Differential recognition of snake venom proteins expressing specific Arg-Gly-Asp (RGD) sequence motifs by wild-type and variant integrin $\alpha_{IIb}\beta_3$: further evidence for distinct sites of RGD ligand recognition exhibiting negative allostery

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Several studies have demonstrated that the amino acid residues flanking the Arg-Gly-Asp (RGD) sequence of high-affinity ligands modulate their specificity of interaction with integrin complexes. Because of the absence of structural data for integrin complexes with bound ligand, the molecular basis for this specificity modulation remains obscure. In a previous paper [Rahman, Lu, Kakkar and Authi (1995) *Biochem. J.* **312**, 223–232] we demonstrated that two genetically distinct venom-derived RGD proteins, kistrin and dendroaspin (both containing the sequence PRGDMP), were simple competitors, indicating the recognition of an identical binding site on the $\alpha_{IIb}\beta_3$ complex. Furthermore, both kistrin and dendroaspin inhibited the binding of the disintegrin elegantin (containing the sequence ARGDNP) via a non-competitive mechanism, suggesting that the binding of elegantin to the $\alpha_{IIb}\beta_3$ complex was at a remote site and down-regulated via an allosteric mechanism. Here we present further evidence for distinct RGD ligand recognition sites on the $\alpha_{IIb}\beta_3$ complex that exhibit a negative allosteric relationship. A panel of well-characterized recombinant dendroaspin and elegantin derivatives were employed for this study. These recombinant molecules were constructed as glutathione S-transferase fusion proteins with either an Ala or Pro residue N-terminal to the RGD sequence in combination with either a Met or an Asn residue immediately C-terminal. Equilibrium competition experiments

showed that elegantin binding to ADP-treated platelets was inhibited by derivatives Eleg. AM (ARGDMP) and Eleg. PM (PRGDMP) via an allosteric competitive mechanism, providing direct evidence that modulation of the RGD motif can alter competitive behaviour. In addition, recombinant kistrin and dendroaspin both inhibited elegantin binding via a non-competitive mechanism, confirming our previous observations. Further evidence for distinct binding sites employing an independent approach was obtained by analysing the binding of the panel of venom proteins to the functionally defective heterodimer $\alpha_{IIb}\beta_3$ Ser¹²³ → Ala expressed on Chinese hamster ovary cells. These studies demonstrated that simple competitors kistrin and dendroaspin bound with high affinity to the variant integrin complex. In contrast, the binding of elegantin and most significantly, recombinant Dendro. PN (PRGDNP) and Dendro. AN (ARGDNP) were abolished. These observations, taken together, are consistent with a model depicting the presence of distinct sites of RGD ligand recognition on the $\alpha_{IIb}\beta_3$ complex that show the preferential recognition of specific RGD motifs. Competition experiments demonstrate a negative allosteric relationship between these RGD recognition sites.

Key words: disintegrin, glycoprotein IIb-IIIa, receptor.

INTRODUCTION

Integrins are a family of cell-surface heterodimeric glycoprotein receptors that mediate a host of cell–cell and cell–matrix interactions underlying numerous physiological processes [1]. Integrin function provides a two-way flow of information between the cell and its external environment, manifested by the capacity of integrin complexes to regulate both the assembly of an extracellular matrix and cellular behaviour in response to ligand engagement [2]. Two processes are fundamental to integrin function: affinity modulation, which physiologically is thought to be predominantly controlled by a process termed ‘inside-out’ signal transduction, and ligand recognition, which is dependent

on the structure of the extracellular domain of the integrin and its cognate ligand [3].

Ligand binding is a complex process involving changes in the conformation of both the receptor and the ligand. 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 use Arg-Gly-Asp (RGD), Leu-Asp-Val (LDV) or a related sequence as a key structural component of their receptor-binding domain [2,4]. NMR and X-ray crystallography studies have demonstrated that these integrin recognition motifs are generally maintained within

Abbreviations used: CHO, Chinese hamster ovary; Eleg. AM, ARGDMP; Eleg. PM, PRGDMP; GST, glutathione S-transferase; mAb, monoclonal antibody; MIDAS, metal-ion-dependent adhesion site.

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solvent-exposed β -loop structures that are located in a variety of different protein modules with distinct three-dimensional structures [5–10].

Details of the structure of the ligand-binding domains of integrin complexes are less well characterized but recent developments have produced significant insights into the mechanisms of ligand engagement. Biochemical studies with I-domain-containing integrins have accrued to establish that this module, located within the α -subunit, recapitulates much of the ligand binding characteristics of the entire integrin complex [11–13]. Complementary mutagenesis and crystallographic approaches have identified a cation co-ordination site termed MIDAS (metal-ion-dependent adhesion site) comprising amino acid residues with oxygenated side chains located at the apex of the I-domain structure [14–16]. Thus cation co-ordination and ligand recognition are intimately associated structurally, providing a firm basis for the well-documented observations of the dependence and regulation of ligand binding by bivalent cation. Biochemical approaches have also identified an analogous region within the β -subunits of both I-domain-containing and non-I-domain-containing integrin complexes [17–20]. Although a model proposing an I-domain-like dinucleotide fold has been suggested [18], the three-dimensional scaffold that engenders the putative β -subunit MIDAS site is currently unknown because the crystal structure of this ligand binding region of the β -subunit has not been resolved. In addition to the MIDAS ligand-binding site, evidence also exists in support of the N-terminal region of the α -subunit in ligand recognition. Cross-linking approaches with both fibrinogen γ -chain and RGD synthetic peptides have also identified regions of the α_{IIB} subunit as putative ligand contact sites, including residues 294–314 [21], 42–73, 696–724 and 752–768 [22]. Residues within the region designated by amino acids 184–193 of the α_{IIB} -subunit have been shown by targeted mutagenesis to be critical for ligand recognition by the $\alpha_{\text{IIB}}\beta_3$ complex [23].

