Modulation of RGD sequence motifs regulates disintegrin recognition of $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$ integrin complexes

Replacement of Elegantin alanine-50 with proline, N-terminal to the RGD sequence, diminishes recognition of the $\alpha_5\beta_1$ complex with restoration induced by Mn^{2+} cation

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Several recent studies have demonstrated that the amino acid residues flanking the RGD sequence of high-affinity ligands modulate their specificity of interaction with integrin complexes. The present study has addressed the role of the residues flanking the RGD sequence in regulating the recognition by disintegrin of the $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$ complexes by construction of a panel of recombinant molecules of Elegantin (the platelet aggregation inhibitor from the venom of *Trimerasurus elegans*) expressing specific RGD sequence motifs. Wild-type Elegantin (ARGDNP) and several variants including Eleg. AM (ARGDMP), Eleg. PM (PRGDMP) and Eleg. PN (PRGDNP) were expressed as glutathione S-transferase (GST) fusion proteins in *Escherichia coli*. The inhibitory efficacies of the panel of Elegantin variants were analysed in platelet adhesion assays with substrates immobilized with fibrinogen and fibronectin. Elegantin molecules containing an Ala residue N-terminal to the RGD sequence (wild-type Elegantin and Eleg. AM) showed strong inhibitory activity towards $\alpha_{IIb}\beta_3$ -dependent platelet adhesion on fibronectin, whereas a Pro residue in this position (Eleg. PM and Kistrin, the inhibitor from the venom of *Calloselasma rhodostoma*) engendered lower activity. The decreased activity could not be attributed to a decrease in the affinity of the disintegrin for the $\alpha_{IIb}\beta_3$ complex because both Eleg. AM and Eleg. PM had similar

K_d (app) values. In contrast, Elegantin molecules into which a Met residue was introduced in place of the Asn residue C-terminal to the RGD sequence showed 10–13-fold elevated inhibitory activity towards platelet adhesion on fibrinogen and this was maintained with either a Pro or Ala residue N-terminal to the RGD sequence. In experiments with the $\alpha_5\beta_1$ complex on K562 cells, the inhibitory efficacies of the panel of Elegantin molecules were analysed under two different cation conditions. First, in the presence of Ca^{2+}/Mg^{2+} , K562 cell adhesion on fibronectin was inhibited equally well by Elegantin and Eleg. AM but inhibited poorly by Eleg. PM and Kistrin. In contrast with platelets, the decreased inhibitory efficacy of the PRGDMP disintegrins was due to poor recognition of the $\alpha_5\beta_1$ complex. In the presence of Mn^{2+} cation, K562 cell adhesion on fibrinogen was observed in an $\alpha_5\beta_1$ -dependent manner. Under these conditions both PRGD and ARGD containing disintegrins were strong inhibitors of K562 cell adhesion on fibrinogen and this was due to a markedly improved recognition of the $\alpha_5\beta_1$ complex by the PRGD molecules. These observations demonstrate the pivotal role of the amino acids flanking the RGD sequence for disintegrin recognition of integrin complexes and highlight the subtle nature by which integrin-ligand binding specificity can be modulated by both cation and adhesive motif.

INTRODUCTION

Integrins are a family of adhesion molecules that mediate cell attachment to various extracellular matrix components [1,2]. They also regulate the formation of the matrix [3] and in turn signal and initiate a variety of cellular processes as a consequence of ligand engagement [4]. Interactions between integrins and their extracellular ligands is a complex phenomenon involving changes in the conformation of both the receptor and the ligand [5,6]. Despite this complexity, some basic principles underlying the recognition between integrins and their ligands have been elucidated by the identification of short peptide motifs within extracellular matrix proteins responsible for integrin-binding activity. Many physiological and non-physiological integrin ligands utilize RGD, LDV or a related sequence as a key structural component of their receptor-binding domain [7]. For

example, components of snake venoms isolated as potent inhibitors of platelet aggregation and integrin-dependent cell adhesion invariably contain an RGD or KGD sequence. These molecules include the disintegrin family [8], derived from viper venoms and a neurotoxin-related molecule Dendroaspin (Mambin) isolated from the venom of the Jamesons mamba (*Dendroaspis jamesonii*) [9–11]. The disintegrin structural scaffold seems to be highly suited for the presentation of integrin recognition sequences as a replacement for the RGD sequence in the inhibitor from the venom of *Calloselasma rhodostoma* (Kistrin) with LDV-engendered binding activity specifically for integrin $\alpha_4\beta_1$ [12]. Furthermore the LDV sequence and an $\alpha_5\beta_2$ recognition motif GPR have recently been located within a novel disintegrin-like domain present in the rotavirus capsid protein VP7 [13]. In addition to snake venom and viral disintegrins, a family of cell-surface molecules, termed ADAMs/MDC proteins, containing a

Abbreviations used: Dendroaspin, inhibitor from the venom of *Dendroaspis jamesonii*; Elegantin, platelet aggregation inhibitor from the venom of *Trimerasurus elegans*; Eleg. AM, ARGDMP; Eleg. PM, PRGDMP; Eleg. PN, PRGDNP; GST, glutathione S-transferase; Kistrin, inhibitor from the venom of *Calloselasma rhodostoma*; mAb, monoclonal antibody; PRP, platelet-rich plasma.

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disintegrin-like domain and a metalloprotease domain have recently been discovered in mammals (reviewed in [14]). The biological function of most of these proteins is largely unknown with the exception of fertilins α and β , which are involved in sperm-egg fusion, and the meltrins, which are involved in myoblast fusion.

Previous studies with disintegrins have established an important function for the amino acid residues immediately flanking the RGD sequence in regulating the specificity of the integrin-ligand interaction [15–17]. For example, Scarborough and co-workers observed that disintegrins with an RGDW motif display a higher avidity for the integrin $\alpha_{\text{IIb}}\beta_3$, whereas disintegrins with an RGDN sequence interact more strongly with $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins [15]. We reported that structurally unrelated venom proteins (Kistrin and Dendroaspin) harbouring identical RGD-flanking residues (PRGDMP) showed analogous inhibitory properties in platelet adhesion assays and could compete with one another in a simple linear competitive manner for binding to the $\alpha_{\text{IIb}}\beta_3$ complex [15,18]. In contrast, the platelet aggregation inhibitor from the venom of *Trimerasurus elegans* (Elegantin; ARGDNP), although structurally related to Kistrin, showed distinct inhibitory properties in platelet adhesion assays and was inhibited by Kistrin (and Dendroaspin) in a non-competitive manner. Definitive evidence for the regulatory function of the RGD-flanking residues was obtained by the introduction of both single and double amino acid substitutions around the RGD sequence in Dendroaspin to the complementary residues of Elegantin [19]. That is, a single Met⁴⁶ → Asn substitution (PRGDMP → PRGDNP) produced a novel and potent inhibitory activity in Dendroaspin directed against platelet adhesion on fibronectin. This engineered activity was correlated with the emergence of a second and distinct class of binding site on platelets, blocked specifically by $\alpha_{\text{IIb}}\beta_3$ -directed monoclonal reagents. Furthermore simultaneous Pro⁴² → Ala and Met⁴⁶ → Asn substitutions (PRGDMP → ARGDNP) enhanced the inhibitory activity towards platelet adhesion on fibronectin and increased the affinity for the second class of binding site on the $\alpha_{\text{IIb}}\beta_3$ complex proportionally.

