α2β1 Integrin Is Not Recognized by Rhodocytin but Is the Specific, High Affinity Target of Rhodocetin, an RGD-independent Disintegrin and Potent Inhibitor of Cell Adhesion to Collagen

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We have recombinantly expressed a soluble form of human α2β1 integrin that lacks the membrane-anchoring transmembrane domains as well as the cytoplasmic tails of both integrin subunits. This soluble α2β1 integrin binds to its collagen ligands the same way as the wild-type α2β1 integrin. Furthermore, like the wild-type form, it can be activated by manganese ions and an integrin-activating antibody. However, it does not bind to rhodocytin, a postulated agonist of αβ1 integrin from the snake venom of Calloselasma rhodostoma, which elicits platelet aggregation. Taking advantage of the recombinantly expressed, soluble α2β1 integrin, an inhibition assay was established in which samples can be tested for their capability to inhibit binding of soluble α2β1 integrin to immobilized collagen. Thus, by scrutinizing the C. rhodostoma snake venom in this protein-protein interaction assay, we found a component of the snake venom that inhibits the interaction of soluble α2β1 integrin to type I collagen efficiently. N-terminal sequences identified this inhibitor as rhodocytin, a recently published antagonist of collagen-induced platelet aggregation. We could demonstrate that its inhibitory effect bases on its strong and specific binding to α2β1 integrin, proving that rhodocytin is a disintegrin. Standing apart from the growing group of RGD-dependent snake venom disintegrins, rhodocytin interacts with α2β1 integrin in an RGD-independent manner. Furthermore, its native conformation, which is stabilized by disulfide bridges, is indispensably required for its inhibitory activity. Rhodocytin does not contain any major collagenous structure despite its high affinity to α2β1 integrin, which binds to collagenous molecules much more avidly than to noncollagenous ligands, such as laminin. Blocking α2β1 integrin as the major collagen receptor on platelets, rhodocytin is responsible for hampering collagen-induced, α2β1 integrin-mediated platelet activation, leading to hemorrhages and bleeding disorders of the snake-bite victim. Moreover, having a widespread tissue distribution, α2β1 integrin also mediates cell adhesion, spreading, and migration. We showed that rhodocytin is able to inhibit α2β1 integrin-mediated adhesion of fibrosarcoma cells to type I collagen completely.

Integrins are cell adhesion molecules that consist of two noncovalently associated subunits, α and β (for review see Refs. 1 and 2). The subfamily of integrins sharing the β1 subunit are well known receptors for extracellular matrix molecules, such as collagens, laminins, and fibronectin. The subfamily of β3 subunit containing cytoadhesins comprise the platelet integrin, αIIbβ3 which binds fibrinogen/fibrin (3) and the vitronectin receptor αVβ3. The latter ones, along with several β1 integrin, such as the fibronectin receptor α5β1 integrin, recognize a linear arginyl-glycyl-aspartyl sequence within their respective ligands, such as fibrinogen, fibronectin, and vitronectin (4). In contrast, the collagen binding integrins α1β1 and α2β1 recognize arginine and aspartate/glutamate residues of different collagen chains, which are in close proximity to each other within the triple helical collagenous framework of the collagen (5–7), thus forming a completely different spatial structure than the linear RGD peptide.

Integrin-mediated cell adhesion not only anchors the cell mechanically within the extracellular matrix of the tissue but also elicits several cellular responses, such as cell spreading and migration, cell proliferation, and differentiation (for review see Ref. 2). A well studied example of cellular response triggered by integrin-ligand interaction is platelet activation and aggregation (8, 9). Thrombocytes abundantly possess the platelet integrin αIIbβ3 on their surface, which unless activated does not bind to fibrinogen/fibrin (3). Ablation of endothelial cells from the blood vessel wall or other injuries of blood vessels make type IV and type I collagen of the basement membrane and the underlying connective tissue, respectively, accessible to platelets. Once getting in contact with collagen, platelets avidly bind to collagen via their collagen receptors (9, 10), such as the α2β1 integrin, GPVI, or indirectly via von Willebrand factor, which binds to both collagen and the vWF receptor on the platelet surface. Receptor-mediated adhesion to collagen elicits a cascade of signals within the platelets, which eventually results in secretion of platelet granula, in platelet aggregation and activation of platelet integrin αIIbβ3 which then binds to fibrin with high affinity. Insoluble fibrin, which has been produced by the enzymatic blood clotting cascade and provides a scaffold, which together with platelets form the blood clot as the first and essential step in hemostasis. The key role of the α2β1 as the sole integrin collagen receptor on platelets is drastically manifested in patients with severe bleeding disorders, caused either by a genetic defect or lack of the integrin α2 subunit (11) or by auto-antibodies against the integrin α2 subunit (12).

Furthermore, snake, leeches, and ticks have developed natural inhibitors of integrin-ligand interactions, called disintegrins, that target at the integrin-mediated platelet adhesion to fibrinogen/fibrin and collagen (9). By inhibiting blood clotting,
αβ1 Integrin, a Specific High Affinity Target of Rhodocetin

The transmembrane and cytoplasmic domain of the integrin αβ1 subunit were substituted for a GSTGSGG spacer and the dimerizing motif of the transcription factor Fos. The cloning strategy started from the construct pUC-HygMT-α-fos, which was described in a previous paper (18). Briefly, the cDNA sequence coding for the αβ1 ectodomain within the pUC-HygMT-α-fos was replaced by the cDNA sequence coding for the ectodomain of the integrin αβ1 subunit. To this end, pUC-HygMT-α-fos was cleaved by SalI and dephosphorylated by calf intestine phosphatase. The 7.2-kilobase pair-long vector fragment still contains the Fos-coding sequence of the original construct pUC-HygMT-α-fos, yet lacking the complete sequence coding for the integrin αβ1 ectodomain. The human cDNA coding for the signal sequence and the N-terminal 912 amino acids of the mature αβ1 ectodomain were excised from pNeo-αβ1 construct (19) using SalI and BglII. The cDNA coding for the C-terminal 131 amino acids of the αβ1 ectodomain and the first few amino acids of the GSTGSGG spacer, the latter one of which contains the SalII restriction site, were obtained by polymerase chain reaction using the αβ1 cDNA of pNeo-αβ1 as template, and the oligonucleotides ATGCTGAAAATCTACCTAACAGATGTACC with the BglII site underlined and GCCGGCCCTGCGCCTGGTCTGCTCGCCGTTCTC with the SalI site underlined as forward and reverse primer, respectively. In a triple ligation the SalI-cleaved vector fragment and the

cDNA fragments for both the N- and C-terminal part of the αβ1 ectodomain were ligated to the pUC-HygMT-α-fos construct coding for the soluble αβ1 ectodomain, which bears at its C terminus the short spacer sequence GGSTGGG and the dimerizing motif of Fos. The pUC-HygMT-αβ1-fus construct was generated as described in a previous paper (18).

