Identification of a novel integrin $\alpha_v\beta_3$ binding site in CCN1 (CYR61) critical for pro-angiogenic activities in vascular endothelial cells

Running title: Novel Integrin $\alpha_v\beta_3$ binding site in CCN1

Ningyu Chen‡, Shr-Jeng Leu†‡, Viktor Todorovic‡, Stephen C.-T. Lam§, and Lester F. Lau‡¶

‡Department of Biochemistry and Molecular Genetics and §Department of Pharmacology
University of Illinois College of Medicine
900 South Ashland Avenue, Chicago, IL 60607-7170

*This work was supported by National Institutes of Health grants CA46565 and CA80080 (to L.F.L.), and HL41793 (to S.C.-T.L.). †Present address: Department of Microbiology and Immunology, Vanderbilt University, Nashville, TN, 37332. ¶To whom correspondence should be addressed: Department of Biochemistry and Molecular Genetics, University of Illinois, College of Medicine, 900 South Ashland Avenue, Chicago, IL 60607. Tel: (312) 996-6978; Fax: (312) 996-7034; E-mail: lflau@uic.edu
ABSTRACT

CCN1 (CYR61) is a matricellular inducer of angiogenesis essential for successful vascular development. Though devoid of the canonical RGD sequence motif recognized by some integrins, CCN1 binds to, and functions through, integrin αvβ3 to promote pro-angiogenic activities in activated endothelial cells. In this study, we identify a 20-residue sequence, V2 (NCKHQCTCIDGAVGCIPLCP), in domain II of CCN1 as a novel binding site for integrin αvβ3. Immobilized synthetic V2 peptide supports αvβ3-mediated cell adhesion; soluble V2 peptide inhibits endothelial cell adhesion to CCN1 and the homologous family members CCN2 (connective tissue growth factor, CTGF) or CCN3 (NOV), but not to collagen. These activities are obliterated by mutation of the aspartate residue in the V2 peptide to alanine. The corresponding D125A mutation in the context of the N-terminal half of CCN1 (domains I and II) greatly diminished direct solid phase binding to purified integrin αvβ3 and abolished αvβ3-mediated cell adhesion activity. Likewise, soluble full-length CCN1 with the D125A mutation is defective in binding purified αvβ3 and impaired in αvβ3-mediated pro-angiogenic activities in vascular endothelial cells, including stimulation of cell migration and enhancement of DNA synthesis. In contrast, immobilized full-length CCN1-D125A mutant binds αvβ3 and supports αvβ3-mediated cell adhesion similar to wild type CCN1. These results indicate that V2 is the primary αvβ3 binding site in soluble CCN1, whereas additional cryptic αvβ3 binding site(s) in the C-terminal half of CCN1 becomes exposed when the protein is immobilized. Together, these results identify a novel and functionally important binding site for integrin αvβ3, and provide a new approach for dissecting αvβ3-specific CCN1 functions both in cultured cells and in the organism.
INTRODUCTION

CCN1 (cysteine-rich 61, CYR61) is a secreted, ECM-associated protein that regulates a broad spectrum of cellular activities, including cell adhesion, migration, proliferation, survival and differentiation in mesenchymal cells (1). Consistent with its expression in the developing vasculature, CCN1 promotes pro-angiogenic activities upon endothelial cells in vitro and induces neovascularization in vivo (2-4). The essential role of CCN1 in vessel development has been established in gene targeting studies, as Ccn1-null mice suffer embryonic death primarily due to vascular defects in both the placenta and the embryo (5). CCN1 can also regulate a set of genes that control angiogenesis and matrix remodeling (6), suggesting that it may modulate these processes in various physiological and pathological contexts. Consistent with this notion, overexpression of CCN1 is associated with cutaneous and skeletal wound repair (6-8), restenosed blood vessels (9,10), advanced atherosclerotic lesions (11,12), endometriosis (13), and human breast cancer (14).

Encoded by a growth factor-inducible immediate-early gene, CCN1 is a cysteine-rich heparin-binding protein of ~40 kDa (15). Upon secretion, CCN1 is non-covalently associated with the ECM and the cell surface, in equilibrium with a fraction that exist in the culture medium (16). A member of the CCN protein family, CCN1 shares sequence homology with five other proteins in vertebrates: CCN2 (connective tissue growth factor, CTGF), CCN3 (nephroblastoma overexpressed, NOV), CCN4 (WISP-1, ELM-1), CCN5 (WISP-2, COP-1), and CCN6 (WISP-3)(1,17,18). Structurally, CCN proteins are characterized by the presence of an N-terminal secretory signal, followed by four distinct modular domains with homology to insulin-like growth factor binding protein (IGFBP, domain I), von Willebrand factor type C repeat (WVC, domain II), thrombospondin type I repeat (TSP1, domain III), and regions of Slit and mucins (CT, domain IV)(Fig. 1A;19). Given their regulatory rather than structural roles, CCN proteins can be considered “matricellular” signaling molecules (20,21). In
keeping with their structural roots in ECM proteins, CCN proteins are ligands of, and act through, distinct integrin receptors in a cell type and context-specific manner (22-26).

Integrin \( \alpha_\text{v} \beta_3 \) is highly expressed in angiogenic endothelial cells and has been implicated in angiogenic processes (27). Although CCN1 does not possess the canonical RGD sequence motif present in a number of \( \alpha_\text{v} \beta_3 \) ligands, it binds \( \alpha_\text{v} \beta_3 \) directly and mediates a number of pro-angiogenic activities through this integrin in activated vascular endothelial cells, including supporting cell adhesion, stimulating chemotaxis, enhancing DNA synthesis, promoting cell survival and inducing tubule formation (22,28). Like CCN1, both CCN2 and CCN3 also bind \( \alpha_\text{v} \beta_3 \), induce angiogenesis in vivo, and stimulate pro-angiogenic activities in endothelial cells through an integrin \( \alpha_\text{v} \beta_3 \)-dependent mechanism (29-31). Therefore, identifying the \( \alpha_\text{v} \beta_3 \) binding site(s) of CCN1 will provide new insights into how these matricellular proteins interact with their receptors, and help to elucidate the \( \alpha_\text{v} \beta_3 \)-specific activities of CCN1 in developmental and pathological angiogenesis.

In this study, we found that CCN1 contains multiple \( \alpha_\text{v} \beta_3 \) binding sites. Using biochemical, functional, and mutational approaches, we identify a novel 20-residue sequence, V2, in the VWC domain of CCN1 as a functional binding site for integrin \( \alpha_\text{v} \beta_3 \). We show that D125 in the V2 sequence is critical for interaction with \( \alpha_\text{v} \beta_3 \), and a single a.a. D125→A substitution in the context of full-length CCN1 is sufficient to selectively impair \( \alpha_\text{v} \beta_3 \)-mediated activities in endothelial cells, including stimulation of cell migration and enhancement of DNA synthesis. Together, these results delineate a novel \( \alpha_\text{v} \beta_3 \) binding site in CCN1, establish its functional significance through mutational analysis, and provide a strategy for dissecting \( \alpha_\text{v} \beta_3 \)-specific CCN1 activities and signaling pathways both in vitro and in the context of the whole organism.
MATERIALS AND METHODS

Proteins, Antibodies, peptides, and reagents. Recombinant mouse CCN1, CCN2, and CCN3 proteins were expressed in Sf9 cells using a baculovirus expression system and purified from serum-free conditioned media by sepharose S column chromatography (32). Human vitronectin and rat tail type-I collagen were from Collaborative Research (Cambridge, MA). Function-blocking mAbs against various integrins, including β₁ (JB1A), α₆ (GoH3), and α₅β₃ (LM609) were purchased from Chemicon, Inc. (Temecula, CA). Rabbit polyclonal anti-Cyr61 antibodies were affinity purified as described (32). Heparin (sodium salt; from porcine intestinal mucosa) was from Sigma. GRGDSP and GRGESP peptides were from American Peptide Company (Sunnyvale, CA). Custom synthetic peptides corresponding to sequences of domain II (vWC domain) of CCN1 were prepared by ResGen, Inc. (Huntsville, AL) and Invitrogen (Carlsbad, CA), followed by purification on reverse-phase high performance liquid chromatography and analysis by mass spectroscopy.

