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Cyclosporin A (CsA)-sensitive Pathway for the Induction of ZAKI-4 Expression by Thyroid Hormone

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Abstract: We identified ZAKI-4 as a thyroid hormone (T3) responsive gene in human skin fibroblasts. Our recent study revealed that two ZAKI-4 isoforms, α and β, are generated from a single gene on chromosome 6. Only expression of α is increased by T3, while that of β is not affected. Both isoforms belong to a family of proteins containing a conserved motif, which interacts with calcineurin and inhibits its activity. Recently, expression of a mouse homologue of the ZAKI-4 family gene (MCIP1) is shown to be induced by the activation of calcineurin in myocytes. We thus studied whether ZAKI-4 expression is also regulated by calcineurin-mediated pathway. Northern blot analysis revealed activation of calcineurin by ionomycin/phorbol 12-myristate 13-acetate (PMA) did not increase the expression of both isoforms. In addition, either basal expression of the two isoforms or T3-induced ZAKI-4α expression was not affected by FK506, a specific calcineurin inhibitor, suggesting the activation of calcineurin is not involved in ZAKI-4 regulation. However, cyclosporin A (CsA), another inhibitor of calcineurin decreased the basal expression of ZAKI-4α. Furthermore, T3-mediated increase of ZAKI-4α was completely blocked by CsA, but not by FK506. It is thus indicated that the two isoforms are differentially regulated, and the regulation of ZAKI-4α involves a CsA-sensitive pathway, which is independent of calcineurin activation.

Key words: ZAKI-4, calcineurin, CsA, FK506, thyroid hormone

ZAKI-4 was identified as a thyroid hormone (T3) responsive gene in human skin fibroblasts.[1] Our recent study demonstrated that three transcripts, α and β1 and β2, are generated from a single gene by differential initiation and splicing. Since β1 and β2 differ in their 5′-non-coding sequences, they encode the same protein product, β.The product of α transcript shares 3′-terminal common region with β isoform. Examination of tissue distribution of ZAKI-4 transcripts revealed a similar expression of β1 and β2 while α expression is different (manuscript in submission), suggesting that the two isoforms, α and β might be regulated differentially. Moreover, the originally identified T3-responsive gene in human skin fibroblasts was shown to be α transcript, while β was not responsive to T3 (manuscript in submission).

ZAKI-4 isoforms belong to a family of proteins containing a conserved motif of ISPPXSPP among different species.[2] The family in human includes ZAKI-4,[1] DSCR1[3] and DSCR1L2.[4] They share 61–68% identity in amino acid sequences, especially in the carboxyl terminal region where the ISPPXSPP motif is present. It has been demonstrated that this region of DSCR1 binds to calcineurin A, the catalytic subunit of calcineurin, and thus inhibits calcineurin activity.[5-7] Our recent results also demonstrated that the common C-terminal region of ZAKI-4α and β associates with calcineurin A and inhibits its activity.[8]

Calcineurin is a serine/threonine protein phosphatase. It plays important roles in immune function, neuronal plasticity and apoptosis, as well as hypertrophy of cardiac and skeletal muscles through activating transcription factors.[9] The enzyme is activated by Ca^{2+}/calmodulin signaling and inhibited by immunosuppressive drugs such as cyclosporin A (CsA) and FK506.[10] These drugs bind to calcineurin and inhibit its activity through forming the complexes with endogenous immunophilins, cyclophilin and FKBP12, respectively. Recently, transcription of a mouse homologue of DSCR1 gene (MCIP1) is reported to be induced by the activation of calcineurin in myocytes, suggesting its role as an endogenous feedback regulator of calcineurin activity.[11] To explore a possible involvement of calcineurin in ZAKI-4 regulation, we investigated whether activation of calcineurin by ionomycin or inhibition by CsA or FK506 could affect the expression of ZAKI-4 in human skin fibroblasts.

Material and Methods

1. Cell culture

Human skin fibroblasts[1] were seeded in 75-cm^2 flasks (Falcon 3111; Becton Dickinson Labware, Franklin Lakes, NJ, USA) in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with

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10% fetal bovine serum (FBS), 50 U/ml of penicillin G and 50 μg/ml of streptomycin at 37°C and cultured in 5% CO2 and 100% relative humidity. After the cells were grown to confluence, the medium was replaced with that containing FBS in which thyroid hormone was depleted by a treatment with activated charcoal.[12] The cells were incubated in this medium for 24 hr. To activate calcineurin, ionomycin (1 μM) and PMA (10 ng/ml) were added. To inhibit the activation of calcineurin, 10 μM CsA or 1μM FK506 was added 15 min prior to the addition of 10^{-8} M T3. The cells were harvested 12 hr after the treatments for total RNA extraction.

