A Peptide Inhibiting the Collagen Binding Function of Integrin \( \alpha_2I \) Domain*

(Received for publication, June 9, 1998, and in revised form, September 15, 1998)

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Integrin \( \alpha_2 \) subunit forms in the complex with the \( \beta_1 \) subunit a cell surface receptor binding extracellular matrix molecules, such as collagens and laminin-1. It is a receptor for echovirus-1, as well. Ligands are recognized by the special “inserted” domain (I domain) in the integrin \( \alpha_2 \) subunit. Venom from a pit viper, Bothrops jararaca, has been shown to inhibit the interaction of platelet \( \alpha_2\beta_1 \) integrin with collagen because of the action of a disintegrin-metalloproteinase named jararhagin. The finding that crude B. jararaca venom could prevent the binding of human recombinant \( \alpha_2I \) domain to type I collagen led us to study jararhagin further. Synthetic peptides representing hydrophilic and charged sequences of jararhagin, including the RSECD sequence replacing the well known RGD motif in the disintegrin-like domain, were synthesized. Although the disintegrin-like domain derived peptides failed to inhibit \( \alpha_2I \) domain binding to collagen, a basic peptide from the metalloproteinase domain proved to be functional. In an in vitro assay, the cyclic peptide, CTRKHKDNACQ, was shown to bind strongly to human recombinant \( \alpha_2I \) domain and to prevent its binding to type I and IV collagens and to laminin-1. Mutational analysis indicated that a sequence of three amino acids, arginine-lysine-lysine (RKK), is essential for \( \alpha_2I \) domain binding, whereas the mutation of the other amino acids in the peptide had little if any effect on its binding function. Importantly, the peptide was functional only in the cyclic conformation and its affinity was strictly dependent on the size of the cysteine-constrained loop. Furthermore, the peptide could not bind to \( \alpha_2I \) domain in the absence of Mg\(^{2+}\), suggesting that the conformation of the I domain was critical, as well. Cells could attach to the peptide only if they expressed \( \alpha_2\beta_1 \) integrin, and the attachment was inhibited by anti-integrin antibodies.

Integrins \( \alpha_1\beta_2 \) and \( \alpha_2\beta_1 \) are the major cellular receptors for native collagens (for review, see Refs. 1 and 2). Like all integrins their interaction with ligands is dependent on divalent cations (3). The \( \alpha_1 \) and \( \alpha_2 \) subunits contain an special inserted domain, the I domain, resembling the A domain found e.g. in von Willenbrand factor (4). It is evident that \( \alpha_1I \) and \( \alpha_2I \) domains are responsible for the primary recognition of collagen by the corresponding integrins (5, 6). Two other ligands for \( \alpha_2\beta_1 \) integrin, namely laminin-1 and echovirus-1, each bind to \( \alpha_2I \) domain, as well. However, echovirus-1 seems to recognize a different site on the \( \alpha_2I \) domain than the matrix proteins do (7).

The binding sites of \( \alpha_2\beta_1 \) and \( \alpha_2\beta_1 \) integrins in collagens have been localized to the triple helical areas of the molecules (8, 9). One peptide sequence derived from the collagen \( \alpha \) chain has been reported to block integrin-collagen interaction (10), but in many studies it has been ineffective and it probably does not represent the actual binding site in collagen (11–13). More likely, collagen-receptor integrins recognize amino acid residues in more than one \( \alpha \) chain. In type IV collagen-\( \alpha_2\beta_1 \) integrin interaction, the importance of one arginine and two aspartic acid residues, all from different \( \alpha \) chains, has been indicated (8). One model of collagen binding to \( \alpha_2I \) domain suggests the interaction of a glutamate residue on the collagen surface with Mg\(^{2+}\) in \( \alpha_2I \) domain (14). The recognition of collagen must, however, include the interaction of several other amino acids. In the structure-function analysis of other integrins, the use of short integrin binding peptides have been of great importance.

Peptides binding to \( \alpha_2I \) domain have so far not been available. Venoms from several snake species contain disintegrin proteins, which block platelet integrin function and are responsible for the anticoagulant effect of the venoms. Many disintegrins harbor the RGD motif and inhibit the function of platelet \( \alpha_{IIb}\beta_3 \) and \( \alpha_2\beta_1 \) integrins. However some toxins, such as jararhagin and catrocollastatin, have a disintegrin-like domain that differs from the disintegrin peptides found in crotalid and vipera venom by the nature of their different disulfide bond structure and the fact that the RGD motif is replaced by an XXCD di sulfide-bonded cysteinyll sequence (with X being any amino acid).

