Inhibitory effects of MLDG-containing heterodimeric disintegrins reveal distinct structural requirements for interaction of the integrin α9β1 with VCAM-1, tenascin-C and osteopontin

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Summary - The integrin α9β1 is expressed on epithelial cells, smooth muscle cells, skeletal muscle and neutrophils and recognizes at least three distinct ligands: VCAM-1, tenascin-C and osteopontin. The α9 subunit is structurally similar to the integrin α4 subunit and α9β1 and α4β1 both recognize VCAM-1 as a ligand. We therefore examined whether the disintegrin EC3, which we have recently shown specifically inhibits the binding of α4 integrins to ligands, would also be a functional inhibitor of α9β1. EC3 and a novel heterodimeric disintegrin we identified, EC6, were both potent inhibitors of α9β1-mediated adhesion to VCAM-1 and of neutrophil migration across TNF -activated endothelial cells. A peptide containing a novel MLDG motif shared by both of these disintegrins also inhibited α9β1 and α4β1-mediated adhesion to VCAM-1. Surprisingly, though, concentrations of EC3 that completely inhibited adhesion of α9-transfected cells to VCAM-1 had little or no effect on adhesion to either of the other α9β1 ligands, osteopontin and tenascin-C. Furthermore, peptides AEIDGIEL and SVVYGLR, that we have previously shown inhibit binding of α9β1 expressing cells to tenascin-C and osteopontin, respectively, had no effect on adhesion to VCAM-1. These data suggest that there are structurally distinct requirements for interactions of the α9β1 integrin with VCAM-1 and the extracellular matrix ligands osteopontin and tenascin-C.

keywords: integrin, α9β1, disintegrins, VCAM-1, osteopontin, tenascin-C, EC3, EC6

running title: Distinct structural requirements for α9β1 ligand binding
Introduction

The integrin α9 subunit forms a single known heterodimer, α9β1, that is widely expressed in epithelia, smooth and skeletal muscle and on neutrophils (1,2). We and others have identified 3 distinct ligands for α9β1, the extracellular matrix proteins tenascin C (3) and osteopontin (4-6), and the inducible endothelial immunoglobulin family member, VCAM-1 (1). We have mapped the ligand binding site in tenasin-C to an exposed peptide loop in the third fibronectin type III repeat containing the sequence AEIDGIEL (7). We have also mapped the α9β1 ligand binding site in osteopontin (6). Although an initial report suggested that α9β1 might bind to an RGD-containing sequence in osteopontin (5), we were able to demonstrate by extensive mutagenesis that the binding site is within the linear peptide sequence SVVYGLR immediately adjacent to the RGD site (6).

Structurally, the α9 subunit is closely related to the α4 subunit, and on the basis of sequence homology α4 and α9 appear to be the only known members of a sub-family of integrin α subunits that lack both an insertional (I) domain and an extracellular disulfide-linked cleavage site (2). Furthermore, both subunits are expressed on leukocytes and mediate leukocyte migration (1,8). Finally, both integrins recognize VCAM-1 as a ligand (1,9). We have shown that both α4β1 and α9β1 contribute to the chemotactic migration of human neutrophils across endothelial cell monolayers activated by tumor necrosis factor-α (TNFα), an effect that is due, at least in part, to interaction of these integrins with VCAM-1 induced in response to TNFα (1).
Disintegrins are a family of low molecular weight, cysteine-rich, anti-adhesive proteins that are present in the venoms of various vipers and selectively block the function of integrins (10,11). Early studies focused on monomeric disintegrins that express an RGD motif within a 13 amino acid putative hairpin loop that is maintained in an appropriate conformation by a disulfide bridge. Not surprisingly, these disintegrins selectively inhibit integrins that bind to ligands through RGD sites, such as β3 integrins (for eristostatin, kistrin and bitistatin) or β3 integrins and the fibronectin receptor, α5β1 (for echistatin and flavoridin). Recently, however, we isolated a disintegrin from the venom of *Echis carinatus* called EC3 that potently and preferentially inhibited the interactions of α4 integrins with the immunoglobulin family members VCAM-1 and MadCAM (12). EC3 is composed of two subunits, A and B, in which the RGD motif is substituted by VGD and MLD sequences, respectively (13). Because of the sequence similarity between α9 and α4, and because both α4 integrins and α9β1 recognize VCAM-1 as a ligand, in this manuscript we examined the effects of EC3 on α9β1-mediated adhesion and neutrophil migration. We also purified a novel, related heterodimeric disintegrin, EC6, from *Echis carinatus* venom, demonstrated that, like EC3, it also has a substitution of MLD for the RGD sequence in one subunit, and examined the effects of this disintegrin on integrin-mediated adhesion and migration.
Methods

Reagents

Bovine serum albumin (BSA), formyl-methionylleucylphenylalanine (FMLP), and dextran were purchased from Sigma (St. Louis, MO). Recombinant human tumor necrosis factor (TNF-α), was obtained from R&D Systems (Minneapolis, MN). A recombinant form of the third fibronectin type III repeat of chicken tenascin-C (14) containing alanine substitution mutations within the RGD site (TNfn3RAA) (14), obtained from Anita Prieto and Kathryn Crossin (Scripps Research Institute, La Jolla, CA), and a recombinant form of the N-terminal fragment of the B splice variant of human osteopontin containing alanine substitution mutations within the RGD site (nOPNb RAA) (6) were prepared in E.Coli. A recombinant VCAM-1/IgG chimera was obtained from Ted Yednock (Elan Pharmaceuticals) and from Biogen, Inc. (15). Ficoll-hypaque plus for isolation of neutrophils from venous blood was purchased from Pharmacia Biotech (Uppsala, Sweden) and used according to the manufacturer’s specifications. Fibronectin was purchased from Calbiochem (San Diego, CA). Highly purified fibrinogen was a gift of Dr. A.Z. Budzyinski (Department of Biochemistry, Temple University). Peptides AEIDGIEL and SVVYGLR and their controls were synthesized using Fmoc (N-(9-fluorenyl) methoxycarbonyl)-chemistry on a peptide synthesizer (model 432A, Perkin Elmer, Foster City, CA) at the Center Laboratory for Research and Education, Osaka University, followed by purification with C18-reversed phase column chromatography. Peptide CKKAMMLDGLNDYC from EC6A and control peptide CKKAMAAGLNDYC were synthesized and purified by Sigma.

