THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 288, NO. 2, pp. 947–955, January 11, 2013

The Collagen-binding Integrin $\alpha2\beta1$ Is a Novel Interaction Partner of the *Trimeresurus flavoviridis* Venom Protein Flavocetin-A

Received for publication, July 10, 2012, and in revised form, November 30, 2012 Published, JBC Papers in Press, November 30, 2012, DOI 10.1074/jbc.M112.399618

Franziska T. Arlinghaus and Johannes A. Eble[1]

From the Center for Molecular Medicine, Department of Vascular Matrix Biology, Excellence Cluster Cardio-Pulmonary System, Frankfurt University Hospital, 60590 Frankfurt, Germany

Background: Snake venoms contain antagonists of the collagen-binding integrin $\alpha2\beta1$.
Results: Flavocetin-A binds to the integrin $\alpha2$A domain and inhibits cell adhesion and migration on collagen.
Conclusion: Flavocetin-A is a novel interaction partner of integrin $\alpha2\beta1$.
Significance: $\alpha2\beta1$ antagonists from snake venoms can be utilized for drug development.

Many snake venoms are known for their antithrombotic activity. They contain components that specifically target different platelet-activating receptors such as the collagen-binding integrin $\alpha2\beta1$ and the von Willebrand factor receptor GPIb. In a search for an $\alpha2\beta1$ integrin-blocking component from the venom of the habu snake (*Trimeresurus flavoviridis*), we employed two independent purification protocols. First, we used the integrin $\alpha2$A domain, a major collagen-binding domain, as bait for affinity purification of an $\alpha2\beta1$ integrin-binding toxin from the crude venom. Second, in parallel, we used classical protein separation protocols and tested for $\alpha2\beta1$ integrin-inhibiting capabilities by ELISA. Using both approaches, we identified flavocetin-A as an inhibitor of $\alpha2\beta1$ integrin. Hitherto, flavocetin-A has been reported as a GPIb inhibitor. However, flavocetin-A inhibited collagen-induced platelet aggregation even after GPIb was blocked with other inhibitors. Moreover, flavocetin-A antagonized $\alpha2\beta1$ integrin-mediated adhesion and migration of HT1080 human fibrosarcoma cells, which lack any GPIb, on collagen. Protein chemical analyses proved that flavocetin-A binds to $\alpha2\beta1$ integrin and its $\alpha2$A domain with high affinity and in a cooperative manner, which most likely is due to its quaternary structure. Kinetic measurements confirmed the formation of a strong complex between integrin and flavocetin-A, which dissociates very slowly. This study proves that flavocetin-A, which has long been known as a GPIb inhibitor, efficiently targets $\alpha2\beta1$ integrin and thus blocks collagen-induced platelet activation. Moreover, our findings suggest that the separation of GPIb- and $\alpha2\beta1$ integrin-blocking members within the C-type lectin-related protein family is less strict than previously assumed.

Integrins are transmembrane receptors that bind molecules of the extracellular matrix, linking the extracellular matrix to the cytoskeleton, and are therefore responsible for cell functions such as adhesion and migration. Integrins are heterodimers of noncovalently associated α and β subunits. 18 α subunits and 8 β subunits form 24 integrins with distinct binding specificities. Of these, four recognize collagen (1). One of them is $\alpha2\beta1$ integrin, which is a collagen-binding receptor found on endothelial and epithelial cells as well as on platelets, where it is the sole collagen-binding integrin. $\alpha2\beta1$ plays a role in physiological and pathological processes mediated by the binding of integrin to collagen, such as wound healing, tumor metastasis, and thrombosis. Identifying antagonists that can be utilized for their potential for tumor progression inhibition as well as for antithrombotic drug development is of great interest.

To our knowledge, three snake venom proteins have been proven to specifically block the A domain (α2A) of the $\alpha2\beta1$ integrin: EMS16 (2), rhodocetin (3), and VP12 (4). This A domain, which is composed of ~200 amino acids, is homologous to the von Willebrand factor (vWF)[2] A domain (5) and is responsible for collagen binding.

EMS16, rhodocetin, and VP12 all belong to the family of C-type lectin-related proteins (CLRPs), which constitutes a large fraction of snake venom proteins (6–8). CLRPs not only target integrin A domains but also other receptors such as the platelet receptor GPIb and blood coagulation factors (9). GPIb is a receptor for the plasma glycoprotein vWF, which initiates platelet adhesion to exposed vascular subendothelium, consequently activating platelets and leading to hemostasis (10, 11). Many CLRPs block the vWF-binding site on the GPIb receptor; however, others enhance the interaction of GPIb and vWF (12–15).

Flavocetin-A was one of the first GPIb-binding CLRPs to be isolated from a snake venom (16, 17). This heterodimer, from the venom of the habu snake (*Trimeresurus flavoviridis*), consists of homologous α and β subunits that oligomerize to form a tetramer, $(\alpha\beta)_4$ (18). Flavocetin-B is composed of the same α and β subunits as flavocetin-A plus an additional γ subunit of unknown sequence (19).

Although flavocetin-A has been known for a long time to block GPIb binding to vWF, we isolated it independently as an

[1] To whom correspondence should be addressed: Center for Molecular Medicine, Dept. of Vascular Matrix Biology, Excellence Cluster Cardio-Pulmonary System, Frankfurt University Hospital, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany. Tel.: 40-69-6301-87651; Fax: 40-69-6301-87656; E-mail: eble@med.uni-frankfurt.de.

[2] The abbreviations used are: vWF, von Willebrand factor; CLRP, C-type lectin-related protein; Ni-NTA, nickel-nitrilotriacetic acid.

inhibitor of α2β1 integrin from the snake venom. Our study proves flavocetin-A to be a novel interaction partner of α2β1 integrin, effectively inhibiting not only collagen-induced platelet aggregation without GPIb involvement but also cell adhesion and migration on collagen. Thus, the concept of a strict separation of inhibitors of GPIb and α2β1 integrin within the CLRP family must be questioned.

EXPERIMENTAL PROCEDURES

Identification of α2β1 Integrin-binding Venom Proteins by Affinity Chromatography—Lyophilized *T. flavoviridis* venom was dissolved in PBS (20 mM sodium phosphate and 150 mM NaCl, pH 7.4) at a concentration of ~100 mg/ml. The oligo-His-tagged integrin α2A domain (termed α2A) was recombinantly expressed as described previously (20). Proteins binding to α2β1 integrin were identified by affinity chromatography with α2A immobilized on a 1-ml HisTrap HP column (GE Healthcare). After elution with an imidazole gradient, integrin A domain-containing fractions were identified and analyzed by SDS-PAGE on a 10–20% polyacrylamide gel.

Ternary Complex Affinity Chromatography—Similarly, α2A was immobilized on a HisTrap HP column, purified flavocetin-A was bound, and platelet lysate containing glycocalicin was applied. The lysate was generated according to Canfield *et al.* (21) with the minor adjustment of excluding the detergent (21). This protein complex was eluted as described before, and fractions were analyzed by SDS-PAGE and Western blotting using anti-human GPIbα antibody (R&D Systems, Wiesbaden, Germany) and alkaline phosphatase-conjugated anti-sheep antibody (Sigma).

Alternative Purification of α2β1 Integrin Inhibitor without α2A—Solubilized *T. flavoviridis* venom proteins (400 mg/ml) were separated using a Superdex 200 10/300 GL gel filtration column (GE Healthcare) at 0.5 ml/min with PBS, pH 7.4. Fractions with α2β1 integrin-inhibiting activity were diluted in 20 mM MES, pH 6.5; loaded onto a Mono S HR 5/5 column (GE Healthcare); and eluted with a linear gradient of 0–50% 20 mM MES and 1 M NaCl, pH 6.5, at a flow rate of 0.5 ml/min. Fractions with integrin-inhibiting activity were concentrated and subsequently separated using a TSKgel G2000SWxl gel filtration column (Tosoh Bioscience, Stuttgart, Germany) at 0.5 ml/min with PBS, pH 7.4. Protein concentration and purity were determined by BCA assay (Thermo Scientific, Dreieich, Germany) and SDS-PAGE, respectively.

