

Kallikrein-related Peptidase 12 Hydrolyzes Matricellular Proteins of the CCN Family and Modifies Interactions of CCN1 and CCN5 with Growth Factors^{*S}

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Kallikrein-related peptidases (KLKs) are an emerging group of secreted serine proteases involved in several physiological and pathological processes. We used a degradomic approach to identify potential substrates of KLK12. MDA-MB-231 cells were treated either with KLK12 or vehicle control, and the proteome of the overlying medium was analyzed by mass spectrometry. CCN1 (cyr61, ctgf, nov) was among the proteins released by the KLK12-treated cells, suggesting that KLK12 might be responsible for the shedding of this protein from the cell surface. Fragmentation of CCN1 by KLK12 was further confirmed *in vitro*, and the main cleavage site was localized in the hinge region between the first and second half of the recombinant protein. KLK12 can target all six members of the CCN family at different proteolytic sites. Limited proteolysis of CCNs (cyr61, ctgf, nov) was also observed in the presence of other members of the KLK family, such as KLK1, KLK5, and KLK14, whereas KLK6, KLK11, and KLK13 were unable to fragment CCNs. Because KLK12 seems to have a role in angiogenesis, we investigated the relations between KLK12, CCNs, and several factors known to be involved in angiogenesis. Solid phase binding assays showed that fragmentation of CCN1 or CCN5 by KLK12 prevents VEGF₁₆₅ binding, whereas it also triggers the release of intact VEGF and BMP2 from the CCN complexes. The KLK12-mediated release of TGF- β 1 and FGF-2, either as intact or truncated forms, was found to be concentration-dependent. These findings suggest that KLK12 may indirectly regulate the bioavailability and activity of several growth factors through processing of their CCN binding partners.

Human tissue kallikrein-related peptidases (KLKs)² belong to a subgroup of secreted serine proteases within the S1 family

of clan SA. So far, 15 members of the family (KLK1–15) have been identified and found expressed in a large number of tissues and cell populations (1). KLKs play important roles in different pathophysiologic processes (2) such as skin desquamation (3), innate immunity (4), semen liquefaction (5), dental enamel formation (6), neuro-degeneration (7), cervicovaginal physiology (8), and angiogenesis (9). Furthermore, dysregulated kallikrein-related peptidase gene and protein expression has been implicated in cancer (10). In particular, numerous clinical studies have linked the differential expression signatures of *KLK* genes and proteins to their potential roles as cancer biomarkers (11). Kallikrein-related peptidases may be involved in different stages of cancer growth and progression including cell growth and survival, differentiation, remodeling of extracellular matrix, invasion, angiogenesis, and metastasis (4, 10, 12).

Elucidation of each protease substrate repertoire is crucial to understand the biological roles of KLKs. Potential substrates for these proteases have been identified both in the extracellular space and at the plasma membrane. Members of the KLK family have been reported to hydrolyze components of the extracellular matrix such as collagens, laminin, fibronectin, and vitronectin (13, 14). Depending on the cellular context, this hydrolysis may promote or inhibit cell migration and invasion. Several lines of data indicate that KLKs may also regulate the bioavailability of hormones and growth factors. For instance, several KLKs (KLKs 4–6, 8, 13, and 14) were shown to cleave human growth hormone yielding to various inactive isoforms (15). Conversely, KLK3 was reported to activate latent TGF- β (16), and KLKs 1, 2, 3, 4, 11, and 14 were proposed to modify the bioavailability of insulin-like growth factors (IGFs) by fragmentation of IGF-binding proteins (IGFBP) (13, 14). Moreover, KLKs have been involved in regulation of cell-cell interactions through hydrolysis of desmosomal cadherins (17) and shedding of E-cadherin (18). Finally, it is currently known that KLKs regulate cell signaling via the protease-activated receptors (19).

KLK12 was originally cloned using the positional candidate gene approach (20). This gene has five coding exons and one 5'-untranslated exon with at least four alternatively spliced forms known to date (21). All spliced forms are predicted to

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^S The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Table S1.

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² The abbreviations used are: KLK, kallikrein-related peptidase; CCN, cyr61, ctgf, nov; IGF, insulin-like growth factor; IGFBP, IGF-binding protein domain; VWC, von Willebrand type C domain; TSP, thrombospondin; CT, cysteine knot; HUVEC, human umbilical vein endothelial cell; ACN, aceto-

nitrile; BMP, bone morphogenetic protein; ECM, extracellular matrix; NSCLC, nonsmall cell lung carcinoma; DTA, data tuning advisor; MMP, matrix metalloproteinase.

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produce secreted proteins; however, the “classical” form of KLK12 (GenBank accession number NM_145894) is the only protein product that represents a typical kallikrein-like enzyme with expected serine protease activity. The classical KLK12 in its mature form consists of 227 amino acids with a predicted mass of 24.5 kDa. Active KLK12 possesses trypsin-like activity, targeting peptide bonds featuring either arginine or lysine at their P1 position. The activity of KLK12 can be tightly regulated by autodegradation, by interaction with zinc ions, and by covalent complex formation with α 2-antiplasmin (22). RT-PCR and ELISA analyses has shown that *KLK12* is highly expressed in a variety of human tissues, such as bone and bone marrow, colon, lung and trachea, prostate, salivary glands, and stomach (1, 20).

Despite substantial progress in the understanding of the pathophysiological functions of KLKs, examination of the *in vivo* role of KLK12 has been minimal, and no biological substrate for this protease is known to date. We acknowledge that modification of cell surface proteins by plasma membrane and soluble proteases can be important for physiological and pathological processes. We therefore recruited a degradomic approach to identify substrates of KLK12 using a cell culture model system. Biochemical analysis of the potential substrates pointed to the matricellular proteins of the CCN family as targets of KLK12 and revealed that proteolytic processing of CCN1 and CCN5 mobilizes several growth factors bound to these proteins. Our findings suggest an involvement of KLK12 in different homeostatic and disease pathways through modulation of growth factor availability and/or activity.

EXPERIMENTAL PROCEDURES

Clinical Samples—Matched samples of tumor and nontumor tissue were obtained from 45 patients who had undergone lung cancer resection as their primary therapy without preoperative radiation or chemotherapy. Tumor and nontumor tissue samples were selected by a pathologist from each fresh surgical specimen, immediately frozen in liquid nitrogen, and stored at -80°C . The control nonmalignant tissue samples were taken from sites at least 3 cm away from the edge of the tumor. Histological diagnosis was performed, and tumor grade was determined in accordance with the WHO classification of lung tumors. The tissue specimens were banked with informed consent in compliance with the Helsinki Accord and French bioethical regulations.

Reagents—ProKLK12, proKLK1, and IGF-2 were from R & D Systems (Minneapolis, MN); CTGF (CCN2) and anti-TGF were from BioVendor GmbH (Heidelberg, Germany) and Clinisciences (Montrouge, France), respectively; whereas all other CCNs, the cytokines, and biotinylated antibodies were purchased from Pepro Tech Ec Ltd. (London, UK). Polyclonal antibody to pTyr996 VEGFR-2 was from Acris Antibodies (Herford, Germany). Recombinant proKLKs 5, 6, 11, 13, and 14 were obtained as described previously (23, 24). Human umbilical vein endothelial cells (HUVECs), endothelial cell growth medium, endothelial cell basal medium, and VEGF were purchased from PromoCell GmbH (Heidelberg, Germany). MDA-MB-231 (from breast cancer; ATCC number HTB-26) and A549 (from lung cancer; ATCC number CCL-185) cells were cultured in RPMI 1640 supplemented with 10% FBS. RPMI

1640, InvitrogenTM CD CHO medium, and FBS were from Invitrogen.

Proteome Analysis of Cell Culture Media—MDA-MB-231 cells were grown in RPMI 1640 supplemented with 10% FBS in culture flasks (surface area, 175 cm^2) until 80% confluency. The cells were extensively washed with PBS to remove extracellular proteins and incubated in 40 ml of CD CHO serum-free medium supplemented with 400 μl of KLK12 activation buffer containing 0.1 $\mu\text{g}/\mu\text{l}$ active KLK12 for 30 min at 37°C . Untreated control cells were similarly incubated in the absence of KLK12. Overlaying media were collected, and cell debris was removed by centrifugation. The media were freeze-dried, and the powdered samples were resuspended in 8 M urea. Protein disulfide bonds were reduced in the presence of 30 mM DTT and 150 mM NH_4HCO_3 at 50°C for 30 min and then alkylated with 125 mM iodoacetamide for 1 h in the dark at room temperature. The samples were then desalted using a NAP-5 column (GE Healthcare Life Sciences) and lyophilized to dryness. The proteins were digested overnight with sequence grade modified trypsin (Promega) (1:50) at 37°C and lyophilized. The peptides were reconstituted in 120 μl of mobile phase (0.26 M formic acid in 10% acetonitrile) and loaded directly onto a Poly-SULFOETHYL ATM column with a 2.0- μm pore size and a 5- μm diameter (The Nest Group Inc.). A 1-h fractionation was performed using HPLC with an Agilent 1100 system. A linear gradient was used with 0.26 M formic acid in 10% acetonitrile (ACN) as running buffer and 1 M ammonium formate as elution buffer. Forty fractions (200 μl) were collected and pooled into eight groups. Each of the eight fraction groups was concentrated to 100 μl using a SpeedVac system. The peptides in each fraction were purified with a ZipTipC₁₈ pipette tip (Millipore) and eluted in 4 μl buffer B (90% ACN, 0.1% formic acid, 10% H_2O , 0.02% TFA). The peptides were initially bound to a 2-cm C₁₈ precolumn with a 200- μm diameter and eluted onto a resolving 5-cm analytical C₁₈ column (75- μm diameter) with a 15-mm tip (New Objective). The liquid chromatography setup was connected to a Thermo LTQ Orbitrap XL mass spectrometer with a nanoelectrospray ionization source (Proxeon). Analysis of the eluted peptides was done by tandem mass spectrometry in positive-ion mode. A two-buffer system was utilized where Buffer A (running) contained 0.1% formic acid, 5% ACN, and 0.02% trifluoroacetic acid in water and Buffer B (elution) contained 90% ACN, 0.1% formic acid, and 0.02% trifluoroacetic acid in water. The MS1 scans were acquired in the Orbitrap at a resolution of 60,000; the MS2 scans were acquired in the Orbitrap at a resolution of 7,500. The Orbitrap was tuned and calibrated according to manufacturer's instructions. All data-dependent scan events had an isolation width set to 3.0. Data analysis DTAs were created using the Mascot Daemon v2.16 and extract_msn (Matrix Science, London, UK). The parameters for DTA creation were as follows: minimum mass, 300 Da; maximum mass, 4,000 Da; automatic precursor charge selection; minimum peaks, 10 per MS/MS scan for acquisition; and minimum scans per group, 1. Mascot (Matrix Science; version 2.1.03) was used to analyze the resulting Mascot Generic Files with a forward and reverse concatenated International Protein Index human database (version 3.62; _167894 entries). No enzyme was selected for the digestion enzyme to accommo-

