Definition of the Native and Denatured Type II Collagen Binding Site for Fibronectin Using a Recombinant Collagen System*

Received for publication, October 28, 2013, and in revised form, December 7, 2013. Published, JBC Papers in Press, December 29, 2013, DOI 10.1074/jbc.M113.530808

Bo An1, Vittorio Abbonante5, Sezin Yigit1, Alessandra Balduini1‡, David L. Kaplan1‡, and Barbara Brodsky1‡

From the Department of Biomedical Engineering, Tufts University, Medford, Massachusetts 02155 and the Department of Molecular Medicine, Biotechnology Research Laboratories, Istituto di Ricerca e Cura a Carattere Scientifico (IRCCS) San Matteo Foundation, University of Pavia, 27100 Pavia, Italy

Background: Sequence requirements for triple-helical collagen to bind fibronectin are not fully understood.

Results: Fibronectin affinity was conferred on a recombinant bacterial collagen by incorporating specific human collagen II sequences.

Conclusion: The chimeric collagen defines the minimum collagen II sequence required for fibronectin affinity.

Significance: This system is useful to investigate sequence/activity relationships for triple-helical collagen and to generate scalable bioactive materials.

Interaction of collagen with fibronectin is important for extracellular matrix assembly and regulation of cellular processes. A fibronectin-binding region in collagen was identified using unfolded fragments, but it is not clear if the native protein binds fibronectin with the same primary sequence. A recombinant bacterial collagen is utilized to characterize the sequence requirement for fibronectin binding. Chimeric collagens were generated by inserting the putative fibronectin-binding region from human collagen into the bacterial collagen sequence. Insertion of a sufficient length of human sequence conferred fibronectin affinity. The minimum sequence requirement was identified as a 6-triplet sequence near the unique collagenase cleavage site and was the same in both triple-helix and denatured states. Denaturation of the chimeric collagen increased its affinity for fibronectin, as seen for mammalian collagens. The fibronectin binding recombinant collagen did not contain hydroxyproline, indicating hydroxyproline is not essential for binding. However, its absence may account, in part, for the higher affinity of the native chimeric protein and the lower affinity of the denatured protein compared with type II collagen. Megakaryocytes cultured on chimeric collagen with fibronectin affinity showed improved adhesion and differentiation, suggesting a strategy for generating bioactive materials in biomedical applications.

Collagen, the most abundant protein in the human body, is distinguished by its signature triple-helical structure based on a (Gly-Xaa-Yaa) repeating sequence (1). So far, 28 types of collagens have been described (2–4). The fibrillar type I collagen, which constitutes more than 90% of the collagen in the body, forms characteristic fibrils with an axial periodicity of 67 nm in tendon, bone, and skin. Type I collagen is a heterotrimer with two α1(II) chains and one α2(I) chain. Type II collagen forms fibrils with the same periodicity in cartilage and vitreous (5, 6), and is a homotrimer composed of three α1(II) chains, which show a high sequence homology to α1(I). Collagen fibrils provide physical support for cells and tissues, and also have a major impact on various cellular processes, such as adhesion, growth, migration, and differentiation. These biological functions are based on the interaction of collagen with various cell surface receptors and with other extracellular matrix proteins (7, 8). An understanding of how extracellular matrix molecules interact and influence each other can shed light on key aspects of extracellular matrix formation, remodeling, and tissue repair. One important example is the interaction between collagen and fibronectin, another abundant matrix protein implicated in cell proliferation as well as matrix assembly.

Fibronectin (Fn) is a large dimeric glycoprotein containing modular domains that mediate self-assembly, binding to cell surface receptors, and interactions with collagen and other extracellular matrix molecules (9, 10). A range of biochemical studies defined a 42-kDa domain containing six modules, 6FnI1–2FnII7FnI and 8–9FnI, to be the gelatin-binding domain (GBD) (11–13), due to its ability to strongly and specifically bind to denatured collagen (gelatin). Two separate fragments of the GBD, 4FnI1–2FnII7FnI and 8–9FnI, have been shown to independently bind denatured collagen, but with a decreased affinity (14–17). High resolution structures have been determined by NMR and x-ray crystallography for the 6FnI1–2FnII7FnI and 8–9FnI subfragments (15, 16), and recently a crystal structure was reported for the zinc-mediated dimer of the intact GBD as well (18).

* This work was supported, in whole or in part, by National Institutes of Health Grant EB01620 (to B. B. and D. K.).
1 To whom correspondence may be addressed. E-mail: david.kaplan@tufts.edu.
2 To whom correspondence may be addressed. E-mail: barbara.brodsky@tufts.edu.
3 The abbreviations used are: FN, fibronectin; GBD, gelatin-binding domain; Hyp, hydroxyproline; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-[hydroxymethyl]propane-1,3-diol; DSC, differential scanning calorimetry; MK, megakaryocytes; CL, collagen-like; SCI-Fn, streptococcal collagen-like protein with Fn-binding site.
Fibronectin Binding in a Bacterial Collagen System

The specific region within collagen responsible for binding to Fn and the nature of this interaction has been a long-standing area of investigation. More than 30 years ago, it was demonstrated that Fn binds denatured collagen more tightly than the native triple-helical form (13, 19), and the binding site on the α1 chain of type I collagen was localized to a cyanogen bromide peptide that includes the collagenase (MMP-1) cleavage site (11, 20). Recently, important advances have been made in defining the interaction of denatured collagen peptides with Fn, using NMR and x-ray crystallography. Short synthetic (Gly-Xaa-Yaa)3 peptides containing sequences from either the α1(I) or α2(I) chains have been employed to further define the required collagen sequence and mode of Fn binding (15–17). NMR monitoring of the perturbation of signals from individual residues within 8–9FnI or 9FnI–2FnII2FnI modules showed that a 24-residue gelatin peptide (α1(I) Gly778–Gly799) bound tightly, and a crystal structure of the 8–9Fn module segment shows residues 784–806QLGQQRGER bound within the longer peptide form an anti-parallel β-strand that interacts with a β-sheet in 8Fn1 (15).

Although the binding of Fn to denatured collagen has been well characterized, the binding of Fn to native collagen has been more difficult to define. Native triple-helical collagen has been shown to interact with Fn by a range of techniques, including rotary shadowing (21), sucrose gradient centrifugation (21), and solid-state binding assays (19), but it is not clear if the same Gly-Xaa-Yaa sequence binds in the triple-helical versus the denatured states. Here, a recombinant bacterial collagen-like protein system is employed to characterize the amino acid sequences in native and denatured collagen required for Fn binding. A collagen-like protein found in Streptococcus pyogenes, which is readily expressed and easily modified in Escherichia coli, was previously shown to have a triple helix structure and stability similar to that of human fibrillar collagens, even though post-translationally formed hydroxyproline (Hyp) is absent (22–24).

