Zic1 is a Transcriptional Repressor Through the Lamin A/C Promoter and has an Intrinsic Repressive Domain

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Zic family proteins are expressed in the cerebellum and play important roles in vertebrate development. However, very few observations have been made regarding the function of Zic proteins in transcription. We previously showed, using P19 cells as a model system, that the lamin A/C promoter has a retinoic acid responsive element (L-RARE), and that Sp1 and Sp3 bind the CACCC box of the L-RARE. Here, Zic1 is identified as a binding protein of the L-RARE by yeast one-hybrid screening. We show that Zic1 can bind to and repress transcription through the CACCC box of the L-RARE. Moreover, reporter assays using various fusion proteins of Zic1 with the GAL4 DNA binding domain reveal that Zic1 has an intrinsic transcriptional repressive and activation domain.

Key words ——— transcriptional gene expression, repressor, lamin A, Zic1, retinoic acid response

INTRODUCTION

Mutations in lamin A and lamin C, which are nuclear intermediate filament proteins expressed in nearly all somatic cells, cause tissue-specific diseases that affect striated muscle, adipose tissue and peripheral nerve and skeletal development.[1–3] Recently, it was reported that lamin A/C gene (LMNA) mutation is involved in Hutchinson-Gilford progeria syndrome, which is a rare genetic disorder resulting in phenotypes suggestive of accelerated ageing.[4,5]

Since the mutation of LMNA is found in the various inherited diseases, it is important to know how expression of the lamin A/C gene is regulated. We previously reported that the lamin A/C promoter contains a retinoic acid responsive element (L-RARE), using P19 cells as s model system of differentiation. We determined that the core motif of the L-RARE is the CACCC box, which is recognized by Sp1and Sp3. Sp1 family proteins activate transcription of the lamin A/C promoter using this CACCC box. Other unidentified binding proteins of L-RARE were detected by EMSA.[6]

Zic1 is a zinc-finger transcription factor that is induced by BMPs in the dorsal region of the neural tube at a specific embryonic stage.[7] It belongs to a family of related proteins including Zic2, Zic3, Zic4 and Zic5, all of which share homology to the Drosophila gene odd paired[7,8] and weaker homology to the Gli family of vertebrate genes and their Drosophila homolog cubitus interruptus.[9] In mouse, Zic1 expression is restricted to the nervous system, with highest levels of expression observed in the cerebellum.[9] Zic1 mutations are found in Joubert syndrome (JS).[10] JS is a rare, autosomal recessive, neurological disorder, involving agenesis or dysgenesis of the cerebellar vermis with accompanying brainstem malformations.[10] Loss-of-function and gain-of-function studies with Zic factors in the neural tissue of Xenopus, mouse, and chicken have suggested that Zic1 inhibits neuronal differentiation and maintains cells as progenitors.[11,12] Although Zic1 has been shown to function as both a transcription activator and repressor, the Zic1 transcriptional mechanism is not yet clarified. In this report, we demonstrate that Zic1 represses gene transcription of the lamin A/C promoter, and that Zic1 has both transcriptional repression and activation domains.

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MATERIALS AND METHODS

Cell Cultures —— 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C. Drosophila Schneider's SL2 cells were cultured in Schneider's insect medium supplemented with 10% fetal bovine serum at 25°C.

Yeat One-Hybrid Screening —— Three tandem copies of the L-RARE were inserted in front of HIS3 and lacZ reporter genes in pHISi-1 and placZi vectors (Clontech), respectively. These 3 × L-RARE-HISi-1 and 3 × L-RARE-lacZ constructs were sequentially integrated into chromosomal DNA of yeast strain YM4271. The reporter-containing YM4271 was transfected with a murine embryonic day 11.0 cDNA library (Clontech). They were selected on histidine-deficient minimal (S.D.) medium that contained 1 mM 3-amino-1,2,4-triazole. The his-positive clones were isolated.

***In vitro* Protein Expression** —— The *in vitro* transcription/translation vector pCI-Zic1, contains a cDNA fragment encoding the Zic1. 1 μg of plasmid DNA was transcribed from the T7 promoter and translated in rabbit reticulocyte lysate with [^{35}S]methionine by using the TnT Coupled Transcription/Translation Kit (Promega, Madison, U.S.A.) according to the manufacturer's instructions.

