A Novel Binding Site in Collagen Type III for Integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$*

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Previously identified high affinity integrin-binding motifs in collagens, GFOGER and GLOGER, are not present in type III collagen. Here, we first characterized the binding of recombinant I domains from integrins $\alpha_1\beta_1$ and $\alpha_1\beta_2$ ($\alpha_1$I and $\alpha_2$I) to fibrillar collagen types I–III and showed that each I domain bound to the three types of collagen with similar affinities. Using rotary shadowing followed by electron microscopy, we identified a high affinity binding region in human type III collagen recognized by $\alpha_1$I and $\alpha_2$I. Examination of the region revealed the presence of two sequences that contain the critical GER motif, GROGER and GAOGGER. Collagen-like peptides containing these two motifs were synthesized, and their triple helical nature was confirmed by circular dichroism spectroscopy. Experiments show that the GROGER-containing peptide was able to bind both $\alpha_1$I and $\alpha_2$I with high affinity and effectively inhibit the binding of $\alpha_1$I and $\alpha_2$I to type III and I collagens, whereas the GAOGGER-containing peptide was considerably less effective. Furthermore, the GROGER-containing peptide supported adhesion of human lung fibroblast cells when coated on a culture dish. Thus, we have identified a novel high affinity binding sequence for the collagen-binding integrin I domains.

Collagen is a major component of the extracellular matrix (ECM). At least 27 genetically different collagen types have been identified, each containing at least one dominant collagenous domain (1). These collagenous domains have a characteristic triple helical structure formed by repeating Gly-X-Y sequences in each participating polypeptide, where X often is proline and Y hydroxyproline. The collagen monomers often assemble into more complex structures of varying organizations, such as fibrils (types I–III, V, and XI), networks (types IV, VIII, and X), and beaded filaments (type VI) (2). The fibrillar collagen types I and III are the major structural components of the ECM of skin, cardiac, and vascular tissues, whereas type II collagen is a major component of cartilage. In addition to contributing to the structural integrity of the tissues, collagens also affect cell behavior through interactions with other matrix proteins and cellular receptors (3–6).

The integrins are a family of heterodimeric cell surface receptors involved in cell–cell and cell–substrate adhesion. They act as bridging molecules that link intracellular signaling molecules to the ECM, controlling cell behavior and tissue architecture through bi-directional signaling (7). Four integrins, $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_1\beta_1$, and $\alpha_1\beta_1$, have been shown to bind collagens (8–10). Of these, the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins have been studied in more detail compared with the others. Collagen-integrin interactions play a role in normal and pathological physiology; these interactions directly affect cell adhesion, migration, proliferation, and differentiation, as well as angiogenesis, platelet aggregation, and ECM assembly (11). The precise molecular events that lead to these activities are not understood. It is possible that different sites in collagens are recognized by different integrins and/or are capable of activating different signaling pathways. Consequently, detailed studies investigating the specificity of the collagen-integrin interactions are essential to further our understanding of these interactions in different biological processes.