Chimeric collagen-like proteins were generated by inserting potential Fn binding sequences from human collagen type II between bacterial collagen modules. The binding of these chimeric constructs in their native and denatured states to Fn was investigated using solid-state binding assays to define the minimal required sequence. The results obtained from the recombinant bacterial collagens allowed comparison with previous studies done on mammalian collagen fragments as well as synthetic collagen-like peptides. Chimeric collagens with in vitro Fn binding ability were also shown to promote adhesion and differentiation of human megakaryocytes, suggesting these recombinant collagens could be developed as biomaterials for biomedical applications.

EXPERIMENTAL PROCEDURES

Molecular Cloning—The protein sequence for the bacterial collagen SCI constructs was based on the Scl2.28 sequence from S. pyogenes with DNA codon optimized for E. coli expression. A His6 tag was introduced at the N terminus for purification purposes. The full DNA sequence encoding the SCI protein was synthesized at Biomatik Corp. (Wilmington, DE). Oligonucleotides encoding various lengths of the type II collagen Fn-binding region were designed and synthesized (Invitrogen). Annealed dsDNA were inserted between the two collagen-like (CL) domains of the SCI constructs through restriction sites XmaI and Apal predesigned in the sequence. The final constructs containing the Fn-binding sites were cloned into the pColdIII vector (Takara Bio Inc.) through NdeI and XbaI restriction sites. All enzymes for cloning were purchased from New England Biolabs (Ipswich, MA). DNA sequencing to confirm fidelity was carried out at the Tufts Core Facility. The DNA constructs and their subsequent proteins were denoted as SCI, for Streptococcus collagen-like proteins, and those recombinant proteins containing type II collagen sequences predicted to binding Fn denoted as SCI-Fn. To define the length of the insertion of type II collagen, we use SCI-FnG784-R792, with # representing the starting residue and #’ representing the final residue of the inserted type II collagen sequence. For example, the putative GLPGQQRGER sequence is located in the type II collagen triple-helix region from residue Gly784 to Arg792, and the recombinant bacterial VCL.CL construct containing this sequence between the two CL domains is denoted as SCI-FnG784-R792.

Protein Purification—All SCI constructs in the pColdIII vector were expressed in E. coli BL21 strain, grown in 20 ml of LB medium with 100 μg/ml of ampicillin overnight at 37 °C. This starting culture was used to inoculate 500 ml of LB-ampicillin media in a shaking flask and grown at 37 °C to an A600 nm = 0.8. To induce protein expression, 1 mM isopropyl β-D-thiogalactopyranoside was added to the culture and the temperature was lowered to 22 °C. After 16 h induction, cells were harvested by centrifugation and resuspended in a His tag purification column binding buffer (20 mM sodium phosphate buffer, pH 7.4, 500 mM NaCl, 10 mM imidazole) containing 0.25 mg/ml of lysozyme and frozen at 80 °C until purification. The expression protocol was modified to decrease the level of truncated products in SCI-FnG769-R789 and SCI-FnG769-R792 expression by using HyperBroth™ (Athena Environmental Sciences, Inc., Baltimore, MD) instead of LB broth, and performing isopropyl β-D-thiogalactopyranoside induction for 4 h. Protease inhibitor mixture (Sigma) was also added to binding buffer during cell resuspension.

Purification of the His-tagged SCI-Fn was carried out by gravity-driven immobilized metal ion affinity chromatography with nickel ion binding and imidazole gradient elution. Frozen cells were thawed and further lysed by sonication. Cellular debris was removed by centrifugation at 8,228 × g at 4 °C. Supernatant containing the soluble target protein was loaded onto equilibrated purification columns packed with nickel-nitrilotriacetic acid beads (Qiagen, Valencia, CA) and run through the column by gravity. The column was then washed sequentially with 2 bed volumes of binding buffer, binding buffer plus 60 mM imidazole, and binding buffer plus 120 mM imidazole. His-tagged protein on the column was eluted by elution buffer (binding buffer plus 400 mM imidazole). Protein purity was checked by SDS-PAGE (NuPAGE™ BisTris 4–12%, Invitrogen) and molecular weight was determined by MALDI-TOF MS. The concentration of protein was determined using an extinction coefficient of ε280 = 9970 M−1 cm−1 after dialysis into phosphate-buffered saline (PBS, pH 7.4). Chemicals used in all experiments are purchased from Sigma, unless otherwise indicated.
Circular Dichroism (CD) Spectroscopy—CD spectra were obtained on AVIV Model 420 CD spectrometer (AVIV Biomedical, Lakewood, NJ) using glass cuvettes with 1-mm path length. Protein solutions were equilibrated for at least 24 h at 4 °C before measurement. Wavelength scans were collected from 190 to 260 nm in 0.5-nm steps with a 4-s averaging time, 1.0-nm bandwidth and repeated three times. Temperature scans were monitored from 15 to 70 °C at 225 nm with a 10-s averaging time and 1.5-nm bandwidth. Samples were equilibrated for 2 min at each temperature, and the temperature was increased at an average rate of 0.1 °C/min.

Differential Scanning Calorimetry (DSC)—DSC was performed on a NANO DSC II model 6100 (Calorimetry Sciences Corp, Lindon, UT). Each sample was re-dialyzed against PBS overnight before measurement to collect the dialyzed buffer as reference in the experiment. Sample solutions were loaded at 0 °C into the cell and heated at a rate of 1 °C/min to 100 °C.

Mass Spectrometry (MS)—Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were acquired on a Microflex LT system (Bruker Corporation, Billerica, MA) with 70% laser intensity using standard LP (linear positive) 60-kDa method provided by the software. MALDI matrix was prepared by saturating sinapinic acid in solution containing 50% (v/v) acetonitrile and 0.3% (v/v) trifluoroacetic acid. A 6-µl aliquot of a 1 mg/ml sample was mixed with 24 µl of matrix, 1 µl of solution was plated onto a 96-spot target plate and allowed to dry.

Trypsin Cleavage Assay—Purified SCI-Fn in PBS buffer was incubated with 0.01 mg/ml (430 nM) of trypsin at 25 °C for 30 min, 60 min, 180 min, and overnight. The reaction was stopped by adding phenylmethylsulfonyl fluoride (PMSF) to 1 mM. Cleavage was visualized on an SDS-PAGE.

