Identification and Cloning of a Connective Tissue Growth Factor-like cDNA from Human Osteoblasts Encoding a Novel Regulator of Osteoblast Functions*

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We have identified and cloned a novel connective tissue growth factor-like (CTGF-L) cDNA from primary human osteoblast cells encoding a 250-amino acid single chain polypeptide. Murine CTGF-L cDNA, encoding a polypeptide of 251 amino acids, was obtained from a murine lung cDNA library. CTGF-L protein bears significant identity (~60%) to the CCN (CTGF, Cef10/Cyr61, Nov) family of proteins. CTGF-L is composed of three distinct domains, an insulin-like growth factor binding domain, a von Willebrand Factor type C motif, and a thrombospondin type I repeat. However, unlike CTGF, CTGF-L lacks the C-terminal domain implicated in dimerization and heparin binding. CTGF-L mRNA (~1.3 kilobases) is expressed in primary human osteoblasts, fibroblasts, ovary, testes, and heart, and a ~26-kDa protein is secreted from primary human osteoblasts and fibroblasts. In situ hybridization indicates high expression in osteoblasts forming bone, discrete alkaline phosphatase positive bone marrow cells, and chondrocytes. Specific binding of 125I-labeled insulin-like growth factors to CTGF-L was demonstrated by ligand Western blotting and cross-linking experiments. Recombinant human CTGF-L promotes the adhesion of osteoblast cells and inhibits the binding of fibrinogen to integrin receptors. In addition, recombinant human CTGF-L inhibits osteocalcin production in rat osteoblast-like ROS 17/2.8 cells. Taken together, these results suggest that CTGF-L may play an important role in modulating bone turnover.

Connective tissue growth factor (CTGF) was originally identified as a major chemotactic and mitogenic factor from endothelial cells (1). CTGF is distinct from, but immunologically related to, platelet-derived growth factor and competes for binding to the platelet-derived growth factor receptor (1). In fibroblasts, transforming growth factor β induces the production of CTGF mRNA and protein, which in turn induces type I collagen gene expression (2). Other members of this family include Fisp12 (3), which is the murine orthologue of human CTGF; human and murine Cyr61 (4, 5); the chicken orthologue of Cyr61, Cef10 (6); and human and Xenopus Nov (7, 8). These genes, with the exception of nov, are immediate-early genes that are induced by serum, growth factors, or certain oncogenes and are collectively referred to as the CCN (CTGF, cef10/cyr61, nov) family (9). The nov gene was identified as a gene that was induced as a result of proviral rearrangement due to insertion of nephroblastosis associated virus (7).

Members of CCN family are cysteine-rich proteins that are organized into four distinct motifs (9). The first motif contains an insulin-like growth factor (IGF) binding domain (GGCC-CXXC) common to all seven known IGF-binding proteins (IGFBPs). The second domain contains a von Willebrand factor type C (VWC) module that is suspected to be involved in oligomerization. The third is a thrombospondin type I repeat (TSP1) that is thought to play a role in cell attachment and binding to matrix proteins and sulfated glycoconjugates (9). The fourth, a C-terminal (CT) domain, has been implicated in heparin binding and dimerization (10). The CT domain (~10 kDa) of CTGF is present in biological fluid and is sufficient for some biological activities (10).

Osteoblasts are specialized mesenchymal cells that are responsible for synthesizing and secreting the complex mixture of collagenous and noncollagenous proteins that make up bone matrix. These cells are also responsible for subsequent mineralization of this matrix (11). During the process of bone formation and remodeling, there is an integrated process of osteoclast-mediated bone resorption and osteoblast-derived bone formation (11, 12). The process of bone formation and remodeling is under tight regulation by numerous factors, including endocrine hormones, cytokines, growth factors, adhesion molecules, and extracellular matrix components.

In the present report, we describe the identification, cloning, expression, and functional characterization of a novel CTGF-like (CTGF-L) cDNA from primary human osteoblast cells. CTGF-L contains the first three domains present in CCN family members but lacks the fourth CT domain. We show that CTGF-L is expressed at high levels in human bone tissue. Recombinant hCTGF-L protein binds to IGFs and promotes adhesion of osteoblast cells. In addition, rhCTGF-L inhibits the
binding of fibrinogen to integrin receptors and inhibits osteocalcin production from rat osteoblast-like cells.

