSHβ promoter activity. The expression of TRβ1 alone did not stimulate the TSHβ gene activity when T_3 was absent, suggesting that TRβ1 itself was not an activator of the TSHβ gene (Figure 1A). This result was confirmed independently by two different researchers repeating the experiments several times. The transfection method, either calcium phosphate or lipofection, did not affect the result (results not shown). A supplement of T_3 decreased the TSHβ ($-128/+37$)–CAT activity dose-dependently, and 1 nM T_3 suppressed it significantly, at approx. 25% of the basal activity without T_3 (Figure 1B). T_3-induced suppression of the TSHβ gene was dependent on the amount of TRβ1 (Figure 1C). Without Pit1 and GATA2, even very large amounts of unliganded TRβ1 did not activate the TSHβ gene.

Nuclear receptor specificity in TSHβ gene regulation

The negative regulation of TSH is specific to T_3 *in vivo*. Since our assay system contains no endogenous nuclear receptor, we examined the effects of other receptors including vitamin D_3 receptor (VDR), RXRβ, oestrogen receptor (ER) α, retinoic acid receptor (RAR) α and peroxisome-proliferator-activated receptor (PPAR) γ2 on the TSHβ($-128/+37$)–CAT activity to see whether or not suppression of the TSHβ–CAT reporter gene was specific to T_3/TR. In the presence of cognate ligand, no significant suppression was detected by any other nuclear receptors, except for ERα, which decreased the CAT activity slightly, but significantly (Figure 2).

The importance of the DBD of TRβ1

The functions of the receptor domains in terms of the negative regulation of the TSHβ gene were examined using various mutant TRβ1s which possessed functional disruption in each domain. The mutant C1, which lacked the N-terminal A/B domain of TRβ1, showed T_3-dependent negative regulation, whereas mutant C2, which lacked the DBD, failed to suppress the TSHβ($-128/+37$)–CAT (Figure 3A). As expected, a truncated TR of the

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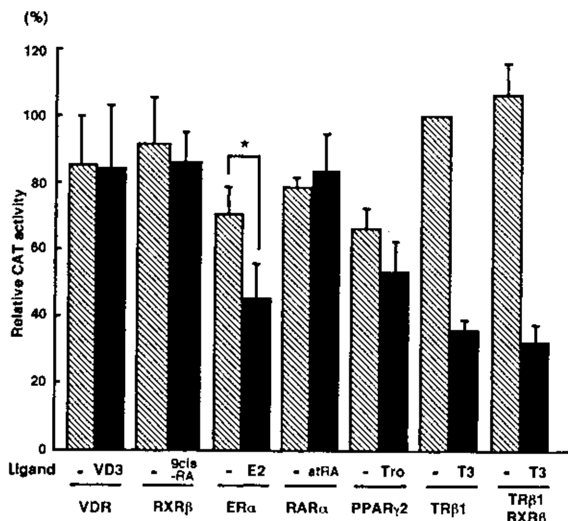

Figure 2 Receptor specificity in the negative regulation of the TSHβ gene promoter

The expression plasmids for VDR, RXRβ, ERα, RARα and PPARγ2 were co-transfected into CV1 cells in the absence (shaded bars) or presence (solid bars) of 1 μM 1α25(OH)$_2$ vitamin D$_3$ (VD3), 9-*cis* retinoic acid (9-*cis* RA), oestradiol (E2), all-*trans* retinoic acid (atRA) and troglitazone (Tro), respectively. The CAT activity of the cells expressed with TRβ1 in the absence of T$_3$ is represented as 100 and other activities are expressed as relative values. The results are means ± S.D. for four experiments. *$P < 0.05$.

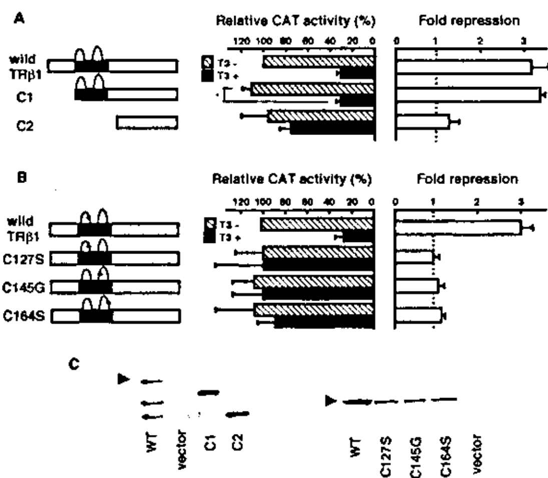

Figure 3 Regulation of TSHβ promoter by mutant TRβ1s possessing deletions in the A/B or A/B + C domains or mutations in the zinc fingers

(A) The effect of the deletion in the A/B domain (C1) or A/B + C domains (C2). **(B)** The effects of mutant TRβ1s which have point mutations in the zinc-finger motifs in the DBD. The CAT activity of the cells expressed with wild-type TRβ1 in the absence of T$_3$ is represented as 100 and other activities are expressed as relative values. The fold repression was calculated from the CAT activity without T$_3$ (shaded bar) divided by that with T$_3$ (solid bar). The results are means ± S.D. for four separate experiments. *$P < 0.05$. **(C)** Expression of wild-type or mutant TRβ1s in CV1 cells. Whole-cell extracts of CV1 cells transfected with equal amounts of expression plasmids for wild-type and mutant TRβ1s were analysed by Western blotting with anti-FLAG antibody (left panel) and the antibody against the C-terminal amino acid sequence of TRβ1 (right panel). The position of wild-type TRβ1 is indicated by the arrowhead.

