Multiple Binding Sites in Collagen Type I for the Integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$

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Integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are two major collagen receptors on the surface of eukaryotic cells. Binding to collagen is primarily due to an A-domain near the N terminus of the $\alpha$ chains. Previously, we reported that recombinant A-domain of $\alpha_1\beta_1$ ($\alpha_1\beta_1$) had at least two affinity classes of binding sites in type I collagen (Rich, R. L., et al. (1999) J. Biol. Chem. 274, 24906–24913). Here, we compared the binding of the recombinant A-domain of $\alpha_2\beta_1$ ($\alpha_2\beta_1$) to type I collagen with that of $\alpha_1\beta_1$ using surface plasmon resonance and showed that $\alpha_2\beta_1$ exhibited only one detectable class of binding sites in type I collagen, with a $K_D$ of $-10 \mu M$ at $-3$ binding sites per collagen molecule. We further demonstrated that $\alpha_1\beta_1$ and $\alpha_2\beta_1$ competed with each other for binding to type I collagen in enzyme-linked immunosorbent assay (ELISA), suggesting that the binding sites in collagen for the two A-domains overlap or are adjacent to each other. By using rotary shadowing, the complexes of $\alpha_1\beta_1$ and $\alpha_2\beta_1$ procollagen were visualized. Morphometric analyses indicated three major binding regions (near the N terminus, in the central part, and near the C terminus) along the type I procollagen molecule for both A-domains. The positions of the respective binding regions for $\alpha_1\beta_1$ and $\alpha_2\beta_1$ were overlapping with or adjacent to each other, consistent with the ELISA results. Analysis of the sequences of type I collagen revealed that GER or GER-like motifs are present at each of the binding regions, and notably, the central region contains the GFOGER sequence, which was previously identified as a high affinity site for both $\alpha_1\beta_1$ and $\alpha_2\beta_1$ (Knight, C. G., et al. (2000) J. Biol. Chem. 275, 35–40). Peptides containing GLOGERGO (peptide I, near the N terminus), GFOGERGQ (peptide II, central), and GASGERGO (peptide III, near the C terminus) were synthesized. Peptides I and II effectively inhibited the binding of $\alpha_1\beta_1$ and $\alpha_2\beta_1$ to type I collagen, while peptide III did so moderately. The N-terminal site in type I collagen has the activity postdoctoral fellowship award (to Y. X.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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** The abbreviations used are: MIDAS, metal ion-dependent adhesion site; MSCRAMMs, microbial surface component recognizing adhesive matrix molecules; SPR, surface plasmon resonance; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; Fmoc, N-(9-fluorenyl)methoxycarbonyl.
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26. Computer modeling using structures of collagen peptides indicated that these triple helical peptides fit into the trenches of $\alpha_1$A and $\alpha_2$A and that a glutamate residue in the collagen could coordinate $\text{Mg}^{2+}$ in the MIDAS site (14, 16). Trench-containing structures have also been observed in the collagen-binding microbial surface component recognizing adhesive matrix molecules (MSCRAMMs) CNA and ACE from the bacteria Staphylococcus aureus and Enterococcus faecalis, respectively (16, 27, 28), and have been implicated in ligand binding. Thus, integrins and MSCRAMMs appear to employ similar binding surfaces for their interactions with collagen. Other similarities in the collagen binding mechanisms of CNA (30–531) and $\alpha_1$A have been observed. Both proteins bind to multiple sites in collagen type I with various affinities. The high affinity class of binding sites in type I collagen for $\alpha_1$A exhibited a $K_D$ of 0.09 ± 0.06 $\mu M$ and occurred 2.5 ± 0.5 times per monomer, whereas CNA (30–531) bound to 1.3 ± 0.1 sites per type I collagen monomer with highest affinity ($K_D = 0.21 ± 0.02$ $\mu M$) (16).

Studies using CNBr or collagenase-generated fragments indicated that $\alpha_1\beta_1$ and $\alpha_2\beta_1$ bind to multiple sites in collagen (29–32). By using synthetic peptides that can adopt a triple helical conformation, Knight and colleagues (33, 34) identified the sequence GFOGER (O represents hydroxyproline) (residues 502–507) as a major binding site in collagen $\alpha_1$(1CB3) for $\alpha_1$A and $\alpha_2$A as well as for the intact receptors. Replacement of residue Glu by Asp causes complete loss of recognition by $\alpha_1$A and $\alpha_2$A, whereas replacement of residue Arg by Lys causes substantial loss of recognition (34). Recently, the GFOGER-containing collagen peptide was co-crystallized in complex with $\alpha_3$A (35). Structural analyses of this complex revealed that residue Glu in the collagen peptide directly coordinates with the metal ion; residue Arg forms a salt bridge to an Asp residue in $\alpha_3$A, and residue Phe makes hydrophobic contact with $\alpha_2$A (35). Thus, in addition to residues Glu and Arg, it seems important that the second residue in this 6-amino acid segment is a hydrophobic residue. Comparison between the collagen-bound structure and $\alpha_1$A without ligand revealed that the formation of a complementary surface for collagen binding involves major conformational changes of the A-domain.

