RESEARCH COMMUNICATION

Arg-Tyr-Asp (RYD) and Arg-Cys-Asp (RCD) motifs in dendroaspin promote selective inhibition of β_1 and β_3 integrins

Beth WATTAM*, Dazhuang SHANG*, Salman RAHMAN†, Sofia EGGLEZOU*, Mike SCULLY*, Vijay KAKKAR* and Xinjie LU*¹

*Thrombosis Research Institute, Manresa Road, London SW3 6LR, U.K., and †The Coagulation Research Laboratory, School of Medicine, Guy's, King's and St Thomas' Hospital (GKT), St Thomas' Campus, Lambeth Palace Road, London SE1 7EH, U.K.

Arg-Gly-Asp (RGD) is a unique minimal integrin-binding sequence that is found within several glycoprotein ligands. This sequence has also been found in snake-venom anti-platelet proteins, including the disintegrins and dendroaspin, a natural variant of short-chain neurotoxins isolated from the venom of *Dendroaspis jamesonii*. In the present study, the motifs RYD and RCD were introduced into the dendroaspin scaffold to replace RGD. Both motifs in dendroaspin caused inhibition of ADP-induced platelet aggregation with IC_{50} values of 200 and 300 nM respectively, similar to that of the wild-type RGD motif (170 nM). In comparison with wild-type dendroaspin, both RYD- and RCD-containing dendroaspins were more selective in the inhibition of the adhesion of K562 cells to laminin rather

than to fibrinogen and fibronectin, even though they were 10–30-fold less potent at inhibiting K562 cell (containing $\alpha_5\beta_1$ integrin) adhesion to laminin compared with wild-type. Interestingly, the RYD motif produced a similar IC_{50} value to the RGD motif at inhibiting A375-SM cell (β_3 integrin) adhesion to collagen, whereas the RCD motif was approx. 2–6-fold less potent compared with either RGD or RYD. These findings show that the selectivity of dendroaspin binding to β_1 and β_3 integrins can be modulated by the introduction of alternative cell recognition sequences.

Key words: cell adhesion, disintegrin, platelet aggregation.

INTRODUCTION

Integrins are a family of heterodimeric class I transmembrane receptors that mediate cell–matrix and cell–cell adhesion phenomena [1]. Two members of the β_3 integrin subgroup are $\alpha_{IIb}\beta_3$, which is essential for platelet aggregation and controls platelet function in haemostasis and thrombosis [2,3], and $\alpha_v\beta_3$, which is expressed on many cells, where it influences cell migration, tumour cell metastasis and angiogenesis [4,5]. Apart from $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$, platelets also contain other integrins, including $\alpha_2\beta_1$ (collagen receptor), $\alpha_5\beta_1$ (fibronectin receptor) and $\alpha_6\beta_1$ (laminin receptor), which mediate platelet functions.

A significant advance in the understanding of integrin function was the identification of the minimal integrin-binding sequences within various ligands. The prototypic example is the Arg-Gly-Asp (RGD) sequence originally identified as the sequence in fibronectin that engages the fibronectin receptor, integrin $\alpha_5\beta_1$ [6], but which is now known to serve as a recognition motif in multiple ligands present within the extracellular matrix (e.g. vitronectin, von Willebrand Factor, collagen, thrombospondin and osteopontin) and is recognized by several different integrins (e.g. $\alpha_3\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_6$ and $\alpha_{IIb}\beta_3$) [7]. The other aspartate-containing motifs, such as AGDV found near the C-terminus of the γ -chain of human fibrinogen [8], DGEA corresponding to residues 435–438 of the $\alpha 1(I)$ chain of collagen [9], and LDV, which is located in an alternatively-spliced region of the fibronectin A chain [10] and was recently introduced into the venom protein kistrin [11], are recognized by integrins $\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$ and $\alpha_4\beta_1$ respectively.

