Substitutions of Proline 42 to Alanine and Methionine 46 to Asparag ine around the RGD Domain of the Neurotoxin Dendroaspin Alter Its Preferential Antagonism to That Resembling the Disintegrin Elegantin*

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Among the integrins, the platelet membrane α_{IIb}β_{3} is the best characterized (3, 5). Upon cell activation, the α_{IIb}β_{3} integrin binds several glycoproteins, predominantly through the Arg-Gly-Asp (RGD) tripeptide sequence (6–8) present in fibrinogen (9), fibronectin (10), von Willebrand factor (11), vitronectin (12), and thrombospondin (13). The nature of the interactions between these glycoprotein ligands and their integrin receptors is known to be complex, and conformational changes occur in both the receptor (14) and the ligand (15).

Recently, many proteins from a variety of snake venoms have been identified as potent inhibitors of platelet aggregation and integrin-dependent cell adhesion. The majority of these proteins which belong to the disintegrin family share a high level of sequence homology, are small (4–8 kDa), cysteine-rich, and contain the sequence RGD (15) or KGD (16). In addition to the disintegrin family, a number of non-disintegrin RGD protei ns of similar inhibitory potency, high degree of disulfide bonding, and small size have been isolated from both the venom of the Elapidae family of snakes (18, 19) and leech homologues (20). All of these proteins are approximately 1000 times more potent inhibitors of the interactions of glycoprotein ligands with the integrin receptors as simple linear RGD peptides, a feature that is attributed to an optimally favorable conformation of the RGD motif held within the protein scaffold. The NMR structures of several inhibitors including kistrin (21–23), floradin (24), echistatin (25–28), abalobin (29), decorisin (30), and dendroaspin (31, 32) have been reported, and the only common structural feature elucidated so far is the positioning of the RGD motif at the end of a solvent exposed loop, a characteristic of prime importance to their inhibitory action.

Recent studies have implied a role for the amino acids around the tripeptide RGD in regulating the ligand binding specificity shown by snake venom proteins. Scarborough et al. (33) examined a range of disintegrins and observed that those containing RGDW were very effective at inhibiting the interactions of fibrinogen to purified α_{IIb}β_{3} but not of vitronectin and fibronectin to purified α_{IIb}β_{3} and α_{IIb}β_{3} respectively, whereas the converse was true for disintegrins containing the sequence RGDNP. Other regions of amino acid sequence divergence may also be contributory (33). We have reported that dendroaspin, a short chain neurotoxin analogue containing the RGD sequence, and the disintegrin kistrin, which show little overall sequence homology but have similar amino acids flanking the RGD sequence (PRGDMP), are both potent inhibitors of platelet adhesion to fibrinogen but poor antagonists of the binding of platelet transmembrane subunits that heterodimerize to produce 20 receptors (6). Among these subunits that heterodimerize to produce 20 receptors (6).

Integrins are a family of cell surface receptors that mediate adhesion of cells to each other or to extracellular matrix substrate (1–5). They are composed of noncovalently associated α and β transmembrane subunits selected from among 16 α and 8 β subunits that heterodimerize to produce 20 receptors (6).

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1 The abbreviations used are: dendroaspin, Dendroaspis Jamesonii kainose platelet aggregation inhibitor; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; GST, glutathione S-transferase; ATP-γ-S, adenosine 5′-O-(3-thiotriphosphate).

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lets to immobilized fibronectin (34). In contrast, elegantin, which has 65% sequence homology to kistrin but markedly different amino acids around RGD (ARGDNP), preferentially inhibited platelet adhesion to fibronectin as opposed to fibrinogen and binds to an allosterically distinct site on αιβ3 complex. These studies suggested that the amino acids around the RGD determine the affinity and selectivity of these RGD proteins. In addition to RGD domains, a number of recent studies have suggested that amino acids at the carboxyl terminus of these proteins may affect their interactions with integrins. Deletion of the PRNP sequence from echistatin has been reported to reduce its ability to inhibit platelet aggregation, implying a reduction in the binding affinity (16). Furthermore, the complete carboxy-terminal peptide (PRNPHKGPAT) of echistatin not only competed with the binding of echistatin to the αιβ3 complex but also enhanced the binding of fibronectin and vitronectin to the purified αιβ3β3, indicating that this non-RGD component of the protein was able to alter the integrin affinity for glycoprotein ligands (35). However, the mechanism by which amino acids at the carboxyl terminus of these proteins interact with their receptors and their binding characteristics are not yet understood.

