COMPETITIVE INTERACTIONS OF COLLAGEN AND A JARARHAGIN-DERIVED DISINTEGRIN PEPTIDE WITH THE INTEGRIN α2-I DOMAIN*

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Running title: Integrin I domain collagen and RKKH peptide binding

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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-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 15N-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 bidirectional through both outside-in and inside-out mechanisms. All integrins are heterodimers comprised of subunits α and β. Different combinations of subunits are expressed on different cell types with the interplay of nineteen α and eight β subunits, generating a family of twenty-five 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, while 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 Mg2+ ion to a metal ion dependent adhesion (MIDAS) motif (6,7). The α-I domain's importance 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-bridged interactions.
interactions, supported by the MIDAS motif residues Asp151, Ser153, Thr221 and Asp254. The crystal structure of a truncated form 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 jararaca, that targets multiple components in haemostasis, 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 effect of the RKKH peptides on the homologous α1β1 integrin have been suggested to reflect the peptide's ability 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 paper 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 (invitrogen.com). \(^{(15)}\text{NH}_4\)\(_2\)\(\text{SO}_4\), \(^{13}\text{C}\)-glucose, and \(\text{D}_2\text{O}\) were purchased from Cambridge Isotopes Laboratories (isotopes.com). Ni-NTA Agarose was from Qiagen (qiagen.com). The reverse phase Delta Pak C18 HPLC column (300x7.8 mm; 300 Å) was obtained from Waters (waters.com). The 24-residue type I collagen peptide with the GFOGER recognition sequence (O=hydroxyproline, Hyp) was purchased in HPLC-purified powder form from Biomer technology (Hayward, CA). The partially purified RKKH peptide was purchased from Genscript (Piscataway NJ). The peptide sequences are shown in Figure 1.

**Expression and purification of integrin α2-I domain.** The recombinant α2-I domain used in these studies corresponds to residues 144-334 of the human sequence (NCBI accession: NP_002194). The DNA coding for the sequence was inserted into the NdeI/XhoI site of the pET-28b expression plasmid (Invitrogen). For eventual chemical derivatization, Ser334, at the C-terminus, was mutated to Cys. Site directed mutagenesis was used to convert Cys150 to Leu. The resulting plasmid, pNHis-α2-I(144-334)C150L, has a (His)_6 tag at the N-terminus for purification. The sequence of the expressed protein is shown in Figure 1.

**Figure 1**

Recombinant α2-I domain was expressed in *E. coli* strain BL21(DE3). To prepare \(^{15}\text{N-}\) and \(^{13}\text{C}\)-labeled protein for NMR experiments, cells bearing pNHis-α2-I(144-334)C150L were grown in minimal M9 media containing \(^{(15)}\text{NH}_4\)\(_2\)\(\text{SO}_4\) and/or \(^{13}\text{C}\)-glucose. Induction at OD\(_{500}\)=1 with 1 mM IPTG for 2 hr at 37°C gave high-level expression of the protein in the soluble fraction. Cells were lysed using a French Press in buffer D (50 mM Tris pH 8.0, 1 M NaCl, 30 mM imidazole), and the α2-I domain was purified by Ni-affinity chromatography on Ni-NTA Agarose, in buffer C (50 mM Tris pH 7, 1 M NaCl, 300 mM imidazole). The purified protein solution was dialyzed against two changes of buffer A (50 mM Tris pH 8.0, 150 mM NaCl) with 1 mM EDTA, followed by two changes of buffer B (50 mM PO\(_4\) pH 7.0, 150 mM NaCl, 5 mM MgSO\(_4\)) with 1 mM.
DTT, using dialysis membranes with a molecular weight cut-off of 10,000. The final yield of purified protein was 20 mg per L of cell culture. For NMR experiments, the protein solution was concentrated by ultrafiltration to 0.7 mM (ε = 17330 cm⁻¹ M⁻¹).