Although both $\alpha_1\beta_1$ and $\alpha_2\beta_1$ bind to collagen types I and IV, their relative affinities for the two collagen types differ. Thus, $\alpha_1\beta_1$ binds to collagen type IV with higher affinity than to type I, whereas $\alpha_2\beta_1$ has a higher affinity for collagen type I than for type IV (36). Similar differences have also been observed in the interaction of the $\alpha_1$A- and $\alpha_2$A-domains with these two collagen types (7, 10, 24, 25). Furthermore, $\alpha_1$A and $\alpha_2$A appear to bind to collagen type I with different affinities although results from different studies are not consistent (7, 24, 25).

Comparison between the structures of $\alpha_1$A and $\alpha_2$A revealed that their trenches differ in dimensions, with the one of $\alpha_1$A being longer, wider, and deeper than that of $\alpha_2$A (16). In both $\alpha_1$A and $\alpha_2$A, a so-called $\alpha C$ helix was found between $\beta$-strand E and helix $f$6 and this $\alpha C$ helix forms a wall of each trench. The $\alpha C$ helix in $\alpha_1$A is more protruding than that of $\alpha_2$A, contributing to the smaller dimensions of its trench. Mutations of residues in the $\alpha C$ helix affect collagen binding of $\alpha_2$A, but the reported results differ perhaps due to the introduction of different amino acids and/or to the different assay systems used (9, 22–25). The MIDAS residues and their spatial arrangement are conserved between $\alpha_1$A and $\alpha_2$A, suggesting a possible common metal-coordinating mechanism in their interactions with collagen. The structural differences observed in the binding trenches, however, raise the possibility of differences in binding characteristics for $\alpha_1$A and $\alpha_2$A.

We previously reported that $\alpha_1$A has at least two classes of binding sites in type I collagen with the highest affinity class having a $K_D$ of 0.09 ± 0.06 $\mu M$ and approximately three binding sites. We now report on the characterizations of the binding of $\alpha_1$A to type I collagen using both surface plasmon resonance (SPR) and ELISAs. We show that $\alpha_1$A binds type I collagen in a slightly different manner compared with $\alpha_1$A. However, the two A-domains seem to target the same or overlapping sites in type I collagen. By using rotary shadowing followed by electron microscopy of mixtures of each A-domain and type I procollagen, we located these sites along the collagen molecule. Amino acid sequences within these regions were selected and incorporated into synthetic collagen peptides. Analyses of these triple helical peptides identified a new high affinity binding site in type I collagen for both $\alpha_1$A and $\alpha_2$A.

**EXPERIMENTAL PROCEDURES**

**Cloning, Expression, and Purification of Integrin $\alpha_1$ and $\alpha_2$A-Domain Proteins**—A DNA fragment encoding the A-domain of $\alpha_1$A was amplified by PCR from a human hepatoma cDNA library and cloned in the expression vector pQE30 (Qiagen Inc., Chatsworth, CA) as described previously (16). The A-domain of $\alpha_2$A was cloned by using a similar strategy. In this case, oligonucleotide primers 5′ CGG ATC CCC TGA TTT TCA CTC CTC AAG C and 5′ GCC ACA GTC AAA TGC TGA AAA C were used to amplify by PCR the DNA fragment encoding $\alpha_2$A-domin from a human hepatoma cDNA library. Amplification was performed using Taq DNA polymerase (PerkinElmer Life Sciences) was used according to the manufacturer’s instructions. The primers contained restriction sites for BamHI and PstI. The PCR product was purified, digested with BamHI and PstI, purified again, and ligated to pQE30 digested with the same enzymes and treated with calf intestine alkaline phosphatase (Life Technologies, Inc.). The ligation mixture was transformed into E. coli JM101. The construct was verified by restriction enzyme digestion and DNA sequencing. The encoded amino acid sequence of the $\alpha_2$A-domain was as published (37).

Large scale expression and purification of $\alpha_1$A and $\alpha_2$A proteins were as described previously using HiTrap Ni+-nitrilotriacetic chromatography (16). Protein concentrations were determined from the absorbance at 280 nm as measured on a Beckman DU-70 UV-visible spectrophotometer. The molecular extinction coefficient at $\lambda_{max}$ of each protein was calculated using the method of Pace et al. (38). Previous analysis showed gradual precipitation of the recombinant A-domains within several days of post-purification. Addition of 5 mM $\beta$-mercaptoethanol delayed the precipitation for several weeks (16). Analysis of immediately purified $\alpha_1$A buffer with or without 5 mM $\beta$-mercaptoethanol gave the same results, indicating that 5 mM $\beta$-mercaptoethanol does not disturb the interaction. Thus, all analyses were performed in the presence of $\beta$-mercaptoethanol and within 2 weeks of purification.