In this study we examined the role of amino acids flanking the RGD sequence by expressing the neurotoxin variant dendroaspin in Escherichia coli and using site-directed mutagenesis. Dendroaspin, unlike echistatin, does not have any appreciable sequence at its carboxy-terminal after Cys57 making it an excellent model to study the functional role of the nature of the amino acids flanking the sequence RGD. We show that recombinant dendroaspin has inhibitory properties identical with native dendroaspin indicating that the expressed protein has the correct folding and disulfide bonding and that substituting Met46→Asn (PRGDMP→PRGDNP) or Met46→Asn and Pro92→Ala (PRGDMP→ARGDNP) dramatically altered the preferential inhibitory properties and binding characteristics of the protein to the αιβ3 complex to that of the diintegrin elegantin containing the sequence ARGDNP. These studies prove that the amino acid flanking sequence RGD provide an extended locus that determines the preferential selectivity of dendroaspin.

**EXPERIMENTAL PROCEDURES**

Materials—Restriction enzymes, T4 polynucleotide kinase, T4 DNA ligase, isopropyl-1-thio-galactopyranoside, and DH5α competent cells were purchased from Life Technologies Ltd. (Paisley, UK) or Promega Ltd. (Southampton, UK). Vent (exo-) DNA polymerase was supplied by New England Biolabs (Herts, UK). Human fibrinogen (grade L) was purchased from Kabi (Stockholm, Sweden). Human fibronectin was supplied by Bioproducts Laboratories (Herts, UK). Lymphoid snake venoms were obtained from either Latoxan (Rosans, France) or Sigma Ltd. (Dorset, UK). The monoclonal reagents PM6/248 and PM6/13, which have specificities for the native αιβ3 complex and β3 subunit, respectively, have been described previously (36). Oligonucleotides were made either in King's College School of Medicine and Dentistry (London, UK) or in Cruachem Ltd. (Glasgow, UK). The gene of dendroaspin (or of mutant dendroaspin) was amplified by polymerase chain reaction, using 1 μl of ligature mixture as a template, 1 μl of two 5′-overhanging oligonucleotides as primers, and 2 units of vent polymerase. The following program was applied: one cycle of 3 min at 94 °C and 1 min at 72 °C, followed by 39 cycles of 30 s at 94 °C, and 2 min at 72 °C. The amplification product was checked and found to be of the expected size (216 bp) as ascertained on a 2% agarose gel and further purified on a 2% low-melting-point agarose gel. The gene of dendroaspin (or of mutant dendroaspin) was digested with EcoRI and BamHI and then cloned into the restricted vector pGX-3X at the carboxyl terminus of the glutathione S-transferase (GST) gene. The factor Xa cleavage sequence was positioned 5′ to the gene coding for the recombinant proteins to produce recombinant plasminoid pGX-endoaspin gene and pGX-mutant-dendroaspin gene. The correct orientation and sequence of the genes of dendroaspin and mutant dendroaspins were confirmed by DNA sequencing using the method of Sanger et al. (38).

Transformation and Protein Expression—The cloned vector was used to transform 50 μl of E. coli DH5α competent cells by standard methods (39). Bacterial culture was carried out as follows; the culture was inoculated with an overnight seed culture (1%, v/v) and grown in LB-medium supplemented with 100 μg/ml (ampicillin) medium (100 μg/ml) and shaken at 37 °C until it reached an A600 of 0.7, then isopropyl-1-thio-β-galactopyranoside was added to a final concentration of 0.1 mM for induction. The cells were grown for an additional 4 h at 30 °C and harvested by centrifugation. In contrast to noninduced transformants, analysis of isopropyl-1-thio-β-galactopyranoside-treated cell lysates by SDS-polyacrylamide gel electrophoresis showed the emergence of a 32-kDa protein corresponding to the GST-fusion protein.