Jararhagin (15) is a potent inhibitor of platelet adhesion to collagen and its effect is based on the inhibition of \( \alpha_2\beta_1 \) integrin function (16). The exact mechanism of its action has been unknown. Intact jararhagin is cleaved in the snake venom-producing jaraceticin, the disintegrin-like domain of jararhagin. The fate of the metalloproteinase domain after this cleavage is unknown. Intact jararhagin is cleaved in the snake venom-producing jaraceticin, the disintegrin-like domain of jararhagin. The fate of the metalloproteinase domain after this cleavage is unknown. The RSECD sequence analogous to the RGD region, had no effect. Our studies reveal a novel integrin binding sequence,
RKK. The role of RKK motif in jararhagin function remains to be shown, but the peptides create new opportunities for structural studies on αβ₂ integrin.

**EXPERIMENTAL PROCEDURES**

**Molecular Modeling of Jararhagin Metalloproteinase Domain and the RKK Peptides** — The sequence of human α₂I domain was aligned with the sequences of the available 1 domain x-ray structures: the 1 domain of human integrin αβ₂ with bound Mg²⁺ at 2.0 Å resolution (17) and the human α₂I domain with bound Mn²⁺ at 1.8 Å resolution (18); using the alignment programs MALGIM and MALFAM (19, 20). Based on this alignment, the α₂I domain was modeled using the programs COMPOSER (Tripos Associates, St. Louis, MO, USA) and MODELLER 4.0 (21) a kind gift of Andrej Sali, Rockefeller University.

From the modeling of Botrops jara-raca the three-dimensional model was built using the same methods as in the modeling of the α₂I domain above. The model was based on the x-ray structure of Actadysin II from Crocutus adamanzues at 2.0 Å resolution (22).

The models were energy minimized in SYBYL 6.3 (Tripos Associates, St Louis, MO, USA) using the TRIPOS force field. In the first application of energy minimization, the backbone was kept rigid and only the side chains were allowed to move. In the second step, all atoms were allowed to move. Energy minimization was performed until all short contacts and inconsistencies in geometry were rectified. The electrostatic static term was not included, as the main purpose was to remove sterical hindrances and to correct bad geometry.

The conformational flexibility of the original cyclic peptide in the cyclic form was assessed using molecular dynamics simulations. The starting conformation of the peptide was taken from the metalloproteinase model structure, cysteines were added to each end, and a disulfide bond was created between them. Peptides derived from the metalloproteinase were first minimized to remove atom-atom clashes and further refined by molecular dynamics simulations. Simulations were performed in vacuum at 300 000 and 20 pS equilibration followed by a 200 pS production run. The SHAKE algorithm was applied to constrain the lengths of all bonds between heavy atoms and hydrogen atoms to allow longer 1 femtosecond time step to be used. Electrostatics were excluded, because small peptides tend to form intramolecular hydrogen bonds to make the structure globular, especially when many charged residues are present within a peptide. All calculations were made using SYBYL 6.3 and the TRIPOS force field using an Oxy II workstation.

**Generation of Human Recombinant Integrin α₂I Domain** — DNA encoding the α₂I domain was generated by polymerase chain reaction using human integrin α₂ DNA as a template (integrin α₂ DNA was a gift from Dr. M. Hemler, Dana-Farber, Boston; Ref. 27). The forward primer was 5' - CACGGCATCCGTTATGCGGCTC - 3' the reverse primer was 5' - GGGCTGGAATTCACAGATCTCTTAATGT - 3'. Primers were designed to introduce two restriction sites into the prod- uct: a BamHI site at the 5'-end and an EcoRI site at the 3'-end. Polymerase chain reaction product and pGEX2T (Amersham Pharmacia Biotech) were digested with BamHI and EcoRI, ligated, and transformed into E. coli BL21 for production of recombinant protein rα₂I. Production and purification of glutathione S-transferase-rα₂I fusion protein were carried out as follows: typically, 400 ml Luria Bertani medium (carbenicillin, 50 µg/ml) was inoculated with 40 ml of overnight culture of BL21/pKJ20 and the culture was grown for 1 h at 37 ºC. Then an inducer, isopropyl-D-thiogalactopyranoside (final concentration 0.1 mM), was added for 4 h. Cells were harvested by centrifugation and pellets were resuspended in phosphate-buffered saline (PBS) pH 7.4. Suspensions were sonicated, centrifuged, and the supernatant was retained. Pellets were resuspended in PBS, sonicated and centrifuged a further two times, and the supernatants were pooled. Glutathione-Sepharose (Pharmacia Biotech) was added to the supernatant and added to the lysate and incubated at room temperature for 30 min by gently agitation. The lysate was centrifuged, the supernatant was removed, and glutathione-Sepharose with bound fusion protein was transferred onto the suitable column. The column was then washed with 10 volumes of PBS, and the fusion protein was eluted with glutathione eluting buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0) (Amersham Pharmacia Biotech). The fusion protein was cleaved with the pre tease thrombin (Pharmacia) (10 units) for at least 2 h at room temperature and dialyzed against PBS to remove glutathione. The cleavage mixture was passed down the glutathione-Sepharose column a second time to remove glutathione S-transferase. rα₂I was collected from the flowthrough. It was necessary to treat the recombinant protein with 5 µM p-Chloromercuribenzenesulfonic acid to allow proper folding, because extra bands were seen without the treatment when analyzed by native polyacrylamide gel electrophoresis (data not shown). The recombinant α₂I domain produced was 223 amino acids long having two nonintegrin amino acids at the amino-terminal (GS), amino acids corresponding to α₂ integrin sequence 124–339 (PDF Q... IEGTV) and six nonintegrin amino acids at the carboxyl-terminal (EFIVTD).