Antibodies, Cells and Cell Culture

Mouse monoclonal antibodies Y9A2 against human α9β1 (16) and AN100226M (100226) against α4 (17) were prepared as previously described. Mouse monoclonal antibodies SAM1
(against α5), Lia (against β1) and HP2/1 (against α4) were from Immunotech (Westbrook, MA). Mouse monoclonal antibody IB4, against the integrin β2 subunit, was prepared from a hybridoma obtained from American Type Tissue Collection (Rockville, MD). Mouse monoclonal antibody 15/7, which recognizes a ligand binding-dependent epitope on the integrin β1 subunit, was obtained from Ted Yednock (Elan Pharmaceuticals, South San Francisco, CA). Human umbilical vein endothelial (HUVE) cells were purchased from Clonetics (San Diego, CA) and grown in endothelial cell growth media (EGM) containing 2% fetal bovine serum (FBS), human recombinant epidermal growth factor (10 ng/ml), gentamycin (50 µg/ml), amphotericin B (50 ng/ml), bovine brain extract (12 µg/ml) and hydrocortisone (1 µg/ml) and were used between passage 4 and 8. α9- and mock-transfected SW480 cells and CHO cells were generated by transfection with the previously described full-length α9 expression plasmid pcDNAIneoα9 (3), or with the empty vector pcDNAneoI (Invitrogen, San Diego, CA.) by calcium phosphate precipitation. Transfected cells were maintained in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal calf serum and the neomycin analog G-418 (1 mg/ml) (Life Technologies, Inc.). Both cell lines continuously expressed high surface levels of α9β1 as determined by flow cytometry with Y9A2. CHO cells expressing αIIbβ3 were a gift from M. Ginsberg (Scripps Research Institute, La Jolla, CA), and all other cell lines were obtained from the American Type Tissue Collection.

**Flow cytometry**

Cultured cells were harvested by trypsinization and rinsed with Tris buffered saline containing 1 nM CaCl2 and 1 mM MgCl2 (TBS). Non-specific binding was blocked with normal goat serum at 4° C for 10 minutes. To evaluate the effects of disintegrins on expression of a ligand binding-dependent epitope on the integrin β1 subunit, mock- or α9-transfected
SW480 cells were incubated with or without various concentrations of disintegrins in DMEM for 15 min at 4° C, then incubated with primary antibodies, 15/7 at 15 µg/ml for 20 min at 4° C, followed by secondary antibodies conjugated with phycoerythrin (Chemicon, Temecula, CA). Between incubations cells were washed twice with TBS. The stained cells were re-suspended in 100 µl of TBS and fluorescence was quantified on 5,000 cells with a FACScan (Becton Dickinson, Rutherford, N.J.).

**Purification of disintegrins**

Monomeric and heterodimeric disintegrins were purified from lyophilized viper venoms provided by Latoxan (Valence, France), by one or two steps of HPLC. Echistatin, EC3 and EC6 were purified from *Echis carinatus suchoreki* venom. Lyophilized venom was dissolved in 0.1% trifluoroacetic acid (30 mg/ml). The solution was centrifuged for 5 min at 37°C to remove insoluble proteins, the pellet was discarded and the supernatant applied to a C-18 HPLC column (Vydac, Hesperia, CA). The column was eluted with an acetonitrile gradient (0-85%) over 45 min. Figure 1 shows that several fractions were eluted from the column under these conditions. Fraction 1 was identified as Echistatin, fraction 3 was called EC3 and fraction 6 was called EC6. To obtain higher purity, fractions were re-chromatographed over the same column with a more gradual gradient. A similar method was used to purify eristostatin and EMF-10 from *Eristochophis macmahoni* venom, kistrin from *Agkistrodon rhodostoma* venom and flavoridin from *Trimeresurus flavoriridis* venom. The purity and molecular mass of each disintegrin was evaluated by SDS-PAGE and mass spectrometry. Amino acid sequencing was carried out by Edman degradation following reduction and ethylpyridylation (13,18). Monomeric disintegrins, tested according to McLane et. al. (19), were potent inhibitors of platelet aggregation. IC50s for echistatin, eristostatin, flavoridan and kistrin were 136, 59, 51 and 40 nM, respectively. All of the heterodimeric disintegrins had IC50s above 1000nM.

**Cell Adhesion Assays**
Initial screening cell adhesion assays were performed at Temple using cells labeled with 5-chloromethyfluorescein diacetate, as described previously (20). Briefly, various ligands, disintegrins or antibodies were immobilized on 96 well microtiter plates (Falcon, Pittsburgh, PA) by overnight incubation in phosphate buffered saline at 4°C. The coating concentrations used were 2 µg/ml for VCAM-1, 10 µg/ml for fibronectin and 10 µg/ml for fibrinogen. Antibodies were coated at 10 µg/ml and disintegrins at 20 µg/ml. Wells were blocked with 1% bovine serum albumin (BSA) at 37°C. Cells were labeled by incubation with 12.5 µM 5-chloromethylfluorescein diacetate in Hanks balanced salt solution containing 1% BSA for 15 min. Unbound label was removed by washing in the same buffer. Labeled cells were added to the well in the presence and absence of inhibitors and incubated at 37°C for 30 min. Unbound cells were removed by washing and bound cells were lysed by the addition of 0.5% Triton X-100. A standard curve was prepared using known concentrations of labeled cells. Plates were read using a Cytofluor 2350 fluorescence plate reader (Millipore, Bedford, MA) with a 485 nm excitation filter and a 530 nm emission filter.

