Targeted Mutagenesis of the Angiogenic Protein CCN1 (CYR61)

SELECTIVE INACTIVATION OF INTEGRIN α6β1-HEPARAN SULFATE PROTEOGLYCAN CORECEPTOR-MEDIATED CELLULAR FUNCTIONS

Received for publication, July 12, 2004, and in revised form, August 9, 2004

Published, JBC Papers in Press, August 17, 2004, DOI 10.1074/jbc.M407850200

Shr-Jeng Leu‡§, Ningyu Chen‡, Chih-Chiun Chen‡, Viktor Todorovic©, Tao Bai‡,
Vladislava Jurii©, Ying Liu‡, Guoqiang Yan‡, Stephen C.-T. Lam©, and Lester F. Lau‡§

From the ‡Department of Biochemistry and Molecular Genetics and §Department of Pharmacology, University of Illinois at Chicago College of Medicine, Chicago, Illinois 60607-7170

The matricellular protein CCN1 (CYR61) regulates multiple cellular processes and plays essential roles in embryonic vascular development. A ligand of several integrin receptors, CCN1 acts through integrin α6β1, and heparan sulfate proteoglycans (HSPGs) to promote specific functions in fibroblasts, smooth muscle cells, and endothelial cells. We have previously identified a novel α6β1, binding site, T1, in domain III of CCN1. Here we uncover two novel 16-residue sequences, H1 and H2, in domain IV that can support α6β1- and HSPGs-dependent cell adhesion, suggesting that these sequences contain closely juxtaposed or overlapping sites for interaction with α6β1 and HSPGs. Furthermore, fibroblast adhesion to the H1 and H2 peptides is sufficient to induce prolonged MAPK activation, whereas adhesion to T1 induces transient MAPK activation. To dissect the roles of the three sites in CCN1 function, we have created mutants disrupted in T1, H1, and H2 or in all three sites in the context of full-length CCN1. We show that the T1 and H1/H2 sites are functionally non-equivalent, and disruption of all three sites completely abolished α6β1-HSPG-mediated cellular activities. All mutants disrupting T1, H1, and H2 fully retain α6β1-mediated pro-angiogenic activities, indicating that these mutants are biologically active and are defective only in α6β1-HSPG-mediated functions. Together, these findings identify and dissect the differential roles of the three sites (T1, H1, H2) required for α6β1-HSPG-dependent CCN1 activities and provide a strategy to investigate these α6β1-HSPG-specific activities in vivo.

The CCN1 family of ECM-associated signaling proteins has recently emerged as developmentally important regulators with diverse functions (1–3). The significance of this protein family is underscored by the finding that targeted disruptions of Ccn1 (cysteine-rich 61, CYR61) and Ccn2 (connective tissue growth factor, Ctgf) both lead to lethality in mice but with very distinct phenotypes (4, 5), and mutations in CCN6 are associated with diverse vascular defects in the placenta and the embryo (4). In keeping with its structural roots in ECM proteins, CCN1 functions as a ligand of integrins, which are heterodimeric transmembrane signaling receptors that mediate diverse cellular responses to ECM proteins (18). CCN1 binds at least five distinct integrins, which mediate its functions in a cell type- and context-specific manner (19–23). In fibroblasts, smooth muscle cells, and endothelial cells, integrin α6β1 and HSPGs act as co-receptors to mediate cell adhesion, and these co-receptors also support smooth muscle cell migration (21, 24, 25). By contrast, the pro-angiogenic activities of CCN1 in activated endothelial cells are all mediated through integrin α6β1 (25).

Focusing on the first three domains of CCN1, we have recently identified a novel integrin α6β1, binding site, T1, in domain III (26). In this study, we have uncovered two additional sites for interaction with α6β1, H1, and H2, which mediate cell adhesion, and these co-receptors also support smooth muscle cell migration (21, 24, 25). By contrast, the pro-angiogenic activities of CCN1 in activated endothelial cells are all mediated through integrin α6β1 (25).
Identification of Two Novel Peptides That Support Integrin αβ₂- and HSPG-dependent Cell Adhesion—Previous analysis was based on the identification of two peptides, GRGDSP and GRGESP, that support integrin-dependent cell adhesion. These peptides were identified by screening a library of synthetic peptides for their ability to inhibit cell adhesion. The GRGDSP peptide was shown to bind to the αIIbβ₃ integrin subunits and to block the interaction of platelets and leukocytes with collagen and fibrinogen, respectively.

Materials and Methods

Antibodies, Peptides, and Reagents—Rat type I collagen, vitronectin, laminin, and VEGF were purchased from Collaborative Biomedical (Bedford, MA). BSA, heparin (sodium salt, from porcine intestinal mucosa), and BSA (50 mM) were from Sigma. GRGDSP and GRGESP synthetic peptides and function-blocking mAbs against integrin β₃ (clone P4C10) were from Invitrogen. mAbs against integrin α₂ (clone LM609, azide-free) and integrin α₃ (clone GoH3, azide-free) were from Chemicon (Temecula, CA) and Immunotech (Marseille, France), respectively. Horseradish peroxidase-conjugated secondary antibodies were obtained from Amersham Biosciences. Antibodies against total and dually phosphorylated p42/p44 MAPKs were from Promega (Madison, WI).

Enzyme-linked Immunosorbent Assay—Microtiter wells coated with GST fusion proteins or BSA (50 µg/ml) overnight at 4 °C followed by blocking with 1% BSA for 2 h at room temperature. Protein-coating efficiency was examined by incubation with an anti-GST mAb (9G). The PCR products were purified on polyacrylamide gels, digested with BamHI and EcoRI, and ligated into the pGEX-4T-2 vector (Amersham Biosciences). All cloning steps were confirmed by sequence analysis. In vivo context.

RESULTS

Identification of Two Novel Peptides That Support Integrin αβ₂- and HSPG-dependent Cell Adhesion—Previous analysis was based on the identification of two peptides, GRGDSP and GRGESP, that support integrin-dependent cell adhesion. These peptides were identified by screening a library of synthetic peptides for their ability to inhibit cell adhesion. The GRGDSP peptide was shown to bind to the αIIbβ₃ integrin subunits and to block the interaction of platelets and leukocytes with collagen and fibrinogen, respectively.