Diagonal Two-dimensional Gel Analysis—Purified venom proteins were submitted to two-dimensional electrophoresis analysis on a 10–20% polyacrylamide gel to determine the complexity of proteins in the eluted fractions. Proteins were separated under nonreducing and reducing conditions in the first and second dimensions, respectively, before detection on the gel by silver staining.

Mass Spectrometry Analysis—Proteins purified by ion exchange chromatography were separated by SDS-PAGE, and bands with molecular masses of 117 and 150 kDa were analyzed by electrospray ionization mass spectrometry.

Inhibition of GST-α2A Binding to Type I Collagen by Flavocetin-A—Type I collagen was immobilized overnight at 4 °C on a microtiter plate at 10 μg/ml in 0.1 M acetic acid. After blocking the plate with 1% BSA in TBS/$MgCl_2$ (50 mM Tris-HCl, 150 mM NaCl, and 2 mM $MgCl_2$, pH 7.4), the GST-tagged α2A domain was allowed to bind to type I collagen in the presence of different fractions from the protein purification for 2 h at room temperature. Similarly, 10 μg/ml soluble α2β1 or α1β1 integrin ectodomain (22) was allowed to bind to immobilized type I collagen (40 μg/ml coating concentration) and CB3 (5 μg/ml), respectively, in the absence and presence of different concentrations of purified flavocetin-A. Bound GST-α2A or integrin was fixed for 10 min with 2.5% glutaraldehyde in HEPES buffer (50 mM HEPES, 150 mM NaCl, 2 mM $MgCl_2$, and 1 mM $MnCl_2$, pH 7.4). The amount of bound GST-α2A or integrin was quantified with rabbit polyclonal antibodies against GST (Molecular Probes, Nijmegen, The Netherlands) or the β1 integrin subunit, followed by alkaline phosphatase-conjugated anti-rabbit antibody (Sigma), used as the primary and secondary antibodies, respectively, each diluted in 1% BSA in TBS/$MgCl_2$. The conversion of *p*-nitrophenyl phosphate (Sigma) was measured at 405 nm in an ELISA reader (BioTek, Bad Friedrichshall, Germany). Nonspecific binding was assessed by binding of GST-α2A to BSA or of α2β1 integrin to collagen in the presence of 10 mM EDTA.

Titration of Flavocetin-A with GST-tagged Integrin A Domains—Flavocetin-A was immobilized overnight at 4 °C on a microtiter plate at 10 μg/ml in TBS/$MgCl_2$. The plate was blocked with 1% BSA in TBS/$MgCl_2$, and subsequently, different concentrations of either GST-α2A or GST-α1A were incubated with the immobilized protein for 2 h at room temperature. Alternatively, a constant concentration of GST-α2A was incubated in the presence of different concentrations of monoclonal antibodies JA218, JA221, and P1E6. Bound GST-α2A was fixed with 2.5% glutaraldehyde in HEPES buffer and detected as described for the inhibition assay.

Surface Plasmon Resonance—A Biacore X system (GE Healthcare) was used to study the interaction between flavocetin-A and the α2A domain. The α2A domain was covalently coupled to a CM5 sensor chip (GE Healthcare) using standard amine coupling chemistry. Sensorgrams were recorded in the presence of different flavocetin-A concentrations in 50 mM HEPES, 150 mM NaCl, and 1 mM $MgCl_2$, pH 7.5, under flow. Following each sensorgram, flavocetin-A was removed by washing with 60 mM DTT in 50 mM Tris-HCl and 300 mM NaCl, pH 9.5. Data were analyzed with BIAevaluation v3.1 software (GE Healthcare).

HT1080 Cell Adhesion Assay—Type I collagen (0.2 μg/ml in 0.1 M acetic acid) was immobilized overnight at 4 °C on a microtiter plate. After blocking with 0.1% BSA in TBS/$MgCl_2$ for 2 h at room temperature, HT1080 fibrosarcoma cells (20,000 cells/well) were seeded onto the plate in both the absence and presence of different concentrations of flavocetin-A or with 10 mM EDTA, a control for nonspecific cell adhesion. After a 30-min incubation at 37 °C, adherent cells were fixed with 70% ethanol and stained for 30 min with 0.2% crystal violet. The crystal violet was extracted from the cells with 70% ethanol for 30 min, and absorbance was read at 560 nm. Cell adhesion signals (means ± S.D.) were corrected for signals measured in the presence of EDTA.

An alternative adhesion assay was performed using the xCELLigence system (Roche Diagnostics). An E-Plate was coated overnight at 4 °C with type I collagen at 5 μg/ml in 0.1 M acetic acid. HT1080 cells were seeded at 20,000 cells/well in the absence or presence of different concentrations of flavocetin-A in DMEM or with 10 mM EDTA. Values were measured every 2 min for 2 h and then every 5 min for another 2 h.

HT1080 Cell Migration Assay—Migration of HT1080 cells was analyzed on a CIM-Plate 16-well device using the xCELLigence system. Briefly, type I collagen was coated overnight at 4 °C onto the bottom face of the CIM-Plate at 10 μg/ml in 0.1 M acetic acid. Different concentrations of flavocetin-A in DMEM were added in duplicates to the bottom chamber. 50 μl of twice-concentrated flavocetin-A were added to the upper wells, and background values were assessed. Subsequently, 50-μl suspensions of 100,000 cells were added to each well, yielding the same flavocetin-A concentrations as in the bottom chambers. After a short incubation of 10 min at room temperature, values were measured every 5 min for 6 h and then every 15 min for 24 h. The assay was evaluated with the software provided with the xCELLigence system.

Platelet Aggregation Studies—Platelet aggregation with flavocetin-A was performed with an aggregometer (Chrono-log Corp.) by measuring light scattering, which reflects the number of platelet aggregates. Washed human platelets were collected as described (23). With continuous stirring at 1100 rpm, washed platelets were preincubated with $CaCl_2$ and $MgCl_2$ before adding the platelet stimulus, collagen, in the absence or presence of different venom proteins (flavocetin-A and rhodocetin) or antithrombotic drugs (*e.g.* Aggrastat).

RESULTS

Identification of an α2β1 Integrin-binding Protein from T. flavoviridis—It was shown previously that snake venoms contain proteins that bind to α2β1 integrin and inhibit integrin binding to type I collagen (2–4). As the A domain of α2β1 integrin is chiefly responsible for collagen binding (5), we developed a method in which the integrin α2A domain is used as bait to identify an α2β1 integrin-inhibiting protein in the venom of the habu snake *T. flavoviridis*. Affinity chromatography was performed by immobilizing an oligo-His-tagged integrin A domain on a nickel-nitrilotriacetic acid (Ni-NTA) column and subsequently allowing proteins from the crude *T. flavoviridis* venom to bind to the integrin. Elution with an imidazole gradient resolved the proteins into four peaks (Fig. 1*A*, *solid line*). Peaks AI and AII are nonspecifically bound protein, as was proven in an alternate experiment in which the crude venom was applied solely to the Ni-NTA matrix (data not shown). Subsequently eluted peaks AIII and AIV contain the α2A domain and venom proteins (Fig. 1*B*). For peak AIII, venom proteins with molecular masses of 13, 18, and 32 kDa were isolated alongside the 26-kDa α2A domain. Peak AIV yielded much higher amounts of the 32-kDa protein, which was eluted from the column without α2A.

