Competitive Interactions of Collagen and a Jararhagin-derived Disintegrin Peptide with the Integrin α2-I Domain

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Integrin α2β1 is a major receptor required for activation and adhesion of platelets, through the specific recognition of collagen by the α2-I domain (α2-I), which binds fibrillar collagen via Mg$^{2+}$-bridged interactions. The crystal structure of a truncated form of the α2-I domain, bound to a triple helical collagen peptide, revealed conformational changes suggestive of a mechanism where the ligand-bound I domain can initiate and propagate conformational change to the full integrin complex. Collagen binding by α2-I and fibrinogen-dependent platelet activity can be inhibited by snake venom polypeptides. Here we describe the inhibitory effect of a short cyclic peptide derived from the snake toxin metalloprotease jararhagin, with specific amino acid sequence RKKH, on the ability of α2-I to bind triple helical collagen. Isothermal titration calorimetry measurements showed that the interactions of α2-I with collagen or RKKH peptide have similar affinities, and NMR chemical shift mapping experiments with $^{15}$N-labeled α2-I, and unlabeled RKKH peptide, indicate that the peptide competes for the collagen-binding site of α2-I but does not induce a large scale conformational rearrangement of the I domain.

The integrins constitute a functionally versatile family of integral membrane receptors that mediate cell-cell and cell-extracellular matrix interactions through their regulation of cell adhesion, differentiation, migration, and the immune response (1–5). Signal transduction is bi-directional through both outside-in and inside-out mechanisms. All integrins are heterodimers composed of subunits α and β. Different combinations of subunits are expressed on different cell types with the interplay of 19 α and 8 β subunits, generating a family of 25 different heterodimers (3, 5).

The integrin receptors share common structural features. The extracellular portions of the α and β subunits combine to form a globular “head” domain that is attached to a pair of membrane-spanning helical “stalks.” Signal transduction is believed to involve an allosteric rearrangement characterized by the separation and reorientation of the stalk segments. The bidirectional nature of signal transduction is complex. Extracellular ligands induce outside-in signals by binding to fixed motifs in the head domain, whereas inside-out signaling ensues from intracellular interactions between relatively short structurally plastic control elements and a large repertoire of cellular proteins.

In nine of the human α subunits, ligand recognition is carried out by a 200-residue structurally conserved inserted (I) domain or a von Willebrand factor A domain (3, 5). The I and A domains adopt a Rossmann dinucleotide-binding fold, with a 6-stranded β-sheet surrounded by seven α-helices, and ligand recognition requires the binding of a single Mg$^{2+}$ ion to a metal ion-dependent adhesion (MIDAS) motif (6, 7). The importance of the α I domain for understanding conformational regulation and ligand binding for all integrins has been reviewed recently (5).

Integrin α2β1 is a member of the collagen/laminin receptor family and is a major receptor required for activation and adhesion of platelets, through the specific recognition of collagen by the α2-I domain (α2-I) (8), which binds fibrillar collagen via Mg$^{2+}$-bridged interactions, supported by the MIDAS motif residues Asp-151, Ser-153, Thr-221, and Asp-254. The crystal structure of the α2-I domain, bound to a triple helical collagen peptide, revealed conformational changes from an unbound “closed” form to a bound “open” form, suggestive of a mechanism where the ligand-bound I domain can initiate and propagate conformational change to the full integrin complex (9).

Collagen binding and fibrinogen-dependent platelet activity can be inhibited by snake venom polypeptide toxins, enhancing the effects of hemorrhagic venom metalloproteases. These so-called disintegrins are functional homologues of the Arg-Gly-Asp (RGD) motif found in extracellular matrix proteins. Integrin α2β1 associates with Jararhagin, a 52-kDa metalloprotease isolated from the venom of the Brazilian pit viper Bothrops jaranca, that targets multiple components in hemostasis, including von Willebrand factor, fibrinogen, and platelet aggregation. Anti-platelet activity is thought to stem from its specificity for the α2β1 integrin (10–14).

