The integrin $\alpha_{IIb}\beta_3$ contains distinct and interacting binding sites for snake-venom RGD (Arg-Gly-Asp) proteins

Evidence that the receptor-binding characteristics of snake-venom RGD proteins are related to the amino acid environment flanking the sequence RGD

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We have previously demonstrated [Lu, Williams, Deadman, Salmon, Kakkar, Wilkinson, Baruch, Authi and Rahman (1994) *Biochem. J.* **304**, 929–936] the preferential antagonism of the interactions of the integrin $\alpha_{IIb}\beta_3$ on activated platelets with three immobilized glycoprotein ligands (fibrinogen, fibronectin and von Willebrand factor) by a selected panel of snake-venom RGD (Arg-Gly-Asp)-containing proteins including the disintegrins kistrin and elegantin, and the neurotoxin variant dendroaspin. Kistrin and dendroaspin, although structurally unrelated, contain similar amino acids flanking the tripeptide RGD and behaved as identical antagonists preferentially inhibiting platelet adhesion to immobilized fibrinogen as opposed to fibronectin. In contrast, elegantin, which shares extensive sequence similarity with kistrin but has different amino acids around the tripeptide RGD, preferentially inhibited platelet adhesion to immobilized fibronectin as opposed to fibrinogen. To develop further insights into the mechanisms underlying the preferential antagonism shown by the venom proteins in the adhesion studies, we, in the present study, sought to determine the binding properties of kistrin, elegantin and dendroaspin to the $\alpha_{IIb}\beta_3$ complex by radioligand kinetic and competition studies. In direct binding experiments, both kistrin and dendroaspin were observed to bind to a single class of binding site on ADP-activated platelets with apparent equilibrium dissociation constant (K_{dapp}) values of 42 ± 2 nM and

21 ± 6 nM respectively. In competition studies, dendroaspin blocked the binding of 125 I-labelled kistrin to ADP-activated platelets in a simple competitive manner, with an apparent equilibrium inhibition constant (K_{iapp}) of 143 ± 14 nM, from which an indirect $K_{dapp} = 22$ nM for dendroaspin was determined. This result suggests that kistrin and dendroaspin bind to the same site on the integrin $\alpha_{IIb}\beta_3$, consistent with their similar inhibitory properties. In contrast, elegantin recognized two classes of binding sites on the $\alpha_{IIb}\beta_3$ complex with K_{dapp} values of 10.5 ± 0.8 nM and 175 ± 10 nM, and, unlike dendroaspin, did not inhibit the binding of 125 I-labelled kistrin to ADP-activated platelets. However, in reciprocal experiments both kistrin and dendroaspin inhibited the binding of 125 I-elegantin to ADP-activated platelets in a non-competitive manner, with K_{iapp} values of 34 ± 3 nM and 21 ± 2 nM respectively. Thus elegantin appears to interact with distinct but interacting sites on the $\alpha_{IIb}\beta_3$ complex from the binding site of kistrin and dendroaspin, consistent with its distinctive inhibitory preferences as shown in platelet adhesion studies. Taken in conjunction with the structural similarities and differences between these three RGD-containing venom proteins, these studies provide indirect evidence that the amino acid environment flanking the residues RGD plays a significant role in determining the binding characteristics and thus functional properties of these ligands.

INTRODUCTION

The platelet integrin $\alpha_{IIb}\beta_3$ (Gp IIb/IIIa) mediates platelet aggregation, and potentially supplements platelet adhesion to components of the subendothelial matrix at sites of vascular injury. In its active conformer, this approx. 230 kDa heterodimer is a receptor for several glycoprotein components of the plasma and extracellular matrix including fibrinogen [1], fibronectin [2], vitronectin [3], von Willebrand factor [4] and thrombospondin [5]. The adhesive interactions of the $\alpha_{IIb}\beta_3$ complex with these physiological ligands operates via a divalent cation-dependent mechanism involving predominantly the ubiquitous adhesive tripeptide RGD (Arg-Gly-Asp) [6,7]; however, a structurally related peptide KQAGDV present exclusively at the C-terminus of the γ -chain of fibrinogen is also thought to play a major role in the formation of fibrinogen cross-bridges leading to platelet aggregation [8–11]. Concurrent with the identification of the minimal adhesive peptides present in these glycoprotein ligands and their putative contact sites on the $\alpha_{IIb}\beta_3$ complex, con-

siderable attention has also been focused in recent years at developing a greater understanding of the binding mechanisms of physiological ligands such as fibrinogen and fibronectin. Results from several studies suggest that binding of these large glycoprotein ligands to their integrin receptors occurs via a sequential, multivalent mechanism involving a rapid, reversible interaction preceded by the formation of a stable, irreversible complex [12–15]. Adhesive regions in fibronectin, distinct from the RGD domain, have been identified but appear to interact relatively weakly with the $\alpha_{IIb}\beta_3$ complex [14], suggesting that they probably comprise secondary sites of contact which assist in stabilizing the receptor–ligand complex. This model of the binding mechanism of ligands has been reinforced by the recent demonstration that changes in the conformation of fibrinogen occur as a consequence of binding to the $\alpha_{IIb}\beta_3$ complex [16]. However, little is known about the biochemistry of ligand specificity and its inclusion into this developing model, that is, does the primary or secondary adhesive interaction confer the specific binding characteristics of a ligand? Furthermore, of the

Abbreviations used: TFA, trifluoroacetic acid; RGD, amino acid sequence arginine-glycine-aspartic acid; K_{dapp} , apparent equilibrium dissociation constant; K_{iapp} , apparent equilibrium inhibition constant; mAb, monoclonal antibody; PRP, platelet-rich plasma; PGE₁, prostaglandin E₁; R_T, total receptor concentration.