Isolation of the α2β1 Integrin-binding Venom Protein Independently of α2A Domain Affinity Chromatography—To characterize the structure of the isolated proteins and to confirm their α2β1 integrin-binding specificity, it was essential to isolate the venom proteins in an α2A-independent manner. This was achieved by a three-step purification procedure with gel filtration and subsequent ion exchange chromatography, followed by another size exclusion chromatography.

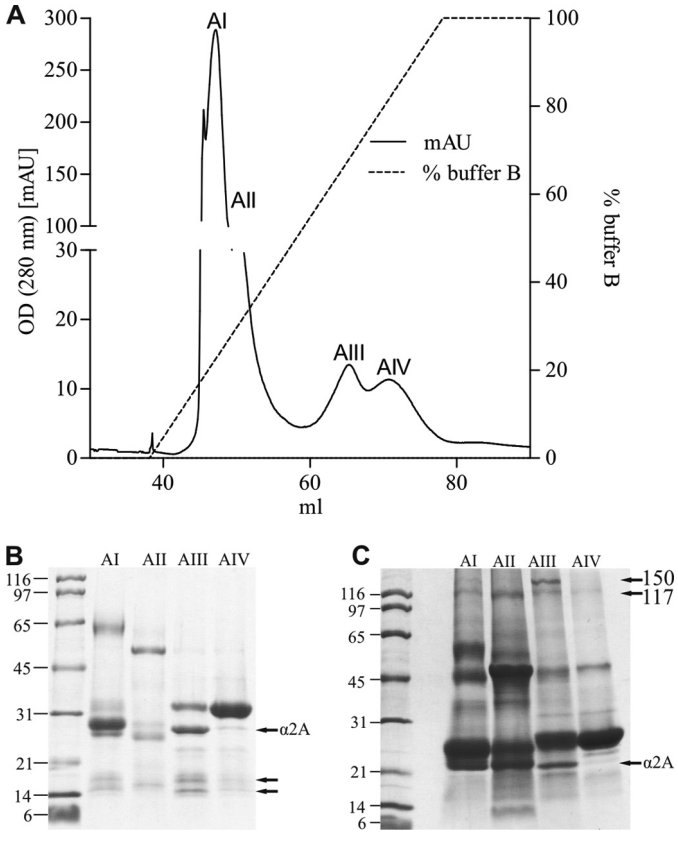

FIGURE 1. **Isolation of an α2β1 integrin-specific venom component.** *A*, elution with an imidazole gradient resolved the *T. flavoviridis* venom into four peaks (*solid line*): one large peak of nonspecifically binding proteins (peak AI) with a shoulder (peak AII), followed by two smaller peaks, the first containing an integrin-venom protein complex (peak AIII) and the next containing only venom protein (peak AIV). Eluate fractions were analyzed by 10–20% gel electrophoresis under reducing (*B*) and nonreducing (*C*) conditions and by Coomassie Blue staining. Peak AIII contains α2A (26 kDa) and two prominent bands at 14 and 18 kDa as well as a protein band at 32 kDa under reducing conditions. In the subsequent peak, the amount of α2A and the low molecular mass bands decreases, whereas the intensity of the 32-kDa band increases. *mAU*, milli-absorbance units.

Crude *T. flavoviridis* venom was separated into three major peaks on a Superdex 200 10/300 GL gel filtration column (Fig. 2*A*, *solid line*), analyzed by SDS-PAGE, and tested for α2β1 binding. Fig. 2*C* illustrates the degree of purity obtained in this gel filtration process. The Superdex column peak GI illustrates a band pattern corresponding to the protein bands isolated by affinity chromatography (Fig. 1*B*, *AIII lane*), as well as other proteins. Neither peak GII nor peak GIII exhibits the band pattern seen in the affinity chromatography.

The biological activity of the eluate fractions was analyzed for their potential to block α2A binding to type I collagen (Fig. 2*A*, *dotted line*). In this inhibition assay, samples were incubated together with the GST-linked α2A domain on immobilized type I collagen. The amount of bound integrin detected gives information on the inhibitory potential of the venom proteins. Integrin binding was reduced by fractions from peak GI. There-

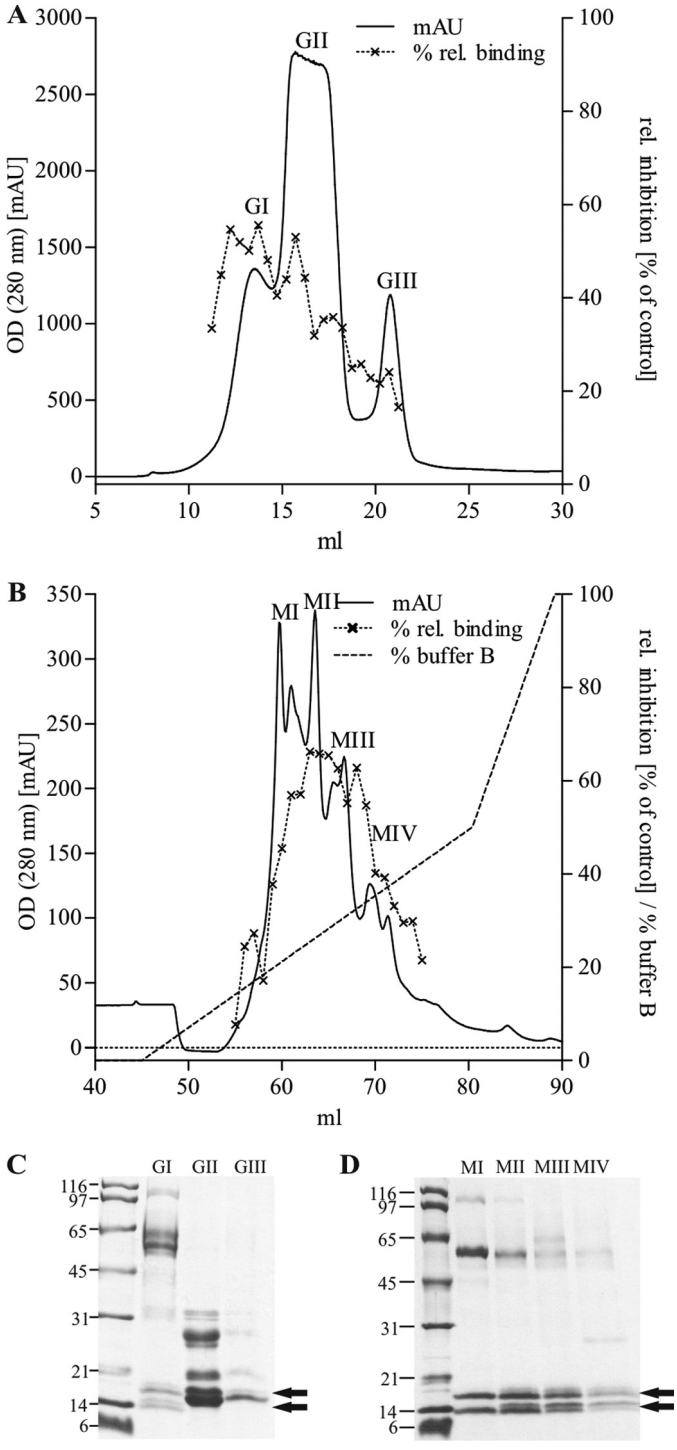

FIGURE 2. **Purification of α2A-binding protein by gel filtration and ion exchange chromatography.** *A*, gel filtration of *T. flavoviridis* on a Superdex 200 10/300 GL column resolved the venom into three peaks (*solid line*). The *dotted line* indicates the percentage of α2β1 inhibition after addition of the respective eluate fractions. *B*, ion exchange chromatography of peak GI from the gel filtration separated the proteins into four peaks (*solid line*) along a sodium chloride gradient (*dashed line*). The potential to inhibit α2β1 binding to collagen was measured for several fractions and is designated by the *dotted line*. *C* and *D*, SDS-PAGE on 10–20% acrylamide gels under reducing conditions and Coomassie Blue staining show venom proteins separated by gel filtration on a Superdex column (*C*) and subsequent Mono S chromatography (*D*). *mAU*, milli-absorbance units; *rel.*, relative.