ligand-binding domain did not exhibit the suppression, since it lost T$_3$ binding (results not shown). To investigate the DBD function further, we examined three different TRβ1s possessing

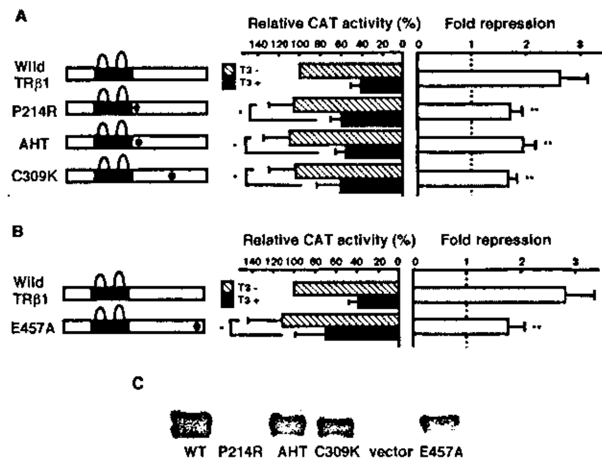

Figure 4 Effects of the mutant TRβ1s, which have impaired co-repressor-binding (A) or co-activator-binding ability (B), on T$_3$-dependent suppression of TSHβ(− 128/ + 37)–CAT reporter gene

The CAT activity of the cells expressed with wild-type TRβ1 in the absence of T$_3$ was represented as 100 and other activities were expressed as relative values. The fold repression was calculated from the CAT activity without T$_3$ (shaded bar) divided by that with T$_3$ (solid bar). The results are means ± S.D. for four to seven separate experiments. *$P < 0.05$. **(C)** Western blot analysis of mutant TRβ1s expressed in CV1 cells performed under the same conditions as in Figure 3(C).

point mutations in the zinc fingers. C127S, C145G and C164S mutants all abrogated the negative regulation, indicating that an intact DBD is essential (Figure 3B). There was no remarkable difference in the expression level among mutant TRβ1s (Figure 3C).

The functions of co-repressors and co-activators

To study whether the co-repressors participate in the T$_3$/TR-dependent suppression of the TSHβ gene, we examined the functions of the mutant TRs, which had impaired co-repressor binding. Two CoR-box mutants, P214R and AHT, the properties of which have been extensively studied [2,22], significantly suppressed the TSHβ(− 128/ + 37)–CAT activity (Figure 4A). Recently, Marimuthu et al. [17] identified TR surfaces that interact with NCoR, reporting that a CoR box is not important. Among the mutant TRs, we confirmed that C309K possessed normal T$_3$-binding activity and did not interact with NCoR or SMRT in the absence of T$_3$. It exhibited no silencing activity, nor dominant-negative function against wild-type TR on pTRE (results not shown). C309K significantly decreased the TSHβ–CAT activity similarly to P214R and AHT (Figure 4A). The strength of suppression by P214R, AHT and C309K was diminished slightly compared with that by wild-type TRβ1. The data suggested that co-repressors are not critical, but may be required for full suppression by T$_3$/TR.

Another mutant TRβ1, E457A, has been known not to bind co-activators, despite its normal T$_3$-binding ability [23]. As shown in Figure 4B, E457A could also decrease the TSHβ(− 128/ + 37)–CAT activity. The extent of the decrease was, however, significantly smaller by E457A than by wild-type TRβ1 (36.6 ± 15.8 % compared with 59.3 ± 13.4 %, $P < 0.05$). This indicates that a co-activator is not indispensable, but is again required for T$_3$/TR-induced full suppression of the TSHβ gene. When we checked the protein levels of TRs by Western blotting, we found unexpectedly that the expression of P214R was low, while that of

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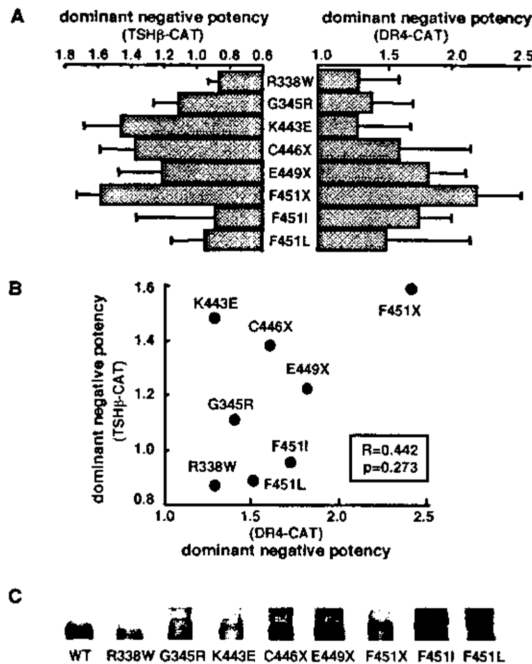

Figure 5 Dominant-negative potency of mutant TRβ1s on the positive and negative TREs

(**A**) Equal amounts (0.4 μg) of expression vectors for wild-type and various mutant TRβ1s were transfected into CV1 cells, and transcriptional activities were measured using DR4-CAT (right) and TSHβ($-128/+37$)-CAT reporter gene (left) in the presence of 1 nM T_3. Dominant-negative potency was calculated from the following ratio: CAT activities of wild-type TRβ1 only/CAT activities of wild-type + mutant TRβ1 on DR4-CAT and CAT activities of wild-type + mutant TRβ1/CAT activities of wild-type TRβ1 only on TSHβ-CAT. Results are means $\pm$ S.D. for three to six experiments. (**B**) Correlation of the dominant-negative potency between DR4-CAT and TSHβ($-128/+37$)-CAT. There was no significant correlation at 1 nM T_3. (**C**) Protein expression of mutant TRβ1s in CV1 cells was analysed under the same conditions as in Figure 3(C).