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several ligand-binding domains identified in both of the subunits of the $\alpha_{IIb}\beta_3$ complex by approaches involving the cross-linking of RGD and fibrinogen γ -chain peptides (reviewed in [17]), it is not known which, or whether all, of these regions are involved in physiological adhesion events, as synthetic peptides from either the binding domains of the receptor or the adhesive domains of ligands tend to display low affinity and probably poor selectivity of interaction in competition experiments. However, recent studies suggest that many of the putative ligand-binding regions of the $\alpha_{IIb}\beta_3$ complex are spatially clustered at the subunit interface (reviewed in [18]).

A further input into our understanding of the operational features of the $\alpha_{IIb}\beta_3$ complex–ligand interaction is emerging from the use of a novel class of RGD proteins present in snake venoms. These proteins, which include the disintegrin family [19–27] and the neurotoxin variant dendroaspin (also referred to as mambin [28–30]), are characterized by their high affinity of interaction (K_d 10^{-9} – 10^{-7} M) with both resting and activated forms of the $\alpha_{IIb}\beta_3$ complex and their capacity to inhibit potently $\alpha_{IIb}\beta_3$ -dependent adhesive events. This potency has been attributed to the positioning of the residues RGD at the apex of a flexible and solvent-exposed loop which protrudes away from the remainder of the protein structure [31–34].

We have previously reported the antagonistic properties of a selected panel of snake-venom RGD proteins, demonstrating the preferential inhibition of the adhesion of ADP-activated platelets to various immobilized glycoprotein ligands [35]. This preferential antagonistic behaviour led us to speculate on the existence of distinct binding sites for these ligands on the $\alpha_{IIb}\beta_3$ complex. In addition, based upon a comparative analysis of their primary structure, we postulated that the amino acid environment flanking the tripeptide RGD in the adhesive domains of these venom proteins may underlie their binding characteristics and thus inhibitory properties. In the present study, we provide further evidence in support of this hypothesis by analysing the binding characteristics of members of this panel of venom proteins to the integrin $\alpha_{IIb}\beta_3$ on platelets through radioligand kinetic and competition studies.

MATERIALS AND METHODS

Materials

Lyophilized snake venoms were obtained from either Latoxan (05150 Rosans, France) or Sigma (Poole, Dorset, U.K.). Fibrinogen was obtained from Kabi (Stockholm, Sweden; Grade L) and its purity and integrity were determined by SDS/PAGE. The $\alpha_{IIb}\beta_3$ -directed monoclonal antibody (mAb) PM6/248 was kindly donated by J. M. Wilkinson (London, U.K. [36] and a polyclonal antibody to the $\alpha_5\beta_1$ complex purified from human placenta was obtained from Gibco/BRL. Radioisotopic Na^{125}I (15.3 mCi/ μg of iodine) was purchased from Amersham (Amersham, Bucks., U.K.). Enzymobead Reagent was obtained from Bio-Rad Laboratories (Hemel Hempstead, U.K.). All other chemicals and reagents were obtained from Sigma and Milligen (Watford, Herts., U.K.).

Purification of venom proteins

Purification of elegantin, kistrin and dendroaspin was performed as previously described [29,37]. Briefly, the snake-venom proteins were purified from crude venoms by reversed-phase HPLC using a 0.1% trifluoroacetic acid (TFA)/acetonitrile solvent system. Lyophilized venom was reconstituted in 0.1% TFA for 10 min on ice and chromatographed on a Vydac C_{18} analytical column. Active fractions were identified by platelet aggregation assays

and rechromatographed to homogeneity. Purified proteins were characterized by either N-terminal sequencing or mass spectrometry.

Iodination of ligands

Iodination of venom proteins was performed using Enzymobead Radioiodination Reagent (Bio-Rad Laboratories) according to the manufacturer's specifications. Briefly, 20 μg of disintegrin was incubated with 1 mCi of carrier-free Na^{125}I (Amersham) in 10 mM sodium phosphate buffer, pH 7.3, containing 0.2% (w/v) glucose, in a volume of 150 μl including an appropriate quantity of Enzymobead suspension for 10–20 min at room temperature. ^{125}I -labelled protein was separated from free iodine by reversed-phased chromatography on Sep-Pak cartridges (Waters). Radio-labelled fractions contained greater than 90% trichloroacetic acid-precipitable isotopic activity and specific radioactivities ranging from $(2-5) \times 10^6$ c.p.m./ μg were routinely obtained. Radiolabelled fibrinogen was kindly donated by Dr. A. Cohen (King's College Hospital). ^{125}I -labelled fibrinogen bound to ADP-treated washed platelets in a saturable and cation-dependent manner with an apparent equilibrium dissociation constant (K_{dapp}) ranging from 300 to 400 nM.