In the present paper we report the generation and characterization of a panel of Elegantin molecules in which the RGD-flanking residues were replaced with the complementary amino acids of Kistrin/Dendroaspin. The molecules were characterized in assays with both $\alpha_{\text{IIb}}\beta_3$ and $\alpha_5\beta_1$ complexes. The results confirm and extend our previous observations with Dendroaspin, confirming that the RGD-flanking residues exert a regulatory effect on ligand binding within the context of a disintegrin scaffold in addition to the neurotoxin structure.

MATERIALS AND METHODS

Materials

Freeze-dried snake venoms were obtained from either Latoxan (Rosans, France) or Sigma. Fibrinogen (grade L) was obtained from Kabi (Stockholm, Sweden) and fibronectin was from Sigma. The purity and integrity of these glycoproteins were determined by SDS/PAGE. A GST-Kistrin clone was generously donated by Dr. Martin Humphries (University of Manchester, Manchester, U.K.). The $\alpha_{\text{IIb}}\beta_3$ -directed monoclonal antibodies (mAbs) 7E3 (β_3 -specific) and 10E5 were gifts from Dr. Barry Collier (Department of Medicine, Mount Sinai Medical Centre, Stony Brook, NY, U.S.A.). mAb PB1 (hamster $\alpha_5\beta_1$) was kindly donated by Dr. Rudy Juliano (University of North Carolina, Chapel Hill, NC, U.S.A.) and mAbs LM609 ($\alpha_v\beta_3$ -specific) and LM142 ($\alpha_v\beta_5$) by Dr. David Cheresch (Scripps Research Institute, La Jolla, CA, U.S.A.). mAbs JBS5, P4C2, and YFC118.3 were

obtained from Chemicon Int. (Harrow, Middx., U.K.). All other chemicals and reagents were obtained from Sigma and Milligen (Watford, Herts., U.K.). Radioisotopic Na¹²⁵I (15.3 mCi/ μ g of iodine) was purchased from Amersham (Little Chalfont, Herts., U.K.). Iodobead Reagent was obtained from Pierce Laboratories (Hemel Hempstead, Herts., U.K.).

Construction of synthetic Elegantin gene and variants

A synthetic Elegantin gene was constructed by ligating 12 complementary and overlapping oligonucleotides coding for the protein sequence of Elegantin [20] with the use of *Escherichia coli* codon data. *Bam*H1 and *Eco*R1 restriction sites were incorporated at the ends of the gene to allow directional cloning into the expression vector pGEX-3X (Pharmacia Biotech, Milton Keynes, Bucks., U.K.). An additional Met codon was inserted into the cDNA sequence N-terminal to Glu¹ to allow chemical cleavage of Elegantin from its GST partner by CNBr. Annealing and ligation conditions were as reported previously [19]. Variant Elegantin constructs were prepared by oligonucleotide-based site-directed mutagenesis with the Transform mutagenesis procedure (Clontech, Basingstoke, Herts., U.K.) in accordance with the manufacturer's instructions. In brief, mutagenic oligonucleotides were designed with the desired amino acid substitution present in the DNA sequence. A selection oligonucleotide was designed to introduce a novel restriction site (*Aat*II → *Sal*I) into the pGEX-3X vector to allow selection of recombinant from parental constructs by digestion with *Aat*II. After annealing, ligation and digestion, the reaction mixture was transformed into *E. coli* mut *S* cells (Clontech) and subsequent colonies were screened by *Sal*I restriction analysis. After two or three rounds of restriction with *Aat*II and transformation, more than 90 % recombinant clones were identified. Mutagenesis was confirmed by dideoxy-sequencing. Variant clones were then transformed into *Episcurian coli* for expression.

Protein purification and analysis

Purification of Elegantin and Kistrin from crude venoms was performed as described previously [16,18]. In brief, the snake venom proteins were purified from crude venoms by reverse-phase HPLC with a 0.1 % (v/v) trifluoroacetic acid/acetonitrile solvent system. Freeze-dried venom was reconstituted in 0.1 % (v/v) trifluoroacetic acid for 10 min on ice and chromatographed on a Vydac C₁₈ analytical column. Active fractions were identified by platelet aggregation assays and rechromatographed to homogeneity. Purified proteins were originally characterized by either N-terminal sequencing or MS.

GST-disintegrins were prepared from the lysates of *Episcurian coli* (DE3)pLysS (Stratagene, Cambridge, U.K.) by affinity chromatography on glutathione-Sepharose (Pharmacia) using a batch procedure. Purified GST-disintegrins were analysed by SDS/PAGE, which indicated that the preparations were more than 95 % homogeneous with minimal proteolytic degradation of the sample observed. GST-disintegrins were observed to form dimers in solution as confirmed by gel filtration. In brief, GST-disintegrins were chromatographed on a Sepharose 6B column (100 cm × 1.5 cm) equilibrated in Tyrode's buffer containing 1 mM MgSO₄ and 2 mM CaCl₂. The column was calibrated with alcohol dehydrogenase (150 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (14 kDa). The working concentrations of the GST-disintegrins were routinely determined by protein estimation with the bicinchoninic acid reagent (Pierce) with BSA as a standard. The molarities of the samples were calculated on the basis of their molecular masses and the observed dimerization of the GST fusion proteins.

Factor Xa cleavage of the GST–disintegrins was performed on resin by incubating the GST fusion protein-adsorbed resin in 50 mM Tris/HCl (pH 7.4)/1 mM CaCl_2 containing an appropriate amount of Factor Xa (enzyme-to-protein ratio of 1:200, w/w). However, in addition to cleavage at the junction between GST and disintegrin, cleavage also occurred within the disintegrin as detected by SDS/PAGE, rendering the protein inactive. Chemical cleavage of GST–Elegantin was performed with CNBr. In brief, CNBr dissolved in formic acid (approx. 10 mg/ml) was added to a freeze-dried GST–Elegantin sample (total CNBr-to-Met molar ratio 200:1) and left for 24 h at room temperature in the dark. The reaction was stopped by the addition of 10 vol. of water and the sample was dried by evaporation under vacuum. The dried sample was then solubilized in 0.1% (v/v) trifluoroacetic acid and chromatographed by reverse-phase HPLC as described above. The molecular mass of the purified Elegantin was confirmed by electrospray MS, yielding a mass ion of 7884, which approximates to the expected molecular mass of 7880 kDa.