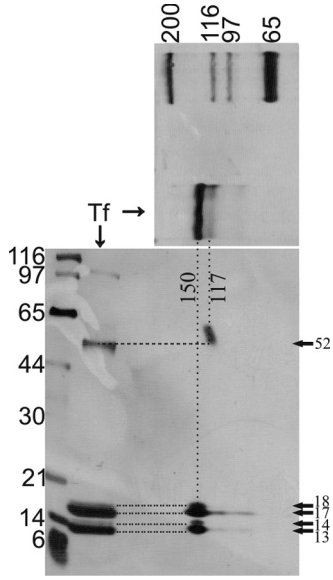

FIGURE 3. **Two-dimensional gel analysis of purified *T. flavoviridis* venom protein.** The active venom protein was separated under nonreducing conditions in the first dimension (*Tf*→, *horizontal lane*). After being reduced, the proteins were separated in the second dimension (*vertical lane*) and silver-stained; the reduced purified protein served as a control (*Tf* ↓).

fore, peak GI contains proteins that are able to bind α2A and inhibit its binding to type I collagen.

The Superdex eluate fractions with α2A integrin-inhibiting activity were pooled and then separated by ion exchange chromatography. Four peaks (Fig. 2*B*, *solid line*) were eluted from the Mono S column with a sodium chloride gradient (*dashed line*). As before, fractions were analyzed by gel electrophoresis and by an inhibition assay. Reducing SDS-PAGE analysis revealed protein bands with apparent molecular masses of 13, 14, 17, 18, 50, 55, and 100 kDa in the different fractions. Although the patterning of the fractions looks very much alike, the inhibition test with α2A indicated that both peaks MII and MIII have inhibitory potential (Fig. 2*B*, *dotted line*).

To gain insights into the complexity of the α2β1 integrin-binding venom protein, two-dimensional gel analyses of both inhibitory peaks (MII and MIII) were carried out with identical results. Fig. 3 shows the results for peak MIII. The first dimension electrophoresis of the nonreduced venom protein (Fig. 3, *horizontal lane*) is shown perpendicular to the second dimension protein separation. Both nonreduced samples were resolved into two high molecular mass bands with apparent molecular masses of 117 and 150 kDa. In the second dimension, the 150-kDa band was separated into two double bands with apparent molecular masses of 13/14 and 17/18 kDa and exhibiting different intensities. The 117-kDa band resulted in one single band of ~52 kDa, which did not appear in the affinity chromatography. These results indicate either that the integrin-binding protein is a complex structure of several subunits, some of which are connected by disulfide links, or that there is still a contaminating protein that needs to be removed. The samples were analyzed by mass spectrometry, and protein fragments were compared with a database of *T. flavoviridis* proteins. The results revealed the isolated 150-kDa protein to be multimeric flavocetin-A (149 kDa) with a coverage of 78 and

88% for the α and β chains, respectively (data not shown). The 117-kDa protein was identified as a zinc metalloproteinase. In the α2A affinity chromatography, only the 150-kDa band was found to bind to the α2A domain, whereas the 117-kDa band was eluted predominantly in peak AII, which did not coelute α2A.

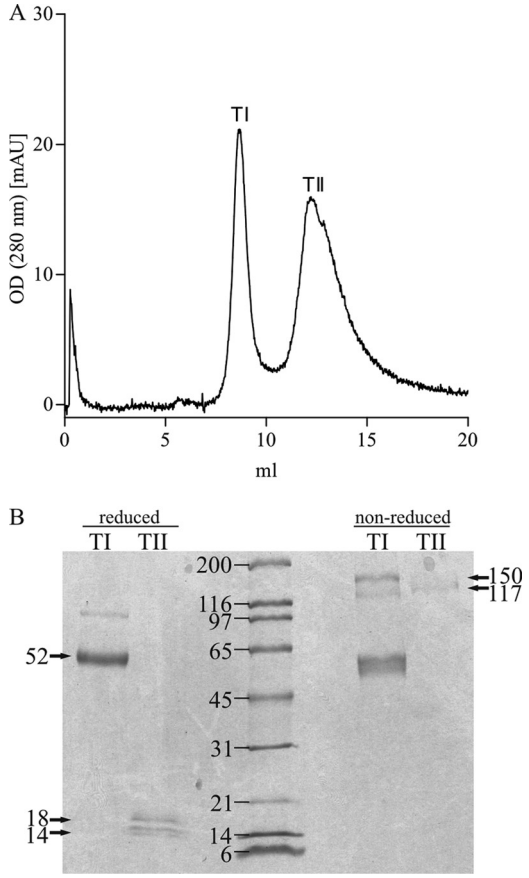

FIGURE 4. **Separation of flavocetin-A from contaminant by gel filtration on a TSK column.** *A*, gel filtration of the contaminant-containing flavocetin-A sample yielded two peaks. *B*, proteins were analyzed by SDS-PAGE on a 10–20% acrylamide gel under reducing and nonreducing conditions and by Coomassie Blue staining. *mAU*, milli-absorbance units.

To separate the 150- and 117-kDa proteins, a additional purification step was introduced. Using a TSK 200 gel filtration column, two peaks were separated, with the second one (peak TII) containing flavocetin-A as shown by gel analysis (Fig. 4). The 117-kDa protein was found to have no effect on platelets, in contrast to the 150-kDa protein.

Platelet Aggregation Studies—Flavocetin-A had initially been reported to bind to and have an effect on the platelet protein GPIb (16, 18). We isolated this same protein as an α2β1 integrin-inhibiting venom component and proceeded to verify its GPIb-binding properties. vWF- and collagen-induced platelet aggregation was challenged with flavocetin-A, purified in our two-step purification procedure. We first showed that flavocetin-A inhibited vWF-induced platelet aggregation, proving its long-known activity as a GPIb-targeting venom component (Fig. 5*A*). Flavocetin-A alone was sufficient to cluster several platelets into small agglutinates by binding to GPIb molecules on different platelets, as shown by aggregometry.

The rhodocetin αβ dimer, a different antithrombotic snake venom component, also binds to GPIb on platelets (24). When this dimer was incubated with platelets before the addition of flavocetin-A, it prevented platelet agglutination (Fig. 5*B*). After pretreatment with rhodocetin αβ, platelets were still activated by type I collagen, which is directly recognized by platelets via α2β1 integrin and GPVI. Stimulation of rhodocetin αβ-pretreated and thus GPIb-blocked platelets with type I collagen was progressively inhibited with higher concentrations of flavocetin-A (Fig. 5*C*, *traces 3–5*).

These results proved that our flavocetin-A indeed was active and interfered with the vWF-GPIb interaction. It also blocked the α2β1 integrin receptor on platelets and inhibited collagen-induced platelet aggregation. As the isolated 117-kDa protein alone did not have any effect on platelets, we concluded that it was rather a contaminant than an α2β1 integrin-inhibiting constituent of the snake venom.