Collagen binding by the four integrins is mediated by the inserted (I) domain, a ~200-amino acid-long segment found between blades 2 and 3 of the $\beta$-propeller domain of the $\alpha$ chains. All four I domains ($\alpha_1$I, $\alpha_2$I, $\alpha_1$I, and $\alpha_1$I) contain a metal ion-dependent adhesion site (MIDAS) that is required for coordinating a divalent cation and is essential for collagen binding. Synthetic collagen peptides containing the type I collagen-derived sequences GFOGER or GLOGER have been reported to bind with a high affinity to $\alpha_1$I, $\alpha_2$I, and $\alpha_1$I; furthermore, synthetic peptides containing these sequences inhibit the binding of I domains to intact collagens (12–14). The crystal structures of apo-$\alpha_1$I and apo-$\alpha_2$I in complex with a collagen peptide containing the GFOGER sequence have been solved and show that the apo-$\alpha_1$I adopts an inactive “closed” conformation and the ligand-bound $\alpha_1$I, an active “open” conformation (15, 16). The Glu residue in the collagen peptide was shown in the structure of the complex to directly interact with a Mg$^{2+}$ ion coordinated by the MIDAS motif, and the Arg residue forms a salt bridge with $\alpha_2$I. The importance of the GER sequence in collagen for integrin binding was confirmed by mutagenesis studies showing that replacing the collagen peptide Glu with an Asp residue completely abolished integrin binding, whereas replacing the Arg with a Lys residue reduced binding by 50% (17). The Phe residue in the collagen sequence appeared to participate in hydrophobic interactions with $\alpha_1$I and presumably can be replaced by a Leu residue. However, changing the Phe residue to a Met or an Ala in the collagen peptide reduced the apparent affinity of the $\alpha$ domains (14). GASGER was also reported to be recognized by the $\alpha$ domains but bind with lower affinity than GFOGER and GLOGER (13, 14, 18). Therefore, GFOGER and GLOGER are the only known collagen-derived sequence motifs that support high affinity binding by the collagen-binding $\alpha$ domains. However, these two motifs are absent in some collagens such as human type II collagen. A previous study showed that Chinese hamster ovary cells expressing $\alpha_1\beta_1$ and $\alpha_2\beta_1$ could adhere to and spread on human type III collagen; furthermore, the...
recombinant proteins of α1(I) and α2(I) could bind to this collagen type (19). We now have examined α1(I) and α2(I) binding to human type III collagen in some detail and identified a previously unrecognized high affinity integrin-binding site.

EXPERIMENTAL PROCEDURES

Recombinant I Domains—Recombinant I domains of integrin α1 and α2 subunits were generated and isolated as previously described (18, 20). Purified recombinant proteins were examined by SDS-PAGE followed by staining with Coomassie blue.

Purification of Recombinant Procollagen—Frozen yeast cells expressing recombinant type I and III procollagens were generously provided by FibroGen (San Francisco, CA). The yeast cells express both genes encoding human collagen and prolyl 4-hydroxylase enabling formation of hydroxyproline residues and thermally stable triple helical collagen. The cells were thawed in an ambient temperature water bath and resuspended in Start Buffer (0.1 M Tris, 0.4 M NaCl, 25 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, pH 7.5). The cells were lysed using a French press and the lysate was centrifuged at 30,000 × g for 30 min at 4 °C. The supernatant was then filtered through a 0.45-μm membrane, and the pH of the filtrate was adjusted to 7.5. An affinity column was prepared by coupling a recombinant collagen-binding MSCRAMM from Staphylococcus aureus, CNA (21), to CNBr-activated Sepharose 4B (Amersham Biosciences). The supernatant was applied to the column and incubated overnight at 4 °C. The column was washed with the Start Buffer, and bound material was eluted with 0.5 M acetic acid. Fractions were examined by SDS-PAGE (4%/8%) under reducing conditions followed by Coomassie Blue staining. Fractions with procollagen were pooled. The concentration of the procollagen was estimated by comparing its band intensity with that of a known concentration of unprocessed procollagen in HBS for 2 h at room temperature. The wells were washed with HBS and incubated with a blocking buffer (HBS containing 0.1% w/v ovalbumin and 0.05% v/v Tween 20) overnight at 4 °C. Varying concentrations of peptides were mixed with fixed concentrations of recombinant I domain in the blocking buffer containing 1 mM MgCl₂ and 5 mM β-mercaptoethanol and then added to the wells. After incubation at 4 °C for 3 h with gentle shaking, the wells were extensively washed with HBS containing 0.05% Tween 20 and 1 mM MgCl₂. Bound α1(I) or α2(I) was detected by incubation with an anti-His monoclonal antibody (Amersham Biosciences) diluted 1:3000 in the blocking buffer containing 1 mM MgCl₂ for 1 h at room temperature, followed by incubation with goat anti-mouse IgG (H + L)-alkaline phosphatase conjugate (Bio-Rad) diluted 1:3000 in the blocking buffer containing 1 mM MgCl₂ for 1 h at room temperature. Bound antibodies were quantified by adding 100 μl of 1.3 M diethanolamine, pH 9.8, containing 1 mM MgCl₂, and 1 mg/ml p-nitrophenyl phosphate (Southern Biotechnology Associates, Birmingham, AL) to each well and measuring the absorbance at 405 nm (A_405nm) after 20–40 min of incubation at room temperature. Background binding to the wells was determined by incubating the I domains in wells that had been pretreated with blocking buffer alone. These values were subtracted from the values generated in the collagen-coated wells to determine collagen specific binding.