Iodination and fluorescent labelling of proteins

Iodination of GST–disintegrins was performed with Iodobeads (Pierce) in accordance with the manufacturer's specifications. In brief, 100 μg (100 μl) of GST–disintegrin was added to a Microfuge tube containing 1 mCi of carrier-free Na^{125}I (Amersham) in 10 mM sodium phosphate, pH 7.3, and one Iodobead in a volume of 100 μl . The mixture was left for 15 min at room temperature. ^{125}I -labelled protein was separated from free iodine by gel filtration on PD10 columns (Pharmacia) and stored at -40°C . Radiolabelled fractions contained more than 90% trichloroacetic acid-precipitable isotopic activity and specific radioactivities ranging from 2×10^6 to 5×10^6 c.p.m./ μg were routinely obtained.

N-Hydroxysuccinimide ester conjugated with fluorescein (5 and 6-carboxyfluorescein, succinimidyl ester; Pierce and Warriner, Chester, Cheshire, U.K.) was used to conjugate GST–disintegrins (1 mg/ml) in 50 mM sodium bicarbonate buffer, pH 8.5. The conjugation reaction was performed at 4°C for 2 h. The fluorescein-conjugated protein was separated from unconjugated fluorescein by gel filtration on PD 10 columns (Pharmacia Biotech). Fluorescence-to-protein ratios were determined for each protein, all proteins were stored at -40°C .

Platelet functional studies

Washed platelets were prepared from platelet-rich plasma (PRP) obtained from healthy individuals who had not taken medication for 9 days before being bled. Adhesion of platelets to immobilized ligands was performed as described previously [16]. Platelet aggregation with PRP was measured with a four-chambered automated aggregometer (PAP-4D Aggregometer; 1 Biodata Corporation). Platelet aggregation was induced with ADP (final concn. 10 μM).

The binding of ^{125}I -labelled GST–disintegrins to washed platelets was performed as described previously [18]. In brief, the incubation mixture was composed of 300 μl of washed platelets (3×10^8 /ml), 10 μl of agonist (1.75 mM ADP) to give a final concn. of 50 μM , 10 μl of ^{125}I -labelled GST–disintegrin protein, 5–20 μl of competitor, inhibitory antibody or buffer and was made to a final volume of 350 μl . In antibody inhibition studies, platelet suspensions were treated with antibody for 30 min before exposure to ADP and the addition of ^{125}I -labelled venom protein; the mixture was incubated at room temperature for a further 60 min. The radioactivities in both pellets and supernatants were determined, indicating the levels of bound and free ligand. Non-

specific binding levels were determined in the presence of a 100-fold excess of unlabelled ligand or 10 mM EDTA. For assays with FITC-labelled disintegrins, washed platelets (3×10^7 /ml) were treated with or without an inhibitor for 30 min at room temperature followed by incubation with the appropriate disintegrin (1 μM) for a further 60 min (total volume 200 μl). Platelets were then fixed in 1% (w/v) paraformaldehyde, washed in Tyrode's buffer and analysed by FACS (Becton and Dickinson FACSscan).

Cell adhesion assays

K562 cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, Wiltshire, U.K.) and maintained in RPMI 1640 plus glutamax, supplemented with 10% (v/v) fetal bovine serum. Harvested cells were washed in PBS containing 1 mM EDTA and resuspended in Tyrode's buffer [10 mM Hepes (pH 7.35)/150 mM NaCl/5 mM KCl] containing either 1 mM MgSO_4 and 2 mM CaCl_2 (standard cation conditions) or an appropriate concentration of MnCl_2 (as indicated in the Results section). The cell adhesion assay was essentially analogous to the platelet adhesion assay previously described in detail [16]. In brief, cells were treated with or without inhibitor (disintegrins or monoclonal reagents) for 30 min before application to microtitre wells (100 μl of 5×10^5 cells/ml) precoated with either fibrinogen or fibronectin (10–20 $\mu\text{g}/\text{ml}$). After being washed, adherent cells were quantified by the measurement of endogenous acid phosphatase.

RESULTS AND DISCUSSION

Production of recombinant Elegantins

Our previous work with the neurotoxin-like platelet aggregation inhibitor Dendroaspin highlighted the pivotal role of the residues flanking the RGD sequence in modulating the specificity of ligand binding to the integrin $\alpha_{\text{IIb}}\beta_3$ on platelets [19]. In the present study, the influence on receptor recognition of the residues flanking the RGD sequence presented within the disintegrin structural scaffold was investigated. A panel of recombinant Elegantin molecules were synthesized with amino acid substitutions around the RGD sequence with the corresponding residues present in both Dendroaspin and the disintegrin Kistlin (Table 1). A synthetic wild-type Elegantin gene was assembled and the variant constructs were prepared by site-directed mutagenesis. Recombinant Elegantins were expressed as GST fusion proteins in *E. coli*. The synthetic Elegantin gene was designed with an additional Met codon positioned N-terminal to Glu¹ to allow the CNBr-mediated removal of the additional vector-derived residues remaining after enzymic cleavage of the GST fusion proteins with Factor Xa (pGEX-3X). Three Elegantin variants were prepared with substitutions around the RGD sequence: two single-residue variants Ala⁵⁰ $\rightarrow$ Pro (Eleg. PN) and Asn⁵⁴ $\rightarrow$ Met (Eleg. AM) and a double-residue variant Ala⁵⁰ $\rightarrow$ Pro plus Asn⁵⁴ $\rightarrow$ Met (Eleg. PM) (see Table 1). Attempts to remove the GST tag from purified preparations by enzymic cleavage with Factor Xa was accompanied by cleavage within Elegantin despite the absence of a consensus cleavage site (results not shown). CNBr cleavage of GST–Elegantin did release the disintegrin from its GST partner although the recovery was low. Analysis by MS of CNBr-released recombinant Elegantin confirmed a molecular species of average mass 7884, in close approximation to the predicted mass of 7880 kDa (results not shown).

Analysis of the recombinant GST–Elegantins as inhibitors of ADP-induced platelet aggregation in PRP indicated that their

Table 1 Amino acid sequences of Kistrin, Elegantin and recombinant Elegantin variants

Abbreviations are: Eg, Elegantin; Ks., Kistrin. Identical residues are represented by dashes. Nomenclature in parentheses is as used in the text.