MATERIALS AND METHODS

Cells and Cell Culture—Primary osteoblasts were grown from explants of human trabecular bone fragments from knee joints taken at surgery (kindly provided by the Rothman Institute, Pennsylvania Hospital, Philadelphia, PA). The osteoblasts were cultured in Eagle’s modified minimum essential medium supplemented with 10% fetal calf serum (HyClone, Logan, UT), 2 mM L-glutamine, and antibiotics for 2–3 weeks as described previously (13, 14). Cells from up to three passages were used for all experiments. Primary human fibroblasts, human osteosarcoma MG 63 and SaOS-2, HeLa, and human mesenchial cells were obtained from ATCC (Manassas, VA). Human stromal TF274 cells have been described previously (15).

RNA Isolation and Northern Blot Analysis—Total RNA was isolated from primary human osteoblasts and different cell lines using Trizol reagent (Life Technologies, Inc.) according to the manufacturer's recommendation. RNA was fractionated by electrophoresis on 1.2% agarose-formaldehyde gels, transferred to Genscreen plus membranes, and cross-linked using an UV Stratalinker-180 (Stratagene, La Jolla, CA). The blots were probed with 32P-labeled CTGF-L cDNA and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) according to standard procedures (16). A commercial human multiple tissue Northern blot (CLONTECH, Palo Alto, CA) containing 2 μg of poly(A)+ RNA from various tissues was processed according to the manufacturer's instructions.

Cloning of CTGF-L cDNAs and Expression of Recombinant Protein—Human CTGF-L cDNA was identified by expressed sequenced tag (EST) analysis (17) of a cDNA library derived from primary human osteoblasts. After sequencing, the clone HOE10893 was found to contain the entire open reading frame of hCTGF-L. An EST (AA754979) from a murine lung library, with high homology to the human CTGF-L cDNA, was identified from the GenBank data base. This EST contained the 3'-untranslated region and encoded about 35 amino acids from the C terminus of CTGF-L protein. Based on the most 5' sequence of this EST, primers and the full-length cDNA was isolated using the 5' rapid amplification of cDNA ends from a marathon cDNA library prepared from murine lung (CLONTECH). For recombinant protein expression, the coding region of hCTGF-L was subcloned into CDN vector, where the expression of hCTGF-L was driven by the cytomegalovirus promoter (18). Two epitope tags, an N-terminal human immunodeficiency virus Gp120 V3 (5SKHRRQGRFRGGR) and His10, were inserted into the signal sequence, and an enterokinase cleavage site was engineered between the epitope tags and CTGF-L protein. The plasmid vector, where the expression of hCTGF-L was driven by the cytomegalovirus promoter, was subsequently transfected into Chinese hamster ovary cells by electroporation, and cells expressing hCTGF-L were bulk-selected in nucleoside-free defined medium supplemented with 10% fetal bovine serum with 100–150 μCi/ml of 35S-labeled cysteine (1000 Ci/mmol, ICN Biomedicals, Costa Mesa, CA). For immunoprecipitation, 5 μl of preimmune or anti-CTGF-L immune-serum was mixed with precleared 35S-labeled conditioned medium and 20 μl of protein A-agarose (Life Technologies, Inc.) and incubated overnight at 4 °C. Immune complex beads were collected by centrifugation and washed three times with PBS (PBS containing 0.1% Tween 20 buffer). The beads were solubilized in sample buffer and resolved through SDS-PAGE, fixed, dried, and processed for fluorography and autoradiography.

Ligand Western Blotting and Cross-linking—Recombinant hCTGF-L (10–300 pmol) was separated by SDS-PAGE under nonreducing conditions. The protein was transferred to nitrocellulose membrane, blocked with 3% BSA in PBS, and probed overnight with 106 cpm of 35S-labeled IGF-I or -II (2000 Ci/mmol, Amersham Pharmacia Biotech) or anti-CTGF-L antibody (1:1000) in PBS containing 0.1% BSA for 1 h. The membrane was then washed 4 times for 15 min each with PBS and either dried and exposed to autoradiographic film for ligand blots or developed with ECL for Western blot (Amersham Pharmacia Biotech). For cross-linking, 300 pmol of rhCTGF-L was mixed with 1 × 106 cpm of 35S-labeled IGF-I or -II for 2 h at 4 °C. Water soluble homobifunctional cross-linker BS3 (Sigma) was added to the reaction mixture at a final concentration of 5 mM for 1 h. The reaction was stopped by addition of SDS-PAGE sample buffer, and the products were separated by 12.5% SDS-PAGE. In some cases, the cross-linking was performed in the presence of excess unlabeled IGF-I or IGF-II (Life Technologies, Inc.).