Establishing a Stable, αβ1 Secreting Schneider Cell Clone—Both constructs were transfected in an equimolar ratio into Drosophila Schneider cells, using TransFast™ Transfection Reagents (Promega, Madison, WI) according to the manufacturer’s instructions. Transfected cells were selected under 0.1 mg/ml hygromycin B. After two rounds of sub-cloning and limited dilution and after screening for positive clones by a sandwich ELISA1 described below, the stable clone αβ1-G1.2 was established, which after induction of the metallothionine promoters upstream of both integrin αβ1 and β1 ectodomain cDNAs secreted soluble αβ1 integrin into the cell supernatant in concentrations of about 40 μg/liter.

To screen hygromycin B-resistant clones for their ability to secrete soluble αβ1 integrin, supernatants of transfected clones were tested in a sandwich ELISA 4–5 days after induction by copper sulfate. For the sandwich ELISA, the mouse monoclonal anti-integrin αβ1 antibody JA218 (kindly provided by Danny Tuckwell, University of Manchester, UK) (20) was immobilized to the plastic surface of a microtiter plate at 8 μg/ml in TBS (50 mM Tris/HCl, pH 7.4, 150 mM NaCl) with MgCl2 (1 mM). After blocking of nonspecific binding sites on the microtiter plate with 1% (w/v) heat denatured BSA in TBS/MgCl2/BST (BSA/TBS/MgCl2), the cell supernatants were added into the coated wells. The antibody JA218 captured the soluble αβ1 integrin, which was then detected by an rabbit anti-human β1 integrin-antiserum as primary antibody and goat anti-rabbit IgG-antibodies coupled to alkaline phosphatase (Sigma) as secondary antibody, diluted 1:300 and 1,600, respectively, in BSA/TBS/MgCl2. Before each antibody incubation and the final enzymatic detection reaction, wells were washed three times with TBS/MgCl2. As substrate of alkaline phosphatase, p-nitrophenylphosphate tablets were used according to the manufacturer’s instructions (Sigma). Absorbance was measured at 405 nm using an ELISA-reader (Dynatech, Burlington, MA).

Isolation of Recombinant Human Soluble αβ1 Integrin—In spinner flasks, αβ1-G1.2 cells were grown in SF900 Medium (Life Technologies, Inc.) containing 0.1 mg/ml hygromycin B and 10% fetal calf serum. Once they had reached a density of about 12 million cells/ml, they were induced by addition of copper sulfate at 0.6 mM. Simultaneously, glucose was added to 0.1% (v/v) and glutamine was added to 0.8 mM. Cell supernatant was harvested 5 days after induction and concentrated by diafiltration in a YM30 membrane cartridge (Amicon, Witten, Germany). Protease inhibitors aprotinin, leupeptin, and pepstatin were added at 1 μg/ml Mn2+ ions that increase integrin affinity to ligands were added to a final concentration of 1 mM. The concentration of dithiothreitol (DTT) was adjusted to 2 mM, before the concentrated cell supernatant was loaded onto the collagen I column. The collagen I column had been generated by covalently coupling bovine type I collagen to cyanogen bromide-activated Sepharose 4B CL according to the manufacturer’s instructions (Amersham Pharmacia Biotech). The loaded collagen I column was washed with TBS containing 2 mM MgCl2, 1 mM MnCl2, and 2 mM DTT (wash buffer A). After a stringent wash with buffer A with a NaCl concentration of 300 mM, the collagen I column was washed with buffer A, before the soluble αβ1 integrin was eluted with TBS containing 20 mM EDTA. Immediately after elution, MgCl2 was added to 30 mM, and the eluate fraction was neutralized with 2 mM Tris/HCl, pH 8.0. The αβ1 containing eluate fractions were concentrated by ultrafiltration.

Diluted with Mono Q buffer A (20 mM Tris/HCl, pH 8.0, 1 mM MgCl2), the αβ1 containing solution was loaded onto a Mono Q column and eluted with a linear gradient of 0 to 50% Mono Q buffer B (1 mM NaCl in Mono Q buffer A) within 60 min. The αβ1 containing eluate fractions were concentrated by centrifugal ultrafiltration using a Centricon 50 tube (Amicon, Witten, Germany). Protein concentration was determined using the bichinonic acid assay according to the manufacturer’s instructions (Pierce). Purity was assessed by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie staining.

Bovine Soluble αβ1 Integrin to Various Extracellular Matrix Molecules—Bovine type I collagen and chicken type II collagen was

1 The abbreviations used are: ELISA, enzyme-linked immunosorbent assay; TBS, Tris-buffered saline; BSA, bovine serum albumin; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; MES, 4-morpholinethanesulfonic acid, MMP, matrix metalloproteinase.
kindly provided by Peter Bruckner (University of Münster, Germany). Type IV collagen, the type IV collagen fragment CB3IV, type V collagen, and murine Laminin-1 (Engельbreth-Holm-Swarm-Laminin) were gratefully obtained from Klaus Kühn, Rupert Timpl, and Albert Ries (Max-Planck-Institute for Biochemistry, Martinsried, Germany). Collagen was excised in 0.1 M acetic acid as except for CB3IV, which like laminin was coated in TBS/MgCl₂ onto the microtiter plate. After the wells were blocked with a BSA/TBS/MgCl₂, the integrin dissolved in the same solution was allowed to bind to the immobilized substratum. The activating monoclonal anti β₁ integrin antibody 9EG7 or EDTA were added as indicated. The activating monoclonal anti β₁ integrin antibody 9EG7 (21) was isolated as a nonpassivating standard protein. Bound 9EG7 hybridoma was kindly provided by Dieter Vestweber (University of Münster, Münster, Germany). After a 2-h incubation at room temperature, nonbound integrin was washed away with HEPES wash buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM MgCl₂, 1 mM MnCl₂) twice. Then collagen-α₁β₁ integrin was covalently cross-linked to the substrate with 2.5% glutaraldehyde solution in HEPES wash buffer for 10 min at room temperature. After washing the plate three times with TBS/MgCl₂, the amount of bound α₁β₁ integrin was measured in an ELISA-like procedure with a rabbit anti-human integrin β₁ subunit antiserum as primary antibody and an anti-rabbit IgG-antibody conjugate as secondary antibody, diluted 1:300 and measured at 405 nm in an ELISA reader.

