FEBS Letters 580 (2006) 1376–1382

CT domain of CCN2/CTGF directly interacts with fibronectin and enhances cell adhesion of chondrocytes through integrin α5β1 ☆

Mitsuhiro Hoshijima[a], Takako Hattori[a], Miho Inoue[a], Daisuke Araki[a], Hiroshi Hanagata[b], Akira Miyauchi[c], Masaharu Takigawa[a,*]

[a] *Department of Biochemistry and Molecular Dentistry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 5-1 Shikata-cho, 2-chome, Okayama 700-8525, Japan*
[b] *R&D Department, Higeta Shoyu Co. Ltd., Choshi, Japan*
[c] *Choshi Laboratory, ProteinExpress Co. Ltd., Choshi, Japan*

Received 31 October 2005; revised 16 January 2006; accepted 17 January 2006

Available online 26 January 2006

Edited by Gianni Cesareni

Abstract **Searching for CCN family protein 2/connective tissue growth factor (CCN2/CTGF) interactive proteins by yeast-two-hybrid screening, we identified *fibronectin 1* gene product as a major binding partner of CCN2/CTGF in the chondrosarcoma-derived chondrocytic cell line HCS-2/8. Only the CT domain of CCN2/CTGF bound directly to fibronectin (FN). CCN2/CTGF and its CT domain enhanced the adhesion of HCS-2/8 cells to FN in a dose-dependent manner. The CCN2/CTGF-enhancing effect on cell adhesion to FN was abolished by a blocking antibody against α5β1 integrin (α5β1), but not by one against anti-αvβ3 integrin. These findings suggest for the first time that CCN2/CTGF enhances chondrocyte adhesion to FN through direct interaction of its C-terminal CT domain with FN, and that α5β1 is involved in this adhesion.**

Keywords: CCN family; CCN family protein 2/connective tissue growth factor; Binding protein; Fibronectin; Integrin; αvβ3; α5β1; Cell adhesion

1. Introduction

CCN family protein 2/connective tissue growth factor (CCN2/CTGF) regulates cell adhesion, migration, proliferation, survival, differentiation, and gene expression in a number of cell types isolated from tissues, including chondrocytes [1]. CCN2/CTGF is a member of the CCN family (Cyr61, CTGF, Nov); it is strongly expressed in growth plate cartilage, especially in hypertrophic chondrocytes [1,2]. Multiple functions of CCN2/CTGF have been reported such as stimulation of cartilage-specific extracellular matrix synthesis, chondrocyte proliferation, and angiogenesis [2,3]. In CCN2/CTGF null mutant mice, osteogenesis is inhibited due to impaired chondrogenesis and growth plate angiogenesis [1]. The axial skeletal defects lead to neonatal respiratory failure and death within minutes of birth. Little is known, however, about the CCN2/CTGF interactions with chondrocytes or about the transmembrane receptors or signalling pathways that are able to transduce CCN2/CTGF-derived signals and mediate their activities in chondrocytes. Recently, it was shown that CCN2/CTGF promotes fibroblast adhesion to fibronectin (FN) and binds to FN as well as to its receptors α4β1 and α5β1 integrins and to syndecan [4]. Furthermore, it induces adhesion of hepatic cells by direct binding to the integrin receptor αvβ3 and to heparansulfate proteoglycan through its C-terminal, heparin-binding domain [5]. There also is a report that CCN2/CTGF signalling occurs through LRP1, an endocytotic receptor [1].

In order to identify additional extracellular or cell-surface targets for CCN2 that may be involved in the regulatory functions of CCN2/CTGF in chondrocytes, we searched for CCN2/CTGF-binding proteins by using the yeast two-hybrid screening assay. A cDNA library derived from human chondrosarcoma-derived chondrocytic cell line HCS-2/8 was screened for CCN2/CTGF binding since this cell line has a differentiated phenotype similar to that of normal chondrocytes in terms of aggrecan- and cartilage collagen secretion and integrin expression profiles, e.g., α2β1 and α5β1 [6]. Among several extracellular matrix proteins we identified *fibronectin 1* gene during the screening, a major cell-surface protein found also on chondrocytes [7].

In this paper, we show for the first time that (1) direct CCN2/CTGF binding to FN occurred through the C-terminal heparin-binding CT domain, the same domain that also binds to integrins αvβ3 and α5β1 (αvβ3 and α5β1) in fibroblasts [5]; (2) CCN2/CTGF as well its C-terminal heparin-binding domain enhanced HCS-2/8 chondrocytic cell adhesion to FN in a dose-dependent manner; and (3) the enhancing activity could be impaired by a monoclonal antibody against the CT domain and by an antibody against α5β1, but not by anti-αvβ3.