Surface Plasmon Resonance (SPR) Measurements—For the analyses of interactions between recombinant I domains and fibrillar collagens, SPR measurements were carried out at ambient temperature using the BLAcore 3000 system (Biacore AB, Uppsala, Sweden) as described previously (20) with the following modifications. First, purified recombinant human procollagen I, procollagen III (described above), or bovine mature collagen II (Sigma) were immobilized on the flow cells of a CM5 BIAcore 3000 system (Biacore AB, Uppsala, Sweden) as described previously (18). Each binding event was measured from the base of the globular domain, and to the middle of the binding spot. The binding events were then binned for every 10 nm along the collagen strand. The percentage of the number of events in each bin over total events counted was calculated and plotted against the length of the collagen strand.

Rotary Shadowing and Electron Microscopy—Rotary shadowing and electron microscopy of I domain-collagen complexes were performed as described previously (18). Each binding event was measured from the C-terminal end of type III collagen, i.e. from the base of the globular domain, and to the middle of the binding spot. The binding events were then binned for every 10 nm along the collagen strand. The percentage of the number of events in each bin over total events counted was calculated and plotted against the length of the collagen strand.

Synthesis and Purification of Collagen Peptides—Peptides were synthesized by a solid phase method on a Tentagel R RAM resin (RAPP Polymere GmbH, Tubingen, Germany) using Fmoc chemistry and a model 396 MBS Multiple Peptide Synthesizer from Advanced ChemTech Inc. (Louisville, KY). Fmoc amino acids were purchased from Novabiochem (San Diego, CA). Coupling of amino acids was carried out twice using diisopropylcarbodiimide/1-hydroxybenzotriazole for 60 min. Fmoc deprotection was carried out using a mixture of 2% (v/v) piperidine and 2% (v/v) 1,8-diazabicyclo-[5.4.0]undec-7-ene in dimethylformamide followed by treatment with 25% piperidine in dimethylformamide. Side chains were protected with the following groups: t-butyl (Glu, Ser, and hydroxy-Pro), 2,2,5,7,8-pentamethyltricyclic-6-sulfonyl (Arg), and trityl (Gln).
After completion of the synthesis, peptide resins were washed thoroughly with dimethylformamide, ethanol, and ether and then dried in a vacuum desiccator. Peptides were released from the resin by treatment with a mixture of trifluoroacetic acid, thioanisole, ethanedithiol, and triethylsilane for 8 h. The resins were filtered, and the peptides were precipitated with cold anhydrous ether. The precipitate was washed with anhydrous ether three times and dried. The cleaved peptides were analyzed by reverse phase HPLC using a Waters Delta Pak C18 column.

**CD Spectroscopy**—Synthetic collagen peptides were analyzed by CD spectroscopy as described previously (18) with the following modifications. Peptides were dissolved in HBS to a concentration of 50 μM. CD spectra were recorded on a Jasco J720 spectropolarimeter (Tokyo, Japan) from 190 to 240 nm, with a bandwidth of 1 nm and integrated for 1 s at 0.2 nm intervals. Samples were measured at room temperature using cuvettes with a 0.02-cm path length. For temperature-dependent denaturation analysis, peptides (30 μM) were added to a thermostatically controlled cuvette with a 0.5-cm path length. Thermal transition profiles were recorded at 225 nm as described above with a temperature slope of 20 °C/h. To calculate the temperature melting points, the thermal transition profiles were fitted with a Boltzmann sigmoidal model using the GraphPad Prism software.