Electrophoretic Mobility Shift Assay (EMSA) —— Binding reactions were carried out using 2 μl of *in vitro* translated Zic1 protein in binding buffer [20 mM Tris–HCl (pH 7.4), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 μg poly (dI-dC) and 5% glycerol]; after addition of 50 fmol of ^{32}P-labeled (2×10^3 cpm/fmol) L-RARE, which served as the probe. The reactants were incubated for 30 min at 25°C. Thereafter, the reaction mixtures were loaded onto 5% polyacrylamide gels. Double stranded oligonucleotides corresponding to the desired lamin A/C promoter sequences (–64 to –33) were ^{32}P end-labeled using a Klenow fragment.[6]

Plasmid Constructions —— The plamA2, which included the region +48 to –100 of the lamin A promoter, was constructed previously.[6] p4 × RARE-, and p4 × M2-TK-luc were derived from pTK-luc, which has a tk minimal promoter.[6] pPac and pPac Sp1 were gifts from Drs. R. Tjian, G. Suske and A. Belayew.[13–15] pCI-Zic1 and pCI-Zic1(1-124) were constructed by PCR. The pRL-TATA, which has Renilla luciferase and a minimal promoter, was constructed as an internal control. Series of pBIND-Zic1 plasimds were constructed by PCR and fused to the yeast GAL4 DNA binding domain using the pBIND vector (Promega).

Transfection and Luciferase Assays —— For transient transfection, 293T cells (2×10^5 cells per well in a 6-well plate) and SL2 cells (2.5×10^5 cells per well in a 24-well plate) were seeded the day before transfection. Cells were incubated with a mixture of DNA and Lipofectamine 2000 reagents (Invitrogen) for 48 hr. The DNA mixtures contained the amount of plasmids indicated on Figure legends. Luciferase assays were performed as described previously.[6] The luciferase activity was normalized to that of pRL-TATA renilla.

RESULTS

Zic1 is a Binding Protein of the L-RARE on the Lamin A/C Promoter

To identify binding protein of L-RARE, we performed yeast one-hybrid screening by using the L-RARE fragment as bait to screen a murine embryonic day 11.0 cDNA library. Screening 1×10^7 transformants gave positive clones, which included Sp1 family proteins (Sp2, Sp3, Sp4, BTEB-1 and TIEG1) and Zic family proteins (Zic1, Zic2). We already reported that Sp1 family proteins bind to the CACCC box of the L-RARE.[6] In order to determin whether Zic1 binds to L-RARE, we carried out an electrophoretic mobility shift assay (EMSA) competition analysis using *in vitro* expressed Zic1 protein (Fig. 1). Zic1 can bind to the L-RARE probe. The band of Zic1 (that bound to the oligos) had decreased intensity in the presence of a 100-fold excess of the unlabeled wild type and M1 mutant forms (TAGAGGAGGG→TAGaggagG) of L-RARE, but not M2 mutant form (CCACCCCCT →CCACtatCT) and non-specific oligonucleotide (Fig. 1). Therefore, Zic1 fusion protein specifically recognizes the CACCC box of the L-RARE.

Zic1 is a Transcriptional Repressor of the L-RARE

It has been reported that Zic1 recognizes specific binding sequence G/ATCCACCC and activates gene transcription.[16] We investigated whether the Zic protein could affect transcription of the L-RARE, by performing reporter assays using L-RARE-TK-luc and M2-TK-luc reporter constructs with Zic1 overexpression in 293T cells (Fig. 2). The luciferase activity of the L-RARE-TK-luc was strongly repressed by the overexpression of Zic1, while M2-

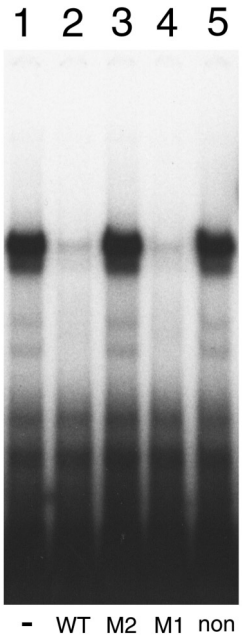

Fig. 1. Zic1 Protein Only Binds to CCACCC Sequence

EMSAs were carried out as described in MATERIALS AND METHODS. *In vitro* translated Zic1 protein was used in each reaction. ^{32}P-labeled (2×10^3 cpm/fmol) L-RARE served as probes. 100-fold excess of unlabeled L-RARE, M2, M1, and non-specific sequence oligonucleotides were used in competition assays.