Flavocetin-A Binds to the α2A Domain—We isolated flavocetin-A by searching for a venom component that inhibits α2A binding to type I collagen. To assess the binding affinity of flavocetin-A for the integrin α2A domain, titration assays were

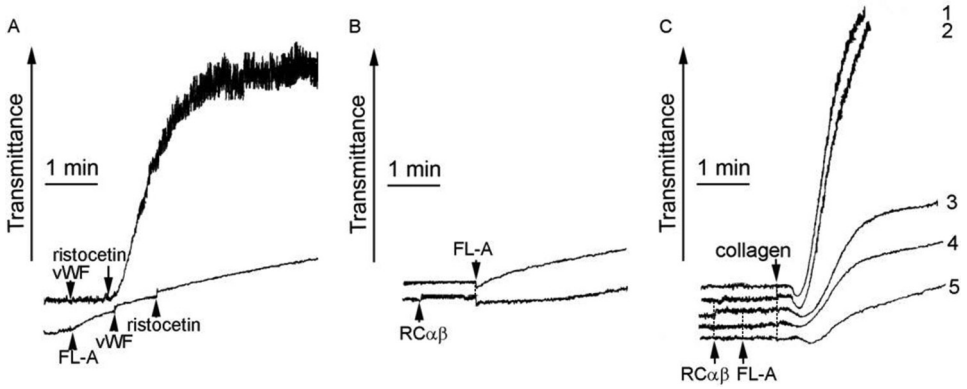

FIGURE 5. **Flavocetin-A inhibits type I collagen-induced platelet aggregation.** *A*, aggregation of washed human platelets was induced by 5 μg/ml human vWF plus 0.5 mg/ml ristocetin but was prevented by 5 μg/ml flavocetin-A (*FL-A*). *B*, without resulting in platelet activation and aggregation, 5 μg/ml flavocetin-A cross-linked several platelets by interacting with GPIb molecules on different platelets, which was confirmed by a small increase in light transmittance. Agglutination was inhibited by 5 μg/ml rhodocetin αβ (*RCαβ*). *C*, platelet aggregation was induced by 2.5 μg/ml collagen (*trace 1*) and also in the presence of 10 μg/ml rhodocetin αβ (*trace 2*), which blocked GPIb. After preincubation of platelets with increasing concentrations of the GPIb-blocking rhodocetin αβ (2.5, 5, and 10 μg/ml), equivalent concentrations of flavocetin-A (*traces 3–5*) showed a dose-dependent inhibition of collagen-induced platelet activation, presumably via α2β1 integrin inhibition.

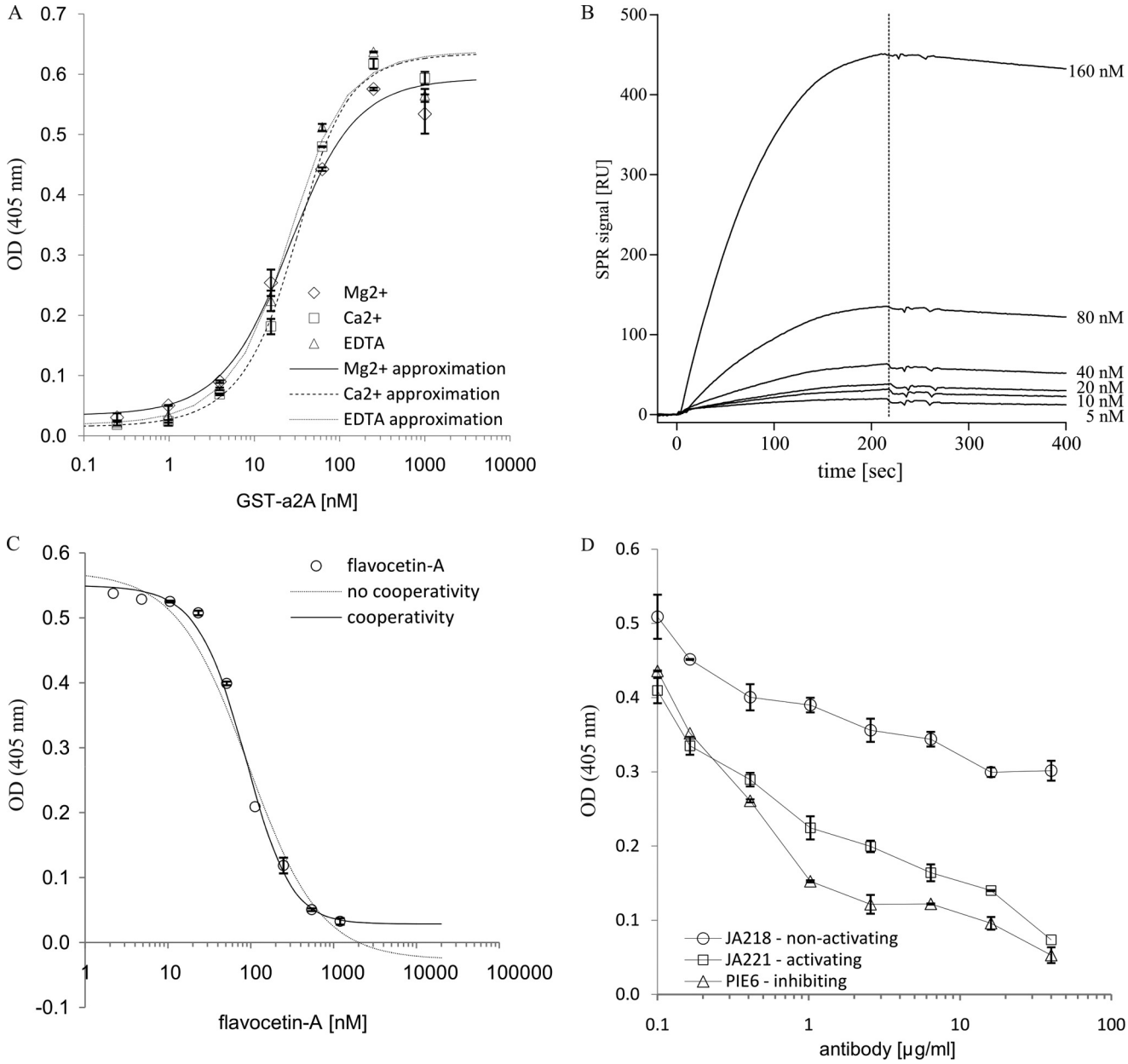

FIGURE 6. **Molecular activity analyses of the isolated venom component.** *A*, flavocetin-A was immobilized and titrated with the α2A domain in the presence or absence of divalent cations. BSA served as background control. The integrin bound to the venom protein in a saturable manner, yielding K_D values of 20.7, 13.6, and 13.8 nM for Mg^{2+}, Ca^{2+}, and EDTA, respectively. *B*, surface plasmon resonance measurement of flavocetin-A and α2A. The A domain was covalently coupled to a CM5 chip. Sensorgrams were recorded in the presence of flavocetin-A beginning at 160 nM with 1:2 dilutions. *C*, flavocetin-A inhibited binding of the soluble α2β1 integrin ectodomain to collagen with an IC_{50} of 88.2 nM. The titration and inhibition curves were iteratively fitted using monovalent or cooperative interaction models, respectively, by χ^2 approximation until $\chi^2 < 5\%$ was reached. *D*, binding of the GST-tagged α2A domain to immobilized flavocetin-A was challenged with increasing concentrations of monoclonal antibodies against the α2A domain. BSA served as a background control. Integrin binding to the venom protein was only slightly reduced by JA218, whereas both JA221 and P1E6 reduced binding considerably.

performed with GST-α2A, which binds to flavocetin-A in a saturable manner. The titration curve in the presence of Mg^{2+} could be approximated, yielding a dissociation constant of 20.7 nM (Fig. 6*A*, *solid line*). Binding was not abolished by deprivation of divalent cations with EDTA. Instead, the presence of EDTA increased the binding signals and the binding affinity (K_d = 13.8 nM). Conspicuously, Ca^{2+} ions had a similar effect (K_d = 13.6 nM) (Fig. 6*A*, *dotted* and *dashed lines*).