Construction and purification of CCN1-D125A, CCN1₁-₂, and CCN1₁-₂-D125A mutants. CCN1 mutant with the D125A substitution in domain II (CCN1-D125A) was constructed by site-directed mutagenesis by PCR, using the sense primer 5’-TGCACATGTATTGCTGGCGCCGTGGGCTGC and the antisense primer 5’- CGGGAATTCCTTTTAGGCTGCTGTACACTGGTTGTC. Mouse CCN1 cDNA was used as template and the PCR product was digested with AflIII and PflMI, cutting at sites flanking the mutated sequence. The AflIII-PflMI fragment of the full-length Ccn1 cDNA in pSG5 was substituted with the mutated PCR product. The resulting construct was confirmed by DNA sequencing. The mutant Ccn1 cDNA was cloned in baculovirus expression vector pBlueBac 4.5 (Invitrogen, Carlsbad, CA). To construct the CCN1₁-₂, PCR reactions upon the mouse Ccn1 cDNA was carried out using primers 5’-CGCGGATCCCGCCGCTCTCCACCTGC and 5’-GGAATTCCCAGGAAGCCTCTTCAGTGAGCTGCC. The PCR product was digested with
BamH1 and EcoR1, and ligated into a modified pBlueBac4.5/V5-His vector (33). The expressed recombinant polypeptide contained the V5 epitope and a polyhistidine tag at the C-terminus, and was purified from Sf9 cells using a serum-free baculovirus expression system through nickel-agarose column as described (26). The mutant CCN1_{1-2}-D125A was constructed by swapping an XmaI-XcmI fragment from CCN1-D125A into the CCN1_{1-2} construct and the recombinant protein was purified as described above.

**Cell culture and cell adhesion assay.** HUVECs were purchased from Cascade Biologics, Inc. (Portland, OR) and grown in medium provided by the company. Primary human foreskin fibroblast 1064SK (CRL-2076) from the American Type Culture Collection was kept in Dulbecco’s modified minimal essential medium (DMEM; high glucose, Invitrogen) with 10% fetal bovine serum (Intergene, Purchase, NY). For adhesion assays, cells were harvested 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) with 2.5 mM EDTA and resuspended in serum-free media (Medium 200 from Cascade Inc. for HUVECs and DMEM for fibroblasts) containing 0.5% BSA as described (23). Microtiter wells (96 well Pro-bind flat bottom assay plate from Becton Dickinson) were coated with proteins or peptides diluted in PBS or H$_2$O overnight at 4°C. After coating, the wells were blocked with 1% BSA for 1 hr at RT. Where indicated, reagents (Antibodies, heparin, peptides, etc.) were mixed with cells and incubated at room temperature for 30 min. before plating. To each well 50 µl of cell suspension was plated and, after incubation at 37°C for 30 min., wells were washed twice with PBS. Adherent cells were fixed with 10% formalin, stained with methylene blue and quantified by dye extraction and measurement of absorbance at 620 nm as described (32).

The amounts of immobilized CCN1_{1-2} and CCN1_{1-2}\text{-}D125A protein were quantified by an ELISA using anti-domain I antibodies as primary antibodies (25). After washing, wells were
incubated with horseradish peroxidase-conjugated secondary antibody (Amersham, Piscataway, NJ), and color reaction was developed using a horseradish peroxidase immunoassay kit (Zymed Laboratories, Inc., San Francisco, CA) with absorbance measured at 420 nm. To determine the coating efficiency of V2 peptides, the plate was blocked with 2% PVA instead of BSA. Immobilized peptides were measured by incubation for 2 h at RT with 10 µM PEO-maleimide-activated biotin (Pierce, Rockford, IL), which reacts with the free sulfhydryl group in the cysteine residue of the peptides. The amounts of coupled biotin were quantified by incubation with horseradish peroxidase-conjugated streptavidin (Santa Cruz Biotech, Santa Cruz, CA). Color reaction was developed using a horseradish peroxidase immunoassay kit (Zymed Laboratories, Inc.) and absorbance measured at 420 nm.

**Solid phase binding assay.** Binding of CCN1 and CNN1-D125A to purified integrin αvβ3 was measured by ELISA as described previously with modifications (31). Microtiter wells (Immulon 2, Dynatech Laboratories, Chantilly, VA) were coated with purified integrin (1 µg/ml in buffer of 20 mM Hepes, pH 7.5 containing 25 mM octylglucoside, 150 mM NaCl, 1 mM CaCl2, and 1 mM MgCl2) and incubated at 4°C overnight. The wells were blocked with 2% heat-inactivated BSA for 2 h at room temperature. Soluble CCN1 and CCN1-D125A were added and allowed to bind at 37°C for 2 hrs; bound ligand was detected using affinity-purified anti-domain I antibodies. For integrin binding to immobilized CCN1,2 and CCN1,2-D125A, microtiter wells were coated with indicated amount of protein at 4°C overnight. After blocking with 2% BSA, purified integrin αvβ3 (1 µg/ml) was added and incubated at 37°C for 2 hrs. After washing, bound integrins were detected with polyclonal anti-integrin β3 (AB1932, Chemicon). Plates were incubated with horseradish peroxidase-conjugated secondary antibody, and color reaction was developed using a horseradish peroxidase immunoassay kit (Zymed Laboratories, Inc.) with absorbance measured at 420 nm. To
block the binding of integrin $\alpha_v\beta_3$ to CCN1 with V2 peptides, integrin $\alpha_v\beta_3$ (1 $\mu$g/ml) was pre-incubated with indicated amount of peptides at RT for 30 min before applied to wells coated with 10 $\mu$g/ml of CCN1. Binding was performed and bound integrins were detected as described above.

**Cell Migration Assay.** Cell migration was monitored as described (28) using Transwell chambers (Corning Costar) with tissue culture-treated filter membranes separating the upper and lower chambers. Subconfluent HUVECs were detached by 2.5 mM EDTA in PBS and then washed and resuspended in serum-free basal medium with 0.5% BSA at $7 \times 10^5$ cells/ml. Cells were stimulated with 100 nM PMA for 30 min and the cell suspension (100 $\mu$l) was placed in the upper compartment of each chamber, and cells were allowed to migrate toward indicated amount of CCN1 or CCN1-D125A diluted in 0.6 ml of assay medium in the lower chamber for 6 h in 5% CO$_2$ during incubation at 37°C. To block cell migration with antibodies, stimulated HUVECs were incubated with 50 $\mu$g/ml of LM609 or regular mouse IgG for 30 min at RT prior to plating. After incubation, unmigrated cells that remained attached to the upper surfaces of the filters were removed by cotton swabs, and cells that migrated to the lower surfaces of the filters were fixed with methanol and stained with hematoxylin. Cells migrated were counted in 10 randomly selected microscopic fields at 200X magnification. Results are expressed as numbers of migrated cells per field.