Subsequent adhesion assays were performed in San Francisco and Hiroshima using unlabelled cells. For these assays, wells of non-tissue culture-treated polystyrene 96-well flat-bottomed microtiter plates (Nunc Inc. Naperville, IL) were coated by incubation with 100 µl of VCAM-1/Ig (10 µg/ml), TNfn3RAA (1 µg/ml), or nOPNb RAA (1 µg/ml) for 1 hr at 37°C. After incubation, wells were washed with PBS, then blocked with 1% BSA in DMEM at 37°C for 30 minutes. Control wells were filled with 1% BSA in DMEM. SW480 or CHO cells were detached using trypsin/EDTA and re-suspended in serum-free DMEM. For blocking experiments, cells were incubated with or without 10 µg/ml of Y9A2 or various disintegrins, or peptides for 15 min at 4°C before plating. The plates were centrifuged (top-side up) at 10 x g for 5 minutes before incubation for 1 hr at 37°C in humidified 5% CO2. Non-adherent cells were removed by centrifugation top-side down at 48 x g for 5 minutes. Attached cells were fixed with 1% formaldehyde and stained with 0.5% crystal violet and the wells were washed with PBS. The relative number of cells in each well was evaluated after solubilization in 40 µl
of 2% Triton X-100 by measuring the absorbance at 595 nm in a microplate reader (Bio-Rad, San Francisco, CA). All determinations were carried out in triplicate.

**Neutrophil transmigration assays**

Neutrophils were purified from human peripheral venous blood containing 20 u/ml of heparin. Neutrophils were isolated by Ficoll-Hypaque density gradient centrifugation, followed by 3% dextran sedimentation (21). Erythrocytes were subjected to hypotonic lysis, remaining neutrophils were washed and re-suspended in PBS. The isolated neutrophils were more than 95% pure and more than 95% viable as assessed by Wright-Giemsa staining and trypan blue exclusion, respectively. Transendothelial neutrophil migration was assessed as described by Cooper and co-workers (22). HUVE cells were plated onto polycarbonate inserts (Transwell, Costar, Cambridge, MA, 6.5 mm diameter, pore size 8 µm for 24 well plate) in serum-containing EGM, and allowed to grow to confluence over 72 hours. 24 hours before assays, upper chambers were washed twice with serum free media and new medium with or without 3 ng/ml of TNF-α was added. Immediately prior to the addition of neutrophils the upper chambers were washed twice with serum-free DMEM and medium in the lower chamber was replaced with 500 µl of serum-free DMEM with or without 10 nM of FMLP. Purified neutrophils were incubated with no antibody, Y9A2 (10 µg/ml), IB4 (20 µg/ml), or various disintegrins for 15 minutes at 4°C, and 2 x 10⁵ cells in 200 µl of media were added to each upper chamber. After 3 hours at 37°C in 5% CO₂, non-adherent cells in the upper chamber were removed. Medium including migrated neutrophils from the lower chamber was collected, the lower chamber was rinsed several times to collect all of the neutrophils that had transmigrated, and the absence of additional adherent neutrophils was confirmed microscopically. The medium and all washes were pooled and resuspended, and cells were counted with a hemocytometer. All determinations were carried out in duplicate and repeated at least twice. Results were analyzed for statistical significance with an analysis of variance followed by the Fisher exact test.
Results

Isolation and peptide sequencing of the novel heterodimeric disintegrin EC6

We have previously described a heterodimeric disintegrin, EC3, that specifically inhibited α4 integrins, with much less effect on β3-integrins or the integrin α5β1, which recognize RGD-containing ligands. In contrast to most of the previously described disintegrins, each chain of EC3 contained a novel sequence in the hairpin loop known to interact with integrins. In one loop the usual RGD (or KGD) sequence was replaced with VGD and in the other with the even more divergent sequence MLD. As part of an ongoing effort to improve the design of integrin inhibitors based on sequences in naturally occurring snake venom disintegrins, we identified and purified a novel heterodimeric disintegrin, EC6, from the venom of Echis carinatus. Purification was performed from crude venom as previously described (19) by reversed-phase high-pressure liquid chromatography using a Vydac C-18 column and an acetonitrile gradient. EC6 eluted as a single peak at an acetonitrile concentration of 60% (Figure 1). After reduction and ethylpyridilation, EC6 yielded two fractions that we called EC6A and EC6B (based on the elution pattern of reduced and alkylated subunits). By mass spectrometry we determined that the molecular mass of EC6 was \(14807 \pm 2\) Da, composed of subunits EC6A (8524 Da) and EC6B (8411 Da, each containing 10 ethylpyridylated cysteine residues. By SDS-PAGE, purified EC6 ran as a single band, and the apparent molecular mass was reduced by ~ 50% upon reduction, consistent with a disulfide linked dimer (Figure 2A). Peptide sequencing after CNBr degradation and proteolytic digestions allowed us to determine the complete amino acid sequence of each subunit, as shown in Figure 2B. Alignment of the sequences of EC6A and EC6B with the previously described heterodimeric disintegrins EC3 and EMF10 and with the monomeric disintegrins
echistatin, eristostatin, kistrin and flavoridin is shown in figure 3. It is apparent that there is considerable homology among all of these disintegrins, including alignment of all 10 conserved cysteine residues in each subunit of each heterodimeric disintegrin (Figure 3). However, like EC3B, EC6A contains an MLDG (instead of RGDX) motif in the putative hairpin loop region. In contrast to EC3, the other subunit of EC6 contains a typical RGD sequence.

Effects of EC6 on adhesion mediated by $\alpha 5\beta 1$ or $\alpha 4\beta 1$ integrins

To determine whether the MLDG motif was the critical disintegrin motif involved in inhibition of $\alpha 4\beta 1$-mediated adhesion to VCAM-1, we performed adhesion assays with Jurkat cells (that express $\alpha 4\beta 1$, but not $\alpha 4\beta 7$). We compared the ability of EC3 and EC6 (that each contain a subunit expressing the MLDG motif) to 5 other disintegrins that lack this motif. In parallel, we evaluated the effects of each of these disintegrins on adhesion of K562 cells to fibronectin (mediated through an RGD site by the integrin $\alpha 5\beta 1$) and of $\alpha IIb\beta 3$ transfected CHO cells to fibrinogen (an effect that has previously been shown to be inhibited specifically by the disintegrin eristostatin). As we have previously reported (12), EC3 potently inhibited the $\alpha 4\beta 1$-mediated adhesion, but was considerably less potent in inhibiting adhesion mediated by $\alpha IIb\beta 3$ or $\alpha 5\beta 1$ (Figure 4). With the exception of eristostatin, the RGD-containing disintegrins preferentially inhibited $\alpha 5\beta 1$-mediated adhesion, with no effect on adhesion mediated by $\alpha 4\beta 1$. However, EC6 inhibited adhesion mediated by $\alpha 4\beta 1$ as well as $\alpha 5\beta 1$. These results suggest that the MLDG motif, uniquely shared by EC3 and EC6, is critical for inhibition of $\alpha 4\beta 1$-mediated adhesion to VCAM-1. The potent inhibition of $\alpha 5\beta 1$-mediated adhesion by EC6 is most likely due to the presence of a typical RGD sequence in the EC6B subunit.