Materials and Methods

Antibodies, Peptides, and Reagents—Rat type I collagen, vitronectin, laminin, and VEGF were purchased from Collaborative Biomedical (Bedford, MA). BSA, heparin (sodium salt, from porcine intestinal mucosa), and BSA (50 mM) were from Sigma. GRGDSP and GRGESP synthetic peptides and function-blocking mAbs against integrin β₃ (clone P4C10) were from Invitrogen. mAbs against integrin α₂ (clone LM609, azide-free) and integrin α₃ (clone GoH3, azide-free) were from Chemicon (Temecula, CA) and Immunotech (Marseille, France), respectively. Horseradish peroxidase-conjugated secondary antibodies were obtained from Amersham Biosciences. Antibodies against total and dually phosphorylated p42/p44 MAPKs were from Promega (Madison, WI).

Enzyme-linked Immunosorbent Assay—Microtiter wells coated with GST fusion proteins or BSA (50 µg/ml) overnight at 4 °C followed by blocking with 1% BSA for 2 h at room temperature. Protein-coating efficiency was examined by incubation with an anti-GST mAb (9G). The PCR products were purified on polyacrylamide gels, digested with BamHI and EcoRI, and ligated into the pGEX-4T-2 vector (Amersham Biosciences). All cloning steps were confirmed by sequence analysis. In vivo context.

RESULTS

Identification of Two Novel Peptides That Support Integrin αβ₂- and HSPG-dependent Cell Adhesion—Previous analysis was based on the identification of two peptides, GRGDSP and GRGESP, that support integrin-dependent cell adhesion. These peptides were identified by screening a library of synthetic peptides for their ability to inhibit cell adhesion. The GRGDSP peptide was shown to bind to the αIIbβ₃ integrin subunits and to block the interaction of platelets and leukocytes with collagen and fibrinogen, respectively.

Materials and Methods

Antibodies, Peptides, and Reagents—Rat type I collagen, vitronectin, laminin, and VEGF were purchased from Collaborative Biomedical (Bedford, MA). BSA, heparin (sodium salt, from porcine intestinal mucosa), and BSA (50 mM) were from Sigma. GRGDSP and GRGESP synthetic peptides and function-blocking mAbs against integrin β₃ (clone P4C10) were from Invitrogen. mAbs against integrin α₂ (clone LM609, azide-free) and integrin α₃ (clone GoH3, azide-free) were from Chemicon (Temecula, CA) and Immunotech (Marseille, France), respectively. Horseradish peroxidase-conjugated secondary antibodies were obtained from Amersham Biosciences. Antibodies against total and dually phosphorylated p42/p44 MAPKs were from Promega (Madison, WI).

Enzyme-linked Immunosorbent Assay—Microtiter wells coated with GST fusion proteins or BSA (50 µg/ml) overnight at 4 °C followed by blocking with 1% BSA for 2 h at room temperature. Protein-coating efficiency was examined by incubation with an anti-GST mAb (9G). The PCR products were purified on polyacrylamide gels, digested with BamHI and EcoRI, and ligated into the pGEX-4T-2 vector (Amersham Biosciences). All cloning steps were confirmed by sequence analysis. In vivo context.

RESULTS

Identification of Two Novel Peptides That Support Integrin αβ₂- and HSPG-dependent Cell Adhesion—Previous analysis was based on the identification of two peptides, GRGDSP and GRGESP, that support integrin-dependent cell adhesion. These peptides were identified by screening a library of synthetic peptides for their ability to inhibit cell adhesion. The GRGDSP peptide was shown to bind to the αIIbβ₃ integrin subunits and to block the interaction of platelets and leukocytes with collagen and fibrinogen, respectively.

Materials and Methods

Antibodies, Peptides, and Reagents—Rat type I collagen, vitronectin, laminin, and VEGF were purchased from Collaborative Biomedical (Bedford, MA). BSA, heparin (sodium salt, from porcine intestinal mucosa), and BSA (50 mM) were from Sigma. GRGDSP and GRGESP synthetic peptides and function-blocking mAbs against integrin β₃ (clone P4C10) were from Invitrogen. mAbs against integrin α₂ (clone LM609, azide-free) and integrin α₃ (clone GoH3, azide-free) were from Chemicon (Temecula, CA) and Immunotech (Marseille, France), respectively. Horseradish peroxidase-conjugated secondary antibodies were obtained from Amersham Biosciences. Antibodies against total and dually phosphorylated p42/p44 MAPKs were from Promega (Madison, WI).

Enzyme-linked Immunosorbent Assay—Microtiter wells coated with GST fusion proteins or BSA (50 µg/ml) overnight at 4 °C followed by blocking with 1% BSA for 2 h at room temperature. Protein-coating efficiency was examined by incubation with an anti-GST mAb (9G). The PCR products were purified on polyacrylamide gels, digested with BamHI and EcoRI, and ligated into the pGEX-4T-2 vector (Amersham Biosciences). All cloning steps were confirmed by sequence analysis. In vivo context.

RESULTS

Identification of Two Novel Peptides That Support Integrin αβ₂- and HSPG-dependent Cell Adhesion—Previous analysis was based on the identification of two peptides, GRGDSP and GRGESP, that support integrin-dependent cell adhesion. These peptides were identified by screening a library of synthetic peptides for their ability to inhibit cell adhesion. The GRGDSP peptide was shown to bind to the αIIbβ₃ integrin subunits and to block the interaction of platelets and leukocytes with collagen and fibrinogen, respectively.