The RGD motif has also been found in proteins isolated from the venoms of crotalid and viperid snakes. The majority of the

RGD-containing snake venom proteins range in length from 49–84 amino acid residues, are known as disintegrins and function as potent inhibitors of platelet aggregation and fibrinogen binding to $\alpha_{IIb}\beta_3$ [12]. The first such disintegrin was trigramin, isolated from the venom of *Trimeresurus gramineus* [13]. Subsequently, many such venom proteins have been identified, including kistrin [14] from the venom of *Calloselasma rhodostoma*, elegantin [15] from the venom of *Trimeresurus elegans* and a natural variant of a short chain neurotoxin, dendroaspin, from the venom of *Dendroaspis jamesonii* [16–18]. Variants of the RGD motif have also been found within the disintegrins, namely KGD in venom proteins of *Sistrurus malaris barbouri* and *Agkistrodon ussuriensis* that binds specifically to $\alpha_{IIb}\beta_3$ [19,20], and MLGD and VGD motifs in the venom protein of *Echis carinatus* which bind to $\alpha_4\beta_1$ and $\alpha_5\beta_1$ [21].

The carboxy group of RGD and the guanidinium group within the RGD motif are postulated to interact with distinct structural elements in the α and β subunits of an integrin [22]. In this respect, the RGD adhesive sequence will give rise to electrostatic as well as hydrogen bonding, for which several hydrogen-bond acceptors and donors from the tripeptide main chain are thought to elicit a variety of contacts with elements in the integrin receptor [23]. Therefore binding specificity between RGD ligands and their cognate integrins may lie in subtle positional requirements of the charged side chains of the arginine and aspartate residues, in addition to the orientation of the peptide backbone.

Our previous studies have shown that dendroaspin can be used as a scaffold for the investigation of determinants of the RGD–integrin interaction [24,25]. To gain further insight into the molecular basis for receptor binding specificity, in the present

Abbreviations used: DTT, dithiothreitol; GST, glutathione S-transferase.

¹ To whom correspondence should be addressed (e-mail xlu@tri-london.ac.uk).

paper we report the characterization of two recombinant dendroaspin molecules in which the RGD motif has been converted into Arg-Tyr-Asp (RYD) or Arg-Cys-Asp (RCD). The molecules were characterized as inhibitors of platelet aggregation and cell adhesion.

MATERIALS AND METHODS

Materials

Restriction enzymes, T4 polynucleotide kinase, T4 DNA ligase, isopropyl β -D-thiogalactoside and DH5 α competent cells were purchased from either Life Technologies (Paisley, Renfrewshire, Scotland, U.K.) or Promega (Chilworth, Southampton, U.K.). Vent (exo-) DNA polymerase was supplied by New England Biolabs (Hitchin, Herts., U.K.). Proteinase Factor Xa was purchased from Boehringer Mannheim (Lewes, East Sussex, U.K.). Oligonucleotides were made by Cruachem Ltd (Glasgow, U.K.). Deoxynucleotide triphosphates, the plasmid pGEX-3X [a vector that expresses a cloned gene as a fusion protein linked to glutathione S-transferase (GST)], and GSH-Sepharose CL-4B were purchased from Amersham Pharmacia Biotech (St Albans, Herts., U.K.). The Gel extraction kit and the Plasmid maxi kit were purchased from Qiagen (Crawley, West Sussex, U.K.). Human fibrinogen and collagen type I were supplied by Calbiochem-Novabiochem Corporation (Nottingham, U.K.). Fibronectin and laminin were purchased from Sigma (Poole, Dorset, U.K.). Monoclonal antibodies 11A1 and P1D6, recognizing the integrin α_5 subunit were purchased from Becton Dickinson UK Ltd (Oxford, U.K.) and Chemicon (Harrow, U.K.) respectively. P2, recognizing α_{11b} , was obtained from Immunotech (Marseille, France). 7E3, recognizing the β_3 subunit, was obtained from the Pharmacy of Royal Brompton Hospital (London, U.K.). The other monoclonal antibodies, including LM609, recognizing human integrin $\alpha_v\beta_3$, JBS2 and P1E6, recognizing $\alpha_2\beta_1$, PC42 and P1H4, recognizing the α_4 subunit, JBS5, recognizing $\alpha_5\beta_1$, 4F10, recognizing the α_6 subunit, 6S6 and P4G11, recognizing β_1 ; YFC118.3, recognizing the β_2 subunit, and P3G8, recognizing α_v , were supplied by Chemicon. The transfected K562 human erythroleukaemia cell line was described previously [26], and the A375-SM human melanoma cell line was a gift kindly provided by Professor M. J. Humphries (School of Biological Sciences, University of Manchester, Manchester, U.K.).