**Surface Plasmon Resonance Spectroscopy**—Analyses were carried out at ambient temperature using the BIACORE 1000 system (BIAcore AB, Uppsala, Sweden) as described previously (16). Brieﬂy, 3000–4000 response units of bovine type I collagen (Vitrogen 100, Collagen Biomaterials, Palo Alto, CA) or chicken type I procollagen were immobilized on one of the flow cells on a CM5 chip. The $\alpha_1$A and $\alpha_2$A proteins at the indicated concentrations in HBS (10 mM HEPES, 150 mM NaCl, pH 7.4) buffer containing 5 mM $\beta$-mercaptoethanol, 1 mM MgCl2, and 0.05% octyl-$\beta$-D-glucopyranoside were run over these surfaces at 5 $\mu$M/min for 4 min. Regeneration of the collagen surface was achieved by running 20 $\mu$M of a solution of 0.02% SDS, 5 mM $\beta$-mercaptoethanol, and 0.05% octyl-$\beta$-D-glucopyranoside through the flow cell at 5 $\mu$M/min. Binding of $\alpha_1$A and $\alpha_2$A to reference flow cells, which had been activated and deactivated without the coupling of collagen, was also measured and was subtracted from the binding to collagen-coated chips. SPR sensorsgrams from different injections were overlaid using the BIAevaluation 2.1 software (BIAcore AB). Data from the equilibrium portion of the sensorsgrams were used for analysis. Based on the correlation between the SPR response and change in soluble A-domain protein binding to the immobilized collagen, values for the binding ratio, $n_{\text{bound}}$, and the concentration of free protein, [P]free, were calculated using the equations described previously (28).

Scatchard analysis was performed by plotting $n_{\text{bound}}$/[P]free against $n_{\text{bound}}$, in which the negative reciprocal of the slope is the dissociation constant, $K_D$, and the X-intercept is the number of binding interactions, $n$. Nonlinear regression was also performed by plotting $n_{\text{bound}}$/[P]free and fitted with the one-binding class or the two-binding class models using the GraphPad Prism™ software (GraphPad Software Inc., San Diego, CA). Results from the two models were compared with respect to the value of $R^2$ and the degree of freedom of the curve fit. $K_D$
values outside the experimental data range were excluded. In the case when the two \( K_d \) values given by the two-class equation were similar to each other, one-binding class equation was used to calculate \( K_d \) and \( n \). Experiments were performed with at least three independent protein preparations and three flow cells coated independently with bovine type I collagen. Purification of type I collagen 1A and 2A runs were performed for each A-domain. The results were reproducible.

**Biotinylation of the Recombinant Proteins—**EZ-Link™ Sulfo-NHS-LC-Biotin (Pierce) was dissolved in distilled H\( \text{H}_2\)O to a concentration of 1 \( \mu \text{g}/\text{mL} \). The solution was immediately added to the recombinant proteins in HBS with 5 mM \( \beta \)-mercaptoethanol at a ratio of 70 \( \mu \text{mol} \) of biotin solution per mg of protein and incubated at room temperature in a hinged end-over-end shaker. The proteins were then dialyzed against HBS containing 5 mM \( \beta \)-mercaptoethanol and 1 mM MgCl\(_2\) at 4 °C with three buffer changes. Final protein concentration was measured as described above.

**Enzyme-linked Immunosorbent Assay (ELISA)—**Microtiter wells (Immulon 4, Dynex Technologies, Chantilly, VA) were coated with 1 \( \mu \text{g} \) of bovine type I collagen or ovalbumin in HBS overnight at 4 °C. The wells were washed with HBS and incubated with blocking buffer (HBS containing 0.1% \( \text{w/v} \) ovalbumin and 0.05% \( \text{v/v} \) Tween 20) for 20 h at room temperature. Varying concentrations of the biotinylated recombinant proteins in blocking buffer containing 1 mM MgCl\(_2\) and 5 mM \( \beta \)-mercaptoethanol were added to the wells. After incubation at room temperature for 45 min, the wells were extensively washed with HBS containing 0.05% Tween 20 and 1 mM MgCl\(_2\). Streptavidin-alkaline phosphatase conjugate (Roche Molecular Biochemicals) was diluted 10,000-fold with blocking buffer containing 1 mM MgCl\(_2\) and added to the wells. After incubation at room temperature for 45 min, the wells were washed with HBS containing 0.05% Tween 20 and 1 mM MgCl\(_2\). For color development, 100 \( \mu \text{l} \) of 1.3 mM diethanolamine, pH 9.8, containing 1 mM MgCl\(_2\), and 1 mg/ml \( \beta \)-nitrophenyl phosphate (Southern Biotechnology Associates, Birmingham, AL) were added to the wells. Absorbance at 405 nm (\( \lambda_{405\text{nm}} \)) was measured using a Thermomax microplate reader ( Molecular Devices Corp., Menlo Park, CA) after 20–30 min of incubation at room temperature. Experiments were performed in triplicate and repeated with independently prepared protein preparations. Binding to ovalbumin-coated wells was considered background and therefore subtracted from binding to collagen. Data were presented as the mean value ± S.E. of \( \lambda_{405\text{nm}} \) from a representative experiment (\( n = 3 \)). Apparent dissociation constants, \( K_{\text{app}} \), were calculated using the non-linear regression method described above, with \( \lambda_{405\text{nm}} \) versus \( [P] \), instead of \( v_{\text{max}} \) and \( [P]_{\text{free}} \) where \( [P] \) is the total protein concentration. Analysis of the results indicated that the one-binding class model rather than the two-binding class model was a better fit for both \( \alpha_2 \)A and \( \alpha_1 \)A. Thus the one-binding class model was used to calculate the apparent dissociation constants (\( K_{\text{app}} \)) of both the \( \alpha_2 \)A and \( \alpha_1 \)A interaction with collagen.