2. Northern blot analysis

Total RNA was extracted by the method reported by Chomczynski and Sacchi.[13] Fifteen micrograms of total RNA per lane was used for Northern blot analysis. The cDNAs specific for ZAKI-4α and β were synthesized by TA-cloning following RT-PCR. The specific primers used are as following: α; 5′-CTCTGCTGTGCTGCCTCAAACTCT-3′ (sense) and 5′-CTCCTGATTGGTAAAGACTCGAC-3′ (antisense) and β; 5′-ATGAGGGGAGAATCATACTTCATC-3′ (sense) and 5′-GCTCTCTTCTCCTTCAAACACTGA-3′ (antisense). After cloning into pGEM-T Easy vector (Promega), the authenticity of the sequences was confirmed before use. The cDNA probes were labeled with [α-^{32}P]dCTP (3000 Ci/mmol; New England Nuclear, Boston, MA) using a random primed DNA labeling kit (Boehringer Mannheim GmbH, Mannheim, Germany). The same membranes were rehybridized with human glyceryldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe. Radioactivity of each mRNA was determined by BAS2000 bioimage analyzing system (Fuji Film Co., Tokyo, Japan). The mRNA levels for human ZAKI-4α and β were normalized by those for GAPDH.

Results

To explore the possible involvement of calcineurin in ZAKI-4 regulation, we stimulated endogenous calcineurin by ionomycin and PMA, and inhibited its activity by CsA. Human skin fibroblasts were treated with CsA (10 μM) or ionomycin (1 μM) and PMA (10 ng/ml) for 12 hr. As shown in Fig. 1A, the basal level of ZAKI-4α was decreased by CsA, while ionomycin and PMA treatment did not affect its expression. On the other hand, β transcript was not affected by preincubation with CsA, and the treatment with ionomycin and PMA resulted in a suppression of its expression (Fig. 1B). The results demonstrate that ZAKI-4α and β are differentially regulated, only α is sensitive to CsA.

CsA (10 μM) or FK506 (1 μM) was added 15 min prior to the addition of 10^{-8} M T3. The cells were harvested 12 hr after the treatments for total RNA extraction. As shown in Fig. 2A, the basal expression of ZAKI-4α was reduced by CsA. Furthermore, T3 treatment markedly increased ZAKI-4α expression, which could be blocked by CsA. However, FK506 had no effect on either basal expression or T3-dependent induction of ZAKI-4α (Fig. 2B). It is thus suggested that CsA-sensitive pathway involved in ZAKI-4α regulation is independent of calcineurin.

