RAPID COMMUNICATION

Intrinsic biological activity of the thrombospondin structural homology repeat in connective tissue growth factor

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Abstract

Connective tissue growth factor (CCN2) is a 349-residue mosaic protein that contains four structural modules (modules 1–4), which are presumptive domains for interactions with regulatory binding proteins and receptors. Module 3, corresponding to residues 199–243, is a thrombospondin structural homology repeat (TSR) and is flanked by regions that are highly susceptible to proteolytic cleavage. To test whether CCN2 module 3 (CCN2₃) has intrinsic biological properties, it was produced recombinantly in *Escherichia coli* (*E. coli*) and examined for its effects on the function of hepatic stellate cells (HSC), the principal fibrogenic cell type in the liver. CCN2₃ stimulated dose-dependent HSC adhesion and activity of p42/p44 mitogen activated protein kinase, the latter of which was antagonized by blocking the activity of focal adhesion kinase. HSC adhesion to immobilized CCN2₃

was attributed to binding interactions with cell surface integrin $\alpha 6\beta 1$. As assessed by RT-PCR or Western blotting, CCN2₃ stimulated production of fibronectin and pro-collagen type IV ($\alpha 5$), both of which are downstream components of HSC-mediated fibrogenesis and which are constituents of high density matrix in fibrotic lesions. These data show that while the full length CCN2 protein is strongly associated with fibrosis and stellate cell function, key integrin-binding properties, signaling, and fibrogenic pathways are exhibited by module 3 alone. These data indicate that module 3 of CCN2 is intrinsically active and suggest that liberation of module 3 following CCN2 proteolysis may contribute to HSC-mediated fibrogenesis, as well as other CCN2-dependent processes.

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Introduction

Connective tissue growth factor (CCN2; also termed 'CTGF') is one of six genes (CCN1–6) that have been classified as members of the CCN family (Brigstock 1999). CCN proteins participate in critical processes, as shown by the fact that CCN2 null mice die after birth due to cardiovascular and skeletal defects while mice that are null for CCN1 (which is about 50% homologous to CCN2) die during embryogenesis due to angiogenic defects (Mo *et al.* 2002, Ivkovic *et al.* 2003).

CCN2 is a 349-residue protein that exhibits a broad spectrum of biological activities and is associated with the regulation of diverse biological processes, many of which have direct relevance to endocrine systems. For example, CCN2 regulates uterine and luteal function; implantation, placentation, growth, development, and differentiation (Brigstock 2003). Many of these functions reflect the ability of CCN2 to exploit integrins as cell surface

signaling receptors (Lau & Lam 1999, Rachfal & Brigstock 2005a), as well as its direct stimulatory effects on the production of extracellular matrix molecules such as fibronectin (FN) and collagen (Brigstock 1999). This latter property has attracted considerable interest because, as an aberration of its normal role, CCN2 has important fibrosis-inducing actions and is highly over-expressed in fibrotic lesions such as those seen in diabetic nephropathy or hepatic fibrosis (Riser & Cortes 2001, Rachfal & Brigstock 2003). Since effective anti-fibrotic therapies are desperately needed, exploring the role of CCN2 in fibrotic pathways may reveal novel therapeutic options.

Structure-function studies of CCN2 are important for determining the location of critical domains in the molecule, especially those that are involved in binding to and signaling in fibrogenic target cells. CCN2 comprises four cysteine-rich structural modules (modules 1–4; Fig. 1A) that may function both independently and inter-dependently (Bork 1993, Brigstock 1999). Isoforms of

CCN2 comprising essentially module 4 alone are bioactive and contain binding sites for the integrins $\alpha v \beta 3$ and $\alpha 5 \beta 1$, which account for the ability of CCN2 to interact with stellate cells in the liver and pancreas,

respectively (Gao & Brigstock 2004, 2005). Stellate cells are normally quiescent, but following injury they become activated and myofibroblastic, expressing high levels of α -smooth muscle actin (α SMA). In the liver, activated

hepatic stellate cells (HSC) are responsible for deposition of excess scar tissue through their production of collagen types I, III and IV, proteoglycans, FN, laminin and the activation of tissue inhibitors of matrix metalloproteases (TIMP), which prevent fibrolysis by inhibiting matrix metalloprotease activity (Britton & Bacon 1999, Burt 1999, Friedman 1999, 2000).