Interaction of EC3 and EC6 with the integrin $\alpha 9\beta 1$
Since \( \alpha_9 \beta_1 \) is the only integrin other than \( \alpha 4 \) integrins that has been reported to recognize VCAM-1 as a ligand, we next sought to determine whether \( \alpha 9 \beta_1 \) would bind to EC3 and/or EC6. As a first step, adhesion assays were performed examining the attachment of \( \alpha_9 \) or mock-transfected SW480 colon carcinoma cells to immobilized EC3, or EC6, or to immobilized antibodies to \( \alpha 9 \beta_1 \) or \( \alpha 5 \beta_1 \) integrins (Figure 5). We also examined attachment to immobilized antibody against the integrin \( \beta_1 \) subunit (as a positive control) and the integrin \( \beta_7 \) subunit (as a negative control). As expected, both cell lines attached to antibodies to \( \alpha 5 \beta_1 \), since this integrin is highly expressed on SW480 cells, but only the \( \alpha 9 \)-transfectants attached to antibody to \( \alpha 9 \beta_1 \). \( \alpha 9 \) transfected cells, but not mock-transfected cells attached to immobilized EC3, an effect that was completely blocked by antibody to \( \alpha 9 \beta_1 \) or antibody to \( \beta_1 \), but unaffected by antibody to \( \alpha 5 \) (data not shown). Both cell types attached to immobilized EC6, consistent with the potent effect of EC6 in inhibiting \( \alpha 5 \beta_1 \)-mediated cell adhesion, and both cell types attached to EMF10, another potent antagonist of \( \alpha 5 \beta_1 \).

Similar results were obtained when disintegrin binding was assessed by flow cytometry using the ligand-binding dependent epitope on the integrin \( \beta_1 \) subunit recognized by monoclonal antibody 15/7. Incubation with EC3 only induced 15/7 binding in \( \alpha 9 \)-transfected cells, whereas EC6 induced expression in both mock- and \( \alpha 9 \)-transfectants (Figure 6). These data are consistent with binding of EC3 preferentially to \( \alpha 9 \beta_1 \). Because SW480 cells also express \( \alpha 5 \beta_1 \), which is potently inhibited by EC6, we could not evaluate interaction of EC6 with \( \alpha 9 \beta_1 \) by these assays.

**Effects of disintegrins on adhesion of \( \alpha 9 \)-transfected SW480 cells to VCAM-1**

We initially screened a range of concentrations of monomeric and dimeric disintegrins for their ability to inhibit the adhesion of \( \alpha 9 \)-transfected SW480 cells to immobilized VCAM-1.
Only EC6 and EC3 potently inhibited adhesion, with IC50 values of approximately 30 nM (Figure 7). To confirm that these effects were not cell-type specific, and to confirm that the inhibitory effect observed was due to inhibition of α9β1-mediated adhesion, we also examined the effects of EC3, EC6 and the control disintegrin, echistatin, on adhesion of mock- or α9-transfected CHO cells to VCAM-1. Both EC3 and EC6, but not echistatin, potently inhibited adhesion of α9-transfected CHO cells, whereas the minimal adhesion of mock-transfected cells was unaffected by any disintegrin (Figure 8).

An MLDG-containing peptide inhibits adhesion of α9- and α4-transfected CHO cells to VCAM-1

EC-3 and EC-6, the two disintegrins we have found to preferentially inhibit α9β1 and α4-integrins, share the novel sequence MLDG within the putative disintegrin loop. To determine whether this sequence is indeed critical, we examined the effects of a range of concentrations of the synthetic peptide, CKKAMLDGLNDYC, corresponding to this loop region, on adhesion of mock-transfected CHO cells, α9-transfectants and α4 transfectants to either VCAM-1 or the irrelevant integrin ligand, fibronectin. We also examined the effects of a mutant form of this peptide in which the MLDG sequence was changed to MAAG (Figure 9). The MLDG-containing peptide caused concentration-dependent inhibition of adhesion of α9- and α4-transfectants to VCAM-1, but had no effect on adhesion to fibronectin. In contrast, the MAAG mutant had no effect on adhesion, even at the highest concentration examined (2 mM). These data support the hypothesis that the sequence MLDG is critical for specific inhibition of α9β1 and α4-integrin mediated interactions with VCAM-1.

EC3 and EC6 inhibit chemotactic neutrophil migration across activated endothelial cells
We have previously shown that both α9β1 and α4β1 are required for optimal migration of human neutrophils across TNFα-activated human umbilical vein endothelial cells. We therefore sought to determine whether EC3 and EC6, which potently inhibit cell adhesion mediated by each of these integrins would also inhibit neutrophil transendothelial migration. In the absence of a chemotactic gradient, there was little neutrophil migration across TNFα-activated HUVE cells, and the low level of baseline migration was unaffected by disintegrins or by antibodies against α9β1 or β2 integrins, as we have reported previously (Figure 10). However, in the presence of a chemotactic gradient of the neutrophil chemoattractant, FMLP, migration was markedly increased, and this FMLP-induced transendothelial migration was potently inhibited by EC3, EC6 or anti-α9β1, but largely unaffected by the control disintegrin, echistatin. It is noteworthy that neither EC3 nor EC6 has any inhibitory effect on β2 integrins at concentrations up to 1 mM (C. Marcinkiewicz, unpublished observations). Numerous previous reports have identified an important role for β2 integrins in neutrophil attachment and migration. However, as we have reported previously (1), under the conditions utilized in these experiments migration is only partially inhibited by a blocking antibody against β2 integrins, allowing us to examine the important roles of α9β1 and α4 integrins in this process.