1	5	10	15	20	25	30	35	40	45	50	55	60	65	70	
KGDGDCSSPENPCCDAATCKLRPGAQCGEGLCCEQCKFSRAGKICRI PRGDM PPDDRCTGQSADCPRYH															Ks
EA-EE---G-----AD---D--R-KKKRT---RA---N-----NGLYS															Eg
EA-EE---G-----AD---D--R-KKKRT---RA---M-----NGLYS															Eg M ⁵⁴ (AM)
EA-EE---G-----AD---D--R-KKKRT---RP---N-----NGLYS															Eg P ⁵⁰ (PN)
EA-EE---G-----AD---D--R-KKKRT---RP---M-----NGLYS															Eg P ⁵⁰ M ⁵⁴ (PM)

Figure 1 Inhibition of platelet aggregation (A) and adhesion (B–D) by GST-disintegrins

(A) PRP (300 μl) was incubated with various concentrations of GST-disintegrin at 37 $^{\circ}\text{C}$ for 2 min before the addition of ADP (10 μM). Aggregation was measured over 2 min (maximal aggregation) and the results are expressed as percentage inhibition relative to aggregation levels observed in the absence of inhibitors. IC_{50} values are shown in Table 2. The figure is representative of three or more experiments with points measured in duplicate (S.D. less than 10%). Symbols: $\square$, GST-Kistrin; $\blacklozenge$, GST-Elegantin; $\blacktriangle$, GST-Eleg. AM; $\circ$, GST-Eleg. PN. Symbols are the same in all figures. (B) Inhibition of platelet adhesion on immobilized fibrinogen (open bars) and fibronectin (filled bars) by mAbs directed against integrin complexes. Washed ADP-stimulated platelets were preincubated with or without the appropriate mAb (2–10 $\mu\text{g}/\text{ml}$) or GRGDS (1 mM) for 30 min before addition to microtitre wells for 1 h at room temperature. Adherent platelets were determined after washing and results are expressed as percentage adhesion compared with untreated platelets. (C, D) Inhibition of platelet adhesion on fibrinogen (C) and fibronectin (D) by GST-disintegrins. Washed platelet suspensions were preincubated with varying concentrations of GST-disintegrin for 15 min before addition to microtitre wells. Results are from two independent experiments with points performed in quadruplicate (S.D. approx. 10%). IC_{50} values are shown in Table 3. Symbols are as in (A).

potencies were comparable to venom-derived Elegantin with the exception of the GST-PN variant (Figure 1A and Table 2). GST-Elegantin, GST-Eleg. AM and GST-Eleg. PM were all observed to inhibit platelet aggregation fully within the nanomolar range characteristic of venom-derived disintegrins. In contrast, GST-Eleg. PN showed a decrease to approx. one fifth in inhibitory activity with an IC_{50} of 1.53 μM (Table 2). The decreased activity of the PN variant was observed in several

different preparations of recombinant material. The potencies of CNBr-released Elegantin (results not shown) and the panel GST-Elegantins in inhibiting platelet aggregation were similar; however, they were less potent than GST-Kistrin, similar to the relative potencies of the venom-derived parent molecules (Table 2). The results indicate that, with the exception of GST-Eleg. PN, the recombinant Elegantin molecules adopted the correct assignment of disulphide bridges and conformation

of the RGD loop when expressed as fusion proteins in *E. coli*; the bridges and loop are essential to the potency of disintegrins. The decrease in inhibitory activity produced by the single amino acid substitution Ala⁵⁰ → Pro might be due to improper folding of the molecule resulting from incorrect assignments of the disulphide bridges and/or an unfavourable conformation of the RGD loop harbouring the PRGDNP motif. The decreased activity of the PRGDNP motif presented within the Elegantin structural template was unexpected and was in contrast with our observations with the Dendroaspis molecule, in which the PRGDNP motif was highly functionally active [19].

Modulation of the specificity of integrin recognition

The functional consequences of the amino acid substitutions flanking the RGD sequence in the recombinant Elegantin molecules were investigated in further functional assays mediated by the $\alpha_{IIb}\beta_3$ complex on platelets and the $\alpha_5\beta_1$ complex expressed on K562 cells.

Preferential antagonism of platelet adhesion ($\alpha_{IIb}\beta_3$ complex)

In previous studies, analysis of the inhibition of platelet adhesion on substrates immobilized with fibrinogen and fibronectin has served to highlight the distinct functional properties of disintegrins expressing specific RGD sequence motifs [16]. The adhesion of ADP-treated washed platelets to immobilized fibrinogen and fibronectin is predominantly $\alpha_{IIb}\beta_3$ integrin-dependent, as demonstrated with several function-blocking mAbs directed towards different integrin complexes (Figure 1B) [16]. mAbs 7E3 and PM6-13 (β_3 subunit) fully blocked platelet adhesion on both fibrinogen and fibronectin. Little or no inhibition was observed when platelets were preincubated with mAb PB1, indicating minimal involvement of the $\alpha_5\beta_1$ complex. Platelet adhesion on both glycoprotein substrates was also inhibited by GRGDS peptide in a non-preferential manner. The inhibitory efficacy of the panel of recombinant disintegrins is shown in Table 3 with examples of the inhibitory profiles shown in Figures 1(C) and 1(D). In contrast with the platelet aggregation data, the inhibitory efficacies of the GST-disintegrins varied considerably with the immobilized substrate. In agreement with our previous work with venom-derived disintegrins [16], GST-Elegantin showed an approx. 6–9-fold greater inhibitory potency towards platelet adhesion on fibronectin than to fibrinogen, promoting more than 90% inhibition of platelet adhesion on either substrate. In contrast with Elegantin, GST-Kistrin showed the reverse antagonistic profile with an approx. 5-fold inhibitory preference towards adhesion on fibrinogen and promoting only a maximum of 80% inhibition of platelet adhesion on immobilized fibronectin. Recombinant GST-Eleg. AM and PM showed markedly elevated (10–13-fold) inhibitory activity towards platelet adhesion to fibrinogen, displaying IC₅₀ values similar to that of Kistrin. Interestingly, the GST-Eleg AM variant also showed an approx. 3-fold improved inhibitory activity towards platelet adhesion on fibronectin, whereas GST-Eleg. PM had marginally decreased activity compared with GST-Elegantin. Thus the single amino acid substitution of Asn⁵⁴ → Met in Elegantin to produce the ARGDMP motif, which to our knowledge is not found naturally, was sufficient to confer strong inhibitory activity against $\alpha_{IIb}\beta_3$ -fibrinogen interactions while retaining potent inhibitory activity against the $\alpha_{IIb}\beta_3$ -fibronectin interaction. However, the presence of the Pro residue N-terminal to the RGD sequence in GST-Eleg. PM maintained potent inhibitory activity towards $\alpha_{IIb}\beta_3$ -fibrinogen interactions but decreased the inhibitory activity towards $\alpha_{IIb}\beta_3$ -fibronectin

Table 2 Inhibition of ADP-induced platelet aggregation in PRP

Values are means ± S.D. for the number of determinations in parentheses.

Inhibitor	IC ₅₀ (μM)
Venom Elegantin	0.330 ± 0.030 (3)
GST-Elegantin	0.363 ± 0.087 (3)
GST-Eleg. AM	0.285 ± 0.041 (4)
GST-Eleg. PM	0.356 ± 0.10 (4)
GST-Eleg. PN	1.54 ± 0.16 (3)
GST-Kistrin	0.11 ± 0.01 (2)
Venom Kistrin	0.181 ± 0.028 (4)

Table 3 Inhibition of platelet adhesion

Results are means ± S.D.; n = 3–5. Abbreviations: Fg, fibrinogen; Fn, fibronectin.