The emerging picture of integrin structure accommodates the potential for allostery in receptor function. Indeed, several studies have identified a role for Ca^{2+} in the negative regulation of ligand recognition by β_3 and β_1 integrins presumably via allosteric modulation of the ligand-binding site [24–28]. Furthermore, ligand binding to the $\alpha_{\text{IIB}}\beta_3$ complex has been shown to promote the expulsion of 2 mol of cation per mol of ligand bound, providing further evidence for a functional linkage between ligand-binding site and cation-binding site [29]. However, the existence of allosteric regulation between high-affinity ligands is less well characterized. In a previous paper we demonstrated that two members of the disintegrin family of RGD ligands (kistrin and elegantin, from the venom of *Calloselasma rhodostoma* and *Trimeresurus elegans* respectively) showed a non-competitive inhibitory mechanism for binding to the $\alpha_{\text{IIB}}\beta_3$ complex on platelets. We postulated that the structural features within the ligand that were predominantly responsible for this allosteric regulation of ligand binding were the amino acid residues flanking the RGD sequence [30]. Furthermore, in these studies, structurally dissimilar venom proteins with identical RGD motifs [kistrin and dendroaspis (the latter from the venom of *Dendroaspis jamesonii*)] bound in a mutually exclusive fashion. Several studies have demonstrated an important regulatory role for the residues flanking the RGD sequence in mediating the specificity of integrin–ligand recognition. Studies with snake venom proteins [31–35], ligand mimetic monoclonal antibodies (mAbs) [36,37] and the observations of integrin-based viral infectivity [38] indicate that the use of this mechanism for modulating integrin recognition occurs in a variety of biological settings mediated by structurally distinct protein ligands. Here we present two in-

dependent lines of evidence supporting a model for two distinct sites of RGD ligand recognition by the $\alpha_{\text{IIB}}\beta_3$ complex. First, direct evidence is presented that modulation of the RGD sequence in the disintegrin elegantin engenders recombinant variants that compete via an allosteric mechanism with their parent. Secondly, we demonstrate the differential recognition of a defective integrin complex $\alpha_{\text{IIB}}\beta_3$ Ser¹²³ → Ala by a panel of venom RGD proteins, emphasizing that modulation of the RGD sequence abrogates the recognition of this complex. Taken together, these results reaffirm and extend our previous observations of snake venom RGD ligand recognition by the $\alpha_{\text{IIB}}\beta_3$ complex and provide further evidence for a two-site, allosteric model of RGD ligand recognition.

MATERIALS AND METHODS

Materials

A glutathione S-transferase (GST)–kistrin clone (pGEX-3T) was generously donated by Dr. Martin Humphries (University of Manchester, Manchester, U.K.). The $\alpha_{\text{IIB}}\beta_3$ -directed mAbs 7E3 (β_3 -specific) and 10E5 were gifts from Dr. Barry Collier (Mount Sinai School of Medicine, New York, NY, U.S.A.). mAbs OPG2 and LM609 ($\alpha_{\text{IIB}}\beta_3$ -specific) were generously provided by Dr. Tom Kunicki and Dr. David Cheresch respectively (Scripps Research Institute, La Jolla, CA, U.S.A.). All other chemicals and reagents were obtained from Sigma and Milligen (Watford, Herts., U.K.). Radioisotopic Na¹²⁵I (15.3 mCi/ μg of iodine) was purchased from Amersham (Little Chalfont, Bucks., U.K.). Iodobead reagent was obtained from Pierce Laboratories (Hemel Hempstead, Herts., U.K.). Chinese hamster ovary (CHO) cell lines expressing wild-type $\alpha_{\text{IIB}}\beta_3$ [39] and β_3 variant Ser¹²³ → Ala [17] have been characterized and described previously.

Construction of synthetic dendroaspis and elegantin genes and variant constructs

Synthetic dendroaspis and elegantin genes were constructed by ligating 10–12 complementary and overlapping oligonucleotides coding for the protein sequences of dendroaspis and elegantin by using *Escherichia coli* codon usage data and cloned into pGEX-3X (Pharmacia). The detailed description and functional characterization of these recombinant venom proteins and their variant derivatives has been reported previously [35,40]. GST–snake-venom proteins were prepared from the lysates of *Epicurean coli* (DE3)pLysS (Stratagene, Cambridge, U.K.) by affinity chromatography on GSH–Sepharose (Pharmacia) with the use of a batch procedure. Purified GST–snake venom proteins were analysed by SDS/PAGE, indicating that the preparations were more than 95% homogeneous; minimal proteolytic degradation of the sample was observed.

Iodination and fluorescent labelling of proteins

Iodination of GST–disintegrins was performed with Iodobeads 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 Na¹²⁵I 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. ¹²⁵I-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; specific radioactivities of $(2\text{--}5) \times 10^6$ c.p.m./ μg were routinely obtained.

N-Hydroxysuccinimide-fluorescein (5- and 6-carboxyfluorescein, succinimidyl ester; Pierce and Warriner Ltd., 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. Ratios of fluorescence groups to protein were determined for each protein, showing a 1:1 stoichiometry; these samples were stored at –40 °C.