Specificity of EC3 and α9β1-recognition peptides from tenasin-C and osteopontin in α9β1-mediated adhesion to each ligand

Based on the adhesion data described above, EC3 appeared to be a highly potent and relatively specific inhibitor of α9β1 and α4 integrins. We therefore expected EC3 to also potently inhibit α9β1-mediated adhesion to the extracellular matrix ligands, tenasin-C and osteopontin. To examine this possibility, we performed adhesion assays with α9-transfected CHO cells on recombinant fragments of tenasin-C and osteopontin that we have engineered.
to be specific ligands for α9β1 (by mutating the RGD site in each fragment to RAA). However, cell adhesion assays with α9-transfected CHO cells demonstrated that concentrations of EC3 up to 100 nM had little effect on adhesion to either of these ligands (Figure 11B and C). We have previously mapped the binding sites of α9β1 in tenasin-C and osteopontin, to the linear peptide sequences AEIDGIEL and SVVYGLR, respectively (6,7). Each of these peptides inhibits, albeit with low potency, α9β1-mediated adhesion to the ligand from which it was derived. The differential effects of EC3 on adhesion to VCAM-1 and tenasin-C and osteopontin suggested that each ligand might bind to different sites on α9β1. If true, this would predict that peptides derived from each ligand would preferentially inhibit adhesion to that ligand. On the other hand, if each of the ligands competes for an identical binding site on α9β1, the order of potency of inhibitors should be similar for inhibition of adhesion to all 3 ligands. The results clearly demonstrate that EC3 preferentially inhibits adhesion to VCAM-1 and that the peptides AEIDGIEL and SVVYGLR preferentially inhibit adhesion to tenasin-C and to osteopontin, respectively, suggesting that each of these 3 ligands interacts with distinct binding sites on α9β1 (Figure 11).
Discussion

We have purified and sequenced a novel heterodimeric disintegrin from the venom of the viper *Echis caranatis* that we call EC6. This disintegrin, like the related disintegrin, EC3, contains a substitution of the sequence MLDG for the usual RGDX present in a putative hairpin loop of one of its two subunits. Like EC3, EC6 potently inhibits adhesion of α4β1-expressing cells to VCAM-1. Furthermore, we now describe that EC3 and EC6 are the only disintegrins examined that inhibit adhesion of the integrin α9β1 to VCAM-1. A peptide composed of the MLDG-containing disintegrin loop of EC6A, but not a mutant peptide containing the sequence MAAG, also inhibits α4β1 and α9β1-mediated adhesion to VCAM-1. EC3 and EC6 also potently inhibit chemotactic transendothelial migration of human neutrophils across TNF-activated endothelial cells, an effect that we have previously shown to be mediated by α4β1 and α9β1 (1). These results establish the sequence MLDG as a defining feature of specific inhibitors of α9β1 and α4-containing integrins, and identify EC3 and EC6 as by far the most potent inhibitors of either of these integrins described to date. In contrast to EC3, EC6 also potently inhibited adhesion mediated by the integrin α5β1. This effect is probably due to the RGD sequence present in the EC6B subunit and the high degree of homology of the disintegrin hairpin loop to other disintegrin inhibitors of α5β1.

Because EC3 specifically inhibited adhesion to each of the α4 integrin ligands examined, we expected that EC3 would also be a potent inhibitor of α9β1-mediated adhesion to each of its ligands. Indeed, our initial identification of VCAM-1 as a ligand for α9β1 was based on the sequence similarity between a critical tripeptide in the α4β1-binding site in VCAM-1 (IDS) and a critical tripeptide in the α9β1 binding site in tenascin-C (IDG). It is worth noting, however, that the precise α9β1 binding site in VCAM-1 has not been definitively mapped. Surprisingly,
EC3 was not found to be a potent inhibitor of α9β1-mediated adhesion to recombinant fragments from the ligand binding sites of either tenasin-C or osteopontin. Examination of the inhibitory effects of peptides derived from the binding sites in each of these ligands on α9β1-mediated adhesion to VCAM-1 made it clear that the order of potency of each of these 3 inhibitors is distinct, with EC3 most potently inhibiting adhesion to VCAM-1, the tenasin-derived peptide most potently inhibiting adhesion to tenasin and the osteopontin-derived peptide most potently inhibiting adhesion to osteopontin. These results are not compatible with a model by which all three ligands compete for an identical binding site on α9β1, but rather suggest that each ligand is interacting with somewhat different sites on the integrin. This conclusion contradicts the simplest model proposed for integrin-ligand interactions, in which a negatively charged amino acid residue in an integrin ligand contributes the final coordination site for a divalent cation within a single binding site created by a "Midas-motif"(23). However, in the absence of a definitive 3 dimensional structural data we cannot determine whether the interaction sites of α9β1 with each ligand are actually distinct or overlapping. The similarities in sequence between the MLDG peptide in EC3 and EC6 and the AEIDGIEL sequence in tenasin-C, as well as the partial inhibition by the AEIDGIEL peptide of α9β1-mediated adhesion to osteopontin (Figure 10) suggest the possibility that there is some overlap among these binding sites.

Although α4–integrins and α9β1 both bind to VCAM-1, and in both cases binding is potently inhibited by EC3 and EC6, there are also considerable differences in ligand binding specificity among these integrins. For example, α4 integrins do not bind to any site on TNfn3, and α9β1 does not bind to the CS-1 site in fibronectin (an α4β1 ligand) or to the α4β7 ligand MadCAM (unpublished observations). These latter findings are of interest, since EC3 and EC6 potently inhibit α4 integrin-mediated adhesion to both CS-1 and MadCAM. These findings are
consistent with numerous reports of dramatic differences among RGD-binding integrins to interact with RGD sequences in different ligands.