AHT, C309K and E457A was comparable. The CV1 cells that expressed P214R exhibited transcriptional activity as high as wild-type TR when T_3 was supplemented (results not shown). It is unlikely that the expression level of P214R was very low; rather the antibody against TR that was used might have failed to recognize the conformation of P214R.

Dominant-negative effect of various mutant TRβ1s identified from patients with RTH on TSHβ($-128/+37$)-CAT

Various mutant TRβ1s identified from patients with RTH express inhibitory effects on the wild-type TRβ1 functions. For example, co-transfection of equimolar amounts of mutant TRβ1s impaired the T_3-dependent transactivation of wild-type TRβ1 on pTREs, such as DR4, as shown in our previous study [24]. Our present assay system has enabled us to examine the negative regulation of the TSHβ promoter by T_3/TR as well as the positive regulation on pTRE using the same CV1 cells under similar assay conditions. We measured the transcriptional activities on DR4-CAT and TSHβ($-128/+37$)-CAT after transfection with an equal amount of wild-type and various mutant TRs. The CAT activity of each mutant TRβ1 was assayed at the physiological T_3 concentration of 1 nM and expressed relative to that of wild-type TRβ1. The dominant-negative potency was calculated from CAT activities of wild-type + mutant TRs and wild-type TR only (Figure 5A). When the dominant-negative potencies were compared between the positive and negative TREs, a correlation was not obtained, suggesting that the negative regulation by T_3/TR might not be a simple mirror image of positive regulation (Figure 5B). The expression levels of wild-type and mutant TRβ1 were confirmed (Figure 5C).

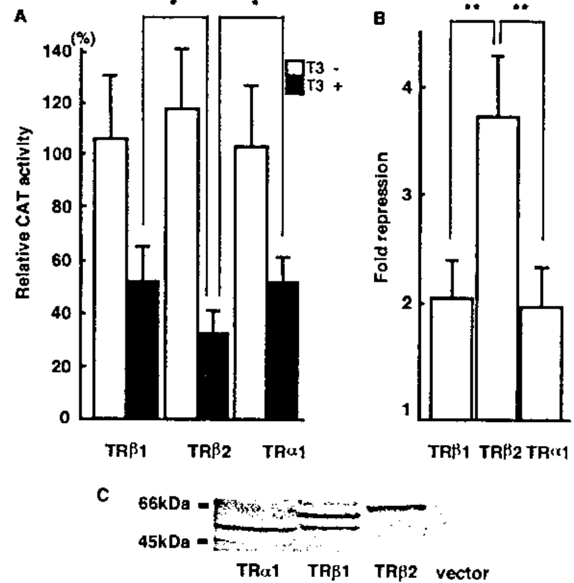

Figure 6 T_3-dependent inhibition of the TSHβ-CAT reporter gene activity by TR isoforms

(**A**) Expression vectors of TRβ1, TRβ2 or TRα1 were transfected into CV1 cells, and the transcriptional activity of the TSHβ($-128/+37$)-CAT gene was assayed in the presence (closed bars) or absence (open bars) of 1 μM T_3. Results are means $\pm$ S.D. for four experiments. (**B**) The fold repression is indicated as the ratio between the CAT activity in the absence and that in the presence of T_3. (**C**) Western blot analysis was performed using the anti-TR antibody, 4B11, raised against the C-terminal 40 amino acids common to TRα1, β1 and β2.

Comparison of suppressive efficiency of the three TR isoforms on the TSHβ promoter

The functional properties of three active TR isoforms, TRβ1, TRβ2 and TRα1, were compared in the context of suppression of the TSHβ promoter (Figure 6). In the presence of T_3, the TSHβ($-128/+37$)-CAT activity was inhibited approx. 50% by TRα1 and TRβ1, and 70% by TRβ2 compared with the basal activity with empty vector. All three TR isoforms are functional and TRβ2 exhibits the strongest inhibition among them. The protein expression was confirmed using antibody against the C-terminal 40-amino-acid sequence common to the three isoforms [20]. Collectively, TRβ2 exhibited the strongest inhibition among them (Figure 6).

Protein expression of three TR isoforms in the pituitary thyrotroph cell line, TαT1

The transfection study in CV1 cells showed the predominant suppressive efficiency of TRβ2. Thus it is desirable to examine the protein expression of the three TR isoforms in pituitary thyrotrophs. Since it is difficult to isolate sufficient amounts of thyrotrophs, we studied the TR protein level using a thyrotroph cell line, TαT1. As positive controls of the Western blot analysis, the whole-cell extracts of CV1 cells transfected with expression

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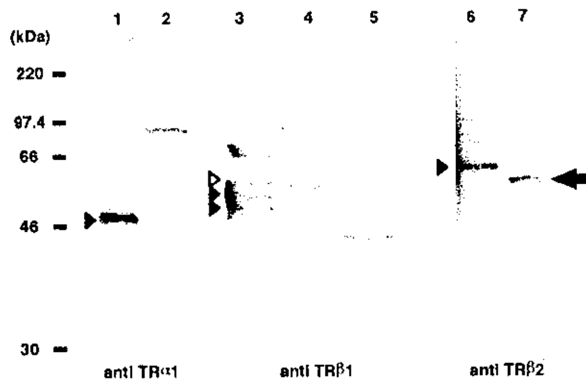

Figure 7 Protein expression of TRα1, β1 and β2 in mouse thyrotroph TαT1 cells

Nuclear extracts (100 μg) from TαT1 cells (lanes 2, 5 and 7) and whole-cell extracts from CV1 cells transfected with empty plasmid (lane 4) or plasmid for human TRα1 (lane 1), β1 (lane 3) or rat β2 (lane 6) were resolved by SDS/PAGE (10% gel). The blot was probed with isoform-specific antibody against TRα1 (lanes 1 and 2), TRβ1 (lanes 3–5) and TRβ2 (lanes 6 and 7), followed by chemiluminescent detection. The protein standards are shown on the left (molecular mass in kDa). The position of wild-type TR isoforms and endogenous TRβ2 in TαT1 cells are indicated as solid arrowheads and an arrow respectively. The open arrowhead indicates the non-specific protein in CV1 cells.