Labeling of rα₂I with europium was carried out as follows: 150 volume 1 M NaHCO₃ (pH 8.5) was added to the purified rα₂I to elevate the pH for labeling with isothiocyanate. The europium-labeling reagent (Wallac) was added at a 100-fold molar excess and incubated overnight at 4 ºC. The unbound label was removed by gel filtration on a Sephadex G50/Sephrose 6B column (Pharmacia), and fractions containing the labeled protein were pooled.

**Binding Assays for Europium-labeled α₂I Domain and Radioactively Labeled EVI** — A sensitive rα₂I binding assay based on the use of europium-labeled rα₂I was developed. The coating of a 96-well immuno- plate (Maxisorp, Nunc) was done by exposure to 0.1 ml of PBS containing 150 µM (5 µg/ml) type I collagen (bovine dermal, Cellon), type IV collagens (Sigma), laminin-1 purified from basement membranes of the Engelbreth-Holm-Swarm mouse tumor, Collaborative Research), fibronectin (human plasma fibronectin, Boehringer Mannheim), or 3.3 µg/ml echovirus-1 or echovirus-7 for 12 h at 4 ºC. Alternatively, peptides and B. jararaca venom (Sigma) or purified jararhagin or jaracetic (a kind gift to us from Dr. Berndt, Baker Medical Research Institute, Australia) were coated at various concentrations on 96-well amine binding plates (Costar) according to the manufacturer's instructions. Residual protein absorption sites on all wells were blocked with 0.1% heat-inactivated bovine serum albumin in PBS for 1 h at 37 ºC. Echo virus-1 (Farouk strain) and -7 (Wallace) were obtained from the ATCC. They were propagated in LLC Mkt cells and purified using the method described by Abraham and Colombo (23). The purified viruses were diluted in PBS containing 0.5 mM MgCl₂ and stored at –70 ºC until used. Europium-labeled rα₂I was added at a concentration of 500 ng/ml in PBS, 2 mM MgCl₂, Delfia enhancement solution (0.1 ml) (Wallace) was added to each well, and the europium signal was measured by fluorometry (Model 1252 Delfia, Wallace). In some experiments, anti-α₂ integrin antibody 12F1 (24) was used. For the virus binding assays, EVI was metabolically labeled with culture medium containing [³⁵S]methionine (Amersham Pharmacia Biotech). Radioactively labeled virus (20,000 cpm) was allowed to bind 3 h in PBS, 2 mM MgCl₂. The wells were washed three times, and the bound radioactivity was measured in a liquid scintillation counter (Wallac).

When peptides were added endogenously, the lophylized peptides were solubilized directly with Eu₃⁺-labeled rα₂I, 500 ng/ml in PBS, 2 mM MgCl₂, 1 mg/ml BSA, and then added to the wells. When EDTA was used instead of MgCl₂, europium-labeled rα₂I was diluted with PBS, 2 mM EDTA, and subsequent washes were performed with this buffer.

**Peptides and Binding Assay Using Biotinylated 229ox** — The jararhagin analog peptides were designed based on the secondary structure prediction of the jararhagin amino acid sequence. Secondary structure prediction was performed using the Pep tideStructural program from the Genetics Computer Group (GCC) Software package (Madison, WI). Surface probability according to the Emini method (25) and hydrophilicity according to the Kyte-Doolittle method (26) were taken into account.