Preparation of washed platelets

Washed platelets were prepared from platelet-rich plasma (PRP) obtained from healthy individuals who had not taken medication for 9 days before bleeding. PRP was obtained from blood containing 0.32% (w/v) trisodium citrate by centrifugation at 200 g for 15 min at room temperature. Platelets were harvested from plasma by centrifugation at 1200 g for 10 min at room temperature and resuspended in wash buffer (36 mM citric acid, 100 mM NaCl, 5 mM KCl, 0.5 mM CaCl_2 , 1 mg/ml glucose, 10 mM Pipes, pH 6.5) containing 1 μM prostaglandin E_1 (PGE_1) and 1 unit/ml apyrase (added just before centrifugation). After re-centrifugation and a further washing cycle in the presence of PGE_1 and apyrase, platelets were finally resuspended in adhesion buffer (145 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 2 mM CaCl_2 , 10 mM glucose, 3.5 mg/ml BSA, 10 mM Hepes, pH 7.35) and adjusted to concentrations of $(3-5) \times 10^8$ cells/ml. Washed platelet suspensions were left to sit for approx. 30 min at room temperature to allow the intracellular cyclic AMP concentrations to return to basal levels and then tested for their ability to aggregate in the presence of exogenously added fibrinogen (1.67 mg/ml) using 10 μM ADP as an agonist before their use in ligand binding experiments.

Platelet aggregation

Platelet aggregation, performed with washed platelets (3×10^8 /ml) in a final reaction volume of 320 μl , was measured as an increase in light transmission as described previously [37]. Platelet aggregation was induced with ADP (final concn. 50 μM) and supported by the addition of 1.67 mg/ml (final concentration) fibrinogen to the resuspension buffer. The experiments were performed using a four-chambered automated aggregometer (DIC Aggregometer II, Daiichi Kagaku Co, Kyoto, Japan) linked to a chart recorder.

Ligand binding studies

The binding of ^{125}I -labelled venom proteins to washed platelets was performed using a modification of the method of Huang et al. [15]. Briefly, the assay allows binding of radioligands to ADP-activated platelets at room temperature in the absence of stirring

or detectable platelet aggregation. 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 ligand, 5–20 μ l of competitor or antibody and made to a final volume of 350 μ l. In antibody inhibition studies, platelet suspensions were treated with antibody for 30 min prior to exposure to ADP and the addition of 125 I-labelled venom protein and the mixture was incubated at room temperature for a further 60 min. For snake venom equilibrium competition studies, 125 I-labelled tracer ligand, competitor ligand and ADP were added simultaneously to the platelet suspension and the incubation period was extended to 60 min (125 I-labelled kistrin) or 80 min (125 I-labelled elegantin) to ensure equilibrium. At equilibrium, the platelet suspension (300 μ l) was then loaded on to a cushion of 25% (w/v) sucrose solution containing 1% (w/v) BSA and centrifuged at 12000 g for 10 min to obtain the platelet pellet. 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 125 I-labelled fibrinogen-binding inhibition experiments a similar assay procedure was used as described above except that higher platelet concentrations of 5×10^8 /ml were used. Both 125 I-labelled fibrinogen (300 nM) and competitor were added simultaneously and suspensions were incubated for 60 min at room temperature. Under these conditions, approx. 19000 fibrinogen dimers were bound per platelet in the absence of competitors.

Derivation of equilibrium constants

The term apparent equilibrium dissociation constant (K_{dapp}) has been adopted throughout the paper as the estimation of dissociation constants were made from saturation binding experiments employing high receptor concentrations, and were not determined as a ratio of the rate constants of the dissociation and association of receptor–ligand complexes. From direct binding experiments (in the absence of competitors) with 125 I-labelled ligands, K_{dapp} values were calculated as the reciprocal of the slope of the isotherm derived by the method of Scatchard [38]. For kistrin/dendroaspin competition studies, an indirect K_{dapp} value for dendroaspin was estimated from the K_{iapp} value (see Figure 2) using the formula:

$$K_{dapp} = K_{iapp}/(A + B)$$

with $A = [2(L^* - L_0^*)/L_0^* + 1 + KL^*]$ and defines a component relating to the occurrence of tracer ligand depletion [39] and $B = (1 + L_t^*/K_{dapp}^*)$ which defines the component relating to the effect of the tracer ligand concentration [40]; where L^* is the free tracer ligand concentration at equilibrium, L_0^* is the free ligand concentration at equilibrium in the absence of competitor, L_t^* is the total tracer ligand concentration and K_{dapp}^* is the apparent equilibrium dissociation constant of the tracer ligand (kistrin). For competition studies analysed by the double reciprocal plot, the K_{iapp} values were calculated from the equation:

$$K_{iapp} = I/([M_1/M_0] - 1)$$

where I is the inhibitor (competitor) concentration, M_1 the gradient of the isotherm derived in the presence of inhibitor at concentration I , and M_0 the gradient of the isotherm derived in the absence of inhibitor.

Analytical procedures

SDS/PAGE was performed according to the method of Laemmli [41]. MS was carried out using a Finnigan MAT Atmospheric Pressure Ionization SSQ 710 C spectrometer.