Materials and Methods

Antibodies, Peptides, and Reagents—Rat type I collagen, vitronectin, laminin, and VEGF were purchased from Collaborative Biomedical (Bedford, MA). BSA, heparin (sodium salt, from porcine intestinal mucosa), and BSA (50 mM) were from Sigma. GRGDSP and GRGESP synthetic peptides and function-blocking mAbs against integrin β₃ (clone P4C10) were from Invitrogen. mAbs against integrin α₂ (clone LM609, azide-free) and integrin α₃ (clone GoH3, azide-free) were from Chemicon (Temecula, CA) and Immunotech (Marseille, France), respectively. Horseradish peroxidase-conjugated secondary antibodies were obtained from Amersham Biosciences. Antibodies against total and dually phosphorylated p42/p44 MAPKs were from Promega (Madison, WI).

Enzyme-linked Immunosorbent Assay—Microtiter wells coated with GST fusion proteins or BSA (50 µg/ml) overnight at 4 °C followed by blocking with 1% BSA for 2 h at room temperature. Protein-coating efficiency was examined by incubation with an anti-GST mAb (9G). The PCR products were purified on polyacrylamide gels, digested with BamHI and EcoRI, and ligated into the pGEX-4T-2 vector (Amersham Biosciences). All cloning steps were confirmed by sequence analysis. In vivo context.
of a C-terminal-truncated CCN1 mutant indicated the presence of an integrin α6β1 binding site within the first three domains (24), leading to the identification of a novel α6β1 binding site, T1, in domain III (26). More recent studies, however, suggested the potential presence of additional α6β1 binding sites in the CT domain (see below). To evaluate this possibility, we constructed a series of six overlapping peptides encompassing the entire CT domain as fusion proteins linked to GST (Table 1). The fusion proteins were purified to apparent homogeneity and showed similar coating efficiency on microtiter wells as detected by enzyme-linked immunosorbent assay using an anti-GST antibody (data not shown). We tested the ability of these fusion proteins to support fibroblast adhesion to the GST-H1 or GST-H2 as well as to GST fusion protein linked to the T1 peptide carrying the K239E mutation (GQKCIVATTTSWSQCES). Fibroblast adhesion to this mutant peptide was assessed. GST-T1 (wild type) and GST-T1-(K239E) have similar coating efficiency as determined by enzyme-linked immunosorbent assay (data not shown). As expected, fibroblasts adhered to the GST-T1 fusion but not to BSA or the GST control (Fig. 2B) (26). GST-T1-(K239E) was completely unable to support cell adhesion, indicating that the single charge-reversed K239E mutation effectively abolished T1 binding to integrin α6β1. We, therefore, constructed the K239E mutation in the context of full-length CCN1, designated SM (Fig. 2A).

We have previously shown that mutations in the basic residues within the H1 and H2 sequences obliterated heparin binding in CCN1 (21). We, therefore, incorporated these mutations, changing KGKKCSSTKKSPEPVR (H1) to AGAC-SATAKSPEPVR and FTYYGCSVKKYRPKY (H2) to FTY-GCSSVVAAAPKY in CCN1 to create the mutant DM (Fig. 2A). In addition, we also created a mutant (TM) that combined the K293E mutation in the DM background, thereby disrupting all three sites for α6β1-HSPG interaction. For consistency, WT CCN1 and the mutants SM, DM, and TM used in this study were similarly constructed with a C-terminal FLAG epitope tag. The recombinant proteins were expressed via a baculovirus vector and purified to apparent homogeneity from insect cell conditioned media (see “Materials and Methods”; Fig. 2C).

**Cell Adhesive Properties of CCN1 Mutants**—Fibroblast adhesion to CCN1 is mediated exclusively through integrin α6β1 with HSPGs as coreceptors (21), thus providing an assay for the interaction of CCN1 with α6β1-HSPGs. Surprisingly, both SM and WT CCN1 supported fibroblast adhesion in a dose-dependent manner with similar efficiency, achieving maximal cell adhesion at a coating concentration of 1 μg/ml (Fig. 3A). Thus, disruption of the T1 binding site for α6β1 did not significantly affect the cell adhesive activity of CCN1, indicating that H1 and H2 are sufficient to support fibroblast adhesion through α6β1-HSPGs.

Disruption of H1 and H2 in DM, however, severely inactivated cell adhesive activity, indicating that H1/H2 are critical for mediating fibroblast adhesion (Fig. 3B). Whereas cell adhesion to CCN1 and SM plateaued when coated at 1 μg/ml (Fig. 3A), DM failed to support cell adhesion at this concentration. Because DM still retained the T1 binding site for α6β1, we postulated that it may be able to support cell adhesion at very high coating concentrations, analogous to the GST-T1 peptide fusion protein (Fig. 2B). Indeed, DM can support fibroblast adhesion when coated at ~50–100-fold higher concentration than WT CCN1, with maximal adhesion observed at 50–100 μg/ml (Fig. 3B). Consistent with this adhesion being mediated through the T1 sequence, further disruption of T1 in TM constructs significantly reduced adhesion.

**Construction of CCN1 Mutants Disrupted in Binding Sites for the α6β1-HSPG Coreceptors**—Results above indicate that CCN1 contains three sequences important for interaction with α6β1 and HSPGs: T1, H1, and H2. To dissect their relative contributions to CCN1 functions, we constructed mutants in the context of full-length CCN1 that are disrupted in T1 (SM, single mutant), H1 and H2 (DM, double mutant), and all three sites (TM, triple mutant) (Fig. 2A).