**Enzyme-linked Immunosorbent Assays—**Competition ELISAs were performed essentially as described above except that biotinylated proteins were mixed with unlabeled proteins in varying ratios and added to the wells. The binding to the blocking agent ovalbumin was subtracted from the binding to collagen. The \( \lambda_{405\text{nm}} \) of biotinylated protein in the absence of competitors was set to 100%. Data were analyzed for any difference in the middle of the binding spot. A total of 376 and 608 binding events were counted for \( \alpha_1 \)A and \( \alpha_1 \)A, respectively. The binding events were then binned for every 5 nm along the collagen strand. The percentage of number of events in each bin over total events counted was calculated and plotted against the length of the collagen strand.

**Purification of Chicken Type I Procollagen—**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 from Novabiochem, San Diego, CA. Coupling of amino acids was carried out twice using diisopropylethylamine and 4-(N,N-dimethylamino)pyridine and once using 2-(4-fluorophenyl)-2-(4-methylphenyl)-1,1-biphenylcarbonitrile (Fmoc-pfp) as activator. Peptides were cleaved from the resin with 95% (v/v) trifluoroacetic acid/thioanisole/ethanedithiol, and triethylsilane (90:5:2.5:2.5 by volume) for 8 h. The resins were filtered, and the supernatants were collected. The peptides were purified by HPLC using a Vydac C18 column. The peptides were then lyophilized in vacuo. The lyophilized powder was dissolved in 0.1 M acetic acid to 50 \( \mu \text{mol} \) (calculated using the molecular weight of a linear peptide). CD data were collected on a Jasco J-720 spectropolarimeter (43) from 210 to 260 nm, with a bandwidth of 1 nm and integrated for 1 s at 0.2-nm intervals. For each sample, data were collected at each of three temperatures (4, 25, and 70 °C) using a cylindrical 0.5-cm path length cuvette. Samples were incubated at each temperature for 20 min before starting a measurement.

**Binding of \( \alpha_1 \)A and \( \alpha_1 \)A Binding to Collagen with Collagen Peptides—**Assays were carried out essentially as described above for competition ELISA with some modifications. 50 \( \mu \text{g} \) \( \alpha_1 \)A and 5 \( \mu \text{g} \) \( \alpha_2 \)A were preincubated with indicated amounts of collagen peptide in HBS containing 5 mM \( \beta \)-mercaptoethanol and 1 mM MgCl\(_2\) for 1 h at 4 °C. The mixtures were added to microtiter wells that had been coated with 1 \( \mu \text{g}/\text{well} \) bovine type I collagen, blocked with ovalbumin, and incubated at 4 °C overnight. All subsequent steps except the color development were carried out at 4 °C using chilled solutions containing 1 mM MgCl\(_2\) as described above. Bound \( \alpha_1 \)A or \( \alpha_2 \)A was detected by incubating with 100 \( \mu \text{g} \) of anti-His monoclonal antibody 7E5 (1 \( \mu \text{g/ml} \) (44) for 3 h, followed by incubation with 100 \( \mu \text{g} \) of goat anti-mouse IgG (H + L) alkaline phosphatase conjugate (Bio-Rad) for 3 h (both were diluted 1000-fold in blocking buffer). Diethanolamine, pH 9.8, containing 1 mM MgCl\(_2\) was added to each well for color development at room temperature. \( \lambda_{405\text{nm}} \) was measured from 5 min to 1 h. The experiment was performed in duplicate. Similar results were obtained when the experiment was repeated.

