EC3, a Novel Heterodimeric Disintegrin from Echis carinatus Venom, Inhibits \( \alpha_4 \) and \( \alpha_5 \) Integrins in an RGD-independent Manner*

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EC3, a heterodimeric disintegrin \( (M_r = 14,762) \) isolated from Echis carinatus venom is a potent antagonist of \( \alpha_4 \) integrins. Two subunits called EC3A and EC3B were isolated from reduced and alkylated EC3 by reverse-phase high performance liquid chromatography. Each subunit contained 67 residues, including 10 cysteines, and displayed a high degree of homology to each other and to other disintegrins. EC3 inhibited adhesion of cells expressing \( \alpha_4\beta_1 \) and \( \alpha_4\beta_7 \) integrins to natural ligands vascular cell adhesion molecule 1 (VCAM-1) and mucosal addressin cell adhesion molecule 1 (MadCAM-1) with IC\(_{50} = 6–30 \) nM, adhesion of K562 cells (\( \alpha_5\beta_1 \)) to fibronectin with IC\(_{50} = 150 \) nM, and adhesion of \( \alpha_IIb\beta_3 \) Chinese hamster ovary cells to fibronogen with IC\(_{50} = 500 \) nM; it did not inhibit adhesion of \( \alpha_5\beta_3 \) Chinese hamster ovary cells to vitronectin. Ethylpyridylthylated EC3B inhibited adhesion of Jurkat cells to immobilized VCAM-1 (IC\(_{50} = 6 \) \mu M), whereas EC3A was inactive in this system. The MLDG motif appeared to be essential for activity of EC3B. Linear MLDG peptide inhibited the adhesion of Jurkat to VCAM-1 in a dose-dependent manner (IC\(_{50} = 4 \) \mu M), whereas RGDS peptide was not active at the same concentration. MLDG partially inhibited adhesion of K562 cells to fibronectin (5–10 \mu M) in contrast to RGDS peptide (IC\(_{50} = 3 \) \mu M), inhibiting completely at 10 \mu M.

Integrins are a family of cell surface proteins that mediate cell-cell interactions and the adhesion of cells to extracellular matrix proteins and other ligands. Integrins are heterodimeric structures composed of noncovalently bound \( \alpha \) and \( \beta \) subunits (1, 2). In humans there are at least 15 different \( \alpha \) subunits and 8 different \( \beta \) subunits, and they can combine to form proteins with diverse ligand specificities and biological activities. The integrins play important roles in many diverse biological processes including platelet aggregation, tissue repair, angiogenesis, bone destruction, tumor invasion, and inflammatory and immune reactions. Integrin \( \alpha_{IIb}\beta_3 \) (glycoprotein IIb/IIIa complex) binds fibrinogen on the platelet surface and mediates platelet aggregation. Integrin \( \alpha_5\beta_3 \) is predominantly expressed on endothelial cells and plays an important role in angiogenesis. It is also expressed on osteoclasts and participates in bone destruction. Integrin \( \alpha_5\beta_1 \) is widely distributed on a variety of cells; it plays a critical role in cell adhesion to extracellular matrix as well as in the formation of tissues and organs during embryonic development (3). All three integrins, \( \alpha_IIb\beta_3 \), \( \alpha_5\beta_3 \), and \( \alpha_5\beta_1 \), recognize RGD sequence in the adhesive ligands (1, 2).

The \( \alpha_4 \) integrins \( \alpha_4\beta_1 \) and \( \alpha_4\beta_7 \) are widely expressed on leukocytes and lymphoid cells and play a major role in inflammation and autoimmune diseases (4). The \( \alpha_4\beta_1 \) integrin (also called VLA-4, very late antigen-4) mediates cell adhesion to vascular cell adhesion molecule 1 (VCAM-1),\(^1\) an adhesive molecule belonging to the immunoglobulin (Ig) superfamily that is expressed on endothelial cells at sites of inflammation. \( \alpha_4\beta_1 \) also binds to alternatively spliced variants of fibronectin that contain connecting segment 1 (CS-1). The \( \alpha_4\beta_7 \) integrin binds to mucosal addressin cell adhesion molecule 1 (MadCAM-1) and to a lesser extent to VCAM-1 and CS-1. Interaction of these integrins with VCAM-1 or MadCAM-1 (which are up-regulated by cytokines) on endothelium mediates leukocyte infiltration, which can lead to tissue and organ destruction (4). Selectins and \( \beta_2 \) integrins (expressed on neutrophils and monocytes) also contribute to this process. Leukocyte engagement via \( \alpha_4 \) integrins is believed to play a significant role in the progression of many diseases including insulin-dependent diabetes, multiple sclerosis, rheumatoid arthritis, ulcerative colitis, arteriosclerosis, asthma, allergy, and re-stenosis of arteries after surgery and angioplasty (4, 5).

