Connective Tissue Growth Factor (CCN2) Induces Adhesion of Rat Activated Hepatic Stellate Cells by Binding of Its C-terminal Domain to Integrin $\alpha_v\beta_3$ and Heparan Sulfate Proteoglycan*

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Connective tissue growth factor (CCN2, also known as CTGF) is a matricellular protein that appears to play an important role in hepatic stellate cell (HSC)-mediated fibrogenesis. After signal peptide cleavage, the full-length CCN2 molecule comprises four structural modules (CCN2$_{1-4}$) and is susceptible to proteolysis by HSC yielding isoforms comprising essentially modules 3 and 4 (CCN2$_{3-4}$) or module 4 alone (CCN2$_4$). In this study we show that rat activated HSC are capable of adhesion to all three CCN2 isoforms via the binding of module 4 to integrin $\alpha_v\beta_3$, a process that is dependent on interactions between module 4 and cell surface heparan sulfate proteoglycans (HSPGs). These findings are based on several lines of evidence. First, integrin $\alpha_v\beta_3$ was detected in HSC lysates by immunoprecipitation and Western blot, and CCN2$_{4}$-mediated HSC adhesion was blocked by anti-integrin $\alpha_v\beta_3$ antibody. Second, as assessed by immunoprecipitation and solid phase binding assay, CCN2$_4$ bound directly to integrin $\alpha_v\beta_3$ in cell-free systems. Third, destruction or inhibition of synthesis of cell surface HSPGs with, respectively, heparinase or sodium chlorate abrogated HSC adhesion to CCN2$_4$. Fourth, prior occupancy of heparin-binding sites on CCN2$_4$ with soluble heparin completely blocked HSC adhesion. These findings indicate that integrin $\alpha_v\beta_3$ functions as a co-receptor with HSPGs for CCN2$_4$-mediated HSC adhesion. Furthermore, by peptide mapping and site-directed mutagenesis we demonstrated that the sequence IRTPKISPIKFLESG within CCN2$_4$ is a unique binding domain for integrin $\alpha_v\beta_3$ that is sufficient to mediate integrin $\alpha_v\beta_3$ and HSPG-dependent HSC adhesion. These findings offer the possibility of developing novel antifibrotic therapies that target the integrin-binding domain.

Connective tissue growth factor (CCN2) is a cysteine-rich protein that stimulates a broad repertoire of cellular responses including proliferation, chemotaxis, adhesion, migration, and extracellular matrix production (1). CCN2 has been implicated in regulating diverse processes in vivo including angiogenesis, placentation, embryogenesis, differentiation, wound healing, and fibrosis, and its target cells include fibroblasts, endothelial cells, smooth muscle cells, epithelial cells, and neuronal cells (1–3). The CCN2 primary translational product comprises 349 residues that are organized into a signal peptide and four structural modules that resemble an insulin-like growth factor-binding domain (module 1), a von Willebrand factor type C repeat (module 2), a thrombospondin type I repeat (module 3), and a C-terminal domain that contains a putative cysteine knot (module 4). The modular structure of CCN2 (CTGF) is conserved in other members of the CCN family that also includes CCN1 (CYR61), CCN3 (NOV), CCN4 (WISP-1), CCN5 (WISP-2), and CCN6 (WISP-3) (1, 3, 4). Studies of the binding properties of the individual modules within CCN proteins, together with their biological properties, have led to the suggestion that they act as matricellular proteins (3, 5), a term originally used to describe a group of unrelated molecules (TSP1, TSP2, SPARC, tenascin C, and osteopontin) that interact contextually with specific cell surface receptors, cytokines, growth factors, and proteases to influence cell function by modulating cell-matrix interactions (6–8).

Recent reports have provided strong evidence of a role for CCN2 in liver fibrosis, especially as a mediator of transforming growth factor-$\beta$ action. Although several cell types produce CCN2 in fibrotic liver (9–11), attention has become focused on hepatic stellate cells (HSC), the liver-specific pericytes located in the space of Disse that lie in close contact with hepatocytes and sinusoidal endothelial cells. Following liver injury, HSC undergo a transition from quiescent vitamin A-rich cells to activated vitamin A-deficient, proliferative, fibrogenic, and contractile myofibroblasts. CCN2 expression by HSC is enhanced during the process of activation as well as by transforming growth factor-$\beta$, vascular endothelial growth factor, platelet-derived growth factor, lipid peroxidation products, and acetaldehyde (10, 12). In response to exogenous CCN2, HSC demonstrate increased migration, proliferation, adhesion, and expression of type I collagen (12–14).