Separation of the Snake Venom Proteins From C. rhodostoma—Snake venom lipoyphilizate from C. rhodostoma (Sigma) was dissolved in TBS, pH 7.4, containing 1 mM EDTA (TBS/EDTA) at a protein concentration of about 200 mg/ml. The proteins were separated by gel filtration on a Superose 6 column HR30/30 (Amerham Pharmacia Biotech) using TBS/EDTA at 0.3 ml/min. Two distinct pools of fractions were able to inhibit the binding of soluble α₁β₁ integrin to immobilized type I collagen. The fractions containing the Low molecular weight Calloselasma inactivator (LMW-CI) was diluted in 20 mM MES/NaOH, pH 6.5 (Mono S buffer), and passed through a Mono S HR5/5 column (Amerham Pharmacia Biotech). The retained proteins were eluted with a linear gradient of 0 to 20 Mono S buffer B (1:0.2 Mono S buffer A) within 60 min. In the third purification step, the LMW-CI containing solution was diluted to pH 8.5 by diluting into 20 mM Tris/HCl, pH 8.5 (Mono Q buffer A). The LMW-CI was eluted from the Mono Q column using a linear gradient of 0 to 50% Mono S buffer B (1:0.2 Mono Q buffer A). The elute fractions containing LMW-CI were concentrated in a Centricon 10 tube by centrifugal ultrafiltration. To reduce contaminating proteins any further, a final gel filtration on a TSK G3000SWXL column (TosoHaas, Stuttgart, Germany) was performed at 0.4 ml/min. The fractions containing rhodocetin were concentrated in a Centricon 10 tube by centrifugal ultrafiltration to reach a concentration of about 0.3 mg/ml. The CD spectrum was recorded from 190 to 260 nm in a 0.1-mm cuvette in a CD spectrophotometer type CD6 (Jobin Yvon, Paris, France). Temperature was controlled by a self-constructed Peltier element cuvette holder. The relative amount of secondary structure (α helix, parallel, and anti-parallel β strands, random coil) were calculated with the deconvolution program of CDNN by Gerhard Böhm (23).

Inhibition of Cell Adhesion to Collagen by Rhodocetin—Monomeric bovine type I collagen at a concentration of 0.2 μg/ml in 0.1 M acetic acid was immobilized onto a microtiter plate at 4 °C overnight. After being washed with TBS/MgCl₂ for three times, the plate was blocked with BSA/TBS/MgCl₂ for 2 h at room temperature. HT1080 fibrosarcoma cells at a density of 500,000 cells/ml in Dulbecco’s modified Eagle’s medium were plated onto the plate for 35 min in a tissue culture incubator at 37 °C in both absence and presence of various concentrations of rhodocetin. Adherent cells were detected by staining with crystal violet (24). Briefly, adherent cells were fixed with 70% (v/v) solution of ethanol for 7 min and stained with a 0.1% (w/v) solution of crystal violet for 15 min destilled water. After washing the wells, cell bound dye was extracted with a 0.2% (v/v) Triton X-100 solution, and its amount was measured in an ELISA reader at 560 nm. Experiments with cells were done in triplicates. The adhesion signal of HT1080 cells measured on BSA was considered nonspecific background and subtracted from the adhesion signals of cells on type I collagen. Adhesion signals in the presence of rhodocetin were normalized to the adhesion signal of the noninhibited cell adhesion to type I collagen without any inhibitor.

RESULTS

Production and Isolation of Recombinant Soluble Human α₁β₁ Integrin—A recombinant soluble human α₁β₁ integrin that consists of the ectodomains of both α₁ and β₁ integrin subunits being noncovalently associated by the dimerizing motif of Fos and Jun, respectively, was secreted by transfected Drosophila Schneider cells. Affinity purification of the cell supernatant on a type I collagen column yielded not only the soluble α₁β₁ integrin but also a protein of 45 kDa as determined without any inhibitor was taken as 100%.

Titration of Immobilized Rhodocetin with Soluble α₁β₁ Integrin—Both native and inactive rhodocetin were coated onto a microtiter plate at 50 μg/ml in TBS/MgCl₂ at 4 °C overnight. Rhodocetin had been inactivated by heat denaturation at 95 °C for 20 min in the presence of 40 μM phenylmethylsulfonyl fluoride and 1,10-phenanthroline. After a 2-h incubation at room temperature, wells were washed twice with HEPES wash buffer. Bound α₁β₁ integrin was fixed, and its amount was determined by ELISA as described above. Nonspecific binding signals measured as α₁β₁ binding to the blocking agent BSA were subtracted from the binding values for α₁β₁ binding to native and denatured rhodocetin, respectively. The titration curves were linearized, and a Kᵣ value was determined according to the algorithm given by Heyn and Weischat (22).

RGD Peptide Inhibition Assay of α₁β₁ Binding to Rhodocetin—Inhibition of α₁β₁ binding to immobilized rhodocetin by RGD peptide was performed similarly to the titration experiments. After the microtiter plate was coated with rhodocetin at 50 μg/ml overnight at 4 °C and blocked with BSA/TBS/MgCl₂ at room temperature for 2 h, soluble α₁β₁ integrin was added either alone or in the presence of various concentrations of the linear GRGDSP peptide (Bachem, Heidelberg, Germany) for 2 h at room temperature. Then unbound α₁β₁ integrin was removed by washing with HEPES wash buffer twice. Bound α₁β₁ integrin was fixed with 2.5% glutaraldehyde in HEPES wash buffer. Its amount was determined by ELISA as described above. The binding signals were corrected for the blank values measured as α₁β₁ binding to BSA and afterward normalized to the noninhibited binding of α₁β₁ to rhodocetin in the absence of GRGDSP peptide (positive control, 100% binding).

Circular Dichroism Spectroscopy of Rhodocetin—The buffer of the rhodocetin solution was changed to 20 mM sodium phosphate, pH 7.0, 50 mM NaCl by gel filtration on a TSK G3000SWXL column (TosoHaas, Stuttgart, Germany). The fractions containing rhodocetin were concentrated in a Centricon 10 tube by centrifugal ultrafiltration to reach a concentration of about 0.3 mg/ml. The CD spectrum was recorded from 190 to 260 nm in a 0.1-mm cuvette in a CD spectrophotometer type CD6 (Jobin Yvon, Paris, France). Temperature was controlled by a self-constructed Peltier element cuvette holder. The relative amount of secondary structures (α helix, parallel, and anti-parallel β strands, random coil) were calculated with the deconvolution program of CDNN by Gerhard Böhm (23).
by SDS-PAGE under reducing conditions (Fig. 1, lane 5). Edman degradation of the latter one revealed its N-terminal sequence as STEFSEDLLDEDDLDIDE and, thus, identified the 45-kDa protein as Drosophila BM40 (GenBank® accession number AJ1333736). Interestingly, BM40 was abundantly expressed by Schneider cells. Like the soluble αβ1 integrin, it bound to type I collagen column in a divalent cation-dependent manner. About 10 times more BM40 than soluble αβ1 integrin was eluted from the type I collagen column by EDTA. However, binding of BM40 to type I collagen did not interfere with αβ1 integrin binding to its collagen ligand. Being a less acidic protein than BM40, the soluble αβ1 integrin was further purified by anion exchange chromatography on a Mono Q column, from which the soluble αβ1 integrin was eluted at lower ion strength than the highly acidic BM40. Yields of soluble αβ1 integrin ranged from 30 to 40 μg/liter of cell supernatant.