Some CCN proteins, notably CCN1 and CCN2, undergo controlled, limited proteolysis via the action of matrix metalloproteases or serine proteases at sites each side of module 3, yielding low mass bioactive CCN2 isoforms (Fig. 1A) (Brigstock *et al.* 1997, Ball *et al.* 1998, Steffen *et al.* 1998, Ball *et al.* 2003a, 2003b, Tam *et al.* 2004, Pendurthi *et al.* 2005). Module 3 of CCN2 (residues 199–243) is a thrombospondin structural homology repeat (TSR) (Bork 1993), an evolutionarily-conserved moiety that occurs in about 40 other unrelated proteins that collectively regulate cell adhesion, migration, communication and tissue remodeling (Adams & Tucker 2000, Silverstein 2002). Previous data have shown that interference with module 3 can compromise functionality of CCN1 or CCN2. For example, site directed mutagenesis has demonstrated a role for module 3 in the activities of CCN1 (Leu *et al.* 2003), while an antibody to module 3 blocks CCN2-stimulated chondrocyte maturation and proteoglycan synthesis (Minato *et al.* 2004). While these studies have demonstrated the functional importance of module 3 in the context of full-length CCN proteins, the 'stand-alone' properties of module 3 have not yet been studied, even though this isoform likely arises from limited CCN2 proteolysis. Thus, to study the intrinsic biological properties of CCN2 module 3 (CCN2₃), we have produced it recombinantly in *Escherichia coli* (*E. coli*) and analyzed its ability to stimulate HSC function.

Materials and Methods

Generation of recombinant CCN2₃

Human CCN2₃ was produced in *E. coli* as a His-tagged protein. Briefly, CCN2₃ cDNA was generated by PCR using primers (forward: 5'-GAAGGATTCATGCCTGGTCCAGACCACAGAGT-3'; reverse: 5'AAGCTTTTCGCAAGGCCTGACCATGCAC-3') that amplified between residues 199 and 243 of a full length human

CCN2 cDNA template (Fig. 1A). CCN2₃ cDNA was cloned into a PCR2.1 TOPO vector for sequence verification and then cloned into pQE30Xa (Qiagen) for expression in competent M15[pREP4] *E. coli*. Colonies were screened to verify CCN2₃ protein production by Western blot using an antiserum previously raised against recombinant human CCN2₃₋₄, a form of CCN2 comprising modules 3 and 4 (Ball *et al.* 2003b). The highest producer was grown up in 1 liter cultures from which cell lysates were prepared using a French® Pressure Cell Press (SLM Instruments Inc., Urbana, IL, USA).

Production, purification and characterization of recombinant CCN2₃

E. coli supernatants were clarified by centrifugation (10 000 × *g* for 30 mins) and subjected to nickel affinity chromatography using a His-bind Quick column (7.5 cm × 1.5 cm; Novagen, Madison, WI, USA), followed by sequential heparin-affinity fast protein liquid chromatography and C₈ reverse phase HPLC, essentially as previously described in Brigstock *et al.* (1997). The presence of CCN2₃ in fractions containing the column eluates was determined by SDS-PAGE and Western blotting of aliquots of the fractions using anti-CCN2₃₋₄ as described above. The protein concentration of purified CCN2₃ samples was determined using a BCA protein assay kit (Pierce Chemical Co, Rockford, IL, USA).

HSC cultures and assays

Rat HSC T6 cells were kindly provided by Dr Scott Friedman (Mount Sinai Hospital, New York, NY, USA). Cell adhesion assays were as described (Gao & Brigstock 2004) except that some incubations were performed in the presence of 25 µg/ml mouse anti-rat integrin α6 IgG (Serotec, Oxford, UK), 25 µg/ml mouse anti-human integrin β1 IgG (Chemicon, Temecula, CA, USA), 25 µg/ml mouse anti-rat integrin α6β1 IgG (Chemicon) or 25 µg/ml normal mouse IgG (Santa Cruz Biotech Inc, Santa Cruz, TX, USA).