Notably, a short cyclic peptide derived from the jararhagin metalloprotease domain, containing the specific amino acid sequence RKKH, is sufficient to prevent binding of type I collagen to α2-I in a competitive manner and is capable of disrupting cell adhesion to type I collagen (15). The cyclic RKKH peptide-binding site coincides with the collagen-binding site, near the I domain MIDAS motif (16). The inhibitory effects of the RKKH peptides on the homologous α1β1 integrin have been...
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suggested to reflect the ability of the peptide to mimic the natural type I collagen ligand by inducing or stabilizing a conformational transition from the closed to the open form of the I domain (17).

In this study we characterize the conformational dynamics of the α2-I domain and its interactions with collagen and RKKH peptides. Our studies show that the RKKH peptide binds the α2-I domain and inhibits its association with type I collagen, without inducing a conformational change in the α2-I domain.

EXPERIMENTAL PROCEDURES

Materials—The pET-28b expression plasmid was from Invitrogen. (15NH4)2SO4, [13C]glucose, and D2O were purchased from Cambridge Isotopes Laboratories. Nickel-nitrilotriacetic acid-agarose was from Qiagen. The reverse phase solution was concentrated by ultrafiltration to 0.7 mM (cut-off of 10,000. The final yield of purified protein was 20 μg/μl against four changes of buffer A. The protein was removed from the dialysis bag, reacted with 10 mM iodoacetamide for 10 h at 4 °C, dialyzed against two changes of buffer B, and concentrated by ultrafiltration.

Formation of Collagen Triple Helix—To obtain a collagen triple helix, the 24-residue collagen peptide powder was suspended in buffer B at 1 mM concentration, heated to 45 °C, and then allowed to equilibrate at 4 °C for at least 12 h, as described previously (18).

Formation of Cyclic RKKH Peptide—Formation of cyclic peptide was obtained by forming a disulfide link between the terminal Cys residues as described (15). The peptide was dissolved at 1 mg/ml concentration in 0.1 M NH4CO3 and incubated at 4 °C for 24 h. The reaction was flash-frozen and the water removed by lyophilization. The cyclized peptide was resuspended in HPLC grade water and purified by reverse phase HPLC. Peak fractions were combined, frozen, and lyophilized to powder. A colorimetric assay using 5,5′-dithiobis(2-nitrobenzoic acid), to test for the presence of free thiol, showed complete conversion to the cyclized product within the limits of detection. The cyclized peptide was suspended in buffer B immediately before experiments.

NMR Spectroscopy—NMR experiments were performed on Bruker AVANCE 600- and 800-MHz spectrometers. The standard 1H/15N fast HSQC pulse sequence was used for experiments with peptides (19). Backbone resonance assignments were made using a standard CBCA(CO)NH experiment (20) and by comparison with the assignments reported previously by Elshorst et al. (21) for the same polypeptide. The chemical shifts are referenced to the 1H2O resonance, set to its expected position of 4.87 ppm at 20 °C (22). The NMR data were processed using NMRPipe (23), and the spectra were assigned and analyzed using Sparky (24). All experiments were performed at millimolar concentrations of collagen or the RKKH to obtain saturation of the α2-I domain, for a single site binding model of interaction and the measured affinity of the peptides for α2-I (see below).

Isothermal Titration Calorimetry (ITC)—The α2-I domain, the triple helix collagen peptide, and the cyclized RKKH peptide were all dissolved in buffer B. The pH of each solution was measured to ensure that no changes were produced by the polypeptide components. For the collagen binding experiments the concentration of α2-I domain in the sample cell was 100 μM and that of the collagen peptide solution 1 mM. For the RKKH binding experiments, the α2-I domain was Cys-alkylated with iodoacetamide, and its concentration in the sample cell was 100 μM. The concentration of cyclized RKKH was 1 mM.