RESULTS

In a previous study [35] we reported that a selected panel of snake-venom RGD proteins comprising the disintegrins kistrin [19], elegantin [20] and albolabrin [20], and the neurotoxin variant dendroaspin [28–30] showed distinctive inhibitory preferences towards the interactions of the $\alpha_{IIb}\beta_3$ complex with the different immobilized glycoproteins as summarized in Table 1. To develop further insights into the mechanisms underlying the inhibitory properties of these venom proteins, we sought to determine the binding characteristics of three members of this panel of inhibitors, kistrin, dendroaspin and elegantin, by radioligand kinetic and equilibrium competition studies.

Kistrin and dendroaspin bind to a similar site on the $\alpha_{IIb}\beta_3$ complex

The similar inhibitory properties of kistrin and dendroaspin as determined by platelet aggregation in PRP [28,29,37] and platelet adhesion studies ([35], Table 1), led us speculate that these two structurally unrelated venom proteins might share similar binding characteristics to the $\alpha_{IIb}\beta_3$ complex. Equilibrium binding studies were therefore performed using an unstirred assay system incorporating washed platelets at a concentration of 3×10^8 /ml and ADP as an agonist. The platelet density, which corresponds to a total receptor concentration (R_T) of approx. 25 nM (assuming 50000 copies of the $\alpha_{IIb}\beta_3$ complex per platelet [42]), was chosen to mimic physiological levels, thereby allowing a more accurate comparison of radioligand binding and functional data. Under the conditions of this assay, the platelets did not aggregate as no increase in light transmission was observed when suspensions were incubated in a Payton aggregometer (results not shown).

The binding of 125 I-labelled kistrin to ADP-treated platelets was saturable and bivalent cation-dependent, with a $K_{dapp} = 42 \pm 2$ nM ($n = 3$, Figure 1a). Analysis of the data by the method of Scatchard [15] produced a simple monophasic isotherm, indicating that kistrin bound to a single class of binding site on the $\alpha_{IIb}\beta_3$ complex (β_{max} , approx. 209000 ± 20000 copies per platelet). Binding of kistrin was reversible as judged by the addition of a 100-fold excess of unlabelled ligand at equilibrium (results not shown). At the high receptor concentrations used in the assay system, depletion of the free kistrin levels was observed. At equilibrium, the bound fraction of the total ligand present in the incubation mixture varied from 0.72, at a kistrin concentration of 10 nM, to 0.33 at apparent saturation (250 nM), manifested as a rightward shift in the observed EC_{50} value of 65 nM (Figure 1a, inset) by a factor approximately equal to

Table 1 Preferential antagonism of platelet adhesion to immobilized glycoprotein ligands^a

Abbreviations: Fg, fibrinogen; Fn, fibronectin; vWF, von Willebrand factor.

Inhibitor	Relative efficacy of inhibition ^b		
	Fg	Fn	vWF
Kistrin	+++++	+	+++++
Dendroaspin	+++++	+	+++++
Elegantin	++	+++	+++++
Albolabrin	+++	+	+++++

^a Summarized from [35].

^b +++++, IC_{50} below 0.3 μ M; ++++, IC_{50} below 1.0 μ M; ++, IC_{50} below 5.0 μ M; +, IC_{50} below 10.0 μ M; ., IC_{50} > 20.0 μ M.

Figure 1 Scatchard analysis of ^{125}I -labelled kistrin and ^{125}I -labelled dendroaspin binding to ADP-treated platelets

Various concentrations (10–250 nM) of ^{125}I -labelled kistrin (a) and ^{125}I -labelled dendroaspin (b) were incubated with ADP-treated washed platelets ($3 \times 10^8/\text{ml}$) for 30 min at room temperature in a volume of 350 μl . Bound and free levels of kistrin/dendroaspin were determined by loading 300 μl of the platelet suspension on to a cushion of 25% (w/v) sucrose/1% BSA and centrifuging for 10 min at 12000 g . Both the radioactivity in the platelet pellets and supernatants were determined. Insets: Saturation isotherms of ^{125}I -labelled kistrin and ^{125}I -labelled dendroaspin binding to ADP-treated washed platelets in the presence (●) and absence (▲) of 10 mM EDTA. The data are representative of three similar experiments with all points determined in triplicate (S.E.M. values were less than 10%)

$0.5 \times R_T$, i.e. 12.5 nM [43]. Compensating for this rightward shift, the EC_{50} value of kistrin is re-adjusted to approx. 42.5 nM, a value that closely correlates with the calculated K_{dapp} . The occurrence of kistrin depletion observed in these equilibrium binding assays is likely to reflect the internalization of $\alpha_{\text{IIb}}\beta_3$ -kistrin complexes previously observed by Handagama and co-workers [44] producing an inflated β_{max} value derived by Scatchard analysis. The occurrence of kistrin depletion would not be expected to affect the affinity of its interaction with the $\alpha_{\text{IIb}}\beta_3$ complex and thus the estimation of the K_{dapp} . In agreement with this notion, Dennis and co-workers [19] have reported a K_{dapp} for kistrin of 1.7 nM, a value obtained using an assay system with a 25-fold lower platelet concentration corresponding to an R_T of approx. 1 nM. Since K_{dapp} has been predicted to increase proportionally with R_T (i.e. tissue concentration [45]), the K_{dapp} value of 42 ± 2 nM determined for kistrin in the present study is, by proportion, in good agreement with that previously reported by Dennis et al.

