The “Linker” Region (Amino Acids 38–47) of the Disintegrin Elegantin Is a Novel Inhibitory Domain of Integrin $\alpha_5$$\beta_1$-Dependent Cell Adhesion on Fibronectin

**EVIDENCE FOR THE NEGATIVE REGULATION OF FIBRONECTIN SYNERGY SITE BIOLOGICAL ACTIVITY**

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Rushika Sumathipala, Cunshuan Xu, Julian Seago, A. Paul Mould, Martin J. Humphries, Sue E. Craig, Yatin Patel, Errol S. Wijelath, Michael Sobel, and Salman Rahman

From the Laboratory of Thrombosis and Vascular Remodelling, Division of Cardiovascular Medicine, King’s College London School of Medicine, St Thomas’ Hospital, Lambeth Palace Road, London SE1 7EH, United Kingdom, the Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, United Kingdom, and the Division of Vascular Surgery, University of Washington School of Medicine and Veterans Affairs Puget Sound Health Care System, Seattle, Washington 98108

Disintegrins are a family of potent inhibitors of cell-cell and cell-matrix adhesion. In this study we have identified a region of the disintegrin elegantin, termed the “linker domain” (amino acids 38–47), with inhibitory activity toward $\alpha_5$$\beta_1$-mediated cell adhesion on fibronectin (Fn). Using a chimeric structure-function approach in which sequences of the functionally distinct disintegrin kistrin were introduced into the elegantin template at targeted sites, a loss of inhibitory function toward $\alpha_5$$\beta_1$-mediated adhesion on Fn was observed when the elegantin linker domain was substituted. Subsequent analysis comparing the inhibitory efficacies of the panel of elegantin–kistrin chimeras toward CHO $\alpha_5$ cell adhesion on recombinant Fn III6–10 fragments showed that the loss of inhibitory activity associated with the disruption of the elegantin linker domain was dependent upon the presence of a functional Fn III, synergy site within the Fn III6–10 substrate. This suggested that the elegantin linker domain inhibits primarily the activity of the Fn synergy domain in promoting $\alpha_5$$\beta_1$ integrin-mediated cell adhesion. Construction of a cyclic peptide corresponding to the entire region of the elegantin linker domain showed that this domain has intrinsic $\alpha_5$$\beta_1$ inhibitory activity comparable with the activity of the RGDS peptide. These data demonstrate a novel biological function for a disintegrin domain that antagonizes integrin-mediated cell adhesion.

Cell adhesion is a complex process involving several classes of molecular complexes, including the integrin family of adhesion receptors. The regulation of integrin-ligand binding is a targeted process for both physiological and pathophysiological mediators. Disintegrins were originally described as inhibitors of platelet aggregation, an integrin-dependent process, isolated as monomeric proteins of 5–8 kDa from viper venoms (1). Subsequently, it has been demonstrated that these molecules are potent integrin ligands and are found in snake venoms as both homo- and heterodimers (2, 3), as fusion proteins with metalloproteases, and in mammals as modules within the disintegrin-metalloprotease family. Currently, the monomeric snake venom disintegrin family can be conveniently divided into three different groupings according to their length and prevalence of disulfide bonds, including short (41–51 residues), medium (~70 residues), and long (84 residues) (4).

An archetypal structure within all disintegrins is the presence of a solvent-exposed $\beta$-loop containing an “integrin-binding” tri-peptide motif (IBM) that almost invariably contains an acidic residue. The exceptions to this are the R/KTS disintegrins, which display specificity for the $\alpha_i$-integrins (5, 6). The most ubiquitous motif observed in disintegrins is the RGD sequence, which is also found in fibronectin (Fn) and other extracellular matrix and plasma proteins. It is, therefore, assumed that disintegrins bind to integrins through an analogous mechanism to physiological ligands whereby the RGD loop interacts with amino acids of both the $\alpha_5$ and $\beta_1$-subunits at the subunit interface of the integrin heterodimer (7, 8).

Structure-function studies with disintegrins have shown that the positioning of the IBM at the apex of the $\beta$-loop and the correct pairing of disulfide bonds are essential features for biological activity (reviewed in Ref. 4). Synthetic peptides corresponding to the entire RGD loop of disintegrins possess only between 6 and 20% of the potency of their parent protein even after cyclization (9). The molecular details of how the structure of disintegrins contribute to their full biological activity still remain obscure, because a high resolution structure of a disintegrin in complex with an integrin receptor is unavailable at present.