Inhibitor	Substrate ...	IC ₅₀ (μM)	
		Fg	Fn
GST-Elegantin		4.7 ± 0.8	0.76 ± 0.11
GST-Eleg. AM		0.35 ± 0.04	0.26 ± 0.12
GST-Eleg. PM		0.4 ± 0.01	1.18 ± 0.32
GST-Eleg. PN		2.35 ± 0.3	1.79 ± 0.70
GST-Kistrin		0.46 ± 0.04	2.64 ± 0.4*

* Maximal inhibition was 80%.

binding. The inhibitory behaviour of the Eleg. PM variant therefore resembled that of Kistrin even though these disintegrin molecules have a sequence variation at position 49 N-terminal to the RGD sequence (see Table 1). GST-Eleg. PN showed an approx. 2-fold improved inhibitory activity towards platelet-fibrinogen interactions compared with GST-Elegantin but was considerably less potent at blocking platelet adhesion to fibronectin. The decreased potency and non-preferential antagonistic behaviour of GST-Eleg. PN (see Table 3) resembled the behaviour of GRGDS peptide and cyclized synthetic peptides derived from the RGD loops of disintegrins, reagents in which the RGD sequence is not thought to assume the precise conformation present within the parent molecule [16,21].

To investigate the biochemical basis for the alteration to the inhibitory preferences of GST-Elegantin by the amino acid substitutions flanking the RGD sequence, equilibrium binding assays were performed with washed platelet suspensions. Binding of ¹²⁵I-labelled GST-disintegrins to ADP-treated platelets was saturable and bivalent cation-dependent (Figure 2, insets). Scatchard analysis of the saturation data showed that GST-Elegantin recognized two classes of binding site on activated platelets (Figure 2A), analogous to venom-derived Elegantin [18], with K_d (app) values of 5.9 ± 3.5 and 324 ± 98.4 nM respectively. The lower-affinity GST-Elegantin-binding site was considerably more abundant than the high-affinity site with 66400 as apposed to 1000 sites per platelet (Table 4). GST-Eleg. AM and GST-Eleg. PM each bound to a single class of binding site on platelets with K_d (app) values of 55 ± 5.5 and 53.6 ± 5 nM respectively, indicating that the single Asn⁵⁴ → Met transition was sufficient to alter the specificity and mechanism of the binding of GST-Elegantin to platelets. The approx. 6-fold increase in the affinity of GST-Eleg. AM compared with the GST-Elegantin lower-affinity site was maintained by GST-Eleg. PM (Table 4), indicating that the Ala⁵⁰ → Pro substitution did

Figure 2 Saturation binding characteristics of GST-disintegrins to platelets

Various concentrations of ^{125}I -labelled GST-disintegrin were incubated with ADP-treated washed platelets ($3 \times 10^8/\text{ml}$) for 60 min at room temperature in a volume of $350 \mu\text{l}$. Bound and free levels of radioligand were determined by loading $300 \mu\text{l}$ of the platelet suspension on a cushion of 25% (w/v) sucrose, followed by centrifugation for 10 min at $12\,000 \text{ g}$. The radioactivity in both the platelet pellets and the supernatants was determined. Scatchard analysis was performed with RADLIG software (Biosoft, Cambridge, U.K.). (A) GST-Elegantin; Scatchard analysis was performed with data compiled from two independent experiments ($r^2 = 0.99$). (B) GST-Eleg. AM ($r^2 = 0.99$). (C) GST-Eleg. PM ($r^2 = 0.99$). (D) GST-Kistrin ($r^2 = 0.99$). Insets: saturation isotherms of ^{125}I -labelled GST-disintegrins showing total (○), specific (□) and non-specific (△) binding. The results are representative of two to four similar experiments, with all points performed in duplicate. (S.D. less than 10%.) Parameters are shown in Table 4.

Table 4 Equilibrium saturation binding parameters of GST-disintegrins to platelets

Abbreviations: H, high-affinity Elegantin-binding site; L, low-affinity Elegantin-binding site; n.d., not determined.

Disintegrin	K_d (app) (nM)	β_{max} (molecules per platelet)
Venom Elegantin	(H) 2.5 ± 3.84 (L) 275 ± 116	1000 ± 620 55000 ± 7600
GST-Elegantin	(H) 5.9 ± 3.5 (L) 324 ± 98.5	1000 ± 560 66600 ± 13340
GST-Eleg. AM	55 ± 5.5	52200 ± 3580
GST-Eleg. PM	53.6 ± 4.9	41160 ± 2800
GST-Eleg. PN	n.d.	
GST-Kistrin	34.4 ± 4.7	53700 ± 3880

not alter the binding affinity significantly. Therefore the effect of the Ala⁵⁰ → Pro substitution in Eleg. AM was to produce a marked decrease in the inhibitory efficacy towards platelet

adhesion on fibronectin without decreasing the overall binding affinity for the $\alpha_{\text{IIb}}\beta_3$ complex. This observation indicates that the specificity of binding of the Eleg. AM and PM variants to the $\alpha_{\text{IIb}}\beta_3$ complex is distinct despite their apparently similar binding profiles (Figure 2). Indeed, competition studies between Eleg. AM and Eleg. PM has revealed a non-linear competitive mode, indicating that these molecules bind to non-identical sites on the $\alpha_{\text{IIb}}\beta_3$ complex (S. Rahman, G. Flynn, A. Aitken and G. F. Savidge, unpublished work). With PGE₁-treated platelets, however, GST-Eleg. AM bound with higher affinity than GST-Eleg. PM (results not shown). GST-Kistrin also bound to a single class of site on ADP-treated platelets with a K_d (app) of $34.4 \pm 4.7 \text{ nM}$, which is similar to that of venom-derived Kistrin [18].

The receptor-binding specificity of the panel of GST-disintegrins to ADP-treated platelets was confirmed by antibody inhibition assays (Figure 3). mAb 7E3 inhibited the binding of all the GST-disintegrins by more than 90%, and mAb 10E5 inhibited binding to various degrees in a ligand-dependent manner. The variation in the inhibition of the GST-disintegrins

Figure 3 Inhibition of binding of GST–disintegrins to ADP-treated platelets

ADP-treated washed platelets ($3 \times 10^7/\text{ml}$) were incubated in the absence or presence of the inhibitory mAb (2–10 $\mu\text{g}/\text{ml}$), as shown in the key, for 60 min at room temperature before the addition of FITC-conjugated GST–disintegrin (1.0 μM). Platelets were incubated for a further 60 min then fixed, washed and analysed by FACS. The results are displayed as median fluorescence and are representative of two experiments giving similar results.

by mAb 10E5 is consistent with the notion that the GST–disintegrins bind distinctively to the $\alpha_{\text{IIb}}\beta_3$ complex and is most probably related to the nature of the 10E5 epitope. In contrast, function-blocking mAbs JBS5 ($\alpha_5\beta_1$ -dependent), LM609 and LM142 (α_v -dependent) were ineffective at blocking disintegrin binding to ADP-treated and resting platelets (results not shown). The results confirm that the GST–disintegrins bind predominantly to the $\alpha_{\text{IIb}}\beta_3$ complex and that their differing inhibitory properties in platelet adhesion experiments are accompanied and underpinned by their distinctive binding characteristics to the $\alpha_{\text{IIb}}\beta_3$ complex (Table 4).