Over the last decade a number of investigators have sought naturally occurring or synthetic peptides that may selectively inhibit integrin-ligand interactions. Research on disintegrins, low molecular weight, cysteine-rich, RGD-containing peptides isolated from viper venoms was stimulated by this long term objective. The first disintegrin described in the literature, tripeptatin, was identified and characterized on the basis of its ability to block platelet aggregation and inhibit fibrinogen binding to \( \alpha_{IIb}\beta_3 \) (6). Subsequently a number of laboratories

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\(^1\) The abbreviations used are: VCAM-1, vascular cell adhesion molecule 1; RGD, Arg-Glu-Asp; CS-1, connecting segment 1; MLDG, Arg-Glu-Asp-Glu-Asp; CHO, Chinese hamster ovary cells; HPLC, high performance liquid chromatography; CHO, Chinese hamster ovary cells; CS-1, connecting segment 1.
have isolated several other RGD containing viper venom disintegrins of similar size, including kistrin (rhodostomin) (7), aplaggin (8), and floravadin (triflavin) (9, 10). Two short (49 amino acids) RGD disintegrins, echistatin (11) and eristostatin (12, 13), have been isolated from the venoms of Echis carinatus and Eristochis macmahoni, respectively. A number of NMR studies on kistrin, echistatin, and floravadin showed that their RGD sequences are located in a mobile loop joining two strands of β sheet protruding from the protein core (reviewed in Ref. 14). The disulfide bonds around the RGD sequence in disintegrins maintain the hairpin loop conformation in each peptide, which is important for their potency and selectivity.

It is known that disintegrin-like and cysteine-rich domains occur in larger venom proteins containing a metalloproteinase domain and that the RGD sequence in these proteins is substituted with other amino acids (15). We considered the possibility that viper venoms may contain low molecular weight disintegrins with anti-adhesive properties mediated by epitopes other than RGD. We fractionated E. carinatus venom on HPLC reverse-phase column, and we tested each fraction for its ability to bind to Jurkat cells, which express αβ1 and αβ5 integrins but do not express β3 integrins. We isolated and characterized a new protein, referred to as EC3, that is selective and a highly potent inhibitor of α4 integrins and shows a low level of interaction with β3 integrins. EC3 is the member of a new protein family called heterodimeric disintegrins, which is first reported in this paper.

EXPERIMENTAL PROCEDURES

MATERIALS—Monoclonal antibodies (mAb) HP2/1 (anti-α4 subunit of VLA-4) and SAM-1 (anti-α5 subunit of VLA-5) were purchased from Immunotech, Inc. (Westbrook, ME). HP2/4 (anti-α4 subunit of VLA-4) was kindly provided by Dr. M. Ginsberg (Scripps Research Institute). Biological effects of HP2/1 and HP2/4 were identical, only data with HP2/1 are shown. Highly purified human fibrinogen was a gift from Dr. A. Budzynski (Temple University, Philadelphia, PA). Recombinant human VCAM-1 (16) was a gift from Dr. M. Renz (Genentech, San Francisco, CA). Human vitronectin and fibronectin were purchased from Calbiochem and Sigma, respectively. GRGDSP and GRGESP peptides were purchased from Bachem (Torrance, CA). E. carinatus suchoreki venom ob-
at concentrations of 20–130 nM potently inhibited in a panel of integrin assays (Table I). As expected, echistatin and the RGD-containing disintegrin echistatin were compared and KRA was substituted in EC3A and EC3B with KRA

VGD let aggregation and IC 50

The hairpin loop sequence of echistatin, KRA

VGD surprisingly, neither EC3 subunit contained an RGD sequence.