Western blotting

Activation of p42/p44 mitogen activated protein kinase (MAPK) in response to treatment with 0–100 ng/ml CCN2₃

Figure 1 Structure and recombinant production of CCN2₃. (A) Structural organization of the 349-residue human CCN2 protein showing the location of the module 3 sequence that was selected for expression cloning. Arrows indicate sites in the native CCN2 molecule that are prone to cleavage by serine proteases or matrix metalloproteases. (B) HPLC purification of CCN2₃ by C₈ reverse-phase HPLC. The identity of the 7.4 kDa protein eluting at 58–59 mins was verified as CCN2₃ by Western blotting of aliquots of column fractions using anti-CCN2₃₋₄ antiserum (inset). (C) Dose-dependency of CCN2₃-supported HSC adhesion. Microtiter wells were coated with the indicated amounts of CCN2₃ or 5 µg/ml CCN2 at 4 °C for 16 h and then blocked with PBS containing 3% BSA. 2.5 × 10⁵ HSC-T6 cells/ml in serum-free Dulbecco's Modified Eagle's medium were plated at 50 µl/well and incubated at 37 °C for 30 min. Non-adherent cells were removed by gentle rinsing and the remaining adherent cells were stained with CYQUANT GR dye and quantified by measuring the fluorescence intensity at Ex 485 nm/ Em 530 nm. Values represent mean ± S.D. of quadruplicate determinations and are representative of three separate experiments.

for up to 5 h was assessed by Western blot of cell lysates using anti-phospho p42/p44 MAPK antibody (Cell Signaling Inc., Beverly, MA, USA) as compared with the total MAPK signal detected using anti-p42/p44 MAPK antibody (Cell Signaling Inc.) (Gao *et al.* 2004). Controls included 50 ng/ml full-length human recombinant CCN2 (Ball *et al.* 2003b) or 2 ng/ml transforming growth factor beta 1 (TGF- β 1; R&D Systems, Minneapolis, MN, USA). Some experiments were performed after 1-hour pre-treatment of the cells with 0–10 μ M 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2; Calbiochem, San Diego, CA, USA), an inhibitor of focal adhesion kinase (FAK) (Hakuno *et al.* 2005). After treatment, HSC were washed twice with ice-cold phosphate-buffered saline and harvested by scraping the cells into cold lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 2 mM EDTA, 1 mM phenyl-methylsulfonyl fluoride, 20 μ g/ml aprotinin, 10 μ g/ml leupeptin, 20 mM β -glycerophosphate, and 2 mM sodium fluoride) supplemented with protease inhibitor cocktail (Sigma Chemical Co.), 1 μ M PMSF and 1 μ M AGP. Cell lysates were clarified by centrifugation at 15 000 $\times g$ for 10 min at 4 °C, and protein concentrations in the supernatants were determined using a BCA protein assay kit (Pierce Chemical Co.). For each sample, 40 μ g total protein were separated on 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose.

Production of FN protein was assessed by Western blotting after stimulating serum-starved HSC for 2 days with 0–100 ng/ml CCN2₃, 2 ng/ml TGF- β 1 or 50 ng/ml CCN2. FN levels were detected using rabbit anti-mouse FN antibody (Chemicon).

Reverse-transcription polymerase chain reaction

HSC were stimulated for 2 days with 0–100 ng/ml CCN2₃ or 2 ng/ml TGF- β 1, after which total RNA was extracted using TRIzol (Invitrogen) according to the manufacturers instructions. Two micrograms of purified RNA were synthesized into cDNA using SuperScriptTM II RNase H⁻ Reverse (Invitrogen) with oligo-dT(18)-primers. 2 μ l of RT products were amplified using Taq DNA polymerase by denaturation at 94 °C for 4 min, 25 cycles of 94 °C for 45 sec, 50 °C for 30 sec and 72 °C for 1 min, and then 10 min extension at 72 °C. The primers used were: FN forward: 5'-GAGAGCACACCCGTTTT CAT-3'; FN reverse: 5'-TGGAGGTTAGTGGGAGC ATC-3'; pro-collagen IV(α 5) forward: 5'-AATGGAC TCCCAGGCTTTGATGGT-3'; pro-collagen IV(α 5) reverse: 5'-CATGTCTGACATATCAACAGTGGCC-3'; β -actin forward: 5'-AGCTTGCTGTATTCCCCTCCA TCGTG-3'; β -actin reverse: 5'-AATTCGGATGGCTA CGTACATGGCTG-3'. β -actin was included as a control for quantity of RNA. PCR products were visualized by ethidium bromide staining.