Fibronectin Binding Assay—The binding of Fn to immobilized SCI-Fn were measured using a solid-state binding assay. 50 µl of 10 or 100 µg of SCI-Fn proteins were coated onto high binding 96-well assay plates (R&D Systems, Minneapolis, MN) overnight at 4 °C. Denatured samples were first incubated in a 90 °C water bath for 30 min before coating. Plates were blocked with 3% BSA for 1 h at RT. 50 µl of human plasma Fn (Millipore FC010–10MG) was subsequently added to each collagen-coated well at a concentration of 100 µg/ml and incubated for 2 h. For dose-response assay, series concentrations of Fn at 100, 60, 20, 6, 2, 0.6, and 0 µg/ml were used. For the gelatin inhibition assay, 100 µg/ml of fibronectin was mixed with 100 µg/ml of denatured collagen type II overnight at 4 °C before being incubated on the collagen-coated plates. Bound Fn on plates were detected by mouse anti-human Fn C-terminal antibody (or mouse anti-human Fn GBD antibody for competitive binding assay, Millipore MAB1935 and MAB1892) at a 1:1000 dilution for 1 h. Secondary anti-mouse HRP antibody (Santa Cruz) at 1:3000 dilution was incubated for 1 h and 50 µl of 3,3,5,5-tetramethylbenzidine solution (Invitrogen) was added for the colorimetric reaction. Plates were washed by 200 µl of PBST 6 times between every step. Color was allowed to develop at room temperature for 5 min and 50 µl of 1 N HCl was added to stop the reaction. A 494 nm was recorded from the 96-well plate using a Spectra Max M2 plate reader (Molecular Devices, Sunnyvale, CA) for data analysis. Appropriate controls (bovine collagen type II and SCI with no insertion) were included using the same setup. All assays were performed in triplicates. Human cellular Fn (Sigma F2518) was also tested in the above mentioned binding assay, no obvious differences were observed compared with human plasma fibronectin, consistent with an early study (19). This is likely due to the fact that they share the same GBD.

Megakaryocyte Cell Cultures—Human umbilical cord blood was purchased from The Cord Blood Bank of New York (Long Island, NY). Megakaryocytes (MK) were differentiated as previously described (25). Briefly, mononuclear cells were separated by layering blood onto Lympholyte (<1077 g/ml, Cedarlane, Hornby, Canada). Cells were then washed twice in phosphate-buffered saline (PBS) and CD34+ cells were separated by the immunomagnetic bead selection technique (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions. CD34+ cells were then cultured in Stem Span medium (Stem-Cell Technologies, Vancouver, Canada), supplemented with 10 ng/ml of TPO, IL-6, and IL-11 (PeproTech, Rocky Hill, NJ) at 37 °C in a 5% CO2 fully humidified atmosphere. At the end of the culture (13 days), 1 × 105 cells were collected and plated per well of a 24-well tissue culture plate. The plates were pretreated with 100 µg/ml of filter-sterilized SCI-FnG769,R792 and SCI-FnG769,R789 solution overnight. The plate was washed twice with PBS and subsequently incubated with 25 µg/ml of human cellular Fn (Sigma F2518). The plate was washed twice with PBS before being seeded with mature human MK.

Data Analysis—All quantitative analyses were performed in triplicate. Results presented were based on the averages of data points and S.D. as error bars. The significance level was determined by a p value using two sample paired Student’s t test between the means of two samples. Raw CD and MS data were processed and plotted in Origin 6.0 (MicroCal Inc.). Results of the binding assay were plotted in Excel 2013 (Microsoft Corporation) and Bmax and Ky values were calculated by fitting the plot to specific saturation binding equation: \( Y = \frac{B_{\text{max}} \times X}{K_d + X} \) in GraphPad Prism 6.0 (GraphPad Software Inc.) with the S.E. obtained from the curve fit.

Note—The human collagen type II sequence used for the recombinant collagen design is based on UniProt entry P02458. Amino acids in the collagen domain are numbered starting from the beginning of the triple-helical region (residues 201–1214 of entry P02458), based on typical collagen notations. “O” or “Hyp” denotes 4-hydroxyproline in the text.

RESULTS

Design and Expression of Chimeric Bacterial Collagens—A collagen-like protein in S. pyogenes (ScI2.28) contains an N-terminal trimerization domain (V domain) followed by a CL domain with 79 Gly-Xaa-Yaa triplets (22, 26, 27). A construct in which the collagen domain was duplicated, VCLCL, was cloned and has been used in this study as the host for human collagen sequence insertions that may mediate binding to Fn. Both VCL and VCLCL constructs form homotrimers with a typical triple-helix structure and a thermal stability of 36–37 °C. These proteins are produced in high yields in E. coli using a cold-shock expression system (28), and were purified on a His tag column. Prolyl hydroxylase is not present in E. coli, so the expressed
protein does not undergo any post-translational modification of Pro residues to Hyp.

The VCLCL construct, denoted as SCl, was modified by recombinant DNA technology to contain potential Fn binding sequences from human collagen, inserting \((\text{Gly-Xaa-Yaa})_n\) sequences, where \(n = 3\) to 8. Because the bacterial system produces homotrimers, sequences from the homotrimeric human \(\alpha_1(II)\) chain rather than the heterotrimeric type I collagen were inserted into the bacterial system (Fig. 1). The human collagen sequences were inserted between two tandem CL domains of bacterial collagen because this design was previously reported to be successful for conferring biological activity (24, 29). Recent studies by Erat et al. (15) indicated a peptide with residues 778–799 from the \(\alpha_1(II)\) chain of type I collagen bound tightly to Fn. These studies on denatured \(\alpha_1(II)\) collagen model peptides were used to guide the design of inserted human \(\alpha_1(II)\) sequences, because \(\alpha_1(II)\) and \(\alpha_1(I)\) are highly homologous in this region.

Initially, the three triplets, GLPGQRGER, shown to participate in the \(\beta\)-sheet with the \(\alpha_1(I)\) module were used to insert the chimeric collagen constructs into the bacterial collagen construct (FnG784-R792) to see if this interacting sequence was sufficient for binding Fn. In further constructs, the human type II sequence was extended in the N-terminal direction to insert 4, 5, and 6 tripleptides, creating constructs SCl-FnG778-R792 and SCl-FnG775-R792. A second set of proteins was constructed to explore the requirements at the C terminus, inserting 4, 6, 7, or 8 tripleptides from the \(\alpha_1(II)\) chain: SCl-FnG772-V783, SCl-FnG769-R786, SCl-FnG769-R789, and SCl-FnG769-R792 (Fig. 1).

The recombinant proteins were expressed in E. coli and purified through a nickel-nitrilotriacetic acid column (immobilized metal ion affinity chromatography), yielding ~50–100 mg/liter of chimeric collagen proteins. The purity of the proteins was confirmed by SDS-PAGE (Fig. 2A). All constructs showed one major band with the exception of the two proteins with the longest 7 and 8 triplet insertions, SCl-FnG769-R789 and SCl-FnG769-R792. On SDS-PAGE, these two proteins showed a major band that represented the full-length of SCl (VCLCL) constructs, together with one lower band corresponding to the size of VCL, suggesting that some portion had been degraded into VCL and CL fragments and/or that limited nutrients in the culture media caused premature termination of protein translation. The level of truncated fragments decreased after the protein production conditions were optimized (richer media: HyperBroth™; shorter induction time; and protease inhibitors during bacteria lysis), but these shorter proteins were never fully eliminated. MALDI-TOF confirmed the identity of all purified proteins (Fig. 2B, Table 1).