The peptides were synthesized on an automated peptide synthesizer (Applied Biosystems 431A) using N-(9-fluorenylmethoxycarbonyl chemistry. The peptides for the alanine substitution series were purchased from Research Genetics (Huntsville, AL). After synthesis, the peptides were oxidized to form disulfide bridges. The peptides were solubilized at 1 mg/ml concentration with 0.1 M ammonium carbonate buffer and incubated for 16–24 h at 4 ºC. The oxidation was checked with both one-phase high pressure liquid chromatography, and the oxidized peptides were lyophilized. In the 261ox peptide, the carboxyl-terminal cysteinyI residue was protected with an acetoamidomethyl group, and the cysteinyI residues at positions 1 and 8 were protected with trityl groups that were removed during cleavage of the peptide from the resin. The isoelectric points (pI) of the various peptides were also determined from the primary sequence using the isoelectric pro-
Biotinylated 229ox was carried out as follows: lyophilized 229ox peptide was solubilized in PBS and 1/5 volume 0.1 M NaHCO3, 0.5 M NaCl (pH 8.0) was added to elevate the pH for biotinylation. Sulfobiotin-NHS (Calbiochem) was added 1:2 (w/v) 229ox/biotin and incubated for 2 h at room temperature. 1/5 volume 0.5 Tris-HCl (pH 8.0) was added to stop the biotinylation reaction.

For the binding assays using biotinylated 229ox peptide, 96-well amine binding plates (Costar) were coated with various concentrations of rα2I domain or rα1 domain-derived peptides according to the manufacturer’s instructions. Residual protein absorption sites on all wells were blocked with 0.1% heat-inactivated bovine serum albumin in PBS for 1 h at 37 °C. 100 µl biotinylated 229ox in PBS, 2 mM MgCl2 and 1 mg/ml BSA was added to the coated wells and incubated for 3 h at 37 °C. Wells were then washed three times with PBS, 2 mM MgCl2 and europium-labeled streptavidin (Wallac) was added at a concentration of 500 ng/ml in PBS, 2 mM MgCl2, 1 mg/ml BSA for 30 min at room temperature. Wells were again washed three times. 0.1 ml of Delfia enhancement solution was added to each well, and the europium signal was measured (Wallac). When EDTA was used instead of MgCl2, europium-labeled rα2I was diluted with PBS, 2 mM EDTA, and subsequent washes were performed with this buffer.

Platelet Aggregation Assay—Human blood was obtained from healthy donors who had not taken any medication within the previous ten days. Blood samples were drawn into a Becton Dickinson Vacutainer 228 containing 0.129 m sodium citrate. The tube was then centrifuged 250 g for 10 min and the platelet-rich plasma was transferred to a clean tube. Platelet aggregation was conducted at 37 °C in an aggregometer (Payton, CO). 450 µl of platelet-rich plasma was preincubated 3 min at 37 °C, and platelet aggregation was quantified by measuring the total amplitude of aggregation at a predetermined time interval following the addition of 50 µl of 2 mg/ml collagen (Sigma). To assay the ability of the synthetic peptides to inhibit platelet aggregation, the antagonists were dissolved in phosphate-buffered saline at pH 7.4, 20 mM MgCl2 immediately before use. The antagonist solution was preincubated with platelet-rich plasma for 4 min at 37 °C before collagen stimulation.

The extent of inhibition of platelet aggregation was assessed by comparison with the maximal aggregation induced by collagen and then expressed as a percentage.

Cell Lines and Construction of the α2I Integrin Expression Plasmid—Human osteogenic cell line SAOS-2 (American Type Culture Collection) were used. HACAT cells are immortalized human keratinocytes originally obtained from Dr. N. E. Fusenig (DKFZ, Heidelberg). Cells were maintained in Dulbecco’s modification of Eagle’s medium supplemented with 10% fetal calf serum. Integrin α2I mRNA and protein.