Purification of Elegantin, Dendroaspin, Recombinant Wild-type Dendroaspin, and Mutant Dendroaspins—Elegantin and dendroaspin were purified using reverse-phase HPLC as described previously (40). Recombinant dendroaspins were purified as follows: the cell pellets were washed with PBS buffer (pH 7.4) containing the protease inhibitors phenylmethylsulfonyl fluoride (1 μM), pepstatin (5 μg/ml), aprotinin (5 μg/ml), trypsin inhibitor (1 μg/ml), 1 mM EDTA, and sonicated on ice. The sonicated mixture was centrifuged at 7,800 × g at 4 °C for 10 min to pellet the cell debris and insoluble material. Recombinant GST-dendroaspin and GST-mutant-dendroaspins from supernatants were purified by affinity chromatography on glutathione-Sepharose CL-4B columns by adsorption in PBS containing 150 mM NaCl and elution with 50 mM Tris-HCl containing 10 mM reduced glutathione (pH 8.0). Elution of the absorbed material with glutathione resulted in the appearance of a major band migrating at 32 kDa (GST-dendroaspin fusion protein) in 12.5% polyacrylamide gels. The appropriate fractions comprising the 32-kDa fusion protein were digested in the presence of 150 mM NaCl, 1 mM CaCl2, and Factor Xa (1:100, v/v, Factor Xa fusion protein) at 4 °C for 24 h. Treatment of the purified GST-proteins with Factor Xa released recombinant proteins migrating as 7-kDa bands, approximating the size of dendroaspin, and free GST appearing as an intensification of a 28-kDa band on SDS-stained gels. The digested Nimrod was combined with a 32-kDa fusion protein and subjected to a Vydac C4 reverse-phase HPLC analytical column (TP104) and eluted with a linear gradient of 0–26% acetonitrile (1.78% per min) containing 0.1% trifluoroacetic acid, followed by 26–36% acetonitrile in 0.1% trifluoroacetic acid (0.25% per min). When necessary, further analytical columns were run under the same conditions. The fractions from HPLC were freeze-dried, dissolved in water, and assayed for inhibition of ADP-induced platelet aggregation.

Electrospray Ionization Mass Spectrometry of the Dendroaspin Proteins—Electrospray ionization mass spectroscopy was used to deter-
mine the molecular sizes of native and mutant dendroaspins. Samples (approximately 50 pmol) were lyophilized and dissolved in 20 μl of acetonitrile/ether (1:1). Ion-mass spectral analysis was performed with a SRS 710 mass analyzer (Finnigan Mat, UK) using an injection rate of 5 μl/min. There were observed molecular masses of 6746.0 ± 2 for native dendroaspin, 6746.6 ± 2 for recombinant wild-type dendroaspin, 6728.2 ± 2 for [Asn]46Dendroaspin and 6704.2 for [Ala]46,Asn]46]-dendroaspin. These are in good agreement with the molecular masses of 6745.66, 6745.66, 6728.5, and 6702.4 (respectively) calculated from their complete amino acid sequences on the basis that all cysteiny1 residues participate in intrachain disulfides.

Measurement of Platelet Aggregation—Platelet aggregation was measured by the increase in light transmission as described previously (34, 40). Briefly, platelet-rich plasma was prepared from citrated human blood obtained from healthy individuals by centrifugation at 2000 (34, 40). Briefly, platelet-rich plasma was prepared from citrated human blood obtained from healthy individuals by centrifugation at 200 g for 15 min. Washed platelets were prepared from platelet-rich plasma and resuspended in adhesion/aggregation buffer (145 mM NaCl, 5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 10 mM glucose, 3.5 mM HEPES, pH 7.35) and adjusted to a count of 3 x 109/ml. Platelet aggregation (320 μl well, and the absorbance values were read at 410/630 nm on an auto-
cord according to the manufacturer’s specifications. The binding of 125I-labeled Reagent (Bio-Rad Laboratories, Ltd., Hertfordshire, UK) ac-
dination onto a 25% (w/v) sucrose, 1% bovine serum albumin cushion and cen-
trifugation at 12,000 χ g for 10 min. Both platelet pellets and superna-

tants were counted to determine the levels of bound and free ligand. Background binding levels were determined in the presence of a 50-fold excess of unlabelled disintegrin or 10 μM EDTA.

