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, $\beta 1$ and $\beta 2$, 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 $\beta 1$ and $\beta 2$ 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 $\beta 1$ and $\beta 2$. 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-4$\beta 1$ and $\beta 2$ have been deposited in the GenBank® database under accession nos. AY034085 and AY034086 respectively.

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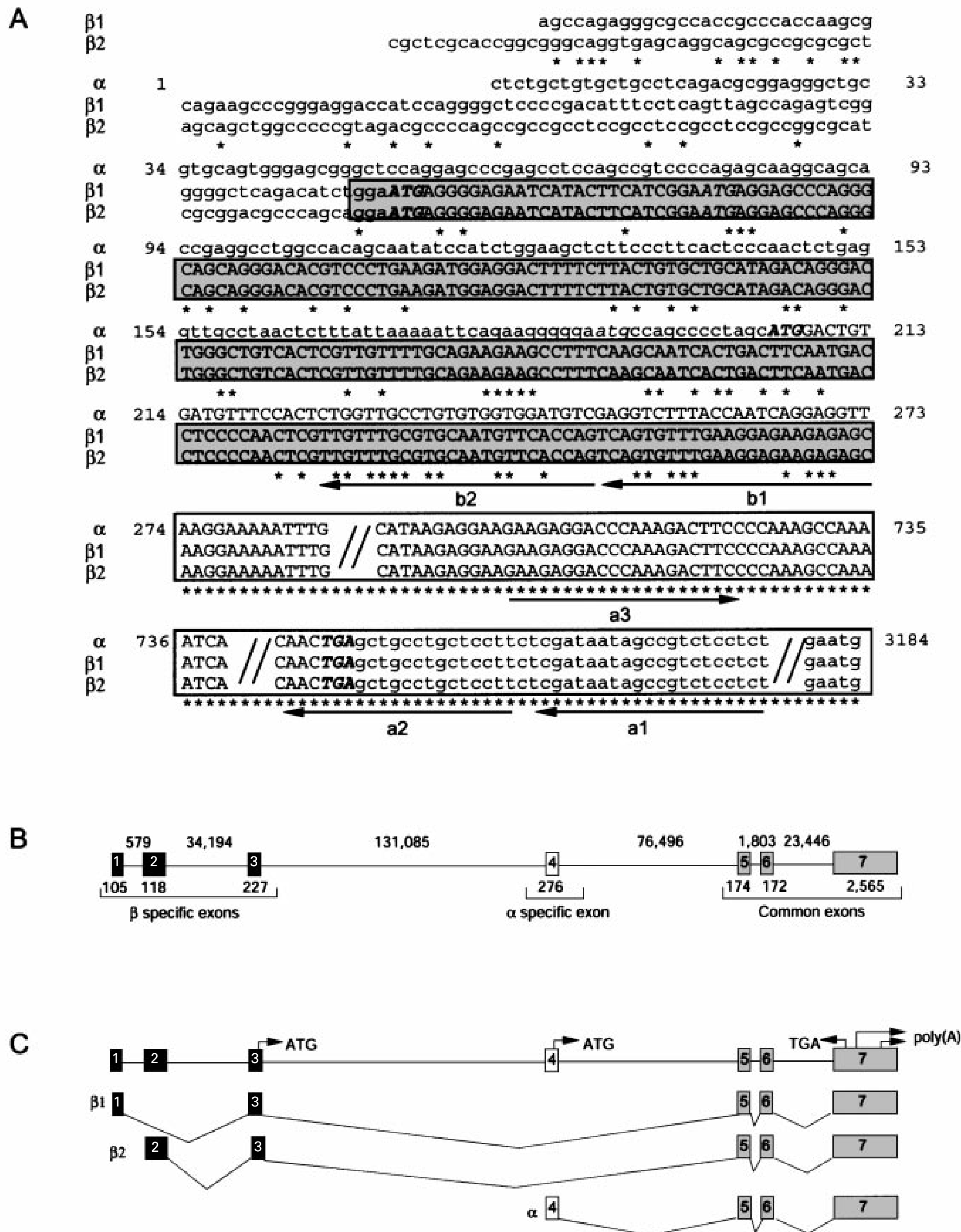

Figure 1 ZAKI-4 gene organization and its transcripts

(**A**) Three transcripts encoded by ZAKI-4 gene. RACE revealed three different transcripts, α, $\beta1$ and $\beta2$, 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, $\beta1$ and $\beta2$, 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 $\beta1$ and $\beta2$ 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. $\beta1$ and $\beta2$ mRNAs are generated by alternative initiation from exon 1 and exon 2 respectively. They are joined to exon 3 shared by $\beta1$ and $\beta2$ 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 $\beta1$ and $\beta2$ 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-CC**ATG**GAAAAATTTGGGGGACTGTTTCGGACTTAT-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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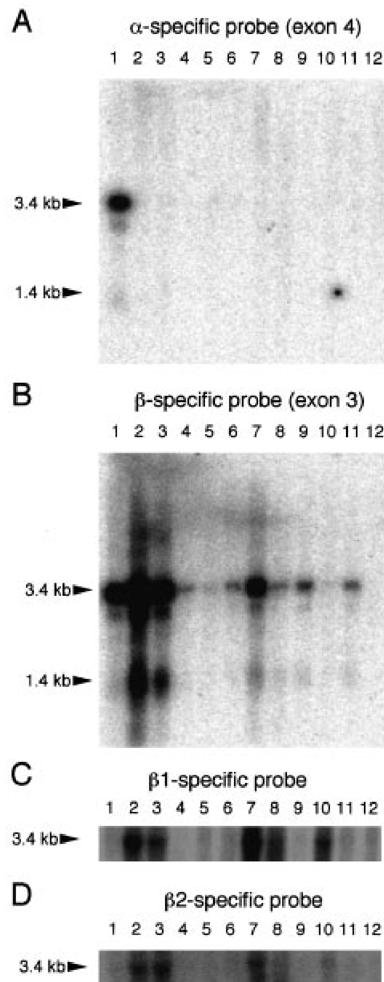