The binding characteristics of dendroaspin to the $\alpha_{\text{IIb}}\beta_3$

Table 2 Binding characteristics of RGD venom proteins

Abbreviations: P.S., present study. All the data correspond to platelets activated with ADP alone or in synergy with other weak agonists.

Proteins	K_{dapp} (nM) ^a	β_{max} ($\times 10^3/\text{platelet}$)	References
Dendroaspin	22	33	P.S.
Kistrin	42	200	P.S.
Halysin	34	45.5	[25]
Trigramin	21–47	16.5	[15]
Applagin	56	42	[21,22]
Flavoviridin	73	35	[26]
Albolabrin	80	90	[23]
Barbourin	140	—	[24]
Elegantin ^b	(H)10.5	4	P.S.
	(L)175	25	
Echistatin	180	32	[21,27]

^a The data were compiled from studies using platelet counts of $10^8/\text{ml}$ or greater (R_T = approx. 25 nM).

^b H, high-affinity binding site; L, low-affinity binding site.

complex were determined both directly using ^{125}I -labelled dendroaspin and indirectly by equilibrium competition studies using ^{125}I -labelled kistrin as a tracer ligand [43,46]. The binding of ^{125}I -labelled dendroaspin to ADP-treated platelets was saturable and bivalent cation-dependent (Figure 1b, inset). Scatchard analysis of the binding data indicated that dendroaspin also bound to a single class of binding site on the $\alpha_{\text{IIb}}\beta_3$ complex with a mean K_{dapp} of 21.3 nM ($n = 2$), with approx. 29000–37000 sites per platelet. Binding of dendroaspin to the $\alpha_{\text{IIb}}\beta_3$ complex was reversible as the addition of a 100-fold excess of unlabelled ligand at equilibrium produced a 50% dissociation of ^{125}I -labelled dendroaspin in 15 min. This dissociation rate is of a similar order of magnitude to that observed for kistrin in the present study and previously reported using ADP-activated platelets [19]. Although depletion of free dendroaspin levels was observed at low concentrations (bound fraction = 0.35 at a total ligand concentration of 10 nM), these levels of ligand depletion were significantly less than those observed for kistrin and were not observed at concentrations approaching saturation (bound fraction = 0.064 at a ligand concn. of 250 nM). The equilibrium binding parameters obtained for both kistrin and dendroaspin with ADP-treated platelets fall within the range observed for disintegrins in other laboratories using a similar assay procedure (Table 2).

In order to assess comprehensively the binding characteristics of both kistrin and dendroaspin, competition studies were performed by analysing the binding of a single concentration of ^{125}I -labelled kistrin in the presence of increasing amounts of dendroaspin (Figure 2a) and, reciprocally, by varying the concentration of ^{125}I -labelled kistrin in the presence of a single concentration of dendroaspin (Figure 2b). In the former analysis, dendroaspin inhibited the binding of ^{125}I -labelled kistrin to ADP-treated platelets in a dose-dependent manner, promoting a maximal 80% inhibition with an IC_{50} of approx. 156 nM (Figure 2a). The incomplete displacement of kistrin by dendroaspin (80% as opposed to 100%) is consistent with the notion of an intracellular pool of ^{125}I -labelled kistrin, produced as a consequence of the internalization of $\alpha_{\text{IIb}}\beta_3$ -kistrin complexes which are inaccessible to external competitor. In contrast with dendroaspin, elegantin did not inhibit the binding of ^{125}I -labelled kistrin to ADP-treated platelets even when present at 8-fold higher concentrations. Using the latter assay design, the in-

Figure 2 Inhibition of ^{125}I -labelled kistrin binding to activated platelets by dendroaspin and elegantin

(a) ^{125}I -labelled kistrin (125 nM) was incubated with ADP-activated washed platelets in the presence of various amounts of dendroaspin (●) and elegantin (▲) for 60 min at room temperature. Bound kistrin levels were determined as explained in the legend to Figure 1. The radioactivity in both pellets and supernatants was determined. Results are expressed as a percentage of the bound kistrin levels determined in the presence of competitors. The data are a compilation of three experiments. Inset: Indirect determination of the binding characteristics of dendroaspin by ^{125}I -labelled kistrin displacement using the method of Scatchard reveals a $K_{\text{dapp}} = 153 \pm 38$ nM using the Enzfitter program. This value of K_i was used to determine the indirect $K_{\text{dapp}} = 22 \pm 5.5$ nM for dendroaspin by application of correction factors accounting for the kistrin concentration and depletion (see the Materials and methods section). (b) Double reciprocal plot of ^{125}I -labelled kistrin binding to activated platelets in the presence and absence of dendroaspin. ^{125}I -labelled kistrin was incubated with ADP-activated washed platelets at various concentrations (10–250 nM) in the absence (□) and presence of dendroaspin at 300 nM (○) and 600 nM (△) for 60 min at room temperature. Bound kistrin levels were determined as described above. The data are representative of two similar experiments with points determined in triplicate, and identical $K_{\text{dapp}} = 143 \pm 10$ nM were obtained from each inhibition curve.

inhibition of ^{125}I -labelled kistrin binding by dendroaspin followed simple linear competitive kinetics as indicated by the analysis of equilibrium binding data by the double reciprocal plot (Figure 2b). The K_{dapp} for dendroaspin, calculated from the slopes of each inhibition curve (300 and 600 nM dendroaspin, see legend), produced identical values of 143 ± 14 nM in accordance with the behaviour of a system conforming with simple linear competitive kinetics, i.e. K_i is independent of the inhibitor concentration [46].