The abbreviations used are: IBM, integrin-binding tri-peptide motif; Fn, fibronectin; Fg, fibrinogen; Eg, elegantin; CHO, Chinese hamster ovary.

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The integrin selectivity of disintegrins has been shown to be dependent upon the composition of the amino acid environment surrounding the acidic residue, which comprises the IBM within the β-hairpin loop. The disintegrin barbourin, which contains a KGD IBM, was shown to possess a high degree of selectivity toward the integrin αIIbβ3 as opposed to the related integrin α5β1 (10), now known to be due to the additional length of the aliphatic lysine side chain (8). Tselepis et al. (11) showed that the substitution of the IBM derived from the CS-1 domain of Fn (LDV) in place of the RGD sequence in kistrin altered the integrin specificity of this disintegrin from the integrin αIIbβ3 toward the integrin α5β1. Furthermore, several studies have demonstrated that the amino acid residues positioned N- and C-terminal to the RGD sequence in disintegrins modulates the specificity of their binding to integrin complexes (12, 13). This specificity has a number of facets, including specificity toward distinct integrin affinity states (12) and the inhibitory preferences toward specific physiological integrin-ligand partnerships (14). Furthermore, recombinant disintegrins with distinct RGD motifs show several mechanisms of competitive behavior for binding to the integrin αIIbβ3 indicative of allosteric modulation (15, 16).

In addition to the β-hairpin loop containing the IBM, other regions of the disintegrin molecule have been postulated to play a role in modulating their binding to integrins. For example, several studies have implicated a role for the residues at the extreme of the C terminus of short disintegrins such as echistatin and eristostatin as regulators of disintegrin binding to integrins (13, 17). These studies illustrate that the C-terminal residues may be involved in the promotion of high affinity conformational states within the integrin complex as detected by the expression of ligand-induced binding site epitopes. However, as studies with Fn have shown, physiological ligands are postulated to interact with integrins in a complex manner involving synergy contacts between receptor and ligand (18–21). Disintegrin inhibition of physiological integrin-ligand partnerships may necessitate down-regulation of these synergy interactions in addition to direct competition between the IBM containing loops of the disintegrin and the physiological ligand. However, little is known about which regions of the disintegrin structure antagonize the activity of these synergy domains.

The purpose of the present study was to test the hypothesis that disintegrins inhibit and thereby mimic receptor-ligand interactions at synergy sites. We also wanted to identify the structure-function relationship of this biological activity. We, therefore, constructed a panel of chimeric disintegrins based upon the structures of two well characterized “medium-sized” family members elegantin and kistrin with differing inhibitory properties toward αIIbβ3-mediated cell adhesion. Kistrin sequences were introduced at sites of sequence variation within the elegantin IBM loop: the C-terminal peptide and the “linker domain” (amino acid 38–47), which connects the N- and C-terminal portions of the molecule. The aim was to identify a potential region of elegantin that functioned as an additional inhibitory domain for the integrin αIIbβ3, supplementing activity of the IBM-containing loop. The data presented here provide compelling evidence that the linker domain of elegantin (amino acids 38–47) harvests inhibitory activity toward the integrin αIIbβ3. This activity is of biological significance through the inhibition of Fn synergy site activity in promoting αIIbβ3-mediated cell adhesion.

Given that the selectivity of the IBM of barbourin underscored its success as a template for the development of a highly successful pharmacological reagent to treat thrombotic episodes, the potential for the linker domain of elegantin to inhibit αIIbβ3-Fn synergy interaction identifies a potential for disintegrin linker domains to serve as potential templates for the construction of a novel integrin antagonists.

**EXPERIMENTAL PROCEDURES**

**Materials**—Fibrinogen was obtained from Kabi (Stockholm, Sweden, Grade L) and fibronectin was from Sigma. The purity and integrity of these glycoproteins were determined by SDS-PAGE. The αIIbβ3-directed monoclonal antibody AP2 was a generous gift from Dr. Tom Kunicki (Scripps Research Institute, La Jolla, CA). All other antibodies were obtained from Chemicon (Harrow, UK).