ITC experiments were performed with a Microcal VP-ITC calorimeter. Measurements were made by titration of collagen or RKKH peptide into the α2-I domain at a temperature of 10 °C. For titration experiments, the α2-I domain was degassed and placed in the 1.4-ml reaction cell. The collagen or RKKH peptides were loaded in the 250-μl injection syringe, and a series of 8-μl injections over 16 s were made, with a spacing of 500 s between injections over 300 min. The reference power was set to 20 μcal/s, and the stirring speed was 300 rpm. Parallel control experiments, to correct for the heat of mixing, were...
performed by adding the peptide to a sample cell containing only buffer without a2-I domain. The thermodynamic data were processed with the ORIGIN program (Microcal) to extract the enthalpic, entropic, and equilibrium constants. Nonlinear least squares fitting was done using a single site binding model.

Size Exclusion Chromatography—Size exclusion chromatography was performed at 4 or 22 °C, using an Acta Prime flow system with a Superdex 75 10/300 column (GE Healthcare), running in buffer B plus 0.25 mM dithiothreitol. Samples of the system with a Superdex 75 10/300 column (GE Healthcare), raphy was performed at 4 or 22 °C, using an Acta Prime flow

thermodynamic ITC characterization of a2-I domain interactions with triple helical collagen (A and C) and cyclic RKKH peptides (B and D). The ITC titration profiles are shown at top (A and B) for the incremental addition of either peptide into 100 μM a2-I at 10 °C. The fits of heat absorbed per mol of titrant are shown at the bottom (C and D).

TABLE 1

| Peptide            | $K_d$ μM | $\Delta G$ kcal/mol | $\Delta H$ kcal/mol | $\Delta S$ kcal/mol |
|--------------------|----------|---------------------|---------------------|---------------------|
| (GPO)$_3$GFOGER(GPO)$_3$-NH$_2$ | 7.8 ± 0.24 | -6.6                | -1.17          | 5.44               |
| CTRKKHDNAQC-NH$_2$ | 8.0 ± 0.33 | -6.6                | -0.254          | 6.35               |

ITC was also performed to determine the affinity of a2-I for a cyclic RKKH peptide whose sequence had been previously identified to have the most potent inhibitory effect on a2-I (Fig. 1C) (15). To enable direct comparison with the affinity determined for collagen, this study was also performed at 10 °C. Fig. 2B shows the titration profile, and the thermodynamic parameters are reported in Table 1. The data fit to a single-site binding model (Fig. 2D) with a $K_d$ of 8.0 μM, consistent with the IC$_{50}$ of 1.2 μM, estimated in competition assays for the inhibition of collagen binding (15).

Characterization of the a2-I-Collagen Complex—To further characterize the formation of the a2-I-collagen complex, we performed size exclusion chromatography at temperatures below or above the melting transition of the collagen triple helix (Fig. 3, A and B). Isolated a2-I elutes with an apparent molecular mass near 30 kDa at both temperatures (peak a), whereas the collagen peptide elutes near 20 kDa (peak b). We attribute the differences between these observed values and those expected from the calculated molecular weights of the proteins (23 kDa for a2-I; 6.8 kDa for triple helical collagen peptide; 2.3 kDa for monomeric collagen peptide) to the hydrodynamic radii of the molecules, which govern the elution profiles. In particular, the elution of collagen is likely to be dominated by the rod-like shape of the triple helix. However, it is interesting to note that the peptide elutes at a slightly higher apparent molecular weight at 4 than 22 °C, reflecting triple helix formation below the melting temperature.

This is further corroborated by the elution profiles of pre-mixed a2-I and collagen. At 22 °C, the elution profile is identi-
cal to that of the individual components, with two resolved peaks corresponding to either a2-I (Fig. 3A, peak a) or collagen (Fig. 3A, peak b). However, when the molecules are combined and eluted at 4 °C, a new peak appears at a higher apparent molecular mass of about 45 kDa (Fig. 3B, peak c), indicating complex formation with triple helical collagen.