**RESULTS**

The \( \alpha_2 \)A-domain Binds to Multiple Sites on Bovine Type I Collagen—SPR was used to examine the binding of \( \alpha_2 \)A and \( \alpha_1 \)A to a surface coated with bovine type I collagen. 10 \( \mu \text{g} \) \( \alpha_1 \)A and \( \alpha_2 \)A, respectively, were passed over the collagen surface. Representative sensorgrams are shown in Fig. 1A. \( \alpha_1 \)A gave a much higher response than \( \alpha_2 \)A, indicating that the latter binds collagen with lower affinity. These ligand affinity differences are probably not due to differences of the affinities for Mg\(^{2+}\) in the A-domains. Previous analysis showed that in 1 mM Mg\(^{2+}\), over 99% of the \( \alpha_2 \)A was in complex with the cation (16). Furthermore, comparison of the binding of \( \alpha_2 \)A to collagen at different concentrations of 1 and 10 mM MgCl\(_2\) did not reveal any significant difference (data not shown). We therefore assume that \( \alpha_2 \)A is essentially saturated with Mg\(^{2+}\) when the cation is added at 1 mM, and subsequent measurements were in the presence of 1 mM MgCl\(_2\). The kinetics of the interactions between the two proteins and type I collagen also differed as indicated by the shape of the sensorgrams. The association and dissociation
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FIG. 1. Analyses of α1A and α2A binding to type I collagen by SPR. A, representative profiles of the relative SPR responses of the binding of 10 μM α1A (solid line) and α2A (dashed line) to immobilized bovine type I collagen in the presence of 1 mM MgCl2. Both profiles have been corrected for the response of proteins over a flow cell containing no collagen. B, nonlinear regression analysis of the binding of varying concentrations of α1A over immobilized bovine type I collagen as measured by SPR. Responses at the equilibrium portion of the sensorgrams were used for the analysis. A one-binding class model was used to fit the data and gave R = 0.9878, K_D = 10.7 ± 1.4 μM, n = 2.6 ± 0.2.

processes of α2A are much more rapid than those of α1A. Attempts to resolve the association and dissociation rates of α1A resulted in a range of rates depending on the concentration used (16); the association and dissociation processes of α2A were too rapid to obtain any meaningful rates.

In order to measure the dissociation constant of α2A, a series of increasing concentrations of α2A (0.1–20 μM) was examined. SPR response at the equilibrium portion of each sensorgram was used for the analyses. The SPR response of α2A at 0.1 to 1.8 μM range was low, and the data points in the Scatchard plot were too scattered to fit any line (data not shown). This suggested that unlike α1A (16), α2A did not have a binding class in type I collagen with a K_D in the order of 10^−7 M. Binding data generated using α2A concentrations from 2 to 20 μM were used to calculate the dissociation constant (K_D) and the number of binding sites (n). When the data points were fitted to a line, a K_D of 10.3 ± 0.8 μM and n of 2.5 ± 1.3 was obtained. These data points when connected directly to each other, however, appeared slightly concave. Nonlinear regression analysis of bound versus [P]_free was carried out, and the one-binding class and two-binding class models were used to fit the data. After comparison of the results, the one-binding class appeared to be a good working model for this analysis, giving a K_D of 10.7 ± 1.4 μM, and n of 2.6 ± 0.2. The curve fit with one-binding class is shown in Fig. 1B. Very similar results were obtained when three independent flow cells were analyzed using different preparations of fresh α2A. Thus α2A exhibited a single class of binding sites in type I collagen with a K_D ~10 μM and with ~3 sites in a collagen molecule.

α1A and α2A Showed Differences in Affinity for Type I Collagen in ELISA—The binding of biotinylated α1A and α2A at increasing concentrations (0.005–25 μM for α1A and 0.005 to 100 μM for α2A) to bovine type I collagen was examined by ELISA (Fig. 2). Both proteins showed dose-dependent and saturable binding. The binding curve of α1A reached a plateau at a much lower concentration than that of α2A, consistent with a higher affinity for the former. For α1A, a K_D(app) = 0.11 ± 0.01 μM was determined that is close to the K_D of the high affinity class of α1A previously determined by SPR (K_D = 0.09 ± 0.06 μM, n = 2.5 ± 0.5) (16). However, we were not able to detect the low affinity binding class of α1A to type I collagen in the ELISA used. For α2A, a K_D(app) = 5.8 ± 0.7 μM, in agreement with the results from the SPR analysis.

α1A and α2A Compete with Each Other for Binding to Type I Collagen—Competition ELISAs were carried out to examine if α1A and α2A bound to the same sites in type I collagen. The amounts of the biotinylated proteins (50 nM α1A and 2 μM α2A) that yielded approximately similar A_450 nm in the ELISAs described above were chosen for the competition assays. The labeled proteins were mixed with increasing concentrations of unlabeled α1A (0.1–50 μM) and α2A (1–100 μM) to examine their inhibitory activity of each other for binding to type I collagen (Fig. 3). A recombinant fragment, CNA-(30–531), of the collagen-binding MSCRAMM from S. aureus, was also used at concentrations from 1 to 100 μM. CNA-(30–531) was previously characterized as having at least two classes of binding sites in type I collagen with K_D1 = 0.21 ± 0.02 μM, n1 = 1.3 ± 0.1, K_D2 = 35 ± 9 μM, and n2 = 19 ± 3 (16). The results indicated that α1A and α2A competed with each other for binding to collagen, suggesting that the binding sites of the high affinity class for α1A are overlapping with or adjacent to the sites recognized by α2A. CNA-(30–531) did not inhibit the collagen binding of either α1A or α2A even at 100 μM of inhibitor concentration, demonstrating that the MSCRAMM and the integrin A-domains bind to different sites in type I collagen. We observed an increased binding of α2A to collagen in the presence of CNA-(30–531). The basis for this effect is unclear.