This report is not the first to suggest that a single integrin can use different sites to interact with different ligands. For example, Trapani-Lombardo, et. al. identified a monoclonal antibody, LJ-P5, that effectively blocked the binding of von Willebrand factor, but not fibrinogen, to the platelet integrin, αIIbβ3, and suggested that these two ligands interacted with distinct sites on the integrin (24). Furthermore, Rahman, et. al. showed that the disintegrin, elagantin, was considerably more effective in inhibiting the adhesion of αIIbβ3 to fibronectin than to fibrinogen, whereas a mutant version of the neurotoxin, dendrospin, was a more potent inhibitor of fibrinogen binding (25). Again the authors suggested that these findings implied that each ligand interacted with somewhat different sites on the same integrin. Perhaps the most convincing evidence in support of this idea comes from recent reports that Echo virus and collagen bind to two distinct sites on the isolated insertional domain from the integrin α2 subunit (26), a finding that has been confirmed by differential effects of substitutions within this domain on adhesion to each ligand (27).

Venomous vipers have evolved remarkable overlapping mechanisms to produce tissue injury and prevent appropriate repair in their victims. Viper venoms have now been shown to contain at least 4 classes of proteins involved in this process. These include: C-lectin like proteins, venom metalloproteinases (reprolysins), that may or may not include a disintegrin domain (28), and monomeric and dimeric disintegrins. Monomeric disintegrins potently inhibit β3-containing integrins, including the platelet integrin αIIbβ3 (10,11), and by interfering with platelet aggregation result in extensive tissue hemorrhage at the site of viper bites. C-lectin like proteins can also potentiate local hemorrhage by interfering with von Willebrand
factor binding to platelet glycoprotein GPIb on platelets and endothelial cells (29,30). In addition, these venom proteins can inhibit the collagen binding integrin, α2β1 (unpublished observations) which could further impair healing of the viper wound. Reprolysins further potentiate local injury by degrading components of the extracellular matrix and in some cases degrading the α2β1 integrin (31). α9β1 and α4 integrins play critical roles in the transendothelial and extravascular migration of neutrophils, lymphocytes, eosinophils and monocytes. Our identification of heterodimeric disintegrins in viper venom that potently inhibit these integrins suggests that vipers have also evolved mechanisms to impair leukocyte-mediated repair of wound sites, further increasing the likelihood that victims will be incapacitated or killed by viper bites. These highly potent, naturally occurring integrin inhibitors provide a basis for the development of more potent drugs that could be used to inhibit the excessive tissue emigration of leukocytes that characterize a number of common diseases.

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References

1. Taooka, Y., Chen, J., Yednock, T. and Sheppard, D. (1999) J Cell Biol 145, 413-420

2. Palmer, E. L., Ruegg, C., Ferrando, R., Pytela, R., and Sheppard, D. (1993) J. Cell Biol. 123(5), 1289-97

3. Yokosaki, Y., Palmer, E.L., Prieto, A.L., Crossin K.L., Bourdon, M.A., Pytela, R. and Sheppard D. (1994) J. Biol. Chem. 269, 26691-26696

4. Smith, L. L., Cheung, H. K., Ling, L. E., Chen, J., Sheppard, D., Pytela, R., and Giachelli, C. M. (1996) J. Biol. Chem. 271, 28485-91.

5. Smith, L. L., Giachelli., C.M. (1998) Exp. Cell Res. 242, 351-60.

6. Yokosaki, Y., Matsuura, N., Sasaki, T., Murakami, I., Schneider, H., Higashiyama, S., Saitoh, Y., Yamakido, M., Taooka, Y., and Sheppard, D. (1999) J. Biol. Chem. 274, 36328-36334

7. Yokosaki, Y., Matsuura, N., Higashiyama, S., Murakami, I., Obara, M., Yamakido, M., Shigeto, N., Chen, J., and Sheppard, D. (1998) J Biol Chem 273, 11423-11428

8. Shang, T., Yednoch, T., and Issekutz, A. C. (1999) J. Leukoc. Biol. 66, 809-816

9. Elices, M. J., Osborn, L., Takada, Y., Crouse, C., Luhowskyj, S., Hemler, M. E., and Lobb, R. R. (1990) Cell 60(4), 577-84

10. McLane, M. A., Marcinkiweicz, C., Vijay-Kumar, S., Wierzbicka-Patynowski, I., and Niewiarowski, S. (1998) Proc. Soc. Exp. Biol. Med, 210, 109-119
11. Niewiarowski, S., McLane, M. A., Kloczewiak, M., and Stewart, G. J. (1994) *Seminars in Hematology* **31**, 289-300

12. Brando, C., Marcinkiewicz, C., Goldman, B., McLane, M. A., and Niewiarowski, S. (2000) *Biochem. Biophys. Res. Comm.* **267**, 413-417

13. Marcinkiewicz, C., Calvete, J. J., Vijay-Kumar, S., Marcinkiewicz, M. M., Raida, M., Schick, P., Lobb, R. R., and Niewiarowski, S. (1999) *Biochem.* **38**, 13302-13309

14. Prieto, A. L., Edelman, G. M., and Crossin, K. L. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 10154-10158

15. Yednock, T., Cannon, C., Vandevert, C., Goldbach, E., Shaw, G., Ellis, D., Liaw, C., Fritz, L., and Tanner, L. (1995) *J. Biol. Chem.* **270**, 28740-50

16. Wang, A., Yokosaki, Y., Ferrand R., Balmas J., and Sheppard, D. (1996) *Am. J. Respir. Cell Mol. Biol.* **15**, 664-72.