**BrdU incorporation assay.** DNA synthesis was assessed using BrdU Cell Proliferation Assay Kit (Oncogene Research Products) following manufacturer’s protocol. Briefly, subconfluent HUVECs were replated on 96-well cell culture plates (Corning, NY) at $2 \times 10^4$ cells/well in complete culture medium for 16 h and starved in serum-free basal medium with 0.5% BSA for 24 h. CCN1, CCN1-D125A, or VEGF were added to the wells alone or in combination in basal assay medium containing BrdU. After 24 h of incubation, cells were fixed, and incorporated BrdU was quantified by ELISA according to the protocol with antibodies and reagents provided with the kit.
RESULTS

Identification of an integrin $\alpha_v\beta_3$-binding peptide within the VWC domain of CCN1. Previous studies showed that a truncation mutant of CCN1 lacking precisely the CT domain (CCN1$_{1-3}$ or CCN1$\Delta$CT) retains the ability to promote DNA synthesis through integrin $\alpha_v\beta_3$, indicating the presence of an $\alpha_v\beta_3$ binding site within the first three domains of CCN1 (26). Moreover, antibodies specific for domain II of CCN1, but not those against domains I or III, inhibited $\alpha_v\beta_3$-mediated endothelial cell migration (34). These observations suggested that a binding site for $\alpha_v\beta_3$ may be located in domain II. To test this hypothesis, we prepared four overlapping synthetic peptides that encompassed the entire domain II (Fig. 1B). The ability of these peptides to support HUVEC adhesion when immobilized on microtiter wells was assessed. Of the four peptides, only V2 (corresponding to a.a. 116-135 of the mouse CCN1) was able to support HUVEC adhesion (Fig. 2A). Furthermore, V2 supported HUVEC adhesion in a dose-dependent manner, with significant cell adhesion evident at a coating concentration of 0.05 mM peptide and cell adhesion was optimal at 0.1 mM peptide (Fig. 2B).

To determine whether cell adhesion to V2 was mediated through integrin $\alpha_v\beta_3$, we evaluated the effects of $\alpha_v\beta_3$ antagonists. EDTA, a general inhibitor of integrin function, blocked HUVEC adhesion to the V2 peptide (Fig. 2C). The peptide GRGDSP, which inhibits ligand binding of specific integrins including $\alpha_v\beta_3$, also abolished cell adhesion to V2. Furthermore, the $\alpha_v\beta_3$-specific mAb LM609 completely abrogated HUVEC adhesion, indicating that V2 supports cell adhesion through integrin $\alpha_v\beta_3$. HUVEC adhesion to CCN1 was inhibited partially by LM609 or GRGDSP peptides, since this process is mediated through both $\alpha_v\beta_3$ and $\alpha_v\beta_3$-HSPGs (28). As expected, cell adhesion to vitronectin was inhibited by EDTA and GRGDSP peptide completely, and by LM609 partially. Together, these results show that the V2 peptide supports dose-dependent and $\alpha_v\beta_3$-mediated HUVEC
adhesion, implicating the presence of an $\alpha_v\beta_3$ binding site in this peptide.

To probe the specificity of V2 in cell adhesion further, we used this peptide to conduct inhibition studies. Soluble V2 peptide was able to block HUVEC adhesion to CCN1 in a dose-dependent manner (Fig. 3A), with significant inhibition observed at 0.2 mM V2 peptide, similar to the concentration required for linear RGD-containing peptides to inhibit ligand interactions with $\alpha_v$ integrins (35). In contrast, no inhibition occurred on cell adhesion to type I collagen, which binds $\beta_1$ integrins. Other members of the CCN family, including CCN2 (CTGF) and CCN3 (NOV) have also been shown to support endothelial cell adhesion through integrin $\alpha_v\beta_3$, and their corresponding V2 sequences are highly conserved (29,31). The V2 peptide was able to block HUVEC adhesion to both CCN2 and CCN3 as well (Fig. 3B), suggesting that the V2 sequences in these CCN proteins contain homologous binding sites for $\alpha_v\beta_3$.

To determine whether the peptide supporting $\alpha_v\beta_3$-mediated cell adhesion can be further defined, we prepared a series of shorter peptides that contain the core sequence of V2 (Fig. 3C). None of these shorter peptides were able to support cell adhesion when immobilized (data not shown). We also used the peptides in a soluble form to inhibit cell adhesion to CCN1. Only V2, but not the shorter peptides, was able to inhibit cell adhesion (Fig. 3D). These results suggest that the full V2 sequence is required to support efficient binding to integrin $\alpha_v\beta_3$.

D125 is critical for recognition by integrin $\alpha_v\beta_3$. Phage display and mutational analyses have indicated that an aspartic acid residue in the ligand plays a critical role in interaction with integrin $\alpha_v\beta_3$ (36,37). Recent structural studies demonstrated direct contact of the aspartic acid with the ligand binding pocket of $\alpha_v\beta_3$ (38,39). The V2 sequence contains D125, an aspartate conserved among all CCN proteins except CCN6. To test whether D125 might be critical for $\alpha_v\beta_3$ binding, we prepared a mutant synthetic V2 peptide (V2-mut) that harbors a single a.a. D→A substitution (Fig. 1B). When
coated as an adhesion substrate on microtiter wells, the V2-mut peptide was completely unable to support HUVEC adhesion (Fig. 2B). Even when coated at 1 mM, a concentration 10-fold higher than necessary for wild type V2 to support optimal cell adhesion, V2-mut had no activity. In control experiments, both the V2 and V2-mut peptides were able to coat plastic surfaces with equal efficiency in a dose-dependent manner (Fig. 2D), showing that the difference in cell adhesion to the peptides was not due to differential coating. Furthermore, V2-mut was unable to inhibit HUVEC adhesion to either CCN1, CCN2, or CCN3 (Fig. 3B). Thus, the D125A mutation abolished the ability of V2 to support $\alpha_v\beta_3$-mediated HUVEC adhesion, indicating that D125 is a critical residue for the V2 peptide to interact with $\alpha_v\beta_3$.

**Cell adhesion to CCN1$_{1-2}$ through integrin $\alpha_v\beta_3$.** CCN1 is organized into four discrete domains (Fig. 1A), with a protease sensitive linker region between domains II and III (32). Proteolytic processing of the structurally related CCN2 has been observed *in vivo*, producing both N- and C-terminal fragments in urine, plasma, and uterine fluids (40-42). In HUVECs, CCN1 supports cell adhesion through both integrin $\alpha_v\beta_3$ and the $\alpha_6\beta_1$-HSPG coreceptors (28), and the binding sites for $\alpha_6\beta_1$-HSPGs have been localized in domains III and IV (23,33). Since identification of the V2 sequence in domain II provided the first evidence of cell adhesive function in the N-terminal half of CCN proteins, we investigated the ability of domains I and II of CCN1 to support cell adhesion. We expressed and purified a CCN1 isoform with only the IGFBP and VWC domains (CCN1$_{1-2}$), thus removing the binding sites for $\alpha_6\beta_1$-HSPGs (Fig. 1A). A mutant harboring the D125A substitution was also produced in the same context (CCN1$_{1-2}$-D125A). Both isoforms retain the N-terminal secretory signal, and a C-terminal polyhistidine tag was added to allow a means of purification. These isoforms were expressed in insect cells via a baculovirus vector and purified to apparent homogeneity from conditioned media. The polypeptides had the expected molecular mass (27 kDa) and were
immunoreactive with polyclonal anti-CCN1 antibodies (Fig. 1C).

