Isolation and Characterization of EMS16, a C-Lectin Type Protein from *Echis multisquamatus* Venom, a Potent and Selective Inhibitor of the α2β1 Integrin†

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ABSTRACT: We have isolated and characterized EMS16, a potent and selective inhibitor of the α2β1 integrin, from *Echis multisquamatus* venom. It belongs to the family of C-lectin type of proteins (CLPs), and its amino acid sequence is homologous with other members of this protein family occurring in snake venoms. EMS16 ($M_r \sim 33$K) is a heterodimer composed of two distinct subunits linked by S−S bonds. K562 cells transfected with α2 integrin selectively adhere to immobilized EMS16, but not to two other snake venom-derived CLPs, echicetin and alboaggregin B. EMS16 inhibits adhesion of α2β1-expressing cells to immobilized collagen I at picomolar concentrations, and the platelet/collagen I interaction in solution at nanomolar concentrations. EMS16 inhibits binding of isolated, recombinant I domain of α2 integrin to collagen in an ELISA assay, but not the interaction of isolated I domain of α1 integrin with collagen IV. Studies with monoclonal antibodies suggested that EMS16 binds to the α2 subunit of the integrin. EMS16 inhibits collagen-induced platelet aggregation, but has no effect on aggregation induced by other agonists such as ADP, thromboxane analogue (U46619), TRAP, or convulxin. EMS16 also inhibits collagen-induced, but not convulxin-induced, platelet cytosolic Ca^{2+} mobilization. In addition, EMS16 inhibits HUVEC migration in collagen I gel. In conclusion, we report a new, potent viper venom-derived inhibitor of α2β1 integrin, which does not belong to the disintegrin family.

A collagen receptor, the α2β1 integrin, is expressed on platelets, endothelial, many epithelial, and some tumor cells. Its α2 subunit contains an I domain, a ∼200 amino acid extracellular region homologous with the von Willebrand factor (vWF)[1] A domain. A homologous domain is also found in other α subunits of integrins such as α1, αM, and αL (*1*−*3*). Ligand binding of these integrins is frequently mediated through the I domain and in most cases is metal dependent (*4*). Together with α1β1, the α2β1 integrin belongs to a subset of integrins that binds to collagen I, collagen IV, and laminin, but with different affinities (*5*, *6*). In view of the importance of α2β1 in physiology and disease, this integrin is now the focus of research in many laboratories. α2β1 expressed on endothelial cells plays a significant role in angiogenesis and neovascularization (*7*). Thus, inhibitors of this integrin may be important in drug development for tumor therapy.

Viper venoms contain a number of proteins showing antagonistic effects on adhesive receptors expressed on the cell surfaces. These proteins belong to two classes: disintegrins and C-lectin proteins (CLPs). Short, RGD-containing disintegrins include echistatin, which inhibits three integrins (αIIbβ3, αvβ3, α5β1), and eristostatin, which is selective to αIIbβ3 (*8*−*10*). The heterodimeric disintegrins are another class, which are more specific for leukocyte integrins. Two of them, EC3 and EMF10, characterized recently (*11*, *12*), inhibit α4β1/α4β7 and α5β1, respectively, with a high degree of selectivity. The metalloproteinase−disintegrin (jararhagin) inhibits collagen-induced platelet aggregation mediated by the α2β1 integrin(*13*). C-lectin proteins represent a separate, large family of snake venom proteins. The prototype of CLPs is the mannose binding protein. The snake venom CLPs demonstrate several biological activities; however, there has been no report of their anti-integrin activity. Echicetin (*14*), agkicetin (*15*), flavocetins (*16*), and tokaracetin (*17*) bind to the GPIb/IX complex and inhibit

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[1] Abbreviations: AP, alkaline phosphatase; BSA, bovine serum albumin; CLP, C-lectin protein; CMFDA, 5-chloromethylfluorescein diacetate; GST, glutathione-*S*-transferase; HBSS, Hanks balanced salt solution; HUVEC, human umbilical vein endothelial cells; mab, monoclonal antibody; MIDAS, metal ion-dependent adhesion site; PBS, phosphate-buffered saline; PRP, platelet rich plasma; TBS, Tris-buffered saline; TFA, trifluoroacetic acid; TRAP, thrombin receptor activating peptide; VCAM-1, vascular cell adhesion molecule 1; vWF, von Willebrand factor.

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vWF binding to this protein, thereby inhibiting platelet agglutination. Alboaggregin A and alboaggregin B activate the GPIb complex, causing platelet agglutination without any cofactor (*18*). Alboaggregin A it is a heterotetramer and alboaggregin B a heterodimer. Other CLPs include botrocetin that binds to the A domain of vWF, and factor IX/X binding protein (*19*). Convulxin is an activator of the platelet collagen receptor GPVI (*20*). This protein, like many other CLPs, exists as a multimeric form of two subunits, α and β. It is a hexamer of three α subunits and three β subunits. Despite having many different activities, the active sites of CLP have not been yet identified. Current structural studies of these proteins (*21*) may help in the localization of their active sites.

In this communication, we report a novel CLP, designated EMS16, isolated from *Echis multisquamatus* venom which is a potent and selective inhibitor of α2β1 integrin.

EXPERIMENTAL PROCEDURES

Isolation of CLPs and Disintegrins from Snake Venoms. Echicetin and alboaggregin (GPIb/IX ligands) were isolated from *Echis carinatus* and *Trimeresurus albolabris* venoms, respectively, as earlier described (*14, 18*). Eristostatin and EMF10 (α5β1 ligands) were isolated from *Eristocophis macmahoni* venom by two steps of HPLC as described previously (*12, 22*). The venoms were purchased from Latoxan (Valence, France).