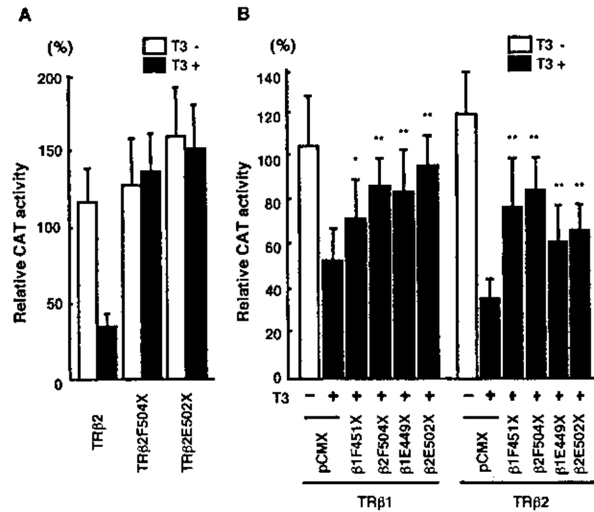

Figure 8 Functional properties of the mutant TRβ2-F504X and E502X which have the C-terminal truncations identical with those of TRβ1-F451X and E449X respectively

(**A**) The effects on the TSHβ promoter (−128/+37). Expression vectors for wild-type or mutant TRβ2s were transfected into CV1 cells and transcriptional activities were measured in the absence (open bars) and presence (closed bars) of 1 μM T_3. The CAT activities are indicated as percentages compared with the activities in which empty vector was co-transfected. Result are means ± S.D. for four experiments. (**B**) The dominant-negative effects by mutant TRβ1s or TRβ2s. Expression vectors of mutant TRβ1s or TRβ2s were co-transfected into CV1 cells together with equimolar wild-type TRβ1 or TRβ2. Transcriptional activities were measured in the absence or presence of 1 μM T_3. The CAT activities are indicated as percentages compared with the activities in which empty vector was co-transfected. Results are means ± S.D. for four experiments. *$P < 0.05$, **$P < 0.01$.

vector encoding wild-type human TRα1 and TRβ1, and rat TRβ2 were used. As shown in Figure 7, the antibodies against each TR isoform detected the cognate receptors expressed in CV1 cells with similar intensity, suggesting that the recognition ability of each antibody is comparable. In TαT1 nuclear extracts, only the protein band of TRβ2 was detected with anti-TRβ2 antibody, the molecular mass of which was identical with that calculated from the reported amino acid sequence of mouse TRβ2 (54 kDa), slightly smaller than that of rat TRβ2 (58 kDa). No signals of TRα1 and TRβ1 were observed, indicating that at least TRβ2 predominantly exists in this thyrotroph cell line.

Mutant TRβ2 exhibited dominant-negative potency

Since TRβ2 exhibited the most potent inhibitory activity in TSHβ-CAT, and since TRβ2 is clearly present in thyrotroph TαT1 cells, we determined whether or not mutant TRβ2s showed dominant-negative effects similar to the mutant TRβ1s. We constructed the mutants TRβ2-F504X and E502X possessing C-terminal truncations identical with those of TRβ1-F451X and E449X respectively. They did not mediate T_3-dependent negative regulation because of the deletion of the T_3-binding activity (Figure 8A), and exhibited similar dominant-negative potencies against wild-type TRβ2, as well as TRβ1 (Figure 8B). No significant difference was observed between the inhibitory effects of mutant TRβ1s and mutant TRβ2s.

TSHβ gene suppression by T_3/TRβ2 using different culture cells or a reporter gene containing a longer promoter region

We confirmed T_3-dependent suppression of the TSHβ gene by TRβ2 using different culture cells or a reporter gene containing a longer human TSHβ promoter. HEK-293T cells, which have no endogenous nuclear receptors, exhibited the enhanced TSHβ (−128/+37)–CAT activity with expression of Pit1 and GATA2. TRβ2 significantly decreased the CAT activity by administration of 1 μM T_3 (Figure 9A). The TSHβ(−1193/+37)–CAT reporter gene activity in CV1 cells also showed augmentation by Pit1 and GATA2, and a significant decrease by 1 μM T_3. These indicate that TSHβ gene suppression by T_3/TR in our assay system does not relate to the cell lines used or small fragment of the promoter used.