Over the last few years, progress has begun to be made in determining the mechanisms that underlie the diverse effect of CCN proteins on cell function. CCN proteins have emerged as novel ligands for integrins and can functionally engage different integrin subtypes to elicit specific biological effects. Integrins are composed of $\alpha$ and $\beta$ subunits, and each $\alpha$-$\beta$ combination has its own binding specificity and signaling properties that can impact cell behavior and cell-matrix or cell-cell interactions (15, 16). Because integrins can transduce extracellular binding events into intracellular signaling cascades (17, 18),

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1 The abbreviations used are: HSC, hepatic stellate cells(s); BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; HSPGs, heparan sulfate proteoglycans; LN, laminin; VN, vitronectin; MBP, maltose-binding protein; PBS, phosphate-buffered saline.

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FIG. 1. Recombinant CCN2 proteins. The figure shows the structures of the various CCN2 isoforms used in these studies. Human recombinant CCN2_{1-4} and CCN2_{1-3} were produced in a Chinese hamster ovary expression system as described (25). Human recombinant CCN2_{1} was produced in Escherichia coli as MBP (14). MBP-CCN2_{1}, fusion proteins harboring alanine substitutions were as follows: I257A/R258A/P259A/P260A (M1), K261A/I262A/S263A/K264A (M2), P265A/K266A/K267A/K268A (M3), and E269A/L270A/S271A/G272A (M4). SP, signal peptide; IGFBP, insulin-like growth factor binding protein domain; VWC, von Willebrand factor C repeat; TSP1, thrombospondin type 1 repeat; CT, C-terminal domain.

The ability of CCN proteins to interact with different integrins provides a mechanistic basis for their broad ranging biological activities (3). To date, the most compelling data have emerged for CCN1 that uses different integrin subtypes to individually regulate adhesion, migration, and proliferation in fibroblasts, endothelial cells, smooth muscle cells, or monocytes (17, 19–24).

Although CCN2 likely functions as a paracrine and autocrine fibrogenic factor for HSC, there is no information regarding the nature of cell surface CCN2 receptors on HSC. In the present study we show that integrin α_{6}β_{3} and cell surface heparan sulfate proteoglycans (HSPGs) are indispensable for adhesion of HSC to CCN2. The integrin α_{6}β_{3} binding property localizes to a unique non-RGD region in module 4 (residues 257–272) that supports HSC adhesion via integrin α_{6}β_{3} and HSPG-dependent mechanisms.

MATERIALS AND METHODS

Antibodies, Peptides, and Reagents—Function-blocking monoclonal anti-human anti-α_{6}, β_{3} (LM609), anti-α_{v} (LM142), and anti-β_{3} (B3A) as well as purified human integrin α_{6}β_{3} were produced from Chemicon, Inc. (Temecula, CA). Heparinase I, chondroitinase ABC, bovine serum albumin (BSA; protease-free and γ-globulin-free), mouse normal IgG, laminin (LN), vitronectin (VN), echistatin, and sodium chloride (NaClO_{4}) were obtained from Sigma. Synthetic peptides GRGDsp, GRGEsp, and Dulbecco's modified Eagle's medium supplemented with 25 mM Hepes buffer and 10% fetal bovine serum. The adhesion assays were performed between the first and third serial passages on three different HSC preparations that had been allowed to autonomously activate in culture.

Cell Adhesion Assay—HSC were detached using 1 ml EDTA in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_{2}HPO_{4}, 1.4 mM KH_{2}PO_{4}, pH 7.3), washed twice in Dulbecco's modified Eagle's medium, and resuspended in serum-free medium containing 0.5% BSA. CCN2 proteins or peptides or control cell adhesion molecules were diluted to the desired concentration in PBS and used at 50 μl/well to precoat 96-well plates (Costar, Corning, NY) at 4 °C for 16 h. The wells were then blocked for 1 h with PBS containing 1% BSA prior to addition of 50 μl of HSC suspension (2.5 × 10^{5} cells/ml) for 20 min at 37 °C. The wells were washed three times with PBS, and adherent cells were fixed with 10% formalin and stained by the addition of 100 μl of Cy3-conjugated goat anti-rabbit IgG (diluted 1:1000) for 1 h. The sample fluorescence intensity was determined using a micro-plate reader (CytoFluor™2350) at an excitation of 485 nm and an emission of 530 nm.

Immunoprecipitation and Immunoblotting—1 × 10^{6} activated HSC were plated in 10-cm culture dishes and incubated for 24 h. The cells were washed with cold PBS prior to addition of 1 ml of Nonidet P-40 buffer (20 mM Tris-HCl, pH 7.4, containing 1% Nonidet P-40, 150 mM NaCl, 1 mM MgCl_{2}, 1 mM CaCl_{2}, 10% glycerol, 1 mM Na_{3}VO_{4}, 1 mM MgCl_{2}, 10% glycerol, 1 mM Na_{3}VO_{4}, 1 mM NaF, and 1 μM leupeptin, and 1 μg/ml aprotinin) with rocked at 4 °C for 1 h. The cell lysates were collected and centrifuged for 5 min at 15,000 × g. The supernatants were incubated with anti-integrin α_{v} (1:200) at 4 °C for 1 h, and the immune complexes were precipitated with 25 μl of protein A-Sepharose beads for 1 h. The beads were washed three times with 1 ml of cold Nonidet P-40 buffer and then boiled for 10 min in sample buffer. The samples were separated on 8% SDS-polyacrylamide gels and transferred onto nitrocellulose. After blocking the membrane with 5% nonfat milk, the filter was incubated with anti-integrin β_{3} (1:1000) for 1 h. The membrane was then incubated with 1 h with horseradish peroxidase-linked anti-mouse IgG (1:4000) diluted in PBS/T (10 mM Tris-HCl, pH 8.0, containing 150 mM NaCl and 0.1% Tween 20) and washed extensively with PBS/T before detection using the ECL system.