For experiments with the RKKH peptide, the surface exposed C-terminal Cys residue of the α2-I domain was alkylated with iodoacetamide as follows. Purified protein was dialyzed against four changes of buffer A. The protein was removed from the dialysis bag, reacted with 10 mM iodoacetamide for 10 hr at 4°C, and 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 NaH₂CO₃, 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/¹³C fHSQC experiment 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 previously reported by Elshorst and coworkers (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 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 injection 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 α2-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°C 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 DTT. Samples of the α2-I domain (0.3 mM in buffer B) were
combined with 0.15 mM of equilibrated collagen peptide at 4°C in buffer B. Injections of the collagen and α2-I domain alone were done using the same buffer. The flow rate was 0.4 mL/min for the 22°C experiments, and 0.55 mL/min for the 4°C experiments. The protein/collagen complexes were detected at UV wavelengths of 254 nm or 215 nm. For size comparison we utilized bovine serum albumin (66.3 kDa) and lysozyme (14.4 kDa).

RESULTS

Association of α2-I with type I collagen peptide and RKKH disintegrin peptide. In activated platelets α2β1 integrin has a high affinity for soluble collagen with an associated Kd of 35-90 nM (25), while the affinity of the α2-I domain for type I collagen measured by surface plasmon resonance is weaker, with a Kd in the low μM range (26,27). Recognition by α2β1 resides totally within the collagen sequence GFOGER, where Glu cannot be replaced by Asp, and sequence recognition is entirely dependent upon the presence of a triple-helical conformation (28). To determine the affinity of α2-I for the triple helical type I collagen peptide we performed ITC experiments where the 26-residue collagen peptide, containing the GFOGER recognition sequence (Figure 1B), was titrated into the α2-I domain. This peptide sequence is similar to that which was co-crystallized with α2-I (9).

To maintain the collagen peptide in its active triple helical form, the ITC cell temperature was kept at 10°C, below the predicted melting temperature of about 20°C (9). The results are shown in Figure 2A, and the free energy parameters are reported in Table 1. Fitting the ITC data to a single-site binding curve (Figure 2C) yields a Kd of 7.8 μM.

Characterization of the α2-I/collagen complex. To further characterize the formation of the α2-I/collagen complex we performed size exclusion chromatography at temperatures below or above the melting transition of the collagen triple helix (Figure 3A, B). Isolated α2-I elutes with an apparent molecular weight near 30 kD at both temperatures (peak "a"), while the collagen peptide elutes near 15 kD at 22°C (Figure 3B, peak "b"), and near 22 kD at 4°C. We attribute the differences between these observed values and those expected from the calculated molecular weights of the proteins (23 kDa for α2-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 higher apparent molecular weight at 4°C than 22°C, reflecting triple helix formation below the melting temperature.

This is further corroborated by the elution profiles of pre-mixed α2-I and collagen. At 22°C, the elution profile is identical to that of the individual components, with two resolved peaks corresponding to either α2-I (Figure 3A, peak "a") or collagen (Figure 3A, peak "b"). However, when the molecules are combined and eluted at 4°C, a new peak appears at a higher apparent molecular weight of about 45 kD (Figure 3B, peak "e"), indicating complex formation with triple helical collagen.

These results are consistent with specific recognition by α2-I of the GFOGER sequence properly displayed in triple helical collagen (28). The size exclusion results in Figure 3 further show that the α2-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 slow-tumbling biomolecular species (see below).
Effects of Mg and collagen on α2-I. The α2-I domain possesses high affinity for type I collagen in the presence of divalent metal cation Mg. The metal binding site consists of MIDAS motif residues Asp151, Ser153, Ser155, Thr221, Asp254 and Glu256 which form an octahedral coordination sphere comprised of direct and water-bridged interactions. The structural role of Mg has been examined using both X-ray and NMR methods in the CD11a/LFA-1 I domain (29-32), where Mg 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 α2-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 metal (9). Collagen binding causes three concerted changes in the I domain: the loops of the MIDAS motif are perturbed due to 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 downwards 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 and collagen binding could be characterized in solution we examined the $^1$H/$^{15}$N HSQC NMR spectrum of α2-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, while much larger changes (>1 ppm) can reflect major conformational rearrangements (33-36).

Figure 4

A sample of metal-free α2-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-bound forms of the α2-I domain are shown in Figure 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 in the MIDAS motif (Figure 5).

Figure 5

This is further highlighted by mapping the frequency changes on the previously determined crystal structure of the α2-I domain (Figure 6C). However, most of the peaks from other residues throughout the protein structure do not change, indicating that Mg binding does not induce a large-scale conformational change of the α2-I domain in solution. This is similar to the results reported for LFA-1 (31).