Platelet functional studies and ligand binding assays

Washed platelets were prepared from platelet-rich plasma obtained from healthy individuals who had not taken medication for 9 days before sampling. The binding of 125 I-labelled GST–snake venom proteins to washed platelets was performed as described previously [18]. In brief, the incubation mixture was composed of 300 μ l of washed platelets (3×10^8 /ml), 10 μ l of agonist (1.75 mM ADP) giving a final concentration of 50 μ M, 10 μ l of 125 I-labelled GST–disintegrin protein and 5–20 μ l of competitor, inhibitory antibody or buffer, made to a final volume of 350 μ l. In ligand competition assays the binding reaction proceeded for at least 120 min and for kinetic experiments for various durations. The radioactivity in both pellets and supernatants was 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 CHO cell binding assays with FITC-labelled molecules, washed CHO cell suspensions were resuspended in Tyrode's buffer (2×10^6 /ml) and treated with or without the inhibitory mAb (2–20 μ g/ml) for 30 min at room temperature followed by incubation with the appropriate FITC-conjugated snake venom molecule (1 μ M) for a further 60 min (total volume 200 μ l). CHO cells were then fixed in 4% (w/v) formaldehyde in PBS, pH 7.4, washed in PBS and analysed by FACS (FACScan; Becton Dickinson). The absolute levels of fluorescence obtained for each FITC-labelled snake venom ligand were approximately equal. Background binding levels were determined by the measurement of cell-associated fluorescence obtained in samples in which 10 mM EDTA was included in the incubation mixture. Background levels were routinely less than 3% of the total fluorescence levels.

Derivation of equilibrium binding constants

The data obtained from equilibrium binding experiments were analysed with the RADLIG program (Kinetic EBDA; Biosoft, Cambridge, U.K.) to obtain values of bound and free ligand. Data were then analysed by both the double-reciprocal plot and the Scatchard plot. Values of competitor K_i (app) were derived from the slopes of the isotherms of the double-reciprocal plots, showing competitive-type inhibition by using the formula:

$$K_i \text{ (app)} = I / ([M_i / M_0] - 1)$$

where I is the inhibitor (competitor) concentration, M_i is the gradient of the isotherm derived in the presence of inhibitor at concentration I , and M_0 is the gradient of the isotherm derived in the absence of inhibitor. Values of tracer K_d (app) were determined from the slopes of isotherms derived by Scatchard analysis.

RESULTS

Recombinant snake venom RGD proteins

In a previous paper [30] we demonstrated by equilibrium competition experiments employing activated platelets that two structurally distinct venom-derived RGD proteins, kistrin and

dendroaspin (both containing the sequence PRGDMP), were simple competitors, indicating the recognition of an identical class of binding site on the $\alpha_{IIb}\beta_3$ complex. Furthermore, both kistrin and dendroaspin inhibited the binding of the disintegrin elegantin (ARGDNP) via a non-competitive mechanism, suggesting that the binding of elegantin to the $\alpha_{IIb}\beta_3$ complex was at a remote site and regulated by a negative allosteric mechanism. To investigate further the role of the amino acids flanking the RGD sequence on the regulation of ligand recognition by allosteric modulation of the $\alpha_{IIb}\beta_3$ complex, we constructed a series of recombinant snake venom RGD proteins expressing specific RGD motifs by GST fusion protein expression technology. Two distinct structural templates were employed for this study, namely the disintegrin elegantin and the neurotoxin homologue dendroaspin. Molecules were constructed with either an Ala or Pro residue N-terminal to the RGD sequence in combination with either a Met or Asn residue immediately C-terminal (Figure 1). The detailed characterization of these molecules in platelet functional assays, including the demonstration of their predominant specificity for the $\alpha_{IIb}\beta_3$ complex on ADP-treated platelets, has been reported previously [35,40]. Equilibrium competition experiments were performed with an identical assay procedure to that used in our previous study [30].

Modulation of residues flanking RGD induces allosteric competition

With 125 I-labelled elegantin as a tracer, unlabelled Eleg. AM, Eleg. PM and kistrin competed for binding to the $\alpha_{IIb}\beta_3$ complex on platelets in a dose-dependent and saturable manner, with IC_{50} values of 76 ± 5 , 99 ± 11 and 58 ± 10 nM respectively (results not shown). To analyse the inhibitory mechanism operating between these ligands, further experiments were designed in which the concentration of 125 I-labelled elegantin was varied in the presence of single doses of competitor. The results of these experiments were analysed by both double-reciprocal and Scatchard plots to determine the variation of competitor K_i (app) and tracer K_d (app) as a function of competitor concentration (Figure 2). Competitors Eleg. AM and Eleg. PM both produced isotherms that resembled a simple competitive-type inhibitory mechanism characterized in both graphical representations by a decrease in the slope of the isotherms as a function of competitor concentration and their convergence at β_{max} . However, an analysis of the variation of the values of competitor K_i (app) and tracer K_d (app) with competitor concentration indicated a clear departure from simple competitive behaviour for both Eleg. AM and Eleg. PM (Figure 3). That is, K_i (app) values for Eleg. PM and Eleg. AM varied in an inverse fashion with their concentration (Figure 3A), whereas the K_d (app) of elegantin (Figure 3B) increased in a non-linear fashion that was consistent with allosteric regulation [41]. The allosteric competitive behaviour between elegantin and its variants highlights the influence of the amino acid residues flanking the RGD sequence on the specificity of binding of presumably structurally similar molecules to the $\alpha_{IIb}\beta_3$ complex. This contrasts the behaviour between kistrin and dendroaspin (both PRGDMP) that we reported previously with an identical assay system [30] (see Figure 3). Despite their distinct structural folds, the mechanism of competition observed between kistrin and dendroaspin fulfilled the criteria for a simple competitive mode, indicative of the mutual recognition of the same binding site. However, the present results indicate that an Asn⁴⁶ → Met substitution (i.e. a Met residue C-terminal to the RGD sequence) in elegantin produces a marked change in the binding characteristics of elegantin to the $\alpha_{IIb}\beta_3$ complex. Interestingly, the degree of non-linearity (Figure 3B) in the competitive