Cell Adhesion Studies—Corning 96-well enzyme-linked immunosorbent assay plates (Corning, NY) were precoated overnight at 4 °C with various concentrations of rhCTGF-L, 0.1 μl of human vitronectin (0.2 μg/ml in PBS), or BSA (3 mg/ml). The plates were washed once with PBS and blocked with 3% BSA in PBS for 1 h at room temperature. Cells were trypsinized and resuspended in RPMI medium and suplemented with 10% FCS to a concentration of 1 × 105 cells/ml, and 0.1 ml of cell suspension was added to each well. Following 1 h of incubation at 37 °C, the cells were fixed by the addition of 25 μl of a 10% formaldehyde solution, pH 7.4, at room temperature for 10 min. The plates were washed three times with 0.2 ml of PBS, and the adherent cells were stained with 0.1 ml of 0.5% toluidine blue for 20 min. The plates were aspirated and the wells were incubated in 0.1 ml of Buffer A (50 mM Tris, 100 mM NaCl, 1 mM MgCl2, 1 mM MnCl2, pH 7.4) containing 3% BSA for 1 h at room temperature. Excess stain was removed by extensive washing with distilled water. The toluidine blue incorporated into cells was eluted by the addition of 0.1 ml of 50% ethanol containing 50 mM HCl and quantitated by measuring absorbance at 630 nm on a microtiter plate reader (Titertek Multiskan MC, Sterling, VA).

Integrin Binding—Vitronectin receptor, α5β1 (0.12 μg), and fibrinogen receptor, αIIbβ3 (1 μg), purified from human placenta and blood, respectively (22) were added to 96-well microtiter plates and incubated overnight at 4 °C. At the time of experiment, the protein solutions were aspirated and the wells were incubated in 0.1 ml of Buffer A (50 mM Tris, 100 mM NaCl, 1 mM MgCl2, 1 mM MnCl2, pH 7.4) containing 3% BSA for 1 h at room temperature to block the nonspecific binding. After aspirating the blocking solution, various concentrations of CTGF-L was added to the wells followed by the addition of 8 nM biotinylated fibrinogen in 100 μl of Buffer A containing 0.1% BSA. The plates were incubated for 1 h at room temperature and washed twice with 100 μl of binding buffer. Anti-biotin antibody conjugated to alkaline phosphatase (1:2000 dilution, Sigma) was then added for 10 min followed by two washes with binding buffer containing 0.1% Tween 20. The reaction was quantitated with alkaline phosphatase substrate (Bio-Rad) by measuring absorbance at 405 nm using a microtiter plate reader.

Osteocalcin Measurement—ROS 17/2.8 cells were seeded in 24-well plates at a concentration of 3 × 104 cells/ml in assay medium (Ham’s F-12 medium supplemented with 1% fetal calf serum, 2 mM l-glutamine, and antibiotics) and incubated for 3–4 h. Fresh medium, or with 10% F-12 medium supplemented with 1% fetal calf serum, 2 mM l-glutamine, and antibiotics, was then added in duplicate, and the cultures were incubated for an additional 48 h. Osteocalcin in the culture supernatants was measured by...
RESULTS

Identification and Cloning of CTGF-L cDNA—By analyzing a human osteoblast cDNA library using expressed sequence tag analysis, we identified an EST (HOEBG39) that contained an IGF binding domain (GGGCCCCC). Complete sequencing of HOEBG39 revealed that it contained an open reading frame encoding a polypeptide of 250 amino acids. Based on sequence analysis, the HOEBG39-encoded protein exhibited significant identity to the CCN family of proteins of which CTGF and Cyr61 are the most fully characterized members. HOEBG39 encoded protein is 30–60% identical to CCN proteins (Fig. 1A) and is most closely related to CTGF (−60% identity). Therefore, we termed it CTGF-L protein. The first 23 amino acids of CTGF-L encode a putative signal sequence followed by three of the four distinct domains that are found in the CCN family of proteins (9). An IGF binding domain is present from residue Gln24 to Leu93. A VWC repeat is found from residue Ser28 to Gly164. A third TSP1 domain is contained within residues Cys194 to Cys337 (Fig. 1, A and B). A fourth CT domain (−100 residues) that has been implicated in the heparin binding and mitogenic activity of CTGF is missing from CTGF-L. An alignment of CTGF-L to various members of the CCN family is shown in Fig. 1A.