**Structural and Stability Characterization of the Chimeric Proteins**—The CD spectra of all chimeric proteins showed a characteristic maximum near 220 nm, indicating that they adopted a triple-helix conformation (Fig. 3A, Table 1). The constructs with the longest insertions generally had somewhat lower MRE\(_{220}\) values, which could reflect the presence of denatured fragments or a distortion of the triple-helix. The thermal stability of the triple-helix was very similar for all constructs. Monitoring the MRE\(_{220}\) versus temperature indicated a sharp thermal transition, with a \(T_m\) near 36.5 °C for all chimeric proteins, except those with the longest 7 and 8 tripeptide insertions, where \(T_m\) values were slightly lower (35.5 and 35.2 °C). The DSC profile of the SCl control showed a sharp transition near 38 °C, with a calorimetric enthalpy of 4200 kJ/mol (Fig. 3B, Table 1). The insertion of 3 tripeptides, GLPGQRGER, in SCl-FnG784-R792 gave a DSC profile with the same \(T_m\) value and only a slight decrease in enthalpy. However, when 6 or 8 tripleptides were inserted, the enthalpies were
Fibronectin Binding in a Bacterial Collagen System

lowered by \( \sim 400-500 \text{ kJ/mol} \). For both of these proteins, SCI-FnG775-R792 and SCI-FnG769-R792, a shoulder of lower temperature was visible in addition to the main peak, consistent with a small fraction of truncated or denatured Protein. The position of the major DSC thermal transition for SCI-FnG769-R792 also dropped \( -1^\circ\text{C} \) compared with the control, similar to the CD \( T_m \) results. All DSC transitions were at higher temperatures than the CD \( T_m \) values because of the faster heating rate (30).

Trypsin digestion was performed on three of the collagen constructs, SCI-FnG775-R792, SCI-FnG778-R792, SCI-FnG784-R792, to ascertain if the inserted human sequences maintained a tight triple-helix (Fig. 3C). The V domain of SCI was cleaved off quickly, yielding a slightly lower band on SDS-PAGE, corresponding to the size of a single CL domain. In contrast, SCI-FnG784-R792 with the 3 triplet insertions was not susceptible at the insertion site, with no single CL unit detectable on the gel.

Minimum Amino Acid Sequence Requirement for Fibronectin Binding—Solid-state binding assays were used to measure soluble Fn binding to solid-phase collagen adsorbed on a plastic surface. Such a solid-phase assay may mimic Fn interactions with insoluble collagen fiber bundles in the extracellular matrix (31, 32). These binding assays were carried out on all 8 chimeric constructs. The collagen samples were coated on ELISA plates and binding was assessed for two different Fn concentrations (Fig. 4A). The binding assays were performed at room temperature, and because all of these assays represent binding of Fn to native recombinant collagens. The collagen control SCl, to determine the minimum amino acid sequence requirement for Fn binding—solid-state binding assays were used to measure soluble Fn binding to solid-phase collagen adsorbed on a plastic surface. Such a solid-phase assay may mimic Fn interactions with insoluble collagen fiber bundles in the extracellular matrix (31, 32). These binding assays were carried out on all 8 chimeric constructs. The collagen samples were coated on ELISA plates and binding was assessed for two different Fn concentrations (Fig. 4A). The binding assays were performed at room temperature, and because all of these assays represent binding of Fn to native recombinant collagens. The collagen control SCl, to determine the minimum amino acid sequence requirement for Fn binding in a bacterial collagen system.

![FIGURE 2. A, SDS-PAGE of SCI-Fn recombinant collagen constructs. Top panel from left to right: marker, SCI-FnG784-R792, SCI-FnG778-R792, SCI-FnG775-R792, and SCI-FnG775-R792, Bottom panel from left to right: SCI-FnG775-R792, marker, SCI-FnG778-R792, SCI-FnG775-R792, and SCI-FnG775-R792. Bottom bands in SCI-FnG775-R792 and SCI-FnG769-R792 represent truncated products with a single CL unit. Triple-helical proteins migrate slower than normal in SDS-PAGE, thus the M, prediction from the protein standard is higher than the actual M, examples of MALDI-TOF mass spectra showing M, of SCI-Fn. SCI-FnG775-R792, top panel; and SCI-FnG769-R792, bottom panel.](image)

### TABLE 1

Biophysical characterization as well as Fn affinity of SCI-Fn constructs and bovine collagen control

The \( K_d \) values were obtained by fitting the binding curves to saturation binding equation: \( Y = B_{\text{max}} \times X/(K_d + X) \), which give an order of magnitude estimation to the relative Fn affinities of SCI-FnG775-R792, SCI-FnG778-R792, and bovine collagen II control in both native and denatured forms. Units for molecular mass is in kDa. The calculated \( M_r \) is based on amino acids sequence in the open reading frame of each construct. The actual \( M_r \) is acquired from MALDI-TOF results. Unit for MRE, \( \text{deg} \times \text{cm}^2 \times \text{dmol}^{-1} \). The units for enthalpy is \( \text{kJ} \times \text{mol}^{-1} \). \( B_{\text{max}} \) is the maximum absorbance of the A_{590} value in arbitrary unit. The \( K_d \) value is in \( \mu\text{g/mL} \). The \( T_m \) value is in \( ^\circ\text{C} \).