To disrupt the T1 site (GQKCIVQTTSWSQCSKS), we turned to previous mutagenesis in which each conserved residue was individually altered (26). Alanine substitution in any of 4 residues (Trp-234, Ser-235, Ser-238, Lys-239) reduced α6β1-mediated cell adhesion by >90%, and mutation in both Thr-231 and Thr-232 completely abolished cell adhesion. However, our attempts to produce mutant CCN1 proteins with alanine substitutions in these critical T1 residues did not yield soluble proteins (data not shown). The expressed mutants aggregated and formed inclusion bodies inside the cell, suggesting that these mutants adopted detrimental conformations that prevented them from being secreted. To avoid mutations that may cause major structural changes, we applied two algorithms, PSIPRED and FRAGFOLD (30, 31), to predict possible structural alterations that may result from mutations. Each of the critical residues in T1 was substituted with each of the other 19 amino acids to predict the protein structure using these algorithms. Consistent with the inability of alanine substitution mutants to be secreted, the T1 region of CCN1 appears highly sensitive to perturbation. Substitutions of the critical residues in T1 with nearly any amino acid resulted in drastic changes in the predicted protein structure. However, we found one mutation, namely K239E, that did not elicit a predicted conformational change. To test the efficacy of this mutation in disrupting interaction with α6β1, we constructed a GST fusion protein linked to the T1 peptide carrying the K239E mutation (GQKCIVATTTSWSQCES). Fibroblast adhesion to this mutant peptide was assessed. GST-T1 (wild type) and GST-T1-(K239E) have similar coating efficiency as determined by enzyme-linked immunosorbent assay (data not shown). As expected, fibroblasts adhered to the GST-T1 fusion but not to BSA or the GST control (Fig. 2B) (26). GST-T1-(K239E) was completely unable to support cell adhesion, indicating that the single charge-reversed K239E mutation effectively abolished T1 binding to integrin α6β1. We, therefore, constructed the K239E mutation in the context of full-length CCN1, designated SM (Fig. 2A).

**Table 1**

| Peptide | Sequence | Residues |
|---------|----------|----------|
| H1      | KGKKCSSTKKSPEPVR | 280–295  |
| H2      | FTYYGCSVKKYRPKY  | 296–311  |
| H3      | GQKCIVQTTSWSQCSKS | 312–334  |
| H4      | TTTYGCSVKKYRPKY  | 334–359  |
| H5      | FTYYGCSVKKYRPKY  | 352–379  |

| Peptide | Sequence | Residues |
|---------|----------|----------|
| H6      | KCNYNCPHPNEASFLRSLYLSFDN1HKFRD | 334–359  |
pletely abolished cell adhesion at any concentration, even when coated at 250 μg/ml. Function-blocking mAbs against integrin α/β_1 (GoH3) or α/β_2 (P4C10) obliterated cell adhesion to CCN1, SM, or DM, whereas mAb against integrin α/β_3 (LM609) had no effect, thus establishing that cell adhesion to these mutants occurs through α/β_1 as in wild type CCN1 (Fig. 3C). Taken together, these results show that α/β_1-HSPG-mediated fibroblast adhesion to CCN1 occurs primarily through H1 and H2, and mutations of these sites decimated this activity. Cell adhesion through the α/β_1 binding site T1 is evident only when H1 and H2 are mutated and at high protein concentrations.

T1, H1, and H2 Peptides Induce Differential Activation of MAPKs—Transient versus sustained activation of p42/p44 MAPKs by extracellular signals are known to specify different biological outcomes (32, 33). Whereas cell adhesion to most ECM proteins induces a transient activation of p42/p44 MAPKs, CCN1 has the unusual ability to induce sustained MAPK activation as an adhesive substrate (7). To test the possibility that specific CCN1 peptides supporting cell adhesion may contribute directly to activation of MAPKs, we examined fibroblast adhesion to CCN1 or to the GST-peptide fusion proteins. As expected, fibroblast adhesion to CCN1 resulted in rapid and sustained MAPK activation lasting for at least 9 h (Fig. 4A). Remarkably, cells adhered to the GST-H2 also showed similar kinetics of MAPK activation. Likewise, cell adhesion to GST-H1 gave similar results (data not shown). By contrast, cells adhered to GST-T1 or laminin induced rapid but transient MAPK activation that peaked 30 min to 1 h after plating and declined to basal level thereafter. These results reveal two distinct kinetics of MAPK activation by CCN1 peptides, one rapid and transient (when cells adhere to T1) and the other rapid and sustained (when cells adhere to H1 or H2).

To address the signaling contributions of the T1, H1, and H2 sites further, we examined MAPK activation in full-length CCN1 mutants disrupted in these sites. CCN1 mutants disrupted in the H1 or H2 sequence were prepared (21) (Fig. 4B). As shown in Fig. 4C, mutations in H1 or H2 did not abolish the ability to induce sustained MAPK activation, which was observed beyond 3 h after plating. In contrast, cell adhesion to laminin induced only transient MAPK activation lasting about 1 h. Thus, the H1 and H2 sites appear to be equivalent and redundant with respect to their ability to bind heparin (21), support α/β_1-HSPGs-mediated cell adhesion (Fig. 1), and induce sustained p42/p44 MAPKs activation (Fig. 4).

Based on the above results, we postulated that mutations in both H1 and H2 may abolish sustained MAPK activation,
Fig. 2. Construction and expression of CCN1 and mutants. A, schematic diagram showing constructs of wild type CCN1 and mutants either bearing the K239E point mutation in T1 (SM), disruptions in H1 and H2 (DM), or combined mutations in T1, H1, and H2 (TM). Each construct is similarly endowed with an N-terminal secretory signal and a C-terminal FLAG epitope tag. Wild type T1, H1, and H2 sequences and specific amino acid changes in the mutants are shown in bold italics. Recombinant proteins were expressed in insect cells via a baculovirus vector. IGFBP, insulin-like growth factor binding protein; VWC, von Willebrand factor type C repeat; TSP1, thrombospondin type I repeat. B, primary human skin fibroblasts were plated on microwell plates coated with either GST, GST-T1, or GST-T1-K239E fusion proteins (50 μg/ml each). Cells were allowed to adhere at 37 °C for 20 min. After washing, adherent cells were fixed, stained with methylene blue, and quantified by absorbance at 620 nm. C, FLAG affinity-purified recombinant proteins (2 μg each) were electrophoresed in 10% SDS-PAGE followed by staining with Coomassie Brilliant Blue. Molecular mass (kDa) of the markers are shown at the left. The gel was immunoblotted with polyclonal anti-CCN1 antibodies and shown in the lower panel.
whereas transient MAPK activation, an activity supported by T1, might be preserved. Indeed, we observed that MAPK activation in cells adhered to CCN1 and SM was sustained for at least 5 h (Fig. 4C). DM, however, lost the ability to induce sustained MAPK activation but instead supported transient activation similar to laminin, with MAPK activation returning
FIG. 4. Effects of CCN1 mutants on MAPK activation and gene expression. A, primary human fibroblasts were serum-starved and resuspended in serum-free medium at $6 \times 10^5$ cells/ml. Cells were plated on dishes (1 ml/35-mm dish) coated with WT CCN1 (10 μg/ml), GST-H2 (50 μg/ml), GST-T1 (50 μg/ml), or laminin (LN, 10 μg/ml) for various times as indicated. Cell lysates were electrophoresed and immunoblotted with antibodies against total or dually phosphorylated p42/p44 MAPKs. B, schematic diagram showing the CCN1-H1 and CCN1-H2 mutants. Mutant sequences shown (bold italic) were constructed in the context of full-length CCN1 (21). C, primary human fibroblasts were prepared as above and plated on dishes coated with WT CCN1 (10 μg/ml), laminin (10 μg/ml), CCN1-H1 (10 μg/ml), CCN2-H2 (10 μg/ml), SM (10 μg/ml), or DM (250 μg/ml) for 1–5 h as indicated. Cell lysates were separated on 10% SDS-PAGE and immunoblotted with antibodies against total or dually phosphorylated p42/p44 MAPKs. IGFBP, insulin-like growth factor binding protein; VWC, von Willebrand factor C repeat; TSP1, thrombospondin type I repeat.
to background level within 3 h. In these experiments, we used a high coating concentration of DM to ensure that similar number of cells were adherent to DM compared with SM and CCN1. Together, these results show that H1 or H2 is sufficient to induce prolonged activation of p42/p44 MAPK, and the presence of either site is necessary for this activity, whereas T1 is sufficient to induce transient MAPK activation.