EMS16 was isolated from *Echis multisquamatus* venom, also obtained from Latoxan, as follows: lyophilized venom was dissolved in 0.1% TFA (30 mg/mL) and centrifuged for 5 min at 5000 rpm to remove the insoluble proteins. The pellet was discarded, and the supernatant was applied on a C18 HPLC column (250 × 10 mm) (Vydac Inc., Hasperia, CA) equilibrated with 0.1% TFA. The elution was performed with a linear gradient of 0−80% acetonitrile over 45 min. The venom was separated into 22 fractions, and α2β1 binding activity was identified in fraction 16, eluted at approximately 70% acetonitrile. This fraction was collected, lyophilized, and dissolved in 20 mM Tris-HCl buffer, pH 7.5. This solution was subjected to a second step of chromatogaphy using a FPLC system. A Mono Q column was equilibrated with 20 mM Tris-HCl buffer, pH 7.5, and elution was performed with increasing concentrations of NaCl from 0 to 0.5 M. The active peak was collected, concentrated using Centriprep (Millipore, Bedford, MA), and dialyzed against water. The purity of EMS16 was tested using SDS−PAGE, mass spectrometry, and N-terminal sequencing. Ethylpyridylated subunits of EMF10 were separated using the method described for heterodimeric disintegrins (*11, 12*).

Cell Lines, Monoclonal Antibodies, and Integrin Ligands. The K562 cell line expressing α5β1 integrin and Jurkat cells expressing α4β1 integrin were purchased from ATCC (Manassas, VA). K562 cells transfected with α2 and α1 integrins were provided by Dr. M. Hemler (Dana Farber Cancer Insitute, Boston, MA). The K562 cell line transfected with α6 integrin was a gift from Dr. A. Sonnenberg (Netherlands Cancer Institute, Amsterdam, Holland). A5 and VNRC3 cells were CHO cells transfected with human αIIbβ3 and α$v\beta$3, respectively, and were kindly provided by Dr. M. Ginsberg (Scripps Research Institute, La Jolla, CA). Monoclonal antibody against α2 (clone AK-7) was purchased from Pharmingen (San Diego, CA). Monoclonal antibodies anti-α5 (clone SAM-1) and anti-β1 (clone Lia 1/2) were purchased from Immunotech (Westbrook, ME). Collagen type I and collagen type IV were obtained from Collaborative Biomedical Products (Bedford, MA) and Chemicon Inc.-(Temecula, CA), respectively. Highly purified fibrinogen was a gift from Dr. A. Budzynski (Temple University, Department of Biochemistry, Philadelphia PA). Fibronectin and laminin were purchased from Sigma Inc. (St. Louis, MO) and ICN (Costa Mesa, CA), respectively. Human recombinant VCAM-1 was expressed and isolated as described (*11*).

Adhesion Studies. Adhesion of cultured cells labeled with CMFDA was performed as described previously (*23*). Briefly, the ligands (EMS16, alboaggregin, or echicetin) were immobilized on 96-well microtiter plates (Falcon, Pittsburgh, PA) in PBS overnight at 4 °C. In the case of collagen I (α2β1 ligand) or collagen IV (α1β1 ligand), 0.02 M acetic acid was used instead of PBS. Wells were blocked with 1% BSA in HBSS buffer without Ca^{2+} containing 5 mM Mg^{2+}. Cells were labeled by incubation with 12.5 μM CMFDA in HBSS buffer without Ca^{2+} containing 5 mM Mg^{2+} at 37 °C for 15 min. Labeled cells (1×10^5/sample) were added to the wells in the presence or absence of inhibitors and incubated at 37 °C for 30 min. Unbound cells were removed by washing the wells, and bound cells were lysed by addition of 0.5% Triton X-100. In parallel, a standard curve was prepared in the same plate using known numbers of labeled cells. The plates were read using a Cytofluor 2350 fluorescence plate reader (Millipore, Bedford, MA) with a 485 nm excitation filter and 530 nm emission filter.

Binding of platelets to collagen I in the liquid phase was performed as described earlier (*24*).

Platelet Aggregation Assay. Platelets were isolated from fresh human blood obtained from drug-free volunteers. Platelet-rich plasma (PRP) was obtained by blood centrifugation at 110*g* for 15 min. PRP was gel filtered on a Sepharose 2B column using calcium-free Tyrode's buffer. U46619 and TRAP (SFLLRN) were obtained from Biomol (Plymouth Meeting, PA) and Bachem (Torrance, CA). Convulxin was prepared according to the procedure described earlier (*25*). Aggregation of gel-filtered platelets using several agonists was performed in a Chronolog aggregometer at 37 °C under stirring conditions.

Intracellular Platelet Ca^{2+} Mobilization. The PRP was incubated with fura-2 acetoxymethyl ester and separated from incorporated label by gel filtration on Sepharose 2B. The platelet suspension was activated by collagen I or convulxin in a Perkin-Elmer LS-5 spectrofluorometer in water-jacketed cuvette, maintained at 37 °C with stirring. Fura-2 fluorescence was monitored continuously using settings of 340 nm (excitation) and 510 nm (emission).

Interaction of the I Domains of α2 and α1 Integrins with EMS16. The α2-I domain and α1-I domain were expressed in the *E. coli* system as GST fusion proteins, as described previously (*26*). These fusion proteins were conjugated to alkaline phosphatase (AP) and used in an ELISA assay as follows: a 96-well, ELISA plate was coated with collagen I or IV by overnight incubation in 4 °C. The plate was washed with 0.1% Tween-20 in PBS buffer. The AP−I domain−GST fusion proteins were added in the presence or absence of EMS16 in TBS containing 1 mM $MnCl_2$ and 1 mg/mL BSA to the wells, and the plate was incubated at room temperature for 1 h. Bound I domain was detected

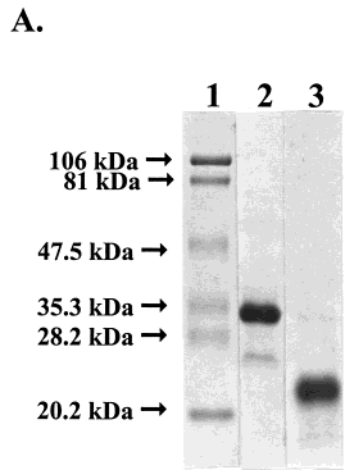

FIGURE 1: SDS−polyacrylamide (10%) analysis of EMS16 (A) and N-terminal sequencing of subunits of EMS16 (B). The gel was stained using Coomassie blue. Lane 1, protein standards; lane 2, purified EMS16 (10 μg) in nonreduced conditions; lane 3, EMS16 (10 μg) in reduced conditions. N-terminal sequencing analysis of ethylpyridylated subunits of EMS16 was performed using an automatic sequencer of Edman degradation.

colorimetrically using *p*-nitrophenyl phosphate (Sigma) as a substrate for AP.