CCN2 isoforms and integrin α_{6}β_{3} binding assay—2 μg of human integrin α_{6}β_{3} were incubated with rocking at 4 °C for 2 h with 4 μg of CCN2_{1-4}, 4 μg of CCN2_{1-3}, or 4 μg of CCN2_{1-2}, in 1 ml of Nonidet P-40 buffer. The mixtures were then incubated at 4 °C for 16 h with 1:100 dilution of an immunoprecipitating polyclonal rabbit anti-CCN2 antibody (previously shown to detect all CCN2 isoforms; see Ref. 25) or 10 μg of mouse IgG followed by the addition of 25 μl of protein A-Sepharose.
beads for 1 h. In inhibition assays, 2 μg of human integrin αvβ3 were preincubated for 1 h with 20 μM echistatin in 1 ml of Nonidet P-40 buffer prior to the addition of 10 μg of CCN2α. The samples were separated on 8% SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane that was incubated with anti-human αvβ3 (1:1000) diluted in TBS/T and developed as described above.

**Solid Phase Binding Assay**—The binding of integrin αvβ3 to immobilized CCN2 isoforms was measured as follows. Microtiter wells (Dynex Technology, Chantilly, VA) were precoated with different CCN2 isoforms at the desired concentrations for 16 h at 4 °C and then blocked at room temperature for 2 h with PBS containing 2% BSA, 1 mM CaCl2, and 1 mM MgCl2. The plate was washed four times with PBS and then incubated at room temperature for 3 h with 1 μg/ml integrin αvβ3 in blocking solution. In inhibition studies, integrin αvβ3 was preincubated with 10 μM individual CCN2α synthetic peptides for 1 h. The plate was developed by the addition of anti-human αvβ3 monoclonal antibody diluted in blocking solution (1:1000) followed by horseradish peroxidase-conjugated goat anti-mouse IgG (1:4000). The color reaction was developed using horseradish peroxidase ELISA reagents (Chemicon, Temecula, CA), and the absorbance at 450 nm was measured using a HTS700 Bio Assay Reader (PerkinElmer Life Sciences).

**RESULTS**

**Involvement of Integrins in Adhesion of Activated HSC to CCN2 Isoforms**—It has previously been shown that cultured HSC produce CCN2 and its low mass isoforms as a function of activation in culture (10) and that CCN2 stimulates HSC proliferation and extracellular matrix production in vitro (12). In view of the emerging role of CCN proteins as cell adhesion molecules (3), we examined the ability of CCN2 to support the adhesion of activated HSC. As previously shown (27), all three CCN2 isoforms promoted dose-dependent HSC adhesion over a comparable concentration range (0.5–2 μg/ml), with maximal adhesion elicited by 2 μg/ml. This effect was comparable to the well characterized cell adhesion molecules, VN and LN (Fig. 2A and Ref. 27).

To assess the possibility that CCN2-mediated HSC adhesion involved cell surface integrins, we examined the effect of divalent cations on cell adhesion. Following incubation of the cells with 10 mM EDTA, CCN2α- and CCN2α-β3-mediated HSC adhesion was decreased by 50%, and CCN2α-mediated HSC adhesion was inhibited by 90% (Fig. 2A). The inhibitory effect of EDTA on the ability of CCN2α to support HSC adhesion was restored by the addition of the divalent cations Ca2+, Mg2+, or Mn2+ (Fig. 2B). In the absence of EDTA, CCN2α-mediated HSC adhesion was supported in the presence of Mg2+ or Mn2+ but completely abolished by Ca2+. Mn2+, but not Mg2+, was able to overcome the inhibitory effect of Ca2+ on HSC adhesion by CCN2α (Fig. 2B). The cation dependence of CCN2-mediated HSC adhesion was consistent with the possible involvement of integrins in this process.

To further investigate the likely role of integrins, we took
the Western blot, which corresponded to the predicted size of the \( \beta_3 \) subunit and thus confirmed that integrin \( \alpha_\beta_3 \) was produced by rat activated HSC. To further clarify a role of integrin \( \alpha_\beta_3 \) in contributing to CCN2-\( \alpha_\beta_3 \)-HSC interactions, a cell adhesion assay was performed in the presence of a specific anti-integrin \( \alpha_\beta_3 \) monoclonal antibody. As shown in Fig. 3B, the antibody inhibited adhesion of HSC to CCN24 by more than 60%, whereas mouse IgG had no inhibitory effect on CCN24-mediated cell adhesion. As expected, anti-\( \alpha_\beta_3 \) completely blocked the cell adhesion to VN but had no effect on the adhesion of HSC to LN, the latter of which is not a ligand for integrin \( \alpha_\beta_3 \).