DISCUSSION

In the present study, we tried to establish a suitable new assay system to examine the T_3/TR-dependent transcriptional repression of the TSHβ gene promoter, considering the following two points: (i) using a conventional culture cell line and (ii) using a CAT, not a luciferase, reporter system. The CV1 cell line is derived from monkey kidney cells and is one of the most frequently used cells for transfection experiments of nuclear receptors because of its good transfection efficiency, ease of handling and lack of endogenous nuclear receptors. In fact, numerous studies have been performed on T_3/TR-dependent transcriptional regulation of a reporter gene containing pTRE using CV1 cells. Unfortunately, no basal activity of the TSHβ gene is usually obtained when simply introduced into these cells. Pit1 and GATA2 have been reported to be important for TSHβ expression [25] and to be determinants of the differentiation from immature pituitary cells to thyrotrophs [26]. We confirmed that the expression of these pituitary-specific transcriptional factors in CV1 cells enhanced the TSHβ promoter activity. The activity was sufficiently high, and when TR was co-expressed, it decreased the TSHβ promoter activity in a T_3-dependent manner. The fold suppression was dose-dependent of the ligand concentration and the receptor amount. In addition,

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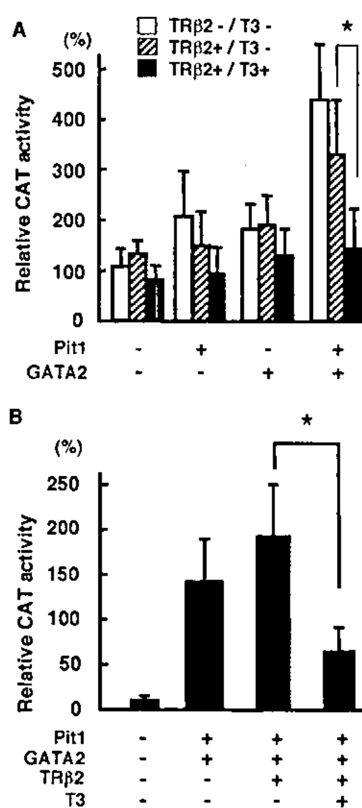

Figure 9 T_3-dependent suppression of the TSHβ gene by TRβ2

(A) TSHβ ($-128/+37$)–CAT plasmid (4.0 μg) was transfected into HEK-293T cells with or without Pit1 (0.2 μg). GATA2 (0.4 μg) and TRβ2 (0.4 μg) plasmids. The experimental procedure was the same as in Figure 1. **(B)** The CAT reporter gene containing TSHβ ($-1193/+37$) promoter was introduced into CV1 cells. Results are means $\pm$ S.D. for four experiments. $^*P < 0.05$.

RARα, VDR, PPARγ2 and RXRβ, nuclear receptors belonging to the same subgroup of TR, did not affect the transcriptional activity, supporting the ligand/receptor specificity in feedback regulation of TSHβ expression and the validity of this assay system (Figure 2).

The second point we considered, using a CAT, not a luciferase, reporter system, is important, although it has often been overlooked. A luciferase reporter gene itself was found to mediate the T_3-dependent decrease in the activity in CV1 [27], JEG3 and other cells [28]. There have been many investigations of the T_3-dependent negative regulation of the TSH gene using a luciferase reporter system [5,6,8,22,29,30]. It may sometimes evoke confusing results of T_3/TR-dependent decrease in the reporter activity caused by the TSH promoter or by the luciferase gene itself.

The important finding in this study is that unliganded TR itself does not enhance the basal transcriptional activity of the TSHβ promoter. No basal TSHβ promoter activity was obtained without co-expression of activator(s) in our assay system and simple introduction of TR did not activate the TSHβ gene. Pit1 and GATA2 were absolutely required to enhance it. Furthermore, co-expression of TR did not stimulate the Pit1/GATA2-driven TSHβ–CAT promoter further. This was confirmed with two different cell line cultures, and with two different shorter and longer TSHβ promoters. Clinically, one may easily misinterpret that unliganded TR itself is an activator of the TSHα and TSHβ genes, since thyroid hormone deficiency enhances TSH production *in vivo*. If this is the case, the absence of TR should cause a decrease in the transcriptional activity of the TSH gene. Knockout mice targeting TRβ alone [31], or both TRα and TRβ [32], did not support this. The serum TSH level and TSHβ mRNA in the pituitary remarkably increased in these mice. Weiss et al. [33] examined precisely TRβ1-knockout mice and concluded that the absence of TRβ does not impair the up-regulation of TSH induced by T_3 deprivation. Collectively, these data, including our findings in the present study, may support the notion that it may not be unliganded TR itself, but another factor that stimulates the TSH gene transcription.

When we studied the functions of each domain of TR in terms of the negative regulation of TSHβ, we found that the intact C domain (DBD) was critical to express the suppression. Mutant TRβ1-C2 with deletion of the A/B and C domains, but not C1 with the A/B deletion, failed to express the inhibition. Three different zinc-finger mutants also demonstrated the absolute necessity of an intact DBD. This may raise a possibility that TR binding to some specific DNA region on the promoter is essential. Immediately downstream of the transcriptional start site in the TSHβ promoter, there is a short sequence, GGGTCA, which is similar to a single half site of pTRE. Several reports have suggested that this sequence might mediate the T_3-dependent negative regulation [34–36]. We are currently examining the significance of this region using various mutated TSHβ reporter genes in our assay system. An alternative possibility is that the DNA-binding activity of TR is not necessarily crucial, but the receptor conformation with an intact DBD is important. In addition to DNA binding, DBD has some other important functions. For example, we reported previously that DBD of TR interacted with HDAC2 in a T_3-dependent manner on the TSHβ promoter [9]. Destruction of DBD may change the conformational structure of the receptor and hinder the recruitment of necessary factor(s) for negative regulation of the TSHβ promoter, resulting in failure of transcriptional repression.