Purified CCN1<sub>1-2</sub> was able to support HUVEC adhesion in a dose-dependent manner (Fig. 4A). By contrast, the CCN1<sub>1-2</sub>-D125A mutant was completely devoid of this activity. These isoforms were able to coat microtiter wells in an equivalent and dose-dependent manner (Fig. 4B), and cell adhesion to them was completely blocked by polyclonal anti-CCN1 antibodies (data not shown). These results show that CCN1<sub>1-2</sub> contains cell adhesive activity, and suggest it most likely contains a single binding site for α<sub>v</sub>β<sub>3</sub> that is abolished by the D125A mutation. To evaluate whether HUVEC adhesion to CCN1<sub>1-2</sub> is mediated through integrin α<sub>v</sub>β<sub>3</sub>, we employed antagonists of α<sub>v</sub>β<sub>3</sub> function. The peptide GRGDSP blocked HUVEC adhesion to CCN1<sub>1-2</sub> completely, whereas the control peptide GRGESP had no effect (Fig. 4C). Furthermore, the anti-α<sub>v</sub>β<sub>3</sub> mAb LM609 obliterated HUVEC adhesion to CCN1<sub>1-2</sub>, whereas control IgG had no effect. Taken together, these results indicate that CCN1<sub>1-2</sub> can support HUVEC adhesion through a single binding site for integrin α<sub>v</sub>β<sub>3</sub> localized within the V2 sequence, and D125 is a critical residue for this binding site.

**Direct solid phase binding to integrin α<sub>v</sub>β<sub>3</sub>.** To demonstrate that the V2 sequence indeed constitutes a binding site for α<sub>v</sub>β<sub>3</sub>, we examined direct binding of CCN1<sub>1-2</sub> to α<sub>v</sub>β<sub>3</sub> in a solid phase binding assay. Microtiter wells were coated with increasing amounts of CCN1<sub>1-2</sub> or CCN1<sub>1-2</sub>-D125A, onto which a constant amount of purified integrin α<sub>v</sub>β<sub>3</sub> was allowed to bind. Bound integrin was then detected by ELISA using an anti-β<sub>3</sub> mAb. The coating of CCN1<sub>1-2</sub> and CCN1<sub>1-2</sub>-D125A was dose-dependent and equivalent (Fig. 4B). As shown in Fig. 5A, immobilized CCN1<sub>1-2</sub> supports α<sub>v</sub>β<sub>3</sub> binding in a dose-dependent manner. By contrast, immobilized CCN1<sub>1-2</sub>-D125A bound α<sub>v</sub>β<sub>3</sub> with much reduced efficiency, and only a low level of binding was detectable at high coating concentrations. Thus, CCN1<sub>1-2</sub> binds directly to α<sub>v</sub>β<sub>3</sub> through a binding site in which D125 is critical.

To establish further the presence of an α<sub>v</sub>β<sub>3</sub> binding site in V2, we used this peptide to inhibit
the direct binding of full-length CCN1 to α,β3. Soluble V2 peptide was able to inhibit direct binding of purified α,β3 to full-length, wild type CCN1 in a dose-dependent manner (Fig. 5B), and complete inhibition was observed at 0.5 mM peptide. In contrast, the V2-a peptide, which does not support HUVEC adhesion, was unable to inhibit CCN1 binding to α,β3. Taken together, these results identified the V2 sequence as the α,β3 binding site within the first two domains of CCN1, and D125 is a critical residue for interaction with integrin α,β3.

To investigate the significance of the V2 binding site for α,β3 in the context of full-length CCN1, we constructed and purified a CCN1 mutant (CCN1-D125A) harboring the D125A mutation in CCN1 (Fig. 1). Direct binding of this CCN1 mutant to integrin α,β3 was examined in two ways: using soluble CCN1 to bind immobilized integrin, and in reverse by using soluble integrin to bind immobilized CCN1. To evaluate the binding of soluble CCN1, purified integrin α,β3 was coated onto microtiter wells, and direct binding of soluble CCN1 or CCN1-D125A was detected by ELISA using anti-CCN1 antibodies. Consistent with previous studies, CCN1 bound to immobilized α,β3, reaching saturation at ~1 µg/ml CCN1 (Fig. 5C;22). In contrast, the CCN1-D125A mutant is impaired in its ability to bind α,β3, showing ~25% binding compared to wild type at 0.8-1.0 µg/ml protein. These results show that the D125A mutation severely blunted the ability of soluble CCN1 to bind α,β3.

To examine the binding of integrin α,β3 to immobilized CCN1, microtiter wells were coated with varying amounts of CCN1 or CCN1-D125A, onto which purified integrin α,β3 was allowed to bind. Surprisingly, α,β3 bound to immobilized CCN1 and CCN1-D125A with similar efficiency and dose response, saturating at a coating concentration of 2 µg/ml protein (Fig. 5D). Thus, the full-length CCN1-D125A mutant is able to bind integrin α,β3 similar to wild type when coated as an immobilized substrate, but binds poorly to α,β3 when presented as a soluble protein. The simplest
interpretation of these results is that V2 is the primary binding site for $\alpha_\text{v}\beta_3$ when the protein is in a soluble form, but a cryptic binding site for integrin $\alpha_\text{v}\beta_3$ becomes available when the protein is immobilized.

**Cell adhesive properties of the CCN1-D125A mutant.** To investigate further the functional impact of the D125A mutation on CCN1 activities, we tested CCN1-D125A in cell adhesion assays. HUVEC adhesion to the CCN1-D125A mutant was similar to wild type and was partially inhibited by RGD peptide and LM609, suggesting that the presence of other binding site(s) for integrin $\alpha_\text{v}\beta_3$ in addition to the V2 sequence (Fig. 6A). This observation is consistent with the binding of $\alpha_\text{v}\beta_3$ to immobilized CCN1 proteins (Fig. 5D). Considering that CCN1$_{1,2}$-D125A is devoid of cell adhesive activity (Fig. 4A), additional $\alpha_\text{v}\beta_3$ binding site(s) most likely resides in domain III or IV of CCN1. This possibility is consistent with a recent report of a sequence in domain IV of CCN2 that can support $\alpha_\text{v}\beta_3$-dependent cell adhesion (43).

CCN1 supports fibroblast adhesion exclusively through the integrin $\alpha_6\beta_1$-HSPGs co-receptors (23), thus providing a convenient assay for $\alpha_6\beta_1$-HSPG-mediated activities. Since the binding sites for $\alpha_6\beta_1$-HSPGs have been localized to domains III and IV (23,33), we do not expect the D125A mutation to impact on $\alpha_6\beta_1$-HSPG-mediated functions. Indeed, using primary human fibroblasts, we found that the CCN1-D125A mutant was able to support cell adhesion similar to wild type CCN1 (Fig. 6B). Furthermore, this adhesion was inhibited by soluble heparin, which can saturate the heparin binding site of CCN1 and thereby preventing its interaction with cell surface HSPGs. In addition, the mAbs against integrin $\alpha_6$ (GoH3) or $\beta_1$ (JB1A) effectively blocked fibroblast adhesion to CCN1 or CCN1-D125A, whereas normal IgG had no effect (Fig. 7B). Therefore, the CCN1-D125A mutant maintains the ability to interact with integrin $\alpha_6\beta_1$ and HSPGs.

**CCN1-D125A is defective in $\alpha_6\beta_3$-mediated stimulation of HUVEC migration and DNA**
synthesis. To elucidate the functional roles of the V2 binding site further, we have examined specific pro-angiogenic activities of the D125A mutant. CCN1 is known to induce HUVEC migration through integrin αvβ3 (3,28). As shown in Fig. 7A, CCN1 induces HUVEC migration in a dose-dependent manner, whereas the CCN1-D125A mutant was much less effective. Nevertheless, a diminished level of activity with a similar dose response was observed with CCN1-D125A, consistent with the low level of αvβ3 binding observed in this mutant protein (Fig. 5C). Furthermore, cell migration to both wild type and mutant was completely blocked by LM609, showing that this activity is mediated through αvβ3 (Fig. 7B). Thus, CCN1 induces HUVEC migration by binding αvβ3 primarily through the V2 sequence.