α1A appeared to be a much more potent inhibitor for both itself and α2A, probably due to its higher affinity for collagen
The IC50 values of bovine type I collagen. In the 2–20 binding in the 0.2–1.8 range, as was observed with mature bovine type I collagen. In the 2–20 μM range, αA showed Kd = 8.8 ± 0.5 μM, n = 3.2 ± 0.1. The Kd values calculated for the binding of αA and αA to chicken type I procollagen are in the same concentration range as those obtained with mature bovine type I collagen described above.

Visualization of Multiple Binding Sites of αA and αA on Type I Procollagen Using EM—In order to locate the binding sites along the collagen triple helix for αA and αA, we incubated type I procollagen with either αA or αA and imaged the binding sites by electron microscopy after rotary shadowing. In the procollagen used for these experiments, the C-terminal propeptide remained intact as a recognizable globular domain, allowing us to determine the orientation of each collagen molecule. The helical portion of the majority of molecules was found to have an average length of 300 nm. In the absence of αA and αA, little, if any, material was found attached to the collagen molecules.

Fig. 5, A and B, are representative micrographs of αA and αA binding to type I procollagen. The complexes appeared as “beads” (the A-domains) attached to a “string” (the procollagen molecule). Multiple binding sites in the helical portion of collagen were observed for both αA and αA. The distance between the base of the C-terminal globular propeptide and the sites where αA (or αA) bound was measured for a large number of procollagen-αA or -αA complexes (Fig. 6). There are three relatively well defined major peaks in the histograms of both αA and αA, suggesting that there are three major binding sites for both proteins. These would reflect the three binding sites for αA of the highest affinity class and the three binding sites for αA. The peaks are somewhat broad, which could be due to errors in measurement or to the presence of more than one binding site in each region. For αA, the highest peak was in the middle of the molecule, and for αA a binding site toward the N terminus seemed to be favored. The third major peak at the C terminus was the smallest for both proteins. There appeared to be additional binding events along the procollagen molecules that may reflect low affinity binding or nonspecific interactions between the A-domains and the procollagen molecules.

Analysis of the Amino Acid Sequences of Binding Sites for αA and αA in Chicken Type I Collagen—Type I collagen is a heterotrimer composed of two α1 [α1(I)] and one α2 [α2(I)] chains. The amino acid sequences of the α1(I) and α2(I) chains of chicken type I collagen were obtained from the Swiss-Prot...
data base using the accession numbers P02457 and P02467. The triple helical sequence of type I collagen is from residues 168 to 1181 (1014 residues) of the α1(I) chain and residues 90–1100 (1011 residues) of the α2(I) chain (45). This corresponds to 3.38 and 3.37 amino acid residues/nm of collagen triple helix for α1(I) and α2(I), respectively. Based on this correlation, the amino acid sequences in the determined binding regions were defined (Table II and Fig. 7). For α1A, the regions at 75–85, 140–155, and 250–275 nm from the C-terminal end of the mature chain were chosen, and for α2A, the regions at 70–60, 155–140, and 265–245 nm were chosen. In the collagen polypeptide α1(I), these regions correspond to amino acid residues (from the N terminus of the mature chain) 84–169 (1A1), 490–541 (1B1), and 726–761 (1C1) for α1A, and 116–186 (1A2), 490–541 (1B2), and 775–829 (1C2) for α2A (Fig. 7A). In the collagen α2(I) chain, they correspond to residues 84–165 (2A1), 488–539 (2B1), and 724–759 (2C1) for α1A, and 118–186 (2A2), 488–539 (2B2), and 775–826 (2C2) for α2A. The first two binding regions for the α-domains in collagen α1(I) and α2(I) overlapped (1A1 and 1A2, 1B1 and 1B2, 2A1 and 2A2, and 2B1 and 2B2), whereas the third binding regions were adjacent to each other (1C1 and 1C2, and 2C1 and 2C2), separated by 15 and 17 amino acid residues, respectively. These localization analyses suggest that the binding sites in type I collagen for the α-domains α1A and α2A are overlapping or adjacent, a conclusion that is consistent with the results of the competition ELISA experiments.

The second sites for α1A and α2A (1B1, 1B2, 2B1, and 2B2) cover the same region. Examination of the sequences of this region in collagen α1(I) chain revealed that it contains the sequence GFOGER (residues 502–507) in the center that was recently identified by Knight et al. (34) to represent a high affinity binding site for both α1A and α2A. We therefore consider it likely that the GFOGER sequence represents the central binding site for both the A-domains.

When the amino acid sequences around 1A1, 1A2, 2A1, and 2A2 were compared, a striking homology was found between residues 111 and 151 in the α1(I) chain and 111 and 151 in the α2(I) chain (correspond to 255–270 nm) (Fig. 7B), which represented a region where three chains have almost identical sequences. Interestingly, the sequence GLOGER (amino acids 127–132) is found in the overlapped segment of the identified binding regions of the two A-domains (1A1 and 1A2). This sequence differs only in the substitution of Leu for Phe from the identified high affinity binding sequence GFOGER. However, unlike the GFOGER sequence, which only occurs in α1(I), the α1(I) chain in this region has the same sequence and could contribute to form a binding site.