Figure 1 Amino acid sequences of native and recombinant disintegrins and dendroaspins

Abbreviations: Eg, elegantin; Ks, kistrin; Dn, dendroaspin; E.b, erabutoxin b. Identical residues are represented by dashes. Nomenclature in parentheses is as used in the text.

Figure 2 Inhibition of ^{125}I -labelled elegantin binding to platelets by Eleg. PM and Eleg. AM

Various concentrations of ^{125}I -labelled elegantin (20–250 nM) were incubated with ADP-treated washed platelet suspensions in the presence or absence of a single dose of Eleg. PM (A, B) and Eleg. AM (C, D). After incubation for 2 h at room temperature, levels of bound and free tracer were determined by centrifugation of the platelets through a sucrose cushion and determination of the radioactivity present in both the pellets and supernatant with a γ -counter. The raw data were processed with RADLIG software. The results are presented as both double-reciprocal plots (A, C) and Scatchard plots (B, D). Symbols: (A, B) ◇, no competitor; △, 100 nM Eleg. PM; □, 200 nM Eleg. PM; ○, 300 nM Eleg. PM; (C, D) ◇, no competitor; △, 100 nM Eleg. AM; □, 200 nM Eleg. AM. The figure represents a single experiment with three additional experiments giving similar results. Each data point is the mean for a duplicate sample; the S.D. was routinely less than 10%. All isotherms were determined by non-linear regression analysis.

behaviour between elegantin and its variants was more pronounced for Eleg. PM than for Eleg. AM, indicating that the Pro residue N-terminal to the RGD sequence also has a role in the specificity of binding to the $\alpha_{\text{IIb}}\beta_3$ complex. This observation is consistent with the differing inhibitory specificities observed between Eleg. AM and Eleg. PM in platelet adhesion experiments [40] and is independent of binding affinity because both molecules have similar K_d (app) values for activated platelets in the absence of competitors. These results suggest that the binding of Eleg. PM and Eleg. AM to the $\alpha_{\text{IIb}}\beta_3$ complex decreases the binding affinity of the tracer elegantin and is consistent with the notion

that both variants bind in a manner distinct from that of elegantin [41].

To analyse further the inhibitory mechanisms operating between elegantin and its variants, competition studies were performed with ^{125}I -labelled Eleg. AM as a tracer. With elegantin as the competitor, analysis of the data by double-reciprocal and Scatchard plots produced isotherms consistent with simple competitive behaviour (Figures 4A and 4B). This notion was confirmed by the fact that the competitor K_i (app) observed at two widely spaced concentrations of elegantin did not vary significantly and the K_d (app) of ^{125}I -labelled Eleg. AM increased

Figure 3 Variation of the ligand binding parameters with competitor concentration distinguishes between simple and allosteric competitive behaviour

(A) Variation of K_i (app) for Eleg. PM (■), Eleg. AM (▲) and elegantin (◇) with inhibitor concentration. K_i (app) values were determined from the slopes of the isotherms produced by the double-reciprocal plots (Figures 2A, 2C and 4A). (B) Variation of K_d (app) of the tracer ligand with competitor concentration. The value for the K_d (app) of 125 I-labelled elegantin with competitor concentration [Eleg. PM (■) and Eleg. AM (▲)] was obtained from the slopes of the isotherms from Figures 2(B) and 2(D) and the K_d (app) values for 125 I-labelled Eleg. AM (◇) were derived from Figure 4(B). For comparison, the pattern of variation of K_i (app) and K_d (app) observed in similar experiments with 125 I-labelled kistrin and unlabelled dendroaspin [30] is shown (○), which is characteristic of simple competitive behaviour.

Figure 4 Inhibition of 125 I-labelled Eleg. AM binding to platelets by elegantin and Eleg. PM

Various concentrations of 125 I-labelled Eleg. AM were incubated with ADP-treated washed platelets in the absence and in the presence of a single dose of elegantin (A, B) or Eleg. PM (C, D) for 2 h at room temperature. The data are presented in the form of double-reciprocal plots (A, C) and Scatchard plots (B, D) and are representative of two independent experiments producing analogous results. Each data point is the mean for duplicate samples (the S.D. was less than 10%). Isotherms were fitted by non-linear regression analysis. Symbols: (A, B) ○, no competitor; △, 150 nM elegantin; □, 450 nM elegantin; (C, D) ○, no competitor; △, 200 nM Eleg. PM; □, 400 nM Eleg. PM.