Characterization of the Recombinant Soluble αβ1 Integrin—In SDS-PAGE, the soluble αβ1 integrin heterodimer was separated into the Fos zipper containing α2-ectodomain, α2-Fos, and the Jun zipper containing β1-ectodomain, β1-Jun, which run at 150 and 95 kDa, respectively, under nonreducing conditions and at 140 and 100 kDa, respectively, after reduction (Fig. 1, lanes 1 and 4). The identity of the α2 band was proven by N-terminal sequencing. Edman degradation revealed the sequence YNVGLPEAKI in agreement with the mature human integrin α2 subunit (19), demonstrating that the human α2 subunit was correctly processed proteolytically within the insect cells. Like the wild-type form on human cells, the human β1-Jun chain expressed by the insect cells was N-terminally blocked and thus inaccessible to Edman degradation. However, it was identified in Western blot by a polyclonal antisemur against the human integrin β1 subunit (data not shown). Unlike other integrin α subunits, the α2-ectodomain is not proteolytically processed into a heavy and light chain. Neither was the human soluble αβ1 integrin cleaved in the heterologous expression system of the Drosophila Schneider cells. Having very similar isoelectric points, the αβ1 integrin and BSA could not efficiently be separated by anion exchange chromatography leading to a slight contamination of BSA in the αβ1 integrin preparation.

The soluble αβ1 integrin was able to bind to collagen types I, II, IV, and V and to laminin-1 (Engelbreth-Holm-Swarm-Laminin) (Fig. 2). The highest binding signals were observed to type I and II collagen, which is in good agreement with results of wild-type αβ1 integrin (25). Like the wild-type form, the soluble αβ1 integrin gave a smaller binding signal on the basal membrane collagen, type IV collagen, and likewise to its triple helical fragment CB3[IV], which comprise the binding sites for both α1β1 and α2β1 integrin (25). A significantly lower binding signal was measured to type V collagen, which together with type I collagen forms the collagen fibrils of the connective tissue. As a ligand without any collagenous triple helix, laminin-1 was bound by the soluble αβ1 integrin, albeit with a much lower binding signal than the collagenous ligands. The latter finding corroborated studies of wild-type αβ1 integrin binding to laminin-1 (26). Identical to cell membrane-anchored wild-type αβ1 integrin, soluble αβ1 integrin required divalent cations to recognize its ligands. Therefore, EDTA abolished αβ1 binding (Fig. 2). The soluble αβ1 integrin seemed to be regulated by extracellular factors in a manner similar to that of the wild-type αβ1 integrin on the cell surface, because integrin-activating Mn2+ ions and the activating monoclonal antibody 9EG7 increase the binding signal of soluble αβ1 integrin to its ligands (Fig. 2). Taken together, the soluble αβ1 integrin showed ligand binding properties similar to the membrane-anchored wild-type αβ1 integrin. However, no detergent was needed to extract the soluble αβ1 integrin or to keep it in solution. Furthermore, unlike the detergent-extracted wild-type αβ1 integrin, soluble αβ1 integrin remained active even after a longer storage period of several months.

Whole Snake Venom of C. rhodostoma Inhibits Binding of Soluble αβ1 Integrin to Immobilized Type I Collagen—The strong binding signal of soluble αβ1 integrin to immobilized type I collagen (Fig. 2) was diminished and completely inhibited by the crude snake venom of C. rhodostoma in a dose-dependent manner with an IC50 value of about 50 μg/ml (data not shown). Like other snake venoms, C. rhodostoma venom contains several proteases that could be detected byzymogram developed with gelatin. Rhodostoxin (kistomin and major hemorrhagin), a metalloprotease (27, 28), and anecrod, a serine protease (29), could be detected in the zymogram among other proteolytic activities (data not shown). To rule out the possibil-
ity that any snake venom protease diminishes the αβ₂ binding signal to immobilized collagen, protease inhibitors directed against all four classes of proteases, such as aprotinin, leupeptin, phenylmethylsulfonyl fluoride, pepstatin, and 1,10-phenanthroline, were added to the venom protein fraction when applied in the inhibition ELISA to test its capability to inhibit binding of soluble αβ₂ to immobilized type I collagen by a nonproteolytic interaction.

Rhodocytin/Aggretin Does Not Inhibit Binding of Soluble αβ₂ Integrin to Type I Collagen—Rhodocytin or aggretin are the two names of a 29-kDa protein of C. rhodostoma venom, which induces activation and aggregation of thrombocytes (15, 30). It was isolated from the snake venom according to Shin and Morita (31). In SDS-PAGE (Fig. 3, lane 1), the purified rhodocytin/aggretin showed a molecular mass of about 29 kDa under nonreducing conditions. Being a disulfide cross-linked heterodimer, it was cleaved under reducing conditions into two subunits of 19 and 15 kDa (Fig. 3, lane 4). The N-terminal sequences of both subunits, GLEDDGFWSYPDYQ(32) and DPSGWSYE(H/G)(H/Y)K, proved their identities as α and β chains, respectively, of rhodocytin/aggretin (14, 31). To test the postulated interaction of soluble αβ₂ integrin with rhodocytin/aggretin, the latter one was immobilized on a microtiter plate, and the binding of soluble αβ₂ was tested. Whereas the soluble αβ₂ binds to immobilized monomeric type I collagen in a divalent cation-dependent manner, no binding to immobilized rhodocytin/aggretin was observed (Fig. 4A). A similar result was obtained when wild-type αβ₂ integrin, which had been purified from platelets (kindly provided by Albert Ries and Rupert Timpl, Max-Planck-Institute for Biochemistry, Martinsried, Germany), was used (data not shown).

Because immobilization may have caused inactivation of rhodocytin/aggretin, binding of soluble αβ₂ to soluble rhodocytin/aggretin was tested by measuring the capability of the snake venom component to inhibit αβ₂ integrin binding to immobilized type I collagen. However, rhodocytin/aggretin does not prevent αβ₂ integrin from binding to collagen (Fig. 4B). Both the binding test and the inhibition test rule out any direct interaction between rhodocytin/aggretin and soluble αβ₂ integrin on the molecular level.