HUVEC Radial Migration Assay. The HUVEC migration experiment was performed using cells isolated from umbilical veins and cultured as described previously (*27*, *28*), with modifications as follows. Bovine collagen I (Vitrogen 100, Collagen Corp., Palo Alto, CA) was polymerized as a thin film on 35 mm tissue culture dishes at a final concentration of 1.5 mg/mL, at 37 °C. A 10 μL droplet containing 15 000 HUVEC was pipetted into a 1 mL layer of mineral oil (Sigma). In this aqueous "bubble", HUVEC attached and spread onto the dried matrix surface at 37 °C in an atmosphere of 5% CO_2, for 60 min, after which cell spreading was complete. After aspirating the oil, the confluent HUVEC circle was washed in Medium 199 and plated in HUVEC serum-free medium with 20 ng/mL basic fibroblast growth factor and 10 ng/mL epidermal growth factor. The net area of HUVEC migration was quantified after 24 h by subtracting the 0 time image from migration after 24 h for each well (*27*). For qualitative analysis of cell migration and HUVEC morphology for longer times, images were recorded after 8 days. Quantification of HUVEC migration was performed using a Nikon Diaphot TMD inverted microscope interfaced with a VCR (Sony, Inc.) CCD camera (Hamamatsu, Inc.) and NIH Image 1.61 software on a Power Macintosh 7600/120 (*27*).

RESULTS

Purification and Structure of EMS16. EMS16 was identified in one fraction of *Echis multisquamatus* venom eluted from the HPLC column using the screening method which was successfully applied previously to identify heterodimeric disintegrins (*11*, *12*). A protein with anti-α2β1 integrin activity was present in fraction number 16. This fraction was collected and applied on a Mono Q column in the FPLC system. The purified protein was called EMS16 (abbreviation from Latin name of snake). The purity of this protein was evaluated on SDS−PAGE (Figure 1A). The EMS16 migrated in a nonreduced gel with a mobility of 33 kDa, and in a reduced gel with a mobility of about 20 kDa. Mass spectrometry analysis of EMS16 revealed a molecular mass of 33 292 kDa. EMS16 is composed of two subunits, EMS16A and EMS16B, with molecular masses of 16−17 kDa, linked with disulfide bonds. The N-terminal sequence of ethylpyridylated subunits is shown in Figure 1B. BLAST search results showed that the N-terminal sequences of EMS16 subunits are homologous to other snake venom-derived CLPs including conserved cysteines. It has a 60% identity with the N-terminus of alboaggregin A (subunit 2), a protein isolated from *Trimeresurus albolabris* venom, which activates GPIb/IX complex on platelets (*29*). A lower level of identity was achieved with echicetin (40%), a CLP isolated from *Echis carinatus suchoreki* (*14*), a species closely related to *Echis multisquamatus* viper.

Activity of EMS16 in Adhesion Assay. The inhibitory effect of EMS16 on the adhesion of cells transfected with genes of various integrins was tested using a cell adhesion assay and compared with two other snake venom CLPs: alboaggregin B and echicetin. K562 cells transfected with α2 integrin adhered to both immobilized EMS16 and immobilized alboaggregin B. Adhesion to echicetin was not observed (Figure 2A). On the other hand control, nontransfected K562 cells did not adhere to EMS16 but still bound to alboaggregin B (Figure 2B). Figure 3A presents the effect of CLPs on the inhibition of α2-K562 cell adhesion to immobilized collagen I. The potent inhibitory effect was