| \( M_r \) measured (calculated) | MRE_{220} | \( T_m \) | Enthalpy | \( B_{\text{max}} \) native | \( B_{\text{max}} \) denatured | \( K_d \) native | \( K_d \) denatured |
|-------------------------------|----------|--------|--------|----------------|----------------|----------------|----------------|
| Bovine collagen II (96.1)      | 4306     | 41.0   |        | 0.74 \( \pm \) 0.2 | 1.36 \( \pm \) 0.1 | 13.22 \( \pm \) 6.6 | 0.41 \( \pm \) 0.1 |
| SCI (55.2)                     | 3502     | 36.8   | 4200   | 38.0          | 4100           | 37.9           |                |
| SCI-FnG774-R792 (56.1)         | 2714     | 36.5   | 3800   | 37.8          | 3700           | 37.1           |                |
| SCI-FnG778-R792 (56.8)         | 2220     | 36.2   |        | 1.33 \( \pm \) 0.1 | 1.51 \( \pm \) 0.1 | 4.82 \( \pm \) 1.0 | 1.27 \( \pm \) 0.2 |
| SCI-FnG778-R792 (56.2)         | 1939     | 36.5   |        | 1.26 \( \pm \) 0.1 | 1.23 \( \pm \) 0.1 | 5.04 \( \pm \) 1.3 | 2.15 \( \pm \) 0.6 |
| SCI-FnG781-R792 (56.3)         | 1918     | 36.2   |        |               |                |                |                |
| SCI-FnG784-R792 (56.1)         | 2714     | 36.5   | 3800   | 37.8          | 3700           | 37.1           |                |
| SCI-FnG775-R792 (56.1)         | 3502     | 36.8   |        | 1.33 \( \pm \) 0.1 | 1.51 \( \pm \) 0.1 | 4.82 \( \pm \) 1.0 | 1.27 \( \pm \) 0.2 |
| SCI-FnG769-R792 (56.1)         | 2220     | 36.2   |        | 1.26 \( \pm \) 0.1 | 1.23 \( \pm \) 0.1 | 5.04 \( \pm \) 1.3 | 2.15 \( \pm \) 0.6 |
| SCI-FnG769-R789 (56.1)         | 1939     | 36.5   |        |               |                |                |                |
| SCI-FnG775-R789 (56.1)         | 1918     | 36.2   | 3800   | 37.8          | 3700           | 37.1           |                |
| SCI-FnG769-R792 (56.1)         | 1939     | 36.5   | 3800   | 37.8          | 3700           | 37.1           |                |
| SCI-FnG769-R789 (56.1)         | 1918     | 36.2   | 3800   | 37.8          | 3700           | 37.1           |                |

\( ^* \) Blank cells in DSC columns indicate value not determined for that sample, blank cells in the Fn binding column indicate no strong binding has been observed for that sample. 

---

This page contains a detailed explanation of the experimental procedures, results, and conclusions related to the interaction between fibronectin and bacterial collagen constructs. The text describes the experimental setup for studying the binding of fibronectin (Fn) to recombinant collagen constructs in a bacterial system. It details the use of CD and DSC techniques to characterize the thermal transitions of the constructs, and MALDI-TOF mass spectrometry to analyze the molecular mass and structural integrity of the proteins. The text also discusses the tryptic digestion of these constructs and the solid-state binding assays performed to measure the binding of Fn to the collagen constructs. The results are presented in a table summarizing the biophysical characterization and binding affinities of the constructs, along with the methods used to determine the minimum amino acid sequence requirement for Fn binding.
collagen CL sequence. In the first set of sequences, Fn did not bind to SCl-FnG784-R792, SCl-FnG781-R792, and SCl-FnG778-R792, indicating the inserted sequences were not sufficient for binding. However, Fn did bind to SCl-FnG775-R792. In the second set of recombinant proteins with a progressive increase of insertion length at the C terminus, SCl-FnG772-V783, SCl-FnG769-P786, and SCl-FnG769-R789 did not bind Fn, whereas SCl-FnG769-R792 did bind. These studies indicated that the 775GLA triplet on the N terminus and the 790GER triplet on the C terminus were required for Fn binding, bracketing the essential sequence. These results define the minimal requirement for Fn binding to human type II collagen as the 6-triplet sequence, GLAGQR-GIVGLPGQRGER, in a triple-helix context. Native bovine type II did bind to Fn using the same binding assay, but the signal observed was weaker than seen for the two recombinant collagens SCl-FnG775-R792 and SCl-FnG769-R792.

To confirm the GBD of Fn was responsible for binding the recombinant collagens, a competitive binding assay was performed that utilized an anti-Fn GBD antibody, as well as an antibody to the distant C terminus of Fn. Fibronectin that bound to plates coated with SCl-FnG775-R792, SCl-FnG778-R792, and SCl-FnG784-R792 on a time course of 0.5, 1, and 3 h and overnight (>16 h).

To investigate if the minimal required Fn binding sequence was the same in the denatured and native states of collagen, solid-state binding assays were performed after incubating all recombinant collagen samples at 90 °C for 30 min. Again, Fn bound only to constructs SCl-FnG775-R792 and SCl-FnG769-R792 in the denatured form (Fig. 4A). This indicated that the minimum amino acid sequence requirement for Fn binding was the same for denatured and native collagens. The binding was also assayed in reverse by immobilizing Fn on the plate and monitoring the binding of soluble SCl-Fn through anti-His tag antibody. Similar results were observed indicating the same sequence requirement for Fn affinity (data not shown), but we were unable to compare this result with bovine collagen type II due to the difficulty in getting His-tagged animal collagen and the lack of an appropriate antibody to detect both animal and bacterial collagen.

To confirm the GBD of Fn was responsible for binding the recombinant collagens, a competitive binding assay was performed that utilized an anti-Fn GBD antibody, as well as an antibody to the distant C terminus of Fn. Fibronectin that bound to plates coated with SCl-FnG775-R792, SCl-FnG778-R792, or type II collagen was detectable by an antibody to the C terminus of Fn, but could not be identified by an antibody to the Fn GBD domain (Fig. 4B). This observation was consistent with the GBD on Fn being occupied by the recombinant collagen proteins or type II collagen, such that the site was no longer accessible to this specific antibody targeting the GBD through a steric exclusion mechanism. Thus, the interaction between SCl-Fn chimeric proteins and Fn was a site-specific interaction that involved the GBD of Fn.

To investigate whether this specific interaction between bacterial collagen and Fn could be disrupted by soluble gelatin, a gelatin inhibition assay was performed by premixing Fn with denatured collagen type II before incubating on collagen-coated plates. The results indicated that soluble gelatin serves as an effective inhibitor for binding between Fn and immobilized collagen. The inhibition effect was more dramatic on recombinant collagen and native collagen II as compared with coated denatured collagen type II (Fig. 4C). This may imply that denatured collagen type II has a lower dissociation constant (K_d) that makes it more effectively compete with recombinant collagen or native collagen type II for the binding site on Fn.
Quantitation of Fibronectin Binding Affinity for Native and Denatured Recombinant Chimeras and Type II Collagen—The two recombinant constructs that bound Fn were SCl-FnG775-R792 and the construct with the same insert plus two additional N-terminal triplets, SCl-FnG769-R792. The Fn binding affinity of these two recombinant constructs, as well as type II collagen, was studied in both the native and denatured states at varying concentrations of Fn, and typical saturation kinetics were observed for all curves (Fig. 5). Fn bound more tightly to SCl-FnG775-R792 and SCl-FnG769-R792 in their denatured states than in their native triple-helical form, demonstrating a preference of Fn for the denatured state in the context of bacterial collagen. The two chimeric proteins showed similar binding in the native state, but the shorter insertion bound somewhat more tightly in the denatured form (Table 1).