Mutations in H1 and H2 Impair Regulation of Gene Expression—CCN1 activates a genetic program in fibroblasts, leading to up-regulation of genes implicated in angiogenesis and matrix metabolism several hours after treatment (9). Although the involvement of integrin αβ3 in gene regulation by CCN1 has not been established, up-regulation of Vegf-A and MMP-1 is inhibited by PD98059, suggesting a dependence on MAPKs (9). To test whether the loss of sustained p42/p44 MAPK activation in DM and TM may alter CCN1-regulated gene expression, serum-starved fibroblasts were treated with soluble CCN1 proteins and analyzed for gene expression. Because activation of gene expression is observed both when CCN1 is used as an adhesion substrate or in a soluble form (7, 9), this assay does not preclude analysis of TM. As shown in Fig. 5, SM up-regulates Vegf-A and MMP-1 expression similar to WT CCN1, whereas DM and TM were defective in this activity. Thus, MAPK-dependent up-regulation of Vegf-A and MMP-1 expression similar to WT CCN1, whereas DM and TM were defective in this activity. This, MAPK-dependent up-regulation of Vegf-A and MMP-1 by CCN1 requires the presence of the H1/H2 sites for αβ3-HSPGs interaction and correlates with sustained MAPK activation through these sites.

Defect in Stimulation of Smooth Muscle Cell Migration—Another CCN1 activity known to be mediated through αβ3-HSPGs is the induction of smooth muscle cell chemotaxis (24). To test the effect of CCN1 mutants, we employed a modified Boyden chamber assay to quantify VSMC migration. Cells were loaded on the upper chamber, and those that migrated through a membrane to the lower chamber where the chemoattractant was placed were counted. As shown in Fig. 6, VSMC migration to soluble CCN1 or SM similarly and in a dose-dependent manner, with maximal migration occurring at 2–4 μg/ml. By contrast, TM was completely inactive at any concentration tested. These results show that the T1 site for αβ3 binding is dispensable for inducing VSMC migration, whereas inactivation of all three sites for αβ3-HSPG interaction completely abolished this activity.

CCN1 Mutants Retain αβ3-mediated Angiogenic Activities—Having lost all three sites for αβ3-HSPG interaction, TM was completely inactive in αβ3-HSPG-mediated activities described above. It may be questioned whether TM is inactive due to possible conformational changes resulting from mutations or due to a specific loss of αβ3-HSPG interaction. Moreover, whether the αβ3-mediated pro-angiogenic activities of CCN1 require interaction with HSPGs is not known (25). To address these questions, we examined the pro-angiogenic functions of CCN1 mutants in activated endothelial cells. HUVEC adhesion to CCN1 occurs through both αβ3 and αβ1-HSPGs (25), and SM supported HUVEC adhesion similar to WT CCN1 (Fig. 7A). Cell adhesion was abolished by EDTA and partially inhibited by GRGDSP peptide or the anti-αβ3 mAb LM609, indicating that cell adhesion to CCN1 and SM was partly mediated through integrin αβ3. Cell adhesion was also partially inhibited by the anti-αα mAb GoH3, consistent with involvement of the αβ3-HSPG coreceptors. In contrast, HUVEC adhesion to DM and TM was completely inhibited by EDTA, GRGDSP peptide, and LM609 but not at all by GoH3. These results indicate that DM and TM support HUVEC adhesion solely through αβ3, having lost αβ1-HSPG interactions.

Induction of endothelial cell migration is a pro-angiogenic activity that correlates with induction of neovascularization in vivo. Using a modified Boyden chamber assay, we found that CCN1, SM, DM, and TM were all able to stimulate HUVEC migration (Fig. 7B). HUVEC migration to CCN1 and all mutants tested was completely inhibited by the anti-αβ3 mAb LM609, whereas the anti-αα mAb GoH3 had no effect. Thus, all CCN1 mutants with disrupted αβ3-HSPG binding sites are still capable of inducing αβ3-dependent HUVEC migration, similar to WT. Consistent with this angiogenic activity, both WT CCN1 and TM were able to induce tubule formation in phorbol 12-myristate 13-acetate-activated HUVEC cultured in collagen gel (Fig. 7C). Preincubation of cells with LM609 inhibited tubule formation, whereas the addition of GoH3 had no effect, showing that tubule formation is αβ3-dependent. Similar results were obtained with SM and DM (data not shown). These findings show that CCN1 mutants defective in αβ3-HSPG interactions are functionally intact and are fully capable of inducing αβ3-dependent angiogenic activities in activated endothelial cells.