Collectively, these data indicated that adhesion of HSC to CCN24 involves binding to integrin \( \alpha_\beta_3 \). However, the inability of integrin \( \alpha_\beta_3 \) antagonists to fully block CCN24-mediated cell adhesion (as compared with their full inhibition of VN-mediated cell adhesion) suggested that other mechanisms are also likely to contribute to the binding of HSC to CCN2.

**Binding of CCN2 to Integrin \( \alpha_\beta_3 \) in Cell-free Systems**—To further characterize the interaction between CCN2 and integrin \( \alpha_\beta_3 \), the three CCN2 isoforms were individually mixed with purified integrin \( \alpha_\beta_3 \) prior to immunoprecipitation with rabbit anti-CCN2 polyclonal antibody and immunoblotting with anti-integrin \( \alpha_\beta_3 \). As shown in Fig. 4A, a direct binding between integrin \( \alpha_\beta_3 \) and each CCN2 protein was demonstrated based on the ability to detect integrin \( \alpha_\beta_3 \) on the Western blot. Additional studies with CCN2 showed that its binding to integrin \( \alpha_\beta_3 \) was completely blocked by echistatin and that the immunoprecipitation occurred specifically in the presence of CCN2 antibodies and not a normal IgG control (Fig. 4A). To confirm the cell-free binding of integrin \( \alpha_\beta_3 \) to CCN2 using an alternative approach, a solid phase binding assay was used in which each CCN2 protein or VN were individually coated onto microtiter wells that were subsequently incubated with purified integrin \( \alpha_\beta_3 \). The presence of immobilized integrin \( \alpha_\beta_3 \) was detected by ELISA using an anti-integrin \( \alpha_\beta_3 \) antibody. As shown in Fig. 4B, each CCN2 isoform or VN was found to bind to integrin \( \alpha_\beta_3 \) with the strongest signal exhibited by CCN24. This effect was confirmed in dose-response experiments (Fig. 4C) that showed a higher level of maximal binding of integrin \( \alpha_\beta_3 \) to CCN24 (saturation at 8 \( \mu \)g/ml CCN24) as compared with VN (saturation at 4 \( \mu \)g/ml VN) Colectively, results from both types of cell-free binding assays clearly indicated that module 4 of CCN2 interacts directly with integrin \( \alpha_\beta_3 \).

**Characterization of a Binding Domain for Integrin \( \alpha_\beta_3 \) in Module 4 of CCN2**—To gain further insight into the interaction of \( \alpha_\beta_3 \) and CCN24, we sought to identify the integrin \( \alpha_\beta_3 \) binding site in CCN24. Synthetic peptides spanning the entire 103 C-terminal residues of CCN24 were assessed for their ability to directly promote HSC adhesion, inhibit CCN24-mediated HSC adhesion, or bind to integrin \( \alpha_\beta_3 \). Peptide P2 (corresponding to residues 257–272 of CCN2) was shown to exhibit agonist activity in the HSC adhesion assay, whereas the other peptides were not effective (Fig. 5A). P2 supported the dose-dependent adhesion of HSC at coating concentrations of 0.5–2 \( \mu \)g/ml (see below). In addition, when HSC were preincubated with P2, this peptide was shown to decrease CCN24-mediated cell adhesion by ~65%, while VN-mediated cell adhesion was reduced to background levels (Fig. 3B). Moreover, when P2 was preincubated with purified integrin \( \alpha_\beta_3 \) prior to performing the solid phase binding assay, the ability of integrin \( \alpha_\beta_3 \) to subsequently bind to either CCN24 or VN was totally abolished (Fig. 5B). Moreover, HSC adhesion to P2 was inhibited by 40% by echistatin and by 29% by anti-integrin \( \alpha_\beta_3 \) (Fig. 5C).

![Fig. 3. Production of integrin \( \alpha_\beta_3 \) by rat activated HSC and its involvement in CCN24-mediated cell adhesion.](image-url)

A. Anti-integrin \( \alpha_\beta_3 \) immunoprecipitates (IP) of lysates from activated HSC were subjected to SDS-PAGE and Western blot analysis using anti-integrin \( \beta_3 \) antibody. The results are representative of three separate experiments. B. P2 (10 \( \mu \)M), anti-integrin \( \alpha_\beta_3 \) (25 \( \mu \)g/ml), mouse IgG (25 \( \mu \)g/ml), or vehicle buffer alone (no add) were added to the cell suspension for 30 min at room temperature. The cells were plated into microtiter wells that had been precoated with MBP (8 \( \mu \)g/ml), CCN24 (8 \( \mu \)g/ml), or VN (4 \( \mu \)g/ml). The data are the means \( \pm \) S.D. of quadruplicate determinations and are representative of three experiments.
Unique Binding Domain for Integrin α₃β₃ in CCN2

Based on these data, it was tentatively concluded that integrin α₃β₃ binds to residues 257–272 in module 4 of CCN2.