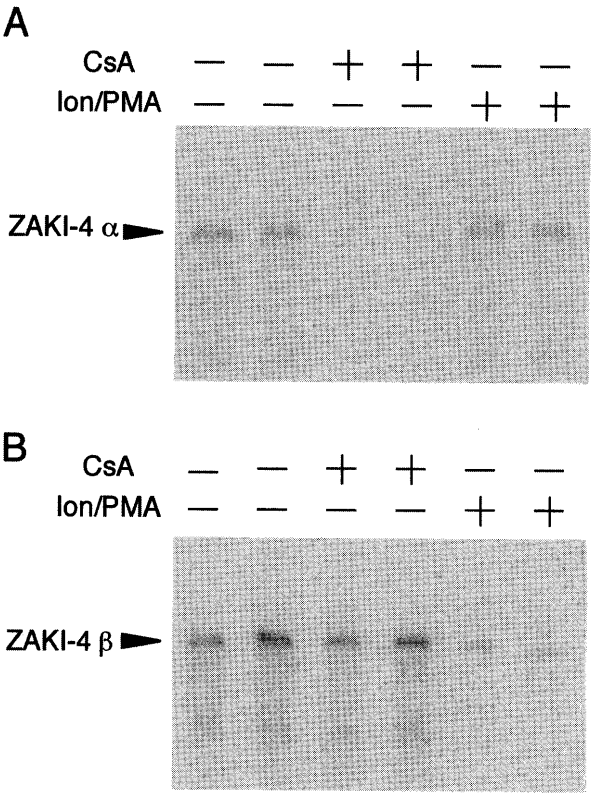

Fig.1 Activation of calcineurin by ionomycin does not increase ZAKI-4 expression.
Human skin fibroblasts were treated with ionomycin (Ion, 1 μM), PMA (10 ng/ml) and cyclosporin A (CsA, 10 μM) as depicted as plus and minus. Twelve hours after the treatments, the cells were harvested, and 15 μg of total RNA per lane was applied for Northern blot analysis. The membrane was hybridized with the probe either specific to α (panel A) or β (panel B). The basal level of ZAKI-4α, but not β was suppressed by CsA, while neither of them was increased by the treatment of Ion and PMA.

Discussion

Our present study revealed that two ZAKI-4 isoforms are regulated through different pathways. Expression of ZAKI-4α was not affected by the activation of Ca^{2+}/calmodulin pathway while it suppressed the expression of β. Suppression of the basal as well as T3-induced ZAKI-4α expression by CsA is likely to be independent of its inhibitory action on calcineurin because FK506 did not affect the expression.

Both CsA and FK506 are immunosupppressants, which directly inhibit calcineurin through complex formation between calcineurin A and B subunit after their binding with cognate immunophilins. However, differences exist in the action of CsA

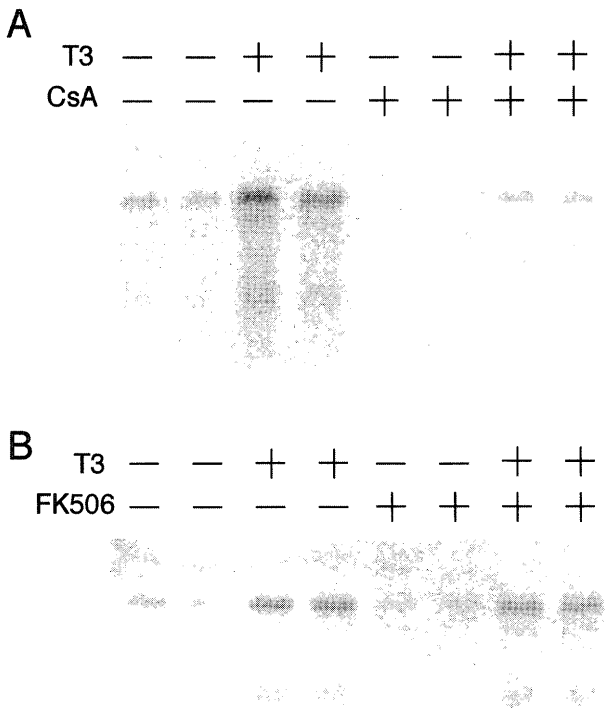

Fig. 2 T3-induced ZAKI-4α expression is inhibited by CsA, but not by FK506.
Human skin fibroblasts were incubated with 10^{-8} M T3 and/or 10 μM CsA or 1 μM FK506 for 12 h. CsA and FK506 were added 15 min prior to T3. The cells were harvested, and the expression of ZAKI-4α was determined by Northern blot analysis with 15 μg total RNA loaded for each lane. T3 increased ZAKI-4α expression significantly, which could be inhibited by pre-incubation with CsA (panel A). However, FK506 had no effect on T3-induced ZAKI-4α expression (panel B).

and FK506. First, CsA, following its binding to cyclophilin, inhibits the release of cytochrome C from membrane permeability transition (MPT) pore of mitochondria,[14,15] while FK506 does not affect the function of MPT pore. Second, CsA, but not FK506 prevents the down regulation of phosphorylated Akt, a serine-threonine kinase downstream target for phosphatidylinositol 3-kinase (PI3-kinase).[16] It remains to be studied whether MPT pore or Akt is related to this CsA-sensitive pathway.

It is of interest that T3 action in inducing apoptosis of cultured tadpole intestinal epithelial cells is inhibited by CsA[17], but not by FK506 although both immunosuppressants block apoptosis in T cells,[18] implicating that the CsA-sensitive pathway is cell type specific. Elucidation of T3 action sensitive to CsA will give us new insights into thyroid hormone-mediated signaling pathway.

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