2. Materials and methods

2.1. Yeast two-hybrid cDNA library screening

Full-length and truncated cDNAs of *ccn2/ctgf* were prepared as baits by PCR amplification and cloned into the pGBKT7 vector containing the DNA-binding domain of GAL4. For screening, a cDNA library

☆ This work was supported by grants from the program Grants-in-Aid for Young Scientists (B) (to T.H.) and Scientific Research (S) (to M.T.) of the Ministry of Education, Science, and Culture of Japan; by a grant from Senri Life Science Foundation (to T.H.); and by internal grants from Okayama University (to T.H. and M.T.).

*Corresponding author. Fax: +81 86 235 6649.
E-mail address: takigawa@md.okayama-u.ac.jp (M. Takigawa).

Abbreviations: CCN2/CTGF, CCN family protein 2/connective tissue growth factor; αvβ3, integrin αvβ3; α5β1, integrin α5β1; FN, fibronectin; BSA, bovine serum albumin

0014-5793/$32.00
doi:10.1016/j.febslet.2006.01.061

from the human chondrocytic cell line HCS-2/8 was constructed in the *Eco*RI site of the pGADT7 vector. A total of 1×10^6 independent cDNA clones with an average insert size of ~2.4 kbp (range: 2.0–4.0 kbp) was obtained. Aliquots of 50 μg of the resulting cDNA library were then used individually to transform AH109 yeast cells that had been transformed with a CCN2/CTGF-bait containing amino acid residues 27–349 and screened on selection plates lacking leucine, tryptophan, histidine, and adenine but containing 3-AT and X-gal. After 3–5 days incubation, positive clones were picked, and plasmid DNA was used to re-transform *Escherichia coli* DH5α strain for further cloning and sequencing. The primers used for amplification of full-length and truncated forms of *ccn2/ctgf* were the following. $CCN2_{full}$ (27–349): 5′-atccgaattccagaactg-cagcgggccgtgccggtgcccg-3′ and 5′-atacggatccctcatgccatgtctccgtacatctt-cctgt-3′; $CCN2_{IGFBP}$ (27–101): 5′-atccgaattccagaactgcagcgggccgtg-ccggtgcccg-3′ and 5′-atacggatccgagcaccatctttggcggtgcacacgccga-3′; $CCN2_{VWC}$ (94–198): 5′-atccgaattcgtgtgcaccgccaaagatggtgctccctgc-3′ and 5′-atacggatccagttggctctaatcatagttgggtctgggc-3′; $CCN2_{TSP}$ (193–258): 5′-atccgaattcactatgattagagccaactgcctggtccaga-3′ and 5′-atacggatccggat-gcacttttgcccttcttaatgttct-3′; $CCN2_{CT}$ (249–349): 5′-atccgaattcaacattaa-gaagggcaaaaagtgcatccgt-3′ and 5′-atacggatccctcatgccatgtctccgtacatcttc-ctgt-3′.

All CCN2/CTGF peptides were expressed as a c-Myc epitope-fused form, and the expression of each protein in yeast was confirmed by Western blotting using anti-Myc antibody and cell lysates from yeast transformants (data not shown).

2.2. Cell culture and DNA transfection

HCS-2/8 human chondrocytic cells were maintained as described before [8,9]. Transient transfection experiments were performed by using Fugene 6 (Roche, Indianapolis, IN). pFlag/mCCN2-3HA, which expressed mouse CCN2/CTGF containing multimerized HA-epitope tag on its C-terminus, was prepared by PCR using primers 5′-ccgcgaattcaatgctcgcctccgtcgcaggtcccatc-3′ and 5′-cccgggatcctctagat-taagatctcgccatgtctccgtacatcttcct-3′, and linkage of *Bam*HI-tacccatac-gatgttccagattacgctagatcc tacccatacgatgttccagattacgctagatcc tacccatac-gatgttccagattacgct-*Bgl*II, and insertion into the pFlag-CMV2 vector.

2.3. Cell attachment and antibody blocking assays

HCS-2/8 cells were trypsinized, washed, and suspended at a cell density of 2×10^5/ml in DMEM containing various concentrations of recombinant CCN2/CTGF [3], or CT [10], or bovine serum albumin (BSA) and/or antibodies. Twelve-well plates were coated with human recombinant FN (Becton Dickinson, NJ) for 16 h and blocked with 1% BSA in PBS, pH 7.3, for 1 h. One milliliter of the cell suspension was introduced into the wells, and the cells were then allowed to attach for 30 min at 37 °C. Unbound cells were removed by washing with PBS 3 times; and the attached cells were then collected, centrifuged, resuspended, and counted. For antibody blocking assays, the following antibodies were used: JMab {a monoclonal antibody against the CT domain which was kindly provided by JT Central Pharmaceutical Research Institute (Takatsuki, Japan), also see [10]}, control antibody (anti-Flag epitope monoclonal antibody, Sigma), anti-αvβ3 (LM609, Chemicon, CA), and anti-α5β1 (JBS5, Chemicon).