To obtain direct evidence that residues 257–272 were involved in integrin α₃β₃ binding in the context of the CCN2 molecule, this region was targeted by site-directed mutagenesis using alanine substitution. The strategy involved spanning residues 257–272 by mutating successive groups of four adjacent amino acids (i.e., residues 257–260 in M1, 261–264 in M2, 265–268 in M3, and 269–272 in M4). All of the mutant proteins exhibited comparable heparin-binding properties to wild-type CCN2 (data not shown). However, each mutant protein exhibited a dramatic loss of binding to integrin α₃β₃ in the solid phase assay (Fig. 6A), showing that residues 257–272 contain critical determinants of integrin α₃β₃ binding. Moreover, M2 was completely unable to support HSC adhesion, whereas M1, M3, and M4 exhibited reduced levels of HSC adhesion that were, respectively, 33, 80, and 46% those of wild type CCN2 (Fig. 6B). These data showed that although HSC adhesion involves the binding of residues 257–272 to integrin α₃β₃, this region of CCN2 likely contains additional determinants involved in HSC adhesion.

Adhesion of Activated HSC to CCN2 requires Cell Surface HSPGs—Because CCN2 is a heparin-binding protein, we explored whether this property was relevant to the process of cell adhesion. As shown in Fig. 7A, adhesion of HSC to CCN2 was completely abrogated by the presence of 2 μg/ml heparin in the plating medium, whereas the same concentration of heparin had little effect on VN-mediated HSC adhesion. These results suggested that prior occupancy by soluble heparin of the heparin-binding sites in CCN2 might cause interference with its ability to bind to cell surface HSPGs. To test this possibility, cell surface HSPGs were removed from HSC by treatment of the cells with heparinase I, an enzyme that acts on highly sulfated HSPGs (31). Heparinase I treatment rendered the cells unable to adhere to CCN2, whereas there was no effect on the ability of HSC to adhere to VN (Fig. 7A). In control experiments, treatment of HSC with chondroitinase ABC failed to inhibit CCN2-mediated HSC adhesion, showing that cell sur-

Fig. 4. Integrin α₃β₃ binds to CCN2 isoforms in cell-free systems. A. 2 μg of human integrin α₃β₃ were individually added to CCN2₃₋₄ (4 μg), CCN2₄₋₄ (4 μg), or CCN2₅ (10 μg) in 1 ml of Nonidet P-40 buffer and mixed at 4 °C for 2 h. For inhibition assays, 2 μg of integrin α₃β₃ were incubated with echistatin (20 μM) for 1 h prior to mixing with CCN2. Each sample was immunoprecipitated (IP) with rabbit anti-CCN2 polyclonal antibody and blotted with anti-human integrin α₃β₃ monoclonal antibody. The results shown are representative of three experiments. B, microtiter wells were individually coated with MBP (8 μg/ml), CCN2₃₋₄ (2 μg/ml), CCN2₄₋₄ (2 μg/ml), CCN2₅ (8 μg/ml), or VN (4 μg/ml) at 4 °C for 16 h and then blocked with 2% BSA for 2 h. 1 μg/ml integrin α₃β₃ was added to the wells and allowed to bind for 3 h at room temperature, and bound integrin α₃β₃ was detected by ELISA. C, microtiter wells were coated with the indicated concentrations of MBP, CCN2₄, or VN at 4 °C for 16 h, and their binding of integrin α₃β₃ was detected by ELISA. The data are the means ± S.D. of triplicate determinations and are representative of three experiments.

Fig. 5. A synthetic CCN2 peptide, IRTPKSPKIPFEISG, contains critical binding sites for α₃β₃-dependent cell adhesion. A, cell adhesion assays were performed on the 96-well plates that had been coated at 2 μg/ml with synthetic peptides spanning the 103 C-terminal residues of CCN2. B, microtiter wells were coated with MBP (8 μg/ml), CCN2₃₋₄ (8 μg/ml), or VN (4 μg/ml) at 4 °C for 16 h and then incubated with 1 μg/ml integrin α₃β₃ alone or after preincubation of 1 μg/ml integrin α₃β₃ with 35 μM P2 for 1 h. The binding of integrin α₃β₃ was quantified by ELISA. C, HSC were preincubated with echistatin (2 μM), anti-α₃β₃ (25 μg/ml), or mouse IgG (25 μg/ml) for 30 min prior to the addition to microtiter wells that were coated with P2 (2 μg/ml) or VN (4 μg/ml). The data are the means ± S.D. of quadruplicate determinations and are representative of three experiments. No Add, no addition.
CCN2 is involved in binding to integrin $\alpha_v\beta_3$ and supporting HSC adhesion. The wells were individually coated at 4 °C for 16 h with MBP, CCN2, M1, M2, M3, or M4 (each at 8 $\mu$g/ml) and then analyzed for their ability to bind to integrin $\alpha_v\beta_3$ (A) or support adhesion of activated HSC (B). The data are the means ± S.D. of quadruplicate determinations and are representative of three experiments.