**Table One**

| Collagen   | α₁I Kd | α₂I Kd |
|------------|--------|--------|
| Type I     | 0.32 ± 0.10 | 0.26 ± 0.08 |
| Type II    | 5.5 ± 1.46 | 3.99 ± 0.82 |
| Type III   | 0.15 ± 0.03 | 1.75 ± 0.09 |
|            | 7.28 ± 1.16 | 16.5 ± 3.89 |
|            | 0.19 ± 0.03 | 0.33 ± 0.03 |
|            | 6.15 ± 0.95 | 14.5 ± 3.41 |

*Kd* was calculated by equilibrium analysis. Data are presented as mean value ± S.E. of three independent studies.

**Reagents and Cell Culture**—The human recombinant mature type III collagen used for cell attachment assays was purchased from FibroGen. All cell culture media components were obtained from Invitrogen. The human lung fibroblast cell line MRC-5 was purchased from American Type Culture Collection (ATCC) (Manassas, VA). The cells were cultured and passaged in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 unit/ml penicillin, and 100 µg/ml streptomycin. The cells were grown to subconfluence and passaged every 2–3 days.

**Cell Attachment Assay**—MRC-5 cells were starved overnight in serum-deficient DMEM containing penicillin and streptomycin, then detached using 1 mM EDTA and 0.025% trypsin at 37 °C for 2 min. The cells were washed with PBS and resuspended in DMEM containing 0.2% BSA supplemented with 2 mM MgCl₂. The cell suspension (100 μl containing ~1.5 × 10⁴ cells) was added to the microtiter wells coated with different concentrations of collagen or collagen peptides and blocked with PBS containing 0.5% (w/v) BSA. After incubation at room temperature for 45 min, the wells were washed with PBS and the attached cells were fixed with 3% p-formaldehyde for 10 min at room temperature. Following washing with cold Tris-buffered saline, pH 7.4, cells were fixed again in 20% methanol for 10 min and stained with 0.5% crystal violet for 5 min. The wells were thoroughly washed with distilled water and air-dried. Sodium citrate (0.1 M) was then added to the wells to dissolve the dye and the absorbance at 590 nm was measured. The maximum cell...
attachment on type III collagen was set to 100%, and residual attachment on BSA was set to 0%.

**Computer Modeling**—The coordinates of the crystal structure of \( \alpha_1I \) in complex with a synthetic collagen peptide were obtained from the Protein Data Bank (code 1dzi) and used as a template for the model studies. First, the Phe residues in both the middle and trailing strands were replaced by Arg residues. Then, the local minimization was carried out in sizes of 5 Å for the best fit. Several basic components (i.e. hydrogen bond, van der Waals, and electrostatic interactions) contributing to the binding energy between \( \alpha_1I \) and the mutated collagen peptide were analyzed. The molecular modeling experiment was carried out under ECEPP/3 force field by using the ICM software (Molsoft, La Jolla, CA).

**RESULTS**

**Characterization of the Binding of \( \alpha_1I \) and \( \alpha_2I \) to Type I, II, and III Collagens**—The interactions between the two \( I \) domains and fibrillar collagens (types I–III) were examined by SPR. Solutions of 1 \( \mu M \) \( \alpha_1I \) or \( \alpha_2I \) were passed over chips containing immobilized collagen types I–III in the presence of 1 mM MgCl\(_2\). Both \( \alpha_1I \) and \( \alpha_2I \) showed binding to all three types of collagen (Fig. 1), consistent with a previous report (19). To determine the dissociation constants (\( K_D \)) for the interactions between the \( I \) domains and each collagen, increasing concentrations of recombinant \( I \) domains (0.01–50 \( \mu M \)) were passed over the collagen surfaces. In our previous SPR studies using the BIAcore 1000 system, we showed...
two classes of binding sites in type I collagen with different affinities for $\alpha_1I$ ($K_{D1} = 0.26 \pm 0.01 \, \mu M$, and $K_{D2} = 13.9 \pm 3.0 \, \mu M$), whereas $\alpha_2I$ appeared to have one class of binding sites ($\sim 10 \, \mu M$) (18, 20). The BIAcore 3000 system used in this study has a higher sensitivity of detection, which enables us to examine the interactions between $\alpha_I$ and collagens within a submicromolar concentration range. Analyses using the SPR responses in the steady state portion of the sensorgrams, which indicates the equilibrium condition, showed that both $I$ domains have at least two classes of binding sites in the three types of collagen. The dissociation constants ($K_D$) of these interactions are summarized in TABLE ONE. $\alpha_1I$ binds all three types of collagen with similar affinities ($K_{D1} \approx 0.15-0.32 \, \mu M$, and $K_{D2} \approx 5.5-7.3 \, \mu M$). The binding affinities of $\alpha_2I$ to the three types of collagen appeared to be slightly more variable. The $K_D$ values for the high affinity binding class range from $\sim 0.3 \, \mu M$ for type I and III collagen to $1.75 \, \mu M$ for type II collagen, whereas the $K_D$ values for the low affinity binding class range from $\sim 4 \, \mu M$ for type I collagen to $\sim 16.5 \, \mu M$ and $\sim 14.5 \, \mu M$ for type II and III collagen, respectively.