Construction of synthetic genes of wild-type, RYD- and RCD-dendroaspin

A synthetic wild-type dendroaspin gene was constructed by ligating ten complementary and overlapping oligonucleotides coding for the protein sequence of dendroaspin, using *Escherichia coli* codon usage data, and was cloned into the pGEX-3X plasmid. A detailed description of gene construction has been reported previously [24]. The RYD- and RCD-dendroaspin genes were amplified by PCR, using a mixture of 2 units of vent polymerase, 1 μ l of the wild-type dendroaspin gene (5 ng) as a template, and 1 μ l of each of two 5'-overhanging deoxyoligonucleotides, consisting of a forward primer 5'-GGATCCAT-ATCGAAGGTCGTCGATCTGCTACAACCATCTTGG-TACTAAAC-3' and one of the following reverse oligonucleotides as a reverse primer: 5'-AATTCTCAAAGGTTGC-ATTTGTCAGATTCGCAGCAGTACGGACCCGGCAT-GTCGTAACGCGGAGTGAAGCA-3' or 5'-CGAGAATTC-TCAAAGGTTGCATTTGTCAGATTCGCAGCAGTACG-GACCCGGCATGTCGCAACGCGGAGTGAAGCA-3' that contain mutations (shown in bold) coding for tyrosine and

cysteine to produce the genes for RYD- and RCD-dendroaspin respectively. The cycling program used was as reported previously [24]. The PCR products were checked and found to be of the expected size as ascertained on a 2% agarose gel, and were further purified on a 2% low-melting-point agarose gel. The purified PCR products were digested with *Bam*HI and *Eco*RI and were then cloned into the restricted vector pGEX-3X. The recombinant plasmids (approx. 5 ng) were then transformed into *E. coli* for expression of GST fusion proteins. The presence of the correct coding sequence of the constructs was verified by complete DNA sequencing of the inserted fragments using the dideoxy chain-termination method [27].

Protein expression and purification

Protein expression was performed as described previously [24]. In brief, recombinant GST-dendroaspin and either GST-RYD- or GST-RCD-dendroaspin from supernatants of sonicated cell debris after centrifugation were purified by affinity chromatography on GSH-Sepharose CL-4B columns. Wild-type, RYD- and RCD-dendroaspin were released from GST fusion proteins after digestion with Factor Xa (Factor Xa/fusion protein, 1:300, w/w) at 4 °C for 12 h, and were then purified by reverse-phase HPLC with a trifluoroacetic acid/acetonitrile (0.1:99.9, v/v) solvent system. Active fractions were identified by a platelet aggregation assay and were re-chromatographed to homogeneity. Purified proteins were originally verified by MS.

Platelet aggregation assay

Platelet aggregation was measured by the increase in light transmission as described previously [24]. Briefly, blood was collected in sodium citrate (final concentration 0.01 M) and centrifuged at 25 °C at 200 g for 15 min. Platelet aggregation was induced with 10 μ M ADP and was measured using a Payton Dual-Aggregometer (Pollution & Process Monitoring Ltd., Borough Green, Kent, U.K.) linked to a chart recorder.