To further characterize the role of HSPGs in CCN2-mediated HSC adhesion, the cells were cultured in the presence of sodium chloride, an inhibitor of 3-phosphoadenosine 5'-phosphosulfate synthesis, to block sulfation of proteoglycans (32). As shown in Fig. 7B, the treated cells were unable to bind to CCN2 yet were unaffected in their ability to bind to VN. The inhibitory effect of sodium chloride on the adhesion of HSC to CCN2 was reversed by the addition of 10 mM Na$_2$SO$_4$ to the culture medium, confirming that this inhibitory effect was mediated through a sulfation block (32). Taken together, these results show that cell surface HSPGs are indispensable for the adhesion of HSC to CCN2 and that HSPGs are involved in the interaction between CCN2 and integrin $\alpha_v\beta_3$. To investigate this aspect further, assays were performed using P2 as an adhesive substrate for HSC in the presence of heparin or after pretreatment of the cells with heparinase. Although P2 was shown to support dose-dependent adhesion of HSC at 0.5–2 $\mu$g/ml, this property was abrogated following heparin or heparinase treatment (Fig. 7C). These data suggested that P2 is able to support HSC adhesion by binding to cell surface HSPGs.

Taken together, these results demonstrate that residues 257–272 in module 4 of CCN2 contain binding sites for integrin $\alpha_v\beta_3$ and HSPGs that impact the ability of HSC to use CCN2 as an adhesive substrate. Moreover, the HSC adhesion that is supported by the interaction of integrin $\alpha_v\beta_3$ with residues 257–272 of CCN2 is HSPG-dependent.

**DISCUSSION**

CCN2 is likely to be important in HSC-mediated fibrosis because this fibrogenic cell type produces CCN2 during activation and shows enhanced migration, proliferation, adhesion, or production of collagen or $\alpha$-smooth muscle actin in response to CCN2 (9–13). Because the mechanistic basis for the interaction of CCN2 with HSC has not been explored previously, we undertook a detailed study of the cell surface molecules involved in CCN2-mediated HSC adhesion and of the particular domains within CCN2 that participate in these interactions. The major findings of this study are that rat activated HSC are capable of adhesion to CCN2 through integrin $\alpha_v\beta_3$ and that this process requires the interaction of CCN2 with cell surface HSPGs. Furthermore, by peptide mapping and site-directed mutagenesis, we demonstrated that residues 257–272 are sufficient to mediate $\alpha_v\beta_3$-dependent HSC adhesion and that this domain also supports cell adhesion via interactions with HSPGs. Previous data have shown that module 4 is of fundamental importance because CCN2 stimulates DNA synthesis, cell adhesion, transdifferentiation, and fibrosis (14, 25, 33). Functional roles for module 4 are also highlighted by the findings that it is involved in regulation of the angiogenic activity of vascular endothelial growth factor by CCN2 and the binding of Notch or fibulin 1C by CCN3 (34, 35).

Activated HSC are myofibroblast-like cells (36) that have previously been shown to express integrins $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_\alpha\beta_3$, and $\alpha_v\beta_3$. Unique Binding Domain for Integrin $\alpha_v\beta_3$ in CCN2

**FIG. 6.** The IRTPKISKPIKFELSG sequence in module 4 of CCN2 is involved in binding to integrin $\alpha_v\beta_3$ and supporting HSC adhesion. A. microtiter wells were coated with MBP (8 $\mu$g/ml), CCN2, (8 $\mu$g/ml), or VN (4 $\mu$g/ml) prior to the addition of HSC that were pre-treated at 37 °C for 30 min with vehicle buffer (No Add), heparinase I (2 units/ml), or chondroitinase ABC (2 units/ml) or that were co-incubated with heparin (2 $\mu$g/ml) prior to plating. B. HSC were cultured in complete medium containing 10 mM NaClO$_3$ for 48 h either in the presence or absence of 10 mM Na$_2$SO$_4$ prior to addition to precoated wells. C, cell adhesion assays were performed on microtiter plates that had been coated with different concentrations of P2 using HSC that had been preincubated at 37 °C for 30 min with vehicle buffer (No Add), heparinase I (2 units/ml), or heparin (2 $\mu$g/ml) prior to plating. The data are the means ± S.D. of quadruplicate determinations and are representative of three experiments.