We have shown that while not mitogenic on its own, CCN1 can enhance growth factor-induced DNA synthesis in endothelial cells through integrin αvβ3 (28,32). Indeed, neither CCN1 nor CCN1-D125A alone stimulated DNA synthesis, whereas wild type CCN1 was able to cooperate with a sub-optimal dose of VEGF to induce DNA synthesis in HUVECs (Fig. 8). The CCN1-D125A mutant, however, was devoid of this activity. These results show that the V2 binding site for αvβ3 is critical for CCN1 to synergize with growth factors to induce DNA synthesis.
DISCUSSION

CCN1 has recently emerged as a matricellular inducer of angiogenesis essential for proper vascular development during embryogenesis (3,5). Though devoid of the canonical RGD sequence recognized by several integrins, CCN1 binds to and acts through integrin $\alpha_v\beta_3$ to promote pro-angiogenic activities in activated endothelial cells (28,44). In this study, we have used functional, biochemical, and mutational approaches to identified a 20-residue V2 sequence, NCKHQCTCIDGAVGCIP, in domain II of CCN1 as a novel binding site for integrin $\alpha_v\beta_3$. Furthermore, we have established the functional significance of this binding site by showing that targeted D125A mutation in the context of full-length CCN1 impairs specific $\alpha_v\beta_3$-mediated pro-angiogenic activities in vascular endothelial cells.

Upon secretion, CCN1, CCN2, and CCN3 are largely associated with the ECM and the cell surface but in equilibrium with a fraction that exist in the culture medium (16,44,45). As immobilized cell adhesion substrates, these proteins induce adhesive signaling and activate intracellular signaling cascades, leading to upregulation of gene expression and promotion of cell survival (28,29,46). When assayed in the soluble form, they can induce cell migration, enhance DNA synthesis, upregulate gene expression, promotion cell survival, and induce differentiation (6,28,29,31). A protease sensitive region exist between domains II and III, and evidence of proteolytic processing has been observed for CCN2 \textit{in vivo}, producing both N- and C-terminal fragments that can be detected in urine, plasma, and uterine fluids (32,40-42). Thus, CCN proteins are biologically active in both immobilized and soluble forms, and proteolytically processed fragments of these modular proteins may possess certain activities.

Previous studies indicated the presence of an $\alpha_v\beta_3$ binding site within the first three domains of CCN1 (26), and domain II-specific antibodies preferentially blocked $\alpha_v\beta_3$-mediated endothelial cell
migration (34). These findings prompted us to conduct further deletion analysis, leading to the finding that the N-terminal half of CCN1 encompassing domains I and II (CCN1 \(_{1,2}\)) is sufficient to mediate \(\alpha_v\beta_3\)-dependent cell adhesion (Fig. 4). In contrast, CCN1 \(_{1,2}\) is unable to support fibroblast adhesion, which occurs through \(\alpha_v\beta_1\) and HSPGs (data not shown). Furthermore, analysis of a panel of synthetic peptides identified the V2 sequence within domain II as an \(\alpha_v\beta_3\) binding site as judged by the following observations: 1) synthetic V2 peptide specifically supports and inhibits \(\alpha_v\beta_3\)-mediated HUVEC adhesion, and inhibits direct binding of CCN1 to \(\alpha_v\beta_3\) (Figs. 2,3, 5B); 2) V2 peptide-supported cell adhesion is abolished by alanine substitution of a single aspartate residue, a critical amino acid that makes contact with the ligand binding pocket of \(\alpha_v\beta_3\) (Fig. 2;38,39); and 3) D125A mutation in the context of the CCN1 \(_{1,2}\) fragment completely obliterated \(\alpha_v\beta_3\)-dependent cell adhesion (Fig. 4A) and impaired direct binding to purified \(\alpha_v\beta_3\) in a solid phase binding assay (Fig. 5A). Further proof that V2 is a functionally important \(\alpha_v\beta_3\) binding site came from the observation that a single a.a. D125A mutation in the context of full-length CCN1 was sufficient to severely reduce direct binding to \(\alpha_v\beta_3\) (Fig. 5C) and impair specific \(\alpha_v\beta_3\)-mediated activities, including stimulation of cell migration and enhancement of VEGF-induced DNA synthesis in vascular endothelial cells (Figs. 7,8).

Originally isolated as the receptor for vitronectin, integrin \(\alpha_v\beta_3\) is known to bind a variety of RGD-containing ligands, including fibronectin, fibrinogen, thrombospondin, osteopontin, and von Willebrand factor. In addition, several non-RGD-containing ligands of \(\alpha_v\beta_3\) are also known, including CD31/PECAM-1, MMP2, FGF-2, tumstatin, and CCN proteins (22,29,31,47-51). Although the V2 peptide does not contain any sequence homology with other known \(\alpha_v\beta_3\) binding sites, it does bear structural features consistent with those found to be important for \(\alpha_v\beta_3\)-ligand interaction. First, an aspartate residue is thought to play important roles in ligand binding to \(\alpha_v\beta_3\) (38,39), and mutational analysis identified D125 within the V2 sequence as critical for binding \(\alpha_v\beta_3\) (Figs. 2,5). Second,
studies on disintegrins and phage display analysis of αvβ3 binding sites have suggested that two cysteine residues flanking the core binding sequence may form disulfide bonds to present the binding site as a loop, thereby enhancing the binding affinity significantly (36,52-55). Consistently, the critical aspartate in CCN1 is in a sequence flanked by cysteines, and the V2-a peptide, which truncates two outermost flanking cysteines, is unable to support or inhibit αvβ3-mediated cell adhesion (Fig. 3D). However, whether these cysteines form disulfide bonds to present the binding site as a loop is currently unknown.

Site-specific mutagenesis of D125 demonstrated that the V2 binding site for αvβ3 is functionally significant. A single a.a. D125A substitution in the context of the full-length CCN1 severely reduced direct binding of soluble CCN1 to integrin αvβ3 and impaired specific αvβ3-dependent activities (Fig. 5C). Thus, the CCN1-D125A mutant is defective in stimulation of endothelial cell migration or VEGF-induced DNA synthesis (Figs. 7,8), activities previously assigned to αvβ3 based on their inhibition by antagonists that disrupted αvβ3 functions (28). The finding that these activities are impaired by a targeted mutation in the binding site for αvβ3 provide further compelling evidence that these CCN1 activities are the consequences of direct CCN1 interaction with integrin αvβ3.

Inasmuch as soluble full-length CCN1 with the D125A mutation has substantially decreased ability to bind immobilized αvβ3 (Fig. 5C), it is likely that V2 is the primary binding site for αvβ3 when CCN1 is in the soluble form. However, immobilized CCN1-D125A is able to bind αvβ3 and support αvβ3-dependent cell adhesion (Fig. 5D, 6A), suggesting that there is a cryptic αvβ3 binding site that becomes available for binding by conformational change upon coating on plastic. An analogous situation has been found with fibrinogen, which undergoes a conformational change to expose a binding site for αvβ3 upon immobilization (56). Since CCN11-2-D125A does not support
HUVEC adhesion (Fig. 4A), potential αβ₃ binding site(s) may be localized in the C-terminal half of CCN1. This possibility is consistent with a recent report of an αβ₃ binding site capable of supporting cell adhesion in domain IV of CCN2 (43), suggesting that a similar αβ₃ binding site may exist in the CT domain of CCN1. However, since this CCN2 αβ₃ binding site is not well conserved in CCN1, whether the corresponding sequence in CCN1 is responsible for αβ₃-dependent cell adhesion remains to be determined.