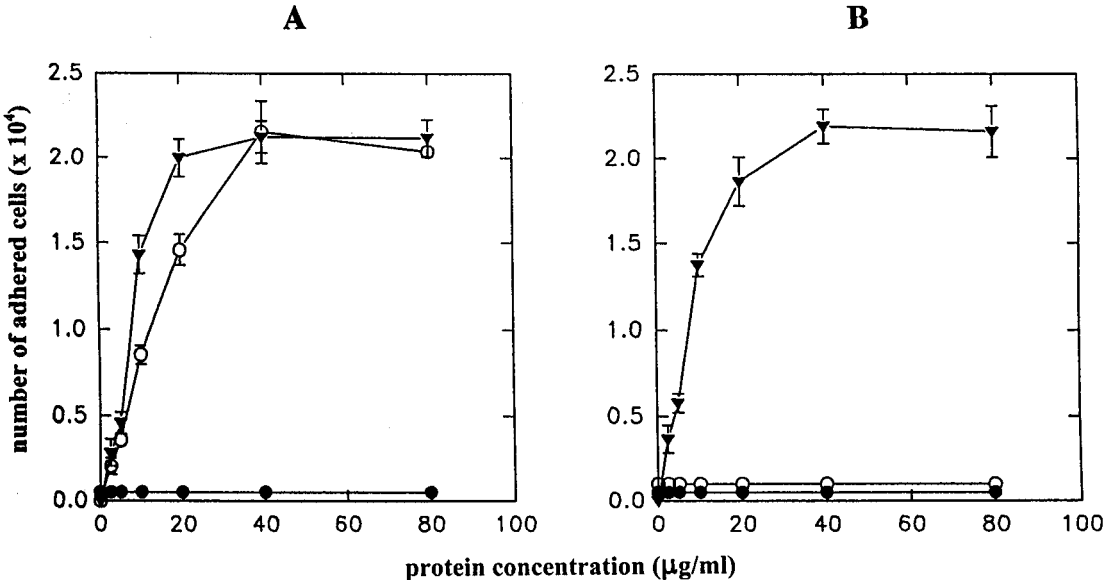

FIGURE 2: Adhesion of K562 cells transfected with α2 integrin (A) and control, nontransfected K562 cells (B) to immobilized EMS16 (open circles), echicetin (filled circles), and alboggregin B (filled triangles). Snake venom-derived proteins were immobilized overnight on a 96-well plate, in PBS buffer at 4 °C. After blocking with HBSS buffer containing calcium and magnesium and 1% BSA, the cells, previously labeled with CMFDA, were added at a concentration 1×10^5 per sample in the same buffer. The plate was incubated at 37 °C for 30 min and washed to remove unbound cells. The bound cells were lysed by addition of 0.5% Triton X-100. In parallel, a standard curve was prepared from known concentrations of lysed CMFDA-labeled cells. The plate was read using a Cytofluor 2350 fluorescence plate reader (Millipore), and the number of adhered cells was calculated from the standard curve. The error bars present the standard deviation from three independent experiments.

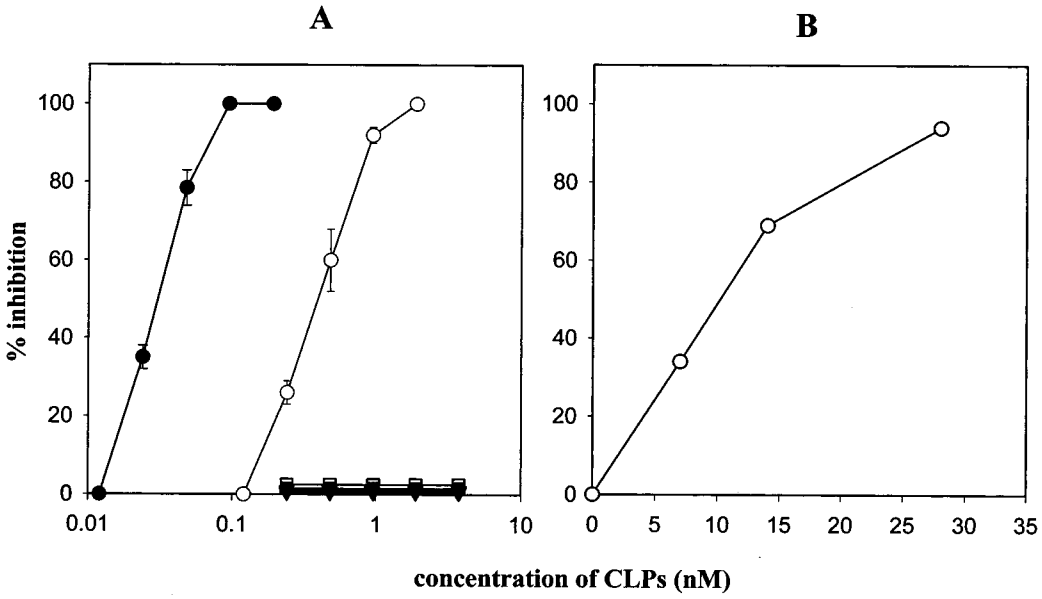

FIGURE 3: Effect of CLPs on adhesion of cells expressing α2β1 integrin to collagen I and collagen IV. Panel A: Effect of EMS16 on adhesion of α2-K562 cells labeled with CMFDA to collagen I (open circles) and collagen IV (filled circles), echicetin to collagen I (open triangles) and collagen IV (filled triangles), and alboaggregin B to collagen I (open squares) and collagen IV (filled squears). The collagens (0.2 μg/well) were immobilized overnight in 0.02 M acetic acid at 4 °C on a 96-well plate. After blocking with HBSS buffer containing 5 mM $MgCl_2$ and 1% BSA, the CMFDA-labeled α2K562 cells were added to the wells in the presence or absence of snake venom proteins in the same buffer. Incubation was performed at 37 °C for 30 min, and unbound cells were washed away from the wells. Bound cells were lysed by addition of 0.5% Triton X-100, and the plate was read using a Cytofluor 2350 fluorescence plate reader. Error bars represent SD from three experiments. Panel B: Effect of EMS16 on adhesion of [^{3}H]oleic acid-labeled platelets to collagen I.

observed only with EMS16, $IC_{50} = 0.5$ nM. Moreover, the inhibitory effect of EMS16 was over 10-fold higher on α2-K562 cell adhesion to collagen IV. Neither alboaggregin B nor echicetin inhibited adhesion of α2-K562 cells adhesion to collagen I. On the other hand, EMS16 did not show any alboaggregin or echicetin-like activity. It did not induce GPIb-dependent platelet agglutination, and it did not inhibit platelet agglutination induced by vWF in the presence of ristocetin (data not shown). EMS16 also inhibited binding of collagen I to [^{3}H]oleic acid-labeled platelets in the liquid-phase assay. In this assay, the adhesion of platelets to collagen IV did not occur (data not shown). An IC_{50} of 12 nM was observed for this binding reaction mediated by platelet α2β1 integrin (Figure 3B). EMS16 did not show any inhibitory activity in the other adhesion assays, including adhesion of cell lines expressing the RGD-dependent inte-