The two recombinant $I$ domains also exhibited different binding kinetics to the collagens as indicated by the shape of the corresponding SPR sensorgrams (Fig. 1). Comparison of the shapes of the SPR sensorgrams of $\alpha_1I$ with those of $\alpha_2I$ indicates a much slower association and dissociation rate of $\alpha_1I$, compared with $\alpha_2I$, in agreement with previous reports (18, 20). However, there was no dramatic difference between each $I$ domain and type I, II, or type III collagen. Thus, the binding characteristics of the interactions between $\alpha_1I/\alpha_2I$ and type III collagen are similar to those of the interactions between $\alpha_1I/\alpha_2I$ and type I/II collagen.

**Localization of a High Affinity $\alpha_1I/\alpha_2I$ Binding Region in Type III Procollagen—**Two sequence motifs, GFOGER and GLOGER, are identified as high affinity binding sites in triple helical collagen for $\alpha_1I$, $\alpha_2I$, and $\alpha_1I$ (13, 14, 18). The fact that these sequences are present in type I and II collagen, but not in type III collagen, suggests the presence of at least one novel high affinity binding site in type III collagen. To locate the high affinity binding region(s) in type III collagen, we examined collagen and $I$ domain complexes by rotary shadowing followed by electron microscopy (EM). Type III procollagen was used in these experiments because it contains a globular-shaped C-terminal propeptide that allows us to determine the orientation of collagen molecules in EM.

Type III procollagen was incubated with $\alpha_1I$ or $\alpha_2I$ under binding conditions and the complexes were then subjected to rotary shadowing and EM. The helical portion of the majority of the collagen molecules was found to be $\sim 300$ nm long, indicating that these molecules are mostly intact, full-length molecules. Multiple binding sites in the helical portion of type III collagen were observed for both $\alpha_1I$ and $\alpha_2I$ (Fig. 2); however, one region at $270-300$ nm from the C-terminal end of the mature chain contained $\sim 75$ and $25\%$ of the total binding events of $\alpha_1I$ ($n = 269$) and $\alpha_2I$ ($n = 299$), respectively (Fig. 3), suggesting that this region contains high affinity binding site(s) for $\alpha_1I$ and $\alpha_2I$. Adding EDTA to the incubation buffer before the rotary shadowing dramatically reduced the number of $I$ domains bound to the procollagens, suggesting that this binding is metal ion dependent (data not shown).