Cell adhesion assays

K562 cells were maintained in RPMI 1640 plus glutamax, supplemented with 10% (v/v) fetal bovine serum. The A375-SM human melanoma cell line was maintained in minimal essential medium supplemented with 10% (v/v) fetal bovine serum, minimal essential medium vitamins (Sigma), non-essential amino acids, 2 mM L-glutamine and 1 mM sodium pyruvate. Harvested cells were resuspended in an appropriate volume of Tyrode's wash buffer (10 mM sodium Hepes, 145 mM NaCl, 5 mM KCl, 2 mM CaCl₂ and 10 mM glucose, pH 7.35). Cells were spun at 1000 g for 10 min and resuspended in Tyrode's wash buffer. Cells were spun as before and resuspended in Tyrode's resuspension buffer (10 mM sodium Hepes, 145 mM NaCl, 5 mM KCl, 10 mM glucose and 3.5 mg/ml BSA, pH 7.35) containing either 1 mM MgSO₄ and/or 2 mM CaCl₂ or an appropriate concentration of MnCl₂ (as indicated in the Results section). The cell adhesion assay was essentially analogous to the platelet adhesion assay described previously [24]. In brief, cells were treated with or without inhibitors [appropriate antibodies (5–10 μ g/ml) or mutants of dendroaspin] for 30 min before application to microtitre wells [100 μ l of (0.8–2) $\times$ 10⁶ cells] precoated with an appropriate glycoprotein ligand. The plates were incubated for 1 h at 25 °C. Wells were subsequently washed with Tyrode's wash buffer and the number of adherent cells was determined by measurement of endogenous acid phosphatase.

RESULTS

Inhibition of ADP-induced platelet aggregation

Our first efforts were aimed at identifying whether the RYD and RCD motifs when incorporated within the dendroaspin molecule were capable of inhibiting platelet aggregation in platelet-rich plasma. Interestingly, both RYD- and RCD-dendroaspin were potent inhibitors of platelet aggregation induced by ADP, producing IC_{50} values of 200 nM and 300 nM respectively. RCD-dendroaspin was slightly less potent (by approx. 2-fold) than either RGD- or RYD-dendroaspin (Table 1). To investigate whether the RYD and RCD motifs exerted inhibitory activity within properly folded molecules, inhibitory activity was measured with samples treated with 1 mM dithiothreitol (DTT) and 0.3% 2-mercaptoethanol and heated to 100 °C for 3 min. A complete loss of their inhibitory activity was observed with the reduced proteins (results not shown). These experiments indicated that the RYD and RCD motifs did not affect the structure of the dendroaspin molecule, which presumably assumed its natural neurotoxin-fold.

Inhibition of K562 cell adhesion ($\alpha_5\beta_1$ -mediated) to fibrinogen, fibronectin and laminin