Statistics

Cell adhesion data are presented as mean $\pm$ S.D. of quadruplicate measurements from 3 assays. Differences were analyzed statistically with paired-sample Student's *t*-test.

Results

Recombinant CCN2₃ was successfully produced in *E. coli* and highly purified using a sequential three-step purification procedure. Typical results for the final C₈ reverse phase HPLC step are presented in Fig. 1B, which shows that the protein was of $M_r \sim 7400$ as expected and was eluted at 58–59 mins. Purified CCN2₃ reacted with a conventional CCN2 antiserum (Fig. 1B) and supported dose-dependent adhesion of HSC (Fig. 1C) showing that critical regions for antibody recognition and biological activity were faithfully preserved, an indication that appropriate protein conformation was achieved. The dose-response and levels of maximal HSC binding were comparable to that of the full length CCN2 protein (Fig. 1C and Gao & Brigstock 2003).

We have previously shown that a module 3 peptide, ²⁰⁴TEWSACSKTCG, can block HSC adhesion to full-length CCN2 (Gao & Brigstock 2003). Since this approximate region in the CCN1 protein has been identified as one that interacts with integrin α 6 β 1 (Leu *et al.* 2003), we explored whether neutralizing antibodies directed against this integrin were able to affect CCN2₃ activity. As shown in Fig. 2, antibodies to the integrin α 6, β 1, or α 6 β 1 subunits were able to completely block CCN2₃-supported HSC adhesion. Binding of the full-length CCN2 protein was also inhibited by anti-integrin α 6 β 1 (Fig. 2A). These data thus showed that interactions of either CCN2 or CCN2₃ with integrin α 6 β 1 on the surface of HSC were critical for their adhesive properties and confirmed that an integrin α 6 β 1 binding site is present in module 3.

We have previously shown that full-length CCN2 promotes activation of p42/p44 MAPK in HSC (Gao *et al.* 2004). To determine whether this response was mimicked by CCN2₃, lysates from CCN2₃-treated HSC were subjected to Western blot using anti-phospho-p42/p44 MAPK. As shown in Fig. 3A, CCN2₃ showed a dose-dependent stimulation of p42/p44 MAPK phosphorylation whereas total p42/p44 MAPK levels remained unchanged. p42/p44 MAPK was also activated by full length CCN2 and TGF- β 1, consistent with previous observations (Reimann *et al.* 1997, Gao *et al.* 2004). Moreover, there was comparable stimulation of MAPK activation by equimolar amounts of each protein as shown by the response to 50 ng/ml CCN2 or 10 ng/ml CCN2₃, the concentrations of which equal ~ 1.3 nM. MAPK activation was evident within 10 mins of CCN2₃ stimulation and was sustained at high levels for 30–120 mins after stimulation (Fig. 3B). As CCN2₃ is an integrin ligand (Fig. 2A),

Figure 2 Integrin $\alpha 6 \beta 1$ -dependency of CCN2- or CCN2₃-mediated HSC adhesion. Microtiter plates were precoated overnight at 4 °C with (A) BSA alone, 16 μ g/ml CCN2₃ or 5 μ g/ml CCN2 or (B) 8 μ g/ml CCN2₃. HSC adhesion assays were then performed in the presence of anti-integrin $\alpha 6 \beta 1$ IgG, anti-integrin $\alpha 6$ IgG, anti-integrin $\beta 1$ IgG, or normal IgG, all of which were tested at 25 μ g/ml. Values are mean $\pm$ S.D. of quadruplicate determinations and are representative of three separate experiments. * $P < 0.01$ versus CCN2₃ stimulation.

we tested the effect of blocking FAK activation by pre-treating the cells with the FAK inhibitor, PP2. CCN2₃-induced phospho-p42/p44 levels were decreased by PP2 treatment, whereas total p42/p44 levels were unchanged (Fig. 3C), showing that MAPK signaling in response to CCN2₃ occurred downstream of FAK activation.