The difference in Fn binding between native versus denatured forms was more striking for collagen type II than for the recombinant proteins. In the native triple-helical form, SCl-FnG775-R792 and SCl-FnG769-R792 bound more efficiently than native bovine collagen type II (Table 1). However, after denaturation, type II collagen showed a dramatic increase in affinity, so that its $K_d$ is now 3–5 times lower than the denatured SCl-FnG775-R792 and SCl-FnG769-R792 values. The higher Fn affinity seen for denatured type II collagen compared with the chimeric collagens could reflect additional Fn-binding sites within mammalian collagen (17, 33, 34).

FIGURE 5. Dose-response of Fn binding to collagens adsorbed onto ELISA plates: SCl-FnG775-R792 (green), SCl-FnG769-R792 (red), bovine collagen type II (blue), and SCl with no insertion (black). Collagen in a native triple-helical state is represented by solid lines, whereas denatured collagen is represented by dashed lines.
Fibronectin Binding in a Bacterial Collagen System

A

SCI-FnG769-R789

SCI-FnG769-R792

Uncotted

CTRL

Type II Col

B

FIGURE 6. Megakaryocytes cultured on different SCI-Fn recombinant collagens. A, human mature MK were plated on two different SCI-Fn constructs: SCI-FnG769-R789, which did not bind Fn, and SCI-FnG769-R792, which did bind Fn. SCI-Fn-coated plates were preincubated with human cellular fibronectin prior to cell plating. After 16 h, MK showed higher adhesion and proplatelet formation on SCI-FnG769-R792 (right panel) compared with SCI-FnG769-R789 (left panel). In parallel controls were performed by seeding MK on uncoated and bovine type II collagen-coated plates. Arrows show proplatelet bearing MK. Scale bar = 100 μm. B, adherent cells were counted by phase-contrast microscopy (magnification ×40). Reported results are the mean ± S.D. of at least 10 randomly chosen fields in three independent experiments.

data demonstrated a pivotal role for these integrins in regulating MK development and interaction with the bone marrow extracellular matrix environment (25, 36, 37). The observed increase in MK adhesion and proplatelet formation on SCI-FnG769-R792 is consistent with its higher Fn affinity in solid-phase binding assays.

DISCUSSION

Collagen sequences from the putative fibronectin-binding region of human collagen type II were included within a triple-helical bacterial collagen context, expressed as recombinant proteins, and studied for their ability to bind Fn. The S. pyogenes bacterial collagen Scl2.28 forms a stable triple-helical structure and has no known biological interactions or activities (38). Previous studies show the insertion of human collagen sequences within the recombinant triple-helical protein conferred biological activity similar to that found in human collagens. For example, recombinant Scl2.28 proteins with inserted integrin binding sequences (GFPGER, GFPGEN, and GLPGER) were shown to bind cell surface integrins in vitro and to mediate cell binding (23, 39), whereas insertion of the GRPGKRGKQGQK sequence led to heparin binding (40). Incorporation of 6 triplets from the human collagen type III MMP cleavage site led to digestion by MMP-1 at the same site as in native type III collagen (29). All of these collagen sequences only have biological activity when in a triple helix context. In contrast, Fn is known to bind to denatured collagen even stronger than native collagen. The bacterial collagen system offers an opportunity to define Fn binding to native and denatured recombinant proteins containing different lengths of human collagen sequences proposed to interact with Fn.

Recent studies on the binding of various unfolded (Gly-Xaa-Yaa), peptides to Fn are extended here to include residues in a recombinant triple-helix context, allowing definition of the minimum sequence requirement for Fn binding in both the native and denatured states. The original bacterial collagen SCI (VCLCL) without genetic modification could not bind Fn, but insertion of sufficient lengths of the amino acid sequence from the human collagen α(II) chain, near the collagenase cleavage site, conferred Fn binding activity. Solid-state binding assays on the chimeric collagen showed that the 6-triplet type II collagen sequence, 77SGLAGQQRGIVGLPGQQRGER792, was the minimal requirement for Fn binding in both triple-helical and denatured conditions. Although residues GLPQQRGER appear to be key in interacting with the 8Fn module in the crystal structure (15), full-length Fn did not bind to the SCI-FnG784-R792 protein, indicating these three triplets may be necessary but are not sufficient for binding to the triple-helical or denatured states in our system. Extending the required 6 triplet sequence two triplets further in the N-terminal direction did not increase the binding to Fn. It is likely that these triplets also represent the binding site in the α(1) chain, which differs from the α(1)(II) sequence in only two residues in this region, L776L and I782V, showing conservative changes in these positions.

The collagen sequence defined here as the minimum for Fn binding falls in a region known to be a ligand binding hot spot in fibrillar collagens (41). Within the required 6-tripeptide Fn binding sequence, the three triplets in GLAGQQRGIV that pre-
Fibronectin Binding in a Bacterial Collagen System

cede GLPGQRGER include the well characterized G\&LA collagenase cleavage site. Human collagenases, such as MMP-1 and MMP-13, cleave between Gly\textsuperscript{775} and Leu\textsuperscript{776} and divide collagen chains into 1/2 and 1/4 fragments (42, 43). A recent study indicated that Leu residues at positions 776 and 785 were critical for MMP activity through direct interaction with the MMP catalytic and hemopexin domains, respectively (44). Because the minimum Fn binding sequence defined here overlaps with the key residues for MMP cleavage, MMP and Fn could compete for binding at this site.

It is well known that Fn binds more tightly to denatured collagen than to native triple-helical collagen (19), and this preference for the unfolded state was retained in the chimeric bacterial collagens studied here. There is much interest in how Fn can bind to collagen in both the denatured as well as the native states, and whether both binding abilities play a physiological role. In our system, the same GLAGQR-GIVGLPGQRGER sequence was found to be required for Fn binding to gelatin or native triple-helical collagen. It is not clear how Fn binds to a sequence within the tightly packed native collagen, which shows resistance to digestion by most enzymes and a lack of immunogenicity. If the GLPGQRGER sequence is to participate in a \(\beta\)-sheet with the Fn module, as suggested from the crystal structure of the \(\beta\)-Fn module with peptide Gly\textsuperscript{778}–Gly\textsuperscript{799} (15), this sequence would have to be pulled out from the supercoil of the triple-helix and extended from the 2.9-Å rise/residue of a triple-helix to the 3.4-Å rise/residue of a \(\beta\) strand. This is not an obvious structural transition because the sequence GLPGQRGER is predicted to have a relatively high propensity for triple-helix formation (45) and a relatively low propensity for a \(\beta\) strand (46). However, a range of observations suggests that this may be a flexible region, which is able to adopt an alternative equilibrium conformation. There is only one imino acid residue within the entire 6 triplet region (6%), and no Pro/Hyp within the first 3 triplets, compared with an overall 20% imino acid content within type I/II collagens. The importance of this imino acid deficiency is illustrated by an early study on transgenic mouse models showing that introduction of a Pro at a residue immediately before or after the Gly\textsuperscript{775} collagenase cleavage site (changing Gln–Gly\textsuperscript{775}–Leu–Ala to either Pro–Gly\textsuperscript{775}–Leu–Ala or Gln–Gly\textsuperscript{775}–Pro–Ala) interferes with both MMP cleavage and Fn binding (47). In addition, two of the first three triplets, GLA and GIV, have a very low triple-helix propensity (45). Computational studies indicate this region has an alternative “vulnerable” conformation in equilibrium with the triple-helical state (48, 49), which is susceptible to collagenases. It is possible that a vulnerable conformation in this relatively loose triple helix region at the MMP cleavage site is transiently extended C terminally to unwind the GLPGQRGER sequence, so it can participate in the Fn \(\beta\)-sheet structure. Any alternative state must exist on a fast time scale in equilibrium with a well folded triple helix. Consistent with this hypothesis, the recombinant chimeric collagens with longer insertions do show partial susceptibility to trypsin at the human sequence insertion site, even though a fraction of the protein remained trypsin resistant after 24 h.