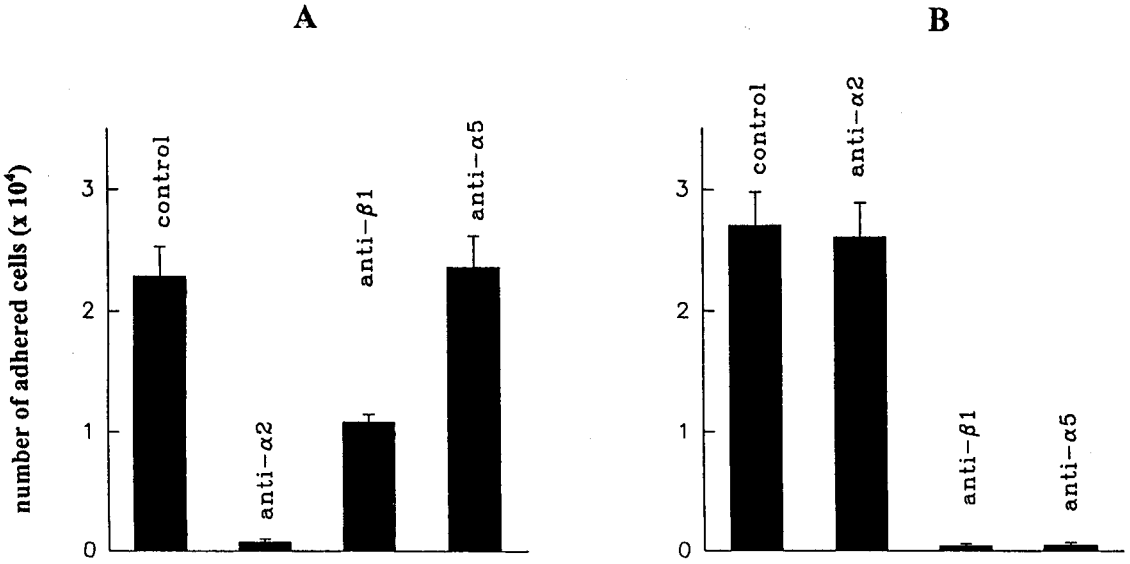

FIGURE 4: Effect of monoclonal antibodies on the adhesion of α2-K562 cells to immobilized EMS16 (A) and EMF10 (B). EMS16 and EMF10 (1 μg/well) were immobilized overnight at 4 °C on a 96-well plate in PBS. After blocking and washing, the CMFDA-labeled cells were added in the presence (10 μg/mL) or absence of monoclonal antibodies. The plate was incubated at 37 °C for 30 min in the HBSS bufer containing 1 mM Ca^{2+} and 1 mM Mg^{2+}. Unbound cells were washed away by the same buffer, and bound cells were lysed using 0.5% Triton X-100. The plate was read using a Cytofluor 2350 fluorescence plate reader. The numbers of adhered cells were calculated from the standard curve prepared from known numbers of cells on the same plate. The error bars represent the standard deviation from three independent experiments.

grins αIIbβ3, αvβ3, and α5β1 to their natural ligands fibrinogen, vitronectin, and fibronectin; α4β1 and α4β7 to VCAM-1 and MAdCAM-1, respectively; and α6β1 expressing cells to laminin (data not shown). It also was interesting that EMS16 had no effect on adhesion of cells expressing the collagen receptor α1β1 integrin to collagen IV (data not shown).

The competition studies with an anti-α2 monoclonal antibody suggest binding of EMS16 to the α subunit of α2β1 integrin, which showed complete inhibition of the adhesion of α2-K562 cells to immobilized EMS16 (Figure 4A). In the same assay, the anti-β1 monoclonal antibody only partially inhibited cell adhesion to EMS16. EMS16 did not interact with α5 integrin, which is abundant on the K562 cells. In contrast, the heterodimeric disintegrin EMF10 did not compete with anti-α2 monoclonal antibody. Both anti-α5 and anti-β1 monoclonal antibodies interfered with the adhesion of α2-K562 cells to immobilized EMF10 (Figure 4B). The experiment on adhesion of α2-K562 cells to EMS16 was performed in the presence of Ca^{2+}. In contrast to collagen I, calcium ions did not inhibit the interaction of EMS16 with α2β1 integrin. Moreover, the α2-K562 cells adhered more extensively to immobilized EMS16 in the presence of Ca^{2+} (data not shown).

Interaction of EMS16 with Purified, Recombinant I Domain of α2 and α1 Integrins. The effect of EMS16 on the binding of the I domain of α2 integrin to immobilized collagen I and the I domain of α1 integrin to immobilized collagen IV was tested in ELISA assays. Binding of the α2 I domain to collagen I was inhibited by EMS16 with an IC_{50} = 6 nM (Figure 5). An inhibitory effect of EMS16 was not observed in the case of binding the α1 I domain to collagen IV.

Effect of EMS16 on Platelet Aggregation and Ca^{2+} Mobilization. The selectivity of EMS16 for α2β1 integrin was also confirmed in a platelet aggregation assay (Figure 6). EMS16 inhibited only collagen I induced platelet aggregation and had no effect on the other agonists: ADP, thromboxane analogue (U46619), and TRAP. Interestingly, EMS16 showed no inhibition of convulxin-induced platelet aggregation. Convulxin, an agonist of GP VI on the platelet surface, and EMS16, an α2β1 inhibitor, belong to the same family of CLPs. However, different interaction with platelets was observed for both proteins. Platelet activation was confirmed by studies of platelet cytosolic Ca^{2+} mobilization

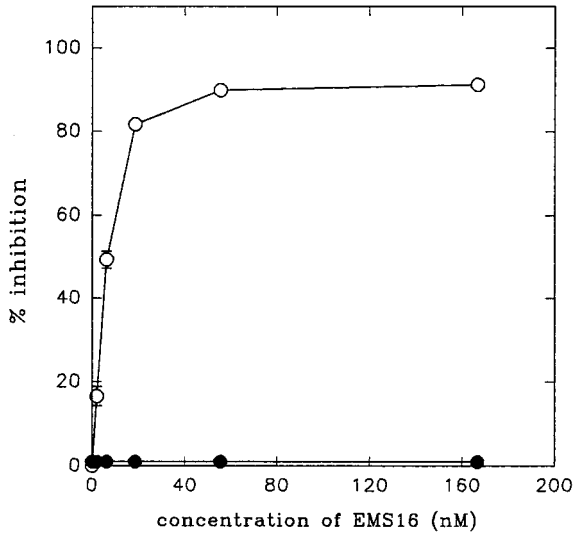

FIGURE 5: Effect of EMS16 on binding of isolated, recombinant I domains of α2 integrin (open circles) and α1 integrin (filled circles) to collagen I and collagen IV, respectively. The experiment was performed with the collagen I or collagen IV immobilized on a 96-well ELISA plate. Purified, recombinant α2−I domain or α1−I domain as GST fusion proteins were conjugated to AP. After blocking the plate, the AP−I domain−GST fusion protein was added in the presence or absence of EMS16 in TBS containing 1 mM $MnCl_2$. The plate was incubated at room temperature for 1 h. Bound I domain was detected colorimetrically using *p*-nitrophenyl phosphate as a substrate for AP.