An EST data base search with hCTGF-L sequence identified a murine EST (AA754979) that exhibited significant identity (−74% at nucleotide level) to hCTGF-L. We cloned the full-length mCTGF-L cDNA from a murine lung cDNA library using marathon 5' rapid amplification of cDNA ends as described under “Materials and Methods.” The protein encoded by mCTGF-L cDNA is −70% identical to hCTGF-L protein (Fig. 1A). Similar to hCTGF-L, the mCTGF-L also encodes a protein of 251 amino acids and contains all but the fourth CT domain (Fig. 1, A and B). Phyllogenetic analysis with all available members of the CCN family grouped both human and murine CTGF-L together in a separate group (Fig. 1C). It appears that *elm* is the oldest gene from which both CTGF-L and other CCN members originated (Fig. 1C). Given the similar arrangement, composition, and length of various domains between the murine and human CTGF-L protein (Fig. 1, A and B), it is clear that the murine cDNA that we isolated is the murine orthologue of human CTGF-L.

Expression of CTGF-L mRNA and Protein—Northern blots containing RNAs from various human tissues and bone-derived cells were hybridized to a 32P-labeled CTGF-L cDNA probe. A 1.3-kilobase CTGF-L mRNA was highly expressed in primary human osteoblasts, fibroblasts, ovary and testes (Fig. 2, A, lanes 1 and 9, and B, lanes 12 and 13). A lower level of expression of CTGF-L was also observed in heart, lung, skeletal muscle, prostate, and colon (Fig. 2, B, lanes 1, 4, 6, 11, and 15). However, CTGF-L was not expressed in human osteosarcoma SaOS-2 or MG 63 cells, stromal TF274 cells, osteoclastoma SaOS-2 cells, osteoclastoma MG63 cells, and mesengial cells (Fig. 2). The expression of CTGF-L in primary osteoblast or fibroblast cultures appears to be constitutive as treatment with a variety of osteotropic agents, including parathyroid hormone, transforming growth factor-β, 1,25(OH)2D3, and estrogen, failed to modulate its expression (data not shown).

*In situ* hybridization indicated strong expression of CTGF-L mRNA in bone-forming osteoblasts on calcified cartilage spicules (primary spongiosa) in fetal bone (Fig. 3, A and B) and human osteophytic tissue (Table I). Discrete alkaline phosphatase positive cells in bone marrow of osteophyte and discrete macrophage-like cells from giant cell tumor also exhibited strong expression of CTGF-L mRNA. Weaker expression was noted at sites of secondary remodeling (secondary spongiosa), chondrocytes, and osteoclasts. These data are summarized in Table I.

Polyclonal antibodies were generated to peptides derived from CTGF-L and used to immunolocalize CTGF-L protein in bone. Intense CTGF-L staining was associated with osteoblasts lining trabecular and periosteal bone surfaces from human fetal bone tissue (Fig. 3, C and D). We also examined the expression of CTGF-L protein in primary human osteoblasts and fibroblasts that show high expression of CTGF-L mRNA. In both cell types, a −26-kDa protein was specifically immunoprecipitated by anti-CTGF-L antibodies from 35S-labeled conditioned medium (Fig. 4, A, lanes 2 and 4) and cell lysate (not shown). The apparent molecular mass (−26 kDa) of immunoprecipitated protein is consistent with the expected size of the protein encoded by the open reading frame of CTGF-L mRNA after cleavage of the signal peptide. A similar sized protein was also immunoprecipitated from HeLa and TF274 cells that were transfected with an expression vector encoding hCTGF-L (data not shown).

Recombinant hCTGF-L was expressed as an N terminus human immunodeficiency virus GP120 V3 and His<sub>6</sub> epitope tag in Chinese hamster ovary cells and was purified by TALON metal affinity chromatography as described under “Materials and Methods.” The purified tagged protein of −30 kDa (expected size due to the tag) was detected by Coomassie Blue staining (Fig. 4B, lane 1) and immunostaining (Fig. 4B, lane 2).

Binding of CTGF-L to IGF-I and IGF-II—The presence of an IGF binding domain within CTGF-L prompted us to investigate the binding of CTGF-L to IGFs using ligand Western blotting and cross-linking. Increasing amounts of rhCTGF-L were separated by nonreducing SDS-PAGE and transferred onto a nitrocellulose membrane. The blot was probed sequentially with 125I-labeled IGF-I, 125I-labeled IGF-II, and anti-CTGF-L antibody. As little as 10 pmol of CTGF-L bound to labeled IGFs (Fig. 5A). rhCTGF-L showed a dose-dependent binding to 125I-labeled IGFs, with IGF-II exhibiting relatively higher binding than IGF-I. Immunostaining confirmed the increasing amounts of rhCTGF-L loaded in each lane (Fig. 5A, bottom panel). As a control, an unrelated His-tagged protein, p38 MAP kinase, did not bind either IGF-I or IGF-II (Fig. 5A, lane 5). In a cross-linking experiment using a homobifunctional cross-linker BS<sub>3</sub>, both 125I-labeled IGF-I and -II bound to rhCTGF-L (Fig. 5B, lane 1). The size of cross-linked product (−37–38 kDa) is consistent with the cross-linking of rhCTGF-L (−30 kDa) to IGFs (−7–8 kDa). We also performed a competitive cross-linking experiment in which the cross-linking was competed with increasing amounts of unlabeled IGF-I (Fig. 5B, lanes 2–5) or IGF-II (lanes 6–9). The signal obtained by cross-linking of rhCTGF-L to 125I-labeled IGF-II was much higher than with 125I-labeled IGF I. In addition, only 30 ng of IGF-II was needed to compete rhCTGF-L cross-linking to IGF-I (Fig. 5B, lane 7, upper panel), whereas even 300 ng of IGF-I could not compete the cross-linking of rhCTGF-L to IGF-II (lane 5, lower panel). These data suggest that both IGF I and IGF II bind to rhCTGF-L and that IGF-II has at least 10-fold higher affinity for CTGF-L compared with IGF-I. It should be noted that the specific activity of both 125I-labeled IGF-I and IGF-II was comparable (−2000 Ci/mmole).