**Construction and Expression of Elegantin-Kistrin Chimeras**—The construction, expression, and characterization of recombinant elegantin and variants constructs has been described previously (12, 16). The elegantin-kistrin chimeric constructs described in the present study were prepared using these elegantin constructs (12) as templates with additional modifications prepared by oligonucleotide-based site-directed mutagenesis using the Transform mutagenesis procedure (Clontech, Basingstoke, Herts, UK) according to the manufacturer’s instructions. In targeting the various regions of the elegantin sequence for amino acid substitution, only non-conserved residues were altered. In some instances, successive rounds of mutagenesis were necessary to obtain full sequence substitution. Recombinant elegantin-kistrin chimeras were subcloned into pGEX-3X (Amersham Biosciences) vectors or pUBHis10 (gift from Dr. T. Butt, LifeSensors Inc., Philadelphia, PA) and expressed. Recombinant fusion proteins were prepared from the lysates of Epicurean coli (DE3)pLysS (Stratagene, La Jolla, CA) by affinity chromatography on glutathione-Sepharose (Amersham Biosciences) or nickel chelating resins (Sigma) using a batch procedure. Purified recombinant disintegrins were analyzed by SDS-PAGE indicating that the preparations were >95% homogeneous with minimal proteolytic degradation of the sample observed.

**Protein Modification and Peptide Synthesis**—NHS-fluorescein (5- and 6-carboxyfluorescein, succinimidyl ester, Pierce & Warner Ltd., Chester, UK) was used to conjugate glutathione S-transferase disintegrins (1 mg/ml) in 50 mM sodium bicarbonate buffer, pH 8.5. The conjugation reaction was carried out at 4 °C for 2 h. The fluorescein-conjugated protein was separated from unconjugated fluorescein by gel filtration on PD 10 columns (Amersham Biosciences). Fluorescence:protein ratios were determined for each protein which was stored at −40 °C. Cyclic synthetic peptides corresponding to the elegantin linker domain were synthesized by Alta Biosciences Ltd. (Birmingham, UK) using conventional Fmoc (N-(9-fluorenylmethoxycarbonyl) solid-phase chemistry. Wild-type elegantin linker sequence peptide (amino acids 38–47) P4 (5-S-CRFKAKRTIC-S-)
and a scrambled version P_{C} (-S-CRRTKIFKKC-S-) were reconstituted in water and stored at −20 °C.

**Cell Adhesion and Binding Assays—**K562 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, UK) and maintained in RPMI 1640 plus Glutamax, supplemented with 10% (v/v) fetal bovine serum. CHO α_{5} and CHO α_{5}/α_{6} cells have been described previously (26) and were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and G418 (200 μg/ml). Harvested cells were washed in phosphate-buffered saline containing and resuspended in Tyrode’s buffer (10 mM Hepes, pH 7.35, 150 mM NaCl, 5 mM KCl) containing either 1 mM MgSO_{4} and 2 mM CaCl_{2} (standard physiological cation conditions) or an appropriate concentration of MnCl_{2} (as indicated under “Results”). For adhesion assays, cells were treated with or without inhibitors (disintegrins, peptide, or monoclonal reagents) for 30 min prior to application to microtitre wells (100 μl of 1 × 10^{6} cells/ml), which were pre-coated with either fibrinogen or fibronectin (10–20 μg/ml) or recombinant Fn III_{6–10} fragments (5 μg/ml). After washing, adherent cells were quantified by measurement of endogenous acid phosphatase. Fluorescent ligand binding were performed as previously described (16). For studies of ligand association, cells (1 ml of 1 × 10^{6}/ml) were suspended in appropriate buffers and incubated with 100 nM of recombinant fluorescein isothiocyanate-conjugated disintegrin for various time periods, and the reaction was stopped by the addition of 2% (v/v) final concentration of ice-cold paraformaldehyde to the incubation mixture. Cells were incubated for 30 min on ice and then washed (four times) in phosphate-buffered saline and analyzed by flow cytometry.

**RESULTS**

**Construction of Elegantin-Kistrin Chimeras—**Elegantin is a disintegrin originally isolated as an inhibitor of platelet aggregation (22) and was subsequently shown to be a highly specific antagonist of the integrin α_{5}β_{1} expressed on K562 cells (12). The substituted domains were expressed as elegantin constructs both in isolation and in various combinations with the construct termed C-P107 comprising the linker, IBM, and C terminus of kistrin thereby differing with the wild-type kistrin sequence by only two non-conserved residues and two additional residues at the N-terminus (Fig. 1B).