The C-terminal binding regions identified for α1A and α2A (1C1, 1C2, 2C1, and 2C2) do not overlap but are adjacent to each other, about 15–17 amino acid residues apart. Although no GFOGER or GLOGER sequences were present in this re-
Bound of A-domains of $\alpha_\beta^1$ and $\alpha_\beta^2$ to Collagen Type I

**TABLE III**

| Peptide     | Sequence (N→C) |
|-------------|----------------|
| I           | (GPO)11(GLOGERGRO)(GPO)6* |
| II          | (GPO)4(GFOGERGVQ)(GPO)4* |
| III         | (GPO)GASGERGPO(GPO)4* |
| Generic     | (GPO)11 |

*O indicates hydroxyproline.

Fig. 7. A, schematic illustration of the three major binding sites in type I collagen for $\alpha_\beta^1$ and $\alpha_\beta^2$. Only the regions in the collagen peptides that form the triple helix were shown. The numbers indicate the amino acid residue positions starting from the N terminus of the triple helical portion of each collagen peptide. Black-shaded boxes represent the major binding sites for $\alpha_\beta^1$ and gray-shaded boxes for $\alpha_\beta^2$. Each binding site was designated a name indicated in parentheses. B, comparison of the amino acid sequences between the $\alpha_\beta^1$ and $\alpha_\beta^2$ chains of collagen at binding region 1 255–270 nm away from the C-terminal end of the triple helical portion. Conserved residues are underlined.

**Discussion**

$\alpha_\beta^1$ and $\alpha_\beta^2$ are two major collagen receptors found on the surface of many eukaryotic cells. Studies suggested that the corresponding A-domains are responsible for binding collagen and that they use similar mechanisms. Crystal structure analyses demonstrate that the two A-domains have very similar tertiary structures and that both exhibit a trench at the binding site into which a rope-like collagen molecule could fit (16). The trench dimensions of the two A-domains differ slightly, with that of $\alpha_\beta^1$ being longer, wider, and deeper. The binding of both proteins to collagen requires a metal ion to coordinate with a glutamate residue in collagen (34). Despite these structural and mechanistic similarities, the data we report here indicate that there are several differences between the collagen-binding behavior of $\alpha_\beta^1$ and $\alpha_\beta^2$. $\alpha_\beta^1$ binds to type I collagen with a much higher affinity than $\alpha_\beta^2$ and where there are at least two classes of binding sites in type I collagen, whereas $\alpha_\beta^2$ only recognizes one class of binding sites. Furthermore, the association and dissociation rates for collagen binding of $\alpha_\beta^1$ are much slower than those of $\alpha_\beta^2$. These differences could be due to the structural differences observed at the trenches. The larger trench in $\alpha_\beta^1$ may allow better contacts between the residues lining the wall of the trench and residues in collagen. The slower dissociation of the $\alpha_\beta^1$-collagen complex could be a reflection of a relatively tighter binding. The significance of the slower association observed for the $\alpha_\beta^1$ binding to collagen is not clear. One possibility is that $\alpha_\beta^1$ undergoes substantial conformational changes upon binding to collagen. Comparison of the crystal structure of $\alpha_\beta^2$ with that of $\alpha_\beta^1$ in complex with a synthetic GFOGER-containing collagen peptide revealed some conformational changes in the integrin upon ligand binding (35). Similar studies have not yet been reported for $\alpha_\beta^1$.

$\alpha_\beta^1$ and $\alpha_\beta^2$ apparently bind to chicken type I procollagen in a similar manner as to mature bovine type I collagen. The number of binding sites in the procollagen for the high affinity class of $\alpha_\beta^1$ and for $\alpha_\beta^2$ are slightly higher compared with the numbers determined for mature bovine type I collagen, 2.9 ± 0.5 versus 2.5 ± 0.5 for $\alpha_\beta^1$ and 3.2 ± 0.1 versus 2.6 ± 0.2 for $\alpha_\beta^2$. The significance of this slight increase is not clear.

The number of collagen binding sites of $\alpha_\beta^1$ (the high affinity binding class) and $\alpha_\beta^2$ revealed by analyses of the SPR response and the number of major binding regions identified by rotary shadowing analyses are in agreement and suggest three binding sites in type I collagen for the highest affinity class of $\alpha_\beta^1$ and $\alpha_\beta^2$. The fact that the two proteins can compete

**Collagen Peptides Inhibit $\alpha_\beta^1$ and $\alpha_\beta^2$ Binding to Bovine Type I Collagen**—If these triple helical peptides contain high affinity binding sites for the integrin A-domains, they should be able to inhibit the binding of $\alpha_\beta^1$ and $\alpha_\beta^2$ to type I collagen. We examined this hypothesis in competition ELISAs where type I collagen was immobilized in microtiter wells. Collagen peptides I and II effectively inhibited the binding of both $\alpha_\beta^1$ and $\alpha_\beta^2$ to collagen in a dose-dependent manner (Fig. 9). This result shows that the GLOGERGRO sequence indeed represented another high affinity binding site in type I collagen. Peptide III had some inhibitory activity compared with the generic peptide but was much less potent than peptides I and II. These results suggest that peptide III is recognized by the A-domains but does not represent a high affinity site.