These results are consistent with specific recognition by a2-I of the GFOGER sequence properly displayed in triple helical collagen (28). The size exclusion results in Fig. 3 further show that the a2-I domain and collagen form a stable long lived complex and help explain the NMR results, where the extreme broadening observed in the presence of collagen reflects the formation of a large slowly tumbling biomolecular species (see below).

Effects of Mg\(^{2+}\) and Collagen on a2-I—The a2-I domain possesses high affinity for type I collagen in the presence of the divalent metal cation Mg\(^{2+}\). The metal-binding site consists of MIDAS motif residues Asp-151, Ser-153, Ser-155, Thr-221, Asp-254, and Glu-256 which form an octahedral coordination sphere composed of direct and water-bridged interactions. The structural role of Mg\(^{2+}\) has been examined using both x-ray and NMR methods in the CD11a/LFA-1 I domain (29–32), where Mg\(^{2+}\) was found to play a role in ligand binding, and its removal did not cause large scale structural change in the CD11a/LFA-1 I domain.

The crystal structure of a2-I bound to triple helical collagen suggests that collagen binding is accompanied by a large conformational rearrangement of the C-terminal helix, coupled with changes in the coordination the Mg\(^{2+}\) metal (9). Collagen binding causes three concerted changes in the I domain; the loops of the MIDAS motif are perturbed because of a rearrangement upon insertion of a collagen Glu side chain into the metal coordination sphere; helices h6 and h7 rearrange to open up the top surface; and helix h7 moves downward to the opposite pole of the MIDAS motif. The rearrangement of helix h7 is thought to produce the large scale conformational changes experienced by the integrin heterodimer during signaling. To see if the protein dynamics and conformation associated with Mg\(^{2+}\) and collagen binding could be characterized in solution, we examined the \(^{1}H/^{15}N\) HSQC NMR spectrum of a2-I in the presence or absence of metal and collagen.

The \(^{1}H\) and \(^{15}N\) chemical shifts from protein backbone amide groups are very sensitive to changes in protein conformation or chemical environment and can be used to monitor the equilibrium exchange between states arising from free and ligand-bound protein (22). If the exchange rate is faster than the difference between the chemical shifts measured for the two states, then the system is in fast exchange, and one peak is observed at the population-weighted average chemical shift of the two states. NMR can be used to detect weak binding or minor conformational rearrangements, and chemical shift changes as small as 0.02 ppm have been reported for minor local effects on protein structure resulting from binding of small molecules or modifications, whereas much larger changes (>1 ppm) can reflect major conformational rearrangements (33–36).

A sample of metal-free a2-I domain (isotopically enriched with \(^{15}N\)) was prepared by exhaustive dialysis against EDTA. The \(^{1}H/^{15}N\) HSQC spectra of the metal-free and Mg\(^{2+}\)-bound forms of the a2-I domain are shown in Fig. 4A. Several peaks undergo measurable frequency changes reflecting metal binding. A plot of the total change in \(^{1}H\) and \(^{15}N\) chemical shifts against amino acid number shows that the peaks with the largest frequency changes are localized to residues involved in direct coordination of Mg\(^{2+}\) in the MIDAS motif (Fig. 5).

This is further highlighted by mapping the frequency changes on the previously determined crystal structure of the a2-I domain (Fig. 6C). However, most of the peaks from other residues throughout the protein structure do not change, indicating that Mg\(^{2+}\) binding does not induce a large scale conformational change of the a2-I domain in solution. This is similar to the results reported for LFA-1 (31).

A potential indicator of close association between the metal-binding site and conformational change in helix h7 is the chemical shift change observed for Glu-318 in the spectra from metal-bound and unbound a2-I. Glu-318 is located in the loop preceding the C-terminal helix and is characterized by a distinct downfield chemical shift (\(^{1}H\sim11.5\) ppm). In the comparison of the structures of the collagen-bound and unbound forms of the a2-I domain, it was noted that Glu-318 in the unbound form of a2-I domain is engaged in a salt bridge interaction with Arg-288, which is not present in the collagen-bound form (9). In addition, Glu-318 and Asp-317 are observed to undergo a pronounced torsion angle change associated with the displacement of helix h7. The observed change in Glu-318 chemical shift upon addition of Mg\(^{2+}\) indicates close association of this residue with the MIDAS-binding site. However, other residues in helix h7, including Asp-317, do not change upon addition of metal, indicating that metal binding alone does not induce the conformational transition from the closed to the open state of the I domain.