date different proteolytic specificities, and searches were performed with fixed carbamidomethylation of cysteines and variable oxidation of methionine residues. A fragment tolerance of 0.1 Da and a parent tolerance of 7 ppm were considered. This operation resulted in eight DAT files for each enzyme-treated and control sample. Scaffold (version Scaffold-2_00_00, Proteome Software Inc., Portland, OR) was utilized to validate MS/MS-based peptide and protein identifications. Peptide thresholds of ion minus identity scores of greater than -7 and ion scores of greater than 10 for 2+ and 3+ ions were utilized. These settings provided one match to the reverse database and 71 matches to the forward database, resulting in a false positive rate of 1.4%. Each protein was assigned a cellular localization based on information from Swiss-Prot and Genome Ontology databases.

Substrate Cleavage Validation—proKLK12 (100 ng/ μ l) was autoactivated by incubation in TCNT buffer (0.1 M Tris-HCl, pH 8.0, 150 mM NaCl, 10 mM CaCl₂, 0.05% Tween 20) overnight at 37 °C. Substrate cleavages were performed in TCNT at 37 °C, and the reaction products were analyzed by 4–12% gradient SDS-polyacrylamide gel electrophoresis (NuPAGE; Invitrogen) and silver-stained using the Silver Xpress Silver staining kit (Invitrogen). The mass of cleavage fragments was determined by MALDI-TOF MS on a Voyager-DE STR biospectrometry work station. N-terminal Edman sequencing was used to identify the neo-N termini of cleavage products.

Surface Plasmon Resonance Spectroscopy—Surface plasmon resonance experiments were performed on BIAcore T100 (GE Healthcare). VEGF₁₆₅ was immobilized at 100–300 resonance units on a CM4 sensor chip (GE Healthcare), using standard amine coupling protocol according to the manufacturer's instructions. Insulin was used for control surface and was immobilized at similar levels. Binding analyses were carried out at a flow rate of 30 μ l min⁻¹ at 25 °C. Intact or KLK12-fragmented CCN1 and CCN5 that were diluted into the running buffer (10 mM Hepes, 150 mM NaCl, 0.05% (v/v) Tween 20) were injected over the control and VEGF surfaces for 180 s. Dissociation was studied for 300 s. Regeneration of the surfaces was performed with 100 mM H₃PO₄ for 40 s followed by two washes with 2 M NaCl for 60 s. The response of the control surface was subtracted from the responses obtained on the VEGF surfaces.

Solid Phase Binding Assays—96-well microtiter plates (black, MaxiSorp; Nunc) were coated overnight at 4 °C with 10 pmol of CCN1 (400 ng) or CCN5 (250 ng) or with blocking solution (casein 0.1% or Pierce superbloc reagent) control diluted in TBS. The plates were then washed three times with TBS containing 0.1% Tween 20, blocked for 4 h at room temperature, and washed three times with TBS-Tween 20. To form the complexes with VEGF, BMP2, TGF- β , or FGF-2, the coated plates were incubated overnight at 4 °C with 100 ng of the protein diluted in the blocking solution and then washed three times with TBS-Tween 20. The immobilized complexes were incubated 3 h at 37 °C with activated KLK12 (using an enzyme/CCN ratio of 1/100 or 1/25) diluted in 100 μ l TCNT. After digestion, the wells were washed three times with TBS-Tween 20, and bound ligands were quantified by ELISA. Biotinylated antibodies were diluted in TBS-Tween 20 and detected with alkaline phosphatase-conjugated streptavidin (Jackson Immuno-

Research Laboratories) and developed with 100 μ l of 4-Methylumbelliferyl phosphate solution (Sigma-Aldrich) according to standard procedures. Substrate formation corresponding to bound ligands was determined by measuring fluorescence at 440 nm using a fluorimeter (WALLAC Victor 2; PerkinElmer Life Sciences).

HUVEC Cell Proliferation—HUVECs (1×10^5 cells/well; third to fifth cell passage) were seeded in 96-well plates and grown for 72 h in endothelial cell growth medium supplemented with VEGF (20 ng/ml) alone or with VEGF preincubated overnight at 4 °C with intact or KLK12-fragmented CCN1 and CCN5 (0.5 μ g/ml). Endothelial cell proliferation was evaluated by MTS assay (Promega; France) as per the manufacturer's instructions.

Western Blotting—HUVECs (1×10^5 cells/ml) in endothelial cell growth medium were seeded on six-well plates. At 70% confluency, HUVECs were serum-deprived for 18 h. Subsequently, the addition of VEGF (10 or 40 ng/ml) alone or VEGF preincubated for 16 h at 4 °C with intact or KLK12-fragmented CCN1 and CCN5 (VEGF: CCN molar ratio of 1:3; 1:10, or 1:40) was performed. After 10 min of incubation at 37 °C, 5% CO₂, in basal medium, the cells were lysed in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM orthovanadate, 1 \times protease inhibitor cocktail (Sigma-Aldrich). The cell lysates were boiled for 7–10 min, electrophoretically separated on 4–12% Bis-Tris NuPAGE gels (Invitrogen) for 1.5 h at 150 V, and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore) for 3 h at 70 V. The membranes were blocked in 5% BSA, 0.1% Tween 20, PBS and incubated overnight at 4 °C with primary antibody against phosphor-VEGFR2 (1:500). SuperSignal® West Dura extended duration chemiluminescent substrate (Pierce) was used for detection. The stripped membranes were re probed with monoclonal β -actin antibody (1:2,000; Sigma-Aldrich) as an indicator of protein integrity and loading control.

Expression Analysis in Lung Cancer Patient of the CCN1, CCN5, and KLK12 Genes—Total RNA was extracted using the RNeasy kit (Qiagen) and used for cDNA synthesis as described previously (25). The KLK12 (forward primer, 5'-CCCCTGCCCAATGACTGTG-3'; reverse primer, 5'-ATGTTGCTCGTGATTCTCCCG-3'; PrimerBank identifier 22208987a3*), CCN1 (forward primer, 5'-GTG ACGAGGATAGTATCAAGGACC-3'; reverse primer, 5'-ATTTCTGGCCTTGTAAGGGTTG-3'), and CCN5 (forward primer, 5'-CACGCTGCCTGGTCTGTCTGGATC-3'; reverse primer, 5'-CACGCATAGGCTTGTATTCAGGAAC-3') primer sets were designed to target two exons. For the quantitative RT-PCR studies, 1 μ l of cDNA was used in a 19- μ l PCR mix containing 1 \times SYBR Premix Ex Taq (Takara) and 0.2 μ M of each primer. Each assay included three no-template controls, cDNA samples in duplicate and calibrator (pool of cDNAs from tumor and nontumor lung tissue samples) in triplicate. Amplification was performed in a Light Cycler 480 (Roche Applied Science) with denaturation for 30 s at 95 °C followed by 45 PCR cycles of denaturation at 95 °C for 5 s and annealing or extension at 60 °C for 20 s. The Light Cycler 480 system software was used to monitor the changes in fluorescence of SYBR Green I dye in every cycle; fluorescent KLK12 product was measured by a single acquisition mode for 15 s at

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84 °C after each cycle. The specificity of the PCR reactions was established through both melting curve and electrophoresis gel analyses. *18 S rRNA* was used for the normalization of the quantity of RNA used. Its C_T value was subtracted from that of the target gene to obtain a ΔC_T value. The difference ($\Delta\Delta C_T$) between the ΔC_T values of the samples and the ΔC_T value of the calibrator was determined. The relative quantitative value was expressed as $2^{-\Delta\Delta C_T}$, representing the amount of target gene expression (normalized to *18 S rRNA*) relatively to the calibrator.

Wound Healing—A549 cells were seeded in 6-well plates and grown in complete medium to 80% confluency before disrupting the cell monolayer by scratching with a 100- μ l sterile tip. Floating cells were washed twice with serum-free medium, and the cells were grown for 24 h in 0.5% FBS medium containing intact or KLK12-fragmented CCNs (1 μ g/ml) prior to migration analysis. The pictures were obtained from the scratching sites, and cell migration analysis was performed with Image J NIH free software.

Endothelial Cell Viability Assay—HUVECs (10,000 cells/well; third to fifth cell passage) were seeded in 96-well plates and grown for 24 h in endothelial cell growth medium. The cells were washed once in endothelial basal medium and cultured for 24 h in 100 μ l of basal medium supplemented with intact or KLK12-fragmented CCNs (0.5, 1, or 2 μ g/ml) or with VEGF (20 ng/ml) pretreated or not with KLK12. Endothelial cell survival was evaluated by MTS assay (Promega, France).

Statistical Analysis—The measured values are expressed as medians \pm quartiles. In the mobilization assay, the difference between KLK12-treated or untreated complexes was analyzed by the Wilcoxon-Mann Whitney test used for unpaired samples. The wound healing and viability assays were analyzed using the Kruskal and Wallis test. The difference between the amounts of transcripts in noncancerous and cancerous tissues was determined using a Wilcoxon matched pairs test. *p* values less than or equal to 0.05 were considered significant.