The contribution of co-repressors such as NCoR and SMRT to the T_3/TR-dependent TSHβ suppression is considered to be small. The two CoR-box mutants, P214R and AHT, could suppress the TSHβ($-128/+37$)–CAT activity. Recently, Marimuthu et al. [17] have identified residues of TR that are critical for NCoR binding by testing more than 100 separate mutations of human TRβ. Since the CoR box was not important for NCoR-binding according to their report, we generated four TR mutants of the receptor surfaces for NCoR binding (results not shown). All of them showed impaired NCoR binding, but three of them also lost T_3-binding ability and were unsuitable for the study of T_3-dependent negative regulation. C309K had T_3-binding ability and significantly decreased the TSHβ($-128/+37$)–CAT activity similarly to P214R and AHT. The fact that two CoR-box mutants and also one TR mutant in the 'co-repressor-binding surface' could exhibit the T_3-dependent suppression of TSHβ($-128/+37$)–CAT activity suggests that co-repressors are not critical.

Tagami et al. [8] proposed a two-step model in negative regulation of the TSH promoter by T_3/TR: (i) unliganded TR, which does not bind to the TSH gene, recruits co-repressors, such as SMRT and NCoR, and withdraws HDAC from the basal promoter to cause activation; (ii) T_3 binding to TR dissociates the co-repressors/HDACs, thereby causing T_3-dependent repression of the TSH gene. In their study, the DBD was not important, and mutation in the CoR-box region was fatal. The results of our present study were very different from theirs and did not support their model. As described, unliganded TR itself did not stimulate the basal transcriptional activity. The DBD of TR is crucial to the TR-mediated suppression. Additionally, RAR that can recruit

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the co-repressors similarly to TR did not affect the basal activity in our experiment. According to their model, liganded RAR should also decrease the TSHβ(− 128/ + 37)–CAT activity. Furthermore, our study showed that co-repressors are not necessarily critical. The reason for the discrepancy between their data and ours is unknown, but two points described previously should be noted. First, TSA201 and JEG3 cells used in their study exceptionally exhibit some degree of the basal transcriptional activity of TSHα and β promoter when introduced, and the mechanism of the activation in these cells is uncertain. It may be conceivable that co-expressed TR interacts with some endogenous factors necessary for the TSH-gene activation, causing augmentation of the basal transcriptional activity in these cells. Secondly, they used the luciferase reporter gene in their assay system, which is known to show a T_3-dependent inhibition of the activity.

E457A, which does not bind co-activators despite its normal T_3-binding ability, significantly decreased the TSHβ(− 128/ + 37)–CAT activity, but not to the extent of wild-type TRβ1. This indicates that co-activators such as SRC-1 (steroid receptor co-activator-1) are not indispensable, but are required to express the full suppression of the TSHβ gene by T_3/TR. The result seems to agree well with a study of SRC-1-knockout mice, which showed partial resistance to T_3 in TSH suppression [37]. The TSHβ mRNA levels in SRC-$1^{+/+}$ and SRC-$1^{-/-}$ mice were comparably high after treatment with low iodine/propylthiouracil, and administration of T_3 resulted in a marked decrease in TSHβ mRNA in SRC-$1^{+/+}$ mice, but blunted the reduction in SRC-$1^{-/-}$ mice significantly. The findings that the T_3-mediated suppression of the TSHβ promoter was attenuated in CV1 cells expressed with E457A and also SRC-1-knockout mice indicate that co-activator SRC-1 partially participates in T_3/TR-dependent transcriptional suppression of the TSHβ gene.

Although all three functional TR isoforms showed the suppression of TSHβ promoter, TRβ2 exhibited the strongest inhibition among them. TRα1 and β1 are expressed ubiquitously in the tissues [38–40], whereas TRβ2 is expressed specifically in the anterior pituitary and hypothalamus [41,42]. A pituitary somatolactotroph cell line GH3 expresses both TRβ1 and β2 equally, but TRβ2 is more abundant than TRβ1 in the rat pituitary gland [41]. Childs et al. [43] demonstrated the expression of the message and protein of TRβ2 in rat thyrotrophs by *in situ* hybridization and immunohistochemical staining. In the present study, we also demonstrated that at least TRβ2 protein exists in a thyrotroph cell line, TαT1, by immunoblotting. In functional experiments, Langlois et al. [44] reported that TRβ2 showed greater T_3-dependent repression than TRα1 and TRβ1 in the negative regulation of the TRH (TSH-releasing hormone) gene. Transgenic mice with targeted disruption of the TRβ2 gene demonstrated elevated basal T_3 and T_4 (thyroxine) concentrations associated with inappropriate TSH production, suggesting that TRβ2 plays an important role in the hypothalamus–pituitary–thyroid axis [45]. These data and ours indicated that TRβ2 has pivotal roles in the negative regulation of TSH and TRH.

Our present assay system enabled us to compare the dominant-negative potency of mutant TRs on the positive and negative target-gene promoters under the same conditions using the same cells. When eight different mutants identified from patients with RTH were studied, no correlation was observed between dominant-negative potency on DR4–CAT and TSHβ–CAT at the physiological concentration of 1 nM T_3 (Figure 5B). Although the interpretation of the data should be cautious, this may suggest that the dominant-negative effect of mutant TRβs on the negative regulation is not the simple mirror image of that on the positive regulation, as previously reported [23].

The mechanism of how TR inhibits the transcriptional activity of the TSHβ promoter in a T_3-dependent fashion remains to be investigated. Pit1 and GATA2, which activate the TSHβ gene, have been known to interact with nuclear receptors. Palomino et al. [46] found a possible interaction between TR and Pit1. The GATA family proteins have been reported to be associated with ER [47], glucocorticoid receptor [48] and steroidogenic factor 1 [49]. We currently postulate a model that liganded TR associated with HDAC2 on the TSHβ gene promoter may interact directly with Pit1 and/or GATA2, resulting in the inactivation of their functions as activators. In this sense, it is interesting that liganded ER, which associates with GATA2, did suppress the TSHβ–CAT activity slightly, but significantly in our study (Figure 2).