**Synthesis and Characterization of Collagen-like Peptides Mimicking Putative High Affinity Binding Sites in Type III Collagen—**Type III collagen is a homotrimer composed of three $\alpha1$ (III) polypeptides, each containing 1029 amino acid residues in the mature chain (GenBank™ accession number P02461). Given that the average collagen molecule measured 300 nm, the average length per residue of collagen is 0.29 nm (3.43 amino acid residues/nm), which is consistent with earlier calculations (22). Based on this correlation, the region located 270–300 nm from C-terminal end of the mature chain corresponds to amino acid residues 168–270 of the $\alpha1$ (III) chain. This stretch of sequence contains...
one GER motif preceded by GROGRO and followed by GLO (Fig. 4C). If the GER sequence is critical for the integrin binding, then this collagen sequence is a potential high affinity site for $\alpha_1$ and $\alpha_2$. There is another GER motif preceded by GAO and followed by GROGLO close to the C-terminal side of the 270–300 nm region. Therefore, peptides $(\text{GPO})_3\text{GROGROGERGLO}(\text{GPO})_3$ (peptide 1) and $(\text{GPO})_3\text{GAOGERGROGLO}(\text{GPO})_3$ (peptide 2) were synthesized and used in I domain binding assays. Peptide 3 composed of $(\text{GPO})_{11}$ was used as a control peptide (Fig. 4B).

The synthetic peptides were examined for their ability to form collagen-like triple helices by CD spectroscopy. The CD spectra of all three peptides showed the characteristic ellipticity maxima at 220–225 nm, indicating that they are capable of forming collagen-like triple helices (Fig. 4C). We followed the temperature-dependent unfolding of the triple helix by monitoring the CD at 225 nm. The reduction of the maxima was seen from about 35 °C with melting points for the triple helix structures of these peptides recorded between 41 and 44 °C (data for peptide 1 are shown in Fig. 4B). These data demonstrate that the peptides occur in triple helix structures at the temperatures (4–25 °C) used in the following experiments.

Inhibition of $\alpha_1$ and $\alpha_2$ Binding to I and III Collagen by Synthetic Collagen Peptides—To determine whether the type III collagen peptides contain high affinity binding sites for $\alpha_1$ and $\alpha_2$, we tested their ability to inhibit the binding of recombinant I domains to type I and III collagen using ELISA type assays. Various concentrations of peptides (0.01–100 μM) were incubated with recombinant I domains before the mixtures were added to microtiter wells coated with type I or III collagen. The results indicated that at 100 μM, peptide 1 inhibited the binding of $\alpha_1$ and $\alpha_2$ to type III collagen by 100 and 80%, respectively (Fig. 5, A and B). Peptide 2 at 100 μM inhibited the binding of $\alpha_1$ and $\alpha_2$ to type III collagen by 40 and 60%, respectively, suggesting that although peptide 2 is recognized by the I domains, it is not a high affinity binding site. The control peptide 3 did not show any inhibitory activity. The IC$_{50}$ values of peptide 1 with $\alpha_1$ and $\alpha_2$ binding to type III collagen were 1.0 ± 0.6 μM and 1.9 ± 0.9 μM, respectively. Similar results were obtained with type I collagen; the IC$_{50}$ values of peptide 1 with $\alpha_1$ and $\alpha_2$ binding to type I collagen were 1.4 ± 0.4 μM and 0.14 ± 0.09 μM, respectively (Fig. 5, C and D).

Characterization of the Binding of I Domains to Collagen Peptide 1—We further investigated the direct binding of the $\alpha_1$ and $\alpha_2$ domains to peptide 1 by SPR. Peptide 1 was immobilized onto a CMS Biacore chip. Increasing concentrations of I domains (0.5–30 nM) were then passed over the surface containing peptide 1. $\alpha_1$ and $\alpha_2$ exhibited similar association rates for peptide 1, 5.6 M$^{-1}$s$^{-1}$ (×10$^4$) and 0.4 M$^{-1}$s$^{-1}$ (×10$^4$), respectively; however, $\alpha_2$ showed a much slower dissociation rate than $\alpha_1$, 1.3 s$^{-1}$ × 10$^{-3}$ for $\alpha_2$ compared with 12 s$^{-1}$ × 10$^{-3}$ for $\alpha_1$ (Fig. 6, A and B, and TABLE TWO). This resulted in a $K_D$ of 23 nM for the interactions between $\alpha_1$ and peptide 1, a $K_D$ of 283 nM for the interactions between $\alpha_2$ and peptide 1 (TABLE TWO). To test whether the binding of $\alpha_1$ and $\alpha_2$ to type III collagen.
Novel Binding Site in Collagen Type III

and α 1 to peptide 1 was metal ion-dependent, each I domain (30 nM) in the presence of either 1 mM MgCl₂ or 2 mM EDTA was passed over a peptide 1-coated surface. The presence of EDTA completely abolished the observed binding, indicating that the interactions were dependent on the presence of divalent cations (Fig. 6C).