Adhesion of Osteoblast Cells to CTGF-L—The TSP1 domain has been implicated in the promotion of cell attachment (9). Therefore, we tested whether rhCTGF-L was able to promote the adhesion of osteoblastic cells. Primary human osteoblast, osteosarcoma MG63, and rat osteoblast-like osteosarcoma ROS 17/2.8 cells attached to immobilized rhCTGF-L in a dose-dependent manner (Fig. 6A). Although maximal adhesion was
observed with Ros 17/2.8 cells, the extent of cell adhesion to rhCTGF-L for all cells was comparable to that observed with vitronectin. Half-maximal adhesion to rhCTGF-L was observed at a coating concentration of 300 ng/ml (10 nM), whereas no cell adhesion was observed using a protein preparation in which CTGF-L was depleted using anti-CTGF-L antibodies or FIG. 1.

A Alignment of CTGF-L with various members of the CCN family of proteins. A, protein sequence alignment was performed using the MEGALIGN program of Lasergene (DNASTAR Inc., Madison, WI) according to the clustal algorithm. The amino acids identical to CTGF are hidden and indicated by dots, whereas dashes indicate gaps. The broken, dashed, solid and dotted lines with arrows indicate the IGF binding domain, the VWC domain, the TSP1 domain, and the CT domain, respectively.

B CCN members

C Phylogenetic relationship between various members of the CCN family.

FIG. 1. Alignment of CTGF-L with various members of the CCN family of proteins. A, protein sequence alignment was performed using the MEGALIGN program of Lasergene (DNASTAR Inc., Madison, WI) according to the clustal algorithm. The amino acids identical to CTGF are hidden and indicated by dots, whereas dashes indicate gaps. The broken, dashed, solid and dotted lines with arrows indicate the IGF binding domain, the VWC domain, and the CT domain, respectively. B, schematic representation of different domains, including signal sequence (SS) of CCN proteins. IGF BD, IGF binding domain. The amino acid number flanking each domain of hCTGF-L is also shown. Note that the CT domain is missing in CTGF-L. C, phylogenetic relationship between various members of the CCN family.
report that Cyr61, another CCN family member, binds α₁β₃ (23) prompted us to examine the effect of CTGF-L on the binding of purified integrins to matrix protein. The purified integrin receptors, α₁β₃, and αIIbβ₃, demonstrated high affinity binding to biotinylated fibrinogen. As shown in Fig. 7, rh-CTGF-L inhibited the binding of fibrinogen to both integrins in a dose-dependent manner. The IC₅₀ for α₁β₃ (Fig. 7, filled triangles) was ~100 nM, whereas weaker inhibition was observed for αIIbβ₃ (IC₅₀ ~1 μM) (open circles), suggesting some degree of selectivity.

Effect of rhCTGF-L on Osteocalcin Production—To determine whether rhCTGF-L could modulate osteoblast function, we examined its effect on osteocalcin production in rat osteoblast-like osteosarcoma, ROS 17/2.8 cells. Osteocalcin is a marker of mineralizing osteoblasts, and its expression is used routinely as a measure of osteoblast function. ROS 17/2.8 cells endogenously secrete low levels of osteocalcin, which can be up-regulated by treatment with 1,25(OH)₂D₃. As shown in Fig. 8, rhCTGF-L inhibited both basal and 1,25(OH)₂D₃-stimulated osteocalcin production in a dose-dependent manner with an IC₅₀ of ~300 ng/ml (~10 nM). Treatment with a CTGF-L-depleted protein preparation or with an unrelated His₆-tagged protein, p38, had no effect on osteocalcin production (data not shown). The inhibition of osteocalcin expression was at the mRNA level because rhCTGF-L also inhibited the osteocalcin mRNA induction by 1,25(OH)₂D₃ both in Ros 17/2.8 and in primary human osteoblasts (data not shown).