The bacterial fusion elegantin-kistrin chimeras were purified to homogeneity, and the genetic substitutions were confirmed at the protein level by mass spectrometry (data not shown). An initial functional screen for activity was performed by assessing the efficacy of the panel of elegantin chimeras as inhibitors of platelet aggregation. Table 1 shows the IC_{50} values for each recombinant disintegrin fell within the range observed for most naturally isolated disintegrins indicating faithful recombinant expression as previously shown (12, 16).

**Elegantin-Kistrin Chimeras Bind to α_{5}β_{1}—**The efficacy of the panel of elegantin-kistrin chimeras as inhibitors of α_{5}β_{1}-mediated K562 cell adhesion was also assessed. Under physiological cation buffer conditions under which the integrin α_{5}β_{1} assumes a low affinity conformation in this cell line (25), the recombinant elegantin-kistrin chimeras expressing the wild-type elegantin RGD motif (50ARGDN54) were strong inhibitors (IC_{50} range, 5–25 nM) of K562 cell adhesion on Fn irrespective of the sequences present in the linker domain or at the C terminus (Fig. 2A). In agreement with our previous observations (12), replacement of Ala^{50} with the corresponding kistrin Pro residue within the RGD loop drastically reduced inhibitory potency (Fig. 2B). The one exception being the chimera designated Eg P107, which contains the elegantin C terminus and showed a more potent inhibitory activity compared with other chimeras containing Pro^{50}. However, this was still considerably weaker (−5- to 10-fold, IC_{50} = 175 nM) than the elegantin chimeras containing the wild-type (Ala^{50}) elegantin RGD motif.

Using an α_{5}β_{1} monoclonal reagent JBS-5 to block disintegrin-α_{5}β_{1} engagement, direct binding of fluorescently labeled elegantin-kistrin chimeras to K562 cells confirmed that the chimeras harboring the kistrin RGD motif failed to interact significantly with K562 cells via the α_{5}β_{1} integrin with the exception of Eg P107 (Fig. 3).

Under buffering conditions containing Mn^{2+} ions, K562 cells adhere on Fg in an α_{5}β_{1}-dependent manner (12). The effect of Mn^{2+} is to moderately activate the integrin through promoting conformational changes in the β-subunit A-domain through displacement of the Ca^{2+} ion coordinating at the ADMIDAS site (7). Although under these conditions, K562 cell adhesion on Fn is enhanced, we observed previously (12) that the integrin dependence of the cell adhesion was no longer solely due to the activity of the α_{5}β_{1} integrin but contained a significant α_{5}β_{1} component, complicating interpretation of the disintegrin inhibitory activity. The α_{5}β_{1}-dependent adhesion on an Fg substrate has been shown to be mediated by the RGD sequence in Fg located at amino acids 572–574 in the Aα chain (24).

Under these experimental conditions, all elegantin-kistrin chimeras containing either the elegantin wild-type or kistrin...
RGD motif, linker, and C-terminal domains were strong and equipotent inhibitors (IC_{50} range, 5–10 nM, Fig. 2, C and D). Therefore, these experiments demonstrate that all the recombinant chimeras retain the capacity to ligate the integrin α_{5}β_{1} and inhibit cell adhesion when a moderately high affinity conformation is induced with Mn^{2+}. They also demonstrate that the kistrin RGD motif can discriminate between different activation states of the integrin. These observations are in agreement with our previous observations that partial activation of the β_{1}-subunit A-domain by Mn^{2+} is necessary to accommodate the Pro residue of the kistrin RGD motif at residue position 50 in the elegantin primary structure (12).