RESULTS AND DISCUSSION

Design and Functional Characterization of Recombinant Wild-type and Mutant Dendroaspins—We had previously noted that dendroaspin and kistrin share similar amino acid residues at positions flanking the tripeptide RGD and speculated that this sequence similarity underpinned the similar functional and binding characteristics of these two structurally unrelated snake venom inhibitors (Table I, Ref. 34). To test this hypo-
thesis, in the present study, we generated dendroaspin variants with specific substitution of the residues at positions flanking RGD to those residues present in elegantin (Table I), a well-defined disintegrin with functional and binding characteristics to the α1β3 integrin complex distinct from those of kistrin and dendroaspin.

The functional characterization of the recombinant wild-type and of the mutant dendroaspins purified from cell lysates of E. coli was determined by platelet aggregation and adhesion as-
says. In order to verify that the expression system generated was satisfactory, we first compared the functional properties of

| Integrin/Ligand Interactions |
|----------------------------|

### Table I

**Sequence alignment of dendroaspin, disintegrins, and short chain neurotaxis**

The numbering corresponds to the sequence of dendroaspin. Residues which are identical with those in dendroaspin are shown as bold. The residues around the tripeptide RGD are shown in the rectangle.

| Disintegrins | Elegandin | Basildin | Batroxostatin | Cereberin | Cotelain | J arracin | Lachexin | Molossin | Viradin | Kistrin | RGD motif |
|--------------|-----------|-----------|---------------|-----------|----------|-----------|----------|----------|---------|---------|-----------|
| EAGEECDGSCPENCCDAATCKLRPGACGACGCLCCDRKFKKKR | ARGDNP | ODDTCQGACDCPNRFLY | EAGEECDGSCPENCCDAATCKLRPGACGACGCLCCDRKFKKKR | ARGDNP | ODDTCQGACDCPNRFLY | EAGEECDGSCPENCCDAATCKLRPGACGACGCLCCDRKFKKKR | ARGDNP | ODDTCQGACDCPNRFLY | EAGEECDGSCPENCCDAATCKLRPGACGACGCLCCDRKFKKKR | ARGDNP | ODDTCQGACDCPNRFLY | GKEECDGSCPENCCDAATCKLRPGACGACGCLCCDRKFKKKR |

**Neurotoxins**

α-Neurotoxin

| RicynnogirwTypKPF--TTECQ---TDCSYKNWTT---FDNIIIRGGCCTF | PRGDMP | GYPDCE-YKCNL |

Erabutoxin b

| RicynnogirwTypKPF--TTECQ---TDCSYKNWTT---FDNIIIRGGCCTF | PRGDMP | GYPDCE-YKCNL |

Toxin S.C10

| RicynnogirwTypKPF--TTECQ---TDCSYKNWTT---FDNIIIRGGCCTF | PRGDMP | GYPDCE-YKCNL |

**Table II**

Inhibition of platelet aggregation expressed as IC50 values

Abbreviations are: WP, washed platelets; PRP, platelet-rich plasma; r.Den., recombinant dendroaspin. Results are means ± S.D. for the numbers of determinations in parentheses.

| RGD-peptides | Sequence around RGD | WP | PRP |
|--------------|---------------------|----|-----|
| Kistrin      | PRGDMP              | 0.040 ± 0.013 (3) | 0.180 ± 0.013 (4) |
| Dendroaspin  | PRGDMP              | 0.075 ± 0.018 (4) | 0.198 ± 0.028 (4) |
| r.Den.       | PRGDMP              | 0.070 ± 0.002 (2) | 0.213 ± 0.04 (2) |
| [Asn]46Dendroaspin | PRGDMP         | 0.064 ± 0.025 (2) | 0.166 ± 0.025 (2) |
| [Ala]46,Asn]46]-Dendroaspin | PRGDMP | 0.170 ± 0.02 (2) | 0.360 ± 0.03 (2) |
| Elegandin    | PRGDMP              | 0.150 ± 0.02 (3) | 0.330 ± 0.03 (3) |
recombinant dendroaspin with that of native dendroaspin purified from snake venom. As shown in Table II, recombinant dendroaspin showed platelet aggregation inhibition as potent as native dendroaspin and displayed similar inhibitory activity toward ADP-induced platelet aggregation both in platelet-rich plasma and washed platelets. This indicated that the protein folded correctly and formed the correct disulfide bondings. The mutant [Asn46]dendroaspin showed an IC50 value similar to that of recombinant dendroaspin, while the mutant with two substitutions, [Ala42,Asn46]dendroaspin, showed an IC50 value similar to that observed with elegantin (Table II).