Figure 5 Association (A) and dissociation (B) of ^{125}I -labelled elegantin from platelets in the presence of competitors

(A) ^{125}I -labelled elegantin (100 nM) was incubated at room temperature with ADP-activated platelets in the absence ($\square$) and in the presence of 50 nM Eleg. AM ($\triangle$), 50 nM Eleg. PM ($\blacklozenge$) and 37.5 nM kistrin ($\circ$) for various durations as shown. Bound and free levels of tracer were determined as described in the Materials and methods section. K_{obs} values were determined for each isotherm by using the KINETIC program (Biosoft) as shown in Table 1. The results are representative of two experiments that gave similar results. (B) ^{125}I -labelled elegantin (50 nM) was incubated with ADP-activated platelets for 1 h at room temperature to achieve equilibrium binding. A concentrated sample of unlabelled elegantin (final concentration 5 μM) without or with unlabelled competitor (100 nM) was added to the platelet suspension and the dissociation of ^{125}I -labelled elegantin was measured by removing aliquots of the suspension after various intervals to determine the levels of bound and free ^{125}I -labelled elegantin. Symbols are as for (A). The dissociation rate constant (K_{-1}) was determined for each set of data as shown in Table 1. Results are representative of two experiments giving very similar results.

in a linear fashion (see Figure 3). The non-reciprocal competitive behaviour between elegantin and Eleg. AM is consistent with the notion that these ligands bind to the $\alpha_{\text{IIb}}\beta_3$ complex in a distinct manner despite their high level of sequence similarity (Figure 1). In contrast, the mode of competition observed between ^{125}I -labelled Eleg. AM and unlabelled Eleg. PM was more complex and distinct from that observed when ^{125}I -labelled elegantin was used as the tracer. The isotherm produced at 200 nM Eleg. PM showed characteristics of simple competitive kinetics, whereas at 400 nM Eleg. PM the mechanism of competition assumed apparently non-competitive characteristics (Figures 4C and 4D). This mixed-type competitive behaviour was also apparent in analogous competition studies with unlabelled kistrin as the competitor (results not shown). The distinct modes of competition observed for Eleg. PM when paired against elegantin or Eleg. AM tracer molecules was also consistent with the distinct specificity of binding of elegantin and Eleg. AM to the $\alpha_{\text{IIb}}\beta_3$ complex.

The non-competitive mechanism observed between Eleg. AM and Eleg. PM was reminiscent of the competition observed when venom-derived ^{125}I -labelled elegantin was incubated in the presence of unlabelled kistrin or dendroaspin [30]. However, in the present study, Eleg. PM, which has the same RGD motif as kistrin (PRGDMP), did not produce a non-competitive profile in competition experiments with elegantin. To establish that the presence of the GST tag was not responsible for this difference in behaviour, increasing doses of ^{125}I -labelled elegantin were incubated in the presence of unlabelled GST-kistrin and GST-dendroaspin (results not shown). These experiments generated binding isotherms that were consistent with a non-competitive mechanism operating between these GST ligands, thereby confirming the reproducibility of the ligand binding assays performed with recombinant GST-venom fusion proteins and our previous observations. Secondary plots generated from these data gave K_i (app) values for kistrin and dendroaspin of approx. 34 and 85 nM respectively. These values are in good agreement with the reported K_d (app) values for these ligands for binding to the $\alpha_{\text{IIb}}\beta_3$ complex in the absence of competitors [30,35]. These data confirm that kistrin and Eleg. PM differ in the mechanism of

Table 1 Kinetic constants for the binding of elegantin in the presence of competitors

Competitor	$10^2 K_{\text{obs}}$ (min^{-1})	$10^3 K_{-1}$ (min^{-1})
None	6.41 ± 0.95	3.8 ± 0.56
Eleg. AM	3.5 ± 0.9	4.02 ± 0.33
Eleg. PM	2.44 ± 0.65	3.2 ± 0.76
Kistrin	6.48 ± 0.63	4.34 ± 0.3

their recognition of the $\alpha_{\text{IIb}}\beta_3$ complex despite showing similar inhibitory properties in functional assays [40] and that these differences relate to the presence of distinct structural features between these disintegrins.

Distinct mechanisms of allosteric competition

To obtain further insights into the distinct nature of the allosteric inhibitor of elegantin binding by variants Eleg. AM, Eleg. PM and kistrin, the effect of these competitors on elegantin association with the $\alpha_{\text{IIb}}\beta_3$ complex (Figure 5A) and dissociation from it (Figure 5B) was investigated. The observed association rate constant (K_{obs}) was determined for elegantin in the absence and presence of competitors (Table 1). Under the experimental conditions used, both Eleg. AM and Eleg. PM decreased the rate of elegantin association by approx. 50–60%, whereas kistrin had little effect. In contrast, the rate of elegantin dissociation from the $\alpha_{\text{IIb}}\beta_3$ complex (K_{-1}) was not significantly altered by the presence of any of the competitors (Figure 5B and Table 1). Therefore the inhibitory mechanism of Eleg. AM and Eleg. PM seems to interfere with the formation of the elegantin–integrin complex and not its stability. Kistrin, in contrast, seems to ablate elegantin recognition of the $\alpha_{\text{IIb}}\beta_3$ complex without affecting the stability of preformed elegantin–receptor complexes, which is consistent with the non-competitive profile obtained from the equilibrium competition studies. These results also confirm that

Table 2 Differential recognition of wild-type and variant $\alpha_{IIb}\beta_3$ complexes

$\alpha_{IIb}\beta_3$ recognition was calculated from $100 \times [(\text{fluorescence in the presence of mAb 7E3}) - (\text{fluorescence in the presence 10 mM EDTA})] / [(\text{fluorescence in the absence of mAb 7E3}) - (\text{fluorescence in the presence of 10 mM EDTA})]$; results are means $\pm$ S.E.M. ($n = 6$).