17. Kent, S. J., Karlik, S. J., Cannon, C., Hines, D. K., Yednock, T. A., Fritz, L. C., and Horner, H. C. (1995) *J. Neuroimmunol.* **58**, 1-10

18. Huang, T.-F., Holt, J. C., Lukasiewicz, H., and Niewiarowski, S. (1997) *J. Biol. Chem.* **262**, 16157-16163

19. McLane, M. A., Kowalska, M. A., Silver, L., Shattil, S., and Niewiarowski, S. (1994) *Biochem. J.* **301**, 289-300
20. Marcinkiewicz, C., Vijay-Kumar, S., McLane, M. A., and Niewiarowski, S. (1997) *Blood* **90**, 1565-1575

21. Gresham, H. D., Clement, L. T., Lehmeyer, J. E., and Griffin, F. M., Jr. (1986) *J. Immunol* **137**, 868-875

22. Cooper, D., Lindberg, F. P., Gamble, J. R., Brown, E. J., and Vadas, M. A. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 3978-3982

23. Lee, J. O., Rieu, P., Arnaout, M. A., and Liddington, R. (1995) *Cell* **80**, 631-638

24. Tripani-Lombardo, V., Hodson, E., Roberts, J. R., Kunicki, T. J., Zimmerman, T. S., and Ruggeri, Z. M. (1985) *J. Clin. Invest.* **76**, 1950-1958

25. Rahman, S., Lu, X., Kakkar, V. V., and Authi, K. S. (1995) *Biochem. J.* **312**, 223-232

26. King, S. L., Kamata, T., Cunningham, J. A., Emsley, J., Liddington, R. C., Takada, Y., and Bergelson, J. M. (1997) *J. Biol. Chem.* **272**, 28518-22

27. Dickeson, S. K., Mathis, N. L., Rahman, M., Bergelson, J. M., and Santoro, S. A. (1999) *J. Biol. Chem.* **274**, 32182-91

28. Bjarnason, J. B., and Fox, J. W. (1994) *Pharmacol. Ther.* **62**, 325-372

29. Tan, L., Kowalska, M. A., Romo, G. M., Lopez, J. A., Darzynkiewicz, Z., and Niewiarowski, S. (1999) *Blood* **93**, 2605-2616

30. Fujimura, Y., Kwasaki, T., and Titani, K. (1996) *Thromb. Haemost.* **76**, 633-639
31. Kamiguti, A. S., Markland, F. S., Zhou, Q., Laing, G. D., Theakston, R. D. G., and Zuzel, M. (1997) *J. Biol. Chem.* **272**, 32599-

32. Gould, R. J., Polokoff, M. A., Friedman, P. A., Huang, T. F., Holt, J. C., Cook, J. J., and Niewiarowski, S. (1990) *Proc. Soc. Exp. Biol. Med.* **195**, 168-171

33. Musial, J., Niewiarowski, S., Rucinski, B., Stewart, G. J., Williams, J. A., and Edmunds, L. H., Jr. (1990) *Circulation* **82**, 261-273

34. Marcinkiewicz, C., Calvete, J. J., Marcinkiewicz, M. M., Raida, M., Lobb, R. R., Vijay-Kumar, S., Huang, Z., and Niewiarowski, S. (1999) *J. Biol. Chem.* **274**, 12468-12473
**Figure Legends**

**Figure 1** - Absorbance at 206 nm of acetonitrile gradient eluant of venom from *Echis carinatus* loaded onto a C-18 HPLC column. The location of the peaks representing Echistatin, EC3 and EC6 are shown.

**Figure 2** – Panel A – Comassie stained non-reduced (lane 1) and reduced (lane 2) SDS-PAGE (20%) demonstrating purity of EC 6. The apparent decrease in molecular mass upon reduction is consistent with the prediction of a disulfide-linked dimer. Panel B - Peptide map of EC6. EC6 was subjected to reduction and ethylpyridylethylation and subunits A and B were separated by HPLC. N-terminal Edman degradation estimated 41 N-terminal peptides. Peptides CNBr 1 and 2 were isolated following EC6A cleavage at methionine 42. Peptides K7, K8, K10 and K14 were isolated following Lys C digestion. Peptides D9, D16a and D16b were isolated following endoproteinase N digestion.

**Figure 3** - Alignment of amino acid sequences of echistatin, eristostatin, kistrin (32), flavoridin (33), EMF-10 (13), EC3A and B (34) and EC6A and B. Conserved cysteine residues are enclosed in boxes.

**Figure 4**- Effects of various concentrations of monomeric and heterodimeric disintegrins on adhesion of Jurkat cells to immobilized VCAM-1 (panel A), K562 cells to immobilized fibronectin (panel B) and A5 CHO cells stabled transfected with αIIbβ3 to immobilized fibrinogen (panel C). Fluorescently-labeled cells (10⁵/well) were mixed with disintegrins, added to 96 well plates coated overnight with the relevant ligand, incubated at 37° C for 30 min and washed. Bound cells were lysed in 0.5% Triton X-100, fluorescence was measured,
and percent inhibition of adhesion was calculated in comparison to fluorescence of adherant cells in the absence of disintegrins. Data are the mean ± SEM of at least 3 experiments.

**Figure 5** – Adhesion of fluorescently-labeled α9-transfected (black bars) and mock-transfected (gray bars) SW480 cells to wells coated with immobilized antibodies against the integrin α9, α5, β1 or β7 subunits, or coated with the heterodimeric integrins EC3, EC6 or EMF10. Bound cells were lysed in 0.5% Triton X-100, fluorescence was measured, and the data expressed as the percentage of added cells that adhered.

**Figure 6** – Effects of disintegrins on expression of the ligand-induced epitope on the integrin β1 subunit recognized by antibody 15/7. Histograms obtained by flow cytometry of fluorescence intensity (plotted on the x axis) of mock- and α9-transfected SW480 cells incubated with EC3 (100 nM) or EC6 (100 nM). Shaded histograms represent 15/7 staining and unshaded histograms are unstained controls.

**Figure 7** - Effects of various concentrations of monomeric and dimeric disintegrins on the adhesion of α9-transfected SW480 cells to immobilized VCAM-1. Fluorescently-labeled cells (10^5/well) were mixed with disintegrins, added to 96 well plates coated overnight with VCAM-1 (2 μg/ml), incubated at 37°C for 30 min and washed. Bound cells were lysed in 0.5% Triton X-100, fluorescence was measured, and percent inhibition of adhesion was calculated in comparison to fluorescence of adherent cells in the absence of disintegrins.
Figure 8 – Effects of various concentrations of disintegrins on the adhesion of α9- and mock-transfected CHO cells to VCAM-1. (Panel A) α9-or mock-transfected CHO cells incubated with or without echistatin (30 nM), EC3 (30 nM) and EC6 (30 nM) were added to 96 well plates coated with VCAM-1/Ig (10 µg/ml). Cells were allowed to attach for 60 min, non-adherent cells were removed by centrifugation, and adherent cells were stained with crystal violet and quantified by measurement of absorbance at 595 nm. Data for a typical experiment are shown and are expressed as the mean (+S.D.) of triplicate measurements. (Panel B) α9-transfected CHO cells incubated with various kinds of disintegrins were added to 96 well plates coated VCAM-1/Ig (10 µg/ml). Percent inhibition of adhesion was calculated in comparison to absorbance of adherent cells in the absence of disintegrins. Percent inhibition of adhesion was calculated in comparison to absorbance of adherent cells in the absence of disintegrins. Data are expressed as the mean (+S.D.) of triplicate measurements.

