RAPID COMMUNICATION

The heparin-binding 10 kDa fragment of connective tissue growth factor (CTGF) containing module 4 alone stimulates cell adhesion

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Abstract

Connective tissue growth factor (CTGF) is a 349-residue mosaic protein that contains four structural modules implicated in protein-protein interactions. To address the functionality of residues 247–349 (containing module 4 alone), this region of CTGF was produced as a maltose binding protein (MBP) fusion protein in E. coli. After removal of MBP, recombinant CTGF commenced at Glu247, was of Mr 10000, was immunoreactive with anti-CTGF[247–260], bound strongly to heparin, and promoted dose-dependent adhesion of fibroblasts, myofibroblasts, endothelial cells, and epithelial cells. An 8 kDa presumptive C-terminally truncated form of CTGF commencing at Glu247 also promoted cell adhesion. CTGF-mediated cell adhesion was abolished by heparin or EDTA. These data demonstrate the presence of heparin-binding and cell-adhesion motifs within the C-terminal 103 residues of CTGF and show that CTGF-mediated cell adhesion is heparin-and divalent cation-dependent. Thus, CTGF isoforms comprising essentially module 4 are intrinsically functional in the absence of the other constituent modules of CTGF.

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Introduction


The CTGF protein is organized into four modules which structurally resemble an insulin-like growth factor binding motif (module 1), a von Willebrand factor type C repeat (module 2), a thrombospondin-1 domain (module 3), and a cysteine knot (module 4) (Bork 1993). This modular structure is conserved in other CTGF-related proteins (CYR61, NOV, WISP-1, -2, -3) that collectively comprise the CTGF/CYR61/NOV (CCN) family (Bork 1993, Brigstock 1999, Lau & Lam 1999, Perbal 2001). Data from several laboratories support an important role for module 4 in the activity of several CCN proteins since it is involved in regulation of mitosis by CTGF, heparin-binding by CTGF or CYR61, regulation of the...
angiogenic activity of VEGF by CTGF, or the binding of Notch or fibulin 1C by NOV (Brigstock et al. 1997, Perbal et al. 1999, Chen N. et al. 2000, Sakamoto et al. 2002). Here we report that a recombinant form of CTGF comprising essentially module 4 alone is a heparin-binding protein that supports heparin- and divalent cation-dependent adhesion of fibroblasts, epithelial cells, endothelial cells, and myofibroblasts. These data emphasize the intrinsic functionality of module 4.

Materials and Methods

**Generation of 10 kDa CTGF fusion protein**

10 kDa human CTGF, corresponding to residues 247–349, was produced in *E. coli* using the maltose-binding protein fusion system. Briefly, cDNA encoding 10 kDa CTGF was generated by RT-PCR of human fibroblast mRNA. The amplified product was blunt end ligated into the *Xmn* I site of pMAL-c2 (New England BioLabs, Beverly, MA, USA) using T4 DNA ligase (GIBCO/BRL, Grand Island, NY, USA) to generate pMAL-c2/10KCTGF. *E. coli* strain BL21 (F*ompT* T*10* m*B*) (Novagen, Madison, WI, USA) was transformed with pMAL-c2/10 KCTGF, plated onto LB agar plates with 100 μg/ml ampicillin, and a CTGF-positive clone, termed DB-10K, was selected.

**Production and purification of recombinant CTGF[247–349] in E. coli**

DB–10K cells were grown in 1 liter of LB broth containing 100 μg/ml ampicillin and 2 g glucose until the optical density at 600 nm was ~4·5. Recombinant protein was induced with 0·3 mM IPTG for 1 h and the bacterial cells were lysed at 16 000 psi in 40 ml amylase column buffer (10 mM Tris–HCl, 200 mM NaCl, 1 mM EDTA) using a French Press. The homogenate was clarified by centrifugation and the supernatant was diluted 1:10 with amylase column buffer and loaded on to an amylase column (5 cm x 10 cm; BioRad, Hercules, CA) at 4 °C. Bound proteins were eluted using 10 mM maltose in amylase column buffer and fractions containing peak protein levels were combined (approx 40 ml) and digested for up to three days at room temperature with 250 μg of Factor Xa. The sample was then clarified using a 0·45 μm filter and subjected to sequential steps of heparin affinity FPLC and C8 reverse-phase HPLC, essentially as previously described (Brigstock et al. 1997).

**Protein analysis**

Aliquots of column fractions were subjected to SDS-PAGE and Western blotting using rabbit anti-CTGF[247–260] peptide antiserum (Brigstock et al. 1997). Automated N-terminal amino acid sequence analysis of HPLC-purified CTGF was performed using an ABI Procise 491 protein sequencer (Applied Biosystems, Foster City, CA, USA).