Inhibition of K562 ($\alpha_5\beta_1$) adhesion to fibronectin

Platelet adhesion experiments demonstrated that the presence of an Ala residue N-terminal to the RGD sequence in Elegantin engendered potent inhibition of integrin $\alpha_{\text{IIb}}\beta_3$ –fibronectin interactions. We therefore sought to determine whether the presence of this Ala residue was favourable for inhibitory activity towards $\alpha_5\beta_1$ –fibronectin interactions. The inhibitory potency of the panel of recombinant disintegrins towards the adhesion of K562 cells on immobilized fibronectin was investigated under standard $\text{Ca}^{2+}/\text{Mg}^{2+}$ buffer conditions. Figure 4(A) illustrates that the adhesion of K562 cells to fibronectin was abrogated only by mAb PB1 (specific for the $\alpha_5\beta_1$ complex) and not by mAbs 7E3 or 10E5, indicating that K562 cell adhesion to fibronectin was $\alpha_5\beta_1$ -dependent under the assay conditions. GST–Elegantin and GST–Eleg. AM potently inhibited K562 cell adhesion with IC_{50} values of 5 and 10 nM respectively, promoting complete inhibition at 20 nM (Figure 4B and Table 5). In contrast, GST–Eleg. PM and PN variants were less potent inhibitors (approx. 1/10 to 1/20 as potent) and promoted only a maximal 50–60% inhibition. Similarly, GST–Kistrin produced a maximal inhibition of only 40% at approx. 0.4 μM (Table 5). The results of the adhesion experiments were corroborated by equilibrium binding analysis of the GST–disintegrins with K562 cells (Figures

4C and 4D). These experiments demonstrated that only GST–Elegantin and GST–Eleg. AM bound in a specific (bivalent cation-dependent) and saturable manner to K562 cells. Scatchard analysis revealed that both molecules bound to a single class of site with K_d (app) values of 4.4 ± 1.0 and 2.6 ± 0.8 nM respectively, closely approximating the observed IC_{50} values in adhesion assays. In comparison, the specific binding of GST–Eleg. PM and GST–Kistrin to K562 cells was so low that Scatchard analysis was not possible (results not shown). The binding of the GST–disintegrins to K562 cells was inhibited by more than 90% by the function-blocking mAb JBS5 ($\alpha_5\beta_1$ complex), indicating the predominant recognition of the $\alpha_5\beta_1$ complex under standard cation conditions (results not shown). These results indicate that the positioning of an Ala residue N-terminal to the RGD sequence in GST–Elegantin is highly optimal for interaction with the $\alpha_5\beta_1$ complex and that the positioning of a Met residue C-terminal to the RGD sequence was not detrimental to binding, analogously to the observations with the $\alpha_{\text{IIb}}\beta_3$ complex on platelets. However, in contrast with the $\alpha_{\text{IIb}}\beta_3$ complex, the presence of a Pro residue N-terminal to the RGD sequence in GST–Elegantin seemed to be unfavourable for binding to the $\alpha_5\beta_1$ complex on K562 cells. The results illustrate that under $\text{Ca}^{2+}/\text{Mg}^{2+}$ cation conditions the PRGDMP motif held within a disintegrin structure is unsuitable for interaction with the ligand-binding pocket of the $\alpha_5\beta_1$ complex on K562 cells.

Inhibition of K562 cell adhesion to fibrinogen

Platelet adhesion studies demonstrated that the GST–disintegrins showed inhibitory preferences for either fibrinogen or fibronectin substrates, depending on the amino acid residues flanking the RGD sequence. However, in contrast with the $\alpha_{\text{IIb}}\beta_3$ complex, the poor inhibitory efficacy of GST Eleg. PM and GST–Kistrin towards K562 cell adhesion on fibronectin was due to the reduced recognition of the $\alpha_5\beta_1$ integrin under the conditions of the assay and not to the specificity of binding to the receptor. To investigate further the differences between the functions of these two integrin complexes, the inhibitory efficacies of the GST–disintegrins for K562 cell adhesion were determined in the presence Mn^{2+} cations (Figure 5), which has recently been shown to support $\alpha_5\beta_1$ –fibrinogen interactions [22]. Indeed, in the presence of Mn^{2+} , K562 cell adhesion on both fibrinogen and fibronectin was maximally augmented by factors of approx. 10-fold and 3-fold respectively (Figure 5A). At a Mn^{2+} concn. of 500 μM , K562 cell adhesion on fibrinogen was predominantly $\alpha_5\beta_1$ -dependent, as only mAb JBS5 promoted high-level inhibition (Figure 5B). Little or no inhibition was observed with mAbs 10E5, 7E3, LM609 or LM142, suggesting no apparent involvement of β_3 or $\alpha_v\beta_5$ integrins as reported for the adhesion of human umbilical-vein endothelial cells on fibrinogen under similar conditions [22]. With these assay conditions, GST–Elegantin and GST–Eleg. AM inhibited K562 cell adhesion maximally with IC_{50} values of approx. 5 nM, indicating that the inhibitory efficacy of these molecules was not effected by the use of Mn^{2+} as a supporting cation or fibrinogen as a substrate (Figure 5C and Table 5). In contrast with experiments performed with $\text{Ca}^{2+}/\text{Mg}^{2+}$ buffer, the inhibitory efficacy of GST–Eleg. PM and GST–Kistrin was markedly elevated under Mn^{2+} conditions, both disintegrins effecting complete inhibition of K562 cell adhesion with IC_{50} values of approx. 10 nM. As expected, the increased inhibitory performance of the PRGD-containing disintegrins in the presence of Mn^{2+} was due to an elevated recognition of the $\alpha_5\beta_1$ complex by GST–Eleg. PM and GST–