DISCUSSION

CCN1 is an angiogenic inducer that plays essential roles in embryonic development (4). Acting through integrins and HSPGs, CCN1 regulates multiple cellular processes. In fibroblasts, smooth muscle cells, and endothelial cells, CCN1 supports cell adhesion through the integrin αβ3-HSPG coreceptors, which also mediate CCN1-stimulated cell migration in
smooth muscle cells (21, 24, 25). This study has identified two novel sequences, H1 and H2, that are responsible for $\alpha_\beta_1$-HSPG-dependent cell adhesion in CCN1. Functional and mutational analyses demonstrate that the H1/H2 sequences and the previously identified $\alpha_\beta_1$ binding site T1 contribute differentially to CCN1 activities including cell adhesion, migration, MAPK activation, and regulation of gene expression. Furthermore, we show that mutation of all three sites completely eliminate $\alpha_\beta_1$-HSPG-mediated activities, which are fully dispensable for $\alpha_\beta_1$-dependent CCN1 functions. Together, these findings identify and dissect the three sites relevant for $\alpha_\beta_1$-HSPG-mediated activities and provide a strategy for analyzing integrin-specific functions of CCN1 in vivo.

Previous studies pointed to the presence of an $\alpha_\beta_1$ binding site within the first three domains, leading to the identification of the T1 sequence in domain III as a novel binding site for $\alpha_\beta_1$ (24, 26). Further studies indicated the presence of additional sites, culminating in the identification of the H1 and H2 sequences as being critical for $\alpha_\beta_1$-HSPGs-dependent CCN1 activities based on the following observations (Fig. 1). 1) Fibroblasts adhere to H1 and H2 peptides, and this adhesion is inhibited by EDTA and Ca$^{2+}$ but supported by Mg$^{2+}$. 2) Anti-$\alpha_\beta_1$ and $\beta_1$ mAbs specifically block cell adhesion to H1 and H2. 3) Fibroblast adhesion to H1 and H2 was abolished by the presence of soluble heparin in the medium or by heparinase treatment of cells. Consistent with these observations is the finding that H1 and H2 contain multiple basic residues, mutations of which eliminate heparin binding activity (21). Analysis of targeted mutations of these sites provided further evidence that H1 and H2 mediate $\alpha_\beta_1$-HSPG-dependent CCN1 activities (Figs. 2–5). These results suggest that H1 and H2 contain closely juxtaposed or overlapping sites for interaction with $\alpha_\beta_1$ and HSPGs.

Compelling evidence demonstrates that the T1 sequence in the TSP1 domain is a bona fide binding site for integrin $\alpha_\beta_1$ (26). Synthetic T1 peptide supports $\alpha_\beta_1$-dependent cell adhesion as an immobilized substrate, blocks $\alpha_\beta_1$-mediated activities as a soluble inhibitor, and purifies $\alpha_\beta_1$ from oligosaccharide extracts of fibroblasts as an affinity matrix. The difference in cell adhesive properties between DM and TM further establishes T1 as an $\alpha_\beta_1$ binding site (Fig. 3B). It is interesting to note that crystallographic analysis revealed the thrombospondin-1 type 1 repeat as an antiparallel, three-stranded fold capped by three disulfide bonds (34). Because alanine substitutions in the T1 sequence had a propensity to adopt apparently detrimental conformations, it is likely that mutations in most residues within T1 may destabilize this highly compact and structured domain. However, the physiological role of the T1 binding site is currently unclear, since disruption of T1 (SM) does not result in any apparent loss of activity. It appears that T1 is not the primary $\alpha_\beta_1$ binding site in CCN1; rather, $\alpha_\beta_1$-HSPGs-dependent CCN1 activities are mediated mainly through H1 and H2. Nevertheless, the possibility that T1 might be critical for $\alpha_\beta_1$-dependent functions in specialized contexts cannot be excluded. For example, the T1 site may become more available or exposed for $\alpha_\beta_1$ binding if the CT domain of CCN1 interacts with another protein or is removed by proteolysis. Existing evidence for proteolytic processing in CCN2 (35, 36) and protein-protein interaction through the CT domain in CCN3 (37–39) are consistent with these possibilities.

A novel and intriguing finding in this study is that cell adhesion to the T1 peptide induces transient activation of p42/p44 MAPKs, whereas cell adhesion to H1 or H2 peptide induces sustained MAPK activation (Fig. 4A). Furthermore, sustained MAPK activation is abolished by mutations in both H1 and H2 (DM) in the context of full-length CCN1 but not by mutations in either H1 or H2 alone (Fig. 4C). Thus, the H1 and H2 sequences appear to be functionally redundant with respect to their ability to bind heparin (21), support $\alpha_\beta_1$-HSPGs-mediated cell adhesion (Fig. 1), and induce prolonged p42/p44 MAPK activation (Fig. 4). In contrast, the presence of the T1 sequence alone, either as a GST fusion protein, or in the context of full-length CCN1 (DM) is sufficient to induce transient but not prolonged MAPK activation. These findings suggest that the T1 and H1/H2 sequences are capable of inducing different cellular responses, since transient versus sustained MAPK activation are known to specify distinct biological outcomes (32, 33). For example, the H1/H2 sequences may play a critical role in CCN1 regulated gene expression, consistent with the observation that DM and TM are concomitantly defective for sustained induction of MAPK and up-regulation of Vegf-A and MMP-1 (9) (Fig. 4).