Figure 6

A potential indicator of close association between the metal binding site and conformational change in helix h7 is the chemical shift change observed in Glu318 in the spectra from metal-bound and unbound α2-I. Glu318 is located in the loop preceding the C-terminal helix, and is characterized by a distinctive downfield chemical shift ($^1$H ~ 11.5 ppm). In the comparison of the structures of the collagen-bound and unbound forms of the α2-I domain, it was noted that Glu318 in the unbound form of α2-I domain is engaged in a salt bridge interaction with Arg288, which is not present in the collagen bound form (9). In addition, Glu318 and Asp317 are observed to undergo a pronounced torsion angle change associated with the displacement of helix h7. The observed change in Glu318 chemical shift upon addition of Mg indicates close association of this residue with the MIDAS binding site. However, other residues in helix h7, including Asp317, 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 α2-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 α2-I domain (Figure 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 hr at 6°C, and then combined with α2-I solution at 10°C for NMR studies, which were also performed at 10°C to maintain the triple helical conformation.

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 (Figure 4B), reflecting the formation of a large slowly tumbling complex and/or slow conformational exchange on the NMR timescale. 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 line broadening for the remaining peaks (see Figure 8A-D) suggests that complex formation leads to the complete disappearance of the signals from α2-I/collagen, rather than a reduction in local backbone dynamics.

A few peaks are also seen to undergo a change in chemical shift frequencies (Figure 7), and for some of these, the presence of two distinct resonances, one shifted and the other at the same frequencies as free α2-I, may reflect slow exchange between the bound and unbound forms of α2-I. Interestingly these peaks map to residues situated in (Gly329) or near (Asp145, Val194, Thr202, Ser236, Ala245, Glu309, Glu318) the C-terminal helix (Figure 6C), and may reflect the conformational change indicated by the crystal structure of collagen-bound α2-I. We note that the crystal structure was obtained with a truncated form of α2-I where the last 13 amino acids in the C-terminus, including 7 residues in helix h7, were deleted, and this form may be more susceptible to stabilization and conformational change induced by collagen binding.

Figure 7

Effect of cyclic RKKH peptide on α2-I.

The ability of recombinant 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 (15,16), suggesting a mechanism of direct competition for the collagen-binding site. Furthermore, the interaction of cyclic RKKH with α1-I has been suggested to induce or stabilize a conformational transition from the closed to the open form of integrin (17). To characterize the α2-I/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 Figure 4C. Favorable linewidths and chemical shift dispersion allowed 12 of the expected 18 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). Three of these peaks correspond to residues (Asn189, Thr221, Asn222) that map to the MIDAS motif of α2-I, near the predicted collagen binding site (Figure 6C), while a fourth peak corresponds to His272, situated on the opposite pole far from the other perturbed residues, which is susceptible to slight changes in buffer conditions.

Notably, Thr221 in the MIDAS motif is absolutely critical for collagen binding to the recombinant α2-I domain, as well as for ligand binding in the αM-I and αL-I domains (9,37,38). The finding that this is the residue most affected by cyclic RKKH peptide indicates that it also plays a role in mediating the interaction of α2-I with RKKH. Thr221 is near Asp219 which is also
critical for collage binding (9). Asp219 was predicted to play a role in RKKH peptide binding (16), however mutating this residue to Arg caused RKKH to bind with higher affinity (16), and the NMR data show no effect on Asp219 due to RKKH binding.

Aside for the small changes observed for these few residues, the near lack of changes in the NMR spectrum does not support the notion of a structural transition induced in the a2-I domain by the RKKH peptide. Instead, the high content of charged residues in the RKKH sequence suggests that charge-charge side chain interactions are responsible for mediating the association between a2-I and RKKH.

To characterize the inhibitory effect of cyclic RKKH on a2-I/collagen binding, we examined the effect of RKKH peptide on the intensity of NMR signals from the a2-I/collagen complex. Addition of RKKH to the a2-I/collagen sample restores some of the a2-I signal intensity which had been lost upon collagen binding. This is illustrated for four isolated NMR peaks in Figure 8 (A-D), however, the effect is uniform across the spectrum of a2-I, reflecting the effective inhibition of the a2-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 a2-I, beyond which no further changes are observed (Figure 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 a2-I.