RESULTS

Identification of CCN1 in Overlying Medium of Cells Treated with KLK12—To screen for KLK12 substrates located at the cell surface, MDA-MB-231 cells were treated either with KLK12 or vehicle control in serum-free medium. After 30 min of treatment, the overlying media were collected, and the protein content was analyzed by mass spectrometry. Seventy different proteins (supplementary data) were identified in the overlying media using high stringency peptide confidence levels (>99%). Among them, 34 proteins were unique to the medium from KLK12-treated cells. Each protein was assigned a cellular localization based on information available from Swiss-Prot, Genome Ontology, or Human Protein Reference database. The largest number of protein identities (32 of 34) corresponded to cytoplasmic and nuclear proteins, indicating that cell death/lysis had occurred during the incubation time. This high proportion of intracellular proteins may be explained by the fact that even small numbers of lysed cells can contribute to a large proportion of proteins in the conditioned medium, whereas the short incubation time is at the same time restrictive for high secreted protein expression. These intracellular proteins do not constitute substrates of a secreted/extracellular protease like

KLK12 and were thus excluded from further analysis. The remaining identities were assigned to two plasma membrane-associated proteins, Annexin A2 (Swiss-Prot P07355) and Cyr61 (CCN1, Swiss-Prot Q00622).

Annexin A2 is one of a dozen annexin family members expressed in humans. The annexins are mainly intracellular, and most play important roles in membrane trafficking, plasma membrane reorganization during signaling, and calcium regulation. Annexin A2 can be directed to membrane surfaces where it binds viruses, bacteria, and several proteins like plasminogen, tissue plasminogen activator, and factor Xa (26).

Cyr61 (CCN1) is a cysteine-rich protein belonging to the CCN family of six related members designated CCN1–CCN6. These members have emerged as extracellular plasma membrane- and matrix-associated proteins that play critical roles in cardiovascular and skeletal development, fibrotic diseases, and cancer. CCNs are typically comprised of four conserved cysteine-rich modular domains that directly interact with integrin receptors, growth factors, and cytokines. The archetypal structure of a CCN protein (see Fig. 2A) consists of an N-terminal secretory signal peptide followed by an IGFBP, a von Willibrand factor type C repeat module (VWC), a thrombospondin type 1 repeat module (TSP), and a cysteine knot-containing module (CT) (27). CCN5 is devoid of the CT module. The N- and C-terminal halves of the proteins are connected by a hinge region varying greatly in length and sequence.

Validation of CCN Proteins as Substrates for KLK12—Because of the well known extracellular localization of CCNs and their emerging role in human diseases, we elected to focus our further analyses on this family. To confirm the cleavage of CCN1 by KLK12, a time course digestion of the recombinant form was carried out and monitored by SDS-PAGE and silver staining. A nearly complete digestion of CCN1 was observed after a 30-min incubation at an enzyme:substrate ratio of 1:100 (w/w) (Fig. 1A). Fragmentation of CCN1 by KLK12 was still visible at a lower enzyme:substrate ratio of 1:1,000 (w/w) (data not shown). Two main fragments were generated and further identified by mass spectrometry (MALDI-TOF): the longer fragment (20,495 Da) corresponded to the N-terminal part, and the shorter fragment (19,378 Da) corresponded to the C-terminal part of CCN1. It should be noted that the apparent masses (Fig. 1A) are greater than those determined by mass spectrometry, possibly indicating aberrant migration of the peptides in gel. Next, we investigated whether KLK12 can cleave other members of the CCN family. As shown in Fig. 1, all of the recombinant CCNs were fragmented following incubation with KLK12. Among the six recombinant CCNs examined, CCN1 was hydrolyzed most efficiently. Interestingly, the fragmentation pattern was unique for each member with a complexity varying from one visible product for CCN5 (Fig. 1E) to nine for CCN4 (Fig. 1D).

N-terminal sequencing analyses of the CCN1 and CCN5 proteolytic fragments generated by KLK12 identified several cleavage sites (Fig. 2B). The major cleavage site in CCN1 was found in the hinge region between the VWC and TSP domains, whereas the major cleavage site identified in CCN5 is located in the TSP domain (Fig. 2B). This domain corresponds to the binding site of integrin $\alpha 6\beta 1$ (28). In CCN1, the Lys⁸⁸–Gly⁸⁹

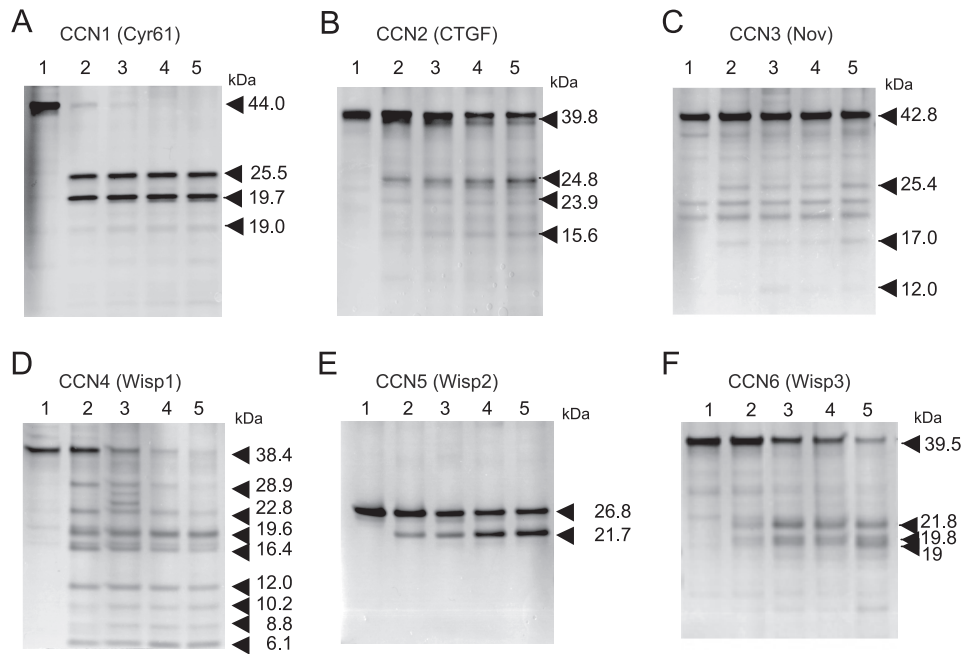


FIGURE 1. **KLK12 proteolytic processing of the six members of the CCN family.** Time course analysis of KLK12 hydrolysis of recombinant CCNs on silver-stained 4–12% SDS-PAGE gels is shown. CCNs were incubated with KLK12 (enzyme:substrate ratio of 1:100, w/w) for 0 (lanes 1), 15 (lanes 2), 30 (lanes 3), 60 (lanes 4), and 120 (lanes 5) min. The arrows indicate full-length CCNs and KLK12-cleaved protein fragments and their apparent molecular masses. A, CCN1. B, CCN2. C, CCN3. D, CCN4. E, CCN5. F, CCN6.

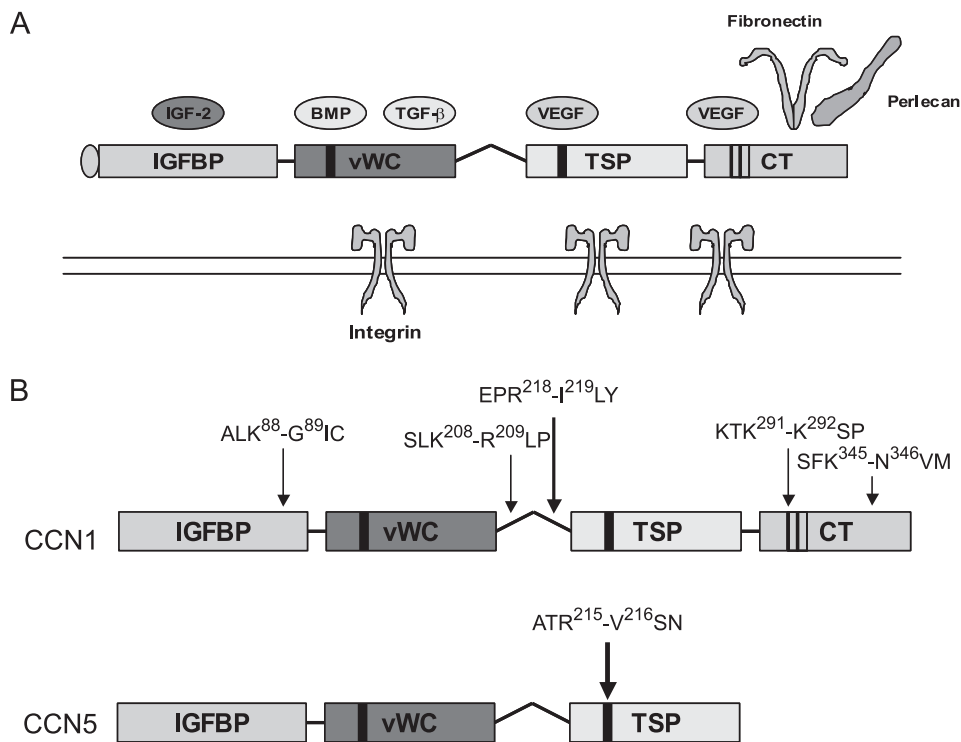


FIGURE 2. **Interaction of CCN proteins with other molecules and localization of the cleavage sites by KLK12.** A, schematics of CCN protein structure and localization of interactions with other molecules. B, localization of the cleavage sites by KLK12. The main cleavage site is indicated in bold arrows.

bond, which was secondarily hydrolyzed by KLK12, is located at the end of the IGFBP domain in a flexible region separating this area from the vWC domain (29). This site is absent in CCN5. A secondary cleavage also occurred at the Lys²⁹¹–Lys²⁹² bond in the α 6 β 1 binding site contained in the CT domain of CCN1 (28).