We previously observed that measurement of ADP-activated platelet adhesion to immobilized glycoproteins highlights selective inhibitory preferences for RGD snake venom proteins that are less easily discernible using the platelet aggregation assay (34, 39). In such experiments, we have shown that dendroaspin and kistrin are potent inhibitors of platelet adhesion to fibrinogen, whereas elegantin preferentially inhibits platelet adhesion to fibronectin (34). Fig. 1 and Table III (showing IC50 values) illustrate the results obtained with the wild-type recombinant and mutant dendroaspins in comparison with the inhibitory properties of native dendroaspin and elegantin. Wild-type dendroaspin showed an identical inhibitory profile toward the inhibition of platelet adhesion to fibrinogen compared to native dendroaspin. Interestingly, the mutants [Asn46]dendroaspin and [Ala42,Asn46]dendroaspin exhibited a progressive decrease in their ability to inhibit platelet adhesion to fibrinogen in both maximal inhibitory levels (using a maximum of 5 μM protein) and IC50 values. Indeed, appropriate substitutions at both flanking positions, i.e. [Ala42,Asn46]-dendroaspin, showed an 8-fold lower IC50 that approached the value obtained with elegantin. Studies using fibronectin as the immobilized ligand showed an even more striking change with respect to antagonistic preference. Both recombinant and native dendroaspins were relatively poor inhibitors of activated platelet adhesion to fibronectin displaying only 40% inhibition at 15 μM. However, the singly substituted Met46 → Asn of dendroaspin has a similar IC50 for platelet adhesion to both fibrinogen and fibronectin, whereas the doubly substituted (Pro42 → Ala and Met46 → Asn) mutant shows an approximately 4-fold preference for inhibition of binding to fibronectin. In particular, the latter showed a maximal extent of inhibition and IC50 values that were markedly similar with those displayed by elegantin. Thus, substituting Pro42 → Ala and Met46 → Asn in the residues immediately flanking the RGD in dendroaspin altered the inhibitory preferences of dendroaspin to that of elegantin. The presence of asparagine adjacent to aspartic acid would be particularly important in inhibiting the interactions of fibronectin with its receptor. This study strongly supports our previous studies (34).

Binding of 125I-labeled recombinant and mutated dendroaspins and of 125I-elegantin to activated platelets was studied to determine whether the alterations in functional properties of the mutated dendroaspins were reflected in their binding characteristics. All four 125I-labeled proteins bound to ADP-activated platelets in a saturable and cation-dependent manner (Fig. 2, insets). Scatchard analysis of the data using the Kinetic, EBDA, Ligand, and Lowry version 4 software programs (BIOSOFT, Cambridge, UK) indicated that recombinant dendroaspin bound to a single class of binding site exhibiting a Kd = 67 nM (Fig. 2A and Table III) with a Bmax equal to approximately 29,100 sites per platelet. The [Asn46]- and [Ala42,Asn46]dendroaspin, however, both produced biphasic isotherms again using the Kinetic, EBDA, Ligand, and Lowry version 4 software with Kd values = 87 nM and 361 nM for [Asn46]dendroaspin (Fig. 2B and Table III) and 33 nM and 371

| RGD-peptides             | Platelet adhesion | Binding characteristics | Binding sites (×10²/platelet) |
|--------------------------|-------------------|-------------------------|-------------------------------|
|                          | Fg                | Fn                      | Kd1                          | Kd2 |              |
| Den.                     | 0.26 ± 0.04       | 20.0 ± 1                | 94.6 ± 0.005                 | 0.067 ± 0.005 | 29.1 (L) |
| r.Den.                   | 0.22 ± 0.05       | 20.0 ± 2                | 0.087 ± 0.004                | 0.361 ± 0.03 | 15 (H)  |
| [Asn46]Dendroaspin       | 0.56 ± 0.06       | 0.88 ± 0.10             | 0.033 ± 0.002                | 0.371 ± 0.015 | 2.5 (H)  |
| [Ala42,Asn46]Dendroaspin | 1.80 ± 0.20       | 0.33 ± 0.04             | 0.018 ± 0.001                | 0.179 ± 0.01    | 5.9 (H)  |
| Elegantin                | 5.40 ± 0.50       | 0.60 ± 0.10             | 0.198 ± 0.001                | 0.179 ± 0.01    | 5.9 (H)  |