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Association between activating mutations of calcium-sensing receptor and Bartter's syndrome

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Bartter's syndrome is a heterogeneous disorder characterised by deficient renal reabsorption of sodium and chloride, and hypokalaemic metabolic alkalosis with hyper-reninaemia and hyperaldosteronaemia. Mutations in several ion transporters and channels have been associated with the pathogenesis of Bartter's syndrome. We describe two hypocalcaemic patients with deficient parathyroid hormone secretion who also showed characteristics of Bartter's syndrome. We found activating mutations of the gene for the calcium-sensing receptor (CASR) in both patients. Activation of this calcium-sensing receptor inhibits the activity of a renal outer-medullary potassium channel that is mutated in type 2 Bartter's syndrome. We therefore suggest that some activating mutations of *CASR* could provide new mechanisms for the development of Bartter's syndrome.

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Bartter's syndrome is a heterogeneous disorder characterised by deficient renal reabsorption of sodium and chloride, and hypokalaemic metabolic alkalosis with hyper-reninaemia and hyperaldosteronaemia. Mutations in several genes have been associated with the disorder. Recessive inactivating mutations of the gene encoding the sodium-potassium-chloride cotransporter (*NKCC2*) cause type 1 Bartter's syndrome, and the gene for the renal outer-medullary potassium channel (*ROMK*) is mutated in type 2 Bartter's syndrome. Type 3 Bartter's syndrome results from deficient function of the chloride channel CLCKB, and type 4 Bartter's syndrome is caused by inactivating mutations of *BSND*, the gene for the β-subunit of CLCKB.[1] Gitelman's syndrome is a hypocalciuric variant of Bartter's syndrome caused by inactivating mutations in the gene for the sodium-chloride cotransporter of the distal convoluted tubule (NCCT).

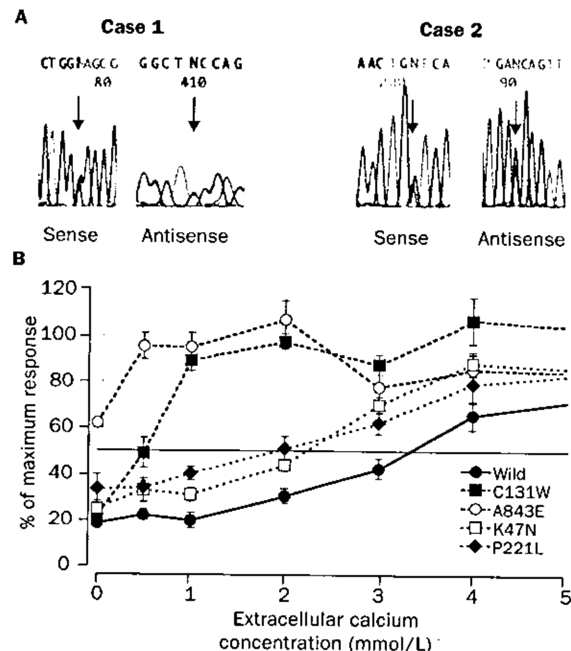
	Case 1			Case 2	Reference range
	Age 6 years	Age 7 years	Age 19 years	Age 26 years	
Na (mmol/L)	145	144	143	141	138–144
K (mmol/L)	2·8	2·8	2·9	3·2	3·5–5·0
Cl (mmol/L)	105	106	100	100	98–107
Ca (mmol/L)	2·0	2·0	1·9	2·1	2·1–2·6
P (mmol/L)	2·1	2·0	1·4	1·2	0·8–1·5
Mg (mmol/L)	0·5	0·5	0·3	0·5	0·7–1·0
Cr (μmol/L)	44	62	203	97	53–97
HCO_3^- (mmol/L)	..	..	30·9	27·1	22–26
Intact parathyroid hormone (ng/L)	..	..	<5	<5	13–53
Plasma renin activity (μg/L/h)	7·2	..	9·2	3·4	0·2–2·7
Aldosterone (ng/L)	141	..	348	150	20–130
FE_{Na} (%)	1·35	1·29	1·50	1·52	1·25–2·50
FE_{K} (%)	26·2	28·3	38·6	14·5	12·5–25·0
FE_{Cl} (%)	2·03	1·91	2·52	2·21	1·62–3·24
FE_{Ca} (%)	3·9	2·2	2·2	3·4	1·0–2·0
FE_{Mg} (%)	13·9	..	14·2	5·2	3·5–7·0

Both patients were being treated with active vitamin D_3. FE=fractional excretion.

Biochemical features of the two patients

The calcium-sensing receptor (CASR) is a G-protein-coupled receptor essential for regulating secretion of parathyroid hormone.[2] In addition to the parathyroid, CASR is expressed in several other tissues, including the kidney. Activation of CASR by high concentrations of extracellular calcium ions inhibits the activity of ROMK in rats.[3] Because potassium secretion by ROMK is essential for maintenance of positive luminal potential in the thick ascending limb of Henle that drives paracellular reabsorption of calcium and magnesium, inhibition of ROMK by calcium via CASR is expected to increase urinary excretion of these cations. In fact, activating mutations of CASR cause autosomal dominant hypocalcaemia (ADH; Online Mendelian Inheritance in Man [OMIM] number 601199) associated with relative hypercalciuria.[3] We describe two patients with ADH who also had features of Bartter's syndrome.