The biological activities of EC3—

Biological Activities of EC3—

The biological activities of EC3 and the RGD-containing disintegrin echistatin were compared in a panel of integrin assays (Table I). As expected, echistatin at concentrations of 20–130 nM potently inhibited αIIbβ3-dependent platelet aggregation and αIIbβ3, αvβ5, and α5β1-dependent cell adhesion (Table I). In contrast, EC3 only weakly inhibited αIIbβ3-dependent interactions (IC 50 = 1 μM for platelet aggregation and IC 50 = 500 nM for A5 cell adhesion to fibronectin) and showed no inhibition of αvβ3-dependent adhesion up to 10 μM, although inhibition of α5β1-dependent adhesion was observed at an IC 50 of 150 μM (Table I). When the two disintegrins were evaluated in a panel of α4 integrin-mediated cell adhesion assays, the specificities were reversed. At concentrations of 25–100 nM, EC3 was a highly potent inhibitor of the interaction of both anchorage-dependent and -independent cells expressing α4β1 with either VCAM-1 or the CS-1 fragment of fibronectin, whereas echistatin showed no detectable activity at 10 μM (Table I). EC3 inhibited to the same extent adhesion of A2 (CHO α4+α5+) cells and α4B2 (CHO α4+α5−) cells to immobilized VCAM-1, confirming direct inhibition of binding to α4β1 integrin. To further extend the data on α4 integrins, the potency of EC3 in assays measuring VCAM-Ig binding directly to either α4β1 on Jurkat cells or α4β7 on JY cells was evaluated. EC3 potently inhibited α4β1 and α4β7 binding at concentrations of 28 nM and 6 nM, respectively. Moreover, adhesion of RPMI 8866 cells was inhibited by EC3 with IC 50 = 17 nM, whereas echistatin was not inhibitory. Cell adhesion assays and direct binding assays yielded similar results. Neither echistatin nor EC3 inhibited adhesion of α6β1-transfected cells to laminin and adhesion of α2β1 cells to collagen (Table I). We also studied biological function of both disintegrins in direct binding assay, confirming the specificity of EC3 for α4β1 and α4β7 integrin.

We also evaluated the biological activity of the EC3A and EC3B subunits after reduction and ethylpyridylethylation. Although residual activity of both subunits was significant, it was decreased by approximately 200-fold. It has been previously reported that reduction and ethylpyridylethylation of flaviridin and albolabrin decreased their ability to inhibit ADP-induced platelet aggregation by approximately 40-fold (25). epEC3B inhibited adhesion of Jurkat cells to immobilized VCAM-1 (IC 50 = 6 μM), whereas epEC3A was inactive in this system. However, epEC3A and epEC3B both inhibited adhesion of K562 cells to fibronectin (IC 50 = 30 μM and 6 μM, respectively) (Fig. 2). This experiment suggests that the specificity of EC3 for α4 integrins likely resides in the MLD sequence in the EC3B subunit, whereas the ability if EC3 to inhibit α5β1 likely resides in both subunits. Obviously, the MLDG sequence in EC3B is replacing the RGDβ motif in monomeric disintegrins. Both RGDβ and MLDG motifs appear to represent integrin binding sites. Fig. 3 shows that MLDG peptide inhibited adhesion of Jurkat cells to immobilized VCAM-1 in a dose-dependent manner approaching saturation at 5–10 mM. Adhesion of K562 cells to immobilized fibronectin showed a similar pattern of inhibition by RGDS. On the other hand RGDS did not cause any significant inhibition of Jurkat cell adhesion to immobilized VCAM-1. Inhibition of K562 to fibronectin by MLDG was only partial at 10 mM. It should be noted that the inhibitory effect on Jurkat cell adhesion to VCAM-1 was increased when longer MLDG-containing peptides were used. For instance CRAMLGDLDNYC caused 50% inhibition at 50 μM (not shown).

Further experiments showed that EC3 competes with mAb HP2/1 for binding to α4 integrin. HP2/1 at a concentration of 1 μg/sample blocked adhesion of Jurkat cells to immobilized EC3, whereas at the concentration of 1 μM, neither the hexapeptide GRGDS nor a control peptide GRGESP had any effect (Fig. 4A). Competition between EC3 and HP2/1 was also confirmed using fluorescence-activated cell sorter analysis. Fig.
EC3, a Heterodimeric Disintegrin from E. carinatus

The data represent the mean from three independent experiments. Fg, fibrinogen; Fn, fibronectin; Vn, vitronectin; Lm, laminin; Coll, collagen; ADP-PA, ADP-induced platelet aggregation; CA, cell adhesion; DBA, direct binding assay; ND, not determined. *, assays performed at Biogen; all other assays were performed at Temple University.

| Cell suspension | Integrin | Ligand | Assay | IC50 (nM) |
|-----------------|----------|--------|-------|-----------|
| Platelets       | αIβ3     | Fg     | ADP PA| 130       |
| A5 (CHO αIβ3+)  | αIβ3     | Fg     | CA    | 50        |
| VNRC3 (CHO αIβ3+) | αIβ3     | Vn     | CA    | 50        |
| K562            | α5β1     | Fn     | CA    | >104      |
| K562 (α6+)      | α6β1     | Lm     | CA    | >104      |
| K562 (α2+)      | α2β1     | Coll   | CA    | >104      |
| A2 (CHO α4+)    | α4β1     | VCAM-1 | CA    | >104      |
| Jurkat          | α4β1     | VCAM-1 | CA    | >104      |
| Jurkat*         | α4β1     | VCAM-1 | CA    | >104      |
| Jurkat*         | α4β1     | CS-1   | CA    | 100       |
| JY*             | α4β7     | VCAM-1 | DBA   | ND        |
| RPMI8866        | α4β7     | MadCAM| CA    | >104      |