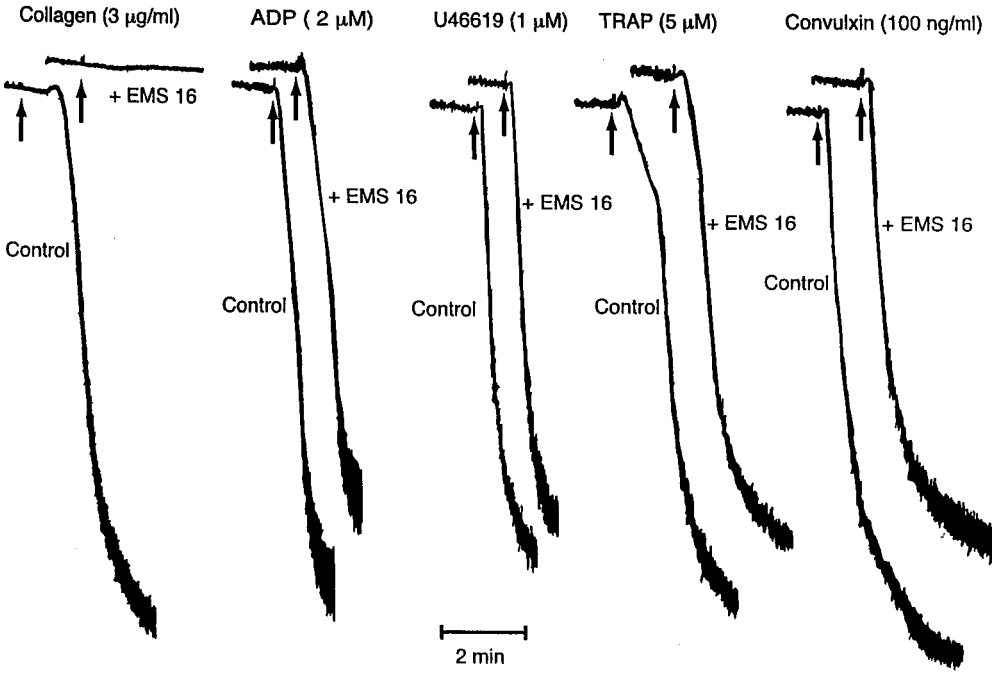

FIGURE 6: Effect of EMS16 on platelet aggregation induced by different agonists. EMS16 (6 nM) was added to washed platelets, followed by agonists: ADP (in the presence of fibrinogen), thromboxane A2 analogue U46619, thrombin receptor activating peptide (TRAP), and convulxin. The curves indicate increases in light transmission after addition of the agonist.

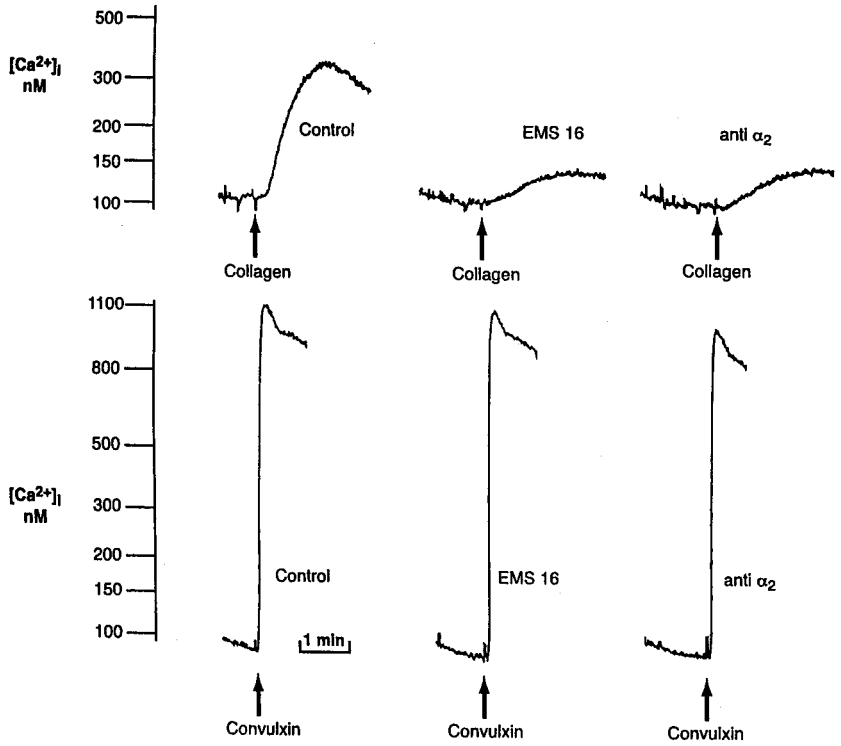

FIGURE 7: Effect of EMS16 (30 nM) and anti-α2 monoclonal antibody (2.5 μg/mL) on platelet cytosolic Ca^{2+} mobilization induced by collagen (50 μg/mL) and convulxin (100 ng/mL). Platelets were activated in a Perkin-Elmer LS-5 spectrofluorometer. Fura-2 fluorescence was monitored continuously using settings of 340 nm (excitation) and 510 nm (emission). F_{max} was determined by lysis of the cells with 40 μM digitonin in the presence of saturating $CaCl_2$. F_{min} was determined by the addition of 2 mM EDTA and 20 mM Tris base. Ca^{2+} concentrations were calculated by meaning F_{max} and F_{min} and using the K_d for fura-2 and Ca^{2+} which is 224 nM.