**FIG. 7.** CCN2 mediates heparan sulfate-dependent HSC adhesion. A, microtiter wells were coated with MBP (8 $\mu$g/ml), CCN2, (8 $\mu$g/ml), or VN (4 $\mu$g/ml) prior to the addition of HSC that were pretreated at 37 °C for 30 min with vehicle buffer (No Add), heparinase I (2 units/ml), or chondroitinase ABC (2 units/ml) or that were co-incubated with heparin (2 $\mu$g/ml) prior to plating. B, HSC were cultured in complete medium containing 10 mM NaClO$_3$ for 48 h either in the presence or absence of 10 mM Na$_2$SO$_4$ prior to addition to precoated wells. C, cell adhesion assays were performed on microtiter plates that had been coated with different concentrations of P2 using HSC that had been preincubated at 37 °C for 30 min with vehicle buffer (No Add), heparinase I (2 units/ml), or heparin (2 $\mu$g/ml) prior to plating. The data are the means ± S.D. of quadruplicate determinations and are representative of three experiments.
$\alpha_4\beta_1$ and $\alpha_5\beta_1$, which function as adhesion receptors for well-known cell adhesion molecules such as collagen, laminin, or fibronectin (37, 38). The results of our study show that HSC also produce integrin $\alpha_4\beta_1$ that acts as a cell adhesion receptor capable of binding CCN2. Because HSC binding to CCN2 was integrin-dependent as shown by its sensitivity to EDTA, divalent cations, RGD peptides, and integrin $\alpha_4\beta_1$ antibodies, module 4 appears to play a fundamental and central role in integrin-mediated HSC adhesion by CCN2. HSC adherence to CCN2 was supported by Mg$^{2+}$ and especially Mn$^{2+}$. It is widely believed that Mn$^{2+}$ induces conformational shifts that mimic the physiological activation of $\beta_1$ and $\beta_2$ integrins as Mn$^{2+}$-induced activation leads to enhanced ligand binding affinity and cell adhesion (39–41).

Although CCN1 and CCN2 can engage a variety of integrin subtypes, both molecules have been shown to bind to integrin $\alpha_4\beta_1$ on fibroblasts, endothelial cells, and breast cancer cells (17, 18, 22, 30, 42). Moreover, the aggressiveness of breast cancer is directly linked to the molecular association of CCN1 with integrin $\alpha_4\beta_1$ on breast cancer cells (42), whereas the angiogenic properties of CCN1 and CCN2 may also be related to the role of integrin $\alpha_4\beta_1$ in the process of neovascularization (43). In addition, CCN3 was recently shown to bind to integrin $\alpha_4\beta_1$ on endothelial cells (29). However, these previous studies did not address the mechanisms underlying the binding of CCN proteins to integrin $\alpha_4\beta_1$. In this regard, whereas integrin $\alpha_4\beta_1$ binds to a broad repertoire of RGD-containing ligands including VN, fibronectin, fibrinogen, von Willebrand factor, and thrombospondin (44–46), CCN1, CCN2, and CCN3 do not contain a RGD sequence, yet they mediate RGD-sensitive cell adhesion (these results and Ref. 3). These data suggest that there is a RGD-induced conformational change in integrin $\alpha_4\beta_1$ that prevents its subsequent binding of CCN proteins. Over the past few years, various other unrelated non-RGD ligands of integrin $\alpha_4\beta_1$ have also been recognized including CD31/PECAM-1 (47), matrix metalloproteinase 2 (48), and FGF-2 (49).

In the case of FGF-2, endothelial cell adhesion to integrin $\alpha_4\beta_1$ was attributed to a DGR motif (i.e., the reverse of the classic RGD cell recognition sequence). Interestingly, CCN2 also contains a DGR motif in module 4 (residues 289–291) that is conserved in CCN1 and CCN3. However, these residues did not appear to be involved in CCN2-mediated HSC adhesion because they were contained within peptide P5, which neither promoted HSC adhesion nor inhibited CCN2-mediated cell adhesion (Fig. 5A and data not shown). Our data show that the CCN2 peptide IRTPKISKPIKFELSG, corresponding to residues 257–272, inhibited adhesion of HSC to CCN2 and was able to support HSC adhesion via integrin $\alpha_4\beta_1$. The peptide also bound strongly and directly to integrin $\alpha_4\beta_1$ in a cell-free binding assay. The involvement of this domain in binding to integrin $\alpha_4\beta_1$ was also shown by the inability of CCN2 proteins harboring mutations within this region to bind to integrin $\alpha_4\beta_1$ in a solid phase binding assay. There is no sequence homology between the integrin $\alpha_4\beta_1$-binding domain identified in CCN2 in these studies and that of other non-RGD integrin $\alpha_4\beta_1$ ligands. Only four of these residues are conserved in CCN1, and only five are conserved in CCN3, and thus the role, if any, of this region in mediating integrin $\alpha_4\beta_1$ binding by these other CCN remains to be explored. Indeed it is possible that other integrin $\alpha_4\beta_1$-binding domains are present elsewhere within other CCN proteins. The presence of additional or alternative binding sites for integrin $\alpha_4\beta_1$ might help to explain the reported interaction of integrin $\alpha_4\beta_1$ with a mutant form of CCN1 lacking module 4 (20), a phenomenon that is clearly distinct from our findings for CCN2. Finally, although a binding site for integrin $\alpha_4\beta_1$ was recently mapped to module 4 in CCN1 (50), this domain corresponds to residues 274–286 of CCN2 and is thus distinct the integrin $\alpha_4\beta_1$-binding domain identified in this study. The only other reported mapping of which we are aware is the determination of an integrin $\alpha_4\beta_1$-binding site in module 3 of CCN1 (21). Collectively, these data demonstrate that the IRTPKISKPIKFELSG sequence in module 4 of CCN2 identified in this study is a unique binding site for integrin $\alpha_4\beta_1$.