To probe conformational changes of the a2-I domain upon binding to type I collagen, a series of NMR titration experiments were performed with a 26-residue collagen peptide containing the GFOGER recognition sequence, similar to that previously co-crystallized with the a2-I domain (Fig. 1B). The
melting temperature for triple helix formation of this peptide is predicted to be around 20 °C (9). The collagen triple helix was allowed to form for 12 h at 6 °C and then combined with allowed to form for 12 h at 6 °C and then combined with 1/5 to account for the 5-fold difference between the chemical shift dispersion allowed.

Additions of increasing concentrations of triple helical collagen lead to a dramatic progressive loss of signal intensity for all peaks in the NMR spectrum of α2-I (Fig. 4B), reflecting the formation of a large slowly tumbling complex and/or slow conformational exchange on the NMR time scale. The formation of a large complex is consistent with the size exclusion data at low temperature. The reduced peak intensity is also exacerbated by slower molecular tumbling rates due the need to perform experiments at 10 °C. Further addition of collagen to α2-I, above 1:1 molar ratio, causes the NMR signals to disappear completely (data not shown). The apparent lack of concomitant measurable frequency changes (ΔN < 0.01 ppm; ΔH < 0.01 ppm). Three of these peaks correspond to residues (Asn-189, Thr-221, and Asn-222) that map to the MIDAS motif of α2-I, near the predicted collagen-binding site (Fig. 6C), whereas a fourth peak corresponds to His-272, situated on the opposite face of the α2-I domain to recapitulate the interaction of the integrin α2β1 heterodimer with RKKH has been demonstrated (15) and provides the basis for the experiments described below. Mutational studies with α2-I indicate that cyclic RKKH peptides bind near the MIDAS motif α2-I (15, 16), suggesting a mechanism of direct competition for the collagen-binding site, and the interaction of cyclic RKKH with α11 has been suggested to induce or stabilize a conformational transition from the closed to the open form of integrin (17). To characterize the α2-1-RKKH interaction, we examined the influence of an RKKH sequence, previously identified to inhibit the association of α2-I with type I collagen (15) on the solution NMR spectra of α2-I.

The effect of RKKH on the 1H/15N HSQC spectrum of α2-I is shown in Fig. 4C. Favorable linewidths and chemical shift dispersion allowed 172 of the expected 188 α2-I resonances to be analyzed. The addition of RKKH (1.5:1 RKKH:α2-I, molar ratio) causes almost no changes in the HSQC spectrum of α2-I, with the exception of four peaks that undergo very small but measurable frequency changes (ΔN < 0.01 ppm; ΔH < 0.01 ppm). These peaks correspond to residues (Asn-189, Thr-221, and Asn-222) that map to the MIDAS motif of α2-I, near the predicted collagen-binding site (Fig. 6C), whereas a fourth peak corresponds to His-272, situated on the opposite
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To characterize the inhibitory effect of cyclic RKKH on α2-I/collagen binding, we examined the effect of RKKH peptide on the intensity of NMR signals from the α2-I/collagen complex. Addition of RKKH to the α2-I/collagen sample restores some of the α2-I signal intensity which had been lost upon collagen binding. This is illustrated for four isolated NMR peaks in Fig. 8 (A–D), however, the effect is uniform across the spectrum of α2-I, reflecting the effective inhibition of the α2-I/collagen interaction by RKKH. The effect of RKKH on peak intensity increases with increasing peptide concentration up to a 2:1 molar ratio of RKKH to α2-I, beyond which no further changes are observed (Fig. 8E). Taken together with the small frequency changes observed upon RKKH binding, these results suggest that RKKH inhibits by competing for the collagen-binding site on α2-I.

