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# Expression of ZAKI-4 in Mammalian Cells

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**Abstract:** ZAKI-4 is a member of a novel gene family that functions as an endogenous calcineurin inhibitor. Two ZAKI-4 isoforms, α and β, display tissue-specific distribution and different hormonal regulation. However, functional differences of each ZAKI-4 isoforms have not been elucidated. Here, we show the subcellular localization of ZAKI-4 isoforms. Both ZAKI-4α and β showed the cytoplasmic distribution. These results suggest that ZAKI-4 seems to regulate calcineurin activity in the cytoplasm.

**Key words:** ZAKI-4, calcineurin inhibitor, subcellular localization

Calcineurin is a $Ca^{2+}$/calmodulin-regulated serine/threonine protein phosphatase that plays an important role in various biological processes, including T cell activation, cardiac and skeletal muscle hypertrophy, neuronal plasticity, and apoptosis (Aramburu et al, 2000). Dephosphorylation of cytosolic NFAT by activated calcineurin results in nuclear translocation of NFAT and subsequent activation of target genes such as IL-2 (Crabtree, 2001). Although the immunosuppressive agents such as CsA and FK506 have been well established as calcineurin inhibitors (Liu et al, 1991), recent studies have led to the identification of a novel gene family, termed ZAKI-4 family, that functions as an endogenous calcineurin inhibitor (Fuentes et al, 2000; Kingsbury et al, 2000; Strippoli et al, 2000a; Rothermel et al, 2000; Cao et al, 2002). In human, there are three ZAKI-4 family genes, including *DSCR1* (Fuentes et al, 1995), *ZAKI-4* (Miyazaki et al, 1995), also known as *DSCR1L1*, and *DSCR1L2* (Strippoli et al, 2000b), all of which bind to and inhibit calcineurin through conserved COOH-terminal region (Fuentes et al, 2000; Kingsbury et al, 2000; Rothermel et al, 2000; Cao et al, 2002). The *ZAKI-4* gene, which was initially identified as a thyroid hormone-responsive gene (Miyazaki et al, 1995), encodes two isoforms, α and β, whose COOH-terminal region is identical (Cao et al, 2002). Northern blot analysis of ZAKI-4 isoforms demonstrated that ZAKI-4α is expressed exclusively in brain, whereas ZAKI-4β is expressed ubiquitously (Cao et al, 2002). In addition, the expression of ZAKI-4α, but not β, is markedly induced by thyroid hormone (Cao et al, 2002). Therefore, the expression of ZAKI-4 isoforms is differentially controlled by tissue-specific as well as signal-dependent regulation. However, it is unknown whether each ZAKI-4 isoforms has unique functions in mammalian cells. Here, we describe the subcellular localization of ZAKI-4 proteins. Both isoforms are located in the cytoplasm and no significant differences in the subcellular distribution were observed. These results suggest that both ZAKI-4 isoforms may function in the cytoplasm to inhibit calcineurin activity.

## Materials and Method

### 1. Cell Culture:

HeLa cells were grown at 37°C under an atmosphere of 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM; Nissui, Tokyo) supplemented with 10% fetal bovine serum.

### 2. Construction of Expression plasmids:

COOH-terminal Myc-tagged ZAKI-4α, ZAKI-4β, and identical region (ZAKI-4c) between ZAKI-4α and β, were generated by PCR with human skin fibroblast cDNAs and the following primers: 5'-GCTAGCCACCATGG ACTGTGATGTTTCCACTCTG-3' and 5'-TATCTCGAG TTGGACACGGAGGGTGGCAGGCC-3' for ZAKI-4α, 5'-GCTAGCATGAGGGGAGACGCCTACTTCATCGGA-3' and 5'- TATCTCGAGTTGGACACGGAGGGTGGCAGGCC-3' for ZAKI-4β, 5'-GCTAGCCACCATGGAAAAATTT GGGGGACTGTTTCGGACTTAT-3' and 5'-TATCTCGAG TTGGACACGGAGGGTGGCAGGCC-3' for ZAKI-4c. The PCR products were digested with EcoRI and XhoI, and subcloned into pCDNA3.1/Myc-His (Invitrogen).

### 3. Immunofluorescence Staining:

HeLa cells grown on glass coverslips in growth medium were transfected with the use of Lipofectamine™ Reagent (Invitrogen life technologies) and prepared as described pre-

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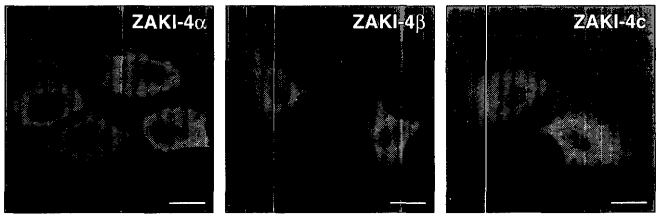


Fig. 1 Subcellular localization of ZAKI-4 isoforms.
HeLa cells were transfected with expression plasmids encoding COOH-terminal Myc-tagged ZAKI-4α (left), ZAKI-4β (middle), and ZAKI-4c (Right). After 48 h, the cells were fixed and stained with anti-Myc antibody to examine the subcellular distribution. ZAKI-4 isoforms are located in the cytoplasm with preferential distribution at perinuclear region. Scale bars, 20 μm.

viously (Miura et al, 1999). In brief, the cells were fixed with 4% formaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature, and then incubated with anti-Myc Ab (9E10; Roche Diagnostic K.K., Tokyo, Japan) at a concentration of 1 μg/ml in PBS containing 0.1% bovine serum albumin and 0.1% saponin for 1 h at room temperature, followed by incubation with FITC-conjugated anti-mouse IgG Ab (CHEMICON) for 1 h at room temperature. The cells were analyzed using Zeiss LSM 510 (Carl Zeiss).