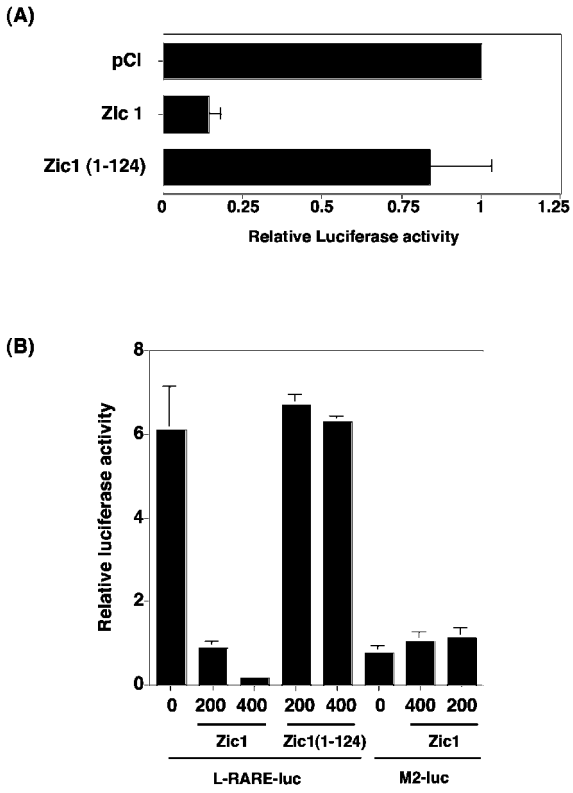

Fig. 2. Zic1 Represses L-RARE-Dependent Gene Transcription

(A) 293T cells were cotransfected with 400 ng of pCI-Zic1 or pCI-Zic1(1-124) and lamin A2 construct. Total amounts of plasmids were adjusted to 1 μg by addition of the pCI-neo vector. Bars depict the mean ± S.D. ($n = 3$) of the luciferase activity relative to the basal activity seen with pCI-neo vector. (B) The indicated amount of pCI-Zic1 or pCI-Zic1(1-124) and L-RARE-luc or M2-Luc were cotransfected into 293T cells. Bars depict the mean ± S.D. ($n = 3$) of the luciferase activity relative to the basal activity seen with pCI-neo vector.

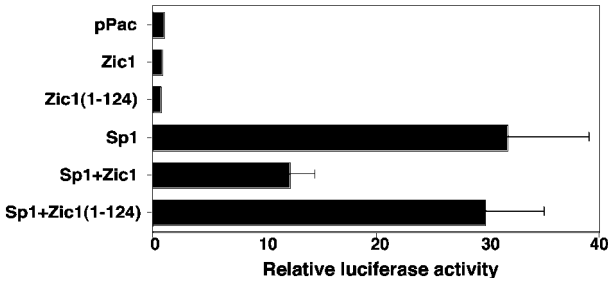

Fig. 3. Zic1 Inhibits the Sp1-Dependent Transcriptional Activation of the L-RARE in SL2 Cells

200 ng of the pPac Sp1 and 400 ng Zic1 or Zic1(1-124) were cotransfected with 100 ng of p4 × L-RARE-TK-luc into SL2 cells. Total amounts of plasmids were adjusted to 1 μg by addition of pPacU. Bars depict the mean ± S.D. ($n = 3$) of the luciferase activity relative to the basal activity seen with pPacU.

TK-luc was not. Thus, Zic1 can function a transcriptional repressor through the CACCC box.

We previously showed that Sp1 and Sp3 work as transcriptional activators through CACCC box of the L-RARE.[6] Because Zic1 also binds to the same element, we tested whether Zic1 can repress the transcriptional activity of Sp1 and Sp3. In SL2 cells, which do not have endogeneous Sp1 activity, cotransfection with Zic1 decreased Sp1 transcription. Zic1(1-124), in which the Zinc finger domains of Zic1 are deleted, did not exhibit repressive activity (Fig. 3). Therefore, Zic1 might act as a repressor of the Sp1 family.