Venom protein	Sequence around RGD	Protein fold	$\alpha_{IIb}\beta_3$ recognition on CHO cells (%)		Loss of recognition by β_3 Ser ¹²³ → Ala variant (%)
			Wild-type $\alpha_{IIb}\beta_3$	β_3 Ser ¹²³ → Ala	
Kistrin	PRGDMP	Disintegrin	47.5 $\pm$ 11	50.8 $\pm$ 5	0
Elegantin	ARGDNP	Disintegrin	33.4 $\pm$ 18	0	100
Eleg. PM	PRGDMP	Disintegrin	54 $\pm$ 16	0	100
Eleg. AM	ARGDMP	Disintegrin	39 $\pm$ 15	0	100
Dendroaspin	PRGDMP	Neurotoxin	56 $\pm$ 3	45 $\pm$ 4	6
Dendro. PN	PRGDNP	Neurotoxin	54 $\pm$ 6	4 $\pm$ 2	94
Dendro. AN	ARGDNP	Neurotoxin	32 $\pm$ 14	0	100

the non-competitive inhibition of elegantin's binding to the $\alpha_{IIb}\beta_3$ complex by kistrin and dendroaspin observed in our previous [30] and present work was not an artifact produced by non-equilibrium conditions in the competition experiments (i.e. by kinetic exclusion of the tracer) because in these experiments the incubation period far exceeded the attainment of steady state observed in association experiments (Figure 5A). These studies demonstrated that the inhibitory mechanism of Eleg. AM and Eleg. PM was distinct from that of kistrin; this was consistent with the results from the equilibrium competition experiments.

Differential recognition of the variant heterodimer $\alpha_{IIb}\beta_3$ Ser¹²³ → Ala by modulation of the RGD motif

The equilibrium competition studies of the present study demonstrated several mechanisms of competition between snake venom RGD ligands, including simple competitive, allosteric competitive and non-competitive inhibition. These observations are consistent with a model depicting at least two distinct RGD-dependent sites of $\alpha_{IIb}\beta_3$ -ligand recognition. To test this hypothesis with an independent approach, we analysed the capacity of the panel of recombinant venom proteins to bind both a wild-type $\alpha_{IIb}\beta_3$ complex and a functionally defective variant, $\alpha_{IIb}\beta_3$ Ser¹²³ → Ala, expressed on CHO cells. This variant complex was selected because previous work had shown that the β_3 Ser¹²³ → Ala substitution produced an $\alpha_{IIb}\beta_3$ lacking the capacity to interact with either fibrinogen or the RGD-ligand-mimetic mAb PAC1. Furthermore, incubation of detergent extracts from cells expressing the variant heterodimer with immobilized GRGDSP peptide failed to adsorb the integrin complex [17]. However, CHO cells expressing $\alpha_{IIb}\beta_3$ Ser¹²³ → Ala were reported to bind the RGD-ligand-mimetic mAb OPG2, an $\alpha_{IIb}\beta_3$ -specific monoclonal reagent. Because CHO cells express high levels of the $\alpha_5\beta_1$ complex, which avidly binds disintegrins containing an Ala residue N-terminal to the RGD sequence [40], the fraction of $\alpha_{IIb}\beta_3$ -dependent recognition was determined by measuring the decrease in the bound levels of ligand in samples preincubated with saturating doses of $\alpha_{IIb}\beta_3$ -function-blocking monoclonal reagents 7E3 and OPG2. The results of this analysis are shown in Table 2.

In these experiments, binding of each recombinant disintegrin and dendroaspin was observed to wild-type $\alpha_{IIb}\beta_3$ complex expressed on CHO cells. Variation in the extent of $\alpha_{IIb}\beta_3$ recognition was observed between different venom proteins and ranged from approx. 30 % to 60 % of the cell-bound ligand. As expected, recognition of the $\alpha_5\beta_1$ complex was evident for ARGD-

containing disintegrins and this was evident in inhibition experiments with the $\alpha_5\beta_1$ -directed mAb JBS5. In addition, significant levels of ligand were bound to one or more unidentified integrin complexes. However, little or no inhibition was observed by preincubation of the cells with mAb LM609, indicating minimal involvement of the hybrid hamster α_v /human β_3 complex, which is assembled by these transfected cell lines [42].

The differences in the levels of $\alpha_{IIb}\beta_3$ recognition were responsive to the amino acid residues flanking the RGD sequence. PRGD molecules showed the highest levels of $\alpha_{IIb}\beta_3$ recognition, irrespective of their structural template (Table 2). A similar pattern of $\alpha_{IIb}\beta_3$ specificity was observed in analogous studies with HEL cells expressing wild-type integrin $\alpha_{IIb}\beta_3$ (results not shown).