Some of the most biologically relevant characteristics of activated HSC are their production of FN or collagen, as these molecules contribute to the high-density matrix that is characteristic of fibrotic lesions. To assess whether CCN2₃ exhibited the ability to stimulate enhanced levels of FN, HSC were treated with 0–100 ng/ml CCN2₃ for 2 days and assessed for FN production by RT-PCR or

Western blot. As shown in Fig. 4A, there was a dose-dependent stimulation by CCN2₃ of FN mRNA and protein levels that was comparable to those attained in response to CCN2 or TGF- $\beta 1$. Under the same conditions, CCN2₃ also stimulated mRNA levels of procollagen type IV($\alpha 5$) (Fig. 4B). These data collectively show that CCN2₃ promotes fibrogenic pathways in HSC.

Discussion

CCN proteins are proposed to exist in a mosaic configuration containing four conserved modules that are evolutionarily conserved in other unrelated extracellular proteins (Bork 1993, Brigstock 1999, Lau & Lam 1999, Perbal 2001). The cysteine residues occur only within each module where they are involved in presumptive intra-modular disulphide bridging. Module 1 is an insulin-like growth factor binding domain, module 2 is a von Willebrand Type C domain, module 3 is a TSR, and module 4 is a C-terminal domain that may contain a cysteine knot. The modular configuration of CCN2 is a provoking model for investigating its function since mosaic proteins are often involved in protein-protein binding (Bork 1993). Indeed, several such interactions are documented, including partnering of integrins with CCN1, CCN2, or CCN3 (Lau & Lam 1999), low density lipoprotein-related protein with CCN2 (Segarini *et al.* 2001, Gao & Brigstock 2003), heparan sulfate proteoglycans with CCN1 or CCN2 (Kireeva *et al.* 1996, 1997, Brigstock *et al.* 1997, Chen *et al.* 2000, Grzeszkiewicz *et al.* 2002, Gao & Brigstock 2004), insulin-like growth factors with CCN2 or CCN3 (Kim *et al.* 1997, Burren *et al.* 1999), fibulin 1C, RNA polymerase II, Notch, or S100A4 (mts1), calcium binding protein with CCN3 (Perbal 1999, Perbal *et al.* 1999, Li *et al.* 2002, Sakamoto *et al.* 2002) and vascular endothelial growth factor with CCN2 (Inoki *et al.* 2002). In many cases, these interactions regulate the activity or bioavailability of the CCN protein or of its binding partner, and are fully consistent with the mosaic nature and matricellular actions of CCN proteins.

While the net activity, half life, and bioavailability of intact, full length CCN proteins reflects the individual and combined effects of their constituent modules, these properties are likely modified as a result of controlled proteolysis which yields bioactive lower mass isoforms. Enzymatic processing of CCN proteins is a feature of diverse biological systems including HSC activation, cyclic uterine remodeling, and tumor cell function (Brigstock *et al.* 1997, Ball *et al.* 1998, Williams *et al.* 2000, Tam *et al.* 2004, Pendurthi *et al.* 2005). CCN proteins, notably CCN2, are particularly susceptible to proteolytic cleavage between modules 2 and 3 or modules 3 and 4. However, while we have previously addressed the

Figure 3 Stimulation of p44/p42 MAPK phosphorylation by CCN2₃. (A) 5.0×10^5 HSC/well were cultured in 6-well plates until they were 80–90% confluent, after which the medium was replaced with serum-free DMEM for 48 h. The cells were then treated with TGF- β 1, CCN2, or CCN2₃ at the indicated doses for 30 min. Cells were harvested, lysed and subjected to Western blot with anti-p42/p44 or anti-phospho-p42/p44 antibodies. (B) Time course of p42/p44 MAPK phosphorylation following stimulation of serum-starved HSC with 50 ng/ml CCN2₃. (C) Serum-starved HSC were pretreated with 0–10 μ M PP2 for one hour and then stimulated with 50 ng/ml CCN2₃ for 30 min prior to Western blot detection of activated or total p42/p44 MAPK. The results are representative of three independent experiments.

