Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 368 (2008) 205–210
BBRC
www.elsevier.com/locate/ybbrc

Ciz, a transcription factor with a nucleocytoplasmic shuttling activity, interacts with C-propeptides of type I collagen

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Received 8 January 2008
Available online 22 January 2008

Abstract

Ciz is a zinc finger transcription factor with nucleocytoplasmic shuttling activity. Ciz-deficient mice show high bone mass phenotype. As a first step to address how Ciz suppresses bone formation, we examined the binding partners of Ciz based on a yeast two-hybrid screening. While Ciz is an intracellular protein, 47% of the positive clones were genes encoding extracellular matrix proteins, including Col1a1, Col1a2, Fbln2, and Rpsa. In vitro coimmunoprecipitation experiments using in vitro translated proteins revealed direct binding of Ciz-ΔZF (zinc finger) to C-propeptides of Col1a1 and Col1a2. In vivo association of the transfected Ciz and C-propeptide of Col1a1 was observed in COS-7 cells based on immunoprecipitation. In terms of intracellular localization, overexpressed C-propeptides of Col1 and Ciz were co-localized in nuclei. These results revealed that Ciz interacts with C-propeptides of type I collagen and this association takes place in nuclei.

Keywords: Ciz; Collagen; C-propeptide; Extracellular matrix; Nucleocytoplasmic shuttling; Yeast two-hybrid; Bone formation; Bone; Osteoblasts; Protein-protein interaction

Ciz (Cas-interacting zinc finger protein) was first identified as a protein that binds to SH3 domain of p130Cas, contains a zinc finger and possesses a nucleocytoplasmic shuttling activity [1]. Ciz was also identified as nuclear matrix protein 4 (Nmp4), which binds to *Col1a1* promoter region and are expressed in osteocytes, osteoblasts, and chondrocytes [2]. As to the function of Ciz in osteoblasts, overexpression of Ciz suppresses BMP-induced osteoblast differentiation in vitro [3]. Ciz-deficient mice show an increase in the levels of bone phenotype-related genes. This was due to enhanced activity of osteoblasts without major alterations in bone resorption activity [4] and [5]. These mice also show impaired spermatogenesis [6]. These in vitro and in vivo analyses on Ciz function demonstrated that Ciz acts as a negative regulator of osteoblast differentiation.

Ciz enhances expression of *MMP* genes [1]. The promoter regions of these genes contain (G/C)AAAAA motif, which was identified as Ciz-binding sequence [1]. Ciz also regulates *MMP-13* gene expression [7]. *MMP-13* expression is induced by parathyroid hormone (PTH) in rat UMR-106 cell line. PTH responsive region in the *MMP-13* promoter contains Ciz-binding cis-element. Basal *MMP-13* promoter activity is enhanced by overexpression of Ciz and the response to PTH is weakened by mutation of the Ciz-binding sequences, suggesting that Ciz regulates

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doi:10.1016/j.bbrc.2008.01.040

MMP-13 gene response to PTH signal. Upregulation of *MMP-13* gene expression by fluid shear stress is mediated by Ciz detachment from *MMP-13* promoter [8]. These results suggest that Ciz would act as a transcriptional modulator in the context of bone formation. However, although Ciz acts as an intracellular adaptor molecule, binding partners for Ciz in bone have not yet been known.

Several binding partners of Ciz have been reported. Human Ciz (ZNF384) binds Zyxin, PCBP1, and vimentin [9]. Human Ciz lacks consensus proline-rich sequence required for interaction with SH3 domain and does not interact with p130Cas directly. Zyxin seems to mediate interaction between p130Cas and Ciz [9]. However, as mentioned above, Ciz-binding proteins in bone cells are still unknown. Therefore, to further understand molecular mechanisms of Ciz function in regulation of osteoblast differentiation, we carried out yeast two-hybrid screening to search for Ciz-interacting protein using cDNA library constructed from mouse newborn calvaria. We found extracellular matrix (ECM)-related proteins as Ciz-interacting proteins. Among these proteins, we confirmed interaction between Ciz and C-propeptides (COLFI domain) of type I collagen in vitro and in vivo. Moreover, Ciz and C-propeptides of Col1 were co-localized in nuclei. These results suggest that Ciz can interact with C-propeptides of Col1 intracellularly.

Materials and methods

Construction of mouse Ciz bait plasmid. Mouse Ciz (mCiz) open reading frame (ORF) was PCR (polymerase chain reaction)-amplified using AccuPrime *Pfx* DNA polymerase (Invitrogen) from one-month-old mouse bone marrow cDNA with the primer set (AAACCATGGAAGA GTCTCACTTCAAT and AAAGTCGACCTAAGAGCTGGCCAGGT GCTC). To construct a bait vector for mCiz, the PCR product was digested with NcoI and SalI and ligated into pGBKT7 (Clontech). The amino acid sequence encoded by this construct was identical to mouse zinc finger protein 384 (NP_780766). To construct pGBKT7-mCiz-ΔZF (amino acid 1–339) lacking zinc finger domain, pGBKT7-mCiz was digested with BamHI and SalI, blunted and self-ligated.

Yeast two-hybrid screening. To construct the cDNA library for yeast two-hybrid screening, total RNA was isolated from mouse newborn calvaria and cDNA was PCR-amplified. The screening was performed using Matchmaker Library Construction & Screening Kits (Clontech) according to manufacture's instruction. The positive clones grown on SD/-Ade/-His/-Leu/-Trp plates were harvested and further grown on SD/-Ade/-His/-Leu/-Trp plates containing X-alpha-gal. cDNA inserts were PCR-amplified directly from the resulting positive yeast colonies and sequenced.