(Figure 7). The same pattern of inhibition was noticed for EMS16 and a monoclonal antibody against α2 integrin in collagen-induced platelet cytosolic Ca^{2+} mobilization. Neither EMS16 nor anti-α2 mab inhibited convulxin-induced platelet Ca^{2+} mobilization.

HUVEC Migration Assay. Previous studies by Senger (*7*) and others (*30*, *31*) implicate the α1β1 and α2β1 integrins in cell attachment, spreading, and migration, as well as angiogenesis in vivo. Therefore, we tested the effect of EMS16 for its ability to inhibit α2β1-dependent HUVEC migration on collagen I. As shown in Figure 8 (upper panel) and quantitated in Table 1, 200 nM EMS16 nearly totally abolished HUVEC migration after 24 h. In contrast, disintegrin eristostatin, which does not recognize any receptor

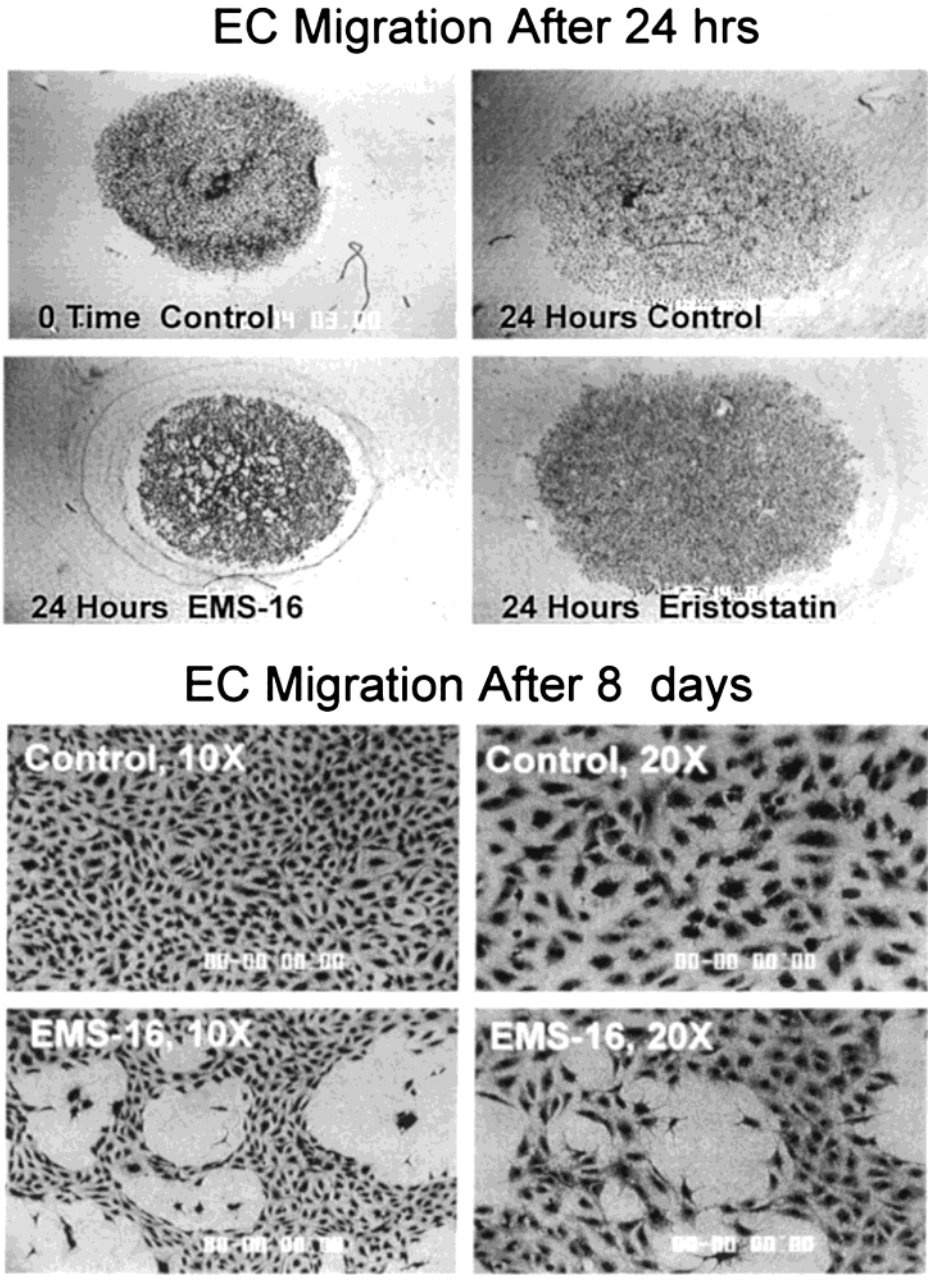

FIGURE 8: Effect of EMS16 on HUVEC migration on a polymerized collagen I substratum. HUVEC migration was monitored in the absence or presence of 200 nM EMS16 or 200 nM eristostatin on a matrix of collagen I under serum-free coditions after 24 h (upper panel) or 8 days (lower panel). Images were recorded and captured at magnification of 2× (upper panel), or 10× or 20× (lower panel).

Table 1: Area of Endothelial Cell Migration after 24 h on Polymerized Type I Collagen[a]

substratum	migration area (mm^2)	% inhibition
collagen alone	5.73 ± 0.18	0
collagen + 150 nM EMS16	2.54 ± 0.21	64
collagen + 200 nM EMS16	0.89 ± 0.18	85
collagen + 200 nM eristostatin	6.01 ± 0.09	0

[a] HUVEC migration was quantified as surface migrated in mm^2 after 24 h as described under Experimental Procedures, in the absence (collagen alone) or presence of EMS16 or eristostatin.

on HUVEC (*32*), had no effect on HUVEC migration at the concentration we tested. EMS16 had another effect on HUVEC adhesion observed after 24 h, causing previously spread cells to pull apart and form small holes in the monolayer. The anti-adhesive effects of EMS16 were observed even after 8 days. In contrast to control HUVEC which had grown to confluency, filling the entire 35 mm tissue culture dish, endothelial cells in the presence of EMS16 continued to pull off the collagen I surface, forming larger holes in the monolayer (Figure 8, lower panel). On the other hand, the growth and spreading of adhered cells was not affected by EMS16.