Cell Adhesion Experiments—The coating of a 96-well amine binding plate (Costar) with 5 µg/cm2 (150 µg/ml) type I collagen (bovine dermal, Cellogene) or the various peptides at the concentrations indicated was done according to the manufacturer’s instructions. Residual protein absorption sites on all wells were blocked with 0.1% heat-inactivated bovine serum albumin in PBS for 1 h at 37 °C. Cells were detached by using 0.01% trypsin and 0.02% EDTA. Trypsin activity was inhibited by washing the cells with 1 mg/ml soybean trypsin inhibitor (Sigma). Cells were suspended in Dulbecco’s modification of Eagle’s medium (Life Technologies, Inc.) containing 1% glycine. When antibodies (anti-β1, Mab13, Becton Dickinson), anti-α2I (5E8, a kind gift from Dr. Bankert, Roswell Park, New York) or peptides were added, the cells were preincubated for 15 min at room temperature before transfer to the wells. 10,000 cells were transferred to each well and incubated at 37 °C for 1 h. Nonadherent cells were removed by washing the wells once with PBS. Adherent cells were fixed with 2% paraformaldehyde, stained with 0.5% crystal violet in 20% ethanol, and washed with distilled water. The bound stain was dissolved in 10% acetic acid and measured spectrophotometrically at 600 nm.
domain, two solid-phase ligand binding assays were performed. First, europium-labeled rα1 domain was allowed to attach to collagen I substratum in the presence of 2 mM Mg²⁺, and the amount of bound rα1 was then determined. The effect of the venom was tested with concentrations ranging from 1 μg/ml to 1,000 μg/ml. The venom inhibited the rα1 domain-collagen interaction efficiently and in a concentration dependent manner (Fig. 1C). Second, we coated microtiter wells with the venom proteins and tested rα1 binding to this substratum. rα1 was found to bind the venom directly in a concentration-dependent manner (not shown).

A Short, Cyclic Jararhagin-derived Peptide Directly Interacts with Integrin rα1 Domain and Inhibits the Ligand Binding.—To study if the jararhagin sequence could be used to find a peptide that would interact with α2I domain, we used a series of short cyclic peptides corresponding to regions along the protein. The most obvious target was to design a peptide corresponding to the RGD-analogous region in the diisintegrin-like domain. The other tested regions were selected based on the following facts: (i) many of the integrin binding motifs in matrix proteins and in snake venom disintegrins are found in loop structures (29, 30); (ii) many of the known integrin binding motifs contain an aspartic acid residue (29, 31–35); and (iii) the published models of integrin-collagen interactions emphasize the role of arginine and glutamic acid residues in addition to aspartic acid residue (8). Peptides corresponding to some of the more promising charged sequences were synthesized (Table I).

To investigate whether any of these peptides could directly interact with α2I domain, the jararhagin peptides along with cyclic RGD peptide, type I collagen, type IV collagen, and fibronectin were coated on microtiter wells and rα1-europium was added. The results show that one of the jararhagin peptides, 229ox, bound to rα1 domain efficiently, whereas other peptides, including 261ox, which is analogous to the atrolysin A functional motif (36), showed no effect (Fig. 2A). The peptides were then tested for their ability to influence rα1 binding to type I collagen at a concentration of 500 μM. Again, only peptide 229ox had any significant effect; it almost completely inhibited the interaction between rα1 domain and collagen (Fig. 2B).

The Sequence of Three Amino Acids, RKK, As Well As The Proper Cyclic Conformation Is Critical for Binding to rα1 Domain.—To reveal which of the amino acid residues of the 229ox peptide are critical for its function, we tested a series of new peptides where amino acids in peptide 229 were replaced one at a time with alanine residues. The peptides were bound to the solid phase and tested for their ability to bind rα1. As predicted based on molecular modeling, the three positively charged amino acids arginine-lysine-lysine (RKK) were found to be essential. In addition, the adjacent histidine showed some effect. The substitution of the aspartic acid or the asparagine residues had no effect (Fig. 3A). Consistent with this, rα1 binding to type I collagen was poorly inhibited with the peptides containing alanine substitutions of the RKK sequence, whereas substitution of the aspartic acid or the asparagine residues did not impair this function (Fig. 3B).

To exclude the possibility that the binding of 229ox to α2I domain was because of nonspecific interactions between positively and negatively charged amino acid side chains, we used both oxidized and linear p229 peptide in our solid-phase binding assay. Both peptides were of identical sequence, but measurements with the non-oxidized 229 were done in the presence of 5 mM dithiothreitol to prevent the formation of a disulfide bond. The cyclic 229ox showed the ability to inhibit rα1 adhesion to type I collagen, whereas the linear form of the peptide had only a small effect (Fig. 4). Thus, the function of the peptide was dependent on the cyclic conformation. Optimization of the loop size of the peptide was done with the help of molecular modeling and dynamic simulations. Predicted three-dimensional structures of RKK-containing peptides with varying