Processing of CCN Proteins by other KLKs in Vitro—To screen for potential processing of all CCNs by representative KLKs, recombinant KLKs 1, 5, 6, 11, 13, and 14 were incubated *in vitro* with CCN1–CCN6 at an enzyme:substrate ratio of 1:100 (w/w). Of these KLKs, KLK14 had the broadest activity, either cleaving or degrading all CCNs (Fig. 3). KLK14 appeared to almost

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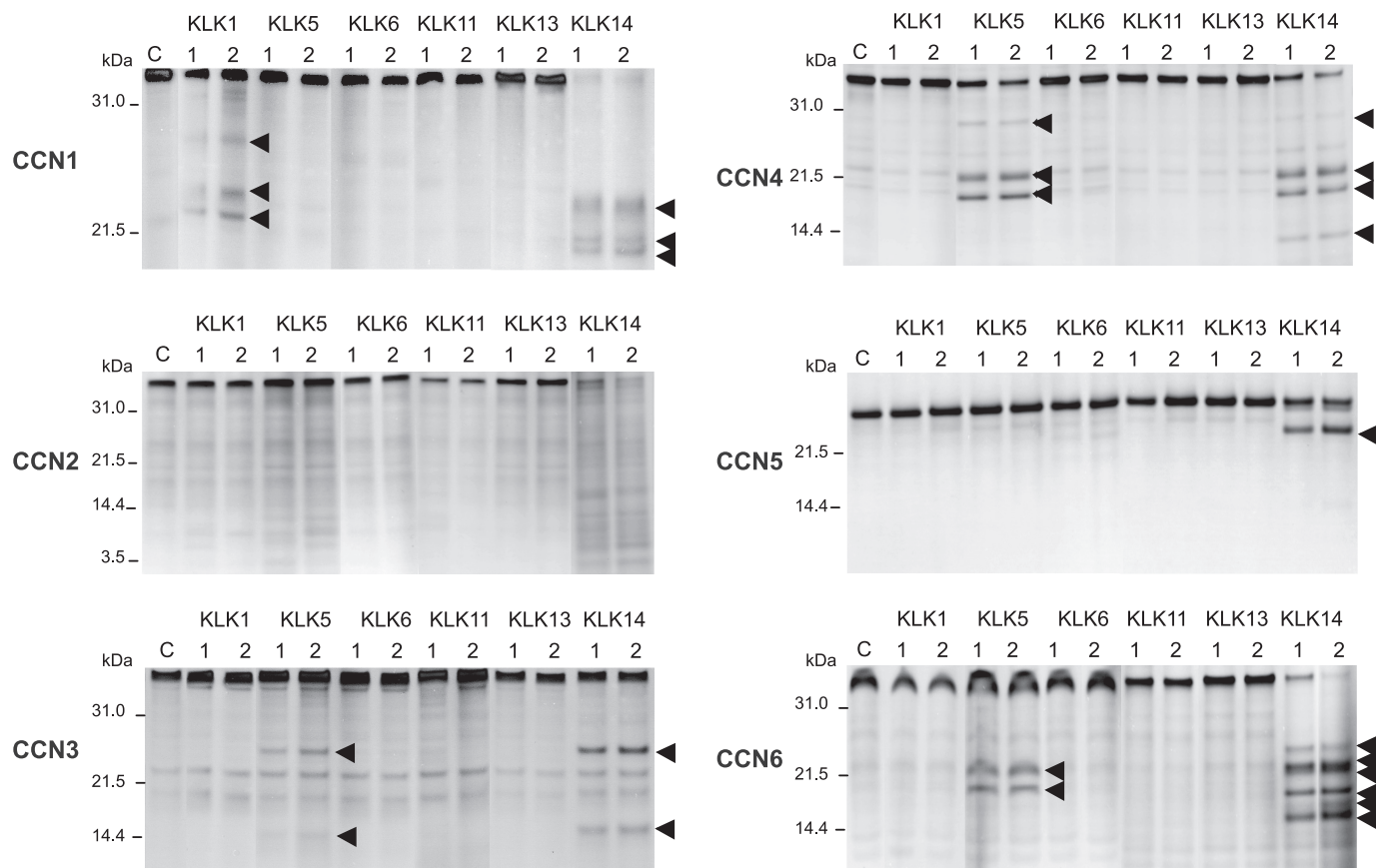


FIGURE 3. **Proteolytic processing of CCNs by different KLKs.** Analysis of KLKs 1, 5, 6, 11, 13, and 14 hydrolysis of recombinant CCNs on silver-stained 4–12% SDS-PAGE gels is shown. CCNs were incubated with the KLKs (enzyme:substrate ratio of 1:100, w/w) for 1 (lanes 1) and 2 h (lanes 2). The arrows indicate KLK-generated protein fragments.

entirely degrade CCN2 in small fragments ranging from 3 to 14 kDa. Except from CCN5, the CCN fragmentation patterns generated by KLK14 were substantially different from those generated by KLK12. KLK5 processed CCN3, CCN4, and CCN6, whereas KLK1 only cleaved CCN1 in a manner different from that of KLK12 and KLK14. Under our assay conditions, we did not detect cleavage of CCNs by KLKs 6, 11, and 13. This points to a distinct specificity of each KLK for CCN cleavage regardless of their common preference for proteolytic targeting of sites following basic residues.

Cleavage of CCN1 or CCN5 Complexes Mobilizes Functional VEGF—Previous studies have showed that CCN proteins have physical interactions with growth factors and modulate their bioavailability and/or their activity. We therefore examined the impact of KLK12 proteolytic activity on the CCN-growth factor interactions. Using both the technique of surface plasmon resonance (Fig. 4, A and B) and a solid phase assay (Fig. 4E), we observed a VEGF₁₆₅ binding to intact CCN1 and CCN5, whereas this interaction was inhibited when CCNs were previously fragmented by KLK12 (Fig. 4, A and B).

Growth factors that bind to CCNs might either mask or stabilize the cleavage sites of CCN1 or CCN5, thus preventing their proteolytic degradation. Therefore, we investigated whether the VEGF-complexed CCNs can be equally fragmented by KLK12. SDS-PAGE analysis showed that identical patterns of cleavage were observed for CCN1 and CCN5 regardless of being coupled with VEGF₁₆₅ (Fig. 4, C and D).

VEGF₁₆₅ is known to be susceptible to degradation by several serine proteases including plasmin, trypsin, chymotrypsin, and elastase (30, 31). However, this growth factor was resistant to the proteolytic action of KLK12 in our settings (Fig. 4, C and D) and was released from CCN1 or CCN5 complexes immobilized to solid matrices upon incubation with the protease (Fig. 4E). Furthermore, VEGF₁₆₅ retained its bioactivity on HUVECs after treatment with KLK12 (Fig. 4F). Subsequently, we examined whether VEGF activity was modified in the presence of intact or KLK12-fragmented CCN1 and CCN5. Treatment of HUVEC with soluble VEGF-CCN1 complexes stimulated both VEGF receptor phosphorylation (Fig. 5A) and cell proliferation (Fig. 5C), as did free VEGF alone or VEGF plus fragmented CCN1. Similar results were obtained with CCN5 (Fig. 5, B and D). Phosphorylation of VEGFR2 was observed even with a large excess of intact CCN (VEGF:CCN ratio, 1:40; data not shown), suggesting that residual-free VEGF is not responsible for stimulation of VEGFR2.

KLK12 Processing of CCN1 or CCN5 Complexes Mobilizes Intact BMP2—It has been shown that CCN2, CCN3, and CCN6 can physically interact with bone morphogenetic proteins (BMP) and inhibit their activity, likely by preventing their binding to BMP receptors (32–34). By contrast, BMP2 binding to CCN4 was reported to enhance BMP2 signaling (35). In this study, we examined whether CCN1 and CCN5 can interact with BMP2 and whether the hydrolysis of these proteins by KLK12 can alter their interactions. Using a solid