DISCUSSION

Here we have described a potent and selective inhibitor of the collagen receptor, an α2β1 integrin. EMS16 belongs to the CLP class of snake venom proteins. It is composed of two covalently linked subunits, which show the same cysteine alignment as subunits of other CLPs. Prior to this work, anti-integrin activity among snake venom proteins has been found to be characteristic for disintegrins. EMS16 is the first example of a different class of proteins, for which an

antagonistic effect on an integrin is well documented. The main difference between the structure of disintegrins and EMS16 reflects in the specificity of their interaction with integrins. Disintegrins are less selective and usually demonstrate interaction with several integrins. For instance, echistatin is a strong inhibitor of all three RGD-dependendent integrins, while eristostatin preferentially binds to αIIbβ3 fibrinogen receptor, weakly interacts with αvβ3, and has no affect on α5β1 integrin (*10*, *23*, *33*). The heterodimeric disintegrin EC3 is a potent inhibitor of α4 integrins; however, it also affects α5β1 and αIIbβ3 integrins (*11*). On the other hand, disintegrins such as echistatin, eristostatin, EMF10 (*5*), kistrin, flavoridin, bitistatin, and EC3 (unpublished data) do not have any inhibitory effect on the α2β1 integrin. EMS16 interacts only with the α2β1 integrin. The experiments with cells expressing other integrins, such as RGD-dependent integrins, leukocyte α4 integrins, α9β1, α6β1, and especially other collagen receptors (α1β1, α3β1), did not show any antagonistic properties of EMS16. The other difference between EMS16 and disintegrins is the nature of the interaction with integrin subunits. EMS16 appears to bind specifically to the I domain of the α subunit. On the other hand, disintegrin EMF10 interacts with both integrin subunits (Figure 4B), and a previous cross-linking study revealed interaction of monomeric disintegrins with the β3 subunit (*34*).

EMS16 has a high level of homology with N-terminal amino acid sequences of other CLPs. The proteins belonging to the snake venom CLP family usually appear as multimers (*19*). EMS16 is a heterodimer and is similar to alboaggregin B and echicetin, proteins binding to the vWF receptor associated with GPIb/IX. However, EMS16 does not show any activities characteristic of these proteins, and alboaggregin B and echicetin had no effect on the α2β1 integrin inhibition (Figures 2 and 3). Recently, Wang et al. (*35*) reported isolation of rhodocetin, a novel collagen-induced platelet aggregation inhibitor, belonging to CLPs. This protein is a noncovalently linked heterodimer, composed of two subunits. However, the mechanism of this inhibitory effect on platelet aggregation was not elucidated.

It was interesting that EMS16 had no effect on adhesion of cells expressing the closely related collagen receptor (the α1β1 integrin to collagen IV). Both integrins α2β1 and α1β1 have very structurally similar I domains, the part of the α subunit that mediates collagen binding. The structures of the I domains of these integrins were recently compared based on their crystal coordinates (*26*, *36*). In ELISA assay, EMS16 inhibited binding of the isolated, recombinant I domain of the α2 subunit to collagen I, but it did not inhibit interaction of the I domain of the α1 subunit with collagen IV (Figure 5).

The selectivity of EMS16 for α2β1 integrin was also confirmed in platelet aggregation assays. It inhibited only collagen I induced platelet aggregation, showing no effect with other agonists. Interestingly, EMS16 showed no inhibition of convulxin-induced platelet aggregation. Convulxin and EMS16 belong to the same family of CLPs. However, different interactions with platelets were observed for both proteins. Convulxin binds to the GPVI collagen receptor, causing platelet aggregation (*37*). EMS16 does not bind to GPVI, but it inhibits another collagen receptor, α2β1 integrin. This conclusion was confirmed by studies of platelet cytosolic Ca^{2+} mobilization. The same pattern of inhibition was noticed for EMS16 and monoclonal antibody against α2 integrin in collagen-induced platelet cytosolic Ca^{2+} mobilization. Neither EMS16 nor anti-α2 mab inhibited convulxin-induced platelet Ca^{2+} mobilization.

The endothelial cell α2β1 integrin has been shown to be of importance in angiogenesis and neovascularization (*7*, *30*, *31*). Taking the specificity of EMS16 for the α2β1 integrin in adhesion assays into consideration, the inhibition of HUVEC migration by EMS16 in our radial assay system is mostly due to EMS16 binding the α2β1 integrin on the endothelial cell surface, blocking α2β1 binding to collagen type I. Thus, further elucidation of EMS16 structure may be of use for the design of angiostatic drugs targeted to block angiogenesis in cancer and other pathological conditions. Since EMS16 did not exhibit an overall toxic effect on endothelial cell growth or morphology, its effects are apparently limited to the inhibition of endothelial cell adhesion and migration. Additionally, the presence of Ca^{2+} in our migration assay system indicates that EMS16 may also be active under physiological conditions, when Ca^{2+} is prevalent. Thus, EMS16 or its derivatives may be particularly good candidates as an inhibitor of angiogenesis in vivo.

In summary, we report a new protein, EMS16, which is a potent and selective inhibitor of collagen receptor, an α2β1 integrin. EMS16 belongs to CLP and is the first snake venom-derived protein with anti-integrin activity which does not belong to the disintegrin family. EMS16 may be also helpful for further investigation of the role of divalent cations in ligand binding to the integrin I domain. This functional region is referred to as MIDAS (*1*) and coordinates the metal ion in I domains of each I domain containing integrins. EMS16 is a unique ligand for α2β1 integrin, because it binds in vitro in the presence of Ca^{2+}. EMS16 may help to explain a possible role of calcium cations in the function of collagen receptor.

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