**Figure 8**

To further test whether RKKH and collagen compete for the same binding site on a2-I we examined the effects of RKKH on a2-I peak intensity in samples where collagen was added to a2-I after RKKH (Figure 8F). While the addition of collagen to a2-I (1:1, collagen:a2-I, molar ratio) dramatically reduces the a2-I signal intensity by as much as 84%, the addition of RKKH to free a2-I (1.5:1, RKKH:a2-I, molar ratio) causes no changes in intensity. When a2-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:a2-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 a2-I. Both RKKH and collagen compete for the same binding site on the a2-I domain, with RKKH exchanging more rapidly than triple helical collagen, however RKKH binding does not induce a conformational change in the a2-I domain.

Pentikainen and coworkers (16), tested three integrin I domain specific antibodies (12F1, 5E8, Gi9) for their ability to inhibit biotinylated RKKH peptide binding. Both 12F1, believed to target a2-I residues 173-199 (39) and/or residues 212-216 (40), and Gi9, believed to target residues 199-216 (41), were able to inhibit RKKH binding. Since the epitopes for 12F1 reside near the MIDAS motif, these results are consistent with an RKKH/collagen competition for the a2-I binding site, as demonstrated in this paper.

**DISCUSSION**

The collagen binding activity of the a2β1 integrin resides entirely in the I domain, and provides the basis for the development of integrin signaling inhibitors. Such inhibitors include the jararhagin RKKH peptide that is the focus of this study. The RKKH inhibitors were originally identified by screening short cyclic peptides for their ability to bind the recombinant a2-I domain and disrupt collagen binding (15).

Our findings confirm the inhibitory properties of the cyclic RKKH peptide. However, they point to a mechanism where the RKKH cyclic peptide inhibits collagen binding without causing a conformational change of the a2-I domain. The jararhagin toxin possesses multiple domains that can inhibit a2β1 integrin and these activities are required to enhance metalloprotease-mediated cleavage of the b1 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 Thr221 is involved in Mg 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 Mg 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 which suggested that RKKH binding causes a conformational change in the α1-I domain (17). A model of RKKH docked on the crystal structure of recombinant α1-I predicted that the cyclic peptide could make extensive interactions with α1-I residues Arg218, Glu255, Ser256, His257, Asn259, Ser291, Glu297, Glu298, and Ser301, 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 Mg, since the removal of Mg has comparatively dramatic effects on the NMR spectrum of α2-I (Figure 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).

REFERENCES


FOOTNOTES.
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Abbreviations: DTT, dithiothreitol; EDTA, Ethylenedinitrilotetraacetic acid; HSQC, heteronuclear single quantum spectroscopy; ITC, isothermal titration calorimetry

TABLE 1

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<th>Peptide</th>
<th>Kd (µM)</th>
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Figure 1. Amino acid sequences of (A) α2-I domain, (B) collagen peptide, and (C) RKKH peptide. In (A) the underlined sequence corresponds to the His tag and thrombin cleavage site which were not removed.

A
MG5SHHHHHSGLVPRGSHMIDVVLDESNSIYPWDAV 162
KNFLEKFVQGLDIPKTQVGLIQYANNPRVFNLYNTYKT 202
KEEMIVATSQSYGGDLNTFQAIQRKYAYSAASGGR 242
RSATKVMVVTQDGSHGMLKAVIDQCNQDNILRF6IAV 282
LGYNRNALDTHNLKEIKIAASIPTERYFFVSNDEAALL 322
EKAGTLGEQIFC 334

B
GPOGPOGPOGFOGERGPOGPOGPO-ΝΗ2 (O=Hyp)