Integrin $\alpha_\beta_1$ is known to bind to a number of substrates, including laminin, meltrin, thrombospondin, CCN proteins, tumstatin, invasin, fibrin, and human papillomavirus-16 (21, 40–45). Interestingly, these ligands are structurally diverse, and no consensus binding site for integrin $\alpha_\beta_1$ has emerged to date. Among the ligands of $\alpha_\beta_1$, CCN1 has an unusual dependence on HSPGs as coreceptors (21, 24, 25). Given the closely juxtaposed or overlapping sites for $\alpha_\beta_1$-HSPG interactions in H1 and H2, it is tempting to speculate that these sites may simultaneously associate with $\alpha_\beta_1$ and HSPGs, and this association may contribute to the unique signaling capabilities of H1/H2 as compared with T1 (Fig. 4). It is noteworthy that cell adhesion to fibronectin is known to require binding of HSPGs for $\alpha_\beta_1$-mediated functions but not for $\alpha_\beta_1$-dependent activities (47). Thus, integrin-specific association with HSPGs may confer signaling specificity of ligands. In this regard, the $\alpha_\beta_1$-mediated pro-angiogenic activities of CCN1 are independent of HSPGs based on functional and mutational studies (Fig. 7) (25).

The mutants SM, DM, and TM retain all pro-angiogenic activities mediated through integrin $\alpha_\beta_2$ in activated HUVEC. Thus, these CCN1 mutants support endothelial cell adhesion, induce cell migration, and induce tubule formation (Fig. 6). In addition, these mutants are also able to enhance HUVEC DNA synthesis induced by VEGF and to promote endothelial cell survival under conditions of growth factor deprivation similar to WT CCN1, both $\alpha_\beta_1$-dependent activities (25). These findings demonstrate that the CCN1 mutants are biologically active, and their functional defects are indeed due to mutation of the specific receptor binding sites rather than structural perturbations. Furthermore, $\alpha_\beta_1$-mediated CCN1 pro-angiogenic activities are fully independent of interaction with $\alpha_\beta_1$ and HSPGs.

Recently, we have identified a novel $\alpha_\beta_2$ binding site within a 20-amino acid sequence (V2) in CCN1 (48). A single amino acid mutation in this V2 sequence in the context of full-length CCN1 impaired both $\alpha_\beta_2$ binding and $\alpha_\beta_1$-mediated activities such as cell migration and DNA synthesis in endothelial cells. It is noteworthy that CCN1 activities previously associated with $\alpha_\beta_1$ are specifically abolished in a targeted mutation of the $\alpha_\beta_2$ binding site (48), and activities associated with $\alpha_\beta_1$-HSPGs are specifically lost in mutants targeting binding sites for these receptors (Figs. 3–7). Thus, there is a complete concordance of results from the current mutational analyses and previous studies that assigned specific integrins to activities using mAbs, agonists, and antagonists (21, 24, 25), thereby providing compelling evidence that the cellular activities of CCN1 are mediated through specific integrins and HSPGs.

The ability of CCN1 to up-regulate Vegf expression is rele-
vant in the context of angiogenesis. Although CCN1 exerts pro-angiogenic activities in activated endothelial cells through binding to αvβ3 (19, 25, 48)(Fig. 7), it may nevertheless act in part through up-regulation of VEGF to induce angiogenesis in vivo (8). This study shows that DM and TM are fully capable of inducing in vitro angiogenesis but are unable to up-regulate VEGF; thus, these events can be dissociated. Future studies examining the angiogenic activities of DM and TM in vivo will likely yield new insight into the angiogenic mechanism of CCN1 and help to dissect its direct action on endothelial cells from potential indirect action through VEGF.

CCN1 is a matricellular protein involved in multiple biological processes. Aside from being essential for successful embryonic vascular development (4), CCN1 expression is also associated with skeletal development (49, 50), cutaneous wound healing (9, 51), restenosed blood vessels (24, 52), advanced atherosclerotic lesions (23, 53), and advanced human breast cancer (10). Although a number of CCN1 activities have been characterized in cell culture systems, it is likely that many of its biological functions may be understood only in the context of an organ or tissue involving the interplay among multiple cell types. Characterization of CCN1 mutants that dissociate αvβ3- and αvβ5-HSPGs-mediated activities will provide a strategy for the in vivo dissection of integrin-specific CCN1 functions using transgenic or gene replacement approaches.