Inhibition of Integrin α_{5}β_{1}-Fn Binding Involves the Elegantin Linker Domain—Although the use of Mn^{2+} ion to promote moderate activation of the α_{5}β_{1} complex expressed on K562 cells allows for recognition of elegantin-kistrin chimeras with the kistrin RGD motif, previous studies have suggested that the interaction of the integrin α_{5}β_{1} with Fn does not involve the

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FIGURE 1. **Design of elegantin-kistrin chimeras.** A, a molecular model of elegantin was constructed from the coordinates of the solution structures of the disintegrins flavoviridin and kistrin using ProMod II software (SwissModel). The molecules are shown as ribbons with the RGD β-loop and C terminus positioned at the top and the N terminus at the bottom. Basic amino acid side chains in the linker domain of elegantin are shown as sticks with spherical orbits. B, amino acid sequences of elegantin, kistrin, and elegantin-kistrin chimeras.
participation of the synergy PHSRN site located in the ninth type III repeat in these cells when the integrin is not fully activated (25). Furthermore, the interaction of the integrin α5β1 with Fg via the RGD site in the Aαc chain does not involve an additional synergy interaction due to the absence of the PHSRN sequence in Fg. Therefore, to assess the impact of the Fn synergy interaction upon the inhibitory efficacy of the panel of elegantin-kistrin chimeras, we exploited a system expressing α5β1 in a different cellular context, which was known to employ the activity of the Fn synergy site (26). Adhesion experiments were performed using CHO cells expressing the human α5- subunit in partner with endogenous hamster β1 (CHO α5). For comparison, the inhibitory efficacy of the panel elegantin-chimeras was also assessed toward the adhesion of CHO cells expressing a human-hamster hybrid α5β1 heterodimer (CHO α5) on Fn, which does not involve interaction with the Fn synergy site (26).

In these experiments, CHO α5 cell adhesion on Fn was inhibited strongly by Eg WT and the chimera Eg C-WT, which differs from native elegantin (Eg WT) only by the inclusion of the kistrin C terminus (Fig. 4A). Therefore, the presence of the kistrin C terminus did not alter the inhibitory efficacy of the chimeric disintegrin toward the integrin-ligand interaction. Conversely, chimeras Eg P106 and Eg C-P106, which both contain the native elegantin RGD motif but in combination with the kistrin linker domain, were weak inhibitors of CHO α5 adhesion on Fn, achieving a maximal inhibition of cell adhesion of only 40% (Fig. 4A). Indeed, these chimeras showed a loss of inhibitory activity of a similar magnitude to the elegantin-kistrin chimeras expressing the kistrin RGD motif (Fig. 4B). This observation, therefore, suggested that the elegantin linker domain was indispensable to the inhibitory efficacy of elegantin toward α5β1 integrin-dependent adhesion on Fn. Significantly, for the CHO α5 cell adhesion on Fn, all elegantin-kistrin chimeras were efficient inhibitors achieving maximal levels of inhibition at ~100 nM albeit with differing IC50 values (Fig. 4, C and D). These data show that, for CHO α5-Fn adhesion, both elegantin and kistrin are efficient antagonists and that the presence of the kistrin linker sequence within the elegantin template does not lead to a loss of inhibitory activity as with CHO α5-mediated adhesion on Fn. These data implied that the inhibitory activity of the linker domain may relate to the antagonism of the function of the synergy domain of Fn in promoting ligation to the α5β1 integrin.

To test this hypothesis, the panel of elegantin-kistrin chimeras was assessed for their capacity to inhibit CHO α5 cell adhesion on the recombinant fragment of Fn comprising type III repeats 6–10 (Fn III6–10) in which the PRSHN site in the ninth type III repeat was mutated to SPSDN, an inactive sequence derived from domain IIIg. However, it is known that the general level of adhesion of cells expressing α5β1 to this recombinant fragment is low compared with the wild-type fragment. This is thought to reflect a reduction in the affinity of the binding interaction between the integrin and its ligand (27). Previous reports (25) have suggested that treatment of K562 cells with the activating monoclonal TS2/16 or phorbol 12-myristate 13-acetate

TABLE 1

| Protein     | Aggregation, IC50 μM |
|-------------|---------------------|
| Kistrin (Ks) | 0.042 ± 0.01        |
| Elegantin (Eg) | 0.350 ± 0.06      |
| Eg C-WT    | 0.340 ± 0.09        |
| Eg IPM     | 0.300 ± 0.03        |
| Eg C-IPM   | 0.290 ± 0.04        |
| Eg P106    | 0.40 ± 0.1          |
| Eg C-P106  | 0.75*               |
| Eg P107    | 0.10 ± 0.015        |
| Eg C-P107  | 0.140 ± 0.02        |

*P < 0.01.