The interactions of many extracellular protein ligands (e.g., heparin-binding growth factors) with their cognate receptors is regulated by their binding to cell surface HSPGs and formation of HSPG-ligand complexes (51). Recently, HSPGs have also been shown to play a role in regulating cell adhesion by acting as co-receptors for integrin ligands. For example, syndecan 1 functions as a co-receptor with integrin $\alpha_4\beta_1$ in the spreading of human breast cancer cells (45), and CCN1-mediated adhesion of fibroblasts occurs via integrin $\alpha_4\beta_1$ and HSPGs (19). In the present studies, we demonstrated that CCN2-mediated HSC adhesion via integrin $\alpha_4\beta_1$ exhibited an absolute requirement for HSPGs because HSC from which HSPGs were enzymatically removed or in which HSPG synthesis was chemically blocked were unable to adhere to CCN2. We also showed that the adhesion of HSC to the P2 peptide (IRTPKISKPIKFELSG) was heparin- and HSPG-dependent because cell adhesion could be blocked by heparin or prior treatment of the cells with heparinase. From solid phase $[^{3}H]$heparin binding data, we have previously reported that although P2 binds modestly to heparin, other regions in CCN2, most notably residues 247–260 (P1) and 273–286 (P4), interact more strongly with heparin (33). Based on deletion mutagenesis studies, the same respective domains in module 4 were also implicated in heparin binding of CCN1 and coincide with a binding domain for integrin $\alpha_4\beta_1$ (19). It is of interest that P1 and P4 failed to support HSC adhesion, showing that heparin binding properties per se are not indicative of a role in cell adhesion. Moreover the relatively lower affinity of P2 for heparin as compared with P1 or P4 (33) did not negate a role for P2 in HSPG binding as shown by its inability to support adhesion of heparinase-treated HSC. Although they are both glycosaminoglycans, heparan sulfate has a more complicated primary structure than heparin in that they mainly differ in the ratio of N-acetylation to O-sulfation (52, 53). Many glycosaminoglycan-binding proteins can be differentially sensitive to variations in glycosaminoglycan structure, and core proteins can also have dramatic ligand-specific influences on protein-proteoglycan interactions (54). Also a recent study demonstrated that although heparin showed preferential binding peptides enriched in R, K, G, and S, heparan sulfate binds preferentially to peptides enriched in R, G, S, and P (55), and these residues are all represented in peptide P2. Although the nature of the HSPG involved in CCN2 binding requires further study, human and rat HSC are known to express syndecans 1, 2, 3, and 4 as well as glypican (56, 57). Syndecans bind to a variety of extracellular ligands via their covalently attached heparan sulfate chains and are thought to play important roles in cell-matrix adhesion, proliferation, migration, and differentiation (45, 58). Because, in the context of CCN2, targeting of the integrin-binding component in HSC adhesion assays using RGD peptides or anti-integrin $\alpha_4\beta_1$ antibodies was not completely successful in blocking HSC adhesion, it is likely that HSPG binding persisted either alone or in combination with as yet other unidentified CCN2 receptors. Indeed the heparin binding characteristics of the mutant CCN2 proteins were comparable with wild type CCN2 (data not shown), consistent with the finding that the principal heparin-binding determinants of CCN2 lie outside residues 257–272 (33).
The interaction of CCN2 with integrin αβ₂ and HSPGs is fully consistent with the proposed role of CCN2 as a matricellular protein. The adhesion of fibroblasts to CCN1 or CCN2 results in a cascade of adhesive signaling events that include formation of filopodia and integrin focal complexes and activation of focal adhesion kinase, paxillin, Rac, and mitogen-activated protein kinase (17). In this manner, and through the cross-talk between integrins, HSPGs, growth factor receptors, proteases, and other signaling molecules, CCN2 may promote the deposition of matrix components and activation of growth factors that support accompanying fibrogenesis, and these effects may be more pronounced for CCN2 than other integrin ligands that interact with HSC. Although speculative, this pathway of CCN2 action offers interesting avenues for further studies. In particular it will be important to analyze the co-expression and coordinated actions of integrins and CCN2 as a function of HSC activation and to investigate the unique integrin αβ₂-binding sequence in module 4 of CCN2 as a potential novel target in the development of new anti-fibrotic therapies.

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