**Adhesion of MRC-5 Cells to Peptide 1 Substrates**—To investigate whether the synthetic collagen peptide 1 is able to support cell adhesion, we used the human lung fibroblast cell line MRC-5 for adhesion assay. MRC-5 cells were shown previously to express comparable levels of α₁β₁ and α₂β₁ integrins (23). Wells were coated with increasing concentrations of type III collagen, peptide 1, or peptide 2; then 1.5 × 10⁴ MRC-5 cells were seeded in each well. The plate was incubated for 45 min at room temperature, and adherent cells were quantified as described under “Experimental Procedures.” The results showed that peptide #1 and type III collagen could support adhesion of MRC-5 cells in a dose-dependent manner, whereas peptide 2 could not (Fig. 7). Considerable cell spreading was observed among cells incubated on peptide 1 at room temperature for 1.5 h, similar to cells seeded on type III collagen (data not shown).

**GROGER Is a Minimal α₁I/α₁I High Affinity Binding Motif**—To determine the minimal binding sequence within peptide 1, a shorter peptide containing the GROGER sequence flanked by three GPO repeats at either end was synthesized. Peptides containing the GFOGER or GLOGER motifs were also made for comparison (Fig. 8A). All three peptides were able to form triple helices as shown by their ellipticity maxima around 225 nm in the CD spectra (data not shown). In addition, all three peptides showed a sharp decrease in their ellipticity at 225 nm as the temperature increased. The melting temperatures (T_m) of the GFOGER, GLOGER, and GROGER peptides were determined to be between 37 and 41 °C.

To test whether GROGER represents a high affinity binding site for α₁I and α₁I, we compared the three peptides ability to inhibit the binding of the two I domains to type III collagen using competition ELISAs. In these experiments, we used recombinant human mature type III collagen instead of procollagen III due to the limited availability of the latter. The recombinant I domains bind to mature type III collagen in a similar way as to type III procollagen (data not shown). The results showed that GROGER, as well as GFOGER and GLOGER, inhibited the binding of α₁I and α₁I to the immobilized collagen (Fig. 8, C and D). The IC₅₀ values of GFOGER, GLOGER, and GROGER are 1.9 ± 0.03 μM, 2.2 ± 0.01 μM, and 3.6 ± 0.09 μM, respectively, for the inhibition of α₁I binding and 1.4 ± 0.1 μM, 12.0 ± 1.1 μM, and 1.1 ± 0.04 μM, respectively, for α₁I binding. The IC₅₀ values determined for the GROGER peptide are in the same range as those recorded for peptide 1, which was...
used as a positive control in this experiment. Thus, GROGER represents a minimal high affinity binding sequence for α3f and α5f.

**Molecular Modeling of the Interactions between α3f and α5f—** The crystal structure of α3f in complex with a synthetic collagen peptide containing the sequence GFOGER has been reported (15). The structure shows that the Glu residue directly interacts with the divalent cation Mg2⁺ coordinated by the MIDAS motif found in the α3f. The Arg residue forms a salt bridge with Asp219 in α3f, and the Phe residue participates in hydrophobic interactions with residues found in the α5f. Another high affinity binding site composed of the sequence GLOGER also contains a hydrophobic residue at the second position. However, here we report an integrin-binding sequence GROGER that contain a charged Arg residue at the second position. To examine how the α3f structure could accommodate this charged residue in place of a hydrophobic residue, computer modeling was performed. In the published α3f-GFOGER complex structure (Protein Data Bank code 1dz1), Phe in the middle strand of the collagen triple helix participates in van der Waals interactions with the side chains of Asn151 and Glu215 of α3f and Phe in the trailing strand participates in van der Waals interactions with Leu286 and Tyr157 of α3f (15). Replacing Phe with Arg in the collagen peptide does not change the positions of neighboring residues in α3f. In the analysis of the molecular interactions between specific residues of α3f and Arg in both middle and trailing strands, the modified complex retains the van der Waals interactions previously described in the interaction with Phe (Fig. 9). Furthermore, a new hydrogen bond interaction is observed between the carbonyl backbone of Gln215 of α3f and the Arg residue in the middle strand with a distance of 2.1 Å (Fig. 9B). Thus, it appears that the second position in the collagen peptide sequence is tolerant to substitutions and that GROGER represents a novel binding motif for the collagen-binding I domains.