The V2 peptide is able to inhibit HUVEC adhesion not only to CCN1, but also to CCN2 and CCN3 (Fig. 4C). Given the high level of conservation of the V2 sequence (16 of the 20 a.a. are identical in CCN2 and 12 in CCN3), it is likely that these CCN proteins interact with αβ₃ through the corresponding sites. If so, our results would suggest that mutations in CCN2 or CCN3 corresponding to D125 of CCN1 may abolish αβ₃ binding at this site, and compromise their αβ₃-mediated activities. CCN6 is uniquely divergent in the V2 sequence among the CCN members, and neither flanking cysteines nor the critical aspartate residue are conserved. At present, it is not known whether CCN6 can interact with αβ₃. Even if CCN6 is an αβ₃ ligand, it is unlikely that the corresponding V2 sequence in CCN6 can mediate this interaction.

To date, several receptor binding sites have been identified in CCN1. A specific binding site for integrin α₆β₁ has been identified in domain III (33), and heparin binding sites have been localized in domain IV (23). Consistently, the D125A mutation in CCN1 does not impair interaction with α₆β₁ and HSPGs (Fig. 6B). Integrin α₅β₂ mediates activation-dependent cell adhesion of monocytes to CCN1, and a specific binding site for this integrin has been identified within domain IV (11). The identification of an αβ₃-binding site critical for stimulation of endothelial cell migration and DNA synthesis in this study extends the dissection of integrin-mediated CCN1 functions and signaling. Since CCN1 also binds integrins αβ₅ and α₁β₃ in distinct contexts (24,26) and these integrins have
similar ligand specificities to those of $\alpha_v\beta_3$, the possibility that the V2 sequence may also serve as binding sites for $\alpha_v\beta_5$ and $\alpha_{III}\beta_3$ merits examination.

CCN1 is an angiogenic inducer important not only for vascular development, but is also implicated in pathological angiogenesis in such conditions as wound healing, vascular diseases, and cancer (6-12). For example, expression of $CCN1$ in tumor cell lines that do not otherwise express $CCN1$ enhances tumorigenicity with increased vascularization of $CCN1$-expressing tumors (3,57,58). The activities of integrin $\alpha_v\beta_3$ have also been associated with pathological angiogenesis (27,59,60), suggesting that CCN1-$\alpha_v\beta_3$ interaction may be important for the role of CCN1 in diseases. The inhibitory function of the V2 peptide may provide the basis for development of therapeutics that specifically target interactions of CCN proteins with $\alpha_v\beta_3$. Furthermore, the identification of an $\alpha_v\beta_3$ binding site important for endothelial cell functions in this study provides a strategy for the dissection of the physiological roles of CCN1-$\alpha_v\beta_3$ interactions in vivo using gene replacement or transgenic approaches.
FOOTNOTES

1The abbreviations used are: BSA, bovine serum albumin; CCN, cysteine-rich 61/connective
tissue growth factor/nephroblastoma overexpressed; DMEM, Dulbecco’s modified Eagle’s medium;
ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; FN, fibronectin; GST,
glutathione-S-transferase; HSPGs: heparan sulfate proteoglycans; HUVECs, human umbilical vein
endothelial cells; IgG, immunoglobulin G; LN, laminin; mAb, monoclonal antibody; PBS,
phosphate-buffered saline; PVA, polyvinyl alcohol; RT, room temperature; SDS-PAGE, sodium
dodecyl sulfate-polyacrylamide gel electrophoresis; VN, vitronectin.
REFERENCES

1. Lau, L. F. and Lam, S. C. (1999) Exp. Cell Res. 248, 44-57
2. O'Brien, T. P. and Lau, L. F. (1992) Cell Growth & Differentiation 3, 645-654
3. Babic, A. M., Kireeva, M. L., Kolesnikova, T. V., and Lau, L. F. (1998) Proc. Natl. Acad. Sci. U.S.A. 95, 6355-6360
4. Fataccioli, V., Abergel, V., Wingertsmann, L., Neuville, P., Spitz, E., Adnot, S., Calenda, V., and Teiger, E. (2002) Hum. Gene Ther. 13, 1461-1470
5. Mo, F. E., Muntean, A. G., Chen, C. C., Stolz, D. B., Watkins, S. C., and Lau, L. F. (2002) Mol. Cell Biol. 22, 8709-8720
6. Chen, C.-C., Mo, F.-E., and Lau, L. F. (2001) J. Biol. Chem. 276, 47329-47337
7. Latinkic, B. V., Mo, F.-E., Greenspan, J. A., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Lau, L. F. (2001) Endocrinology 142, 2549-2557
8. Hadjiargyrou, M., Ahrens, W., and Rubin, C. T. (2000) J. Bone Miner. Res. 15, 1014-1023
9. Wu, K. J., Yee, A., Zhu, N. L., Gordon, E. M., and Hall, F. L. (2000) Int. J Mol. Med. 6, 433-440
10. Grzeszkiewicz, T. M., Lindner, V., Chen, N., Lam, S. C. T., and Lau, L. F. (2002) Endocrinology 143, 1441-1450
11. Schober, J. M., Lau, L. F., Ugarova, T. P., and Lam, S. C. (2003) J. Biol. Chem. 278, 25808-25815
12. Hilfiker, A., Hilfiker-Kleiner, D., Fuchs, M., Kaminski, K., Lichtenberg, A., Rothkotter, H. J., Schieffer, B., and Drexler, H. (2002) Circulation 106, 254-260
13. Absenger, Y., Hess-Stumpp, H., Kreft, B., Kratzschmar, J., Haendler, B., Schutze, N., Regidor, P. A., and Winterhager, E. (2004) Mol. Hum. Reprod. 10, 399-407
14. Menendez, J. A., Mehmi, I., Griggs, D. W., and Lupu, R. (2003) *Endocr. Relat Cancer* **10**, 141-152

15. O'Brien, T. P., Yang, G. P., Sanders, L., and Lau, L. F. (1990) *Mol. Cell. Biol.* **10**, 3569-3577

16. Yang, G. P. and Lau, L. F. (1991) *Cell Growth & Differentiation* **2**, 351-357

17. Brigstock, D. R. (1999) *Endocr.Rev.* **20**, 189-206

18. Perbal, B. (2001) *Mol.Pathol.* **54**, 57-79

19. Bork, P. (1993) *FEBS Lett.* **327**, 125-130

20. Bornstein, P. (1995) *J.Cell Biol.* **130**, 503-506

21. Bornstein, P. and Sage, E. H. (2002) *Curr. Opin. Cell Biol.* **14**, 608-616

22. Kireeva, M. L., Lam, S. C. T., and Lau, L. F. (1998) *J. Biol. Chem.* **273**, 3090-3096

23. Chen, N., Chen, C. C., and Lau, L. F. (2000) *J. Biol. Chem.* **275**, 24953-24961

24. Jedsadayanmata, A., Chen, C. C., Kireeva, M. L., Lau, L. F., and Lam, S. C. (1999) *J Biol. Chem.* **274**, 24321-24327

25. Schober, J. M., Chen, N., Grzeszkiewicz, T. M., Emeson, E. E., Ugarova, T. P., Ye, R. D., Lau, L. F., and Lam, S. C. T. (2002) *Blood* **99**, 4457-4465

26. Grzeszkiewicz, T. M., Kirschling, D. J., Chen, N., and Lau, L. F. (2001) *J Biol. Chem.* **276**, 21943-21950

27. Eliceiri, B. P. and Cheresh, D. A. (2000) *Cancer J.* **6** Suppl **3**, S245-S249

28. Leu, S.-J., Lam, S. C. T., and Lau, L. F. (2002) *J. Biol. Chem.* **277**, 46248-46255

29. Babic, A. M., Chen, C.-C., and Lau, L. F. (1999) *Mol. Cell. Biol.* **19**, 2958-2966

30. Shimo, T., Nakanishi, T., Nishida, T., Asano, M., Kanyama, M., Kuboki, T., Tamatani, T., Tezuka, K., Takemura, M., Matsumura, T., and Takigawa, M. (1999) *J. Biochem.(Tokyo.)*
31. Lin, C., Leu, S. J., Chen, N., Tebeau, C. M., Lin, S. X., Yeung, C. Y., and Lau, L. F. (2003) *J. Biol. Chem.* **278**, 24200-24208

32. Kireeva, M. L., Mo, F.-E., Yang, G. P., and Lau, L. F. (1996) *Mol. Cell. Biol.* **16**, 1326-1334

33. Leu, S. J., Liu, Y., Chen, N., Chen, C. C., Lam, S. C., and Lau, L. F. (2003) *J. Biol. Chem.* **278**, 33801-33808

34. Grzeszkiewicz, T. M. (2002) Cyr61-induced Cellular Activities in Wound Healing: Impact of Structure on Function. Ph.D. thesis. University of Illinois at Chicago.