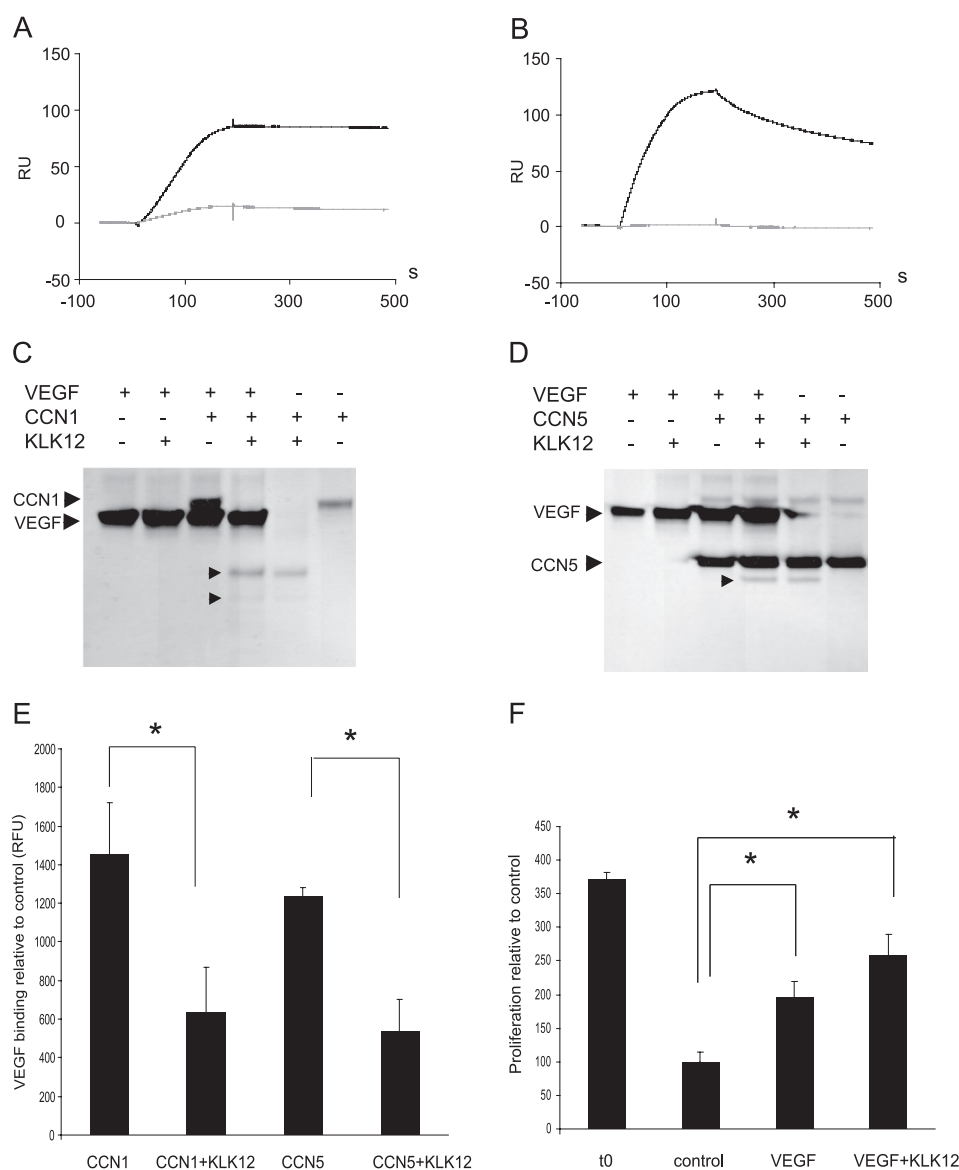


FIGURE 4. KLK12 prevents VEGF₁₆₅ binding to CCN5 and mobilizes intact VEGF from VEGF-CCN complexes. *A* and *B*, sensorgrams of surface plasmon resonance analysis showing the binding of 12.5 nM intact (black curve) or fragmented (gray curve) CCN1 (*A*) or CCN5 (*B*) with immobilized VEGF. *RU*, resonance units. *C* and *D*, CCN1 or CCN5 complexes with VEGF were formed by incubation for 12 h at 4 °C and then incubated with KLK12 for 3 h at 37 °C. VEGF, CCN1, and CCN5 were also incubated with KLK12 for 3 h at 37 °C. The resulting proteolytic fragments were analyzed by 4–12% SDS-PAGE and silver-stained. The arrows indicate full-length and cleaved protein fragments. *E*, CCN1 or CCN5 was complexed with VEGF on a microtiter plate for 12 h at 4 °C and prior to a 3-h incubation at 37 °C with activated KLK12 (KLK12:CCN ratio of 1:100, w/w). VEGF ELISA was used to quantify the residual VEGF bound to CCN1 or CCN5 after KLK12 cleavage. *RFU*, relative fluorescence units. *F*, HUVECs were adhered on tissue culture dishes in complete medium (supplemented with serum) and then cultured with VEGF or KLK12-processed VEGF (20 ng/ml) in basal medium (in the absence of serum). Cell viability was measured at the time of medium change (*t*₀) and after 24 h of treatment using a MTS assay. *, Kruskal and Wallis test values ($p < 0.05$, four independent experiments).

phase assay, we observed binding of BMP2 to CCN1 but not to CCN5 (Fig. 6*A*). Treatment of either soluble or immobilized CCN1-BMP complexes resulted in the release of intact BMP2 (Fig. 6, *A* and *B*).

TGF- β and FGF-2 Are Relatively Sensitive to KLK12 Hydrolysis—CCN2 has been reported to bind TGF- β 1 through the VWC domain and subsequently enhances the binding of TGF- β 1 to all three TGF- β receptors (32). In the current study, we observed a binding of TGF- β 1 to intact CCN1 and CCN5 that were immobilized to solid matrices (Fig. 6*C*). In the case of both CCNs, treatment of the CCN-TGF- β complexes with KLK12 resulted in a reduction of the amount of immobilized TGF- β 1 (Fig. 6*C*). As shown in Fig. 6*D*, soluble TGF- β was

susceptible to proteolysis by KLK12 at a low enzyme:substrate ratio (1:25 w/w) but appeared resistant to hydrolysis at a higher ratio (1:100 w/w). This was also the case when TGF- β was engaged in complexes with CCN proteins (Fig. 6, *E* and *F*). Interestingly, at high enzyme concentration, bound TGF- β was more sensitive to KLK12 hydrolysis than free TGF- β 1 (Fig. 6*F*). Interaction of TGF- β 1 with either CCN1 or CCN5 did not protect these proteins from KLK12 hydrolysis because identical patterns of cleavage were observed for CCN1 and CCN5 regardless of their binding to TGF- β 1 (Fig. 6, *E* and *F*).

It has been previously shown that CCN1 enhances FGF-2-induced DNA synthesis in HUVECs likely because of FGF-2 displacement from extracellular matrix (36). To determine

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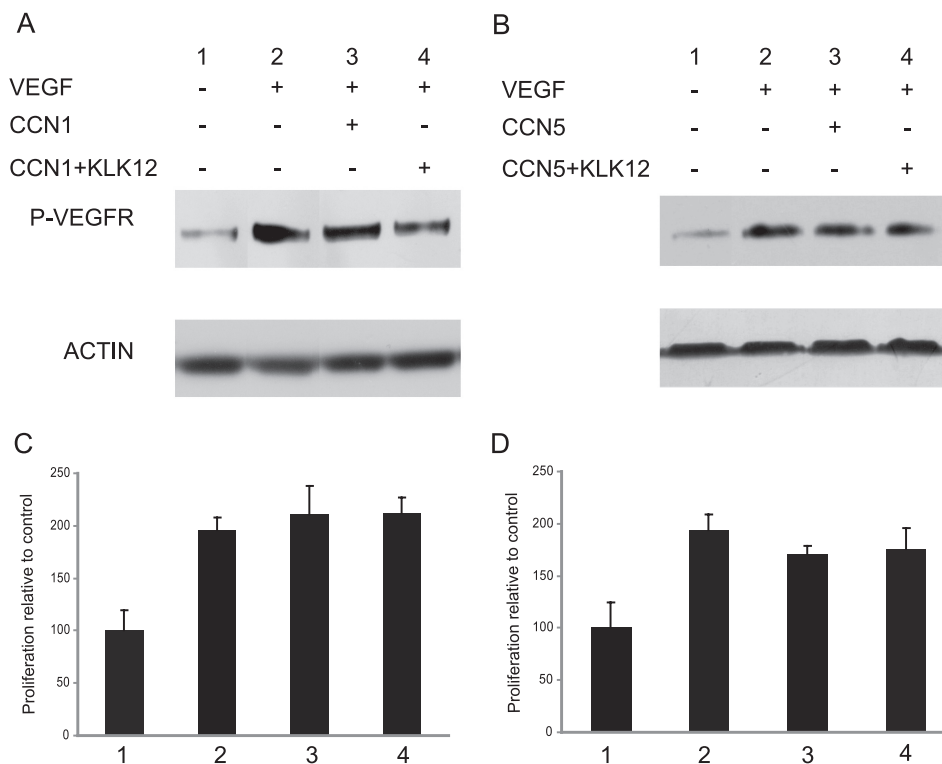


FIGURE 5. Effects of intact or fragmented soluble CCNs on the VEGF-induced responses of endothelial cells. *A* and *B*, phosphorylation of VEGFR2 in response to VEGF. A representative Western blot is shown for untreated HUVEC, HUVEC treated with VEGF₁₆₅ alone, or HUVEC treated with VEGF₁₆₅ preincubated with intact or KLK12-fragmented CCN1 (*A*) and CCN5 (*B*) (VEGF:CCN molar ratio 1:3). Stripped membranes were reprobed with monoclonal β -actin antibody to confirm protein loading levels and integrity. *C* and *D*, proliferation of HUVECs in response to VEGF. HUVECs were cultured for 72 h in endothelial cell growth medium without additive (*lanes 1*), with VEGF₁₆₅ alone (20 ng/ml; *lanes 2*), with VEGF₁₆₅ preincubated with intact (*lanes 3*), or with KLK12-fragmented (*lanes 4*) CCN1 (*C*) or CCN5 (*D*) (VEGF:CCN molar ratio of 1:3). The data were obtained from three independent experiments.

whether CCN1 and CCN5 can directly interact with FGF-2, CCN proteins were immobilized to solid matrices and incubated with soluble FGF-2. Formation of FGF-CCN complexes was observed in the presence of immobilized CCN1 and CCN5 (Fig. 7A). FGF-2 was shown to be sensitive to trypsin (37); therefore, we analyzed the effects of KLK12 on the integrity of FGF. As shown in Fig. 7B, soluble FGF-2 was processed by KLK12 at a low enzyme:substrate ratio (1:25 w/w) but appeared relatively resistant to hydrolysis at a higher ratio (1:100, w/w) (Fig. 7B). FGF-2 is often associated with heparan-sulfate proteoglycans in the extracellular matrix and binding to heparin protects FGF-2 from tryptic cleavage (37). We therefore examined the impact of heparin on the FGF-2 hydrolysis by KLK12. We observed that heparin can protect FGF-2 from KLK12 cleavage (Fig. 7C). This protective effect was fully abolished when the interaction of FGF-2 with heparin was prevented by heat denaturation of the FGF molecule (Fig. 7D). These observations indicate that the protective effect of heparin depends on its interaction with FGF-2 and not with the protease. Next, we investigated whether FGF-2 binding to CCN1 or CCN5 protects the growth factor from degradation by KLK12. FGF-2 was cleaved by KLK12 using an enzyme:substrate ratio of 1:25 (w/w) either when it was incubated alone with KLK12 or when it was coupled with CCN1 (not shown) or CCN5 (Fig. 7E). KLK12 treatment of FGF-CCN complexes using an enzyme:substrate ratio of 1:100 (w/w), which leaves FGF-2 intact, reduced the amount of FGF-2 bound to CCN5, whereas FGF-2 binding to CCN1 remained unchanged (Fig. 7A). However, as observed in Fig. 7F, FGF-2-

complexed CCN1 was fragmented by KLK12. At the same time, we detected a binding of FGF-2 to CCN1 previously fragmented by KLK12 (Fig. 7A). Taken together, these findings indicate that CCN1 fragments conserve the binding capacity to FGF-2 regardless of proteolysis by KLK12. Further experiments are required to identify the FGF-2 binding site(s) of the CCN proteins.