FIGURE 2. Elegantin-kistrin chimeras differentially inhibit K562 cell adhesion. A and B, K562 cells (1 × 10^6/ml) suspended in Ca^2+/-Mg^2+ supplemented buffers were preincubated with varying concentrations of the recombinant disintegrins as shown for 30 min prior to addition to microtiter wells coated with Fn. After 1 h at room temperature, adherent cells were quantified by colorimetric measurement of endogenous acid phosphatase. Background cell adhesion was determined by incubation of a sample of the cell suspension in the presence of 10 mM EDTA. Results are expressed as percent inhibition. All data points were performed in quadruplicate ± S.E. Data shown are for a representative experiment (n = 3). C and D, as for panels A and B except K562 cells were suspended in adhesion buffer supplemented with 50 μM Mn^2+ promoting α5β1-dependent adhesion on Fg (12) (n = 3).
can restore equivalent levels of adhesion to both wild-type and mutant fragment. However, in our hands we were unable to achieve a significant improvement in the level of adhesion of K562 cells to Fn III6–10 SPSDN by treatment with either TS2/16 or phorbol 12-myristate 13-acetate or both reagents in combination (data not shown). Furthermore, CHO α5 cells also did not adhere to Fn III6–10 SPSDN in a comparable manner to Fn III6–10 PRSHN in a Ca\(^{2+}\)/Mg\(^{2+}\) buffer (Fig. 5A). However, when CHO α5 cells were suspended in a buffer containing 100 μM Mn\(^{2+}\), similar levels of adhesion on both substrates were observed with the retention of α5β1 specificity (Fig. 5B).

Under the experimental conditions shown in Fig. 5, the inhibitory efficacy of the panel of elegantin chimeras toward CHO α5 cell adhesion on both substrates was assessed. These studies showed that adhesion on Fn III6–10 PHSRN was inhibited strongly by Eg WT and Eg C-WT. However, chimeras Eg P106 and Eg C-P106 were comparatively poor inhibitors achieving a maximal inhibition of cell adhesion of only 65% and with ~3-fold elevated IC\(_{50}\) values (Fig. 6A). In contrast, all four elegantin-kistrin chimeras were potent inhibitors of CHO α5 cell adhesion on Fn III6–10 SPSDN achieving a maximal level of inhibition of cell adhesion of 100% at 100 nM with similar IC\(_{50}\) values (Fig. 6B). These data, therefore, in agreement with the observations for CHO α5 cell adhesion on Fn under Ca\(^{2+}\)/Mg\(^{2+}\) conditions (Fig. 4), confirm that the loss of inhibitory function toward CHO α5 cell adhesion associated with the substitution of the elegantin linker domain is only apparent when a Fn substrate contains a functioning synergy site in the III\(_{9}\) module.

We postulated that, if the elegantin linker domain antagonized cell adhesion dependent upon the activity of the Fn III\(_{9}\) synergy site, then the linker domain and the Fn synergy site may make contact with the same region of the α5β1 complex. Previously, Mould et al. (26) showed, using a chimeric integrin α\(_v\) subunit comprising the N-terminal residues 1–232 of human α\(_v\) stably expressed in CHO cells with endogenous β1 (CHO α\(_v\)/α\(_v\) cells), that a region comprising the Fn synergy interaction site on the integrin α\(_v\) subunit was contained within this N-terminal region. In that study, the CHO α\(_v\)/α\(_v\) cell adhesion profile on the Fn III\(_{6–10}\) fragments mimicked that of CHO α5 cells. We, therefore, tested the inhibitory efficacy of our elegantin-kistrin chimeras upon CHO α\(_v\)/α\(_v\) cell adhesion on Fn III\(_{6–10}\) PHSRN and Fn III\(_{6–10}\) SPSDN. As shown in Fig. 6C, the cell adhesion on the Fn III\(_{6–10}\) PHSRN fragment was strongly inhibited by

![Figure 3](image-url) Differential recognition of low affinity α\(_v\)β1 conformation by elegantin-kistrin chimeras. K562 cells (1 × 10\(^5\)/ml) suspended in Ca\(^{2+}\)/Mg\(^{2+}\)-supplemented buffers were preincubated with 1 μM fluorescein isothiocyanate-conjugated recombinant disintegrin as shown for 60 min at room temperature in the absence (open bars) or presence (closed bars) of 5 μg/ml monoclonal antibody JBS-5. Cells were fixed in paraformaldehyde, washed, and analyzed by flow cytometry. Data are presented as specific bound disintegrin measured as mean fluorescence intensity having subtracted background levels measured in the presence of 10 mM EDTA. Data are for a representative experiment of two experiments giving similar results with each point performed in triplicate.