Zic1 has a Transcriptional Repressive and Activating Domain

Although Zic1 repressed transactivation of Sp1 and Sp3, its mechanism of transcriptional repression is unclear. To elucidate the domain of Zic1 repressive activity, a series of fusion proteins containing the DNA binding domain (DBD) of Gal4 and various regions of Zic1 was constructed. In these studies, various Zic1 N-terminal regions were cloned into pBIND, a Gal4 DBD expression vector. These Gal4/Zic1 fusion constructs were cotransfected into 293T cells with the reporter construct pRL-luc, containing five copies of the Gal4 binding site. Since amino acids (a.a.) 224-409 of Zic1 contain five zinc finger domains, we hypothesized that a.a. 1-223 of

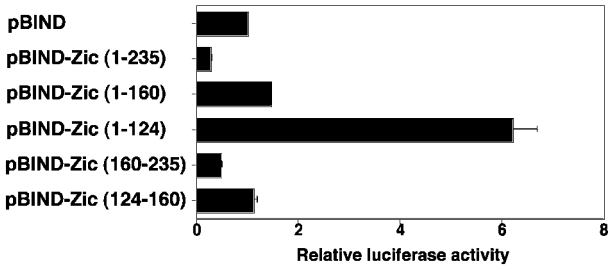

Fig. 4. Zic1 has a Repressive and Activative Domain

pBIND, pBIND-Zic1(1-235), pBIND-Zic1(1-160), pBIND-Zic1(1-124) pBIND-Zic1(124-160) or pBIND-Zic1(160-235) were co-transfected with pFL-luc (a reporter construct) into 293T cells. Total amounts of plasmids were adjusted to 1 μg by empty vector. Bars depict the mean ± S.D. (n = 3) of the luciferase activity relative to the basal activity seen with pBIND vector.

Zic1 are the regulatory domain. pBIND-Zic1(1-235) strongly repressed the transcriptional activity of the reporter (Fig. 4). In order to identify the repressive domain, we constructed four additional constructs of Zic1-Gal4 fusion protein expression plasmids. In addition to pBIND-Zic(1-235), pBIND-Zic(160-235) also repressed the luciferase activity, whereas pBIND-Zic(1-160), pBIND-Zic(1-124) and pBIND-Zic(124-160) did not (Fig. 4). These results strongly suggest that the region containing a.a. 160-235 has a repressive activity. Interestingly, pBIND-Zic(1-124) activated the transcription of the reporter, suggesting this region might be an activating domain. This finding is in agreement with reports that Zic1 can activate gene transcription.[16,17] Therefore, we conclude that Zic1 a.a. 160-235 and 1-124 are transcriptional repression and activation domains, respectively.

DISCUSSION

In this report, we show that Zic1 recognizes and represses the L-RARE and that Zic1 has a repression and activation domain in its regulatory region. Zic1 recognizes the GTCCACCC sequence with high affinity.[16] Since Zic1 binds to L-RARE, but not the M2 mutant (CCACCCCCT→CCACtatCT), Zic1 may recognize the ATCCACCC sequence in the L-RARE of the lamin A/C promoter. Indeed, overexpression of Zic1 can repress transcription of the lamin A/C promoter through the L-RARE.

Zic1 can transactivate the apolipoprotein E, SV40, CMV, and TK promoters,[16,17] but represses transcription of Math1.[18] Our data suggests that Zic1 a.a. 160-235 are a transcriptional repressive domain and 1-124 are an activating domain However, we could not find any conserved motifs within these regions. It was reported that Zic1 activates or inhibits (depending on the cell type) the function of Gli1 by direct interaction.[16] Other zinc finger transcriptional factors, such as Sp3 and HUB1, also have activating and repressive domains, which allow them to either activate or inhibit gene transcription.[19,20] Interestingly, Zic1 binds the same L-RARE sequence as Sp1, suggesting Zic1 may modulate other transcription factors by both direct interaction and by blocking their binding at the promoter.

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