Figure 4 Inhibition of K562 cell adhesion on fibronectin

(A) Inhibition by mAbs. K562 cells were suspended (10^6 /ml) in Tyrode's buffer supplemented with $\text{Ca}^{2+}/\text{Mg}^{2+}$ and incubated in the presence or absence of the inhibitory mAb ($2\text{--}5\text{ }\mu\text{g}/\text{ml}$) or 10 mM EDTA for 30 min before addition to microtitre wells coated with fibronectin. Cells were left to adhere for 60 min at room temperature and the number of adherent cells was determined by a colorimetric method observing linearity over the range of adherent cells (see the Materials and methods section). Results are expressed as percentage adhesion with points performed in quadruplicate (means $\pm$ S.D.). (B) Inhibition by disintegrins. K562 cells were preincubated with various concentrations of GST-disintegrin for 10 min at room temperature before addition to microtitre wells. The graph shown is representative of two experiments giving similar results with points performed in quadruplicate (S.D. approx. 10%). (C, D) Saturation binding analysis of GST-Elegantin and GST-Eleg. AM with K562 cells. Cells (10^6) were incubated with a single concentration of ^{125}I -labelled GST-disintegrin in the presence of increasing concentrations of unlabelled ligand for 60 min at room temperature. Samples were processed as described in the legend to Figure 2. Results were analysed and Scatchard analysis was performed with RADLIG software. Both GST-Elegantin and GST-Eleg. AM bound a single site on K562 cells with K_d (app) values of 4.4 nM ($58\,200 \pm 7000$ sites per cell) and 2.6 nM ($50\,700 \pm 6900$ sites per cell) respectively. Insets: saturation isotherms of ^{125}I -labelled GST-disintegrins showing total (○), specific (□) and non-specific (△) binding. Points were performed in duplicate (S.D. less than 10%).

Table 5 Inhibition of K562 cell adhesion

Results are means; $n = 2$. Abbreviation: n.d., Not determined.

Inhibitor	IC_{50} (μM)		
	$\text{Ca}^{2+}/\text{Mg}^{2+}$	Mn^{2+}	
	F _n	F _n *	Fg†
GST-Elegantin	0.005	0.016	0.004
GST-Eleg. AM	0.010	0.020	0.004
GST-Eleg. PM	0.145‡	0.025	0.010
GST-Eleg. PN	0.264‡	n.d.	n.d.
GST-Kistrin	0.4§	0.050	0.010

* Cell adhesion performed in the presence of 500 μM Mn^{2+} and mAb P4C2, or 50 μM Mn^{2+} alone.

† Cell adhesion performed in the presence of 500 μM Mn^{2+} .

‡ Maximal inhibition was 60%.

§ Maximal inhibition was 40%.

Kistrin (Figure 5D). The binding of both GST-Eleg. PM and GST-Kistrin to K562 cells was significantly augmented compared with standard cation conditions, and the integrin specificity was established by the ability of mAb JBS5 to decrease the binding of both disintegrins to K562 cells significantly compared with mAb 7E3, which had little effect (Figure 5D). Interestingly, approx. 50 % of the GST-Kistrin bound to K562 cells was not blocked by either of the mAbs JBS5 or 7E3, implying that recognition of an additional unidentified receptor had been induced by Mn^{2+} (see below). However, because mAb JBS5 blocked K562 cell adhesion by more than 90 % (Figure 5B), the influence of this additional receptor is unlikely to have a significant bearing on the inhibition of K562 cell adhesion on fibrinogen by GST-Kistrin.

The inhibitory profiles shown by the panel of disintegrins towards K562 cell attachment on both fibrinogen and fibronectin indicate that the antagonistic preferences observed in platelet adhesion experiments were not evident. For example, GST-Elegantin showed no inhibitory preference for $\alpha_5\beta_1$ -fibronectin interactions as opposed to fibrinogen (Table 5). To confirm this

Figure 5 Inhibition of K562 cell adhesion on fibrinogen

(A) Effect of Mn^{2+} on K562 cell adhesion on fibrinogen (filled bars) and fibronectin (open bars). K562 cells ($10^6/ml$) were resuspended in Tyrode's buffer supplemented with either Ca^{2+}/Mg^{2+} or various concentrations of Mn^{2+} as shown and then added ($100 \mu l$) to coated microtitre wells. Cells were allowed to attach for 1 h at room temperature and were quantified as detailed in the Materials and methods section. Results are expressed in cell numbers after the subtraction of background attachment levels in the presence of 10 mM EDTA. Points were performed in quadruplicate (bars indicate S.D.). (B) Specificity of K562 cell adhesion on fibrinogen. K562 cells were resuspended in 500 μM Mn^{2+} and incubated in the presence or absence of various inhibitory mAbs or EDTA for 30 min at room temperature before application to microtitre wells coated with fibrinogen. Cell attachment was performed and quantified as detailed above, with points performed in quadruplicate (bars indicate S.D.). (C) Inhibition of K562 cell adhesion on fibrinogen by GST-disintegrins. GST-disintegrins were incubated with K562 cell suspensions containing 500 μM Mn^{2+} for 10 min before addition to microtitre plate wells coated with fibrinogen. The results are representative of two experiments giving similar results. Symbols are as in Figure 1. Data points were performed in quadruplicate (S.D. approx. 10%). (D) Effect of Mn^{2+} on the binding of GST-Kistrin and GST-Eleg. PM to K562 cells. K562 cells were resuspended in Tyrode's buffer supplemented with Ca^{2+}/Mg^{2+} , 500 μM Mn^{2+} or 10 mM EDTA (background control). Cell suspensions were incubated with FITC-conjugated GST-Kistrin or GST-Eleg. PM for 1 h at room temperature, then washed and analysed by flow cytometry (filled bars). In further experiments the cell suspensions were incubated in the presence of either mAb 7E3 (striped bars) or JBS5 (open bars) for 30 min before the addition of the FITC-conjugated disintegrins. The results are expressed as median fluorescence of samples after the subtraction of background fluorescence.

conclusion, the inhibition of K562 cell adhesion on fibronectin by the panel of GST-disintegrins was determined in the presence of Mn^{2+} (Figure 6). As shown in Figure 5(A), K562 cell attachment on fibronectin was more than 2-fold higher in the presence of 500 μM Mn^{2+} than in standard Ca^{2+}/Mg^{2+} buffer. However, in contrast with a fibrinogen substrate, in the presence of 500 μM Mn^{2+} , K562 cell attachment on fibronectin was no longer predominantly $\alpha_5\beta_1$ -dependent, as mAb JBS5 produced only a maximum of 65% inhibition (Figure 6A). Significantly, mAb P4C2 (α_4 subunit) inhibited K562 cell adhesion by approx. 25% when used alone but completely ablated adhesion in combination with mAb JBS5, demonstrating that the additional receptor was an α_4 subunit-containing integrin complex. In contrast, YFC118.3 (β_2 -subunit) and mAb 7E3 (β_3 -subunit), although promoting low levels of inhibition when used alone, did not further inhibit K562 cell adhesion when used in combination with mAb JBS5. In addition, decreasing the Mn^{2+} concentration to 50 μM , which decreased overall adhesion levels (see Figure 5A), restored $\alpha_5\beta_1$ predominance to the assay system, as shown

by the ability of mAb JBS5 to block K562 cell adhesion by 90% (Figure 6B). To circumvent the adhesive component contributed by the α_4 subunit-containing complex, the inhibitory efficacy of the panel of recombinant disintegrins was assessed in buffer containing 500 μM Mn^{2+} supplemented with mAb P4C2 (Figure 6C) and in buffer with 50 μM Mn^{2+} (Figure 6D). Both assay conditions produced essentially similar inhibitory profiles consistent with the predominance of $\alpha_5\beta_1$ -fibronectin interaction on cell attachment. In these experiments, GST-Elegantin and GST-Eleg. AM inhibited K562 cell adhesion maximally but required higher doses than for the adhesion on fibronectin in Ca^{2+}/Mg^{2+} buffer, producing IC_{50} values of approx. 16 and 20 nM respectively (Table 5). The requirement for higher doses of GST-Elegantin and GST-Eleg. AM most probably reflects the increased avidity of the $\alpha_5\beta_1$ complex for fibronectin in the presence of Mn^{2+} (see Figure 5A), and confirms that no antagonistic preference for a fibronectin substrate was evident for GST-Elegantin. Therefore the presence of the Ala residue N-terminal to the RGD sequence did not confer an inhibitory