In contrast, selective recognition of snake venom ligands was observed in CHO cells expressing the $\alpha_{IIb}\beta_3$ Ser¹²³ → Ala complex. Notably, simple competitors kistrin and dendroaspin (both PRGDMP) bound with high affinity to the variant heterodimer shown by the inhibition of binding of both ligands by mAb 7E3 by approx. 50 % (Table 2). The specificity of recognition of the $\alpha_{IIb}\beta_3$ variant complex by kistrin and dendroaspin was confirmed by using mAb OPG2 as an inhibitor, which engendered similar inhibitory profiles to those for mAb 7E3. A K_d (app) for kistrin binding of approx. 240 nM was determined by saturation binding analysis with mAb 7E3 to determine the level of $\alpha_{IIb}\beta_3$ recognition (results not shown). This value, although lower than the observed K_d (app) for the binding of kistrin to activated platelets (approx. 40 nM), was of a similar order of affinity observed for the binding of kistrin to integrin $\alpha_{IIb}\beta_3$ expressed on HEL cells (120 nM), which is in a lower affinity state (results not shown). In contrast with kistrin and dendroaspin, the binding of elegantin to the $\alpha_{IIb}\beta_3$ Ser¹²³ → Ala complex was not detectable, indicating that β_3 Ser¹²³ is a critical residue for recognition by elegantin of the $\alpha_{IIb}\beta_3$ integrin. Surprisingly, binding of Eleg. PM and Eleg. AM to the $\alpha_{IIb}\beta_3$ Ser¹²³ → Ala complex was also not detectable, indicating that, with respect to the elegantin template, modulation of the RGD motif could not rescue recognition of the variant complex by elegantin. However, of greater significance was the observation that little or no binding of Dendro. PN (PRGDNP) nor Dendro. AN (ARGDNP) to the $\alpha_{IIb}\beta_3$ Ser¹²³ → Ala complex was detected, even though binding of these molecules to the wild-type $\alpha_{IIb}\beta_3$ complex was clearly evident (Table 2). This observation indicates that replacement of Met⁴⁶ with Asn in dendroaspin abolished ligand recognition of the variant $\alpha_{IIb}\beta_3$ complex. Thus, in contrast with elegantin, recognition of the variant $\alpha_{IIb}\beta_3$ heterodimer could be regulated by modulation of the RGD motif within dendroaspin.

DISCUSSION

The results of the present study have provided two independent lines of evidence supporting the existence of distinct binding sites on the $\alpha_{\text{IIb}}\beta_3$ complex for RGD-dependent snake venom ligands. Equilibrium competition studies with both disintegrin and dendroaspin molecules demonstrated several distinct modes of competition for binding to the $\alpha_{\text{IIb}}\beta_3$ complex. Coupled with our previous work [30,35], these radioligand binding studies have established that the amino acid residues flanking the RGD sequence of either structurally similar or distinct venom proteins regulate ligand binding to the $\alpha_{\text{IIb}}\beta_3$ complex, suggesting the existence of distinct ligand interaction sites. For example, the structurally unrelated molecules kistrin and dendroaspin, possessing identical RGD motifs (PRGDMP), show simple competitive behaviour in competition assays, i.e. they bind to an identical site on the $\alpha_{\text{IIb}}\beta_3$ complex. True simple competition between ligands is characterized by the observation that the receptor binding parameters of one partner can be determined by using the other as a tracer in competition experiments (saturation by competitor displacement of the tracer). This relationship was established between kistrin and dendroaspin in a previous study [30]. Furthermore, the observation that both kistrin and dendroaspin inhibited elegantin binding via a non-competitive mechanism is consistent with this notion. Significantly, in the present work both kistrin and dendroaspin bound with high affinity to the variant complex $\alpha_{\text{IIb}}\beta_3$ Ser¹²³ → Ala, whereas no binding of elegantin was detectable. Although this result indicates that the binding site of elegantin on the variant integrin was either disrupted or inaccessible, these combined observations provide compelling evidence for the overlapping nature of the kistrin- and dendroaspin-binding site on the $\alpha_{\text{IIb}}\beta_3$ complex. However, modulation of the residues around the RGD sequence of elegantin, by even a single amino acid substitution, produces variant molecules that show an allosteric competitive mechanism in equilibrium competition experiments with their parent. Indeed, for allosteric competitive behaviour, the competitor (Eleg. AM or Eleg. PM) is predicted to interact with both the overlapping and distinct, negatively co-operative, binding sites with similar affinity [41]. Consistent with this notion was the observation that monophasic Scatchard plots were obtained from the saturation binding analysis of both Eleg. AM and Eleg. PM, indicating that distinct receptor–ligand populations were not distinguishable by this approach [40]. However, in our previous work with the dendroaspin series of molecules [35], with the use of similar equilibrium binding assays with activated platelets, the mutant Dendro. PN produced a biphasic Scatchard isotherm. The appearance of a distinct population of higher-affinity sites (approx. 15000 sites per platelet) was observed in addition to the repertoire of sites observed for the parent dendroaspin. The ligand binding profile of Dendro. PN to activated platelets exemplifies the dual $\alpha_{\text{IIb}}\beta_3$ –RGD ligand recognition in which the difference in affinity of Dendro. PN for each $\alpha_{\text{IIb}}\beta_3$ binding site was sufficiently large for each receptor–ligand population to be discernible by saturation ligand-binding analysis. Similar observations of selective recognition of $\alpha_{\text{IIb}}\beta_3$ populations have been reported with recombinant Fab fragments of the ligand mimetic monoclonal reagent OPG2 in which the residues flanking the RGD sequence were interchanged with those flanking the RGD sequence of mAb PAC 1 [36].