## Results and Discussion

To examine the subcellular localization of ZAKI-4 proteins, expression vectors encoding COOH-terminal Myc-tagged ZAKI-4α, ZAKI-4β, and ZAKI-4c were prepared. Immunofuluorescence analysis of transfected HeLa cells revealed that ZAKI-4α as well as ZAKI-4β was localized to the cytoplasm with preferential distribution at perinuclear region (Fig. 1). Both isoforms were not detected in the nucleus. ZAKI-4c, which is shared by ZAKI-4α and β, thereby lacking different $NH_2$-terminus of the two isoforms, also showed similar localization as ZAKI-4α and β, suggesting that $NH_2$-terminal different region of ZAKI-4 isoforms is dispensable for the cytoplasmic localization of ZAKI-4 proteins. ZAKI-4 isoforms directly bind to and inhibit calcineurin, and $Ca^{2+}$ stimulation did not affect the subcellular localization of ZAKI-4 isoforms (data not shown), suggesting that ZAKI-4 isoforms may function in the cytoplasm to inhibit calcineurin. As for another endogenous calcineurin inhibitor, DSCR1, its subcellular localization seems to be primarily in nucleus (Rothermel et al, 2000; Pfister et al, 2002). Unlike ZAKI-4 isoforms, DSCR1 is known to be upregulated by activation of calcineurin thorough an increase in intracellular $Ca^{2+}$ (Fuentes et al, 2000; Yang et al, 2000). One report demonstrated the mobilization of DSCR1 from nucleus to cytoplasm by the activation of calcineurin (Rothermel et al, 2000), while another denied the redistribution (Fuentes et al, 2000). It is thus still to be studied whether $Ca^{2+}$-signaling changes the subcellular localization of DSCR1. In conclusion, the function of ZAKI-4 family proteins may be regulated by differential intracellular distribution in addition to tissue-specific and signal-dependent expression.

## References

Aramburu J, Rao A, Klee CB. Calcineurin: from structure to function. Curr Top Cell Regul 2000; 36: 237–295.

Cao X, Kambe F, Miyazaki T, et al. Novel human ZAKI-4 isoforms: hormonal and tissue-specific regulation and function as calcineurin inhibitors. Biochem J 2002; 46: 40–42.

Crabtree GR. Calcium, calcineurin and the control of transcription. J Biol Chem 2001; 276: 2313–2316.

Fuentes JJ, Pritchard MA, Planas AM, et al. A new human gene from the Down syndrome critical region encodes a proline-rich protein highly expressed in fetal brain and heart. Hum Mol Genet 1995; 4: 1935–1944.

Fuentes JJ, Genesca L, Kingsbury TJ, et al. DSCR1, overexpressed in Down syndrome, is an inhibitor of calcineurin-mediated signaling pathways. Hum Mol Genet 2000; 9: 1681–1690.

Kingsbury TJ, Cunningham KW. A conserved family of calcineurin regulators. Genes Dev 2000; 14: 1595–1604.

Liu J, Farmer JD, Jr., Lane WS, et al. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell 1991; 66: 807–815.

Miura M, Hatakeyama S, Hattori K, et al. Structure and expression of the gene encoding mouse F-box protein, Fwd2. Genomics 1999; 62: 50–58.

Miyazaki T, Kanou Y, Murata Y, et al. Molecular cloning of a novel thyroid hormone-responsive gene, ZAKI-4, in human skin fibroblasts. J Biol Chem 1996; 271: 14567–14571.

Pfister SC, Machado-Santelli GM, Han SW, et al. Mutational analyses of the signals involved in the subcellular location of DSCR1. BMC Cell Biol 2002; 3 (1): 24.

Rothermel B, Vega RB, Yang J, et al. A protein encoded within the Down syndrome critical region is enriched in striated muscles and inhibits calcineurin signaling. J Biol Chem 2000; 275: 8719–8725.

Strippoli P, Petrini M, Lenzi L, et al. The murine DSCR1-like (Down syndrome candidate region 1) gene family: conserved synteny with the human orthologous genes. Gene 2000a; 257: 223–232.

Strippoli P, Lenzi L, Petrini M, et al. A new gene family including DSCR1 (Down Syndrome Candidate Region 1) and ZAKI-4: characterization from yeast to human and identification of DSCR1-like 2, a novel human member (DSCR1L2). Genomics 2000b; 64: 252–263.

Yang J, Rothermel B, Vega RB, et al. Independent signals control expression of the calcineurin inhibitory proteins MCIP1 and MCIP2 in striated muscles. Circ Res 2000; 87: E61–68.

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