Gene Expression of KLK12, CCN1, and CCN5 in Paired Normal and Cancerous Tissue Samples from Patients with Lung Cancer—In a preliminary effort to assess the relevance of the newly identified enzyme-substrate pair (KLK12-CCNs), we determined the expression patterns of the genes encoding KLK12, CCN1, and CCN5 in the lung, a tissue known to express KLK12 (1). We applied the comparative CT quantification ($\Delta\Delta C_T$ method) of quantitative RT-PCR for comparing changes in expression of the three genes between nontumor and tumor lung specimens. Relative quantification was performed using *18 S rRNA* as an endogenous control gene. Our analysis revealed significant down-regulation of both *CCN1* and *CCN5* in tumor compared with nontumor tissue samples (Fig. 8A). Conversely, *KLK12* expression was increased in lung tumor.

Biological Role of KLK12-derived CCN Fragments in Cell Culture Models—CCN proteins regulate diverse cellular behavior through integrins, heparan sulfate proteoglycans, and lipoprotein receptor-related proteins (Fig. 2) pathways (27, 38). In the present study, we examined the effects of CCN1 and CCN5 fragmentation by KLK12 on cells of the tumor microenviron-

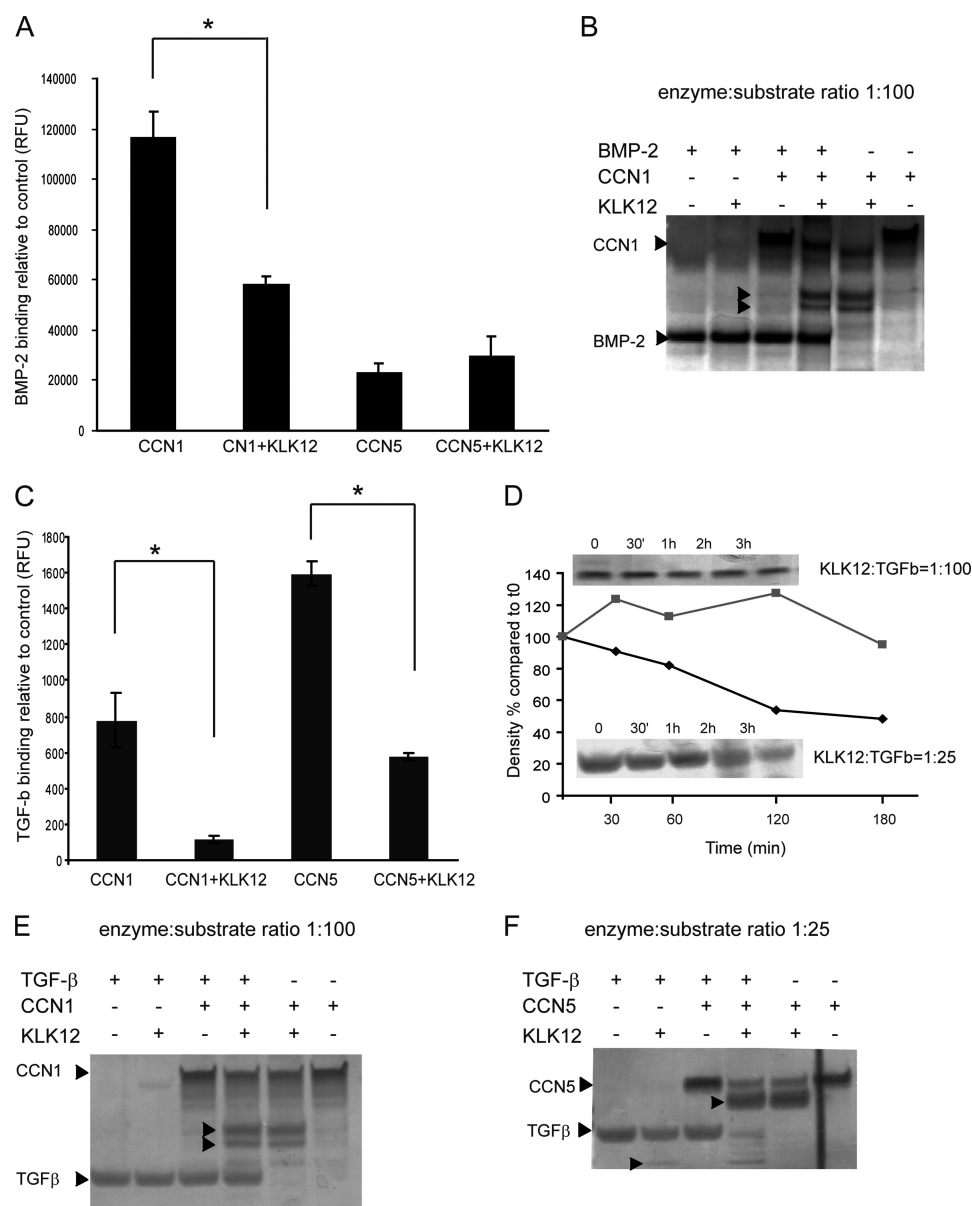


FIGURE 6. Cleavage of BMP2 or TGF- β 1-CCN complexes by KLK12. *A*, CCN1 or CCN5 was complexed with BMP2 on a microtiter plate for 12 h at 4 °C and prior to a 3-h incubation at 37 °C with activated KLK12 (KLK2:CCN ratio of 1:100, w/w). BMP2 ELISA was used to quantify the residual BMP2 bound to CCN1 or CCN5 after KLK12 cleavage. *B*, CCN1 was complexed with BMP2 for 12 h at 4 °C and then incubated with KLK12 for 3 h at 37 °C. BMP2 and CCN1 were also incubated with KLK12 for 3 h at 37 °C. The resulting cleavage fragments were analyzed by 4–12% SDS-PAGE and silver-stained. The *arrows* indicate proteolytically generated CCN1 fragments. *C*, CCN1 or CCN5 was complexed with TGF- β 1 on a microtiter plate for 12 h at 4 °C and prior to a 3-h incubation at 37 °C with activated KLK12 (KLK2:CCN ratio of 1:100, w/w). TGF- β 1 ELISA was used to quantify the residual TGF- β 1 bound to CCN1 or CCN5 after KLK12 cleavage. *D*, TGF- β 1 was incubated with KLK12 (enzyme:substrate ratios of 1:25 and 1:100, w/w) for 0, 30, 60, 120, and 180 min at 37 °C. The residual TGF- β 1 was analyzed by 4–12% SDS-PAGE and quantified by densitometry after silver staining. *E* and *F*, CCN1 or CCN5 complexes with TGF- β 1 were formed by incubation for 12 h at 4 °C and then incubated with KLK12 for 3 h at 37 °C. TGF- β 1, CCN1, and CCN5 were also incubated with KLK12 for 3 h at 37 °C (enzyme:substrate ratio of 1:100 w/w for *E* and 1:25 w/w for *F*). The resulting proteolytic fragments were analyzed by 4–12% SDS-PAGE and silver-stained. The *arrows* indicate molecular species corresponding to fragmented proteins.

ment, including neoplastic and endothelial cells (Fig. 8). First, we investigated the impact of CCN hydrolysis on migration of lung cancer cells using a wound healing assay. Intact CCN1 significantly decreased A549 cell migration, whereas KLK12-fragmented CCN1 was inefficient to do so (Fig. 8*B*). Intact or KLK12-fragmented CCN5 did not affect migration of A549 cells in our experimental conditions (Fig. 8*C*).

Subsequently, we analyzed the effects of CCN fragmentation on endothelial cell survival. Unstimulated, early passage HUVECs were adhered on tissue culture dishes before the addition of solu-

ble intact or KLK12-cleaved CCNs. Cell viability was measured after 24 h of serum withdrawal. As shown in Fig. 8 (*E* and *F*), cells were partially protected from death upon serum withdrawal in the presence of VEGF but not in the presence of intact CCN1 or CCN5. This observation is consistent with previous data showing that integrin $\alpha v \beta 3$ is required for CCN1-mediated endothelial cell survival and that this integrin is poorly expressed in unstimulated HUVECs (39). By contrast KLK12-fragmented CCN1 and CCN5 significantly reduced HUVEC survival (Fig. 8, *E* and *F*). All of these data suggest that KLK12 fragmentation of CCNs may modify the

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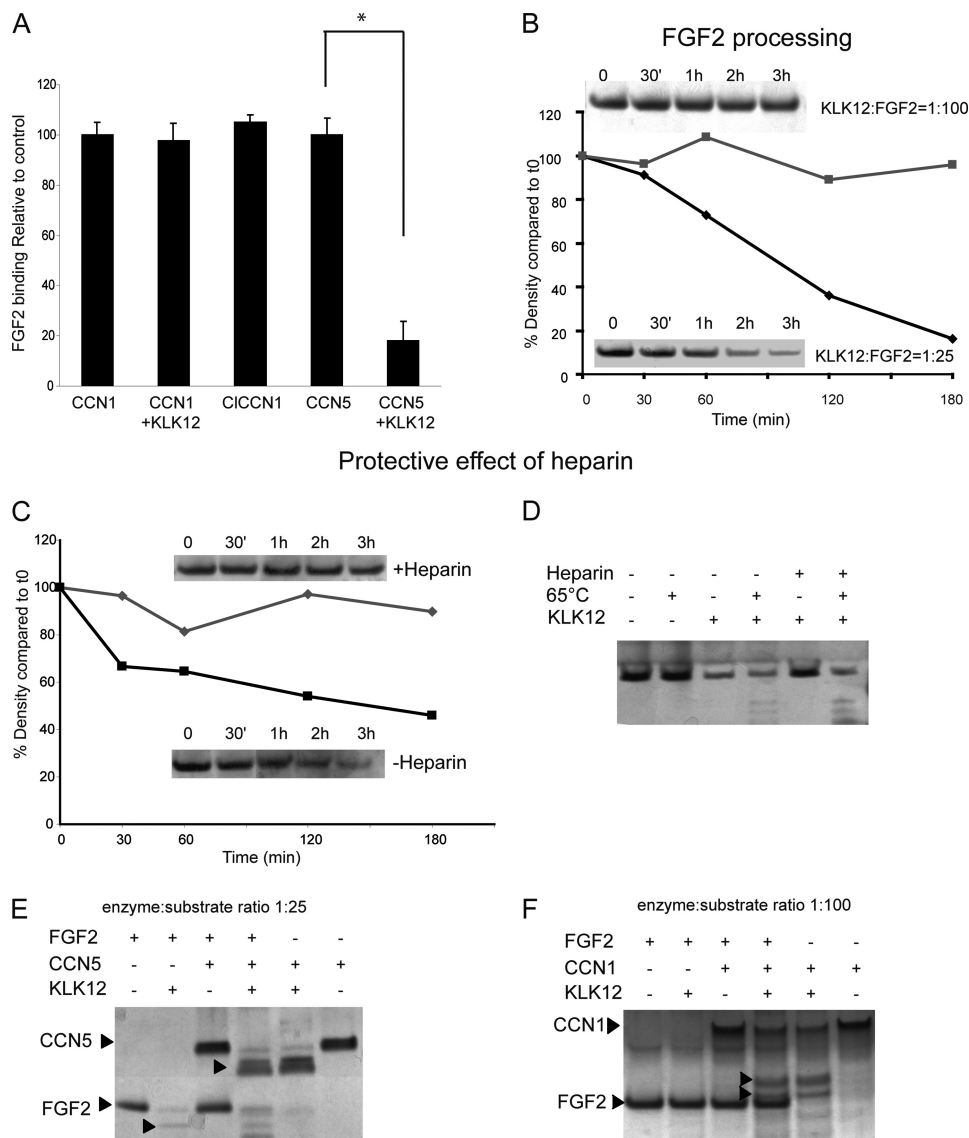


FIGURE 7. KLK12 effects on free FGF-2 or on FGF-2 complexed with CCN1 and CCN5. *A*, intact CCN1 or CCN5 was complexed with FGF-2 on a microtiter plate for 12 h at 4 °C and prior to a 3-h incubation at 37 °C with activated KLK12 (KLK2:CCN ratio of 1:100, w/w). CCN1 cleaved in solution (*C/CCN1*) was also coated on the plate and complexed with FGF-2 in the same conditions. FGF-2 ELISA was used to quantify the residual FGF-2 bound to CCN1 or CCN5 after KLK12 cleavage. *RFU*, relative fluorescence units. *B*, FGF-2 was incubated with KLK12 (enzyme:substrate ratios of 1:25 and 1:100, w/w) for 0, 30, 60, 120, and 180 min at 37 °C. The residual FGF-2 was analyzed by 4–12% SDS-PAGE and quantified by densitometry after silver staining. *C*, FGF-2 was incubated with KLK12 at an enzyme:substrate ratio of 1:25, w/w for 0, 30, 60, 120, and 180 min at 37 °C in the presence or absence of heparin. The residual FGF-2 was analyzed by 4–12% SDS-PAGE and quantified by densitometry after silver staining. *D*, free FGF-2 was treated or not at 65 °C for 5 min prior to a 3-h incubation with activated KLK12 in the presence of heparin. *E* and *F*, CCN1 or CCN5 complexes with FGF-2 were formed by incubation for 12 h at 4 °C and then incubated with KLK12 for 3 h at 37 °C. FGF-2, CCN1, and CCN5 were also incubated with KLK12 for 3 h at 37 °C. The resulting proteolytic fragments were analyzed by 4–12% SDS-PAGE and silver-stained. The *arrows* indicate molecular mass species corresponding to fragmented proteins.

cellular functions of these matricellular proteins in a cell type- and context-specific fashion.

DISCUSSION

This study suggests that the matricellular proteins belonging to the CCN family are novel substrates of kallikrein-related peptidases. Fragmentation of CCN1 by KLK12 was demonstrated both *in vitro* and in functional assays using cultured cells. Interestingly, the fragmentation pattern was unique for each CCN member and varied according to the KLK used. This could be explained by the extended but unique substrate specificity of the KLKs (23, 40) associated with the sequence and structural variability of the CCN proteins (27, 29). For example, the hinge region of CCN5 was not

targeted by KLK12 because of the absence of the arginine and lysine residues that are required at the P1 position for hydrolysis by KLK12 (22). The R-V scissile bond cleaved by KLK12 in CCN5 is conserved in the TSP domain of all CCN proteins; however, the corresponding Arg²⁵⁰–Val²⁵¹ bond in CCN1 did not consist a target of KLK12. This indicates that the efficiency of cleavage primarily depends on the accessibility of the scissile bond and the residues that surround the cleavage site regardless of the presence of conserved basic residues at the P1 position. Our findings also demonstrate that proteolysis of CCNs can potentially affect both flexible linkers connecting structural domains and exposed functional sites in these domains.

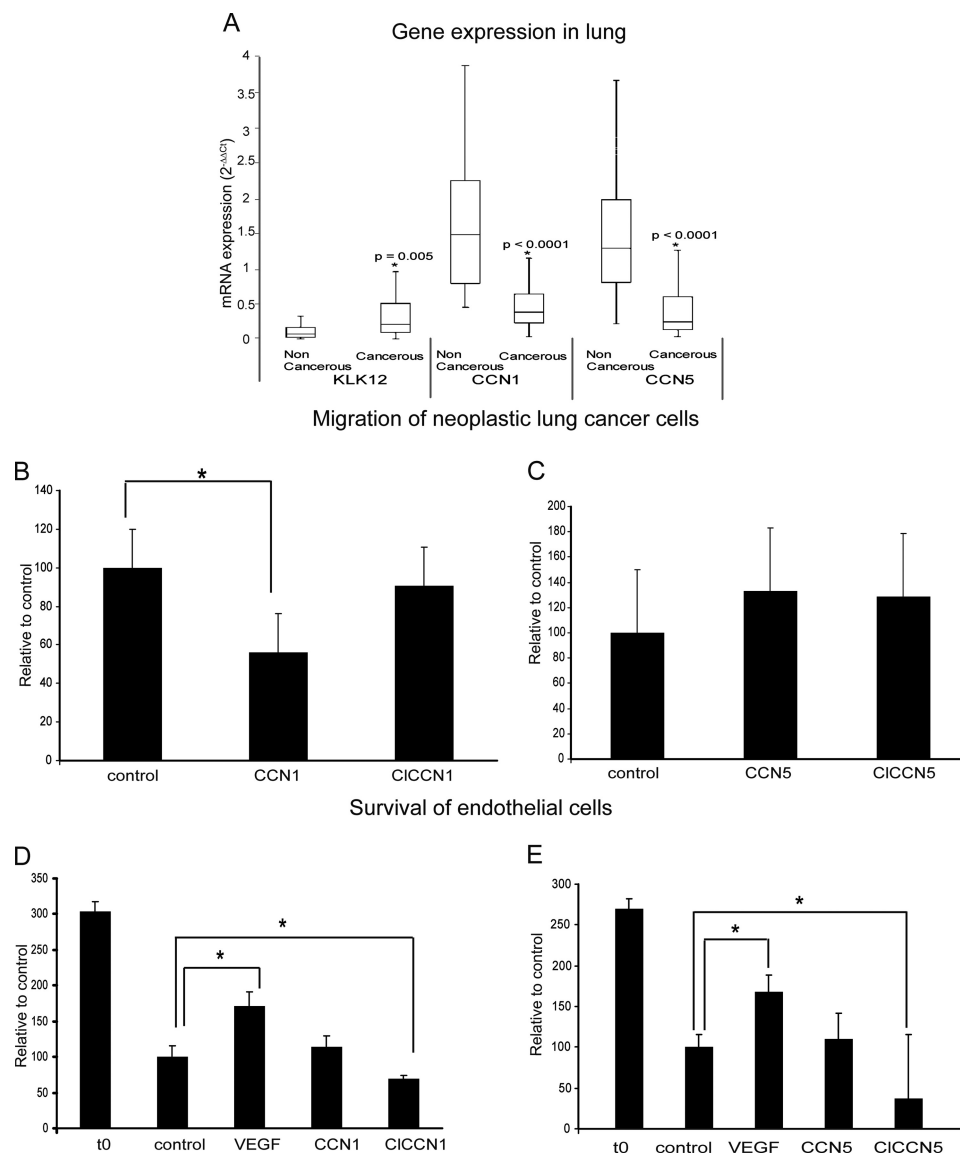


FIGURE 8. Gene expression in patients with lung cancer and biological role of KLK12-derived CCN fragments in cell culture models. *A*, box and whisker plots of quantitative real time RT-PCR assays of KLK2, CCN1, and CCN5 transcripts in 43 noncancerous and matched cancerous samples from patients with primary NSCLC. The relative quantitative value was expressed as $2^{-\Delta\Delta C_T}$, representing the amount of target gene expression (normalized to *18S rRNA*) relatively to the calibrator. The *box* refers to the range defined by the first and third quartile. The *thick line* refers to the range defined by the 10th and 90th percentile. The difference between the amounts of transcripts in noncancerous and cancerous tissues was determined using a Wilcoxon matched pairs test. *B*, wound healing analysis of A549 cells migration in the presence of 1 μ g/ml intact CCN1 or CCN5 alone or previously treated with KLK12. The *bars* represent the means \pm S.D. of three independent experiments. *, relative to control, $p < 0.05$, Kruskal and Wallis test. *D* and *E*, MTS analysis of HUVEC survival after serum withdrawal. HUVECs were adhered on tissue culture dishes in complete medium (supplemented with serum) and then cultured in basal medium (in the absence of serum) containing 20 ng/ml intact VEGF or 2 μ g/ml intact or KLK12-cleaved CCN1 (*D*) or CCN5 (*E*). Cell viability was measured at the time of medium change (*t*₀) and after 24 h of treatment using a MTS assay. *control*, basal medium without additive; *CICC*, KLK12-cleaved CCN. The *bars* represent the mean \pm S.D. of five independent experiments. *, $p < 0.05$, Kruskal and Wallis test.

Although not studied thus far in detail, several lines of evidence indicate that proteolysis of CCNs is a common physiological phenomenon. Various forms of CCN2 were detected in pig uterine flushings resulting from *in utero* proteolytic digestion of the full-length protein (41–43). In rats, an N-terminally truncated CCN3 form predominates in brain tissue and in cerebrospinal fluid (44). Proteolytic processing of CCN2 and CCN3 is also evidenced by observations showing that fragmented forms were present in the culture medium of cells overexpressing the recombinant full-length protein (43, 45). In humans, CCN2 fragments were detected in several fluids such as normal sera, pregnancy sera, cerebrospinal, amniotic, follicular,

and peritoneal fluids (46), and CCN3 fragments were observed in cerebrospinal fluid, amniotic fluid, and prepubertal and pubertal urine samples (47). Finally, N-terminal CCN2 fragments have been found in the plasma of patients with diabetic nephropathy (48) and multiple myeloma (49) and in the interstitial fluid and plasma of patients with scleroderma (50). Because CCNs are sensitive to various secreted proteases (trypsin; chymotrypsin; elastase; plasmin; plasma kallikrein; kallikrein-related peptidases 1, 5, 12, and 14; and matrix metalloproteinases-1, -2, -3, -7, -9, and -13) (Refs. 31, 51, and 52 and this paper), the proteolysis of CCNs can potentially occur within a wide variety of tissues and in diverse physiological and

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pathological settings. In particular, KLKs 1, 5, 12, and 14 have been found in various fetal and adult normal tissues (1), and dysregulation of their expression has been reported in several malignant diseases (ovarian, prostate, breast, and lung cancer) (11). The presence of CCNs in tissues characterized by high expression of KLKs coupled with the high proteolytic efficiency of some of these proteases for these substrates point to a potential role for KLK enzymes in CCN fragmentation *in vivo*.

Both CCNs and KLK12 have been involved in angiogenesis. Blockage of KLK12 activity with specific anti-KLK12 antibodies was reported to reduce proliferation, migration, and formation of branching cords of microvascular endothelial cell *in vitro*. Furthermore, its expression is down-regulated in endothelial cells from patients with systemic sclerosis displaying defective angiogenesis compared with endothelial cells from normal subjects (9). Because KLK12 seems to play a critical role in controlling normal angiogenesis, we sought to investigate the relations between KLK12, CCNs, and factors regulating angiogenesis. Using different approaches, we identified or confirmed direct interactions between VEGF₁₆₅, BMP2, TGF- β 1, FGF-2, and CCN1. With the exception of BMP2, all of these factors can also form complexes with CCN5 in solution or when immobilized on solid matrices. We report for the first time that KLK12-mediated proteolysis of CCN1 and CCN5 can reduce or abolish the binding of VEGF, BMP2, and TGF- β 1. Because cleavage sites were not related to the domains proposed to be responsible for binding of these growth factors, we suggest that the observed changes in function may reflect conformational changes of CCN1 or CCN5 because of proteolysis. This study also provides evidence that binding of VEGF₁₆₅, BMP2, TGF- β 1, and FGF-2 to CCN1 or CCN5 does not mask the proteolytic sites targeted by KLK12, thus allowing the KLK12-mediated CCN fragmentation. VEGF₁₆₅ and BMP2 alone were resistant to the proteolytic action of KLK12, whereas this protease was able to cleave TGF- β 1 and FGF-2 in a concentration-dependent manner. We also observed a change in the hydrolytic sensitivity when TGF- β 1 was complexed with CCN proteins. This suggests that binding of TGF- β 1 would modify its conformation appropriately, further exposing potential sites that are sensitive to hydrolysis by KLK12. Such a change in the conformation of TGF- β 1 following binding to CCN2 was previously reported by Abreu *et al.* (32).

Complex formation of VEGF₁₆₅ with CCN2 was shown to inhibit VEGF₁₆₅-induced tube formation of endothelial cells *in vitro* and angiogenesis *in vivo* (53). Unexpectedly, our study indicated that free VEGF₁₆₅ and VEGF complexed with CCN1 or CCN5 equally trigger VEGFR2 phosphorylation and HUVEC proliferation. At first sight, our data seem to contradict the observations of Inoki *et al.* (53). An explanation for that may lie in the ability of the CCN proteins to bind to extracellular matrix (ECM) components like fibronectin, collagen, vitronectin, and aggrecan (54–57). When functional studies are performed in the presence of ECM, as in the tube formation assay or *in vivo*, a large fraction of CCNs is likely immobilized in ECM sequestering the VEGF₁₆₅ away from its receptors. Conversely, complexes would remain predominantly in a form accessible to receptors when cells are grown directly on plastic as herein. Our results indicate that addition of VEGF₁₆₅-CCN complexes

in the culture medium do not prevent VEGFR2 stimulation by VEGF₁₆₅. One explanation would be that VEGFR2 competes with the soluble CCNs for VEGF binding and becomes activated by VEGF dissociated from the VEGF-CCN complexes. This hypothesis is consistent with data showing that VEGFR2 binds VEGF₁₆₅ with an affinity approximately 3 orders of magnitude greater than those of CCNs (picomolar range *versus* nanomolar range) (53, 58). Therefore, the mode of action of CCNs is expected to be different from that of related proteins: the IGFBPs. Indeed, IGFBPs bind IGF-I and IGF-II with affinities that are equal to or greater than those of the IGF receptor and sequester the factor away from the receptor, resulting in a blockage of the IGF signaling. Signaling is restored following proteolytic processing of IGFBPs, which decreases IGFs binding affinities by 2 orders of magnitude. An alternative explanation of our findings would be that intact CCN1 and CCN5 act as cofactors to potentiate VEGF₁₆₅ effects on VEGFR2 signaling. As a proof of principle, CCNs have already been described to potentiate the activities of TGF- β 1, FGF-2, and BMP2 (32, 35–36).

In our conditions, KLK12-induced fragmentation of CCN1 and CCN5 did not modify the response of HUVEC to VEGF₁₆₅. Functional consequences of proteolysis of VEGF-CCNs complexes appear different when endothelial cells are surrounded with ECM. Hashimoto *et al.* (31) demonstrated that angiogenic activity of VEGF₁₆₅ blocked in the VEGF₁₆₅-CCN2 complex is reactivated to original levels after CCN2 digestion of the complex by MMPs both *in vitro* using a tube formation assay and *in vivo* using a Matrigel injection model in mice. Taken together with our findings, these observations suggest that the CCN proteins may regulate VEGF signaling mostly by sequestering VEGF₁₆₅ in ECM and that proteolysis of the CCNs engaged in immobilized VEGF-CCN complexes may release VEGF in the soluble compartment, allowing interaction with its receptor. We also revealed that BMP2 and TGF- β 1 immobilized in complexes with CCNs are released in the soluble phase after KLK12-induced proteolysis of the CCNs. Thus, KLK12 could regulate the CCN-dependent distribution of these growth factors between the soluble and insoluble compartments of the cellular microenvironment. The case of FGF-2 is more complex, because the CCN1 fragments appear to conserve the binding capacity to FGF-2 regardless of proteolysis. Only a few studies have addressed the impact of CCNs on the biological functions of BMPs, TGF- β 1, and FGF-2. Binding of these growth factors to CCN2, CCN3, or CCN4 were reported to either inhibit or enhance their activities depending on the partners under consideration and the models studied (32, 35, 53, 59, 60). Further studies are required to determine the functional impact of complexes interaction with the substratum and the subsequent biological consequences of this regulation in the proteolysis of CCNs.

KLK12 is expressed in numerous organs including (but not limited to) colon, esophagus, lung, prostate, and stomach, where aberrant expression of CCNs is observed in cancerous tissues. Thus, it is tempting to speculate that KLK12 modulates the promoting or inhibiting effects of CCNs on the tumorigenesis of these organs. In particular in nonsmall cell lung carcinoma (NSCLC), this idea is supported by our findings showing

that: (a) KLK12 is coexpressed with several CCNs in the cancerous tissue of patients and (b) full-length CCN1 and KLK12-released CCN1 fragments have different effects on the migration of the A549 lung cancer cell line *in vitro*. Previous data have indicated that CCN1 may act as a tumor suppressor in NSCLC *in vitro* and *in vivo* (61, 62). Collectively, these findings suggest that two mechanisms affecting CCN1 could act simultaneously to facilitate NSCLC development. The first one corresponds to the decreased expression of CCN1 in the tumor site, and the second one refers to the suppression of the tumor inhibitory effects of the residual CCN1 activity through KLK-mediated proteolysis. In addition to *CCN1*, *CCN2* (62) and *CCN5* (present study) are down-regulated, whereas *CCN4* (62) is up-regulated in NSCLC. These findings indicate that members of the CCN family may play important but contrasting roles in NSCLC progression. Because KLK12 was found to fragment all CCNs *in vitro*, an extensive investigation is needed to determine the final outcome of KLK12-mediated hydrolysis of several members of the CCN family on the NSCLC progression *in vivo*.

We present evidence that a great variety of CCN fragments can be generated by the proteolytic action of KLKs; some of these fragments exhibit intact domains, whereas others display truncated domains. Functional studies of naturally occurring CCN fragments and recombinant truncated forms revealed that they can act as agonists or antagonists of the full-length CCN proteins depending on the involvement of each domain in mediating a specific function (45, 54–56, 63–65). Taken together with these findings, our data support the hypothesis that limited proteolysis has a major role in modulation of the biological functions of these multi-modular proteins. Because activity of proteases is tightly regulated, as is the expression of CCNs and their binding partners, a detailed understanding of the functions of CCNs requires taking into account the tightly controlled spatiotemporal regulation of proteolysis and the impact of the distinct CCN fragments. In summary, our findings suggest that the KLK12-mediated hydrolysis of CCNs may be involved in the modulation of the bioavailability and/or activity of several growth factors including VEGF₁₆₅, BMP2, TGF- β 1, and FGF-2. Because KLKs 1, 5, 12, and 14; CCNs; and the aforementioned growth factors coexist in several situations, fragmentation of CCNs by KLKs can be expected to play a role in diverse physiological and pathological processes including angiogenesis, bone and bone marrow homeostasis, and tumorigenesis.

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