**Fig. 2.** Effect of reduced and ethylpyridylethylated EC3A and EC3B on adhesion of Jurkat cells to immobilized VCAM-1 (A) and K562 cells to immobilized fibronectin (B). Recombinant VCAM-1 (0.5 mg/ml) or fibronectin (0.5 mg/ml) were immobilized overnight at 4 °C on a 96-well plate in phosphate-buffered saline buffer. After blocking, the 5-chloromethylfluorescein diacetate-labeled cells were added to each well in the presence or absence EC3 subunits. The adhesion was performed as described under “Experimental Procedures.” Open circles and closed circles indicate different concentrations of EC3A and EC3B, respectively. Error bars indicate S.D. from three independent experiments.

**Fig. 3.** Effect of MLDG and RGDS peptides on the adhesion of Jurkat cells to immobilized VCAM-1 (A) and on the K562 cells adhesion to immobilized fibronectin (B). The experiment was performed as described in the legend to Fig. 2. The inhibitory effects of MLDG peptide (open circles) and RGDS peptide (closed circles) are shown. Error bars indicate S.D. from three independent experiments.

**Fig. 4.** Competition of EC3 with RGD peptides and mAb HP2/1. A, effect of GRGDSP, GRGESP, and HP 2/1 mAb on the adhesion of Jurkat cells to immobilized EC3. An adhesion study was performed using 5-chloromethylfluorescein diacetate-labeled Jurkat cells in the absence or presence of 1 μM GRGDSP, 1 μM GRGESP, or 10 μg/ml HP 2/1. Error bars represent S.D. from three independent experiments. B, effect of EC3 on the binding of HP2/1 mAb to α5-deficient CHO cells transfected with α4 integrin. Cells were incubated with 10 μg/ml of HP 2/1 in the absence (□) or presence (●) of 60 μg/ml at 30 min. The samples were incubated for another 30 min at room temperature. The samples were fixed by the addition of 1% paraformaldehyde before measurement of fluorescence intensity by flow cytometry. The control binding of mouse IgG is shown in the unfilled trace.

**DISCUSSION**

The experimental data described in this paper identify a novel, heterodimeric disintegrin in the venom of *E. carinatus*. This disintegrin, named EC3, is a potent and relatively selective antagonist of α4 integrins, which inhibits their interaction with ligands in an RGD-independent manner. EC3 is composed of two covalently linked subunits A and B, which show a high degree of homology (including alignment of conserved cysteines) with other viper venom disintegrins. It is likely that the integrin binding sites of EC3 are located in two loops encompassing 13 amino acids (Cys-38 to Cys-50), corresponding to hairpin loops extending from Cys-20 to Cys-32 in echistatin and from Cys-45 to Cys-57 in kistrin and flavoridin. It is well known that the hairpin loops in disintegrins are maintained in appropriate conformation by S-S bridges (14, 15, 26), and the same appears to be true regarding EC3. The biological activity of this protein is decreased by 2 orders of magnitude after reduction and alkylation (Fig. 3). In contrast to all other viper venom disintegrins, EC3 contains neither RGD nor KG sequences. In fact, the RGD motif is substituted with VGD in EC3 (data not shown).
ever, only EC3B was active in the inhibition of α4β1/VCAM-1 interactions. This observation suggests, that MLG is the active sequence in EC3 mediating its anti-α4 effects. The experiment with synthetic peptides (Fig. 3) confirmed this expectation. MLDG linear peptide blocked adhesion of Jurkat cells to VCAM-1. In contrast, RGDS peptide, which is a very well known inhibitor of several β1 and β3 integrins (27), was not significantly active in this system. The MLDG peptide partially inhibits adhesion of α5β1-expressing cells to fibronectin (Fig. 3). This is consistent with the dual inhibitory effect of EC3 and of EC3B subunit containing MLDG (Fig. 2). The inhibitory effects of anti-α4 and anti-α5 inhibitory antibodies are in agreement with this suggestion.