functionality of CCN2₄ and CCN2_{3–4} (Brigstock *et al.* 1997, Steffen *et al.* 1998, Ball *et al.* 1998, 2003a, 2003b, Gao & Brigstock 2003, Gao & Brigstock 2004, 2005), there have been no detailed investigations of CCN2₃. The data presented in this report show that CCN2₃ is intrinsically active and utilizes integrin α 6 β 1 as an adhesion receptor on HSC. Moreover, the CCN2₃ effects on HSC include stimulation of FAK-dependent MAPK activation as well as promotion of fibrogenic pathways that lead to enhanced production of FN or collagen IV. These data show that module 3 of CCN2 has intrinsic biological activities in the absence of the other constituent modules, suggesting that this region of CCN2 retains functionality when liberated from larger CCN2 isoforms. Our data clearly implicate the TSR as mediating – and actually mimicking – some of the properties of the full length CCN2 protein.

TSR-containing proteins are generally involved in cell adhesion, migration, communication and tissue remodeling (Bork 1993, Adams & Tucker 2000, Silverstein 2002). The TSR module maps to a 50–60 residue domain that contains a conserved WSxWSxWS motif. In CCN2, this is a WSxCSxTCG sequence at residues 206–214 and is contained in the peptide ²⁰⁴TEWSACSKTCG which we previously showed blocked HSC adhesion to CCN2 isoforms that contain module 3 (Gao & Brigstock 2003). These observations support the findings of earlier studies that documented direct binding interactions between integrin α 6 β 1 and full length CCN1, with involvement of the same respective region of module 3 (Chen *et al.* 2000, Leu *et al.* 2003).

A very strong case has begun to emerge that links CCN2 with liver fibrosis, irrespective of the underlying etiology (Rachfal & Brigstock 2003). With respect to

Figure 4 Promotion of pro-fibrogenic pathways in HSC by CCN2₃. 2.5×10^5 HSC/well were cultured in 6-well plates until they were 80–90% confluent, after which the medium was replaced with serum-free DMEM for 48 h. The cells were treated with TGF-β1 (2 ng/ml), full-length CCN2 (50 ng/ml) or CCN2₃ (50, 100 ng/ml) in serum-free medium for 48 h at the indicated concentrations and then extracted for protein or RNA analysis by RT-PCR or Western blot, respectively. (A) FN mRNA (upper panel), β-actin mRNA (middle panel) or FN protein (lower panel) and (B) Pro-collagen type IV(α5) mRNA (upper panel) and β-actin mRNA (lower panel).

HSC responses, CCN2 induces adhesion, migration and proliferation, the latter of which is associated with transient induction of c-fos activation and activation of the MAPK signaling pathway (Paradis *et al.* 2002, Gao & Brigstock 2004, Gao *et al.* 2004). In addition, CCN2 induces expression of α-SMA and collagen in HSC, consistent with a role in activation and fibrogenesis (Paradis *et al.* 2002). Adhesive signaling by CCN2 in HSC increases expression of collagen, FN and TIMP-1, all of which are upregulated in hepatic fibrosis (Rachfal & Brigstock 2005b). While module 4 of CCN2 has previously been implicated in eliciting many of these responses via interactions with integrin αvβ3 (Gao & Brigstock 2004), it is striking that module 3 can have very similar effects and utilizes integrin α6β1 as its principal receptor. It remains to be determined to what extent there is redundancy, synergism, or specificity in the signaling pathways downstream of these integrins, whether additional integrins or other receptors are involved, and whether the response is dependent on how the module is presented in the context of a specific CCN2 isoform. Nonetheless, these studies highlight the fact that a single target cell can engage different parts of the CCN2 molecule via distinct integrin receptors. This complex mode of action will require careful consideration in the development of anti-fibrotic therapeutics that target CTGF receptor pathways.

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