C
CTRKKHDNAQC-ΝΗ2
Figure 2. Thermodynamic ITC characterization of α2-I domain interactions with (A, C) triple helical collagen and (B, D) cyclic RKKH peptides. The ITC titration profiles are shown at top (A, B), for the incremental addition of either peptide into 100 µM α2-I at 10°C. The fits of heat absorbed per mole of titrant are shown at the bottom (C, D).
Figure 3. Characterization of the α2-I/collagen interaction by size exclusion chromatography. Samples of α2-I (0.3 mM; dotted line), collagen peptide (0.15 mM; broken line), or combined α2-I/collagen (0.3 mM/0.15 mM; solid line), were chromatographed at either (A) 22°C or (B) 4°C. For each temperature, the apparent molecular weights were estimated using bovine serum albumin (66.3 kD) and lysozyme (14.4 kDa) molecular weight standards. The arrows indicate elution of α2-I (a), collagen (b), or the α2-I/collagen complex (c).
Figure 4. Effects of (A) Mg, (B) triple helical collagen, and (C) cyclic RKKH peptide, on the $^1$H/$^{15}$N HSQC spectrum of α2-I domain. (A) The spectra of Mg-bound (black) or Mg-free (red) α2-I (0.5 mM) were obtained at 20°C and 600 MHz, with either 1.75 mM or 0 mM MgCl$_2$, respectively. Selected peaks that undergo significant frequency changes are labeled. (B) The spectra of collagen-free (black) and collagen-bound (red) α2-I (0.3 mM) were obtained at 10°C and 600 MHz, with 0.3 mM triple-helical collagen peptide (0.9 mM monomeric collagen peptide; 1:1 collagen:α2-I molar ratio). (C) The spectra of RKKH-free (black) and RKKH-bound (red) α2-I (0.5 mM) were obtained at 10°C and 800 MHz, with 0.75 mM peptide (1.5:1 RKKH:α2-I molar ratio). (B, C) The spectra of free and bound α2-I were obtained in the presence of saturating Mg concentration.
Figure 5. Total change in α2-I domain backbone amide chemical shifts induced by the addition of Mg from 0 mM to 1.7 mM (96% saturation). The total combined change in chemical shift (Δ) for each residue was calculated by adding the changes in $^1$H (ΔH) and $^{15}$N (ΔN) chemical shifts, according to the equation $\Delta = [(\Delta H)^2 + (\Delta N/5)^2]^{1/2}$, where the $^{15}$N chemical shift is scaled by 1/5 to account for the 5-fold difference between the chemical shift dispersions of $^{15}$N and $^1$H (35,36). Residue numbers are indicated for peaks with $\Delta \geq 0.1$. 
Figure 6. Molecular backbone representations of α2-I domain. The coordinates of the crystal structure (7) were obtained from the Protein Data Bank (PDB code: 1AOX). The Mg atom bound to the MIDAS motif is shown in yellow, and helix h7 is shown in green. Residues shown in red have $^{1}H/^{15}N$ HSQC peaks that undergo measurable frequency changes due to α2-I association with (A) Mg at saturating concentration, (B) triple helical collagen at 1:1, collagen:α2-I, molar ratio, and (C) RKKH peptide at 1.5:1, RKKH:α2-I, molar ratio. Their Cα atoms are shown as spheres. (A) Residues in red have peaks with $\Delta>0.1$ ppm. (B) Residues in red correspond to peaks that move in Figure 7. (C) Residues in red have peaks that undergo minor but measurable frequency changes.
Figure 7. Expanded regions of the superimposed $^1$H/$^{15}$N HSQC spectra of α2-I domain, obtained in the absence (black), or presence (red), of triple helical collagen (1:1, collagen:α2-I, molar ratio) at 10°C. The spectra show examples of the frequency changes observed upon collagen binding.
Figure 8. Isolated $^1$H NMR peaks and peak intensities for four selected residues (Thr221, Met249, Gly260, Glu318), showing the inhibition of $\alpha$2-I collagen binding by cyclic RKKH. (A-D) Peaks were extracted from the $^1$H/$^{15}$N HSQC spectra of $\alpha$2-I (0.3 mM), obtained at 10°C without collagen or RKKH (solid line), after addition of 0.3 mM triple helical collagen (dotted line), and after the subsequent addition of 0.78 mM RKKH (broken line). (E) Plot of the peak intensity relative to that of free $\alpha$2-I (a), measured after addition of 0.3 mM triple helical collagen (b), followed by addition of 0.3 mM(c), 0.45 mM (d), or 0.6 mM (e) RKKH. Peak intensities are corrected for sample dilution. (F) Plot of the peak intensity relative to that of free $\alpha$2-I (a), measured after addition of 0.45 mM RKKH (b), followed by 0.3 mM (c), 0.45 mM (d), or 0.6 mM (e) collagen. Peak intensities are corrected for sample dilution.