REFERENCES

1. Lau, L. F., and Lam, S. C. (1999) Exp. Cell Res. 248, 44–57
2. Bigstock, D. R. (1999) Endocrinol. Rev. 20, 189–206
3. Perbal, B. (2001) Mol. Pathol. 54, 57–79
4. Mo, F. E., Montevis, A. G., Chen, C. C., Stolz, D. B., Watkins, S. C., and Lau, L. F. (2002) Mol. Cell. Biol. 22, 8799–8720
5. Ivkovic, S., Yoon, B. S., Popoff, S. N., Safadi, F. F., Libuda, D. E., Stephenson, R. C., Daluisio, A., and Lyons, K. M. (2003) Development 130, 2779–2791
6. Hurvitz, J. R., Suwara, W. M., Van, H. W., El-Shanti, H., Superti-Furga, A., Raudner, J., Holderbaum, D., Pauli, R. M., Herd, J. K., Yan, H. E., Rezai-Deluhi, H., Legius, E., Le, M. M., Al-Alami, J., Bahabri, S. A., and Warman, M. L. (1999) Nat. Genet. 23, 94–98
7. Chen, C.-C., Chen, N., and Lau, L. F. (2001) J. Biol. Chem. 276, 10443–10452
8. Babic, A. M., Kireeva, M. L., Kolesnikova, T. V., and Lau, L. F. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6355–6360
9. Chen, C.-C., Mo, F.-E., and Lau, L. F. (2001) J. Biol. Chem. 276, 47329–47337
10. Menendez, J. A., Mehmi, I., Grigg, D. W., and Lupu, R. (2003) Endocrinol. Relat. Cancer 10, 141–152
11. O’Brien, T. P., Yang, G. P., Sanders, L., and Lau, L. F. (1990) Mol. Cell. Biol. 10, 3569–3577
12. Yang, G. P., and Lau, L. F. (1991) Cell Growth Differ. 2, 351–357
13. Bork, P. (1993) FEBS Lett. 327, 125–130
14. Borshtein, P. (1995) J. Cell Biol. 130, 503–506
15. Borshtein, P., and Sage, E. H. (2002) Curr. Opin. Cell Biol. 14, 608–616
16. Armstrong, L. C., and Borshtein, P. (2003) Matrix Biol. 22, 63–71
17. Fataccioli, V., Abergel, V., Wingertsmann, L., Neveille, P., Spitz, E., Adnot, S., Calenda, V., and Teiger, E. (2002) Hum. Gene Ther. 13, 1461–1470
18. Miranti, C. K., and Brugge, J. S. (2002) Nat. Cell Biol. 4, 83–90
19. Kireeva, M. L., Lam, S. C. T., and Lau, L. F. (1998) *J. Biol. Chem.* **273**, 3090–3096.
20. Jedsadayanmata, A., Chen, C. C., Kireeva, M. L., Lau, L. F., and Lam, S. C. (1999) *J. Biol. Chem.* **274**, 24321–24327.
21. Chen, N., Chen, C. C., and Lau, L. F. (2000) *J. Biol. Chem.* **275**, 24953–24961.
22. Grzeszkiewicz, T. M., Kirschling, D. J., Chen, N., and Lau, L. F. (2001) *J. Biol. Chem.* **276**, 21943–21950.
23. 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.
24. Grzeszkiewicz, T. M., Lindner, Y., Chen, N., Lam, S. C., and Lau, L. F. (2002) *Endocrinology* **143**, 1441–1450.
25. Leu, S.-J., Lam, S. C. T., and Lau, L. F. (2002) *J. Biol. Chem.* **277**, 46248–46255.
26. Leu, S.-J., Liu, Y., Chen, N., Chen, C. C., Lam, S. C., and Lau, L. F. (2003) *J. Biol. Chem.* **278**, 33801–33808.
27. Koskinen, P., Lehvaslaiho, H., MacDonald Bravo, H., Alitalo, K., and Bravo, R. (1990) *Oncogene* **5**, 615–618.
28. Kireeva, M. L., Mo, F.-E., Yang, G. P., and Lau, L. F. (1996) *Mol. Cell. Biol.* **16**, 1326–1334.
29. Falk, W., Goodwin, R. H., Jr., and Leonard, E. J. (1980) *J. Immunol. Methods* **33**, 239–247.
30. McGuffin, L. J., Bryson, K., and Jones, D. T. (2000) *Bioinformatics* **16**, 494–495.
31. Jones, D. T. (2001) *Proteins* **45**, Suppl. 5, 127–132.
32. Marshall, C. J. (1995) *Cell* **80**, 179–185.
33. Murphy, L. G., Smith, S., Chen, R. H., Fingar, D. C., and Blenis, J. (2002) *Nat. Cell Biol.* **5**, 556–564.
34. Tan, K., Duquette, M., Liu, J. H., Dong, Y., Zhang, R., Joachimiah, A., Lawler, J., and Wang, J. H. (2002) *J. Cell Biol.* **159**, 373–382.
35. Ball, D. K., Surveyor, G. A., Diehl, J. R., Steffen, C. L., Uzumcu, M., Miranda, M. A., and Bridgstock, D. R. (1998) *Biol. Reprod.* **59**, 824–835.
36. Tan, E. M., Morrison, C. J., Wu, Y. I., Stack, M. S., and Overall, C. M. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 6917–6922.
37. Perbal, B., Martinier, C., Sainsen, R., Werner, M., He, B., and Roizman, B. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 869–874.
38. Sakamoto, K., Yamaguchi, S., Ando, R., Miyawaki, A., Kahasawa, Y., Takagi, M., Li, C. L., Perbal, B., and Katsube, K. (2002) *J. Biol. Chem.* **277**, 29399–29405.
39. Fu, C. T., Hechberger, J. F., Ozog, M. A., Perbal, B., and Naus, C. C. (2004) *J. Biol. Chem.* **279**, 36943–36950.
40. Almeida, E. A., Huvovila, A. P., Sutherland, A. E., Stephens, L. E., Calarco, P. G., Shaw, L. M., Mercurio, A. M., Sonnenberg, A., Primakoff, P., and Myles, D. G. (1995) *Cell* **81**, 1095–1104.
41. Sonnenberg, A., Linders, C. J., Daams, J. H., and Kennel, S. J. (1990) *J. Cell Biol.* **116**, 207–217.
42. Maeshima, Y., Colorado, P. C., and Kalluri, R. (2000) *J. Biol. Chem.* **275**, 23745–23750.
43. Isberg, R. R., and Leong, J. M. (1990) *Cell* **60**, 861–871.
44. Calzada, M. J., Sipes, J. M., Krutzsch, H. C., Yurchenco, P. D., Annis, D. S., Mosher, D. F., and Roberts, D. D. (2003) *J. Biol. Chem.* **278**, 40679–40687.
45. Kertesmaa, J., Turchenco, P., and Tryggvason, K. (2000) *J. Biol. Chem.* **275**, 14853–14859.
46. Schober, J. M., Lau, L. F., Ugarova, T. P., and Lam, S. C. (2003) *J. Biol. Chem.* **278**, 25808–25815.
47. Mostafavi-Pour, Z., Askari, J. A., Parkinson, S. J., Parker, P. J., Ng, T. T., and Humphries, M. J. (2003) *J. Cell Biol.* **161**, 155–167.
48. Chen, N., Leu, S.-J., Todorovic, V., Lam, S. C. T., and Lau, L. F. (2004) *J. Biol. Chem.* **279**, 44166–44176.
49. O’Brien, T. P., and Lau, L. F. (1992) *Cell Growth Differ.* **3**, 645–654.
50. Wong, M., Kireeva, M. L., Kolesnikova, T. V., and Lau, L. F. (1997) *Dev. Biol.* **192**, 492–508.
51. 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.
52. Wu, K. J., Yee, B. A., Zhi, N. L., Gordon, E. M., and Hall, F. L. (2000) *Int. J. Mol. Med.* **6**, 433–440.
53. Hilfiker, A., Hilfiker-Kleiner, D., Fuchs, M., Kaminski, K., Lichtenberg, A., Rothkotter, H. J., Schaeffer, B., and Drexler, H. (2002) *Circulation* **106**, 254–260.