Construction of expression vectors for type I collagen. To construct pcDNA3.1-HA-cpCol1a1 (HA-cpCol1a1) and pcDNA3.1-HA-cpCol1a2 (HA-cpCol1a2) for the transfection experiment, the positive clones encoding Col1a1 (1048–1225) and Col1a2 (1171–1372) in pGADT7-Rec vector was digested with BglII and XbaI, and the DNA fragment was ligated into BamHI and XbaI sites of pcDNA3.1 (+) (Invitrogen). HA tag had been fused to the N-terminal to Col1a1 and Col1a2 in the original pGADT7 vector upon preparation of the cDNA library. To construct pCMV-SPORT6-Col1a1-HA, the DNA fragment was PCR-amplified using pCMV-SPORT6-Col1a1 (IMAGE Clone ID: 4225038) as a template with the primer sets (Col1a1-PstI, 5′-AGGCATAAAGGGTCATCGTG-3′ and 5′-ATCTGGTACGTCGTATGGGTACACGAAGCAGGCAG GGCCAAT-3′) and then PCR-amplified with Col1a1-PstI primer and NotI-HA primer (5′-GCTCTAGACTCGAGGCGGCCGCTTAAGC GTAATCTGGTACGTCGTATGGGTA-3′). The resulting PCR product was digested with PstI and NotI and ligated into PstI and NotI sites of pCMV-SPORT6-Col1a1. To construct pCMV-SPORT6-Col1a2-HA, the DNA fragment was PCR-amplified using pCMV-SPORT6-Col1a2 (IMAGE Clone ID: 3586272) as a template with the primer sets, Col1a2-BglII (GTCTTGCTGGCCTACATGGT) and Col1a2-HA (ATCTGGT ACGTCGTATGGGTATTTGAAACAGACGGGGCCAAC) and then Col1a2-BglII primer and NotI-HA primer. The resulting PCR product was digested with BglII and NotI and ligated into BglII- and NotI-digested pCMV-SPORT6-Col1a2. All constructs were verified by sequencing.

Immunoprecipitation. For in vitro binding assay, protein was in vitro translated using TnT$^{(R)}$ T7 Quick for PCR DNA (Promega) with the PCR products (HA-tagged) as templates or with pGBKT7-mCiz-ΔZF (myc-tagged). The proteins were mixed and incubated for 1 h at room temperature. Then EZview Red Anti-c-Myc Affinity Gel (Sigma) was added and the mixture was incubated for 1 h at room temperature. The agarose beads were washed five times in RIPA buffer (150 mM NaCl, 1.0% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl (pH 8.0)). The bound proteins were eluted in SDS sample buffer with heating at 95 °C for 2 min. The proteins were electrophoresed in SDS–PAGE. The HA-tagged and myc-tagged proteins were detected by anti-HA (Y-11) (Santa Cruz Biotechnology) and anti-c-myc 9E10 (Roche), respectively, using ECL Plus Western Blotting Detection System (GE Healthcare). For in vivo binding assay, full size rat Ciz-FLAG (fCiz) or rat Ciz-ΔZF-FLAG [1] were transfected into COS-7 cells together with pcDNA3.1-HA-cpCol1a1 construct using Lipofectamine 2000 (Invitrogen). Cells were lysed in cell lysis buffer (1% Triton X-100 (w/v), 50 mM Tris–HCl, pH 7.5, 150 mM NaCl) supplemented with complete mini (Roche) and protein extracts were cleared by centrifugation. Immunoprecipitation was performed using ANTI-FLAG® M2 Affinity Gel (Sigma) and the bound proteins were detected with Anti-HA-Peroxidase, High Affinity (Roche). Input FLAG-tagged proteins were detected with ANTI-FLAG M2® Monoclonal Antibody-Peroxidase Conjugate (Sigma).

Immunocytochemistry. Cells were cultured on coverslips and fixed in 4% paraformaldehyde in PBS at 37 °C for 15 min and then they were washed in PBS twice. The cells were permeabilized in 0.1% Triton X-100 in PBS for 5 min and washed in PBS twice. The cells were incubated with blocking buffer (10% normal goat serum in PBS) for 30 min to block non-specific binding sites. The cells were incubated with mouse monoclonal anti-FLAG antibody (Sigma, F 1804) and rat monoclonal anti-HA antibody (Roche 3F10) diluted in the blocking buffer at 1:500 and 1:2000, respectively, for 30 min and then washed twice in PBS. The cells were incubated with anti-mouse IgG antibody conjugated with Alexa Fluor®488 and anti-rat IgG antibody conjugated Alexa Fluor®594 (Invitrogen) diluted in blocking buffer at 5 μg/ml for 30 min and then washed twice in PBS. The cells were mounted in ProLong® Gold Antifade Reagent (Invitrogen). Images were captured using confocal laser microscope (LSM5 PASCAL, Zeiss).

Results

Yeast two-hybrid screening for Ciz-interacting proteins identifies ECM proteins including Col1a1 and Col1a2

To identify novel Ciz-binding proteins in bone tissues, we constructed cDNA library from mouse newborn calvaria and performed yeast two-hybrid screenings. A preceding study indicated that yeast two-hybrid screening using N-terminal half of ZNF384 (human ortholog of Ciz) successfully identified the interacting proteins including zyxin and PCBP1 [9]. Therefore, we performed yeast two-hybrid screening using N-terminal half of Ciz as a bait that lacks zinc finger domain (mCiz-ΔZF) but contains serine-rich domain (SRD), leucine zipper-like domain (LZD),