There is only one Pro in the 6 triplets needed for Fn binding, at position 786 in Gly-Leu-Pro\textsuperscript{786}, and it is located in the Y position, where it normally undergoes hydroxylation to Hyp in type I collagen (50). The bacterially expressed recombinant collagen, which bound Fn did not undergo any post-translational modification and thus did not contain Hyp at position 786. These results indicate that Hyp is not essential for collagen or gelatin binding to Fn, just as it was found not to be essential for collagen binding to integrin or heparin or for MMP-1 cleavage. However, it is possible that the absence of Hyp may be responsible for some differences in Fn affinity observed between the recombinant collagen and type II collagen, because Hyp is known to affect the triple helix structure and stability and to form direct interactions with some proteins and receptors (51, 52). It is expected that the absence of Hyp\textsuperscript{786} will decrease local stability and rigidity, and in the context of Fn binding, a looser triple helix should lead to tighter binding. This is consistent with the ability of trypsin to digest the recombinant constructs with 5 and 6 tripeptides, but not type II collagen, and the apparent tighter binding of the native triple-helical bacterial constructs with 5–6 tripeptides compared with type II collagen.

Because SCI-Fn was produced in a E. coli system, the scalability and easy modification of the recombinant system also raise the possibility for this chimeric protein to be a novel biomaterial. Previously, the potential to obtain collagenous proteins with controllable biological activity modules using the bacterial collagen system has been demonstrated, leading to improved human mesenchymal stem cell growth in two- and three-dimensional cultures (24). To further demonstrate SCI-Fn as a suitable bioactive material, human megakaryocytes were cultured on SCI-Fn in the presence of Fn. SCI-Fn could effectively immobilize soluble Fn in the cell culture and subsequently support the attachment and differentiation of megakaryocytes. Fibronectin has been known to play a key role in supporting hemopoiesis (53), megakaryocyte development, and platelet release (54). It has been previously demonstrated that megakaryocytes are involved in the process of fibronectin fibrillogenesis and that, in turn, fibronectin plays a pivotal role in regulating megakaryocyte-type I collagen interaction in the bone marrow environment (37). Thus, SCI-Fn, as a Fn binding biomaterial, could support megakaryocyte function with the potential for bone marrow modeling and platelet production.

In conclusion, Fn binding affinity was conferred on bacterial collagen by incorporating a minimum of 6 triplets of human collagen type II sequence from residue Gly\textsuperscript{775}–Arg\textsuperscript{792}. We demonstrated the potential of this bacterial collagen system as a model to study collagen and Fn binding, and its practical use in promoting cell attachment and differentiation. Defining this fibronectin binding sequence and demonstrating its function in the recombinant system can help guide future recombinant protein design to build biomaterials with selective Fn binding ability.

Acknowledgments—We thank Dr. Jean Schwarzbauer from the Department of Molecular Biology, Princeton University, for valuable reagents and suggestions; Dr. John Ramshaw from the Commonwealth Scientific and Industrial Research Organisation (CSIRO) of Australia for helpful discussions; and Dr. David Wilbur, Tufts University Chemistry Department, for allowing us to access the MALDI-TOF MS equipment.
Fibronectin Binding in a Bacterial Collagen System

REFERENCES

1. Brodsky, B., and Ramshaw, J. A. (1997) The collagen triple-helix structure. *Matrix Biol.* 15, 545–554

2. Kiely, C. M., and Grant, M. E. (2002) The Collagen family: structure, assembly and organization in the extracellular matrix. In *Connective Tissue and Its Heritable Disorders. Molecular, Genetic, and Medical Aspects* (Royce, P. M., and Steinmann, B., eds) 2nd Ed., pp. 158–222, Wiley-Liss, New York.

3. Veit, G., Kobbe, B., Keene, D. R., Paulsson, M., Koch, M., and Wagener, R. (2006) Collagen XXVIII, a novel von Willebrand Factor A domain-containing protein with many imperfections in the collagenous domain. *J. Biol. Chem.* 281, 3494–3504

4. Shoulders, M. D., and Raines, R. T. (2009) Collagen structure and stability. *Annu. Rev. Biochem.* 78, 929–958

5. Piez, K. A., and Reddi, A. H. (1984) *Extracellular Matrix Biochemistry,* Elsevier, New York.

6. Leitinger, B., and Hohenester, E. (2007) Mammalian collagen receptors. *Matrix Biol.* 26, 146–155

7. Dessau, W., Adelmann, B. C., and Timpl, R. (1978) Identification of the a005041 sequence motif that mediates binding to human collagen receptors, integrin aIIIB1 and aIIIB1. *Cell* 106, 4195–4200

8. Di Lullo, G. A., Sweeney, S. M., Korkko, J., Ala-Kokko, L., and San Antonio, M. J. (2013) Engineering multiple biological functional motifs into a collagen-like protein template from Streptococcus pyogenes collagen. *J. Biol. Chem.* 288, 27312–27318

9. Brodsky, B., Lobody, A., Malara, A., Amin, R., Lukomski, S., Inouye, M., and Balduini, C. (2007) Mechanism of stabilization of a bacterial collagen triple helix in the absence of hydroxyproline. *J. Biol. Chem.* 282, 29757–29765

10. Yoshizumi, A., Yu, Z., Silva, T., Thiagarajan, G., Ramshaw, J. A., Inouye, M., and Brodsky, B. (2009) Self-association of *Streptococcus pyogenes* collagen-like constructs into higher order structures. *Protein Sci.* 18, 1241–1251