![Figure 4](image-url) Substitution of the elegantin linker domain causes loss of inhibitory activity for integrin α\(_v\)β1-Fn interaction. A and B, CHO α5 cells (1 × 10\(^5\)/ml) suspended in Ca\(^{2+}\)/Mg\(^{2+}\)-supplemented buffers were preincubated with varying concentrations of the recombinant disintegrins as shown for 30 min prior to addition to microtiter wells coated with Fn. After 1 h at room temperature, adherent cells were quantified by colorimetric measurement of endogenous acid phosphatase. Background cell adhesion was determined by incubation of a sample of the cell suspension in the presence of 10 mM EDTA. Results are expressed as percentage inhibition. All data points were performed in quadruplicate ± S.E. Data shown are for a representative experiment (n = 3). C and D, the same experiments were performed as for panels A and B employing CHO α\(_v\) adhesion on Fn (n = 3).
Elegantin Linker Domain Has Intrinsic Integrin α₅β₁ Inhibitory Activity—Because the inhibitory activity of the linker domain depends upon the biological action of the Fn synergy site within the fragment Fn III₆₋₁₀ (Figs. 4 and 6), the mechanism of inhibition of Fn binding to α₅β₁ by elegantin could be based upon direct competition with the III₉ synergy domain. However, Takagi et al. (34) have proposed that the Fn III₉ synergy domain does not comprise an extended contact surface with the α₅-subunit but rather promotes the association of the III₉ RGD site with the integrin. If the linker domain of elegantin assumes a similar role, then the weak inhibitory activity of Eg P106 and Eg C-P106 in our adhesion experiments using Fn III₆₋₁₀ PHSRN as a substrate could be accounted for by a reduced on-rate of the chimeras lacking the elegantin linker sequence thereby reducing the disintegrin competitive effectiveness. We decided, therefore, to measure the comparative rates of association of the elegantin chimeras with CHO α₅ cells. As shown in Fig. 8A, rather than having a reduced association, Eg P106 and Eg C-P106 binding to CHO α₅ cells was considerably faster than both Eg WT and Eg C-WT. The data demonstrate that the presence of the linker domain in elegantin slows the interaction of the disintegrin with α₅β₁ suggesting that it may participate in a multicontact binding mechanism. These data, therefore, rule out the possibility that the loss of function observed in elegantin-kistrin chimeras lacking the elegantin linker domain sequence is due to reduced on-rates leading to poorer competitiveness. These data are consistent with the contention that the elegantin linker domain is inhibitory toward the Fn synergy domain biological activity of enhancing Fn interaction with the integrin α₅β₁.

To test this hypothesis, we synthesized a cyclic peptide encompassing the entire linker domain sequence (amino acid 38–47), including flanking cysteines that were cyclized

Eg WT and Eg C-WT with 100% maximal inhibition attained at 150 nM. In contrast, chimeras Eg P106 and Eg C-P106 were comparatively poor inhibitors with only a maximal 35% inhibition of cell adhesion at 200 nM. In contrast, CHO αv/α₅ cell adhesion on FN III₆₋₁₀ SPSDN was inhibited strongly by all four elegantin-kistrin chimeras with or without the wild-type linker sequence, displaying a maximal inhibition of 100% at 150 nM (Fig. 6D). However, in contrast with CHO α₅ cell adhesion upon FN III₆₋₁₀ SPSDN, Eg WT and Eg C-WT did appear to be slightly more efficacious inhibitors than Eg P106 and Eg C-P106, which displayed a 2- to 3-fold increase in IC₅₀ values. In spite of this difference between the two types of CHO cell lines, the results of the CHO α₅/α₅ studies largely parallel those obtained with CHO α₅ cells highlighting a loss of inhibitory efficacy toward α₅β₁-dependent cell adhesion when the Fn substrate contains an active synergy sequence within module III₉. The results also suggest that the elegantin linker domain binding site on the integrin α₅β₁ is also located within the N-terminal 232 residues of the α₅-subunit, because the linker domain inhibitory activity was not significantly altered in CHO α₅/α₅ cells.