To further test whether RKKH and collagen compete for the same binding site on α2-I, we examined the effects of RKKH on α2-I peak intensity in samples where collagen was added to α2-I after RKKH (Fig. 8F). Although the addition of collagen to α2-I (1:1, collagen:α2-I, molar ratio) dramatically reduces the α2-I signal intensity by as much as 84%, the addition of RKKH to free α2-I (1.5:1, RKKH:α2-I, molar ratio) causes no changes in intensity. When α2-I is combined first with RKKH and then collagen is added, the peak intensity decreases only by 22%, in a manner distinctly different from the addition of collagen alone. Further addition of collagen peptide up to 2:1 (collagen:α2-I, molar ratio) shows that as the RKKH concentration is exceeded, the peak heights are reduced to a level similar to that of the addition of collagen alone.

Thus we conclude that the mechanism of RKKH inhibition involves direct binding of RKKH to α2-I. Both RKKH and collagen compete for the same binding site on the α2-I domain, with RKKH exchanging more rapidly than triple helical colla-
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RKKH peptide. However, they point to a mechanism where the RKKH cyclic peptide inhibits collagen binding without causing a conformational change of the α2-I domain. The jararhagin toxin possesses multiple domains that can inhibit α2β1 integrin, and these activities are required to enhance metalloprotease-mediated cleavage of the β1 subunit (13, 14) with the net effect of inhibition of collagen-induced activation of platelets. Jararhagin disintegrin possesses multiple motifs, and its activity is complex. Evidence supports an ability to induce α2β1-mediated integrin signaling in platelets and fibroblasts. However, neither the RKKH motif nor the parent jararhagin metalloprotease domain has been demonstrated to induce activation of α2β1. Rather, if the RKKH motif anchors the jararhagin metalloprotease to the α2 subunit, it may serve to facilitate other jararhagin motifs to play more direct roles in integrin activation.

Our results also indicate a mechanism of direct competition of the RKKH ligand for the collagen-binding site. The α2-I residue MIDAS Thr-221 is involved in Mg²⁺ metal coordination and is slightly perturbed by RKKH, suggesting that the RKKH-binding site coincides with the MIDAS motif and the collagen-binding site. The NMR perturbation data do not offer structural restraints for docking the RKKH ligand on α2-I. However, the small chemical shift change for a residue that directly coordinates Mg²⁺ metal suggests that the interaction of RKKH with α2-I is mediated by charge-charge or water-bridged contacts to the metal-binding site.

In contrast, the NMR results do not support the conclusions of a previous study that suggested that RKKH binding causes a conformational change in the α1-1 domain (17). A model of RKKH docked on the crystal structure of recombinant α1-1 predicted that the cyclic peptide could make extensive interactions with α1-1 residues Arg-218, Glu-255, Ser-256, His-257, Asn-259, Ser-291, Glu-297, Glu-298, and Ser-301, near the MIDAS motif and in helix h6 (17). However, we did not detect any NMR peak perturbations for the corresponding residues in the homologous α2-I domain.

The inhibition of collagen binding by RKKH is metal dependent, and clearly the RKKH ligand does not displace Mg²⁺, because the removal of Mg²⁺ has comparatively dramatic effects on the NMR spectrum of α2-I (Fig. 4). Water-bridged or charge-charge contacts with the binding site would also account for the ability of RKKH to inhibit collagen binding to the free α2-I domain, and its inability to disrupt the α2-I-collagen complex once formed.

Although the thermodynamic data show that the affinities of cyclic RKKH and collagen are comparable, the types of molecular interactions and possible conformational changes experienced by the α2-I domain may contribute to a disparity in the apparent off rate. The ability to mimic triple helical collagen with a simpler ligand would offer a powerful tool to study integrin signaling in solution and pave the way to drug development (42, 43).

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