To investigate the inhibition of glycoproteins binding to their receptors, the inhibitory efficacies of both the wild-type and two mutant dendroaspins on K562 cell adhesion were determined in the presence of Mn^{2+} cations as described previously [26]; Mn^{2+} cations have recently been shown to support $\alpha_5\beta_1$ -ligand(s) interactions via fibrinogen and fibronectin [28]. In a similar manner, K562 cell adhesion to laminin was maximally augmented in the presence of Mn^{2+} at a concentration of 500 μ M (Figure 1A). In contrast, little or no adhesion was observed in the absence of either ligand (laminin) or Mn^{2+} cations, or in the presence of 2 mM Ca^{2+} , 1 mM Mg^{2+} or a mixture of Ca^{2+} and Mg^{2+} , suggesting that K562 cell adhesion was solely dependent on the presence of laminin and Mn^{2+} . The receptor-binding specificity under such conditions was analysed by utilizing different monoclonal antibodies in inhibition experiments (Figures 1B, 1C and 1D). Monoclonal antibodies JBS5 ($\alpha_5\beta_1$ -specific) and 11A1 (α_5 -specific) produced a high degree of inhibition of fibrinogen-, fibronectin- and laminin-mediated adhesion to K562 cells as did the 6S6 monoclonal antibody (β_1 -specific) towards fibrinogen- and laminin-mediated adhesion to K562 cells, suggesting that adhesion of K562 cells to these ligands was predominantly $\alpha_5\beta_1$ -dependent. Additionally, there was a high degree of inhibition by JBS2 ($\alpha_2\beta_1$ -specific) and PC42 (α_4 -specific) towards laminin-mediated adhesion. Medium degrees of inhibition were observed with JBS2 (60% inhibition) and YFC118.3 (40% inhibition; β_3 -specific) with respect to fibrinogen-mediated adhesion, and 40% inhibition was observed with PIE6 (α_2 -specific) with respect to laminin-mediated adhesion. The latter observation indicates the involvement of other integrins in K562 cell adhesion besides $\alpha_5\beta_1$. Little inhibition of K562 cell adhesion mediated by fibrinogen, fibronectin and laminin was observed with monoclonal antibodies 4F10 (α_6 -specific), LM609 ($\alpha_v\beta_3$ -specific), 7E3 (β_3 -specific) and P2 ($\alpha_{IIb}\beta_3$ -specific), indicating that there is no apparent involvement of α_6 , α_{IIb} and $\alpha_v\beta_3$ integrins in K562 cell adhesion to these ligands. Under the same assay conditions, RGD-, RYD- and RCD-dendroaspin produced distinctive patterns of inhibition of K562 cell adhesion to immobilized fibrinogen. RYD- and RCD-dendroaspin were 75–300-fold less potent than RGD-dendroaspin ($IC_{50} = 10$ nM) when fibrinogen was used as an immobilized ligand, producing IC_{50} values of 750 nM and 3300 nM respectively (Table 2 and

Table 1 Inhibition of ADP-induced platelet aggregation in platelet-rich plasma

Values are means $\pm$ S.D.

Inhibitor	IC_{50} (nM)
RGD-dendroaspin	170 $\pm$ 20 ($n = 4$)
RYD-dendroaspin	200 $\pm$ 20 ($n = 3$)
RCD-dendroaspin	300 $\pm$ 30 ($n = 3$)

Table 2 Inhibition of K562 cell adhesion

Results are average values; $n = 2$ or 3.

Inhibitor	IC_{50} (μ M)			Fibrinogen/ laminin	Fibronectin/ laminin
	Fibrinogen	Fibronectin	Laminin		
RGD-dendroaspin	0.01	0.25	0.004	2.5	62.5
RYD-dendroaspin	0.75	5.00*	0.040	18.8	125.0
RCD-dendroaspin	3.30	5.00†	0.150	22.0	33.0

* Maximal inhibition was 30%.
† Inhibition was 10% at 5 μ M.

Figure 1E). Uniquely, all three proteins were potent inhibitors of K562 cell adhesion to laminin, promoting significant levels of inhibition at nanomolar concentrations (Table 2 and Figure 1F). Conversely, all three proteins were considerably weaker inhibitors of K562 cell adhesion to fibronectin, producing minimal or low potency inhibition (Table 2). Despite the fact that the RYD and RCD motifs showed reduced potency (by approx. 10–30-fold) at inhibiting K562 cell adhesion to laminin compared with their RGD counterpart, both motifs showed significant preferential inhibition of this cell line to the three ligands tested, with ratios of 1:19:125 (laminin/fibrinogen/fibronectin) and 1:22:33 (laminin/fibrinogen/fibronectin) respectively. In contrast, the RGD motif was 63-fold more selective towards the adhesion of K562 cell to laminin than to fibronectin, but showed little selectivity between fibrinogen and laminin. The results suggest that both RYD- and RCD-dendroaspin are preferential inhibitors of $\alpha_5\beta_1$ binding to laminin than to either fibrinogen or fibronectin, whereas RGD-dendroaspin is a potent inhibitor of $\alpha_5\beta_1$ binding to both laminin and fibrinogen, but not fibronectin.