EC3 is a new naturally occurring ligand for α4 integrins. The LD motif from its B subunit is also present in other ligands of α4 integrins. An ILDV sequence was found in alternatively spliced connective segment I of fibronectin (28, 29), and KLDAPT is present in the fibronectin type III repeat (30). The LDT sequence occurring in MadCAM (31) appears to be important for its ability to bind to α4β1. Recently Tselpits et al. (32) produced a number of mutants of recombinant kistrin and demonstrated that ILDV kistrin (kistrin in which PRGD sequence was substituted with ILDV) inhibited binding of the LDV-containing fibronectin fragment to immobilized α4β1, with an IC50 close to 0.1 μM. It is difficult to compare activities of EC3 with LDV-kistrin and synthetic peptides because preparations have been tested in different assay systems; however, in our hands LDV-kistrin was some 50-fold less active than EC3 in the direct binding assay. Until now, no MLDG motif has been identified and functionally characterized. Most investigators have achieved better inhibitory effects for tested α4 inhibitors in the presence of Mn2+ (data not shown).

Because EC3A, EC3B, kistrin, and florodrin show identical alignment of cysteines (Fig. 1) and because the pattern of S-S bonds in kistrin and florodrin is well established (26, 33–35), it is possible to deduce a hypothetical structure of EC3. Assuming that both subunits of EC3 may have the S-S pattern of kistrin/florodrin, we propose that Cys-7 and Cys-12 may be involved in two intermolecular bridges. The intramolecular disulfides are likely formed between Cys-6–Cys-29, Cys-20—Cys-26, Cys-25—Cys-50, and Cys-38—Cys-57. On the other hand, if one uses the S-S bonding pattern of albomarin (25), then Cys-6 and Cys-7 will form intermolecular bridges, and the intramolecular S-S bonds would correspond to Cys-7—Cys-12, Cys-26—Cys-20, Cys-25—Cys-50, and Cys-38—Cys-57. Clearly, further structural studies are needed to establish the S-S pattern of EC3.

EC3 inhibits quite selectively adhesion of α4β1-expressing cells to immobilized VCAM-1. Its effect on β1 and on αIIbβ3 appears to be lower by 1 and 2 orders of magnitude, respectively. The effect of EC3 on α4β1 does not appear to be related to the inhibition of α5β1 integrin, because this disintegrin inhibited to the same extent the adhesion to VCAM-1 of CHO cells transfected with α4 and of α5-deficient CHO cells transfected with α4. EC3 also inhibits adhesion of α4β7-expressing cells to MadCAM. Because mAb HPI2/1 competes with EC3 for binding to α4, it appears that EC3 may bind to the N-terminal domain of α4, where the epitope of this antibody also resides (18, 36, 37).

It should be noted that EC3 weakly inhibited ADP-induced platelet aggregation and the binding of CHO cells transfected with αIIbβ3 to fibrinogen, although it had no significant effect on αvβ3-mediated adhesion. This is in agreement with other observations that the RGD motif in disintegrins is not absolutely required for expression of platelet aggregation inhibitory activity. For instance, Jia et al. (38) expressed in insect cells the disintegrin/cysteine-rich domain of atrolysin A from Crotalus atrox and demonstrated that the recombinant protein inhibited collagen and ADP-induced platelet aggregation. This recombinant protein contained RSEC instead of the RGDF motif.

Trikha et al. (39) and Clark et al. (40) isolated a homodimeric, RGD-containing protein, contortrostatin, from the venom of Agkistrodon contortrix contortrix. The amino acid sequences of this protein, which appears to be a disintegrin, have not been reported. As determined by mass spectrometry, molecular mass of nonreduced contortrostatin is 13,505 Da, and the molecular mass of reduced and pyridylethylated contortrostatin is 8,000 Da. This bivalent protein is a potent inhibitor of platelet aggregation, and in contrast to monomeric disintegrins, it induces tyrosine phosphorylation of platelet proteins. In addition, contortrostatin is a potent inhibitor of β1 integrin-mediated melanoma cell adhesion in vitro and lung colonization in vivo. Most recently we isolated three other dimeric disintegrins, EMF10 from E. macmahoni and CC5 and CC8 from Cerasus cerasus venom, which all seem to be heterodimeric disintegrins with an molecular mass of 14–15 kDa. We are in the process of determining the amino acid sequences and function of these novel disintegrins. Further studies are required to establish how the bivalent structure of dimeric disintegrins affects the biological properties of the individual subunits.

In conclusion, we describe a novel dimeric disintegrin, EC3, that is a potent inhibitor of α4 integrin binding to VCAM-1 and moderately inhibits α5β1 integrins. We propose that the activity of EC3 is associated with the MLDG sequence in the putative hairpin loop of this disintegrin.

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