2.4. Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were done as described previously [8,9]. For in vitro binding, 0.3 μg of recombinant CCN2/CTGF and/or 2 μg of recombinant FN were incubated in binding buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% NP-40, 0.5% BSA) containing 3 μg of anti-FN antibody (BD Biosciences, CA) at 4 °C for 30 min. For in vivo binding, HCS-2/8 cells transfected with Flag and HA-tagged CCN2/CTGF were harvested in lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1% Triton-X100) and incubated with 2 μg of anti-HA antibody (Covance, CA). Immuno-complexes were precipitated by protein G agarose (Amersham Biosciences, NJ).

2.5. Solid-phase binding assay

Maxisorp ELISA plate wells were coated with 100 μl of 1 μg/ml recombinant CCN2/CTGF and CT peptide in 50 mM $NaHCO_3$ buffer (pH 9.6) at 4 °C overnight, and blocked with 200 μl of binding buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2% BSA, 0.05% Tween20) for 3 h at 37 °C. Biotinylated and/or unlabeled FN were added to the wells in a total volume of 100 μl of binding buffer and incubated for 6 h at 37 °C. The wells were washed with binding buffer and then incubated with 100 μl of streptavidin-HRP (R&D Systems, MN). Bound HRP was monitored using TMD peroxidase substrate kit (Bio-Rad, CA).

2.6. Immunofluorescence of cell-surface proteins

HCS-2/8 cells transfected with Flag and HA-tagged CCN2/CTGF were grown on glass slides, and cell-surface immunofluorescence staining was done as previously [8,9]. After mounting, the protein localization was observed by using a confocal Laser scanning microscope (Bio-Rad).

3. Results

3.1. FN interacts with CCN2/CTGF through the CT domain

To identify CCN2/CTGF-binding proteins, we carried out GAL4-based yeast two-hybrid screening using human chondrocytic cell line, HCS-2/8 and *ccn2/ctgf* cDNA fragment (a.a.res. 27–349) as a bait. As a result, *fibronectin 1* gene product was identified as one of the CCN2/CTGF-binding proteins among 50 000 individual clones. The C-terminal half of FN was sufficient for binding to CCN2/CTGF (Fig. 1A). To confirm the specificity of this interaction, we sought to identify the FN-interacting sites in the CCN2/CTGF molecule which consists of 4 domains. For this purpose, full-length CCN2/CTGF (“full”, Fig. 1A) and its subdomains were expressed in AH109 yeast cells as GAL4-DNA-binding domain (GAL4 BD)-fusion proteins (Fig. 1A). The strongest binding to FN1 was achieved with the full-length CCN2/CTGF ($CCN2_{full}$) and a fragment containing the CT domain ($CCN2_{CT}$). Relatively weaker binding was obtained with the VWC domain ($CCN2_{VWC}$), whereas the IGFBP ($CCN2_{IGFBP}$) and TSP1 ($CCN2_{TSP1}$) domains did not show any interaction (Fig. 1B). Direct binding between recombinant CCN2/CTGF and FN in vitro was confirmed in a pull-down assay using anti-FN and anti-CCN2/CTGF (Fig. 1C). Additionally, FN binding to CCN2/CTGF was shown in vivo in a pull-down assay with HA-tagged CCN2/CTGF expressed in HCS-2/8 cells (Fig. 1D). We also investigated the ability of full length or CT domain of CCN2/CTGF to bind FN in a solid phase (Fig. 1E). Biotinylated recombinant FN was bound effectively to full length CCN2/CTGF or CT domain coated on microplate wells. In the presence of unlabeled competitor, FN binding decreased in a dose-dependent manner (Fig. 1E). Taken together, these results confirm a direct interaction between FN and CCN2/CTGF, especially with the CT domain.

3.2. CCN2/CTGF enhances cell adhesion to FN

In order to test the possibility that the affinity of CCN2/CTGF for FN affected the cell adhesion of chondrocytes to FN, we plated HCS-2/8 cells on microtiter dishes coated with increasing amounts of FN in the presence or absence (“control”) of CCN2/CTGF and allowed them to adhere for 30 min. Fig. 2A shows that HCS-2/8 cells attached to FN in a dose-dependent manner and that this adhesion was enhanced in the presence of CCN2/CTGF. Similarly, adhesion of HCS-2/8 cells to a constant amount of FN was enhanced by increasing amounts of CCN2/CTGF (Fig. 2B).