Figure 6 Inhibition of K562 cell adhesion on fibronectin

(A) Receptor specificity of K562 cell adhesion on fibronectin in 500 μ M Mn^{2+} determined by mAbs. K562 cells (10^6 /ml) were suspended in Tyrode's buffer supplemented with 500 μ M Mn^{2+} and incubated in the presence or absence of various inhibitory mAbs (2–5 μ g/ml) or 10 mM EDTA for 30 min before addition to microtitre wells coated with fibronectin. (B) Receptor specificity of K562 cell adhesion on fibronectin in 50 μ M Mn^{2+} determined by mAbs. Experimental conditions were as for (A) except for the lowering of the Mn^{2+} concentration to 50 μ M. See the text for the receptor specificities of the mAbs. Absorbance ('extinction') was measured at 405 nm. (C, D) Inhibition of K562 cell adhesion on fibronectin by the recombinant disintegrins. In (C) K562 cells were resuspended in buffer supplemented with 500 μ M Mn^{2+} and an appropriate concentration of mAb P4C2 before incubation in the presence or the absence of various concentrations of recombinant disintegrin. In (D) the buffer was supplemented with 50 μ M Mn^{2+} only. Cell adherence was determined and the results were calculated and expressed as percentage inhibition compared with untreated cells. The results shown are a single representative of two determinations.

preference for $\alpha_5\beta_1$ –fibronectin interactions supported by Mn^{2+} . Similarly, the presence of a Met residue C-terminal to the RGD sequence of GST–Elegantin did not confer an inhibitory preference for $\alpha_5\beta_1$ –fibrinogen binding, in contrast with the inhibition of $\alpha_{IIb}\beta_3$ –fibrinogen interactions.

The elevated inhibitory performance of GST–Eleg. PM and GST–Kistrin induced by Mn^{2+} was also evident with fibronectin as the immobilized substrate; both disintegrins promoted approx. 80 % inhibition of K562 cell adhesion, with IC_{50} values ranging from 25 to 50 nM depending on the assay conditions (Figures 6C and 6D, and Table 5). The elevated IC_{50} values obtained with fibronectin compared with those for fibrinogen are of a similar order to that observed for GST–Elegantin. These results therefore demonstrate that the use of Mn^{2+} as a supporting cation engendered the high-affinity recognition of both Eleg. PM and Kistrin by the $\alpha_5\beta_1$ complex. Although the precise mechanism of the induced recognition of the PRGDMP motif was not elucidated in the present study, considerable evidence exists for the Mn^{2+} -mediated augmentation of $\alpha_5\beta_1$ integrin–ligand binding in other studies [23,24]. The effect of Mn^{2+} is thought to occur as a consequence of the induction of conformational changes in the

extracellular domains of integrins. The recent crystal structures showing different conformations of the α_M I-domains obtained by crystallization from solutions supplemented with either Mg^{2+} or Mn^{2+} provides compelling evidence for this notion [25]. The conformational change in the extracellular domains of integrins induced by Mn^{2+} has been likened to the physiological activation (inside-out signalling) that classically discriminates macro-molecular from peptidomimetic ligands. However, the present results highlight the subtle nature of the different conformations of the ligand-binding pocket of the $\alpha_5\beta_1$ complex that discriminates between the Eleg. AM and Eleg. PM molecules, which are of similar overall sizes. The difference in the structure of the disintegrins induced by the presence of a Pro residue N-terminal to the RGD sequence is unknown but, in view of its close proximity, it could cause a change in the orientation of the RGD amino acid side chains.

In summary, the present work has examined the effect of modulating the amino acid residues flanking the RGD sequence on the specificity of disintegrin recognition and the inhibition of function of the $\alpha_{IIb}\beta_3$ and $\alpha_5\beta_1$ integrin complexes. The results demonstrate that the residue positioned both N-terminal and

C-terminal to the RGD sequence can have a major role in determining the specificity of integrin recognition. The results confirm several studies that have highlighted the importance of the residue C-terminal to the RGD sequence in regulating the integrin-binding specificity of disintegrins [15,16,26] and Dendroaspins [19]. In addition, the present results have also demonstrated the importance of the residue immediately N-terminal to the RGD sequence on the recognition by disintegrin of both the $\alpha_{\text{IIB}}\beta_3$ and $\alpha_5\beta_1$ integrin complexes. To our knowledge, all snake venom disintegrins reported so far accommodate either an Ala or a Pro residue at this position. The regulatory influence of RGD-flanking residues on ligand binding to integrins is not only restricted to snake venom-derived molecules but has also been reported for RGD-mimetic monoclonal reagents [27], virus capsid components [28] and recently the disintegrin domain of ADAM/MDC-15 [29]. Therefore the modulation of residues flanking the RGD sequence seems to be a widespread mechanism for manipulating the specificity of integrin–ligand interactions. Finally, the present work has demonstrated distinct characteristics in the inhibition of function of the $\alpha_{\text{IIB}}\beta_3$ and $\alpha_5\beta_1$ complexes by the disintegrin panel, indicative of distinct mechanisms of disintegrin recognition by these integrins. The inhibitory performance of the disintegrin panel, assessed in $\text{Ca}^{2+}/\text{Mg}^{2+}$ cation buffer for the $\alpha_{\text{IIB}}\beta_3$ complex and Mn^{2+} cation for the $\alpha_5\beta_1$ complex, allows a comparison under conditions in which high-affinity recognition of all disintegrins and glycoprotein substrates occurred with both receptors. The inhibitory preferences exemplified by GST–Elegantin were evident with the $\alpha_{\text{IIB}}\beta_3$ complex but not with the $\alpha_5\beta_1$ complex. Thus, consistently with our previous observations with venom-derived disintegrins [15,18] and recombinant dendroaspins [19], the preferential antagonism of $\alpha_{\text{IIB}}\beta_3$ function highlights distinct recognition specificities of RGD sequence motifs for this integrin complex even when these adhesive motifs are presented within different structural templates.

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