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**Table I**

Sequences of the synthetic peptides used in this study

| Peptides      | Amino acid sequence          | Residue no. |
|---------------|------------------------------|-------------|
| Jararhagin    |                              |             |
| 192ox         | C*WSNGDKIC*                  | 212–219     |
| 195ox         | C*EQQRYDPYKC*                | 151–159     |
| 197ox         | C*KLPDSEAHAC*                | 103–111     |
| 223ox         | C*HYSPDGREIC*                | 46–54       |
| 225ox         | C*PADVFHIKNC*                | 441–449     |
| 229ox         | C*TRKKHDNAQC*                | 241–249     |
| 231ox         | C*YSNDEHEKGC*                | 537–545     |
| 248ox         | C*TRKKHDIC*                  | 241–246     |
| 252ox         | C*TRKKHDNAC*                 | 241–248     |
| 261ox         | C*RASMSECFDPAEHC(Acm)        | 421–434     |
| Other         |                              |             |
| RGD           | GAC*RGDC*LGA                 | Ref. 39     |
| RGE           | GAC*RGEC*LGA                 | Ref. 39     |

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**Fig. 2.** Binding of europium-labeled rα1 to adhesion proteins and jararhagin-derived peptides in a solid-phase assay. Amine binding microtiter plate wells were precoated with various peptides, type I collagen, type IV collagen and fibronectin. The data are means ± S.D. from a representative experiment done in triplicate showing rα1 binding to different substrata in the presence of 2 mM MgCl₂ (A). Microtiter plate wells were precoated with BSA and type I collagen, and the europium-labeled rα1 was allowed to attach for 3 h in the presence of 2 mM MgCl₂ and 500 μM peptide (B).
numbers of residues were compared with the predicted structure of the RKK-loop found in the molecular model of the jararhagin metalloproteinase domain. Computer-based simulations on the dynamic movements available to the cyclic peptides suggested that 248ox could maintain conformation very similar to the corresponding loop region of the jararhagin metalloproteinase model structure. The 248ox is more rigid than the two-residue longer 229ox peptide, but both peptides have similar conformations, and the side chains are similarly positioned. In contrast, the eight-residue-long peptide (252ox) did not mimic the loop in the jararhagin model but after extended molecular dynamics simulations was seen to be kinked.

Peptides of six (248ox) and eight (252ox) residues (we do not count the artificially added cysteines) were synthesized and tested for their ability to inhibit \( \alpha_2I \) adhesion to type I collagen. As shown in Fig. 5, soluble type I collagen was the most potent inhibitor with an apparent IC\(_{50}\) value of 0.004 ± 0.002 \( \mu \text{M} \). In agreement with the modeling data, peptide 248ox with six residues was the most potent peptide inhibitor of \( \alpha_2I \) domain adhesion to type I collagen having an apparent IC\(_{50}\) of 2.3 ± 0.2 \( \mu \text{M} \) compared with the approximate IC\(_{50}\) values of 52 ± 20 \( \mu \text{M} \) and greater than 10 \( \mu \text{M} \) for peptides 229ox and 252ox, respectively.

The requirement of the correct architecture of the \( \alpha_2I \) domain for 229ox binding was shown in binding assays performed in the presence of EDTA instead of \( \text{Mg}^{2+} \). Interaction of the two molecules was tested by having either 229ox or \( \alpha_2I \) domain bound to the solid phase. In both cases, EDTA completely
prevented the binding of the molecules to each other (Fig. 6, A and B). Thus, the molecular recognition of α1 domain by 229ox was strictly dependent on the proper three-dimensional structure of both components.

Some of the peptides were also tested for their ability to inhibit collagen-induced platelet aggregation. With up to 1 mM concentrations, peptides 225ox and 261ox showed no effect, at 100 μM concentration 248ox showed 88% and RGD peptide 62% of the aggregation measured in the controls. At higher peptide concentrations, RGD showed complete inhibition of aggregation, whereas increasing 248ox concentrations were only slightly more effective.

The RKK peptides seem to be potent inhibitors of α1 domain suggesting a role for the metalloproteinase domain in α1 domain binding. However, the previous hypothesis (37) has been that the jararhagin disintegrin domain or jaracetin are responsible for α1 domain binding. To test this, type I collagen was coated to the microtiter plate wells and rα1-europium was added in the presence of 100 μg/ml jaracetin. No interaction between α1 domain and jaracetin was detected (not shown). The venom proteins were also bound to the solid-phase along with type I collagen, and rα1-europium was added. The α1 domain bound to collagen but showed no binding to jaracetin (not shown). It was not possible to study the role of the metalloproteinase domain of jarahragin in α1 domain binding, because the purification of the domain has not been described, and the corresponding cDNA was not available for us to use in the production of a recombinant protein. Our preliminary experiments with the purified intact jararhagin protein failed to show binding to α1 domain (not shown). We did not have access to jararhagin protein in amounts allowing optimization of the test conditions, but the result may indicate that the disintegrin domain masks the highly charged RKK motif in the metalloproteinase domain.