Inhibition of A375-SM cell adhesion (β_3 -mediated) to fibrinogen and collagen

Platelet aggregation studies demonstrated that RYD-dendroaspin, like RGD-dendroaspin, is a potent inhibitor of $\alpha_{IIb}\beta_3$ -mediated platelet aggregation. A study of A375-SM cell adhesion was also employed to identify whether RYD- and RGD-dendroaspin are inhibitors of β_3 -integrin cell adhesion. A375-SM cell adhesion to fibrinogen was determined in the presence of 500 μ M Mn^{2+} as described in a recent paper from our laboratory [29]. A375-SM cell adhesion to collagen was maximal at a Mn^{2+} concentration of 500 μ M (Figure 2A). In contrast, there was little or no absorbance either in the absence of collagen or in the presence of 2 mM Ca^{2+} , suggesting that A375-SM cell adhesion was dependent on the presence of collagen and Mn^{2+} . Under these assay conditions, the effects of function-blocking anti-integrin antibodies were also tested to determine the integrin receptor involvement in cell adhesion to collagen (Figure 2B).

Figure 1 Inhibition of K562 cell adhesion to fibrinogen, fibronectin and laminin

(A) Effect of Mn^{2+} on K562 cell adhesion to laminin. K562 cells $[(0.8-2) \times 10^6/\text{ml}]$ were resuspended in Tyrode's buffer supplemented with either Ca^{2+} (2 mM), Mg^{2+} (1 mM), Ca^{2+} and Mg^{2+} , or various concentrations of Mn^{2+} as shown. The cells were subsequently added (100 μl) to microtitre wells coated with laminin. Cells were incubated for 1 h at 25 $^{\circ}\text{C}$ and were quantified as described in the Materials and methods section. The number of adherent cells, determined by measurement of endogenous acid phosphatase, is shown as absorbance. (B) Inhibition of K562 cell adhesion to fibrinogen by monoclonal antibodies. (C) Inhibition of K562 cell adhesion to fibronectin by monoclonal antibodies. (D) Inhibition of K562 cell adhesion to laminin by monoclonal antibodies. K562 cells were suspended in Tyrode's buffer supplemented with Mn^{2+} and incubated in the presence of the inhibitory monoclonal antibodies (5–10 $\mu\text{g}/\text{ml}$) or 10 mM EDTA for 30 min before addition to microtitre wells coated with the appropriate ligand. Cells were treated as detailed in (A). The results are expressed as percentage inhibition (means $\pm$ S.D., $n = 4$). (E) Inhibition of K562 cell adhesion to fibrinogen by RGD-, RYD- and RCD-dendroaspin. (F) Inhibition of K562 cell adhesion to laminin by RGD-, RYD- and RCD-dendroaspin. K562 cells were suspended in Tyrode's buffer supplemented with Mn^{2+} and incubated in the presence of dendroaspins with different motifs at various concentrations, and incubated for 30 min before addition to microtitre wells coated with either fibrinogen or laminin. Cells were treated as described in (A). The results are expressed as percentage inhibition (means $\pm$ S.D., $n = 4$).

Monoclonal antibodies LM609 ($\alpha_3\beta_3$ -specific) and 7E3 (β_3 -specific) substantially blocked A375-SM cell adhesion to collagen, whereas P1E6 (α_2 -specific), P1H4 (α_4 -specific), P1D6 ($\alpha_5\beta_1$ -specific) and P4G11 (β_1 -specific) were less effective than both LM609 and 7E3, producing approx. 5–40% inhibition, indicating minimal involvement of $\alpha_2\beta_1$, $\alpha_4\beta_1$ and $\alpha_5\beta_1$. In contrast, P2 (α_{IIIb} -specific) and P3G8 (α_v -specific) were ineffective at blocking A375-SM cell adhesion to collagen, suggesting that there is no apparent involvement of the α subunits. A375-SM cell adhesion to fibrinogen [29] and collagen, therefore, was predominantly dependent on β_3 -associated integrins (Figure 2B).