Case 1 was a 19-year-old man who had shown tetany soon after birth and was found to have striking hypocalcaemia. He has been treated with active vitamin D_3 since then. Because of nephrocalcinosis, his renal function gradually deteriorated (table). He also had hypomagnesaemia, hypokalaemia with metabolic alkalosis, hyper-reninaemia, and hyperaldosteronaemia (table). Fractional excretion of potassium, calcium, and magnesium was high. His parents' serum electrolytes showed no abnormality. Trichlormethiazide was given periodically to reduce urinary calcium between the ages of 8 and 18 years. Case 2 was a 26-year-old woman who had



Analysis of DNA sequence for CASR gene and function of mutant CASRs

A: DNA sequence of *CASR* gene shows heterozygous substitution of GAA for GCA at codon 843 in case 1, and heterozygous substitution of TGG for TGC at codon 131 in case 2 (arrows). B: Production of inositol 1,4,5-trisphosphate (IP_3) in response to extracellular calcium in COS-1 cells transfected with cDNA for two mutant CASRs associated with characteristics of Bartter's syndrome (C131W and A843E), and two mutant CASRs not associated with Bartter's syndrome (K47N and P221L). Results are expressed as a percentage of response by wild-type CASR. Horizontal line=half-maximum effective concentration (EC_{50}).

also shown tetany and hypocalcaemia soon after birth. She had been treated with vitamin D metabolites and calcium. She also had nephrocalcinosis, hypomagnesaemia, hypokalaemia with metabolic alkalosis, hyper-reninaemia, and hyperaldosteronaemia (table). Fractional excretion of potassium and magnesium was normal, but fractional excretion of calcium was high. She had never taken diuretics. Her parents were heathly. Neither patient had hypertension.

The association between severe hypocalcaemia and characteristics of Bartter's syndrome has not been reported previously, suggesting that clinical features of these patients are not derived from mutations known to cause Bartter's syndrome. Hypocalcaemia with suppressed secretion of parathyroid hormone (table), high fractional excretion of calcium, and nephrocalcinosis suggested activating mutations of *CASR*.

Genomic DNA was isolated from peripheral leucocytes, and the DNA sequence of *CASR* was determined as described previously.[4] Complementary DNAs (cDNAs) for mutant CASRs were transfected into COS-1 cells. Production of inositol 1,4,5-trisphosphate (IP_3) in response to increasing concentrations of extracellular calcium was determined by measuring radioactivity in the IP_3 fraction after ion-exchange chromatography.[5] Results were expressed as a percentage of the maximum response of cells containing wild-type CASR to extracellular calcium at a concentration of 16 mmol/L.

After informed consent was obtained, direct sequencing of all coding exons of *CASR* revealed that case 1 had a heterozygous substitution of adenine (GAA) for cytosine (GCA) at codon 843 (figure), which would result in substitution of glutamic acid for alanine (A843E) in the protein. Case 2 also showed heterozygous substitution of guanine (TGG) for cytosine (TGC) at codon 131 (figure), resulting in a cysteine to tryptophan (C131W) aminoacid substitution. These substitutions were not found in 60 healthy controls.

Because of the presence of hypocalcaemia, the results suggest that these substitutions are activating mutations. However, characteristics of Bartter's syndrome have not been reported in patients with ADH. Since we have also seen two families with ADH without clinical features of Bartter's syndrome in whom two activating mutations of CASR (K47N and P221L) were present, we compared the functional properties of CASR in the present two cases with those in patients with ADH but not Bartter's syndrome. Production of IP_3 increased in a dose-dependent manner, with a half-maximum effective concentration (EC_{50}) of 3·4 mmol/L when cDNA for wild-type CASR was transfected (figure). When CASR cDNA with the K47N or P221L substitutions (ie, those from families without Bartter's syndrome) was transfected, the dose-response curve shifted to the left, with an EC_{50} of 2·0–2·2 mmol/L (figure). When CASR cDNA found in the cases with both ADH and Bartter's syndrome were transfected, IP_3 production was almost maximum at 1 mmol/L calcium, with an EC_{50} of less than 0·5 mmol/L (figure). These results indicate that A843E and C131W mutations in CASR are activating mutations with the lowest EC_{50} reported so far,[3] and that these mutant CASRs are fully activated by physiological concentrations of calcium.

We have not examined whether these patients have mutations in the genes for NKCC2, ROMK, CLCKB, BSND, or NCCT. However, our results show that two unrelated patients with ADH caused by different mutations of *CASR* also had unique clinical features of Bartter's syndrome. Although nephrocalcinosis might have contributed to the salt-losing tendency in the late stage of patient 1, it cannot explain persistent hypokalaemia from the early stages, with almost normal renal function. Therefore, these results agree with the idea that only mutations of *CASR* that result in proteins that are almost fully activated by physiological concentrations of calcium cause ADH associated with Bartter's syndrome. Although activation of CASR inhibits ROMK in rats, other factors such as changes of prostaglandin production might also have contributed to the phenotype of these patients. Furthermore, the results also suggest that other molecules that inhibit activity of NKCC2, ROMK, CLCKB, BSND, or NCCT might be additional causes of Bartter's or its related syndromes.

Contributors

S Watanabe did most of the experimental work and wrote the first draft of the report. Y Takeuchi, Y Hasegawa, R Okazaki, and N Chikatu examined patients and collected clinical data. T Fujita managed the study. H Chang helped interpret the data. S Fukumoto designed the study, helped with the experimental work, and refined the report.

Conflict of interest statement

None declared.

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