Furthermore, the K_{dapp} value of 143 nM is in close agreement with the IC_{50} value of 156 nM obtained from the inhibition profile of dendroaspin shown in Figure 2(a). Making the assumption that a single molecule of dendroaspin displaces a single molecule of kistrin, the data presented in Figure 2(a) were used to determine the free and bound concentrations of dendroaspin and analysed by the method of Scatchard (Figure 2a, inset). The resulting monophasic isotherm is consistent with the results obtained from the direct binding studies with ^{125}I -labelled dendroaspin, confirming that dendroaspin binds to a single class of binding site on the $\alpha_{IIb}\beta_3$ complex and produced a $K_{\text{dapp}} = 153 \pm 36$ nM. However, this analysis produced an inflated estimate of the number of dendroaspin binding sites of approx. 100000 copies per platelet, probably as a consequence of the tracer ligand (kistrin) depletion. Using the K_{dapp} value of 153 nM, a K_{dapp} for dendroaspin of 22 nM was determined by application of correction factors accounting for tracer ligand (kistrin) depletion [39], and the Cheng–Prusoff factor relating to tracer ligand concentration ([40], see the Materials and methods section). Thus, the estimation of K_{dapp} for dendroaspin by the method of kistrin displacement is in excellent agreement with the value obtained by the direct binding of ^{125}I -labelled dendroaspin to the $\alpha_{IIb}\beta_3$ complex. These observations provide strong evidence that kistrin and dendroaspin bind to an identical site on the $\alpha_{IIb}\beta_3$ complex.

Elegantin recognizes two classes of binding site on the $\alpha_{IIb}\beta_3$ complex

Platelet adhesion studies indicated elegantin as having distinct inhibitory preferences from those exhibited by kistrin and dendroaspin (Table 1). The binding characteristics of this disintegrin to the $\alpha_{IIb}\beta_3$ complex were, therefore, determined for comparison. In common with kistrin and dendroaspin, ^{125}I -labelled elegantin bound to ADP-treated washed platelets in a saturable, cation-dependent manner (Figure 3a, inset). However, analysis of the equilibrium binding data by the method of Scatchard revealed that elegantin recognized two classes of binding site on the platelet surface; a high-affinity site of $K_{\text{dapp}} = 10.5 \pm 0.8$ nM with approx. 4000 sites per platelet, and a lower-affinity site of $K_{\text{dapp}} = 175 \pm 10$ nM with approx. 25000 sites per platelet. To determine whether both classes of elegantin-binding sites were present on the $\alpha_{IIb}\beta_3$ complex, the efficacy of the $\alpha_{IIb}\beta_3$ -specific mAb PM6.248 [36] at blocking the binding of both low (30 nM) and higher (175 nM) concentrations of ^{125}I -labelled elegantin to ADP-treated platelets was investigated (Figure 3b). The results of these experiments demonstrated that the monoclonal reagent PM6.248 was an effective inhibitor of ^{125}I -labelled elegantin binding to platelets, promoting a maximal 80 % inhibition at both low and high concentrations of ^{125}I -labelled elegantin. By contrast, a polyclonal reagent with specificity for the $\alpha_5\beta_1$ integrin complex was comparatively ineffective. Taken together, these results suggest that both classes of elegantin-binding sites identified by the equilibrium binding analysis are present on the $\alpha_{IIb}\beta_3$ complex. Further evidence in support of this notion was obtained from the analysis of the association kinetics of elegantin (Figure 3c). Bound levels of elegantin at pre-equilibrium were maximally (at 2 min) 3-fold higher than bound levels at equilibrium (40 min). In contrast, the association kinetics of kistrin and dendroaspin followed a simple rectangular hyperbola with equilibrium attained at approx. 5 min. The complex association kinetics of elegantin confirms that its mechanism of binding to the $\alpha_{IIb}\beta_3$ complex is distinctive from that of kistrin and dendroaspin, in accordance with the presence of additional binding sites, but the precise underlying

Figure 3 Binding characteristics of ¹²⁵I-labelled elegantin to ADP-treated platelets