Kinetics were measured by surface plasmon resonance. As shown in Fig. 6*B*, flavocetin-A bound quickly to the α2A domain, whereas dissociation of the resulting complex could not be observed. In fact, reducing agents and high pH were necessary to remove flavocetin-A from the immobilized α2A domain. An association rate constant (k_a) of $2.9 \times 10^5\ M^{-1}\ s^{-1}$ was obtained by approximating a dissociation rate constant for the best fit of the data. This resulted in a K_d of 6.4 nM. The very low dissociation rate constant and the corresponding high affinity indicate an almost irreversible blocking of the integrin α2A domain.

Flavocetin-A Demonstrates Cooperative Binding to α2β1—To test whether purified flavocetin-A inhibits not only α2A but also the soluble α2β1 integrin ectodomain, its binding to immobi-

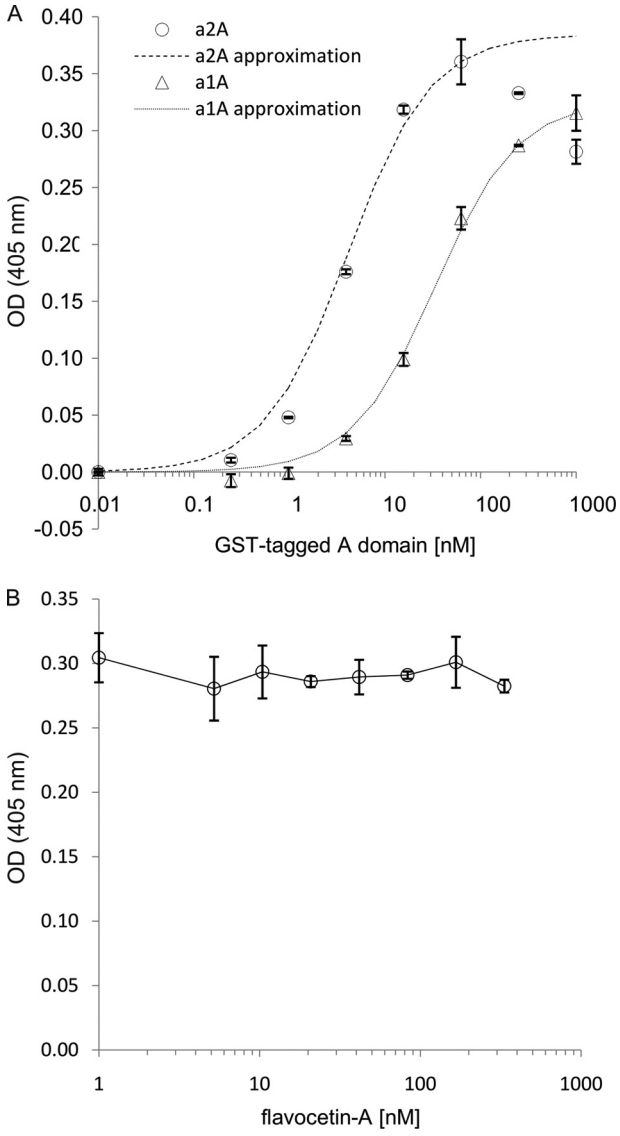

FIGURE 7. **Specificity analyses of flavocetin-A for the α2A domain.** *A*, flavocetin-A was immobilized and titrated with the α2A and α1A domains. BSA served as a background control. Both A domains bound the venom protein in a saturable manner; however, α1A had a much lower affinity than α2A. *B*, flavocetin-A did not inhibit binding of the soluble α1β1 integrin ectodomain to CB3.

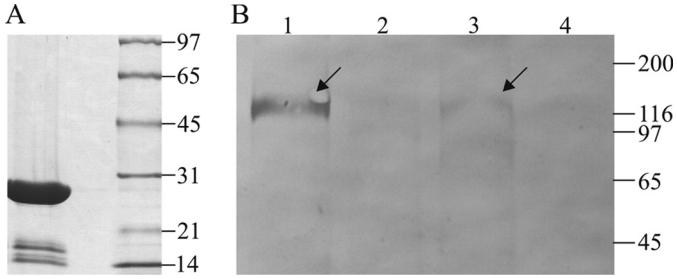

FIGURE 8. **Flavocetin-A binds α2A and GPIb with different binding sites.** *A*, oligo-His-tagged α2A (~26 kDa) was immobilized on a Ni-NTA column. After binding flavocetin-A (13–17-kDa bands), the protein complex was eluted as a single peak. *B*, Western blot analysis of the proteins eluted when platelet lysate was added to the complex of immobilized α2A with flavocetin-A (*lanes 1* and *2*) and only immobilized α2A (*lanes 3* and *4*).

lized collagen was challenged with increasing concentrations of flavocetin-A. Evaluation revealed an accurate fit of the data with an IC_{50} of 88.2 nM and a cooperativity of $n \sim 2$ (Fig. 6*C*).

Flavocetin-A Prefers the Inactive α2A Conformation—Monoclonal antibodies were utilized to determine whether flavocetin-A binds to the active or inactive conformation of the A domain. The non-activating antibody JA218 showed only a slight decrease in the binding signal of α2A to flavocetin-A (Fig. 6*D*). In contrast, the activating antibody JA221 and the inhibitory antibody P1E6 reduced the binding signal to a similar extent. These results provide evidence for a preferential binding of the toxin to the inactive α2A conformation.

Flavocetin-A Shows Very Low Affinity for the α1A Domain—To further determine the specificity of flavocetin-A for the α2A domain, the recombinantly expressed α1A domain was tested for binding to the toxin. Although α1A bound to flavocetin-A (Fig. 7*A*), its affinity was much lower, and it failed to interact with the entire α1β1 integrin ectodomain (Fig. 7*B*), therefore ruling it out as a natural target of flavocetin-A.

Flavocetin-A Has Different Binding Sites for α2A and GPIb—To assess whether flavocetin-A utilizes the same binding site for the integrin A domain and the platelet receptor GPIb, we performed affinity chromatography employing all three compounds. Fig. 8*A* shows that the oligo-His-tagged α2A domain immobilized to a Ni-NTA column bound flavocetin-A. An increase in imidazole eluted the complex of the α2A domain with flavocetin-A. Intriguingly, all four bands of flavocetin-A were eluted, indicating that all putative subunits stay together in complex with the α2A domain. In the next step, platelet lysate containing glycocalicin, which is the extracellular part of the GPIb receptor, was applied to the column. Two peaks were eluted from the column. The first peak at 60 mM imidazole contained glycocalicin as shown in Fig. 8*B* (*lane 1*). The second peak at 120 mM imidazole (*lane 2*) contained α2A and flavocetin-A. When flavocetin-A was omitted from the α2A-loaded column (Fig. 8*B*, *lanes 3* and *4*), negligible amounts of glycocalicin were eluted from the column (*lane 3*), whereas the α2A domain was eluted later (*lane 4*). These results imply that flavocetin-A was able to form a ternary complex with both the α2A domain and GPIb. Moreover, as α2β1 integrin and GPIb did not mutually exclude each other from binding to flavocetin-A, flavocetin-A must have different sites to interact with both platelet receptors simultaneously.

Flavocetin-A Inhibits α2β1 Integrin-mediated Cell Adhesion and Migration—To assess whether flavocetin-A has any cellular effect, possibly enabling it to inhibit α2β1 integrin-mediated cell adhesion or migration *in vivo*, two independent methods were employed. First, adhesion of HT1080 cells to type I collagen was analyzed in the presence of flavocetin-A, which inhibited cell adhesion to type I collagen entirely and efficiently with an IC_{50} of 6.3 ± 1.5 nM (Fig. 9*A*) as shown by real-time impedance measurement. An independent experiment in a microtiter plate with crystal violet staining showed that flavocetin-A was unable to influence the binding of HT1080 cells to laminin-111 or fibronectin (Fig. 9*C*), underlining the selectivity of flavocetin-A for α2β1 integrin.