Searching for the Component of C. rhodostoma Venom That Inhibits the Interaction of Soluble αβ₂ Integrin with

Collagen—Although rhodocytin/aggretin did not inhibit αβ₂ binding to collagen (Fig. 4), the whole snake venom hampered binding of soluble αβ₂ integrin to immobilized type I collagen. Taking advantage of the inhibition ELISA, the constituent of C. rhodostoma venom that is responsible for the inhibition of αβ₂ integrin binding to type I collagen was searched. In the first purification step, the venom proteins were separated according to their molecular masses by gel filtration on a Superose 6 column (Fig. 5A). When the eluate fractions were screened for their capability to inhibit αβ₂ binding to immobilized type I collagen, two peaks of inhibitory activity could be identified (Fig. 5B). Because of their different molecular masses, they were referred to as high molecular weight and low molecular weight Calloselasma inhibitor. Purification and identification of the LMW-CI activity was further pursued. Ion exchange chromatography both on Mono S and Mono Q could clearly separate rhodocytin/aggretin from the αβ₂ integrin inhibitory activity of LMW-CI. The Mono S column retained LMW-CI at pH 6.5 up to a ionic strength of 105 mM NaCl, whereas rhodocytin/aggretin barely bound to Mono S at pH 6.5 and was washed off the column at very low ionic strength. In the opposite elution order, LMW-CI was eluted from the Mono Q column at pH 8.5 at low ionic strength of about 100 mM NaCl, whereas rhodocytin/aggretin remained bound to the Mono Q resin at NaCl concentrations of up to 300 mM NaCl. In conclusion, the isoelectric point of LMW-CI must be higher than the one of rhodocytin/aggretin, although the isoelectric points of both proteins must be in a pH range of 6.0–8.5. As final purification step of LMW-CI, another gel filtration chromatography on a TSK G3000SWXL was performed, resulting in a highly purified band at 27 kDa in SDS-PAGE. Furthermore, coprecipitation experiments with αβ₂ integrin showed that the 27-kDa protein binds to the αβ₂ integrin, suggesting that the 27-kDa protein is the LMW-CI (data not shown).

2 Brackets indicate two possible amino acids that could not be clearly identified in the sequencing cycle. Parentheses indicate a less likely amino acid when the Edman degradation cycle did not give a clear identification but an option of two or more possible amino acids.
Characterization of LMM-CI—Under nonreducing conditions, LMM-CI shows an apparent molecular mass of 27 kDa in SDS-PAGE (Fig. 3, lane 2). LMM-CI is a heterodimer, which upon reduction falls apart in two subunits of 16 and 14 kDa (Fig. 3, lane 5). The N-terminal sequences of both LMM-CI subunits were identified by Edman degradation with the N terminus of the 16-kDa subunit being D/(F)/PD(G/S)/WSSTKSYVR[P/(R)][F/P]/[K/F]/[E/K][R/E]).3 and the N terminus of the 14-kDa subunit being DFRPTTWMSKLY[-/(S)]YKPF(K). These N-terminal sequences clearly showed that the LMM-CI is distinct from the rhodocytin/aggretin. However, these sequences disclosed that LMM-CI is identical to rhodocytin, a recently published inhibitor of collagen-induced platelet aggregation (16).

On a molecular level, rhodocytin inhibited binding of soluble α₁β₁ integrin to immobilized type I collagen in a dose-dependent manner (Fig. 6), thus proving that, in contrast to rhodocytin/aggretin, the effect of rhodocytin on whole platelets (16) can indeed be imitated on a molecular scale, i.e., on the interaction of isolated α₁β₁ integrin to collagen. With increasing concentrations, LMM-CI/rhodocytin decreased the binding signal of the collagen receptor to its ligand and eventually abolished it entirely. From Fig. 6, an IC₅₀ value of about 30 nM could be determined.

Rhodocytin is a Disintegrin That Directly and Specifically Binds to α₁β₁ Integrin—Addition of various protease inhibitors to the inhibition ELISA ruled out the possibility that the decrease of α₁β₁ binding to collagen was caused by proteolytic digestion of either binding partner by a snake venom protease.

Therefore, a direct, yet nonenzymatic binding interaction of LMM-CI/rhodocytin with either α₁β₁ integrin or with the integrin-binding site on type I collagen must be responsible for its inhibitory effect. To test a direct interaction of rhodocytin with the soluble α₁β₁ integrin, rhodocytin was immobilized onto a microtiter plate, and binding of soluble α₁β₁ was measured. As shown in Fig. 7, the soluble α₁β₁ integrin directly bound to rhodocytin, thereby qualifying it to be a disintegrin. The binding signal could be increased slightly by addition of 1 mM MnCl₂ and the integrin-activating antibody 9EG7. However, in contrast to other integrin ligands, binding of α₁β₁ to rhodocytin did not require any divalent cations, because addition of EDTA did not abolish α₁β₁ binding to rhodocytin. A binding signal similar to the one of soluble α₁β₁ integrin was obtained when detergent-extracted wild-type α₁β₁ integrin from human platelets was applied (data not shown). Another soluble integrin, the laminin-5 receptor α₅β₁ integrin (18), did not bind to immobilized, native rhodocytin, although it showed binding activity to laminin-5 (Fig. 7). The soluble α₅β₁ integrin had been produced in our lab by insect cells similarly to the soluble α₁β₁ integrin (18). Even more striking, another widespread collagen receptor, α₁β₁ integrin, which had been isolated from human placenta according to Kern et al. (25) and was tested biologically active by its binding to type I and IV collagen, entirely fails to bind to rhodocytin (Fig. 7), proving the specificity of LMM-CI/rhodocytin to recognize α₁β₁ integrin selectively.

It is noteworthy that the ability of LMM-CI/rhodocytin to interact with α₁β₁ integrin depends on its disulfide bridges, which stabilize both its quartenary and tertiary structure. Precipitation of LMM-CI at DTT concentrations higher than 0.016 mM without any thermal denaturation resulted in a strong decrease of α₁β₁ binding (Fig. 8A). However, when scrutinized by SGS-PAGE (Fig. 8B), the partially reduced LMM-CI/rhodocytin run as stable heterodimer even up to 10 mM DTT. Amazingly, rhodocytin does not possess any intercatenary disulfide bridges (16), but its subunits stayed together even under the harsh denaturating condition of the SDS-PAGE sample buffer containing 2% SDS. Reduction of the intracatenary disulfide bridges at DTT concentrations higher than 10 mM made the rhodocytin heterodimer dissociate. Although lacking an intercatenary disulfide bridge, quartenary structure of rhodocytin is very stable and depends on the tertiary structure of...
both subunits, which is stabilized by intracatenary disulfide bridges. As the binding signal of soluble $\alpha_2\beta_1$ integrin gradually decreased with increasing DTT concentrations higher than 0.016 mM and is entirely lost at 10 mM DTT, it can be envisioned that the intracatenary disulfide bridges are of paramount importance in maintaining the native tertiary structure of rhodocetin, which is essential for $\alpha_2\beta_1$ integrin binding. Furthermore, the tertiary structure of its subunits as evidenced by its inherent binding function seems to be even more sensitive to denaturation than its quaternary structure, i.e. association of its two subunits.

To determine the binding affinity of rhodocetin to $\alpha_2\beta_1$ integrin, both native and denatured rhodocetin were immobilized onto a microtiter plate and titrated with soluble $\alpha_2\beta_1$ integrin (Fig. 9). Treatment of rhodocetin with 40 mM DTT in addition to thermal denaturation entirely abolished its binding activity to the integrin, again demonstrating that the specific interaction of rhodocetin with $\alpha_2\beta_1$ integrin requires the disulfide-stabilized native conformation of rhodocetin. For binding of soluble $\alpha_2\beta_1$ integrin to native rhodocetin, saturation was achieved at $\alpha_2\beta_1$ concentrations of about 100 nM. From such titration curves, an apparent $K_D$ value of LMW-CI/rhodocetin binding to $\alpha_2\beta_1$ integrin was calculated to be $10.3 \text{nM}$.

**Rhodocetin Does Not Contain a Triple Helical Collagen Domain**—Being essential for its inhibitory activity, the native conformation of LMW-CI/rhodocetin was further studied by CD. We were especially interested in whether or not LMW-CI contains any triple helical collagenous motifs, because high affinity ligands of $\alpha_2\beta_1$ integrin are mostly collagenous molecules. Laminin-1, which lacks any collagenous structure, is bound by $\alpha_2\beta_1$ integrin with much lower affinity. Although LMW-CI/rhodocetin competed with the high affinity binding of
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**Fig. 10.** Binding of the disintegrin LMW-CI/rhodocetin to \( \alpha_2 \beta_1 \) integrin does not depend on an RGD peptide sequence. LMW-CI was coated onto a microtiter plate at 40 \( \mu \)g/ml. After being blocked with heat denatured BSA, wells were incubated with soluble \( \alpha_2 \beta_1 \) integrin for 2 h in the absence and presence of the linear peptide GRGDSP at concentrations indicated in the plot. After wells had been washed twice, bound receptor was chemically fixed, and its amount was determined by ELISA. \( \alpha_2 \beta_1 \) binding to heat denatured BSA was taken as blank and subtracted from the binding values. Binding signals were normalized to the noninhibited binding signal measured in the absence of peptide. Values were determined in duplicate. Standard deviations are indicated.

\( \alpha_2 \beta_1 \), to type I collagen, the CD spectrum of LMW-CI (data not shown) did not reveal any collagenous triple helical structure within the disintegrin. Although lacking a collagen domain, rhodocetin possesses a distinct native structure, because deconvolution of the CD spectrum recorded at 20 °C disclosed a high content of 59.5% \( \beta \)-sheet and a minor amount of 10.3% \( \alpha \)-helical secondary structure for rhodocetin. Heat denaturation abrogated any secondary structural signals in the CD spectrum, leaving a spectrum typical of random coil.

**Rhodocetin Is an RGD-independent Integrin—** Many disintegrins bind to RGD-dependent integrins in an RGD peptides inhabitable manner. However, the linear GRGDSP peptide failed to inhibit the interaction of \( \alpha_2 \beta_1 \) integrin with immobilized LMW-CI/rhodocetin (Fig. 10). Even at concentrations of 4 mM, which represented an 800,000-fold molar surplus to the soluble \( \alpha_2 \beta_1 \) integrin in the inhibition experiment, the GRGDSP peptide did not affect the \( \alpha_2 \beta_1 \) disintegrin interaction, thus showing that LMW-CI/rhodocetin belongs to the small group of RGD-independent disintegrins.

**Effect of Rhodocetin on \( \alpha_2 \beta_1 \)-mediated Adhesion of Fibroblasts—** Whether LMW-CI/rhodocetin can be used in vivo, e.g. to inhibit \( \alpha_2 \beta_1 \)-mediated cell adhesion and migration or to influence other cellular reactions triggered by the \( \alpha_2 \beta_1 \)-collagen interaction, the effect of the isolated rhodocetin on adhesion of HT1080 cells onto immobilized type I collagen was examined. HT1080 is a human fibrosarcoma cell line that abundantly expresses \( \alpha_3 \beta_1 \) integrin on its surface and that heterologously expressed by Drosophila \( C. rhodonoma \), does not interact with \( \alpha_2 \beta_1 \) integrin, one of the collagen receptors on the surface of blood platelets. However, from the same snake venom, we have isolated and characterized LMW-CI, which is identical to rhodocetin, a recently published inhibitor of collagen-induced platelet aggregation (16). We show that LMW-CI/rhodocetin is a disintegrin that specifically and avidly binds to \( \alpha_2 \beta_1 \), integrin. Independently of Wang et al. (16), we have purified LMW-CI/rhodocetin as a component of \( C. rhodonoma \) venom, which inhibits the binding of \( \alpha_2 \beta_1 \) integrin to immobilized collagen on the molecular level. We used recombinantly expressed and purified, soluble \( \alpha_2 \beta_1 \) integrin in an inhibition ELISA to screen the snake venom for components that specifically and nonproteolytically inhibit the interaction of \( \alpha_2 \beta_1 \) integrin with collagen. Although commonly used, the method of using whole platelets to test for integrin agonists and antagonists may be biased by the presence of various other collagen receptors on the platelet surface. Furthermore, here we describe our detailed studies on the interaction of the novel disintegrin LMW-CI/rhodocetin with \( \alpha_2 \beta_1 \) integrin.

**DISCUSSION**

Here, we report that rhodocytin, an inducer of platelet aggregation from the hemorrhagic snake venom of \( C. rhodonoma \), does not interact with \( \alpha_2 \beta_1 \) integrin, one of the collagen receptors on the surface of blood platelets. However, from the same snake venom, we have isolated and characterized LMW-CI, which is identical to rhodocetin, a recently published inhibitor of collagen-induced platelet aggregation (16). We show that LMW-CI/rhodocetin is a disintegrin that specifically and avidly binds to \( \alpha_2 \beta_1 \) integrin. Independently of Wang et al. (16), we have purified LMW-CI/rhodocetin as a component of \( C. rhodonoma \) venom, which inhibits the binding of \( \alpha_2 \beta_1 \) integrin to immobilized collagen on the molecular level. We used recombinantly expressed and purified, soluble \( \alpha_2 \beta_1 \) integrin in an inhibition ELISA to screen the snake venom for components that specifically and nonproteolytically inhibit the interaction of \( \alpha_2 \beta_1 \) integrin with collagen. Although commonly used, the method of using whole platelets to test for integrin agonists and antagonists may be biased by the presence of various other collagen receptors on the platelet surface. Furthermore, here we describe our detailed studies on the interaction of the novel disintegrin LMW-CI/rhodocetin with \( \alpha_2 \beta_1 \) integrin.

Identification of LMW-CI/rhodocetin as disintegrin, which specifically binds to \( \alpha_2 \beta_1 \) integrin, was made feasible by the recombinant production of a soluble \( \alpha_2 \beta_1 \) integrin and its purification in sufficient amounts. Soluble \( \alpha_2 \beta_1 \) integrin was generated by replacing the transmembrane and cytoplasmic domain of both integrin subunits \( \alpha_3 \) and \( \beta_1 \) with the dimerizing motifs of the transcription factor Fos and Jun, respectively. Lately, a similar attempt to produce soluble \( \alpha_2 \beta_1 \) integrin had been successful (18). However, yields of soluble \( \alpha_2 \beta_1 \) integrins were generally lower than with soluble \( \alpha_2 \beta_1 \) integrin in compliance with the comparatively lower expression of membrane-bound \( \alpha_2 \beta_1 \) on transfected mammalian cells, such as erythroblastic leukemia K562 cells. Although the human integrin ectodomains were heterologously expressed by Drosophila cells, both sunbunits were correctly processed proteolytically, because the leader sequences were cleaved off to give the N termini of both mature human subunits. Whereas the N-terminal amino acid sequence of mature human \( \alpha_2 \) subunit was accessible to Edman degra-

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dation, the β₁ subunit was N-terminally blocked. However, loss of signal sequence of the β₁ subunit and its subsequent N-terminal blockage reaction to a pyroglutamate residue also occurs in the homologously expressed human β₁ subunit, indicating a correct processing of the soluble human β₁ integrin subunit by the insect cells. Furthermore, the ectodomain heterodimer of the collagen receptor α₂β₁ integrin binds avidly to monomeric type I collagen, suggesting that the integrin is not only correctly processed but also correctly folded. The binding signals of soluble α₂β₁ to different types of collagen and laminin-1 demonstrated a ligand preference similar to the wild-type α₂β₁ integrin, with decreasing binding signals in the order of type I, type II, type IV, and type V collagen and laminin-1 (25, 26). Binding affinities of soluble α₂β₁ integrin could be increased with Mn²⁺ ions and an activating antibody 9EG7, an observation that resembles the activity regulation of wild-type α₂β₁ integrin.

A great advantage of the soluble α₂β₁ integrin in comparison with the membrane-bound wild-type α₂β₁ integrin, which was isolated by extracting blood platelets with the mild detergent octylglucoside, is its stability and the comparatively high yield. Whereas the detergent-extracted wild-type α₂β₁ integrin lost activity within days after preparation, the soluble α₂β₁ integrin remained stable for several weeks. However, we have not found any explanation for this observation yet.

Having sufficient amounts of a stable, soluble α₂β₁ integrin at hand, we could address the question of which component of the hemorhagic snake venom of C. rhodostoma is responsible for inhibiting the interaction of α₂β₁ integrin with type I collagen. This interaction is of major importance for platelet reactions to collagen. Collagen becomes accessible to platelets after damage of the blood vessels or tissue injury. It initiates the activation of platelets (8, 30, 33), which results in degranulation and an increase in number and/or affinity of other cell adhesion molecules on the platelet, such as the major platelet integrin α₁β₁β₃ (3), which eventually leads to platelet aggregation and blood clotting. RGD-containing disintegrins, such as rhodostomin (kistrin) from C. rhodostoma (27, 34), inhibit the interaction of the RGD-dependent α₁β₁β₃ integrin with fibrin, thereby impairing a later step in the blood clotting mechanism. Moreover, snake venoms contain several proteases, such as the metalloprotease rhodostoxin (kistomin and major hemorrhagin) (27, 28), and the serine protease ancord (29) from C. rhodostoma, which cleave fibrinogen/fibrin or prothrombin, thus again interfering in the blood clotting cascade and resulting in bleeding and hemorrhages.

Being the initial step of collagen-triggered platelet activation and aggregation, α₂β₁ integrin is of paramount importance in hemostasis (9, 10, 17). Previous studies with the hemorrhagic snake venom of C. rhodostoma were performed on whole platelets. However, platelets contain various collagen receptors on their surface with different characteristics, e.g., α₂β₁ integrin (GPIa/IIa) and GPVI (9, 10, 17). Whereas GPVI mainly recognizes type I collagen molecules, which are bundled into collagen fibrils, in a divergent cation-independent manner, α₂β₁ integrin mainly binds to monomeric type I collagen molecules in the presence of divalent cations (35–37). The importance of α₂β₁ integrin in normal hemostasis is corroborated by severe bleeding disorders in patients, who either lack the α₂β₁ integrin receptor on their platelets (11) or who have developed inhibiting autoantibodies against the α₂ integrin subunit (12).

Possessing various collagen receptors and being easily activated by several stimuli other than collagen, such as ADP, thrombin, etc., whole platelets are rather coarse targets to screen hemorrhagic snake venoms for specific inhibitors to the α₂β₁ integrin-collagen interaction, inasmuch as snake venoms by themselves contain a whole battery of various agents interfering with platelet activation and blood clotting. Based on such studies with whole platelets, rhodocytin/aggretin have been published to be an activator of α₂β₁ integrin-mediated platelet aggregation (15, 31). However, we have isolated rhodocytin/aggretin, proved its identity by N-terminal sequencing, and could not see any interaction of rhodocytin with α₂β₁ integrin. Nor could we observe any influence of rhodocytin on the integrin-collagen interaction. Therefore, a direct interaction of rhodocytin/aggretin with the soluble α₂β₁ integrin ectodomain on the protein level can be ruled out. Alternatively, its effects on platelets aggregation may be caused by protein-carbohydrate interactions. Wild-type α₂β₁ integrin on the platelets surface may differ from recombinantly expressed soluble α₂β₁ integrin in their carbohydrate side chains, because Drosophila Schneider cells are unable to process the N-linked carbohydrate side chains of proteins from high mannose type into complex type carbohydrate antennary structures (18). Because rhodocytin/aggretin belongs to the family of C-type lectins bearing homology to the carbohydrate recognition domains of Ca²⁺-dependent lectins (31), it can be surmised that rhodocytin cross-links several α₂β₁ integrins on the platelet surface via their carbohydrate side chains, thereby imitating the recruitment of integrins into focal contact-like structures, which eventually leads to platelet activation and aggregation. However, because both recombinantly expressed soluble α₂β₁ integrin and wild-type α₂β₁ integrin extracted from platelets that are likely to bear high-mannose type and complex-type N-linked carbohydrate side chains, respectively, fail to interact with rhodocytin/aggretin in our binding tests, even in the presence of Ca²⁺ ions, a mechanism involving a direct interaction of rhodocytin/aggretin with α₂β₁ integrin to explain platelet activation and aggregation by rhodocytin can be considered very unlikely.