**DISCUSSION**

During the past few years, several proteins belonging to the expanding CCN family have been described (9). Most members of this family are immediate-early genes that are induced by treatment with serum or growth factors (1, 3, 5, 24, 25). Most members of this family contain four distinct domains, including the IGF binding domain, the VWC domain, the TSP1 domain, and the CT domain.

We have identified human (and murine) CTGF-L as a novel member of the CCN family. There are two striking features that distinguish CTGF-L from the other CCN family members. First, CTGF-L lacks the fourth CT domain found in all other family members. Second, CTGF-L is not induced by serum or growth factors. Although an alternatively spliced form may exist, we have been unable to identify a CTGF-L variant that encodes a protein containing the CT domain in libraries derived from cells and tissues expressing CTGF-L.

In addition, immunoprecipitation of CTGF-L by anti-CTGF-L antibodies detected only a single ~26-kDa protein in both osteoblasts and fibroblasts. We did not detect a protein of
were labeled with [35S]cysteine, and the conditioned medium was immunoprecipitated with preimmune (PI) or immune (IM) anti-CTGF-L antibody. The location of purified rhCTGF-L as detected by Coomassie Blue staining indicates negative signal; + to +++ indicates moderate to very strong signal obtained with antisense probe. No signal was obtained with sense probe.

b non-bone-forming giant cell tumors were negative.

We have examined the activity of rhCTGF-L in several assays to elucidate potential functions for various domains of this protein, including cell adhesion, IGFs and integrin binding, and osteocalcin production. IGFBPs regulate the activity and availability of IGFs in bone and other tissues (26). Although we have unequivocally shown that rhCTGF-L binds to both IGF-I and IGF-II, to date, no significant change in IGF action has been observed in the presence of CTGF-L. This suggests that CTGF-L may serve not to modulate IGF activity, but rather to increase the local concentrations of IGFs. Although cross-linking and ligand Western blotting studies did not allow a precise determination of binding affinities, it appears that the relative affinity of rhCTGF-L for IGF-II is at least 10-fold higher than for IGF-I. In this regard, it has been reported that human osteoblasts produce and respond better to IGF-II, whereas rodent osteoblasts produce and are more responsive to IGF-I (26). In a recent report, rhCTGF was also shown to bind both IGF-I and IGF-II (27) but with much lower affinity than the binding of IGFBP3 to IGFs. Unlike IGFBPs, no reports on modulation of IGFs activity by CCN family of proteins have been described.

Whereas the binding of CTGF-L to IGFBPs is likely mediated via the IGF binding domain similar to the IGFBPs', cell adhesion is most likely mediated via the TSP1 domain, perhaps through binding to integrin receptors.

Figure 4. Expression of endogenous and recombinant CTGF-L protein. A, primary human fibroblasts (Fib.) and osteoblasts (Obs.) were labeled with [35S]cysteine, and the conditioned medium was immunoprecipitated with preimmune (PI) or immune (IM) anti-CTGF-L antibody. The location of ~26-kDa CTGF-L is indicated. B, recombinant CTGF-L was expressed as N terminus GP120/His6-tagged protein in Chinese hamster ovary cells and purified by TALON metal affinity chromatography. Molecular weight markers (in thousands) and the location of purified rhCTGF-L as detected by Coomassie Blue staining (lane 1) and Western blot (lane 2) are shown.

Higher molecular mass in the anti-CTGF-L immunoprecipitates that might contain the fourth CT domain. Furthermore, murine CTGF-L cDNA encodes an open reading frame that terminates at the same amino acid residue as human CTGF-L. CTGF-L without the CT domain, therefore, is the functional protein being produced and secreted by osteoblast and fibroblast cells.

Another feature that distinguishes CTGF-L from all other members of the CCN family is the inability of serum or various growth factors to induce its expression. The expression of CTGF-L in primary osteoblast or fibroblast cultures appears to be constitutive, and to date, we have not been able to modulate its expression by treatment with a variety of osteotropic agents, including parathyroid hormone, transforming growth factor-β, 1,25(OH)2D3, and estrogen. An additional feature is the absence of CTGF-L mRNA expression in various tumor cell lines, including osteosarcoma, and it appears that its expression is restricted to primary cells, such as osteoblasts and fibroblasts.