Under the same assay conditions, RGD-, RYD- and RCD-dendroaspin were potent inhibitors of A375-SM cell adhesion to both fibrinogen and collagen, promoting high levels of inhibition with IC_{50} values of 7, 78 and 40 nM for fibrinogen and IC_{50} values of 6.5, 8.7 and 14 nM for collagen respectively. Interestingly, the RYD-dendroaspin was approx. 9-fold more selective towards the A375-SM cell adhesion to collagen than to fibrinogen, whereas both RGD- and RCD-dendroaspin were less or non-selective when fibrinogen and collagen were used as immobilized ligands (Figures 2C and 2D, and Table 3). Surprisingly, the RYD-dendroaspin showed a similar potency of

Figure 2 Inhibition of A375-SM cell adhesion to fibrinogen and collagen

(A) Effect of Mn^{2+} on A375-SM cell adhesion to collagen. A375 cells $[(0.8-2) \times 10^6/\text{ml}]$ were resuspended in Tyrode's buffer supplemented with either Ca^{2+} (2 mM), Mg^{2+} (1 mM), Ca^{2+} and Mg^{2+} , or various concentrations of Mn^{2+} , as for K562 cell adhesion. (B) Inhibition of A375-SM cell adhesion to collagen by monoclonal antibodies. A375-SM cells were resuspended in Tyrode's buffer supplemented with Mn^{2+} and incubated in the presence of the inhibitory monoclonal antibodies (5–10 $\mu\text{g}/\text{ml}$) or 10 mM EDTA for 30 min before addition to microtitre wells coated with collagen. (C) Inhibition of A375-SM cell adhesion to fibrinogen by RGD-, RYD- and RCD-dendroaspin. (D) Inhibition of A375-SM cell adhesion to collagen by RGD-, RYD- and RCD-dendroaspin. A375-SM cells were resuspended in Tyrode's buffer supplemented with Mn^{2+} and incubated in the presence of dendroaspins with different motifs at various concentrations, and incubated for 30 min before addition to microtitre wells coated with fibrinogen or collagen. Results are expressed as percentage inhibition (means $\pm$ S.D., $n = 4$).

Table 3 Inhibition of A375 cell adhesion

Results are average values; $n = 2$ or 3.

Inhibitor	IC_{50} (μM)		
	Fibrinogen	Collagen	Fibrinogen/collagen
RGD-dendroaspin	0.007	0.0065	1.08
RYD-dendroaspin	0.078	0.0087	8.96
RCD-dendroaspin	0.040	0.0140	2.80

inhibition of A375 cell adhesion to collagen as RGD-dendroaspin, in spite of being less effective than the RGD-motif at inhibiting A375-SM cell adhesion to fibrinogen.

DISCUSSION

In the present study we have sought to use dendroaspin as a template to test the effect of the replacement of the RGD sequence with RYD and RCD. The following considerations were taken into account, namely that dendroaspin is a unique RGD-containing protein structurally belonging to a neurotoxin family [30] that behaves functionally as a potent inhibitor of platelet aggregation and $\alpha_{IIb}\beta_3$ -integrin-mediated platelet ad-

hesion [31]. Like other RGD-containing venom proteins, the RGD motif is at the apex of a solvent-exposed loop, a characteristic of prime importance to its inhibitory action. Dendroaspin, therefore, represents a useful structural scaffold to analyse the effective interaction between an adhesive motif and a target integrin. We have previously shown that dendroaspin has a well-defined secondary structure [30], making it a suitable template for structure–function studies. Dendroaspin, therefore, provides a useful scaffold for the presentation of effective motifs, such as RYD and RCD, for integrin–ligand interaction studies.

The selection of the RYD motif to replace RGD in dendroaspin was made, since the RYD sequence has been found in glycoprotein gp63 of *Leishmania* [32], bacterial biotin-binding protein streptavidin [33] and three murine monoclonal antibodies OP-G2, LJ-CP3 and PAC-1 [34]. The RCD motif was chosen since it was uncertain as to whether the cysteine residue (which normally contributes to disulphide bonds) could be a component of a cell recognition sequence.