35. Pierschbacher, M. D. and Ruoslahti, E. (1987) *J. Biol. Chem.* **262**, 17294-17298

36. Healy, J. M., Murayama, O., Maeda, T., Yoshino, K., Sekiguchi, K., and Kikuchi, M. (1995) *Biochemistry* **34**, 3948-3955

37. Plow, E. F., Haas, T. A., Zhang, L., Loftus, J., and Smith, J. W. (2000) *J. Biol. Chem.* **275**, 21785-21788

38. Arnaout, M. A., Goodman, S. L., and Xiong, J. P. (2002) *Curr. Opin. Cell Biol.* **14**, 641-651

39. Xiong, J. P., Stehle, T., Zhang, R., Joachimiak, A., Frech, M., Goodman, S. L., and Arnaout, M. A. (2002) *Science* **296**, 151-155

40. Ball, D. K., Surveyor, G. A., Diehl, J. R., Steffen, C. L., Uzumcu, M., Mirando, M. A., and Brigstock, D. R. (1998) *Biol Reprod.* **59**, 828-835

41. Roestenberg, P., van Nieuwenhoven, F. A., Wieten, L., Boer, P., Diekman, T., Tiller, A. M., Wiersinga, W. M., Oliver, N., Usinger, W., Weitz, S., Schlingemann, R. O., and Goldschmeding, R. (2004) *Diabetes Care* **27**, 1164-1170
42. Gilbert, R. E., Akdeniz, A., Weitz, S., Usinger, W. R., Molineaux, C., Jones, S. E., Langham, R. G., and Jerums, G. (2003) *Diabetes Care* **26**, 2632-2636

43. Gao, R. and Brigstock, D. R. (2004) *J Biol. Chem.* **279**, 8848-8855

44. Kireeva, M. L., Latinkic, B. V., Kolesnikova, T. V., Chen, C.-C., Yang, G. P., Abler, A. S., and Lau, L. F. (1997) *Exp. Cell Res.* **233**, 63-77

45. Perbal, B., Martinerie, C., Sainson, R., Werner, M., He, B., and Roizman, B. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96**, 869-874

46. Chen, C.-C., Chen, N., and Lau, L. F. (2001) *J. Biol. Chem.* **276**, 10443-10452

47. Piali, L., Hammel, P., Uherek, C., Bachmann, F., Gisler, R. H., Dunon, D., and Imhof, B. A. (1995) *J. Cell Biol.* **130**, 451-460

48. Brooks, P. C., Silletti, S., von Schalscha, T. L., Friedlander, M., and Cheresh, D. A. (1998) *Cell* **92**, 391-400

49. Rusnati, M., Tanghetti, E., Dell’Era, P., Gualandris, A., and Presta, M. (1997) *Mol. Biol. Cell* **8**, 2449-2461

50. Ruoslahti, E. (1996) *Ann. Rev. Cell Dev. Biol.* **12**, 697-715

51. Maeshima, Y., Colorado, P. C., and Kalluri, R. (2000) *J Biol. Chem.* **275**, 23745-23750

52. McLane, M. A., Vijay-Kumar, S., Marcinkiewicz, C., Calvete, J. J., and Niewiarowski, S. (1996) *FEBS Letters* **391**, 139-143

53. Yamada, T. and Kidera, A. (1996) *FEBS Letters* **387**, 11-15

54. Koivunen, E., Gay, D. A., and Ruoslahti, E. (1993) *J. Biol. Chem.* **268**, 20205-20210

55. Koivunen, E., Wang, B., and Ruoslahti, E. (1994) *J. Cell Biol.* **124**, 373-380

56. Ugarova, T. P., Budzynski, A. Z., Shattil, S. J., Ruggeri, Z. M., Ginsberg, M. H., and Plow, E. F. (1993) *J Biol. Chem.* **268**, 21080-21087
57. Xie, D., Miller, C. W., O'Kelly, J., Kakachi, K., Sakashita, A., Said, J. W., Gornbein, J.,
and Koeffler, H. P. (2001) *J Biol. Chem.* **276**, 14187-14194

58. Tsai, M. S., Bogart, D. F., Castaneda, J. M., Li, P., and Lupu, R. (2002) *Oncogene* **21**, 8178-8185

59. Reynolds, L. E., Wyder, L., Lively, J. C., Taverna, D., Robinson, S. D., Huang, X.,
Sheppard, D., Hynes, R. O., and Hodivala-Dilke, K. M. (2002) *Nat. Med.* **8**, 27-34

60. Taverna, D., Moher, H., Crowley, D., Borsig, L., Varki, A., and Hynes, R. O. (2004) *Proc. Natl. Acad. Sci. U.S.A* **101**, 763-768
FIGURE LEGENDS

Fig. 1. CCN1 isoforms and peptides. A, schematic representation of CCN1 and mutant constructs, showing the secretory signal (SP) and four modular domains: IGFBP (domain I), VWC (domain II), TSP1 (domain III), and CT (domain IV). For constructs of the CCN1_1-2 domains, a polyhistidine tag (HIS) has been added to the termini. A hatched box indicates the D125A mutation in domain II. B, sequences of synthetic peptides encompassing domain II; residue numbering refer to their positions in the mouse protein. C, ion-exchange sepharose column-purified CCN1 (lanes 1), CCN1-D125A (lanes 2), and nickel-agarose-purified CCN1_1-2 (lanes 3), and CCN1_1-2-D125A (lanes 4) were electrophoresed on a 12% SDS-PAGE, followed by Coomassie Brilliant Blue staining or immunoblotting with anti-CCN1 antibodies. Molecular mass of markers (MW) are shown in kDa.

Fig. 2. Immobilized V2 peptide supports integrin αvβ3-dependent HUVEC adhesion. A, cell adhesion assays were performed with washed HUVECs detached with 2.5 mM EDTA and resuspended in serum-free Medium 200 at 5×10^5 cells/ml. 50 µl of cell suspension was plated on each microtiter well coated with CCN1 (10 µg/ml) or 1 mM V1, V2, V3, or V4 peptides and blocked with 1% BSA. After incubation at 37°C for 30 min, adherent cells were fixed and stained with methylene blue, and extracted dye was quantified by absorbance at 620 nm. B, microtiter wells were coated with indicated amounts of V2 or V2-mut peptides and blocked with 1% BSA. HUVEC adhesion was determined as described above. C, microtiter wells were coated with CCN1 (10 µg/ml), V2 peptide (0.5 mM), or VN (0.5 µg/ml). Detached HUVECs were treated with EDTA (5 mM), GRGDSP peptide (0.2 mM), or LM609 (50 µg/ml) for 30 min. prior to plating; cell adhesion assays were performed as described above. D, V2 or V2-mut peptides were coated on microtiter wells as above and blocked with 2% PVA. Peptide coating was determined by incubation with PEO-maleimide.
activated biotin (10 µM) for 2 h at 22°C followed by horseradish peroxidase-conjugated streptavidin. The color reaction was developed and absorbance was measured at 420 nm. Data shown for all panels are mean ± S.D. of triplicate determinations and are representative of three experiments.