11. Balduini, A., Pallotta, I., Malara, A., Lova, P., Pecci, A., Viarengo, G., Balduini, C. L., and Torti, M. (2008) Adhesive receptors, extracellular proteins and myosin II A orchestra platelet formation by human megakaryocytes. *J. Thromb. Haemost.* 6, 1900–1907

12. Mohs, A., Silva, T., Yoshida, T., Amin, R., Lukomski, S., Inouye, M., and Brodsky, B. (2007) Mechanism of stabilization of a bacterial collagen triple helix in the absence of hydroxyproline. *J. Biol. Chem.* 282, 29757–29765

13. Singh, P., Carracher, C., and Schwarzbauer, J. E. (2010) Assembly of fibronectin extracellular matrix. *Annu. Rev. Cell Dev. Biol.* 26, 397–419

14. Akiyama, S. K., and Yamada, K. M. (1983) Fibronectin in disease. *Monogr. Pathol.* 24, 55–96

15. Ingham, K. C., Brew, S. A., and Migliorini, M. (2002) Type I collagen contains at least 14 cryptic fibronectin binding sites of similar affinity. *Arch. Biochem. Biophys.* 407, 217–223

16. Guidry, C., Miller, E. J., and Hook, M. (1990) A second fibronectin-binding region is present in collagen α chains. *J. Biol. Chem.* 265, 19230–19236

17. Fox, N. E., and Kaushansky, K. (2005) Engagement of integrin aIIIB1 enhances thrombopoietin-induced megakaryopoiesis. *Exp. Hematol.* 33, 94–99

18. Mazhari, A., Thomas, S. G., Dhanjal, T. S., Buckley, C. D., and Watson, S. P. (2010) Critical role of Src-Syk-PLCγ2 signaling in megakaryocyte migration and thrombopoiesis. *Blood* 116, 793–800

19. Malara, A., Gruppi, C., Rebuzzini, P., Visai, L., Perotti, C., Moratti, R., Balduini, C., Tira, M. E., and Balduini, A. (2011) Megakaryocyte-matrix interaction within bone marrow. New roles for fibronectin and factor XIII-A. *Blood* 117, 2476–2483

20. Caswell, C. C., Barczyk, M., Keene, D. R., Lukomska, E., Guiberg, D. E., and Lukomska, S. (2008) Identification of the first prokaryotic collagen sequence motif that mediates binding to human collagen receptors, integrins αIIIB1 and αIIIB1. *J. Biol. Chem.* 283, 36168–36175

21. Kosgriff-Hernandez, E., Hahn, M. S., Russell, B., Wilems, T., Munoz-Pinto, D., Browning, M. B., Rivera, J., and Höök, M. (2010) Bioactive hydrogels based on designer collagens. *Acta Biomater.* 6, 3969–3977

22. Peng, Y. Y., Stoichevska, V., Schacht, K., Werkmeister, J. A., and Ramshaw, J. A. (2013) Engineering multiple biological functional motifs into a blank collagen-like protein template from *Streptococcus pyogenes*. *J. Biomol. Mater. Res. Part A*, A10.1002/jbm.a.34898

23. 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) Candidate cell and matrix interaction domains on the collagen fibril, the predominant protein of vertebrates. *J. Biol. Chem.* 283, 21187–21197

24. Visse, R., and Nagase, H. (2003) Matrix metalloproteinases and tissue inhibitors of metalloproteinases. Structure, function, and biochemistry. *Circ. Res.* 92, 827–839

25. Sant, A. M., Kuznetsova, N. V., DeRidder, A. M., Sutter, M. B., Losert, W., Phillips, C. L., Visse, R., Nagase, H., and Leikin, S. (2010) Mo-
molecular mechanism of type I collagen homotrimer resistance to mammalian collagens. J. Biol. Chem. 285, 22276–22281
44. Manka, S. W., Carafoli, F., Visse, R., Bihan, D., Raynal, N., Farndale, R. W., Murphy, G., Enghild, J. J., Hohenester, E., and Nagase, H. (2012) Structural insights into triple-helical collagen cleavage by matrix metalloproteinase 1. Proc. Natl. Acad. Sci. U.S.A. 109, 12461–12466
45. Persikov, A. V., Ramshaw, J. A., and Brodsky, B. (2005) Prediction of collagen stability from amino acid sequence. J. Biol. Chem. 280, 19343–19349
46. Sen, T. Z., Jernigan, R. L., Garnier, J., and Kloczkowski, A. (2005) GOR V server for protein secondary structure prediction. Bioinformatics 21, 2787–2788
47. Dzamba, B. J., Wu, H., Jaenisch, R., and Peters, D. M. (1993) Fibronectin binding site in type I collagen regulates fibronectin fibril formation. J. Cell Biol. 121, 1165–1172
48. Nerenberg, P. S., Salsas-Escat, R., and Stultz, C. M. (2008) Do collagenases unwind triple-helical collagen before peptide bond hydrolysis? Reinterpreting experimental observations with mathematical models. Proteins 70, 1154–1161
49. Stultz, C. M., and Edelman, E. R. (2003) A structural model that explains the effects of hyperglycemia on collagenolysis. Biophys. J. 85, 2198–2204
50. Fietzek, P. P., Rexrodt, F. W., Hopper, K. E., and Kühn, K. (1973) The covalent structure of collagen. 2. The amino-acid sequence of α1-CB7 from calf-skin collagen. Eur. J. Biochem. 38, 396–400
51. Perret, S., Eble, J. A., Sijjander, P. R., Merle, C., Farndale, R. W., Theisen, M., and Ruggiero, F. (2003) Prolyl hydroxylation of collagen type I is required for efficient binding to integrin α1β1 and platelet glycoprotein VI but not to α2β1. J. Biol. Chem. 278, 29873–29879
52. Asselin, J., Knight, C. G., Farndale, R. W., Barnes, M. J., and Watson, S. P. (1999) Monomeric (glycine-proline-hydroxyproline)10 repeat sequence is a partial agonist of the platelet collagen receptor glycoprotein VI. Biochem. J. 339, 413–418
53. Jiang, F., Jia, Y., and Cohen, I. (2002) Fibronectin- and protein kinase C-mediated activation of ERK/MAPK are essential for proplateletlike formation. Blood 99, 3579–3584
54. Matsunaga, T., Fukai, F., Kameda, T., Shide, K., Shimoda, H., Torii, E., Kamiunten, A., Sekine, M., Yamamoto, S., Hirota, T., Kubuki, Y., Yokokura, S., Uemura, M., Matsuoka, A., Waki, F., Matsumoto, K., Kanaji, N., Ishii, T., Imataki, O., Dobashi, H., Bandoh, S., and Shimoda, K. (2012) Potentiated activation of VLA-4 and VLA-5 accelerates proplatelet-like formation. Ann. Hematol. 91, 1633–1643