**Collagen Peptide Sequences**

| Peptide | Sequence (N→C) |
|---------|----------------|
| I       | (GPO)11(GLOGERGRO)(GPO)6* |
| II      | (GPO)4(GFOGERGVQ)(GPO)4* |
| III     | (GPO)GASGERGPO(GPO)4* |
| Generic | (GPO)11 |

*O indicates hydroxyproline.
pared with peptide I and II and does not appear to represent a high affinity site. This could be due to the fact that alanine does not have a long hydrophobic side chain to make contact with a residue in the A-domains. It may be that additional residues flanking the sequence GASGER are required to stabilize the interaction or that different sequences in the vicinity are responsible for the binding events that we observed in this region. Although replacement of residue Glu with Asp and Arg with Lys in the GFOGER-containing collagen peptide causes substantial loss of recognition by α1A and α2A (34), the possibility that Asp or Lys can function in a different sequence context cannot be ruled out. A third possibility is that residues from both α1(I) and α2(I) chains of type I collagen may be involved. As reported earlier, the high affinity interaction between type IV collagen with integrin α2β1 involves three amino acid residues located on different chains of the collagen molecule, Arg-473 α1(IV) and two Asp-461 α1(IV) (46, 47). Knight et al. (34) reported that although binding of α1A to type I collagen was effectively inhibited by GFOGER-containing collagen peptides, binding to type IV collagen was poorly inhibited, suggesting that the latter interaction involved site(s) with higher affinity than the GFOGER-containing site. Our results also indicated that peptides I and II essentially completely inhibited the binding of α1A and α2A to type I collagen. Thus, it appears that if the C-terminal site in type I collagen involves residues from different collagen chains, this binding site has a lower affinity and the residues are likely to be different from those in type IV collagen. This is in agreement with mutagenesis studies by Käpylä et al. (24) that indicated that α1A recognized type I collagen with a different mechanism than type IV collagen. Binding of α1A or α2A to type I collagen requires divalent metal ions such as Mg$^{2+}$ and Mn$^{2+}$. The MIDAS site is so far the only metal ion-coordinating site identified in α1A and α2A. Therefore, it seems likely that interactions with the C-terminal site in type I collagen also involves the MIDAS site in the two A-domains. Further studies are required to determine the C-terminal binding site for α1A and α2A.

A search through the protein data base at the National Center for Biotechnology Information using GFOGER and GLOGER sequences indicate that the two sequences are present in different genetic collagen chains from a variety of animals. The GFOGER sequence is present in the α1 chain of collagen types I, II, V, VII, and XI, the α2 chain of collagen type XI, and the α3, α4, α5 chain of type IV collagen, whereas the GLOGER sequence is present in the α1 chain of collagen types I–III and VII, and the α2 chain of collagen types I and VIII. Type II, III, and VII collagens are homotrimers, and types V and VIII may occur as homotrimer or heterotrimer, and types I, IV, and XI are heterotrimer. It seems likely that the two integrin A-domains may target these two sequences and bind to many different types of collagen. More studies will be needed to determine if other similar sequences also can support high affinity integrin binding.

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Fig. 8. Circular dichroism spectra of the synthetic collagen peptides at indicated temperatures. A, peptide I; B, peptide II; C, peptide III; and D, the generic peptide. Solid line, 4 °C; dashed line, 25 °C; and dotted line, 70 °C.

Fig. 9. Inhibition of the binding of α1A and α2A to type I collagen by synthetic collagen peptides. α1A (A) and α2A (B) were preincubated with the indicated amounts of each peptide and then added to microtiter wells coated with collagen. The binding in the absence of any peptides was set to 100%. Data are presented as mean ± S.D. (n = 4).

with each other for binding to collagen in ELISAs and the position of the major regions further allowed us to conclude that the respective binding sites for α1A and α2A are overlapping or located very close to each other. The rotary shadowing results suggest that the third sites (toward the C terminus) for α1A and α2A (1C1 and 1C2, 2C1, and 2C2) may not overlap. However, since the peaks are relatively broad, we cannot completely exclude the possibility that α1A and α2A share a high affinity binding site in this region.

Analysis of amino acid sequences in the three binding regions identified indicated the presence of GER or GER-like sequences in each of the regions. The central binding region contains the GFOGER sequence identified by Knight et al. (34). Thus we have arrived at the same binding sequence using a different approach. Synthetic collagen peptide I (containing GLOGER) inhibited the binding of both α1A and α2A to collagen, as effectively as peptide II, which contains the GFOGER sequence. This indicated that GLOGER represents a second high affinity site in collagen type I. Thus peptide I may represent a native high affinity binding site from type I collagen for α1A and α2A. A search of the entire type I collagen sequence showed that this sequence is present at only one location.

Fig. 9 shows that peptide III (GASGER) is a less potent inhibitor of both α1A and α2A binding to collagen type I.