**Cell adhesion**

50 μl PBS containing CTGF samples were incubated over night at 4 °C in 96-well round-bottom ELISA plates (Corning Inc, Reynoldsburg, OH, USA). Wells were...
Figure 2 Purification of recombinant human CTGF[247–349]. The figure shows sequential steps of (A) amylose-affinity chromatography followed by Factor Xa cleavage of the eluted fusion protein, (B) heparin-affinity chromatography of the cleavage reaction; and (C) C8 reverse-phase HPLC of the 0.8 M NaCl heparin eluate. The insets show CTGF immunoreactivity in 10 µl (A,C) or 5 µl (B) of selected fractions that were analyzed by SDS-PAGE on 15% (A) or 18% (B,C) gels followed by Western blotting using anti-CTGF[247–260] peptide antiserum. 3 µl (B) or 20 µl (C) of selected fractions were diluted to a total volume of 100 µl in PBS and tested for their ability to promote adhesion of Balb/c 3T3 cells to U-bottom wells of non-tissue culture 96-well plates.
blocked with 200 µl PBS containing 3% BSA and then incubated for 1 h at 37 °C with 100 µl PBS containing approximately 5 x 10⁴ Balb/c 3T3 cells, intestinal epithelial cells (IEC-6), bovine aortic endothelial cells (BAEC), or myofibroblastic hepatic stellate cells (HSC-T6; kindly provided by Scott Friedman M.D., Mount Sinai Hospital, New York, NY, USA). Some incubations were done in the presence of 5 µg/ml heparin or 10 mM EDTA. Adherent cells were then fixed for 15 min with 5% formaldehyde and non-adherent cells were removed by washing each well three times with PBS. The remaining cells were measured by fluorescent emission from the wells at 520 nm following addition of 100 µl Cytoquant reagent (Molecular Probes, Eugene, OR, USA) in lysis buffer.

Results

A ~52 kDa CTGF-immunoreactive protein doublet, representing the MBP-10 kDa CTGF fusion protein, was produced in DB-10K cells following treatment with IPTG (Figure 1). In the presence of Factor Xa, a 10 kDa cleavage product was generated in a time-dependent manner, with complete production by 48–72 h (Figure 1). The fusion protein was isolated by amylose-affinity purification (Figure 2A) after which it was cleaved with Factor Xa and subjected to heparin-affinity FPLC. Uncleaved fusion protein and 10 kDa CTGF were eluted from the heparin column by, respectively, ~0.5 M NaCl (fractions #7–9) and ~0.8 M NaCl (fractions #16–19) (Figure 2B), the

Figure 3 N-terminal sequence analysis of recombinant CTGF proteins and their relationship to full length CTGF. The figure shows the modular structure of the 349-residue primary translational product of CTGF as well as the structure of native and recombinant N-terminally truncated CTGF. (1) Native 10 kDa CTGF from ref (Brigstock et al. 1997); (2) Recombinant 10 kDa CTGF present in HPLC fraction #43 (see Figure 2C): Initial yield 144 pmol, repetitive yield 63%; (3) Recombinant 8 kDa CTGF protein in HPLC fraction #39 with possible C-terminal truncation (see Figure 2C): Initial yield 314 pmol, repetitive yield 64%.

Figure 4 Dose-dependent stimulation of Balb/c 3T3 cell adhesion by 10 kDa CTGF
latter of which is an identical elution position as that of the native 10 kDa CTGF protein (Brigstock et al. 1997). Both the fusion protein and 10 kDa cleavage product supported adhesion of 3T3 cells to non-tissue culture plastic (Figure 2B). C8 reverse-phase HPLC of the 0.8 M NaCl eluate resulted in the separation of several CTGF proteins in the 8–10 kDa range, all of which promoted 3T3 cell adhesion (Figure 2C). N-terminal sequence analysis of the most abundant 8 kDa and 10 kDa proteins demonstrated that they both commenced at Glu247 (Figure 3) suggesting that the 8 kDa protein was C-terminally truncated.

HPLC-purified 10 kDa CTGF promoted 3T3 cell adhesion in a dose-dependent manner, with maximal binding at 8 ng/well (Figure 4). 10 kDa CTGF also promoted the dose-dependent adhesion of several additional cell types including endothelial, epithelial, and myofibroblastic cells (Figure 5). The binding of each cell type to 10 kDa CTGF was completely inhibited by 5 µg/ml heparin (Figure 5), suggesting that cell surface heparan sulfate proteoglycans (HSPGs) act as adhesion receptors for 10 kDa CTGF. In view of the recent findings that full-length CTGF or CYR61 bind to cell-surface integrins and that this process is dependent on divalent cations (Lau & Lam 1999), we next tested the effect of EDTA on 10 kDa CTGF-mediated cell adhesion. Binding of all cell types to 10 kDa CTGF was substantially reduced by EDTA treatment, though 3T3 cells were less affected as compared with the other cell types (Figure 5).

Discussion

Although CTGF and other CCN proteins were recognized as modular proteins almost a decade ago (Bork 1993), there has been very little progress in understanding the functional significance of their constituent modules, either collectively or individually. This aspect of CCN biology has been confounded by the discovery that the ‘4-module’ structure is not rigorously conserved in all CCN proteins. For example, WISP-2 completely lacks module 4 (Zhang et al. 1998, Kumar et al. 1999) while module 2 is absent in a short form of WISP-1 called WISP1 v (Tanaka et al. 2001). Additionally, N-terminal truncations have been described that result in the absence of module 1 or modules 1 and 2 from NOV (Joliot et al. 1992, Perbal 1999) or in the absence of modules 1 and 2 or modules 1, 2, and 3 from CTGF (Brigstock et al. 1997, Ball et al. 1998). Although the biological significance of this structural heterogeneity remains largely unexplored, it may be a means of generating variants that exhibit a diverse range of agonistic and antagonistic activities, as well as bioavailabilities. Based on the structure of their constituent modules, CCN proteins are predicted to bind to a diverse variety of other molecules (Bork 1993). Among the interactions that have been identified are partnering of integrins with CTGF or CYR61 (Lau & Lam 1999), low density lipoprotein-related protein with CTGF (Segarini et al. 2001), heparin with CTGF or
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