Expression of α2β1 Integrin on the Cell Surface Is Essential for the Recognition of the RKK Peptide—Two different cell lines were used to study the effect of the RKK peptide (248ox; CTRKKHNC). HACAT keratinocytes express α2β1 integrin, but α2β1 integrin was not detectable on their surfaces. Human osteosarcoma SAOS-2 cells express α2β1 integrin, but not α2β1 integrin. To test whether cells bind to the RKK peptide in the absence of α2β1 integrin, we assayed for adhesion with SAOS-2 cells. These cells could bind to type I collagen, but no cell adhesion occurred on the RKK peptide, thus showing that α2β1 integrin does not recognize the RKK motif (Fig. 7A). Cell adhesion on type I collagen was prevented by anti-β1 integrin antibody. When α2 integrin cDNA was transfected into SAOS-2 cells and cell clones overexpressing α2β1 integrin were tested, cell adhesion on the RKK peptide and on type I collagen increased significantly. Anti-β1 integrin antibody could decrease cell adhesion on both the RKK peptide and on collagen (Fig. 7A).

HACAT keratinocytes were selected for a second set of experiments, because in these cells anti-α2 integrin antibody (5E8) could completely block cell adhesion to type I collagen (Fig. 7B), suggesting that α2β1 integrin is the only receptor used by these cells in collagen binding. In these cells, the RKK peptide was almost as effective an inhibitor of cell adhesion as the 5E8 antibody (Fig. 7B). These experiments showed that the expression of α2β1 integrin is needed on the cell surface for the recognition of the RKK peptide and that other integrins, including the α3β1, cannot replace α2β1 integrin.

Peptide 229ox Activates the Echovirus-1 Recognition Site in Integrin α2β1 Domain—In addition to type I collagen, rα1 domain also binds type IV collagen and laminin-1. The 229ox peptide inhibited the binding of rα1 to these ligands, whereas the control peptide 225ox of the same length and conformation together with a similar pI value had no effect (Fig. 8). This suggests that the α2 domain binds all of these ligands by the same mechanism, and that 229ox inhibits the binding either by interacting directly with the ligand recognition site or by altering the native three-dimensional structure of the α2 domain to an inactive one.

Integrin α2β1 also functions as a virus receptor, mediating cell surface attachment and infection by a human pathogen, echovirus-1 (7). Matrix proteins and echovirus-1 have been found to interact with the integrin in a different manner (7), but the binding site for echovirus-1 is also located on the I domain of the α2 subunit. As described above, the rα1 domain showed weak binding to the solid-phase bound echovirus-1, but the addition of RKK peptide increased this binding about 10-fold, and the control peptide 225ox had no effect (Fig. 9A). Importantly, the RKK peptides did not directly bind to the virus itself (Fig. 9B). This result indicates that binding of the RKK peptide to the α1 domain may induce a structural change in the protein that increases the binding affinity of rα1 to echovirus-1.

DISCUSSION

The identification of the RGD motif (31) started an intensive search for other similar short recognition sequences in the integrin binding proteins. Peptides mimicking these sequences have played a central role in the studies on structure-function relationship of many integrins. The regions in collagens recognized by α3 and α2 integrin I domains contain three polypep-
The cyclic conformation, the length of the fourth amino acid in the sequence, may also be important for integrin binding motif, RKK or RKKH, because histidine, the

Mutational analysis of the peptide sequence revealed a novel antibody (1 μg/ml) (A). HACAT cell adhesion on type I collagen in the presence of 229ox and RKK peptides and 5E8, a functional anti-α2β1, integrin antibody (B). Type I collagen (5 μg/cm²; 150 μg/ml), BSA (1 mg/ml), or RKK peptide (1 mg/ml) was coated on amine binding immunoplates. In the inhibition assays, the cells were preincubated with antibodies (1 μg/ml) or peptides (500 μM) for 15 min at room temperature. 10,000 cells in Dulbecco’s modification of Eagle’s medium with 0.1% glycerine were added to each well and incubated at 37 °C for 1 h. Nonadherent cells were removed by washing the wells once with PBS. Adherent cells were fixed with 2% paraformaldehyde, stained with 0.5% crystal violet in 20% ethanol, and washed with distilled water. The bound stain was dissolved in 10% acetic acid and spectrophotometrically measured at 600 nm. The data shown are the mean values ± S.D. of a representative experiment done in quadruplicate.