Further evidence consistent with the notion that Eleg. AM and Eleg. PM interact with either of two distinct but interacting sites on the $\alpha_{\text{IIb}}\beta_3$ complex was evident from the results of the competition experiments between these molecules. ¹²⁵I-labelled Eleg. AM and Eleg. PM competed in a complex manner, i.e.

distinct from the competition observed between Eleg. PM and ¹²⁵I-labelled elegantin. These results indicate subtle differences between Eleg. AM and Eleg. PM in their binding properties to the $\alpha_{\text{IIb}}\beta_3$ complex. Indeed, saturation studies with these ligands showed that although Eleg. AM and Eleg. PM produced similar profiles for binding to ADP-activated platelets, the binding of Eleg. PM to resting platelets was of approx. 2.5-fold lower affinity, whereas that of Eleg. AM was unaltered (S. Rahman, unpublished work). In contrast with Eleg. PM, the observation that elegantin inhibited the binding of ¹²⁵I-labelled Eleg. AM in a simple competitive manner indicates that elegantin exerts a competitive effect through preferential occupancy of a single site on the $\alpha_{\text{IIb}}\beta_3$ complex. Importantly, this observation indicates that elegantin occupancy of the $\alpha_{\text{IIb}}\beta_3$ complex restricts the binding of Eleg. AM to an overlapping site; that is, it prevents Eleg. AM from interaction with a second remote site.

In addition to the allosteric competitive behaviour observed between elegantin and its derivatives, the non-competitive inhibition of elegantin by both kistrin and dendroaspin reported previously [30] was confirmed with the recombinant GST fusion proteins used in the present work. Although Eleg. PM has the same RGD motif (PRGDMP) as kistrin and dendroaspin, the distinct mechanisms of inhibition of elegantin– $\alpha_{\text{IIb}}\beta_3$ interaction observed between Eleg. PM and kistrin suggest that the binding of kistrin (and therefore dendroaspin) to the $\alpha_{\text{IIb}}\beta_3$ complex occurs predominantly at a single site. Kinetic studies confirmed the distinct inhibitory mechanisms between Eleg. PM and kistrin because the former caused a decrease in the on-rate of elegantin but the latter had no effect. In addition, Eleg. PM and kistrin also differed in their recognitions of the variant $\alpha_{\text{IIb}}\beta_3$ Ser¹²³ → Ala complex. Whereas kistrin bound to the variant integrin with high affinity, the presence of the PRGDMP motif within the elegantin template did not promote ligand recognition. Taken together, these observations suggest that regions of the disintegrin molecule outside the RGD loop might also contribute to the disintegrin–integrin recognition mechanism. A role for residues at the C-terminus in integrin recognition for the disintegrin echistatin has been reported [43,44] but whether sequence variation between kistrin and Eleg. PM at their C-termini underlie their differing binding properties is not known. Because kistrin exerts a non-competitive inhibitory effect on the binding of elegantin to the $\alpha_{\text{IIb}}\beta_3$ complex via interaction predominantly with a single site, the inhibitory mechanism might involve either a negative allosteric transition within the elegantin-binding site or the prevention of a conformational change in the $\alpha_{\text{IIb}}\beta_3$ complex that is necessary to allow elegantin accessibility to its recognition site. Alternatively, kistrin and elegantin might bind to similar regions within the $\alpha_{\text{IIb}}\beta_3$ complex but, by virtue of the presence of distinct residues flanking the RGD sequence, display different orientations of this motif. Thus the ligand-binding properties of these disintegrins to the $\alpha_{\text{IIb}}\beta_3$ complex, although distinct, might involve shared contact residues within the receptor's active site. Such a scheme might also produce the non-competitive kinetics observed between kistrin and elegantin. A non-competitive inhibitory mechanism was recently reported to operate between factor X and a factor X peptide for binding to the integrin $\alpha_{\text{M}}\beta_2$ on THP-1 cells, indicating that such inhibitory mechanisms operate on other integrins besides $\alpha_{\text{IIb}}\beta_3$ [45]. The results of our equilibrium competition studies indicate that the inhibition of elegantin binding to the $\alpha_{\text{IIb}}\beta_3$ complex can occur by distinct mechanisms involving remote but physically coupled ligand-binding sites.

At present it is not possible to state whether the distinct RGD sites on the $\alpha_{\text{IIb}}\beta_3$ complex predicted by the present study can be correlated with the previously reported γ -chain and RGD sites

identified by chemical cross-linking approaches [21,22,45]. However, because our previous studies [32,35,40] demonstrated that venom proteins with the PRGDMP sequence preferentially inhibit $\alpha_{IIb}\beta_3$ -fibrinogen interactions, whereas venom proteins with the ARGDNP sequence preferentially inhibit $\alpha_{IIb}\beta_3$ interaction with fibronectin, these observations raise the possibility that the allosteric regulation of physiological binding events between plasma components such as fibrinogen and fibronectin might occur *in vivo* during platelet aggregation and adhesion to the sub-endothelial matrix. At present, little is known about the role of the amino acids flanking the RGD sequence motif in regulating the binding of physiological ligands to the $\alpha_{IIb}\beta_3$ complex. However, the present study has further demonstrated their importance in regulating the binding of high-affinity RGD ligands, illustrated by the change in the ligand-binding properties of elegantin to the wild-type $\alpha_{IIb}\beta_3$ complex and by the loss of recognition of the variant complex $\alpha_{IIb}\beta_3$ Ser¹²³ → Ala by the Met → Asn substitution C-terminal to the RGD sequence in den-droasp. The further analysis of $\alpha_{IIb}\beta_3$ integrin complexes with mutations in putative ligand-binding domains with snake venom ligands should elucidate further details of the mechanisms of integrin-ligand recognition and the refinement of present models that might also be extended to other integrin complexes.

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