Human tissues expressing the highest levels of CTGF-L mRNA are bone, ovary, and testes. We have shown that both CTGF-L mRNA and protein are expressed at high levels in osteoblasts forming bone in the periosteum and primary spongiosa of human fetal and osteophytic bone. These bone formation areas are zones of appositional and longitudinal bone growth. Lower levels of expression were observed in osteoblast lining the relatively slower remodeling trabeculae of the secondary spongiosa. Thus, CTGF-L mRNA was highly expressed by osteoblasts at sites of high bone turnover (Table I) associated with net gain in bone mass.

Table I

| Tissue type | Cell type | mRNA expression |
|-------------|-----------|-----------------|
| Osteophyte, including synovium and granulation tissue | Osteoblasts | +
|                  | Primary spongiosa | +
|                  | Secondary spongiosa | +
|                  | Chondrocytes | +/
|                  | Synovial macrophages | +
|                  | Marrow cells | + to +++
|                  | Osteoclasts | +/
| Fetal bone | Osteoblasts | +
|                  | Primary spongiosa | +
|                  | Secondary spongiosa | +
|                  | Chondrocytes | +
|                  | Myocytes | +
| Giant cell tumor, including areas of bone formation and alkaline phosphatase-positive stroma | Stromal cells | +
|                  | Osteoblasts | +
|                  | Osteocytes | +
|                  | Marrow cells | +/
|                  | Osteoclasts | –
|                  | Blood vessels | –
|                  | Macrophages (discrete) | +++

* – indicates negative signal; + to +++ indicates moderate to very strong signal obtained with antisense probe.
strated that rhCTGF-L directly inhibits fibrinogen binding to purified integrin receptors. Fibrinogen, like many extracellular matrix proteins, contains an RGD motif that is crucial for integrin binding. However, no RGD sequence is present in CTGF-L or Cyr61, which has also been shown to bind \( \alpha_\text{v} \beta_3 \) integrin (23). Therefore, CTGF-L binding must occur through an RGD-independent mechanism. Consistent with this notion, the adhesion of osteoblasts to vitronectin, but not to CTGF-L, was inhibited by the RGD-containing snake venom protein echistatin (data not shown). Therefore, CTGF-L may inhibit matrix/integrin interaction by binding close to the RGD recognition site due to steric hindrance or by inducing a conformational change. Similarly, the effect of CTGF-L on osteocalcin release may occur through the TSP1 domain and integrin binding because it is known that integrin binding to extracellular matrix proteins can result in signal transduction leading to changes in cell proliferation, gene expression, and cellular differentiation (28). It is possible that these changes may be
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...role in wound repair and/or fibrosis (1, 10, 31–33). Cyr61 has been shown to be an extracellular matrix-associated molecule that has mitogenic activity when used in conjunction with basic fibroblast growth factor and can also promote adhesion and migration of fibroblasts and vascular endothelial cells (34, 35). Recently, it has been determined that Cyr61 mediates the adhesion of cells via αvβ3 integrin binding and has been suggested to promote tumor growth and angiogenesis (36, 37). Elm, another family member, is expressed at low levels in metastatic cancer and suppresses metastasis (25).

While this report was in preparation, two laboratories reported the cloning of murine and rat CTGF-L. WISP-2, the murine orthologue of hCTGF-L, was cloned by subtractive hybridization as a Wnt-1 inducible gene, the expression of which was reduced in some tumors (38). The rat orthologue of CTGF-L, rCOP-1, was cloned by differential display analysis as a gene of which the expression was lost as a result of cell transformation (39). An identical sequence has also been submitted to the GenBank™ data base as a CTGF-related protein, CT 58, which was found in a yeast two-hybrid screen from epithelial cell library using mucin, Muc1, as a bait (GenBank™ accession number AF074604). Mucin is a family of highly glycosylated secreted proteins that are aberrantly expressed in epithelial tumors, including breast carcinomas (40). Although these studies suggest a role for CTGF-L in tumorigenesis possibly as a tumor suppressive gene, they are yet to be confirmed using purified protein. Given the range of activities associated with this class of proteins, the exact function(s) of CTGF-L is not readily predictable.

We have shown that rhCTGF-L is active at nanomolar concentrations in assays measuring the inhibition of osteocalcin production, the promotion of cell adhesion, and integrin binding, suggesting that these could be among the major functions of this protein. We do not yet know whether CTGF-L is deposited in bone matrix, where it might facilitate osteoblast adhesion leading to osteoid formation. By virtue of its ability to promote cell adhesion and binding to IGFs, CTGF-L may increase the local concentration of IGFs to augment osteoblast activity. In this regard, it is tempting to speculate that CTGF-L may also function to maintain or extend the osteoblast matrix maturation phase by inhibiting the production of osteocalcin, a standard marker of mineralizing osteoblasts. The high expression of CTGF-L in bone and suggested tumor suppressive activity of some CCN protein suggests that it may also play a role in preventing tumor metastasis to bone and other tissues. The identification of CTGF-L adds to the diversity of the CCN family and suggests their potential role in bone metabolism. Further studies with CTGF-L will help elucidate its role in bone and other tissues.