It was important to demonstrate that RYD- and RCD-dendroaspin could still functionally inhibit platelet aggregation. Our data highlighted that these mutations did not affect the inhibition of platelet aggregation significantly, indicating that recognition of $\alpha_{IIb}\beta_3$ is not altered significantly. The total inhibition of platelet aggregation within the nanomolar range by RYD- and RCD-dendroaspin, and a complete loss of platelet aggregation inhibition following reduction, as described in the

Results section, indicates that the folding of these molecules is correct. Because of the thiol group of the cysteine residue it was also important to analyse whether the RCD-dendroaspin underwent dimerization. The observed molecular masses of 6791 for RCD-dendroaspin and 6851 for RYD-dendroaspin before DTT treatment and of 6799 for RCD-dendroaspin and of 6859 for RYD-dendroaspin after DTT treatment were observed using electrospray ionization MS. An increase in mass of 8 Da after DTT treatment corresponds to the reduction of four disulphide bonds, and the results are in excellent agreement with the calculated molecular masses (6799.8 for RCD-dendroaspin and 6859.8 for RYD-dendroaspin). These results confirm that both molecules are monomeric and that correct folding of the molecules has occurred. In K562 adhesion experiments, the interaction of the $\alpha_5\beta_1$ integrin with immobilized fibrinogen and laminin was inhibited less effectively by the mutant dendroaspins than by wild-type dendroaspin, yet RYD-dendroaspin was almost equally effective as an antagonist as wild-type dendroaspin in the platelet aggregation assay. In comparison with wild-type dendroaspin, the reduction in inhibitory activity of RCD-dendroaspin was only seen towards K562 cell adhesion ($\alpha_5\beta_1$ -dependent) [there was no significant effect on A375 cell adhesion (β_3 -dependent)], suggesting that RYD- and RCD-dendroaspin are selective inhibitors in our assay system. More striking features are reflected in the selectivity of RYD-dendroaspin at inhibiting the β_3 integrin binding to its ligands between fibrinogen and collagen and in the selectivity of RYD- and RCD-dendroaspin at inhibiting integrin $\alpha_5\beta_1$ binding to fibrinogen, fibronectin and laminin. It should be noted that all three dendroaspin molecules, with their different motifs, are potent antagonists of β_3 binding to either fibrinogen or collagen, promoting significant levels of inhibition at nanomolar concentrations.

A very important corollary of our results is the functional role of the middle residue in the RXD motif using a dendroaspin template. It has been reported that a RYD-to-RGD motif conversion within the framework of the OP-G2 antibody did not alter the specificity or affinity of the recombinant Fab fragment binding to the integrin $\alpha_{IIb}\beta_3$ [35]. In contrast, the present study showed that in dendroaspin the middle residue can have an effect on receptor inhibition, indicating that the three motifs may recognize distinct binding sites on the various receptors or can induce different conformational changes in the receptor that promote inhibition of the interaction with the immobilized ligand e.g. an allosteric effect.

In view of the above findings, the RYD and RCD tripeptides are novel cell recognition sequences for predominantly binding to β_1 and β_3 integrins in addition to RGD, a universal motif. Our results demonstrate that the selectivity of dendroaspin binding to β_1 and β_3 integrins can be modulated by the introduction of alternative cell recognition sequences. However, the structural explanation of these three motifs binding to integrins remains unknown, since the middle residues of these three tripeptides are significantly different as RGD contains a glycine residue without a side chain, RYD contains the bulky aromatic side chain of a tyrosine residue and RCD contains a thiol-group containing cysteine residue, which could potentially form a disulphide bridge with another counterpart. It has been demonstrated that an RGD-to-LDV motif conversion within the disintegrin kistrin generates an antagonist of $\alpha_4\beta_1$ integrin instead of $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins [11]. More recently, it was reported that motifs other than RGD can be recognized by $\alpha_5\beta_1$, e.g. an RHD sequence found in the Alzheimer's-disease causing peptides A β 40 and A β 42 [36]. The present findings show that dendroaspin provides a versatile and robust protein scaffold to investigate the effect of

the replacement of glycine upon the specificity of integrin–ligand interactions.

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