Second, the effect of flavocetin-A on migration of HT1080 cells was investigated. HT1080 cells migrated through a filter along a haptotactic gradient of type I collagen. Impedance values increased over time when the cells covered the electrodes

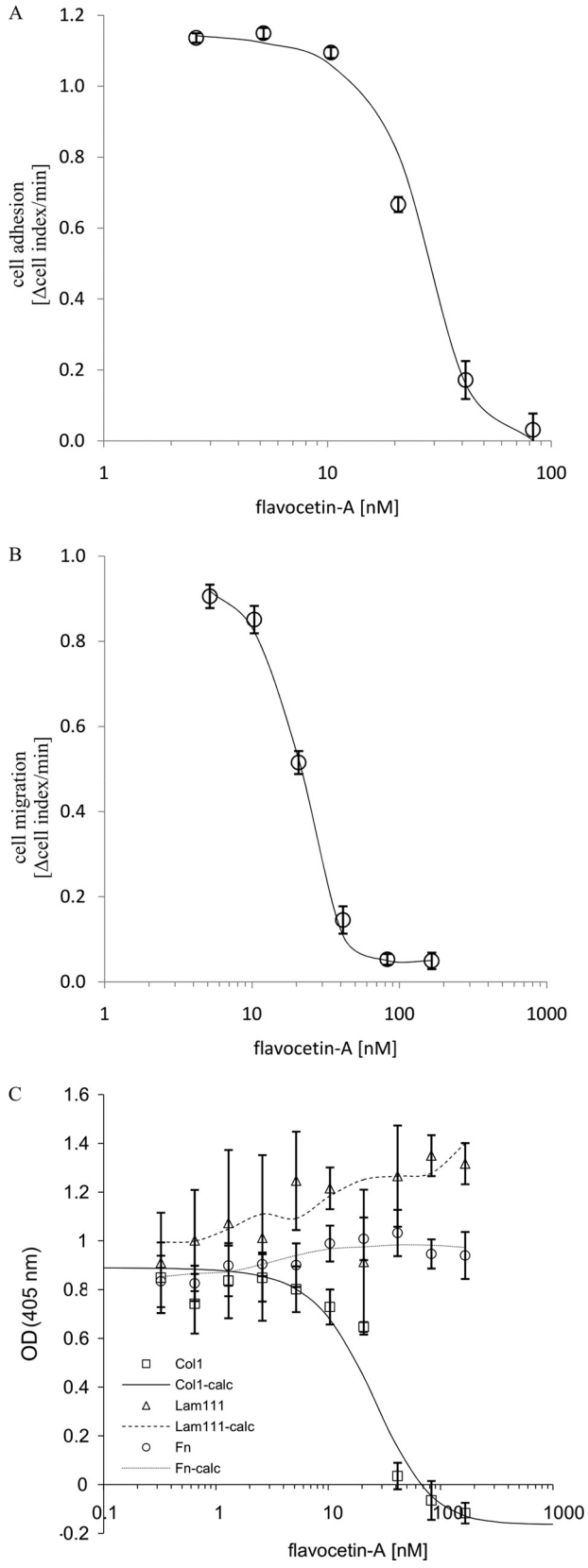

FIGURE 9. **Cellular analyses of the isolated venom component.** *A*, venom protein activity was assessed at the cellular level by adhesion of HT1080 cells to type I collagen using the xCELLigence system. Adhesion in the absence of flavocetin-A served as a positive control for cell binding to collagen, whereas the presence of 10 mM EDTA corresponded to no binding. Cell adhesion to collagen was reduced by flavocetin-A in a concentration-dependent manner with an IC_{50} of 6.3 ± 1.5 nM. *B*, cell migration analysis was performed using the xCELLigence system and its diagnostic software. Snake venom protein inhibited cell migration with an IC_{50} of 3.98 ± 1.9 nM, calculated as the area under the sigmoidal dose-response curve. *C*, the specificity of flavocetin-A for α2β1 integrin was assessed in an adhesion assay with crystal violet staining. HT1080 cells did not adhere to laminin-111 (*Lam111*) or fibronectin (*Fn*) but did adhere to type I collagen (*Col1*). Curves were fitted by χ^2 approximation. *calc*, calculated values of curve approximation.

on the bottom face of the filter, with the slope in the graph (Fig. 9*B*) representing HT1080 cell migration. With increasing flavocetin-A concentrations, impedance decreased with an IC_{50} of 3.98 ± 1.9 nM (Fig. 9*B*).

DISCUSSION

We have shown that the CLRP flavocetin-A binds to the A domain of α2β1 integrin and acts antagonistically on adhesion and migration of cells. Furthermore, our results suggest that flavocetin-A may actually be a more complex molecule than initially proposed.

We were able to establish a method to quickly screen venoms for α2β1 integrin-binding proteins that affect the integrin-collagen interaction. By using affinity chromatography with the collagen-binding A domain of α2β1 integrin, proteins were specifically captured from the crude venom of *T. flavoviridis*. Although, in this process, a protein of 32 kDa was eluted alongside the protein complex of α2A and flavocetin-A, this same protein was not present in the A domain-independent purification. Similarly, a 117-kDa protein was co-purified with flavocetin-A (150 kDa) in the α2A-independent purification protocol. This 117-kDa protein affected neither α2β1 integrin on platelets nor GPIb. After effective removal by high resolution gel filtration without loss of α2β1 function, we concluded that the 117-kDa protein was a contaminant.

To date, flavocetin-A has been recognized only as a GPIb-binding protein that induces platelet agglutination. Our study confirmed that the α2β1 integrin-binding venom component purified by us is indeed the GPIb-binding protein flavocetin-A.

According to our data, flavocetin-A efficiently binds to the integrin α2A domain. Molecular analyses indicated that flavocetin-A binds to α2A with an affinity constant (K_d) of 20.7 nM. Surface plasmon resonance studies confirmed these results and further revealed that association not only occurs very rapidly but also is very strong, requiring harsh measures to remove flavocetin-A from α2A.

Furthermore, the results also indicate that this CLRP not only inhibits binding of the GST-linked α2A domain and the recombinant α2β1 integrin ectodomain to type I collagen *in vitro* but also inhibits wild-type integrin. This was demonstrated at the cellular level by adhesion and migration of HT1080 cells, which express high amounts of α2β1 integrin but no GPIb. Flavocetin-A was unable to affect cell adhesion to laminin-111 or fibronectin, thus revealing the specificity of flavocetin-A for α2β1 integrin.

When flavocetin-A was first discovered, it was shown to be composed of two subunits, the α and β chains (19). Despite the fact that mass spectrometry showed our purified protein to be flavocetin-A, gel analysis of our data indicated the presence, under reducing conditions, of two additional low molecular

mass bands, which formed one single band under nonreducing conditions. In the original study (19), flavocetin-B was also isolated and shown to consist of the same two α and β chains as flavocetin-A plus an additional γ chain. Flavocetin-B has the same properties as flavocetin-A; it is actually able to abolish binding of flavocetin-A to platelets (19). We propose that one of the additional proteins visible in our gel analysis may be the γ chain from flavocetin-B, which would not be recognized by mass spectrometry as only the primary structures of flavocetin-A chains are known. Also, the fourth protein might be a δ chain, indicating that flavocetin-A may actually be composed of four different chains forming a tetramer of two different heterodimers, generating an $(\alpha\beta)_2(\gamma\delta)_2$ structure. In this constellation, it can be assumed that the αβ heterodimer is responsible for binding to GPIb based on the data in the original publication (19); the lack of α2β1 integrin binding by the αβ heterodimer suggests that the γδ heterodimer is the α2β1 integrin-binding moiety. The results from our affinity chromatography with the immobilized α2A domain corroborate this proposition. The proposed $(\alpha\beta)_2(\gamma\delta)_2$ flavocetin structure has binding sites for both α2β1 integrin and GPIb, which sterically do not overlap and allow simultaneous binding of both platelet receptors. This would make flavocetin a further heterotetrameric CLRP, once again demonstrating the high versatility of this protein family. Another CLRP that targets both GPIb and α2β1 integrin is rhodocetin, and its heterotetrameric nature has been characterized by our group (20). However, the two different heterodimers of rhodocetin associate noncovalently only to form a dimer; in contrast, the flavocetin subunits appear to be covalently associated with each other. Furthermore, bilinexin also binds these same two receptors and is thought to be a heterotetramer (25). Under nonreducing conditions, the bilinexin subunits associate to form a single band just like flavocetin, as shown in our gel analysis. In conclusion, it is obvious that the diversity within the CLRP family refers to both structure and function.