Without using whole platelets, we have established a cell-free inhibition assay as a tool to search for an inhibitor of the integrin-collagen interaction on the molecular level without any interfering cellular reactions that occur on platelets during or after their activation. Furthermore, the use of soluble α₂β₁ integrin instead of detergent-extracted wild-type α₂β₁ integrin even allows us to work not only in a cell-free but also in a detergent-free test system. Therefore, we could identify the disintegrin LMW-CI from C. rhodostoma venom, which specifically binds to α₂β₁ integrin in an RGD-independent manner, thereby inhibiting the interaction of α₂β₁ integrin with immobilized, monomeric type I collagen. N-terminal sequencing of LMW-CI revealed its identity with rhodocytin (16). We called this inhibitor low molecular weight Calloselasma inhibitor to distinguish it from another α₂β₁ integrin inhibiting activity of the C. rhodostoma venom that was found in an earlier eluate fraction, i.e., higher molecular mass fraction, of a size exclusion column. However, we have not yet characterized the latter one, which we referred to as high molecular weight Calloselasma inhibitor. Although binding with high affinity and specificity, the LMW-CI/rhodocetin does not need any divalent cations to be bound by the integrin. Its native three-dimensional structure, which is stabilized by disulfide bridges, is essential for α₂β₁ binding. Unlike the other high affinity collagen ligands of α₂β₁ integrin, LMW-CI/rhodocetin lacks a collagenous triple helical conformation. Nevertheless, it binds to α₂β₁ integrin avidly and even competes with collagen efficiently. Because we had included protease inhibitors into the test assay and proved absence of protease activities in the LMW-CI/rhodocetin preparation by zymogram, the inhibitory effect of LMW-CI/rhodocetin is not caused by proteolytic activity.
α2β1 Integrin, a Specific High Affinity Target of Rhodocetin

LMW-CI/rhodocetin is a heterodimer with an apparent molecular mass of 27 kDa consisting of two subunits of 16 and 14 kDa, which are firmly attached. Despite lacking any covalent, intracatenary disulfide bridges (16), the two subunits remained associated under the harsh denaturation conditions of the SDS-PAGE sample buffer, containing 2% SDS. However, reduction of intracatenary disulfide bridges leads to destruction of the tertiary structure and subsequently to the dissociation of the two subunits. Judging the native tertiary structure by its capability to bind to α2β1 integrin, we found that the native tertiary structure of rhodocetin, which is required for integrin binding, is lost at much lower concentrations of reducing agents than the quartenary structure, detected as dissociation of the two subunits in SDS-PAGE under nonreducing conditions.

Interestingly, both rhodocetin/aggregrin and LMW-CI/rhodocetin belong to a family of snake venom proteins that bear similarity to the carbohydrate recognition domain of C-type lectins (16, 31). However, platelet-derived wild-type α2β1 integrin and recombinantly expressed, soluble α2β1 integrin bind equally well to immobilized LMW-CI/rhodocetin, although the two integrins may vary in their glycosylation pattern of N-linked carbohydrate side chains, suggesting that the interaction of the novel disintegrin LMW-CI/rhodocetin bases on a protein-protein interaction. This direct interaction then causes the inhibition of collagen binding to α2β1 integrin.

It is noteworthy that LMW-CI/rhodocetin does not bind to α2β1 integrin, the other collagen-binding integrin with a widespread tissue distribution. Nor does this disintegrin interact with α2β1 integrin. Therefore, LMW-CI/rhodocetin differs from other, mainly RGD-dependent disintegrins in its unique specificity toward α2β1 integrin. Interestingly, LMW-CI/rhodocetin does not require divalent cations to bind to α2β1 integrin, because, in contrast to other integrin ligands, deprivation of divalent cations by EDTA does not abolish α2β1 binding to LMW-CI/rhodocetin. This suggests a binding mechanism distinct from the integrin binding mechanism to collagen. Nevertheless, LMW-CI can completely abolish α2β1 integrin binding to collagen. Either LMW-CI binds at a site within α2β1 integrin distinct from the ligand binding site that leads to a conformational change and to an allosteric inactivation of the integrin, or LMW-CI binds to a site within α2β1 integrin, which is overlapping or even identical to the collagen binding site, thereby inhibiting collagen binding sterically. Future structural studies will help to answer this question.

As a prerequisite for its binding activity to α2β1 integrin, LMW-CI indispensably needs its native conformation, which is stabilized by disulfide bridges. Further structural insights into LMW-CI were gained by CD spectroscopy. The CD spectrum of LMW-CI is in good agreement with the CD spectrum provided by in Refs. 16. It clearly demonstrated that LMW-CI/rhodocetin does not bear any structural resemblance to a collagenous triple helix, which is typical of high affinity ligands of α2β1 integrin. Still, LMW-CI/rhodocetin avidly binds to α2β1 integrin and efficiently competes with type I collagen.

Having found and characterized LMW-CI/rhodocetin as snake venom disintegrin that specifically recognizes α2β1 integrin and preventing it from binding to its collagen ligand, we eventually returned to whole cells to study the α2β1-related functions in the cellular context. α2β1 integrin not only is a pivotal trigger in hemostasis, but its widespread distribution on other cell types also suggests a much broader biological role in the organism (17, 38). The presence of α2β1 integrin on endothelial cells of newly grown blood capillaries suggests a potential role in angiogenesis (39). Fibroblasts also bear α2β1 integrin and use it to exert mechanical forces to a surrounding collagen gel, which in vivo takes place in connective tissue to maintain the shape of tissues and organs, during wound contraction and scar formation (40). Furthermore, ligand occupancy of α2β1 integrin on fibroblasts and epithelial tumor cells elicits expression of various matrix metalloproteases (MMPs) (40), such as interstitial collagenase (MMP-1) (41, 42), stromelysin-1 (MMP-3) (43), collagenase-3 (MMP13), and membrane-bound metallomatrixproteinase-1 (MT1-MMP, MMP14) (44). The latter one itself proteolytically activates gelatinase A (MMP-2) (40). α2β1 integrin-triggered secretion of MMPs is a key point in tumor invasion and metastasis, because these proteases degrade extracellular matrix proteins, among them basal membrane proteins, thus opening the path for invading tumor cells. To manipulate such α2β1-triggered effects, LMW-CI/rhodocetin may be a valuable tool because of its unique specificity and high affinity toward α2β1 integrin. Another advantage of LMW-CI/rhodocetin is its high solubility under physiological conditions compared with the poor solubility of collagen, which because of its high tendency to aggregate and precipitate cannot be applied as soluble inhibitor. Furthermore, because of its independence of divalent cations, LMW-CI is likely to bind in vivo as effectively as in the cell-free test, whereas α2β1 integrin binds less avidly to collagen in vivo because of the presence of β1-integrins attenuating Ca2+ ions under physiological conditions. With HT1080 fibrosarcoma cells, which adhere to type I collagen mainly via α2β1 integrin, we demonstrated that LMW-CI indeed inhibits cell adhesion to type I collagen as the initial step of integrin-mediated cell migration, gene activation, and anchorage-dependent growth. LMW-CI/rhodocetin efficiently and completely inhibits α2β1-mediated cell adhesion to type I collagen, proving its suitability as specific α2β1 integrin inhibitor in vivo. Thus, LMW-CI may be a useful agent to study and influence α2β1 integrin-triggered cell function, like cell adhesion, cell migration, or secretion of MMPs. Therefore, it may help not only in treating thrombosis but also in treatments aimed to prevent tumor invasion and metastasis.

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