**Fig. 3. Soluble V2 peptide inhibits cell adhesion to CCN proteins.** A, microtiter wells were coated with CCN1 (10 µg/ml) or type I collagen (Col.I, 5 µg/ml) and blocked with 1% BSA. Washed HUVECs were pre-treated with indicated amount of soluble V2 peptide for 30 min prior to plating and cell adhesion assay. B, microtiter wells were coated with CCN1, CCN2 or CCN3 (10 µg/ml) and blocked with 1% BSA. Washed HUVECs were pre-treated with 0.2 mM V2 or V2-mut peptides for 30 min prior to cell adhesion assay as described. C, sequences of variants of the V2 peptide used. D, microtiter wells were coated with CCN1 (10 µg/ml) or type I collagen (Col.I, 5 µg/ml) and blocked with 1% BSA. Washed HUVECs were pre-treated with V2, V2-a, V2-b, or V2-c peptides (0.2 mM) prior to cell assay as described. Data are means ± S.D. of triplicate determinations and are representative of three experiments.

**Fig. 4. CCN1<sub>1-2</sub> supports integrin α<sub>4</sub>β<sub>3</sub>-dependent HUVEC adhesion.** A, microtiter wells were coated with indicated amounts of CCN1<sub>1-2</sub> or CCN1<sub>1-2</sub>-D125A, and HUVEC adhesion assays were performed as described above. B, microtiter wells were coated with indicated amounts of CCN1<sub>1-2</sub> or CCN1<sub>1-2</sub>-D125A and blocked with 1% gelatin. Protein-coating was determined by ELISA using affinity-purified anti-CCN1 polyclonal antibodies. The color reaction was developed using a horseradish peroxidase immunoassay kit, and absorbance was measured at 420 nm. C, microtiter wells were coated with CCN1 (10 µg/ml), CCN1<sub>1-2</sub> (10 µg/ml), or VN (0.5 µg/ml). Detached HUVECs were treated with GRGDSP or GRGESP peptides (0.2 mM), LM609 (50 µg/ml) or normal
mouse IgG (50 µg/ml) for 30 min. prior cell adhesion assay. Data shown for all panels are mean ± S.D. of triplicate determinations and are representative of three experiments.

Fig. 5. **D125 is critical for direct binding of CCN1-2 to purified integrin αvβ3.** A, microtiter wells were coated with indicated amount of CCN1-2 or CCN1-2-D125A and blocked with 2% BSA. Purified integrin αvβ3 (1 µg/ml) was allowed to bind at 37°C for 2 hrs. Bound integrin was detected by ELISA using anti-β3 antibodies. B, purified integrin αvβ3 (1 µg/ml) was pretreated with indicated concentration of V2 peptide for 30 min. at room temperature before plating onto microtiter wells coated with CCN1 (10 µg/ml) protein. C, microtiter wells were coated with purified integrin αvβ3 (1 µg/ml) and blocked with 2% BSA. Binding of varying concentrations of CCN1 and CCN1-D125A was detected by ELISA using affinity-purified polyclonal anti-CCN1 antibodies. D, Indicated amounts of CCN1 or CCN1-D125A were coated on microtiter wells, and purified integrin αvβ3 (1 µg/ml) was allowed to bind at 37°C for 2 hrs. Bound integrin was detected by ELISA using anti-β3 antibodies. Data shown for all panels are mean ± S.D. of triplicate determinations and are representative of three experiments.

Fig. 6. **Adhesive properties of CCN1-D125A mutant.** A, microtiter wells were coated with of CCN1 (10 µg/ml), CCN1-D125A (10 µg/ml), or VN (0.5 µg/ml). Detached HUVECs were treated with GRGDSP or GRGESP peptides (0.2 mM), LM609 (50 µg/ml), or normal mouse IgG (50 µg/ml) for 30 min. prior to plating and cell adhesion assay was performed as described. B, microtiter wells were coated with 1 µg/ml of CCN1, CCN1-D125A or VN. Detached primary human skin fibroblast were incubated with heparin (1 µg/ml), anti-α6 mAb GoH3 (50 µg/ml), anti-β1 mAb JB1A (50 µg/ml)
or normal mouse IgG (50 µg/ml) for 30 min. prior to plating and cell adhesion assay was performed as described.

**Fig. 7.** CCN1-D125A is impaired in stimulation of α5β3-mediated HUVEC migration. A, migration of HUVEC was measured using Transwell chambers. HUVECs were treated with PMA (100 nM) for 30 min. to activated integrins before plating in the upper chamber. Indicated amounts of CCN1 or CCN1-D125A were added to the lower chamber as chemoattractants. Cells migrated to the lower chamber were counted in 10 high power fields. B, HUVECs were prepared as above, and treated with either LM609 (25 µg/ml) or normal mouse IgG (100 µg/ml) before being added to Transwell chambers. CCN1 or CCN1-D125A (10 µg/ml) were used as chemoattractants, and cell migration was quantified as above.

**Fig. 8.** CCN1-D125A is defective in promoting VEGF-induced DNA synthesis and cell survival in HUVECs. The effect of soluble CCN1-D125A on VEGF-induced mitogenesis under serum-free condition was assessed on HUVECs attached to 96-well plates. Serum-starved cells were treated with VEGF (1 ng/ml), CCN1 or CCN1-D125A (5 µg/ml), or a combination of VEGF and CCN1 or CCN1-D125A. Assay medium was supplied with BrdU and incubation was for 21 h. BrdU incorporation was measured by ELISA using anti-BrdU antibodies. Data shown are mean ± S.D. of triplicate determinations and are representative of three experiments.
**A**

| SP | IGFBP | VWC | TSP1 | CT |
|----|-------|-----|------|----|
| I  |       |     |      |    |
| II |       | III | IV   |    |

- **CCN1**
- **CCN1-D125A**
- **CCN1_{1,2}**
- **CCN1_{1,2}-D125A**

**B**

| Peptide | Sequence | Residues  |
|---------|----------|-----------|
| V1      | RIYONGESFQPNCKHQCTCI | 105-124   |
| V2      | NCKHQCTCIDGAVGCIPLCP | 116-135   |
| V2-mut | NCKHQCTCIAGAVGCIPLCP | 116-135   |
| V3      | IPLCPQELSLPNLGCNPNR  | 131-149   |
| V4      | PNPRLVKVSGQCCCEWVCDE | 146-165   |

**C**

- **Comassie Blue staining**
- **Immunoblotting**

**Fig. 1**
Fig. 2
A

Fig. 3

Peptide Sequence

| Peptide  | Sequence                  |
|----------|---------------------------|
| V2       | NCKHQCTCIDGAVGCIPLCP      |
| V2-mut   | NCKHQCTCIAGAVGCIPLCP      |
| V2-a     | KHQCTCIDGAVGCIPL          |
| V2-b     | TCIDGAVGCI                |
| V2-c     | IDGAVG                    |
Fig. 5
Fig. 6

A

B

Downloaded from http://www.jbc.org/ by guest on March 24, 2020
Fig. 7
Fig. 8
Identification of a novel integrin αvβ3 binding site in CCN1 (CYR61) critical for pro-angiogenic activities in vascular endothelial cells
Ningyu Chen, Shr-Jeng Leu, Viktor Todorovic, Stephen C.-T. Lam and Lester F. Lau

J. Biol. Chem. published online August 10, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406813200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts