⌘ *Author's Choice*

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 285, NO. 46, pp. 35803–35813, November 12, 2010

The Streptococcal Collagen-binding Protein CNE Specifically Interferes with $\alpha_V\beta_3$-mediated Cellular Interactions with Triple Helical Collagen*[S]

Received for publication, May 19, 2010, and in revised form, August 10, 2010 Published, JBC Papers in Press, September 13, 2010, DOI 10.1074/jbc.M110.146001

Tijs van Wieringen[‡], Sebastian Kalamajski[‡], Åsa Lidén[‡§], Dominique Bihan[¶], Bengt Guss[‖], Dick Heinegård[**], Richard W. Farndale[¶], and Kristofer Rubin[‡1]

*From the [‡]Department of Medical Biochemistry and Microbiology, Uppsala University, BMC Box 582, SE-751 23 Uppsala, Sweden, the [§]Department of Biomedicine, University of Bergen, Jonas Lies vei 91, N-5009 Bergen, Norway, the [¶]Department of Biochemistry, University of Cambridge, Downing Site, Cambridge CB2 1QW, United Kingdom, the [‖]Department of Microbiology, Swedish University of Agricultural Sciences, Box 7025, SE-750 07 Uppsala, Sweden, and the **Department of Clinical Sciences Lund, BMC C12 Lund University, SE-221 84 Lund, Sweden*

Collagen fibers expose distinct domains allowing for specific interactions with other extracellular matrix proteins and cells. To investigate putative collagen domains that govern integrin $\alpha_V\beta_3$-mediated cellular interactions with native collagen fibers we took advantage of the streptococcal protein CNE that bound native fibrillar collagens. CNE specifically inhibited $\alpha_V\beta_3$-dependent cell-mediated collagen gel contraction, PDGF BB-induced and $\alpha_V\beta_3$-mediated adhesion of cells, and binding of fibronectin to native collagen. Using a Toolkit composed of overlapping, 27-residue triple helical segments of collagen type II, two CNE-binding sites present in peptides II-1 and II-44 were identified. These peptides lack the major binding site for collagen-binding β_1 integrins, defined by the peptide GFOGER. Peptide II-44 corresponds to a region of collagen known to bind collagenases, discoidin domain receptor 2, SPARC (osteonectin), and fibronectin. In addition to binding fibronectin, peptide II-44 but not II-1 inhibited $\alpha_V\beta_3$-mediated collagen gel contraction and, when immobilized on plastic, supported adhesion of cells. Reduction of fibronectin expression by siRNA reduced PDGF BB-induced $\alpha_V\beta_3$-mediated contraction. Reconstitution of collagen types I and II gels in the presence of CNE reduced collagen fibril diameters and fibril melting temperatures. Our data indicate that contraction proceeded through an indirect mechanism involving binding of cell-produced fibronectin to the collagen fibers. Furthermore, our data show that cell-mediated collagen gel contraction does not directly depend on the process of fibril formation.

Collagen fibrils packed in the quarter-staggered fashion expose specific domains that specifically interact with other molecules or molecular assemblies of the interstitial matrix, or with cells (reviewed in Ref. 1). These domains, summarized by Sweeney *et al.* (2), are reflected in specific binding between, on the one hand, the constituent tropocollagen monomers and, on the other, collagen receptors and other extracellular matrix (ECM)[2] components. The introduction of Toolkits of defined synthetic triple helical peptides covering the Col1 domains of collagen types II and III, including hydroxylated proline residues, has enabled the identification of collagenous motifs that interact with other ECM proteins and cells (3).

Interstitial fluid pressure (IFP) plays an important role in control of tissue fluid homeostasis (4). Lowering of IFP occurs during acute inflammation or anaphylaxis and contributes to formation of edema (5). Cell-mediated collagen gel contraction has been used as an *in vitro* model for studying control of IFP in loose connective tissues (6–8), but also for wound contraction (9). Several substances that stimulate collagen gel contraction *in vitro* also increase IFP *in vivo* and, conversely, substances that inhibit contraction lower IFP. Cell-mediated collagen gel contraction can be mediated by collagen-binding β_1 integrins (9–12) and the collagen-binding integrin $\alpha_2\beta_1$ is of particular importance for control of IFP in rat dermis during homeostasis (13). Contraction by cells lacking collagen-binding β_1 integrins, *e.g.* cells from the murine myoblast cell line C2C12, is induced by PDGF-BB and uses the $\alpha_V\beta_3$ integrin to contract collagen gels (14–16). This is paralleled *in vivo* by the observation that PDGF-BB and insulin normalize IFP that has been lowered as a result of mast cell degranulation by a process dependent on β_3 integrins (16, 17). Available data suggest that collagen-binding β_1 integrins are involved in control of IFP and thereby fluid volume during homeostasis, whereas integrin $\alpha_V\beta_3$-mediated contractions are involved in IFP control during inflammatory processes.

Streptococcus equi subspecies *equi* (*S. equi*) causes a serious and highly contagious disease in the upper respiratory tract of horses. Cells of *S. equi* grown *in vitro* express collagen-binding activity, and a collagen-binding protein called CNE, displaying typical features of a cell surface-anchored protein, has previ-

* This work was supported by grants from the Swedish Science Council (to K. R.), the Swedish Cancer Foundation (to K. R.), the King Gustaf the V:s 80-årsfond (to K. R.), the Swedish Research Council for Environment, Agricultural Sciences and Spatial planning Grant FORMAS 221-2006-989 (to B. G.), Swedish Horse Board Grant H0747197 (to B. G.), and the Wellcome Trust and Medical Research Council supported synthesis of the Toolkits (to R. W. F.).

⌘ *Author's Choice*—Final version full access.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

[1] To whom correspondence should be addressed. Tel.: 46-18-4714116; Fax: 46-18-4714673; E-mail: Kristofer.Rubin@imbim.uu.se.

[2] The abbreviations used are: ECM, extracellular matrix; IFP, interstitial fluid pressure.

ously been isolated and characterized (18). We have previously shown that among a set of ECM-binding proteins from *S. equi* the collagen- and fibronectin-binding protein FNE modulates collagen gel contraction (8). FNE, which is secreted, stimulates collagen gel contraction and normalizes IFP lowered as a result of anaphylaxis. The mechanism by which FNE stimulates collagen gel contraction involves binding of fibronectin to collagen fibers and subsequent adhesion of cells to the complex by a mechanism dependent on the integrin $\alpha_V\beta_3$ (8). As determined by rotary shadowing, FNE binds collagen type I at a region located around 120 nm from the C terminus and therefore presents a high affinity, indirect binding site for fibronectin on the collagen fiber at a domain of collagen that is not known to interact with fibronectin (8). In the current study we have explored the potential to use the collagen-binding streptococcal protein CNE in combination with Toolkits to delineate domains in collagen fibers and molecular mechanisms that are operative in $\alpha_V\beta_3$-mediated contraction.

EXPERIMENTAL PROCEDURES

Cells and Reagents—The murine myoblasts C2C12 were provided by Dr. Anna Starzinski-Powitz (Goethe-Universitaet, Frankfurt am Main, Germany). These cells lack expression of collagen-binding integrins but express the β_1-integrin subunit. C2C12 cells stably expressing human α_2-integrin subunit have been described before (19). Human diploid AG1518 skin fibroblasts (Genetic Mutant Cell Repository, Camden, NJ) were used between passages 18 and 24. Cells were propagated in DMEM with Glutamax (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (PAA Laboratories GmbH, Pasching, Austria) and 50 μg/ml of gentamycin (Invitrogen) at 37 °C and 5% CO_2. Preparation of recombinant protein CNE has previously been described (18). Recombinant PDGF-BB was purchased from Invitrogen. The specific α_V-integrin inhibitor cyclo-(Arg-Gly-Asp-Phe-Val) was obtained from Bachem (Bubendorf, Switzerland). Bovine dermal collagen type I (Purecol, 3 mg/ml) was from Inamed (Fremont, CA). Pepsin-solubilized calf nasal collagen type II, rat skin collagen type III, and Engelbreth-Holm-Swarm-sarcoma collagen type IV were produced according to established protocols (20–22). Human plasma fibronectin and vitronectin were purified as described (23, 24). Rabbit anti-PDGFR-β and rabbit anti-phosphotyrosine IgG were from Santa Cruz (Santa Cruz Biotechnology, CA). The rabbit anti-integrin β_1 IgG has been described elsewhere (25). Anti-mouse integrin β_3-subunit (HMβ3), anti-mouse integrin α_5-subunit (MFR5), and FITC-labeled anti-hamster IgGs were from BD Pharmingen (San Jose, CA). FITC-labeled anti-rabbit IgG and anti-rat IgG were from Vector Laboratories (Burlingame, CA). 1-Hydroxysuccinimide biotin ester was purchased from Sigma and PD-10 columns from GE Healthcare. Avidin-conjugated alkaline-phosphatase and streptavidin-conjugated horseradish peroxidase (HRP) were from Vector Laboratories and AnaSpec, respectively. Tetramethylbenzidine substrate for HRP was from Pierce. Receptor tyrosine kinase inhibitor AG1296 and MMP inhibitor GM6001 were from Merck (Merck, NJ), and both were used at 10 μM. Recombinant human pro-MMP-1 and aminophenyl mercury acetate were from Sigma (Sigma). Coomassie Brilliant Blue G-250 was from Roth (Karlsruhe, Germany). The anti-human fibronectin polyclonal IgG has been described elsewhere (10). siRNA directed against murine fibronectin was from Sigma-Proligo and the transfection agent N-TER Nanoparticles was from Sigma.

Solid Phase Assay—Microtiter plates were coated with fibronectin (10 μg/ml), native or denatured (56 °C for 30 min), collagen types I, II, III, or IV (50 μg/ml) or collagen type II triple helical peptides (10 μg/ml) and incubated overnight at 4 °C or, to prevent renaturation of collagens, at 56 °C. Plates were incubated overnight with 2% BSA at 4 or 56 °C to prevent unspecific binding. Proteins were biotinylated with 1-hydroxysuccinimide biotin ester overnight at 4 °C, followed by a single desalting step with PD-10 columns in PBS with 0.1% BSA and 0.02% azide as preservative. Biotinylated proteins were diluted in 0.5% BSA in PBS and incubated in the coated wells for 2 h at 37 °C. After three washes with PBS supplemented with 0.05% Tween 20 (PBS-Tween), plates were incubated with avidin-conjugated alkaline phosphatase (1:500) for 2 h at room temperature. Plates were washed three times with PBS-Tween and developed with *p*-nitrophenyl phosphatase substrate (Sigma) (0.6 mg/ml in ethanolamine solution, pH 9.8) at 37 °C until A_{405} was between 0.1 and 1.0 absorbance units.

CNE Binding Assay Using Collagen Toolkit—The triple helical collagen type II 27-amino acid peptide library has been described previously (26). Microtiter plates were coated with triple helical peptides at 10 μg/ml in a volume of 100 μl/well. Plates were blocked with 10% BSA in Tris-buffered saline (TBS) for 1 h at room temperature. Plates were washed three times with TBS substituted with 0.1% Tween 20 (TBST). Wells were incubated with 75 nM CNE-biotin (assuming 70% recovery from PD-10 column) in 100 μl of PBS without $CaCl_2$ and $MgCl_2$ (pH 7.4) for 1 h at room temperature. Wells were washed three times with TBST before incubation with streptavidin-HRP (1:5000 in TBS) for 1 h at room temperature. Wells were washed 3 times and developed by adding 100 μl of tetramethylbenzidine substrate. Adding 100 μl of 2.5 M H_2SO_4 stopped development and wells were measured at A_{450}.

Collagen Gel Contraction—Collagen gel contraction was performed and quantified as described elsewhere (10). Briefly, 96-well plates were blocked in 2% BSA and a collagen solution was prepared from 2× DMEM, HEPES, and collagen types I or II. One part cell suspension (10^6 cells/ml) was mixed with 9 parts collagen solution. When indicated, recombinant proteins, antibodies, and/or inhibitors were added to the cell-collagen solution. Cell-collagen gels (100 μl) were allowed to form and were subsequently detached by ejection of 100 μl of DMEM in the absence or presence of 40 ng/ml of PDGF-BB into the wells. The relaxed, free-floating gels were further incubated at 37 °C and gel diameters were measured microscopically at the indicated time points.

Zymography—Recombinant human pro-MMP-1 was activated with 1 mM aminophenyl mercury acetate in TCNB buffer (50 mM Tris, 10 mM $CaCl_2$, 150 mM NaCl, 0.05% Brij-35, pH 7.5). 10 μg of triple helical collagen type I was preincubated in the presence or absence of 2.2 μg of CNE (dissolved in PBS with 0.5% BSA) for 1 h at 37 °C (equimolar ratio). Preincubated collagen was incubated for the indicated times with 25 ng of activated MMP-1. Digestion reactions were terminated by adding

4× SDS sample buffer. Protein samples were separated with SDS-PAGE and gels were stained with Coomassie Brilliant Blue to detect banding patterns.

Adhesion Assay—24-Well plates were coated overnight at 4 °C, or, to prevent re-naturation of collagen, at 56 °C with native or heat-denatured collagen type I (50 μg/ml), vitronectin (10 μg/ml), or Toolkit II peptides (10 μg/ml) in Buffer 3 (140 mM NaCl, 4.7 mM KCl, 0.65 mM $MgSO_4$, 1.2 mM $CaCl_2$, 10 mM HEPES, pH 7.4). Plates were washed three times in the same buffer and cells were diluted to 125,000 cells/ml. 50,000 cells were allowed to adhere at 37 °C and where indicated PDGF-BB and/or CNE were added together with the cells at concentrations of 20 ng/ml and 350 nM, respectively. Non-adherent cells were removed and the wells were gently washed twice with pre-warmed Buffer 3. The relative amount of adhered cells was quantified using a hexosaminidase assay, as described previously (27).

Fibril Formation—Fibrillogenesis of pepsin-extracted bovine skin collagen type I and nasal cartilage collagen type II was monitored by change in turbidity at 400 nm at 4-min intervals. Fibrillogenesis was monitored for 720 min. Four times concentrated buffer (80 mM HEPES, 0.6 M NaCl, pH 7.4), CNE, and 0.012 M NaOH, in a volume equal to neutralize the collagen solution, were mixed, and water was added to a final volume of 238 ml. Twelve ml of a solution of collagen types I or II, purified after pepsin digestion in either 0.012 M HCl or 0.1 M acetic acid was added, yielding a final collagen concentration of 144 μg/ml. Collagen was added just prior to initiation of absorbance readings. The cuvettes were placed in a Beckman DU640 scanning spectrophotometer with a temperature controlled six-place cuvette chamber equilibrated to 37 °C.

Electron Microscopy and Differential Scanning Calorimetry—Scanning electron microscopy (EM) was performed on collagen gels with or without cells prepared as described above. Gels were dehydrated, critical-point dried, gold-sputtered, and analyzed in a PHILIPS 515 electron microscope. Fibril diameter was quantified with ImageJ software (NIH). Differential scanning calorimetry measurements were performed on collagen fibrils formed *in vitro*, in the presence or absence of CNE, for 5 h in 37 °C in PBS (molar ratio collagen:CNE was 10:1). The differential scanning calorimetry thermograms were recorded in VP-DSC (MicroCal), at a scan rate of 0.5 °C/min, and medium feedback. Each thermogram was corrected by subtraction of a linear baseline based on a blank buffer sample, and normalized for collagen concentration.

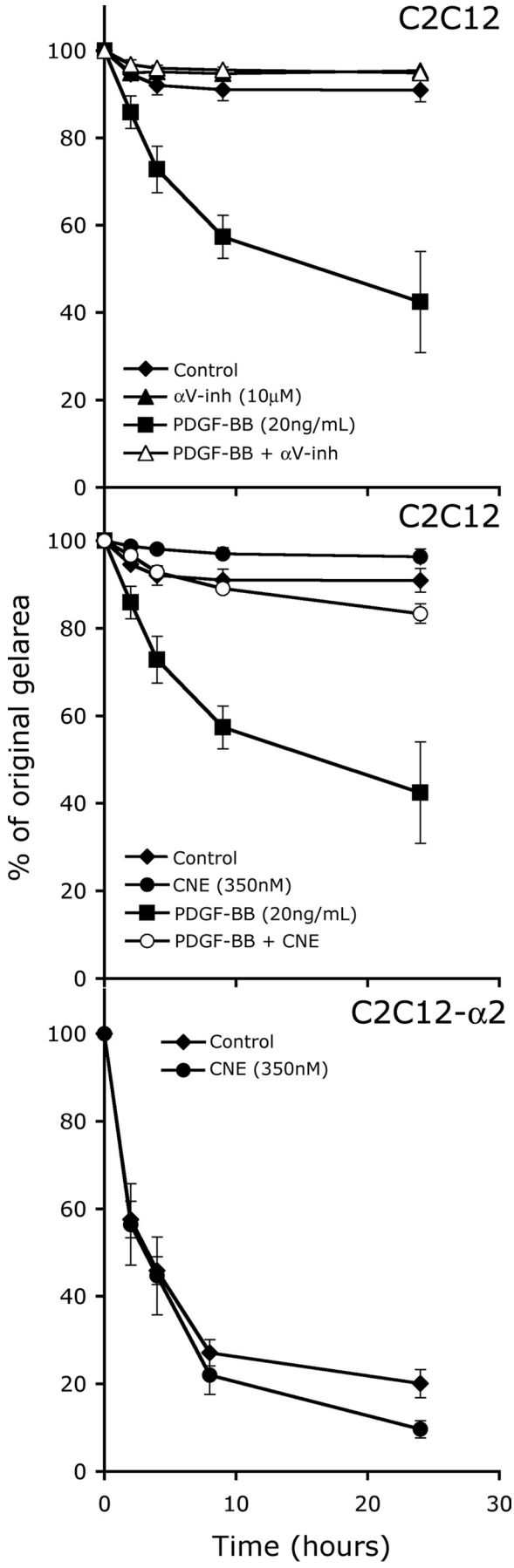
Knockdown of Fibronectin with siRNA—C2C12 cells were seeded in 24-well plates at 90,000 cells per well and grown for 24 h in antibiotic-free DMEM supplemented with 10% FBS. Cells were transfected with a final concentration of 20 nM siRNA directed against murine fibronectin mRNA (PubMed accession number NM_010233) or with control siRNA that has no binding interaction with any known mRNA. A second control consisted of cells that were exposed only to the transfection agent, N-TER™ Nanoparticles, which were used according to the manufacturer's instructions. Cells were harvested at 24 h and fibronectin protein levels were assessed by separating equal amounts of cleared cell lysates with SDS-PAGE and Western blotting with rabbit anti-fibronectin polyclonal IgG. Transfected cells were used in collagen gel contraction assays.

PDGFR-β Phosphorylation—C2C12 cells were seeded in 6-well plates at a density of 500,000/well. Cells were allowed to spread and then serum starved in DMEM with 0.1% FBS for 12–18 h. Cells were pre-treated with 350 nM CNE in DMEM for 2 h and subsequently stimulated with PDGF-BB (20 ng/ml) for 10 min in the presence of 350 nM fresh CNE. Wells were washed twice in ice-cold PBS and lysed on ice in solubilization buffer (50 mM Tris, 150 mM NaCl, 2 mM EGTA, 1 mM Na_3VO_4, 1% Nonidet P-40, 0.25% sodium deoxycholate and protease inhibitors). Supernatants were pre-cleared with normal rabbit IgG for 1 h at 4 °C. PDGFRβ was immunoprecipitated with 5–10 μg of rabbit anti-PDGFR-β for 1.5 h. Proteins were separated on 7.5% polyacrylamide gels, transferred to nitrocellulose, and blocked in 5% BSA overnight at 4 °C. Membranes were probed with rabbit anti-phosphotyrosine IgG (1:1000) and HRP-labeled donkey anti-rabbit IgG (1:5000), and protein bands were visualized with luminol.

Flow Cytometry—Cells were trypsinized and washed twice with PBS. 500,000 cells were resuspended in 50 μl of primary antibody (10 μg/ml) diluted in 0.5% BSA in PBS together with 10 μg/ml of normal IgG of the same origin as the secondary antibody and incubated on ice for 1 h. Cells were washed two times in cold PBS and resuspended in 50 μl of secondary antibody (diluted 1:50 in 0.5% BSA in PBS) followed by a 30-min incubation on ice. After washing, cells were resuspended in 0.5% BSA in PBS and cell-bound antibodies were detected in a BD Biosciences FACS scan.

RESULTS

Inhibition of PDGF BB-induced $\alpha_V\beta_3$-dependent Collagen Gel Contraction by CNE—C2C12 cells lack collagen-binding β_1 integrins but express other β_1 integrins such as the fibronectin-binding integrin $\alpha_5\beta_1$. PDGF-BB induces integrin $\alpha_V\beta_3$-dependent collagen gel contraction by these cells (8). In agreement with these earlier reports, C2C12 cells contracted collagen gels only after stimulation by PDGF-BB (Fig. 1, *upper panel*). C2C12 cells with forced expression of $\alpha_2\beta_1$, after transfecting the cells with full-length human α_2-integrin subunit (C2C12-α_2), efficiently contracted collagen lattices, even in the absence of external stimuli (Fig. 1, *lower panel*). During initial experiments with a panel of recombinant streptococcal proteins (data not shown), only protein CNE was found to inhibit $\alpha_V\beta_3$-mediated collagen gel contraction. At a final concentration of 350 nM, CNE inhibited PDGF BB-induced contraction of C2C12 cells by an average of 86 ± 5% (Fig. 1, *middle panel*), but had no inhibitory effect on contraction mediated by C2C12-α_2 cells (Fig. 1, *lower panel*). AG1518 fibroblasts normally contract collagen lattices by using collagen-binding β_1 integrins but not $\alpha_V\beta_3$ (supplemental Fig. S1). The monoclonal anti-human β_1 integrin antibody M13, which blocks β_1 integrin function, inhibited AG1518 human fibroblast-mediated collagen gel contraction, an effect that could be overcome by addition of PDGF-BB. This effect of PDGF-BB was in turn dependent on $\alpha_V\beta_3$ integrin, because the contraction was blocked by a cyclic RGD peptide, which specifically inhibits $\alpha_V\beta_3$ integrin function at the concentrations used here. Similarly, in the presence of M13,



the effect of PDGF-BB was abolished by addition of CNE. However, addition of CNE had no effect on control contraction in the absence of M13 (supplemental Fig. S1, *A* and *B*). When taken together, our data show that CNE specifically inhibited $\alpha_V\beta_3$ integrin-mediated collagen gel contraction but had no effect on collagen-binding β_1 integrin-mediated contraction.

Binding of CNE to Native Collagens—Recombinant streptococcal CNE bound to native interstitial collagen types I, II, and III but not to collagen type IV in solid phase assays (Fig. 2*A*). In these assays, collagens were coated at neutral pH and 37 °C allowing for fibril formation in the plates. Denaturation of the collagens by heating to 56 °C at neutral pH reduced CNE affinity below the detection limit (Fig. 2*B*). Average avidities for the binding of CNE to the various collagen fibrils were estimated to ~125 nM for collagen type I, ~50 nM for collagen type II, and ~100 nM for collagen type III based on data from the solid phase experiments. These findings show that CNE only bound to native triple helical collagen chains.

CNE Inhibits Collagen Fibril Formation and Reduces Diameter as well as Denaturation Temperature of in Vitro Formed Collagen Fibrils—Because CNE bound native collagens, it could possibly interfere with the formation of collagen fibrils, thereby changing the biomechanical properties of collagen gels and in such a manner hamper the contractibility of the collagen matix. We tested the ability of CNE to inhibit fibril formation of collagen types I and II by monitoring change in turbidity. CNE effectively inhibited fibril formation in a dose-dependent fashion (Fig. 3, *A* and *B*). Furthermore, we tested the ability of CNE to reduce fibril diameter of collagen type I and II fibers reconstituted *in vitro*. As revealed by scanning electron microscopy (Fig. 3, *C–F*), CNE reduced the average fibril diameter from 130 to 90 nm (Fig. 3, *G* and *H*). Because collagen fibril formation is influenced by CNE *in vitro*, we also analyzed its effect on collagen denaturation. After incubating collagen types I or II with CNE and allowing fibrils to form *in vitro*, the samples were run in a differential scanning calorimeter to determine the collagen denaturation curves. During denaturation, two melting peaks were produced; the early peak corresponds to denaturing free collagen monomers (triple helices), and the later peak due to denaturation of the collagen fibrils. Addition of CNE did not affect denaturation of free collagen monomers at 40 °C but lowered the melting point of fibrils by about 5 °C from 50 to 45 °C for collagen type I (Fig. 3*I*) and about 2.5 °C from 50 to 47.5 °C for collagen type II (Fig. 3*J*). These findings demonstrate that

FIGURE 1. **CNE inhibits PDGF BB-induced $\alpha_V\beta_3$-mediated, but not $\alpha_2\beta_1$-mediated collagen gel contraction.** C2C12 cells were seeded in collagen type I gels in the presence or absence of CNE or an inhibitor to α_V-integrins. *Top panel*, C2C12 cells did not contract collagen gels in the absence of exogenous factors. Addition of PDGF-BB at a final concentration of 20 ng/ml induced efficient contraction of C2C12 cells that was abolished by adding a final concentration of 10 μM function-blocking inhibitor to α_V-integrin subunit (αV-inh). *Middle panel*, addition of CNE at a final concentration of 350 nM nearly completely blocked PDGF BB-induced $\alpha_V\beta_3$ integrin-mediated contraction. Note that the control and PDGF-BB values in the *two upper panels* are identical. *Bottom panel*, in contrast to $\alpha_V\beta_3$ integrin-mediated contraction, contraction mediated via integrin $\alpha_2\beta_1$ (C2C12-α_2 cells) was carried out efficiently in the absence of any exogenous factors. Addition of CNE at a final concentration of 350 nM did not inhibit this process. In all three panels values represent averages of at least three independent experiments and *error bars* are S.E.

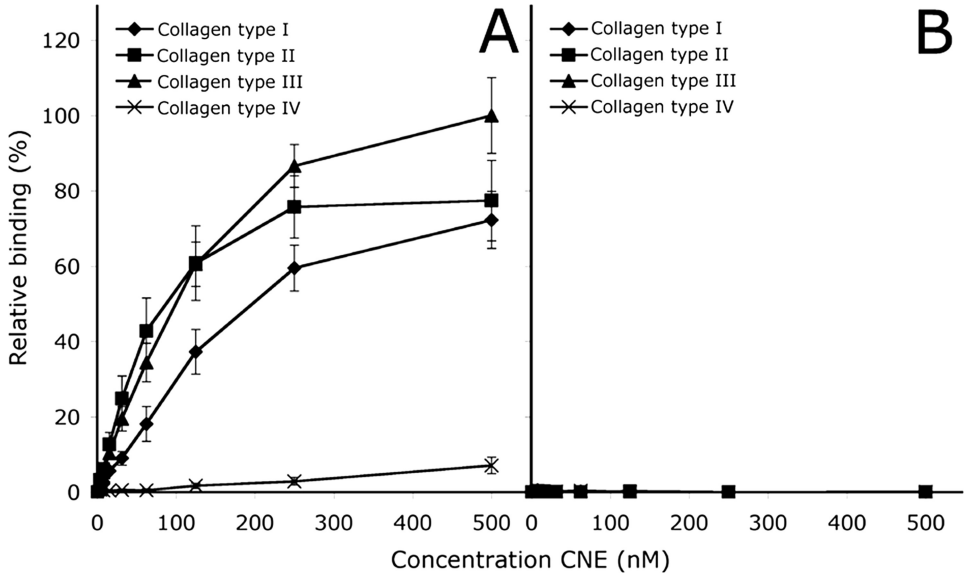


FIGURE 2. **CNE only binds native interstitial triple helical collagens.** Biotinylated CNE was allowed to interact with immobilized collagen types I, II, III, and IV in a solid phase approach where bound CNE was detected with an avidin-alkaline phosphatase conjugate. *A*, CNE bound efficiently to native collagen types I, II, and III fibrils, but not to IV. Maximum binding for the three reactive collagens was reached at around 250–500 nM CNE. At maximum binding, CNE had the highest affinity to collagen type III. *B*, when collagens were denatured the binding ability for CNE was lost in all collagens. Graphs represent averages of four independent experiments and *error bars* are S.E.

CNE could modulate the biomechanical properties of collagen gels.

MMPs Are Not Required for PDGF BB-induced $\alpha_V\beta_3$ Integrin-mediated Contraction—We investigated the possibility that PDGF-BB acted on MMPs that cleave a specific site in the collagen triple helix, thereby relaxing the triple helix that can result in exposure of nearby RGD sequences in a conformation that could be recognized by $\alpha_V\beta_3$. In such a scenario, CNE could exert its effects by inhibiting MMP-induced exposure of RGD sequences and subsequent abrogation of contraction. Therefore, we first tested whether CNE could inhibit enzymatic digestion of collagen by MMPs. Using zymography, we found no inhibitory effect of CNE on MMP-1 collagenase activity at the CNE to collagen ratio used here, 1:1 (Fig. 4*A*). To further test whether MMP activity affected PDGF BB-induced $\alpha_V\beta_3$-mediated contraction we made use of the MMP inhibitor GM6001 that selectively blocks activities of MMP-1, -2, -3, -8, and -9 or MMP inhibitor III that selectively blocks activities of MMP-1, -2, -3, -7, and -13. Neither of the inhibitors blocked PDGF BB-induced $\alpha_V\beta_3$ integrin-mediated contraction (Fig. 4*B* and data not shown). However, GM6001 potentiated the PDGF BB-induced $\alpha_V\beta_3$ integrin-mediated contraction, suggesting that MMPs have an inhibitory rather than stimulatory effect on collagen gel contraction.

CNE Inhibits PDGF BB-induced Adhesion of C2C12 Cells to Fibrillar Collagen Type I—We investigated the possibility that CNE exerts its inhibitory action on $\alpha_V\beta_3$–mediated collagen gel contraction through interference with the function of integrins, *e.g.* by blocking ligand-binding sites on these cell adhesion receptors. C2C12 and C2C12-α_2 cells were seeded on plastic dishes coated with vitronectin, heat-denatured or native collagen type I in the presence or absence of CNE. C2C12 cells adhered to heat-denatured collagen and vitronectin, but not significantly to native collagen type I within a 30-min time frame. Addition of CNE at a concentration of 350 nM had no inhibitory effect on adhesion to heat-denatured collagen or vitronectin (Fig. 5*A*, *upper panel*). These findings demonstrate that CNE did not inhibit the function of $\alpha_V\beta_3$. As expected, C2C12-α_2 cells adhered effectively to native collagen type I, as well as to vitronectin and heat-denatured collagen (Fig. 5*A*, *lower panel*). Adhesion of C2C12-α_2 cells to any of the tested ligands was not affected by the presence of CNE (Fig. 5*A*, *lower panel*). Upon longer incubation of C2C12 cells on native collagen type I (>2 h), cells started to adhere (Fig. 5*B*). Interestingly and in analogy to collagen gel contraction, PDGF-BB significantly stimulated the latter process and this stimulatory effect was reduced to control levels when CNE, at a concentration of 350 nM, was present in the incubation medium (Fig. 5*B*). These data show that CNE did not interfere with the functionality of the investigated integrin but with a PDGF BB-stimulated process that possibly involves *de novo* protein synthesis.

CNE Specifically Binds Two Triple Helical Amino Acid Sequences in Collagen Type II—So far, we reported on the effects of CNE on modulation of collagen gel contraction brought about by integrin $\alpha_V\beta_3$ and modulation of biomechanical properties of collagen gels. To study the underlying mechanisms of these two distinct actions it was important to identify binding sites in collagen for CNE. For this purpose we took advantage of Toolkit II, comprising the full-length human collagen type II sequence, consisting of synthetic triple helix peptides 27 amino acids in length (26). The last nine amino acids of each peptide overlap with the first nine of the next peptide so that the middle nine amino acids are unique sequences. Both ends of each peptide comprise GPP pentamers (GPP_5) to induce triple helical folding of the insert. In this approach 56 peptides were created that comprise Toolkit II. First we established that C2C12-mediated contraction of collagen type II gels shared characteristics with contraction of collagen type I gels. PDGF-BB-induced contraction of collagen type II gels and this effect was abolished by CNE (Fig. 6*A*). CNE bound 2 different peptides, peptides II-1 and II-44 from Toolkit II with a signal to noise ratio above 3 (Fig. 6*B*). Several II-44 variant peptides (amino acid substitutions and shorter variants of the peptide) bound CNE, even with distinct and non-overlapping sequences (data not shown), suggesting either two different binding sites within peptide II-44, or a lack of requirement for all the amino acids in the sequence. This means that binding would be dependent on either specific structure- and/or charge-dependent features of the amino acid sequence in triple helical collagens. To further establish

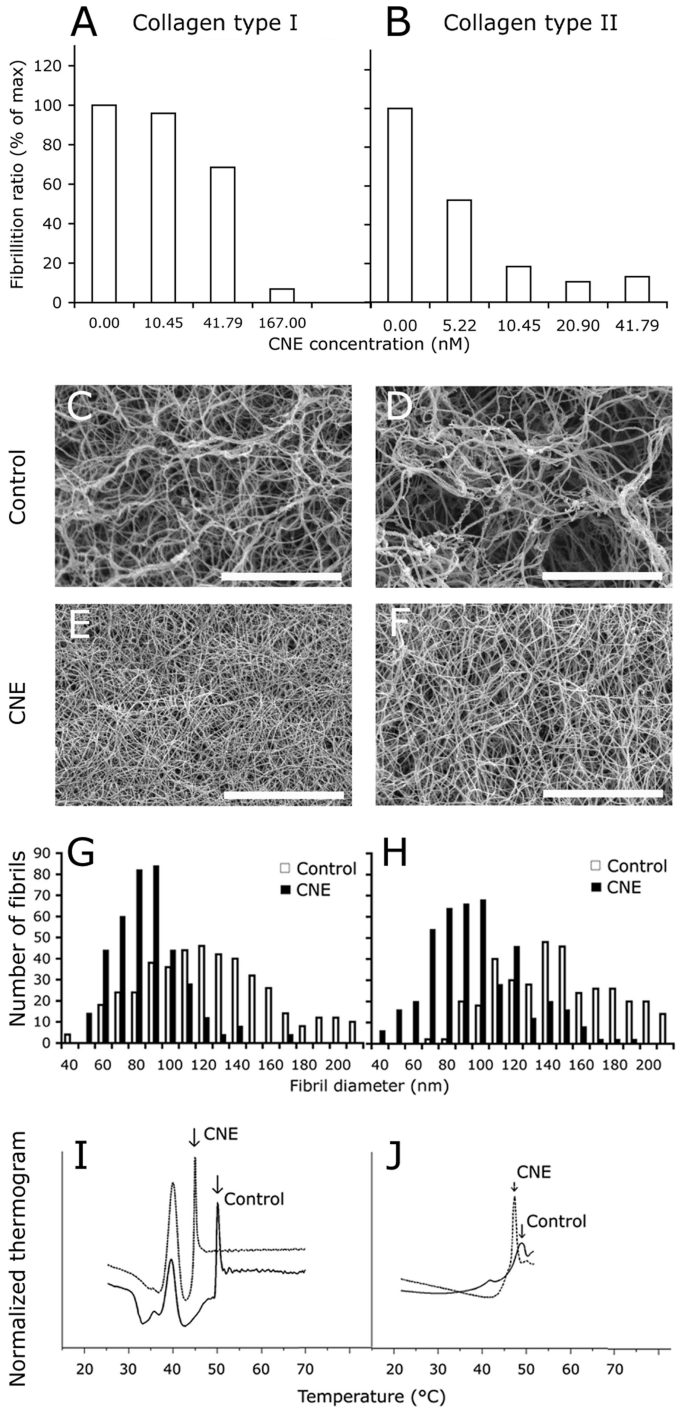


FIGURE 3. **CNE inhibits fibrillogenesis and reduces collagen fibril diameter and melting temperature.** Fibrillogenesis of bovine collagen types I and II was monitored by change in turbidity at 400 nm at 4-min intervals. Addition of increasing amounts of CNE inhibited fibrillogenesis in both collagen types tested (*A* and *B*). Maximum inhibition of fibrillogenesis for collagen types I and II was observed at 167 and 21 nM CNE, respectively. Collagen gels consisting of collagen types I or II were prepared in the absence (*C* and *D*) or presence (*E* and *F*) of 350 nM CNE. Scanning electron microscopy revealed reduced fibril diameters (from 120–130 to 90 nm) when CNE had been present in the gels (*G* and *H*). Differential scanning calorimetry revealed that collagen type I (*I*) and II (*J*) fibrils formed in the presence of CNE (10:1 molar ratio) had no altered monomer melting temperature (40 °C) but had a 5 (collagen type I) and 2.5 °C (collagen type II) lowered fibril melting temperature when compared with fibrils formed in the absence of CNE. *Bar* in *C–F* is 10 μm.

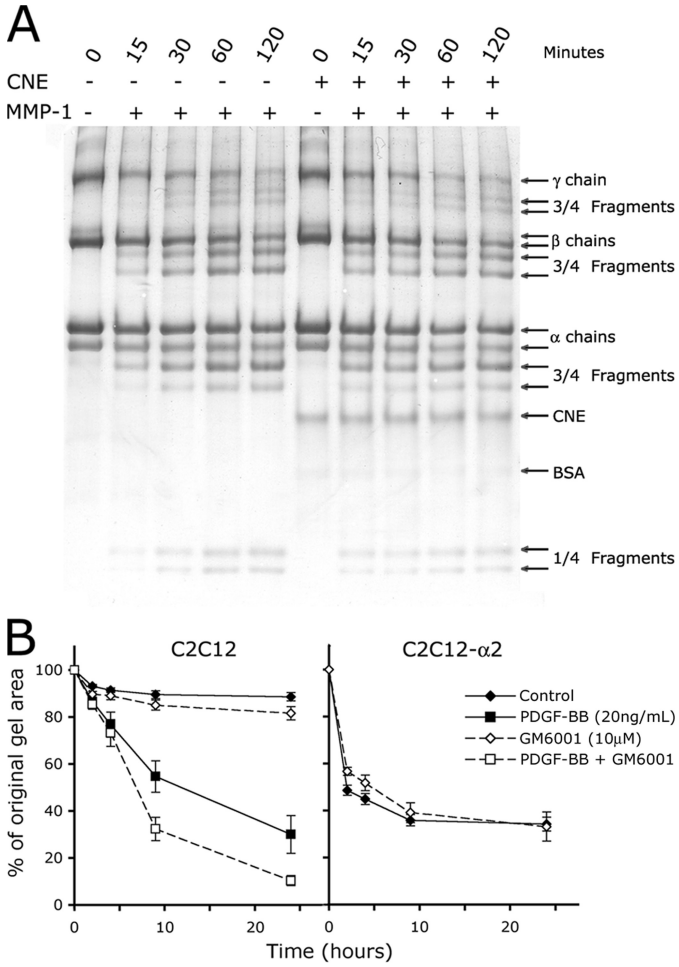


FIGURE 4. **MMP activity is not inhibited by CNE and is not required for PDGF BB-induced $\alpha_V\beta_3$ integrin-mediated contraction.** *A*, 10 μg of collagen type I was preincubated with 2.2 μg of CNE (equimolar ratio) or with buffer only for 1 h at 37 °C. Immediately afterward the mixture was incubated with 25 ng of aminophenyl mercury acetate-activated MMP-1 for the indicated times and reactions were stopped by adding sample buffer. No change in digestion pattern was observed when collagen was pre-treated with CNE. Collagen chains and reaction products are indicated to the *right*. *B*, collagen gel contraction was performed as described under "Experimental Procedures." GM6001 at 10 μM did not inhibit PDGF BB-induced C2C12 cell-mediated contraction, but substantially potentiated the effect of PDGF-BB. GM6001 had no effect on C2C12-α_2-mediated contraction. Graphs represent averages of three independent experiments and *error bars* are S.E.

whether peptides II-1 and/or II-44 contain domains crucial for C2C12 cell binding, adhesion assays were performed on plates coated with peptide II-1, II-44, or control peptide GPP_{10}. C2C12 cells bound effectively to peptide II-44, whereas binding to peptide II-1 was of similar low magnitude as binding to GPP_{10} (Fig. 6*C*). Because contraction of collagen type I and II gels were similarly modulated by PDGF-BB and CNE, and because the α1(I) collagen chain of collagen type I has a significant amino acid sequence homology with type II, we reasoned that the CNE-binding sites in collagen type I might be translated from the Toolkit II peptides. The bovine α1(II) collagen is, respectively, 96 and 100% identical with human α1(II) collagen in the amino acid sequences that encompass Toolkit peptides II-1 and II-44. The bovine collagen type I that has been used in this study has a high amino acid sequence homology with human col-

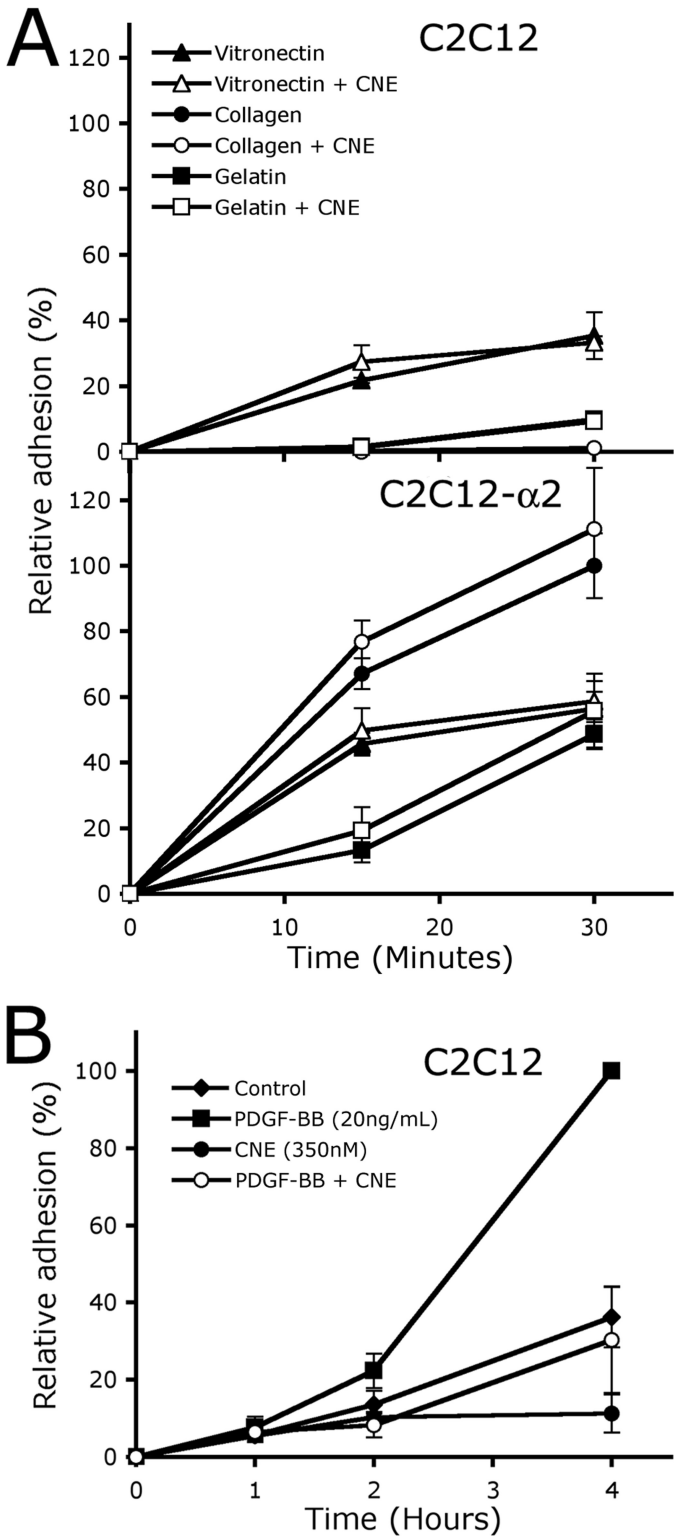


FIGURE 5. **CNE inhibits PDGF BB-induced adhesion of C2C12 cells to immobilized collagen type I.** C2C12 and C2C12-α_2 cells were allowed to adhere to different immobilized ECM ligands in the presence or absence of 350 nM CNE. *A*, *upper panel*, up to 30 min, C2C12 cells bound moderately to vitronectin and gelatin (denatured collagen type I), although there was no significant adhesion to collagen type I. Addition of 350 nM CNE had no effect on C2C12 adhesion to any of these ligands. *A*, *lower panel*, C2C12-α_2 cells bound effectively to collagen type I and to a lower extend to vitronectin and gelatin. Addition of 350 nM CNE had no effect on C2C12-α_2 adhesion to any of these ligands. *B*, upon prolonged exposure to collagen type I, C2C12 cells started to bind moderately. This effect became apparent after 2–4 h of incubation. Addition of 20 ng/ml of PDGF-BB increased the baseline adhesion around 150%. Addition of 350 nM CNE alone almost completely abolished the baseline adhesion and reduced the increase in adhesion brought about by PDGF-BB down to control levels. Data represent averages of three independent experiments and *error bars* are S.E. For *A*, results in both panels are normalized to maximal binding of C2C12-α_2 to collagen at 30 min. For *B*, results are normalized to 4 h adhesion in the presence of PDGF-BB.

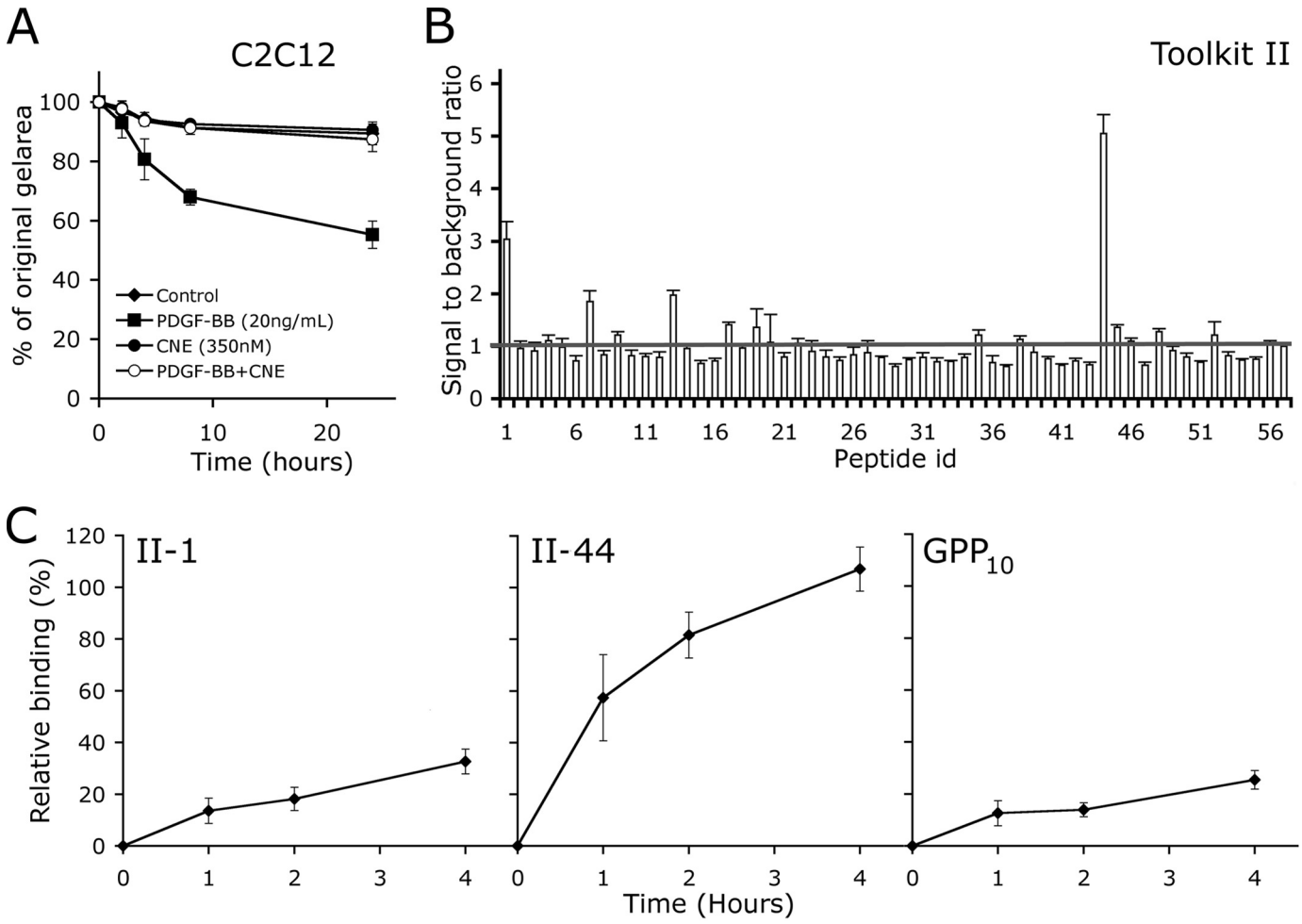
lagen type II. Indeed, the sequence homology between bovine α1(I) collagen and human α1(II) collagen corresponding to peptides II-1 and II-44 are 85 and 93%, respectively (Fig. 6*D*), suggesting that it is likely that these sequences in collagen type I also mediate binding to CNE.

Distinct Effects on Collagen-related Processes of the Two CNE-binding Peptides—So far we have demonstrated two effects of CNE, namely inhibition of $\alpha_V\beta_3$-mediated PDGF BB-induced collagen gel contraction and inhibition of fibrillogenesis of collagen. We addressed the issue whether these two activities could be attributed to two distinct collagen peptides that were recognized by CNE. Soluble peptide II-44 affected collagen gel contraction such that it inhibited contraction at a dose of 50 μg/ml, whereas peptide II-1 had no effect at the same dose (Fig. 7). Conversely, whereas peptide II-1 inhibited fibrillogenesis measured by change in turbidity of dilute collagen type I or II solutions incubated at 37 °C, peptide II-44 slightly stimulated fibrillogenesis (data not shown). These findings suggest that the collagen region defined by peptide II-44, and not peptide II-1, is involved in $\alpha_V\beta_3$-mediated PDGF BB-induced contraction, whereas this region is not involved in fibrillogenesis.

Fibronectin and CNE Compete for Binding to Collagen—Previously, our laboratories have identified several proteins that bind peptide II-44, including DDR2, SPARC (osteonectin) (26, 28), and fibronectin.[3] Peptide II-44 also contains the MMP cleavage site. Together, these data indicate that the collagen locus defined by peptide II-44 contains a broad-specificity binding region. Furthermore, PDGF-BB is known to induce increased synthesis of several ECM proteins including fibronectin (8). Therefore we asked whether fibronectin was involved in PDGF BB-induced $\alpha_V\beta_3$-mediated collagen gel contraction. A requirement for fibronectin in this *in vitro* system would imply that CNE interferes with binding between fibronectin and collagen. Binding of human plasma fibronectin and CNE to Toolkit peptides II-1 and II-44 was investigated in solid phase assays (Fig. 8). As expected CNE bound both peptides, but fibronectin only bound peptide II-44 (Fig. 8*A*). Furthermore, CNE inhibited binding of biotin-labeled fibronectin to immobilized collagen type I in a dose-dependent manner (Fig. 8*B*). In addition, biotin-labeled CNE was unable to bind to immobilized fibronectin (Fig. 8*C*). Together these data suggest that the two proteins compete for the same binding site or closely neighboring binding sites.

Fibronectin Is Required for PDGF BB-induced $\alpha_V\beta_3$-mediated Collagen Gel Contraction by C2C12 Cells—The potential role of fibronectin in PDGF BB-induced adhesion to native collagen type I by C2C12 cells was investigated using an anti-fibronectin IgG that binds and blocks adhesive processes mediated by fibronectin (10). This IgG effectively blocked PDGF

[3] D. Bihan, unpublished data.



D

Peptide II-1

IIα1	*Homo Sapiens*	G	P	M	G	P	M	G	P	R	G	P	O	G	P	A	G	A	O	G	P	Q	G	F	Q	G	N	O
IIα1	*Bos Taurus*	G	P	M	G	P	M	G	P	R	G	P	O	G	P	A	G	A	O	G	P	Q	G	F	Q	G	N	**P**
Iα1	*Bos Taurus*	G	P	M	G	P	**S**	G	P	R	G	**L**	O	G	P	**O**	G	A	O	G	P	Q	G	F	Q	G	**P**	O
Iα2	*Bos Taurus*	G	P	M	G	**L**	M	G	P	R	G	P	O	G	**A**	**S**	G	A	O	G	P	Q	G	F	Q	G	**P**	O

Peptide II-44

IIα1	*Homo Sapiens*	G	L	A	G	Q	R	G	I	V	G	L	O	G	Q	R	G	E	R	G	F	O	G	L	O	G	P	S
IIα1	*Bos Taurus*	G	L	A	G	Q	R	G	I	V	G	L	O	G	Q	R	G	E	R	G	F	O	G	L	O	G	P	S
Iα1	*Bos Taurus*	G	**I**	A	G	Q	R	G	**V**	V	G	L	O	G	Q	R	G	E	R	G	F	O	G	L	O	G	P	S
Iα2	*Bos Taurus*	G	L	**L**	G	**A**	**O**	G	**F**	**L**	G	L	O	G	**S**	R	G	E	R	G	**L**	O	G	**V**	**A**	G	**S**	**V**

FIGURE 6. **Identification of CNE-binding collagen sequences.** The validity of using collagen type II peptides for comparative analysis was tested by collagen type II gel contraction. *A*, C2C12 cells did not contract collagen type II gels in the absence of exogenous factors. However, addition of 20 ng/ml of PDGF-BB induced contraction of the gels to a similar extent as observed for collagen type I gels (Fig. 2). This effect was abrogated by addition of 350 nM CNE. *B*, wells were coated with synthetic triple helical collagen peptides from Toolkit II and blocked with BSA as described under "Experimental Procedures." Wells were incubated with 2 μM CNE-biotin and bound CNE was detected using an avidin-HRP conjugate. Among the Toolkit II peptides, CNE bound with the highest affinity to peptide II-44 (5-fold signal-to-background ratio). Peptide II-1 was bound with intermediate affinity (3-fold signal-to-background ratio), whereas peptides II-7, II-13, II-17, II-35, II-45, and II-48 were bound with $<$2-fold signal-to-background ratio. The Gly-Pro-Pro decamer (GPP_{10}) was used as negative control. Shown values are signal-to-background ratios (A_{450}) where binding to GPP_{10} was set to 1. The *horizontal line* represents the GPP_{10} value for easy comparisons. Values for each peptide are averages of six samples and *error bars* are S.D. For the Toolkit II peptide library sequence, see Ref. 26. *C*, C2C12 cells were allowed to adhere to plates coated with 10 μg/ml of peptides II-1, II-44, or GPP_{10} and blocked with 2% BSA. Cells adhered poorly to peptide II-1, similarly as their adhesion to control peptide GPP_{10}. However, peptide II-44 supported efficient adhesion of C2C12 cells. *D*, the representative amino acid sequences within peptides II-1 and II-44 in human collagen IIα1 chains are identical to those in bovine collagen IIα1 chains. Compared with human and bovine collagen IIα1, bovine collagen Iα1 has 4 mismatches in peptide II-1 (85% homology), of which 2 mismatches are within the unique middle region of the peptide. For peptide II-44, 2 mismatches are found (93% homology) of which none are found within the unique middle region. Mismatches to the collagen type II peptides are *bold*.

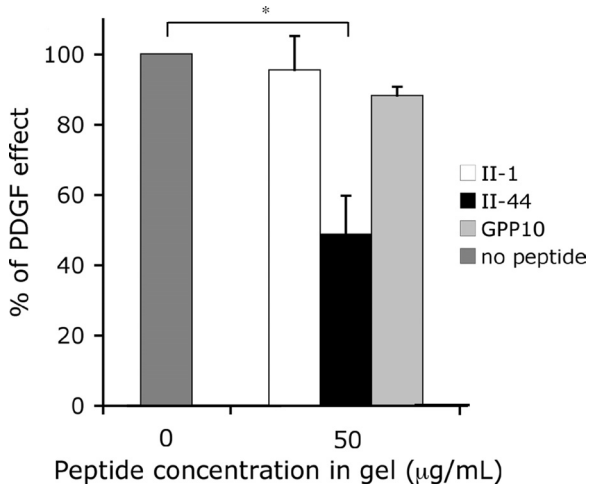


FIGURE 7. **Peptide II-44 inhibits $\alpha_V\beta_3$-dependent contraction and peptide II-1 inhibits fibril formation.** C2C12 cells were seeded in collagen type I gels in the presence or absence of peptides II-1, II-44, and GPP_{10} at a final concentration of 50 μg/ml. Gels were floated with a final concentration of 20 ng/ml of PDGF-BB. Addition of peptide II-1 had no significant effect on the PDGF-BB effect, whereas peptide II-44 reduced the effect with more than 50%. Control peptide GPP_{10} had a significant but small inhibitory effect on the PDGF-BB effect. The average magnitude of contraction by addition of 20 ng/ml of PDGF-BB in the absence of peptides was set to 100% and defined as the gel area of control gels minus gel area of PDGF-BB-treated gels after a 24-h incubation. *Error bars* are S.E. * indicates $p < 0.05$ (Student's *t* test).

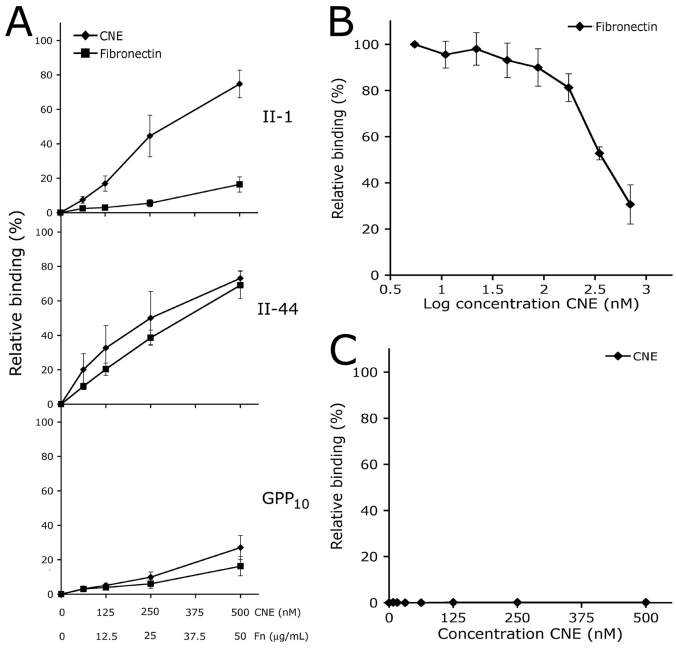


FIGURE 8. **Fibronectin and CNE compete for binding to collagen.** *A*, increasing concentrations of biotinylated fibronectin or biotinylated CNE were incubated with coatings of peptide II-1 or II-44 (1 μg/well) for 2 h. After washing, bound proteins were detected with avidin-alkaline phosphatase. Both fibronectin and CNE did not bind to the control peptide GPP_{10}. Biotinylated fibronectin bound significantly to peptide II-44, whereas binding to peptide II-1 was of similar magnitude as binding to GPP_{10}. CNE bound to both peptides II-1 and II-44. Binding was normalized where "100%" binding was set to the amount of binding of the respective biotinylated proteins to a coating of 1 μg of native collagen type I. *B*, microtiter plates were coated with native collagen type I (10 μg/ml). Coatings were incubated with 0.23 μg/well of biotinylated fibronectin in the presence of increasing concentrations of CNE. CNE inhibited binding of biotinylated fibronectin to collagen type I in a dose-dependent manner with half-maximum inhibition at a concentration of around 350 nM. *C*, microtiter plates were coated with human plasma fibronectin (50 μg/ml) and incubated with different amounts of biotinylated CNE. Biotinylated CNE did not bind to immobilized fibronectin, even at the highest concentration tested. Values in all panels are averages of three experiments and *error bars* are S.E.

BB-induced adhesion suggesting a role for fibronectin in $\alpha_V\beta_3$-mediated PDGF BB-induced adhesion to native collagen (Fig. 9*A*). Note that the control and PDGF-BB values in Fig. 9*A* are identical to those shown in Fig. 5*B*. To investigate the requirement of endogenously produced fibronectin for PDGF BB-induced C2C12 cell-mediated collagen gel contraction we took the approach to down-regulate expression of fibronectin in these cells by siRNA. siRNA directed against fibronectin knocked down protein levels of fibronectin in C2C12 cells to around 35% as compared with a scrambled oligonucleotide (siRNA-) or transfection agent alone (N-TER) (Fig. 9*B*). siRNA-transfected cells were put in collagen gels and gel diameters were measured after 24 h. Fibronectin siRNA inhibited PDGF BB-induced contraction by C2C12 cells, whereas siRNA- and N-TER still allowed for reduction of gel diameter after PDGF-BB stimulation (Fig. 9*C*). These results demonstrate that fibronectin is required for efficient PDGF BB-induced $\alpha_V\beta_3$-mediated collagen gel contraction.

DISCUSSION

We have investigated cell-mediated integrin $\alpha_V\beta_3$-dependent collagen gel contraction and adhesion using the collagen-binding protein CNE from *S. equi* subspecies *equi*. This bacterial cell-surface protein bound native fibrillar collagen types I, II, and III with high affinities (apparent K_d values ranging from 50 to 125 nM) but not denatured collagens. Based on the finding that CNE specifically inhibited $\alpha_V\beta_3$ integrin-mediated contraction and adhesion to native collagen, as well as fibrillogenesis, we reasoned that identification of the site(s) in collagen to which CNE binds might offer new insight into these processes. In our efforts to detect the CNE-binding sites we made use of the previously described collagen type II Toolkit (26, 29). CNE bound effectively to two peptides from Toolkit II and to a few additional peptides but with low signal to noise ratio. The two high affinity peptides were located at the N terminus of the triple helical part of collagen type II, *i.e.* peptide II-1, and three quarters toward the C terminus, *i.e.* peptide II-44.

The inhibitory effect of CNE on integrin $\alpha_V\beta_3$-mediated collagen gel contraction could potentially have been due to one or more of several possibilities. Thus, the effect could have been due to the fact that CNE binds and impairs function of $\alpha_V\beta_3$, this possibility could be ruled out by the finding that CNE had no effect on adhesion of cells to vitronectin, a process that is strictly dependent on $\alpha_V\beta_3$. Furthermore, because $\alpha_V\beta_3$-mediated contraction had to be induced by PDGF-BB, the inhibitory effect could have been due to that CNE negatively affected ligand binding or activation of the PDGF receptors. The effect of PDGF-BB on $\alpha_V\beta_3$-mediated contraction required activation of PDGF receptors because the tyrosine kinase inhibitor AG1296 blocked PDGF BB-induced contraction by C2C12 cells (supplemental Fig. S2*A*). However, CNE at a concentration that inhibited PDGF BB-induced contraction had no effect on PDGF β-receptor phosphorylation (supplemental Fig. S2*B*). PDGF BB-induced $\alpha_V\beta_3$ integrin-mediated contraction was furthermore, not likely dependent on changes in cell surface

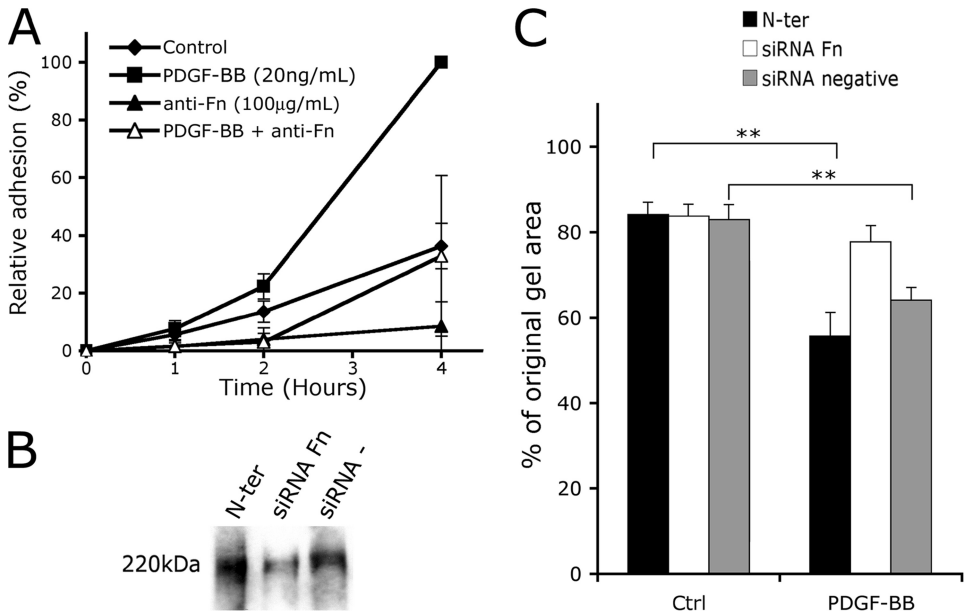


FIGURE 9. **PDGF BB-induced collagen gel contraction and adhesion to collagen are dependent on fibronectin.** *A*, C2C12 cells were allowed to adhere to a coating of collagen type I in the presence or absence of PDGF-BB (20 ng/ml) and presence or absence of anti-fibronectin IgG (100 μg/ml) for the indicated times. Anti-fibronectin IgG significantly inhibited both control- and PDGF BB-stimulated adhesion of C2C12 cells to collagen. *B*, transfection with siRNA directed against murine fibronectin specifically reduced fibronectin synthesis in C2C12 cells to around 35% (as according to pixel intensity) compared with mock-transfected cells. *C*, siRNA-transfected C2C12 cells were subjected to collagen gel contraction. When compared with mock-transfected cells, cells transfected with siRNA directed toward fibronectin were significantly less responsive to PDGF BB as measured after 24 h. In *A*, control and PDGF-BB values are identical to the values shown in Fig. 2*B*. Other values are average of two experiments and *error bars* are S.E. In *B*, values are the average gel area after 24 h contraction of 5 experiments and *error bars* are S.E. ** indicates $p < 0.01$ (Student's *t* test).

expression of β_1, β_3, or α_5 integrin subunits because stimulation of C2C12 cells with PDGF-BB did not show differences in expression levels of these integrins (supplemental Fig. S2, *C* and *D*). The possibility that the effect of CNE on $\alpha_V\beta_3$-mediated collagen gel contraction was restricted to C2C12 cells or to cells lacking collagen-binding β_1 integrins seems less likely based on collagen gel contraction experiments using AG1518 fibroblasts that effectively contract collagen gels also in the absence of exogenous stimulators and that utilize collagen-binding β_1 integrins for this contraction. Monoclonal anti-β_1 integrin IgG inhibited contraction mediated by human AG1518 diploid fibroblasts, an effect that could be overcome by PDGF-BB. This effect could in turn be abolished either by CNE or a cyclic peptide that blocks $\alpha_V\beta_3$-mediated cell interactions (supplemental Fig. S1*B*). CNE had, however, no effect on contraction by AG1518 cells in the absence of anti-β_1 integrin IgG (supplemental Fig. S1*A*). Together, our data show that CNE specifically inhibits $\alpha_V\beta_3$-mediated contraction by binding to the native collagen fibers. The fact that peptide II-44 but not peptide II-1 supported adhesion of C2C12 cells and that soluble peptide II-44 but not peptide II-1 inhibited $\alpha_V\beta_3$ integrin-mediated contraction of collagen type I gels suggests that this site, recognized by CNE, constitutes a major recognition site in collagen for $\alpha_V\beta_3$ integrin-mediated cell interactions.

Previous studies using Toolkits have identified several proteins that bind to peptide II-44, including DDR2 and SPARC (osteonectin) (26, 28). MMPs are also known to bind and cleave collagen at this position, within the first few residues of peptide II-44. In the present studies we have presented evidence that argues against involvement of MMPs in PDGF BB-induced $\alpha_V\beta_3$-mediated contraction. In fact, inhibition of MMPs rather stimulated contraction, potentially by protecting the collagen fibers from cleavage during the contraction process, suggesting that contraction is optimally executed when collagen fibers are non-cleaved. Furthermore, our data speak against the idea that $\alpha_V\beta_3$-directed adhesion depended on exposed RGD sequences. This is based on the fact that peptide II-44 lacks RGD sequences. It remains possible that GM6001 exerts its effect here by displacing MMPs from collagen, allowing enhanced fibronectin binding and in consequence, greater gel contraction. We can, however, not exclude a role for DDRs in $\alpha_V\beta_3$-directed contraction. It is possible that DDR1 or -2 participates in activation of $\alpha_V\beta_3$ on C2C12 cells; however, data showing that DDR receptors do not activate β_1-integrins on C2C12 cells have been reported (30). This speaks against the idea of a general mechanism for contraction based on activation of integrins by DDRs.

The observation that CNE inhibited fibrillogenesis of pepsin-solubilized collagen opens the possibility that $\alpha_V\beta_3$-mediated collagen gel contraction depends on the fibrillar status of the collagen matrix. The data presented herein speak, however, against this possibility. First and most importantly, contraction mediated by $\alpha_2\beta_1$ occurred equally well in the presence of CNE as in its absence. Second, whereas soluble peptide II-44 inhibited $\alpha_V\beta_3$-mediated contraction it had little effect on fibril formation. Thus, even though CNE could modulate the biomechanical properties of collagen gels, it specifically inhibited collagen gel contraction mediated by $\alpha_V\beta_3$ integrins, whereas it did not affect contraction mediated by collagen-binding β_1 integrins. Because CNE bound native collagens, this integrin-specific inhibition of contraction suggested that, during the respective contractile processes, $\alpha_2\beta_1$ and $\alpha_v\beta_3$ integrins would be recruited to different domains on native collagen. Indeed, the key $\alpha_2\beta_1$-binding sites, GLOGER, GFOGER, and GMOGER (31), are located in peptides II-7/8, II-28, and II-31, respectively, whereas $\alpha_V\beta_3$ is considered to bind the collagen triple helix only indirectly, as described here.

Many studies have reported on the effects of PDGF-BB on cells, including increased cytoskeletal dynamics (32) and synthesis of ECM proteins including fibronectin (8). The $\alpha_V\beta_3$ integrin-mediated attachment of cells to native collagen was increased by stimulation of the cells with PDGF-BB and proceeded only after a lag phase of around 120 min. This is in sharp contrast to attachment mediated by $\alpha_2\beta_1$ that typically was completed within 30–60 min. Furthermore, $\alpha_2\beta_1$-mediated

contraction and adhesion proceeded in the absence of PDGF-BB. Data presented herein indicate that *de novo* synthesis of fibronectin is required for $\alpha_V\beta_3$-directed and PDGF-BB-induced contraction. Because PDGF-BB is known to induce production of fibronectin (8), it is thus possible that PDGF-BB induced the synthesis of fibronectin that has either a bridging or adaptive function. Our data strongly suggest that $\alpha_V\beta_3$ integrin-mediated contraction by C2C12 cells depends on binding of endogenously produced fibronectin to a site in collagen corresponding to peptide II-44. These findings are in agreement with that one fibronectin-binding site in collagen located adjacent to the MMP-binding site and thus to the region encompassing peptide II-44 (33–35). It is noteworthy that adhesion of cells to plates coated with peptide II-44 occurred effectively also without stimulation of cells by PDGF-BB. This is most likely due to that in the absence of exogenous factors C2C12 cells express low, but sufficient amounts of cell-surface fibronectin that allow for attachment to coated substrates offering high-density binding sites. Previous studies from our laboratory show that suspended C2C12 cells indeed express cell-surface fibronectin (36), in line with such an assumption. In light of this, a low density of fibronectin-binding sites in fibrillar collagen gels could reflect the requirement for PDGF-BB to increase availability of fibronectin in $\alpha_V\beta_3$-directed contraction. Therefore, we suggest that induction of an increase in fibronectin expression leading to supportive cell-collagen fiber adhesions, together with increased cytoskeletal dynamics brought about by PDGF-BB could possibly be the mechanistic background to the effect of PDGF-BB on C2C12 cell-mediated collagen gel contraction. Fibronectin is present in physiological and pathological connective tissue compartments. Studies are ongoing to determine whether fibronectin is also required during $\alpha_V\beta_3$-directed normalization of IFP *in vivo*.

In a previous report we showed that the secreted collagen and fibronectin-binding protein FNE from *S. equi* has a possible function in blocking inflammatory driven edema formation that is part of the innate immune response. CNE would function in the opposite direction: by blocking $\alpha_V\beta_3$-dependent normalization of the lowered IFP, CNE would promote long standing edema. It is possible that bacteria can modulate edema response in either direction by expressing and shedding different surface components thereby modeling tissue responses during different phases of the infection.

Acknowledgment—We thank Viveka Tillgren for expert technical assistance.

REFERENCES

1. Herr, A. B., and Farndale, R. W. (2009) *J. Biol. Chem.* **284,** 19781–19785
2. Sweeney, S. M., Orgel, J. P., Fertala, A., McAuliffe, J. D., Turner, K. R., Di Lullo, G. A., Chen, S., Antipova, O., Perumal, S., Ala-Kokko, L., Forlino, A., Cabral, W. A., Barnes, A. M., Marini, J. C., and San Antonio, J. D. (2008) *J. Biol. Chem.* **283,** 21187–21197
3. Farndale, R. W., Lisman, T., Bihan, D., Hamaia, S., Smerling, C. S., Pugh, N., Konitsiotis, A., Leitinger, B., de Groot, P. G., Jarvis, G. E., and Raynal, N. (2008) *Biochem. Soc. Trans.* **36,** 241–250
4. Aukland, K., and Reed, R. K. (1993) *Physiol. Rev.* **73,** 1–78
5. Reed, R. K., Lidén, A., and Rubin, K. (2010) *J. Mol. Cell Cardiol* **48,** 518–523
6. Berg, A., Ekwall, A. K., Rubin, K., Stjernschantz, J., and Reed, R. K. (1998) *Am. J. Physiol.* **274,** H663–671
7. Ahlén, K., Berg, A., Stiger, F., Tengholm, A., Siegbahn, A., Gylfe, E., Reed, R. K., and Rubin, K. (1998) *Cell Adhes. Commun.* **5,** 461–473
8. Lidén, A., van Wieringen, T., Lannergård, J., Kassner, A., Heinegård, D., Reed, R. K., Guss, B., and Rubin, K. (2008) *J. Biol. Chem.* **283,** 1234–1242
9. Grinnell, F. (2003) *Trends Cell Biol.* **13,** 264–269
10. Gullberg, D., Tingström, A., Thuresson, A. C., Olsson, L., Terracio, L., Borg, T. K., and Rubin, K. (1990) *Exp. Cell Res.* **186,** 264–272
11. Tingström, A., Heldin, C. H., and Rubin, K. (1992) *J. Cell Sci.* **102,** 315–322
12. Langholz, O., Röckel, D., Mauch, C., Kozlowska, E., Bank, I., Krieg, T., and Eckes, B. (1995) *J. Cell Biol.* **131,** 1903–1915
13. Rodt, S. A., Ahlén, K., Berg, A., Rubin, K., and Reed, R. K. (1996) *J. Physiol.* **495,** 193–200
14. Cooke, M. E., Sakai, T., and Mosher, D. F. (2000) *J. Cell Sci.* **113,** 2375–2383
15. Grundström, G., Mosher, D. F., Sakai, T., and Rubin, K. (2003) *Exp. Cell Res.* **291,** 463–473
16. Lidén, A., Berg, A., Nedrebø, T., Reed, R. K., and Rubin, K. (2006) *Circ. Res.* **98,** 635–641
17. Svendsen, O. S., Lidén, A., Nedrebø, T., Rubin, K., and Reed, R. K. (2008) *Am. J. Physiol. Heart Circ. Physiol.* **295,** H555–560
18. Lannergård, J., Frykberg, L., and Guss, B. (2003) *FEMS Microbiol. Lett.* **222,** 69–74
19. Tiger, C. F., Fougerousse, F., Grundström, G., Velling, T., and Gullberg, D. (2001) *Dev. Biol.* **237,** 116–129
20. Miller, E. J., and Rhodes, R. K. (1982) *Methods Enzymol.* **82,** 33–64
21. Rubin, K., Johansson, S., Höök, M., and Obrink, B. (1981) *Exp. Cell Res.* **135,** 127–135
22. Lundgren, E., Gullberg, D., Rubin, K., Borg, T. K., Terracio, M. J., and Terracio, L. (1988) *J. Cell. Physiol.* **136,** 43–53
23. Miekka, S. I., Ingham, K. C., and Menache, D. (1982) *Thromb. Res.* **27,** 1–14
24. Hayashi, M., Akama, T., Kono, I., and Kashiwagi, H. (1985) *J. Biochem.* **98,** 1135–1138
25. Gullberg, D., Terracio, L., Borg, T. K., and Rubin, K. (1989) *J. Biol. Chem.* **264,** 12686–12694
26. Konitsiotis, A. D., Raynal, N., Bihan, D., Hohenester, E., Farndale, R. W., and Leitinger, B. (2008) *J. Biol. Chem.* **283,** 6861–6868
27. Landegren, U. (1984) *J. Immunol. Methods* **67,** 379–388
28. Giudici, C., Raynal, N., Wiedemann, H., Cabral, W. A., Marini, J. C., Timpl, R., Bächinger, H. P., Farndale, R. W., Sasaki, T., and Tenni, R. (2008) *J. Biol. Chem.* **283,** 19551–19560
29. Raynal, N., Hamaia, S. W., Siljander, P. R., Maddox, B., Peachey, A. R., Fernandez, R., Foley, L. J., Slatter, D. A., Jarvis, G. E., and Farndale, R. W. (2006) *J. Biol. Chem.* **281,** 3821–3831
30. Vogel, W., Brakebusch, C., Fässler, R., Alves, F., Ruggiero, F., and Pawson, T. (2000) *J. Biol. Chem.* **275,** 5779–5784
31. Siljander, P. R., Hamaia, S., Peachey, A. R., Slatter, D. A., Smethurst, P. A., Ouwehand, W. H., Knight, C. G., and Farndale, R. W. (2004) *J. Biol. Chem.* **279,** 47763–47772
32. van Wieringen, T., Kimani, S. G., Hultgård-Ekwall, A. K., Forsberg, J., Reyhani, V., Engström, A., and Rubin, K. (2009) *Exp. Cell Res.* **315,** 1745–1758
33. Hohenester, E., Sasaki, T., Giudici, C., Farndale, R. W., and Bächinger, H. P. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105,** 18273–18277
34. Kleinman, H. K., McGoodwin, E. B., Martin, G. R., Klebe, R. J., Fietzek, P. P., and Woolley, D. E. (1978) *J. Biol. Chem.* **253,** 5642–5646
35. Dzamba, B. J., Wu, H., Jaenisch, R., and Peters, D. M. (1993) *J. Cell Biol.* **121,** 1165–1172
36. Lidén, A., Karlström, A., Lannergård, J., Kalamajski, S., Guss, B., Rubin, K., and Rydén, C. (2006) *Biochem. Biophys. Res. Commun.* **340,** 604–610