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...cells were treated with different concentrations of rhCTGF-L in the absence (open circles) or presence (filled triangles) of 10 nM 1,25(OH)2D3. Osteocalcin present in 48-h culture supernatants was measured by radioimmunoassay. The data are representative of three triplicate determinations.

Although the VWC domain has been implicated in oligomerization, we have not observed either dimers or any other higher order species; nor have oligomers been reported for other CCN proteins. CTGF-L lacks the CT domain, which alone is sufficient for heparin binding and mitogenic activity of CTGF (10). This suggests an interesting possibility that CTGF-L may antagonize the effect of CT domain in one or more CCN proteins. As osteoblasts are capable of expressing various CCN members, including CTGF (2), Cyr61 (24), and CTGF-L.

Due to the multidomain structure of CTGF-L, antisera generated against peptides derived from the VWC domain and the C terminus of CTGF-L were nonneutralizing and, therefore, were not useful for blocking experiments. Alternatively, we used a similarly tagged but unrelated protein, p38, or a preparation in which rhCTGF-L was depleted with anti-CTGF-L antibodies. Neither protein preparations had any effect in any of the assays tested, suggesting that the functional results obtained were due to CTGF-L protein. Similarly, we have also confirmed various activities reported here using a rhCTGF-L preparation in which the epitope tags were removed by enterokinase digestion, suggesting that the presence of epitope tags did not have any effect on the rhCTGF-L protein activity (data not shown).

The multidomain structure of the CCN family of proteins, of which Cyr61 and CTGF are the best characterized members, suggests that they may have multiple functions (9). CTGF protein has mitogenic and chemotactic activity and may have a role in wound repair and/or fibrosis (1, 10, 31–33). Cyr61 has been shown to be an extracellular matrix-associated molecule that has mitogenic activity when used in conjunction with basic fibroblast growth factor and can also promote adhesion and migration of fibroblasts and vascular endothelial cells (34, 35). Recently, it has been determined that Cyr61 mediates the adhesion of cells via αvβ3 integrin binding and has been suggested to promote tumor growth and angiogenesis (36, 37). Elm, another family member, is expressed at low levels in metastatic cancer and suppresses metastasis (25).

While this report was in preparation, two laboratories reported the cloning of murine and rat CTGF-L. WISP-2, the murine orthologue of hCTGF-L, was cloned by subtractive hybridization as a Wnt-1 inducible gene, the expression of which was reduced in some tumors (38). The rat orthologue of CTGF-L, rCOP-1, was cloned by differential display analysis as a gene of which the expression was lost as a result of cell transformation (39). An identical sequence has also been submitted to the GenBank™ data base as a CTGF-related protein, CT 58, which was found in a yeast two-hybrid screen from epithelial cell library using mucin, Muc1, as a bait (GenBank™ accession number AF074604). Mucin is a family of highly glycosylated secreted proteins that are aberrantly expressed in epithelial tumors, including breast carcinomas (40). Although these studies suggest a role for CTGF-L in tumorigenesis possibly as a tumor suppressive gene, they are yet to be confirmed using purified protein. Given the range of activities associated with this class of proteins, the exact function(s) of CTGF-L is not readily predictable.

We have shown that rhCTGF-L is active at nanomolar concentrations in assays measuring the inhibition of osteocalcin production, the promotion of cell adhesion, and integrin binding, suggesting that these could be among the major functions of this protein. We do not yet know whether CTGF-L is deposited in bone matrix, where it might facilitate osteoblast adhesion leading to osteoid formation. By virtue of its ability to promote cell adhesion and binding to IGFs, CTGF-L may increase the local concentration of IGFs to augment osteoblast activity. In this regard, it is tempting to speculate that CTGF-L may also function to maintain or extend the osteoblast matrix maturation phase by inhibiting the production of osteocalcin, a standard marker of mineralizing osteoblasts. The high expression of CTGF-L in bone and suggested tumor suppressive activity of some CCN protein suggests that it may also play a role in preventing tumor metastasis to bone and other tissues. The identification of CTGF-L adds to the diversity of the CCN family and suggests their potential role in bone metabolism. Further studies with CTGF-L will help elucidate its role in bone and other tissues.

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