Fig. 1. Inhibition of platelet adhesion to immobilized glycoproteins by RGD-containing proteins. Washed platelet suspensions were incubated with various concentrations of native dendroaspin (•), recombinant wild-type dendroaspin (○), [Asn46]dendroaspin (●), [Ala42,Asn46]dendroaspin (■), or elegantin (□) for 3 min prior to application to microtiter wells coated with either 10 μg/ml fibrinogen (A) or fibronectin (B). The number of adherent platelets were determined by measurement of endogenous acid phosphatase as described previously (34). Results are expressed as percent inhibition relative to the number of adherent platelets observed in the absence of inhibitors. All points were performed in quadruplicate, and the mean ± S.E. are shown in Table III (n = 2–4).
nM for [Ala42,Asn46]dendroaspin (Fig. 2 and Table III). In agreement with the results obtained with the adhesion experiments, the mutated dendroaspinsshowed a progressive shift in their binding characteristicstoward those of elegantin (K_d values 518 nM and 179 nM) as shown in Fig. 2D and Table III.

Considering that [Ala 42,Asn46]dendroaspin and elegantin share little sequence homology (Tables I and II) and have structures unrelated, except for the (A)RGD(N) domain, the close similarity of the dissociation constants is striking.

To confirm that both binding sites occupied by the two dendroaspinmutants on ADP-treated platelets were present on...
the α1β3 integrin complex, the effects of two inhibitory antibodies on radioligand binding were monitored. PMEL24, a monoclonal antibody with specificity for the native α1β3 complex (36), effectively inhibited in a dose-related manner the binding of all these [125I]-labeled recombinant dendroaspins by 80--100% (Fig. 3). In contrast, an anti-α5β1 antibody was comparatively ineffective, confirming that the binding parameters observed were specifically associated with the α1β3 complex.

Further evidence for the close similarity in the binding of [Ala42,Asn46]- and [Asn46]dendroaspin and of elegantin to the α1β3 complex was obtained by examining the association kinetics of three ligands (Fig. 4). Native and recombinant dendroaspin show simple and rapid binding, reaching equilibrium by 5 min. However, elegantin, [Asn46]- and [Ala42,Asn46]-dendroaspin showed complex association kinetics with approximately 3- to 4-fold higher binding before equilibrium than at equilibrium. The reasons for this complex association pattern are not known at present, but are not due to internalization of the ligand as the binding was fully reversible (data not shown). That [Asn46]- and [Ala42,Asn46]dendroaspin, but not native dendroaspin, behaved in this manner points to this property being solely due to the presence of the ARGDN sequence, and whether other ARGDN-containing disintegrins, e.g. viridin, jararacin, cotiarin (Table I), behave in a similar manner remains to be examined.

These studies report that the amino acids around the RGD motif regulate the affinity and selectivity of the RGD protein dendroaspin and support our earlier studies (34) and those of Scarborough et al. (33). Further details of the mechanisms of integrin/ligand interactions will benefit greatly from the analysis of both wild-type and mutant dendroaspins by x-ray crystallography or NMR spectroscopy. Until the receptor-ligand lysis of both wild-type and mutant dendroaspins by x-ray crystallography or NMR spectroscopy. Until the receptor-ligand interactions will benefit greatly from the analysis of both wild-type and mutant dendroaspins by x-ray crystallography or NMR spectroscopy. Until the receptor-ligand lysis of both wild-type and mutant dendroaspins by x-ray crystallography or NMR spectroscopy. Until the receptor-ligand interactions will benefit greatly from the analysis of both wild-type and mutant dendroaspins by x-ray crystallography or NMR spectroscopy. 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