Figure 9 - Effects of various concentrations of either wild type (MLDG) or mutant (MAAG) peptides derived from the disintegrin loop of EC6A on the adhesion of α9- (Panels A and B), α4- (Panels C and D) and mock-transfected CHO cells (Panels E and F) to VCAM-1 (Panels A, C and E) or fibronectin (Panels B, D and F). CHO cells incubated with or without (-) a range of concentrations of each peptide were added to 96 well plates coated with VCAM-1/Ig (10 µg/ml) or fibronectin (10 µg/ml). Cells were allowed to attach for 60 min, non-adherent cells were removed by centrifugation, and adherent cells were stained with crystal violet and quantified by measurement of absorbance at 595 nm. Data are expressed as the mean (+S.D.) of triplicate measurements from a single experiment.

Figure 10 - Effects of disintegrins on transmigration of neutrophils across activated-human endothelial cells monolayers. Purified human neutrophils that had been incubated with or without echistatin (30 or 300 nM), EC3 (30 or 300 nM), EC6 (30 or 300 nM), anti- α9β1 antibody
(Y9A2, 10 µg/ml) or anti-β2 antibody (IB4, 20 µg/ml) were added to upper chambers above confluent monolayers of HUVE cells that had been incubated with TNF-α (3 ng/ml) for 24 hrs. Serum free DMEM containing FMLP (10 nM) or serum free DMEM alone was added to the lower chamber. After 3 hr incubation at 37° C in 5% CO2, neutrophils that had migrated across the monolayer were collected from the lower chamber and counted. Data are expressed as the mean (+S.D.) of quadruplicate measurements from 2 separate experiments. * p<0.05, ** p<0.01

Figure 11 – Effects of disintegrins and synthetic peptides on the adhesion of α9- transfected CHO cells to VCAM-1, TNfn3RAA and nOPNb RAA. α9- transfected CHO cells incubated with or without disintegrins (30 or 100 nM) or synthetic peptides (1 mM) were added to 96 well plates coated with VCAM-1/Ig (10 µg/ml) (Panel A), TNfn3RAA (1 µg/ml) (Panel B) or nOPNb RAA (1 µg/ml) (Panel C). Cells were allowed to attach for 60 min, non-adherent cells were removed by centrifugation, and adherent cells were stained with crystal violet and quantified by measurement of absorbance at 595 nm. Data for one of three typical experiments are shown and are expressed as the mean (+S.D.) of triplicate measurements.
A

B

EP-EC6A

1 5 10 15 20 25 30 35 40 45 50 55 60 65 68

NSVHPCCDPVTEPREGESPRCCRNCKFLNAGTICKKAMLDDLNDYCTGISSDCPRNRYKGKEDD

|------------------------| N-terminal |-----------------| K10              |

CNBr-1                    | K14               |

CNBr-2

EP-EC6B

1 5 10 15 20 25 30 35 40 45 50 55 60 65 69

NSVHPCCDPVTCKPKRGHCASGPPCCENCYYIVGVGTYCNPARGDWNDNDCTGVSSDCPPNPWNGKPSDN

|------------------------| N-terminal |-----------------| K8              |

K7 | D16a | D16b | D9


EC3A  NSVHPCCDPVTCEPREGEHCCISGPCCRNCFLRAGTVCKRA  VGD DVDDYCSGITPDPNRYKGD
EC3B  NSVHPCCDPVTCEPREGEHCCISGPCCRNCFLNAGTCKRA  MLD GLNDYCTGKSSCPNPRNYKGD
EC6A  NSVHPCCDPVTCEPREGEHCCISGPCCRNCFLNAGTCKKA MLD GLNDYCTGISSCPNPRNYKGD
EC6B  NSVHPCCDPVTCKPKRGGCHASGPCCENCYIVGVGTCPNA RGD WNDNCTGVSSCDPNNPWNKPD
EMF10A MNSAPCCDPITCKPKGEHCISGPCCRNCFLNPGTCKGA RGD NLNDYCTGVSSCPNPWKSS
EMF10B ELLQNSGNCPPDPVTCKPRRGEHCISGPCCRNCFLNAGVCPNA MGD WNDYCTGISSCPNPRFP
Flavoridin GEECDGSPSNPCDAACTKLRPGAQCADGCLCCQCRFKKKGKRIA RGD FPDDRCTGSLNDGPRWN
Kistrin  GKECDGSPPNPCDAACTKLRPGAQCCGELCCQCKFSRAGKICRIIP RGD MPDDRCTGQSADGPRY
Eristostatin  QEEPCATGPPCPRRCKFKRAGKVORVA RGD WNDYCTGKSSCDPNNPH
Echistatin  ESESAPCPPCRCFLKEGTICRKA RGD DMDDYCNBGKCDPRNPCHKG
% inhibition

Concentration of disintegrins (nM)

A

B

C

10         100       1,000     10,000

1      10     100   1,000   10,000

100

80

20

0

60

40

100

80

20

0

60

40

Echistatin
EMF-10
Flavordin
Kistrin
Eristatin
EC3
EC6
SW480 $\alpha^9$

SW480 Mock
A

absorbance @ 595 nm

|        | CHO α9 | CHO Mock |
|--------|--------|----------|
| Echistatin |       |          |
| EC6     |       |          |
| EC3     |       |          |

B

% inhibition

Concentration of disintegrins (nM)

- Echistatin
- EC3
- EC6
Inhibitory effects of MLDG-containing heterodimeric disintegrins reveal distinct structural requirements for interaction of the integrin α9β1 with VCAM-1, tenascin-C and osteopontin

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