(a) Various concentrations of ¹²⁵I-labelled elegantin were incubated with ADP-treated washed platelets (3×10^8 /ml) for 45 min at room temperature. Bound and free levels of ¹²⁵I-labelled elegantin were determined as described in the legend to Figure 1 and analysed by the method of Scatchard. The biphasic isotherm indicates two classes of binding sites of $K_{dapp} = 10.5 \pm 0.8$ nM (approx. 4000 sites per platelet) and 175 ± 10 nM (approx. 25000 sites per platelet). Inset: Saturation isotherm for ¹²⁵I-labelled elegantin binding to ADP-treated platelets in the presence (●) and absence (▲) of 10 mM EDTA. (b) Inhibition of ¹²⁵I-labelled elegantin binding to ADP-treated platelets by mAb PM6/248. Platelet suspensions were incubated with various concentrations of the $\alpha_{IIb}\beta_3$ -specific mAb PM6/248 (filled symbols) or a polyclonal antibody raised against the integrin $\alpha_5\beta_1$ (open symbols) for 30 min before addition of ¹²⁵I-labelled elegantin at 30 nM (▲) and 170 nM (●) and ADP. Platelet suspensions were then incubated at room temperature for a further 60 min. Bound levels of ¹²⁵I-labelled elegantin were determined as explained in the legend to Figure 1. The results are expressed as percentage inhibition relative to bound levels of ¹²⁵I-labelled elegantin observed in the absence of antibody. (c) Association kinetics of ¹²⁵I-labelled elegantin (●), ¹²⁵I-labelled kistrin (▲) and ¹²⁵I-labelled dendroaspin (□) to ADP-treated platelets. ¹²⁵I-labelled venom proteins (30 nM) were incubated with ADP-treated platelets for various lengths of time before determination of the bound and free ligand concentrations as described previously. Results are expressed as percentage ligand bound relative to bound levels at equilibrium. All data shown comprise either a representative or a compilation of two independent experiments with points determined in triplicate.

Figure 4 Purity of the elegantin preparations

(a) SDS/PAGE analysis of elegantin (lane 2) and kistrin (lane 3) preparations using a 20% (w/v) separating gel. Approx. 3.0 μg of each purified disintegrin was loaded. Lane 1 contains molecular mass standards as indicated in kDa. (b) HPLC chromatogram of a typical elegantin preparation using a reversed-phase TFA/acetonitrile buffer system.

mechanism is unknown. As this mode of tracer association kinetics has been observed in the presence of competitor ligands [43], the elegantin preparations were carefully monitored for the presence of contaminants. Two independent criteria, SDS/PAGE analysis and reverse-phase HPLC (Figures 4a and 4b) indicated that the preparations were indeed homogeneous. Similarly to kistrin, the binding of elegantin to the $\alpha_{IIb}\beta_3$ complex was reversible with 49% dissociation occurring 15 min after the addition of a 100-fold excess of unlabelled ligand at equilibrium (results not shown) but, unlike kistrin, depletion of the free ligand was not observed.

The lower-affinity elegantin-binding site is distinct from the kistrin/dendroaspin-binding site

Competition studies using ¹²⁵I-labelled kistrin as a tracer ligand revealed that, in contrast with dendroaspin, elegantin was unable to compete for binding to the $\alpha_{IIb}\beta_3$ complex, even at concentrations (1 μM) that would compensate for differences in the K_{dapp} between the two ligands (Figure 2a). To investigate this behaviour further, competition experiments with ¹²⁵I-labelled elegantin were performed using a tracer concentration range corresponding to the limits of the lower-affinity site (see Figure 3a). In these experiments, both kistrin and dendroaspin effectively

Figure 5 Inhibition of ^{125}I -labelled elegantin binding to ADP-treated platelets by dendroaspin and kistrin

Various concentrations of ^{125}I -labelled elegantin (25–250 nM) were incubated with ADP-treated platelets in the absence and presence of the competitors dendroaspin (a) and kistrin (b) for 80 min at room temperature. In (a), ^{125}I -elegantin was incubated in the absence ($\square$) and presence of 100 nM ($\circ$) and 400 nM ($\triangle$) dendroaspin. In (b), ^{125}I -labelled elegantin was incubated in the absence ($\circ$) and presence of 50 ($\bullet$), 100 ($\triangle$) and 150 nM ($\square$) kistrin. Bound and free levels of ^{125}I -labelled elegantin were determined as described for Figure 1. Both dendroaspin and kistrin inhibited ^{125}I -labelled elegantin binding in a non-competitive manner with $K_{\text{app}} = 21 \pm 2$ and 34 ± 3 nM respectively. All points were determined in triplicate (S.E.M. less than 10%).

inhibited the binding of ^{125}I -labelled elegantin to ADP-treated platelets (Figures 5a and 5b). In contrast to the inhibitory mechanisms observed between kistrin and dendroaspin, both of these ligands inhibited the binding of ^{125}I -labelled elegantin in a non-competitive manner, i.e. reducing the availability of elegantin-binding sites (lowering the value of β_{max}) without altering the affinity status of remaining sites. Notably, the K_{app} values obtained for kistrin and dendroaspin in these experiments ($K_{\text{app}} = 34 \pm 3$ and 21 ± 2 nM respectively) are in excellent agreement with the K_{dapp} values obtained for these ligands in direct ^{125}I -labelled ligand binding experiments (Figures 1 and 2), indicating that the binding affinities of both kistrin and dendroaspin were unaffected in the presence of elegantin. These findings are consistent with the observation that elegantin did not inhibit the binding of ^{125}I -labelled kistrin (Figure 2a), indicating a 'one-way' inhibitory mechanism. Furthermore, the observation that both kistrin and dendroaspin inhibit the binding of ^{125}I -labelled elegantin to the $\alpha_{IIb}\beta_3$ complex via a similar non-competitive mechanism provides yet further evidence that these

Figure 6 Inhibition of platelet aggregation and ^{125}I -labelled fibrinogen binding to washed platelets by venom proteins