The aim of this study was to find a single amino acid chain ligand molecule interacting with the α2β1 domain and to use this to design a peptide that could inhibit the function of the collagen-binding α2β1 integrin. Previous studies have shown that venom from B. jararaca can inhibit platelet adhesion to collagen by blocking the function of α2β1 integrin (16). Here, the crude B. jararaca venom could directly interact with the recombinant α2β1 domain. This led us to synthesize and test a number of jararhagin-derived peptides. We tested a peptide from the disintegrin-like region in jararhagin corresponding to the RGD loop found in other disintegrins and also other hydrophilic and charged regions along the molecule. In a solid-phase assay, the peptide derived from the disintegrin-like domain showed no interaction with the α2β1 domain, whereas a peptide, CTRKKHDNAQC, containing amino acids 241–249 from the metalloproteinase domain bound tightly to recombinant α2β1 domain inhibiting its interaction with other ligands. None of the other peptides tested showed any binding to α2β1 domain. Mutational analysis of the peptide sequence revealed a novel integrin binding motif, RKK or RKKH, because histidine, the fourth amino acid in the sequence, may also be important for the full function. The cyclic conformation, the length of the cyclic peptide and presence of Mg2⁺ was critical for the binding.

Another snake venom protein catrocollastatin from Crotalus atrox, also an inhibitor of α2β1 integrin function (38), has an identical RKK motif in its metalloproteinase domain. However, the role of the RKK motif in the function of the metalloproteinase domains of jararhagin and catrocollastatin remains to be shown. On the basis of the present knowledge, it is not possible to name the structural basis of jararhagin binding to α2β1 integrin. A recent paper presents a hypothesis that jararhagin binds to α2β1 domain by its disintegrin domain and then degrades the β1 subunits by its metalloproteinase domain (37). However, no direct interaction between α2β1 domain and jararhagin disintegrin domain has been shown, and also our experiments failed to show it. In agreement with this, a peptide representing the jararhagin sequence corresponding to the collagen-induced platelet aggregation blocking disintegrin-like motif in atrolysin (36) was nonfunctional. Thus an alternative model for jararhagin action must be considered. One possibility is that the disintegrin domain and the metalloproteinase domain, present also as separate peptides in snake venom, have independent functions. Our data support the idea that the metalloproteinase domain can block the function of α2β1 domain. The metalloproteinase domain of jararhagin as such could not be tested because the cDNA was not available to us for the production of the recombinant protein, and the cleavage product of jararhagin containing the metalloproteinase domain has not been purified. The fact that intact jararhagin did not interact with the α2β1 domain, in our experiments, can mean that the disintegrin-like domain adjacent to the metalloproteinase domain folds in a way that it masks the highly charged RKK loop and therefore inhibits the function of the RKK motif. Finally,
we cannot exclude the possibility that the snake venom contains limited. Therefore, we chose two different cell lines that differ in the number of different integrins expressed on platelets and the presence of other integrins, including α1β1 integrins. In addition, we used function-blocking antibodies specific to β1 and α2 integrin subunits. Results support the conclusion that the RKK peptide is specific for α2β1 integrin. Most importantly, human osteosarcoma cell line SAOS-2, which lacks α4β1, has several other integrins, including α1β1, α3β1, α6β1, α6β3, and αVβ5. The side chain was transfected with α2 integrin cDNA and forced to express α2β1 on the cell surface. These cells showed β1 integrin mediated binding to the RKK peptide. Our preliminary results indicate that RKK peptide can still bind to recombinant α2β1 domain, but the peptide inhibits only slightly the binding of α1β1 domain to collagen.

The fact that the RKK motif can bind to α2β1 domain and inhibit its collagen recognition function opens new possibilities to study the structure-function relationship of α2β1 domain. We have docked the jararhagin metalloproteinase domain structure manually onto the surface of the α2β1 domain. The side chains of the RKK motif consist of three positively charged residues that are oriented roughly with the positively charged groups occupying the corners of a triangle. We found a region on the α2β1 domain surface that provided a complimentary set of acidic residues. Mutation of one of these amino acids, Asp-219, generated an α2β1 domain that bound to collagen, but its function could not be inhibited by the RKK peptides. Asp-219 is located near the predicted collagen binding site close to metal ion binding MIDAS site. On the basis of the data presented here, it is evident that the RKK peptides also altered the interaction between α2β1 domain and echovirus-1. This was seen as the activation of the echovirus-1 binding function. This phenomenon can be used in future studies to unravel the molecular details of the α2β1 domain-virus interaction.

Acknowledgments—We thank Dr. M. Hemler for cDNA, Drs. V. Woods and R. Bankert for the antibodies, and Dr. Berndt for the purified jararhagin and jaracetin proteins. We are grateful for technical assistance from M. Potila.

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