Acknowledgment—We thank Dr. Heide (Molecular Bioenergetics Department, University Hospital Frankfurt) for mass spectrometry analysis.

REFERENCES

1. Eble, J. A. (2005) Collagen-binding integrins as pharmaceutical targets. *Curr. Pharm. Des.* **11,** 867–880
2. Marcinkiewicz, C., Lobb, R. R., Marcinkiewicz, M. M., Daniel, J. L., Smith, J. B., Dangelmaier, C., Weinreb, P. H., Beacham, D. A., and Niewiarowski, S. (2000) Isolation and characterization of EMS16, a C-lectin type protein from *Echis multisquamatus* venom, a potent and selective inhibitor of the α2β1 integrin. *Biochemistry* **39,** 9859–9867
3. Eble, J. A., Niland, S., Dennes, A., Schmidt-Hederich, A., Bruckner, P., and Brunner, G. (2002) Rhodocetin antagonizes stromal tumor invasion *in vitro* and other α2β1 integrin-mediated cell functions. *Matrix Biol.* **21,** 547–558
4. Staniszewska, I., Walsh, E. M., Rothman, V. L., Gaathon, A., Tuszynski, G. P., Calvete, J. J., Lazarovici, P., and Marcinkiewicz, C. (2009) Effect of VP12 and viperistatin on inhibition of collagen receptor-dependent melanoma metastasis. *Cancer Biol. Ther.* **8,** 1507–1516
5. Emsley, J., Knight, C. G., Farndale, R. W., Barnes, M. J., and Liddington, R. C. (2000) Structural basis of collagen recognition by integrin α2β1. *Cell* **101,** 47–56
6. Morita, T. (2005) Structures and functions of snake venom CLPs (C-type lectin-like proteins) with anticoagulant-, procoagulant-, and platelet-modulating activities. *Toxicon* **45,** 1099–1114
7. Clemetson, K. J. (2010) Snaclecs (snake C-type lectins) that inhibit or activate platelets by binding to receptors. *Toxicon* **56,** 1236–1246
8. Arlinghaus, F. T., and Eble, J. A. (2012) C-type lectin-like proteins from snake venoms. *Toxicon* **60,** 512–519
9. Clemetson, K. J., Navdaev, A., Dörmann, D., Du, X. Y., and Clemetson, J. M. (2001) Multifunctional snake C-type lectins affecting platelets. *Haemostasis* **31,** 148–154
10. Clemetson, K. J., and Clemetson, J. M. (2008) Platelet GPIb complex as a target for antithrombotic drug development. *Thromb. Haemost.* **99,** 473–479
11. Sakariassen, K. S., Bolhuis, P. A., and Sixma, J. J. (1979) Human blood platelet adhesion to artery subendothelium is mediated by factor VIII-von Willebrand factor bound to the subendothelium. *Nature* **279,** 636–638
12. Peng, M., Lu, W., and Kirby, E. P. (1991) Alboaggregin-B: a new platelet agonist that binds to platelet membrane glycoprotein Ib. *Biochemistry* **30,** 11529–11536
13. Peng, M., Lu, W., Beviglia, L., Niewiarowski, S., and Kirby, E. P. (1993) Echicetin: a snake venom protein that inhibits binding of von Willebrand factor and alboaggregins to platelet glycoprotein Ib. *Blood* **81,** 2321–2328
14. Andrews, R. K., Booth, W. J., Gorman, J. J., Castaldi, P. A., and Berndt, M. C. (1989) Purification of botrocetin from *Bothrops jararaca* venom. Analysis of the botrocetin-mediated interaction between von Willebrand factor and the human platelet membrane glycoprotein Ib-IX complex. *Biochemistry* **28,** 8317–8326
15. Hamako, J., Matsui, T., Suzuki, M., Ito, M., Makita, K., Fujimura, Y., Ozeki, Y., and Titani, K. (1996) Purification and characterization of bitiscetin, a novel von Willebrand factor modulator protein from *Bitis arietans* snake venom. *Biochem. Biophys. Res. Commun.* **226,** 273–279
16. Fukuda, K., Mizuno, H., Atoda, H., and Morita, T. (1999) Crystallization and preliminary x-ray studies of flavocetin-A, a platelet glycoprotein Ib-binding protein from the habu snake venom. *Acta Crystallogr. D Biol. Crystallogr.* **55,** 1911–1913
17. Taniuchi, Y., Kawasaki, T., and Fujimura, Y. (2000) The high molecular mass, glycoprotein Ib-binding protein flavocetin-A induces only small platelet aggregates *in vitro*. *Thromb. Res.* **97,** 69–75
18. Fukuda, K., Mizuno, H., Atoda, H., and Morita, T. (2000) Crystal structure of flavocetin-A, a platelet glycoprotein Ib-binding protein, reveals a novel cyclic tetramer of C-type lectin-like heterodimers. *Biochemistry* **39,** 1915–1923
19. Taniuchi, Y., Kawasaki, T., Fujimura, Y., Suzuki, M., Titani, K., Sakai, Y., Kaku, S., Hisamichi, N., Satoh, N., and Takenaka, T. (1995) Flavocetin-A and -B, two high molecular mass glycoprotein Ib-binding proteins with high affinity purified from *Trimeresurus flavoviridis* venom, inhibit platelet aggregation at high shear stress. *Biochim. Biophys. Acta* **1244,** 331–338
20. Eble, J. A., Niland, S., Bracht, T., Mormann, M., Peter-Katalinic, J., Pohlentz, G., and Stetefeld, J. (2009) The α2β1 integrin-specific antagonist rhodocetin is a cruciform, heterotetrameric molecule. *FASEB J.* **23,** 2917–2927
21. Canfield, V. A., Ozols, J., Nugent, D., and Roth, G. J. (1987) Isolation and characterization of the α and β chains of human platelet glycoprotein Ib. *Biochem. Biophys. Res. Commun.* **147,** 526–534
22. Eble, J. A., Beermann, B., Hinz, H. J., and Schmidt-Hederich, A. (2001) 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. *J. Biol. Chem.* **276,** 12274–12284
23. Navdaev, A., Dörmann, D., Clemetson, J. M., and Clemetson, K. J. (2001) Echicetin, a GPIb-binding snake C-type lectin from Echis carinatus, also contains a binding site for IgMκ responsible for platelet agglutination in plasma and inducing signal transduction. *Blood* **97,** 2333–2341
24. Nuyttens, B. P., Thijs, T., Deckmyn, H., and Broos, K. (2011) Platelet adhesion to collagen. *Thromb. Res.* **127,** Suppl. 2, S26–S29
25. Du, X. Y., Navdaev, A., Clemetson, J. M., Magnenat, E., Wells, T. N., and Clemetson, K. J. (2001) Bilinexin, a snake C-type lectin from *Agkistrodon bilineatus* venom, agglutinates platelets via GPIb and α2β1. *Thromb. Haemost.* **86,** 1277–1283