Figure 2 Expression of ZAKI-4α and β mRNAs in various human tissues

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

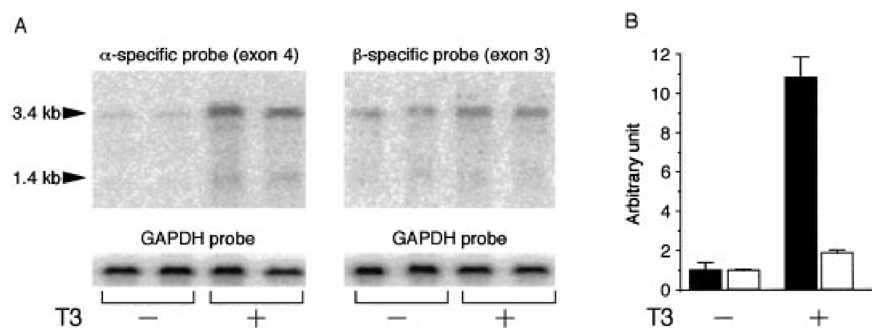

Figure 3 Differential regulation of ZAKI-4α and -4β expression by T_3 in human skin fibroblasts

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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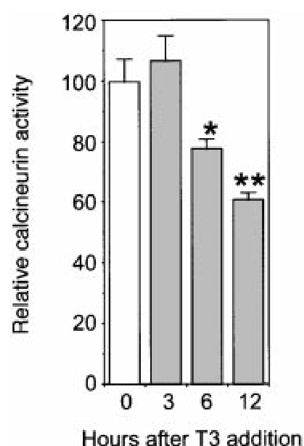

Figure 4 T_3 treatment results in a decrease in endogenous calcineurin activity

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 $\pm$ S.D. Statistical significances for 0–3 h and 0–6 h are $^{*}P < 0.05$ and $^{**}P < 0.001$ respectively.

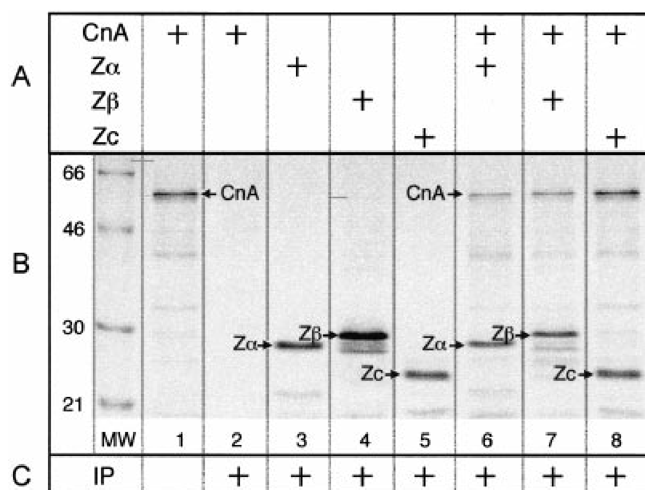

Figure 5 *In vitro* protein–protein binding assay of ZAKI-4 isoforms with calcineurin

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).

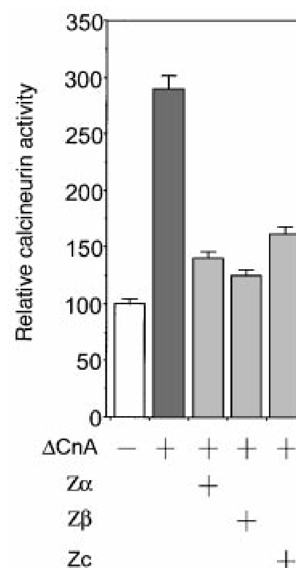

Figure 6 Inhibition of calcineurin activity by ZAKI-4 isoforms

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.

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.

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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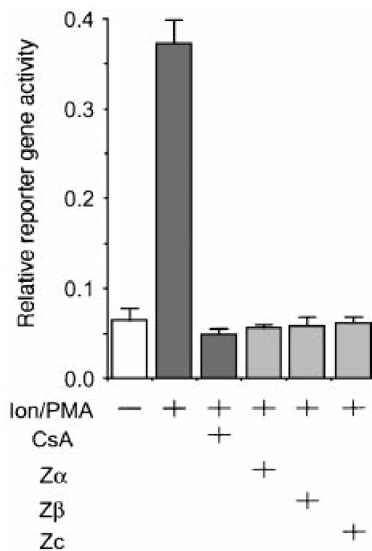

Figure 7 Inhibition of calcineurin-mediated activation of NF-AT by ZAKI-4 isoforms

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 $\pm$ 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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