**DISCUSSION**

A crucial aspect for our understanding of the molecular interactions between collagens and integrins is to identify the sequence motifs in different types of collagen that are recognized by the collagen-binding integrins. In this study, we identified a novel sequence motif, GROGER, from human type III collagen and characterized its binding to the I domains of the α1 and α2.

Type III collagen is a homotrimeric molecule and is a member of the fibrillar collagen family. It co-localizes with type I collagen in tissues such as blood vessels and skin and plays a role in the development of these tissues (3, 24). *In vitro*, it has been reported that type III collagen was able to support adhesion and spreading of cells expressing integrin α1β1, or α2β1 (19). However, human type III collagen does not contain the two previously identified high affinity integrin-binding motifs, GFOGER and GLOGER. We therefore compared the binding of α1f and α1f to types I–III collagen. SPR analyses showed that all three collagen types contain at least two classes of binding sites for the two I domains. A high affinity integrin-binding site was located by rotary shadowing of I domains in complex with type III procollagen, and a synthetic collagen triple helix peptide containing the GROGER sequence was shown to bind with high affinity to the I domains and could serve as a substrate for integrin dependent cell adhesion. Other I domain-binding sites in type III collagen indicated by the rotary shadowing experiment may represent low affinity sites.

Siljander et al. (14) recently studied the role of hydrophobic residues at the second position in GFOGER and GLOGER sequences and examined the interactions between α3f and a number of GXGGER-containing collagen peptides differing only in the second or third positions. They found that with respect to the second position, the order of the inhibition potency was F=G=L=M>A (14). All of the residues tested were hydrophobic or non-polar. However, the GROGER sequence we have identified herein contains a charged residue at the second position. Furthermore, by comparing the apparent affinity of GROGER to integrin I domains with that of GFOGER and GLOGER using the peptide inhibition assay, we tentatively show that GROGER exhibits a somewhat...
Novel Binding Site in Collagen Type III

higher affinity than GFOGER and GLOGER for $\alpha_1\text{I}$ and slightly lower affinity for $\alpha_2\text{I}$. This observation may suggest that different integrins recognize different sites in collagen with different affinities. To investigate how the presence of a charged residue would affect the interactions with the $I$ domains, computer modeling was performed based on the published structure of $\alpha_2\text{I}$ in complex of a GFOGER-containing collagen peptide. Interestingly, the change from Phe to Arg does not affect the positions of the neighboring amino acid residues in $\alpha_2\text{I}$; furthermore, there is an additional hydrogen bond interaction between the Arg residue and the carbonyl backbone of Gln$^{115}$ in $\alpha_2\text{I}$. This additional contact may explain the slightly higher observed affinity of $\alpha_2\text{I}$ for GROGER compared with GLOGER. If this change affects the downstream signaling events by integrins is not yet known.

A search of different collagen sequences for the presence of GROGER indicates that it is present in a variety of collagen types (TABLE THREE). Noticeably, it is present in all the type I collagens examined; yet, this sequence was not identified as a high affinity binding sequence in previous studies with type I collagen (18). A more detailed examination revealed that it is only present in the $a_2$ chain of type I collagens from bovine or chicken, which were the sources of type I collagens in the previous studies. As type I collagen is composed of two $a_1$ chains and one $a_2$ chain, the presence of GROGER in the $a_2$ chain only may not provide sufficient interactions with residues in the $I$ domains to allow a high affinity binding.

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A Novel Binding Site in Collagen Type III for Integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$

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