(a) Washed platelets ($3 \times 10^8/\text{ml}$) were incubated for 3 min at 37°C with various concentrations of either kistrin ($\triangle$), dendroaspin ($\square$) or elegantin ($\bullet$) before addition of ADP ($50 \mu\text{M}$), and aggregation was measured for 2 min (maximal aggregation). Results are expressed as percentage inhibition relative to levels of aggregation observed in the absence of inhibitors. The data are a compilation of two separate experiments with points determined in duplicate. (b) Washed platelets ($5 \times 10^8/\text{ml}$) were incubated with ^{125}I -labelled fibrinogen (300 nM) and various concentrations of venom protein as indicated above in the presence of ADP ($50 \mu\text{M}$) for 60 min at room temperature. Bound and free levels of ^{125}I -labelled fibrinogen were determined as described in the legend to Figure 1. Results are expressed as percentage inhibition relative to the levels of bound ^{125}I -labelled fibrinogen in the absence of inhibitors. The data are a compilation of two separate experiments with points determined in triplicate (S.E.M. approx. 10%).

two structurally unrelated proteins bind to an identical site on the $\alpha_{IIb}\beta_3$ complex.

Inhibition of the interactions of fibrinogen with the $\alpha_{IIb}\beta_3$ complex by snake-venom RGD proteins

The efficacy of snake venom-derived RGD proteins at inhibiting the binding of ^{125}I -labelled fibrinogen to the $\alpha_{IIb}\beta_3$ complex and abrogating platelet aggregation is well documented [21–27]. In the present study, we analysed the inhibition of the interaction of soluble fibrinogen with the $\alpha_{IIb}\beta_3$ complex by the venom proteins in both platelet aggregation and ^{125}I -labelled fibrinogen equilibrium competition studies. In contrast with the observations of the platelet adhesion studies using immobilized fibrinogen ([35]; Table 1), kistrin, dendroaspin and elegantin were all potent inhibitors of ADP-induced platelet aggregation in washed platelet suspensions producing IC_{50} values of approx. 40, 50 and 120 nM respectively (Figure 6a). In addition, the venom

1	5	10	15	20	25	30	35	40	45	50	55	60	65	70	
EAGEDCDCGSPANPCCDAATCKLLPGAQCGEGLCCDQCSFMKKGTICRRARGDDLDYYCNGISAGCPRNPLHA															Ab
-----R-----R-E-R-----NP--R-T-Q--D-----GLY															Eg
-K---S-E-----K-S-A-K--IP--MP--R-T---D---YH															Ks.
RI-YNHLGTKPPTTET-Q EDS-YKNIWTFD NIIRRGCG-FTP--MPGP--CESDKCNL															Dn
---F--QS-Q-Q--K--SPG-S---HKQ-SDFRGT--E-----P-VKPGIKLS -----V-N															E.b

to the structural similarities and differences between the snake-venom ligands characterized. The finding that the structurally unrelated proteins kistrin and dendroaspin, which have identical amino acid residues flanking the tripeptide RGD (Table 3), bind to identical sites on the $\alpha_{\text{IIb}}\beta_3$ complex implies an important role for these amino acids in regulating the binding characteristics of this class of ligand. In addition, kistrin and elegantin share a high degree of amino acid sequence similarity and are likely to share significant structural homology, yet these two disintegrins appear to exhibit distinctive binding and functional characteristics. It is noteworthy that kistrin and elegantin have different amino acid residues flanking the sequence RGD (Table 3), further highlighting an important functional role for these residues. Consistent with this view are the studies of Dennis and co-workers [54] who demonstrated, by alanine replacement mutagenesis, that residues outside of the RGD loop of kistrin do not appear to regulate the function of this protein provided that the native structure is not perturbed. Although alanine replacement of residues flanking the sequence RGD in kistrin had a minor effect on functional performance, only replacement of Arg⁴⁹ and Asp⁵¹ produced a drastic reduction in the activity of this disintegrin. Thus, residues flanking the sequence RGD in disintegrins appear not to be critical for integrin binding as such, but in light of our observations and those of others [55,56], we speculate that they act as specificity regulators by providing a specific chemical environment and/or promoting a specific conformation in which the reactive nucleus provided by the tripeptide RGD is presented. Further support for this notion is emerging from the biochemical characterization of integrin-mediated viral infection [57,58]. For example, Reider et al. [57] have observed that natural variants of the foot and mouth disease virus (FMDV), which bound poorly to cultured BHK cells compared with tissue-culture-adapted viruses, contained amino acid sequence variation of residues adjacent to the sequence RGD in the primary structure of the capsid protein VP1. Furthermore, these workers noted that the continued passage of naturally isolated viruses in culture engendered the rapid selection of variants with high cell binding activity (infectivity) resulting from variation of the amino acid sequence adjacent to the tripeptide RGD to those sequences prevalent in tissue-culture-adapted viruses. Taken in conjunction with the findings of the present work, these observations indicate that the variation of the amino acids immediately flanking, or in close linear proximity to, the residues RGD is possibly a widespread mechanism by which the specificity of RGD-dependent ligands towards their receptors can be modulated. The functional characterization of recombinant snake-venom proteins with specific modifications to amino acids flanking the residues RGD is currently in progress.

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