The significance of the elegantin linker domain in promoting efficacious inhibition of CHO α₅ cell adhesion on Fn was further supported by the employment in this experimental system of a structurally unrelated snake venom-derived integrin antagonist dendroaspin, which has no structural domain comparable to the linker domain of elegantin (14). This antagonist is a naturally occurring inhibitor of the integrin α₅β₁ and has an identical RGD motif to kistrin rendering it unable to ligate low affinity integrin α₅β₁. In a previous study (28), we substituted the wild-type dendroaspin RGD motif with the elegantin RGD motif by site-directed mutagenesis to generate a variant dendroaspin (Ala₄², Asn₄⁶) with a capacity to engage low affinity α₅β₁. In adhesion assays, dendroaspin (Ala₄², Asn₄⁶) showed very weak inhibitory efficacy toward CHO α₅ adhesion on Fn III₆₋₁₀ PHSRN (Fig. 7). In contrast, dendroaspin (Ala₄², Asn₄⁶) was a comparable inhibitor to the elegantin-kistrin chimeras at blocking CHO α₅ adhesion upon Fn III₆₋₁₀ SPSDN. This observation supports the contention that the potent inhibitory efficacy of elegantin toward α₅β₁-Fn-mediated adhesion is associated with its dual abrogation of the RGD and synergy site activities.

Dendroaspin (Ala₄², Asn₄⁶) has 50- to 100-fold greater inhibitory activity than wild-type dendroaspin toward CHO K1 and K562 cell adhesion on Fn (S. Rahman, unpublished data).
through oxidation of their free thiol groups. We assessed the capacity of the linker domain peptide, designated P_l, to block CHO α5β1 adhesion on Fn III<sub>6–10</sub> PHSRN and Fn III<sub>6–10</sub> SPSDN (Fig. 8, B and C). In these experiments, we observed that P<sub>L</sub> was unable to inhibit CHO α5β1 cell adhesion on Fn III<sub>6–10</sub> PHSRN to any measurable extent at concentrations up to 1.0 mM when the substrate was coated at 5.0 µg/ml. Under the same experimental conditions, RGDS at similar peptide concentrations was also unable to block cell adhesion to any measurable extent (data not shown). This suggested that the coating density promoted an adhesion strength that could not be competed by both peptides under the assay method. We, therefore, reduced the coating density of the Fn III<sub>6–10</sub> PHSRN substrate to 0.75 µg/ml, which supported cell adhesion to ~30–40% of that observed at 5.0 µg/ml (data not shown). At this adhesion strength, both P<sub>L</sub> and RGDS blocked CHO α5β1 cell adhesion on Fn III<sub>6–10</sub> PHSRN with similar efficacy with an approximate maximal inhibition of 80% and IC<sub>50</sub> values of ~50 µM (Fig. 8B). By comparison, P<sub>L</sub> strongly inhibited CHO α5β1 cell adhesion on Fn III<sub>6–10</sub> SPSDN to a maximal level of 100% with an IC<sub>50</sub> of ~10 µM even when the substrate coating was maintained at 5.0 µg/ml (Fig. 8C). A scrambled cyclic peptide P<sub>C</sub>, comprising the same residues between the cysteines showed no measurable inhibitory activity to either Fn III<sub>6–10</sub> PHSRN or Fn III<sub>6–10</sub> SPSDN substrates. Under these experimental conditions RGDS showed approximately similar inhibitory potency with P<sub>L</sub>. These data, therefore, demonstrate that the elegantin linker domain can block integrin α5β1-Fn binding in the presence and absence of the synergy domain indicating that the linker sequence has intrinsic integrin α<sub>5</sub>β<sub>1</sub>-inhibitory activity.

**DISCUSSION**

This study has demonstrated that the medium-sized disintegrin elegantin, a potent RGD-based antagonist of the integrin α<sub>5</sub>β<sub>1</sub>, contains a region encompassing amino acids 38–47 that contributes to its efficacy as an inhibitor of cell adhesion on Fn. Our data suggest that this region of elegantin binds to the α<sub>5</sub>β<sub>1</sub> complex and inhibits the interaction with Fn through a mechanism that antagonizes primarily the biological activity of the Fn synergy site located in module III<sub>9</sub>. This conclusion is based upon the following observations. Elegantin-kistrin chimeras lacking the elegantin linker domain but retaining the elegantin RGD loop with Ala<sup>39</sup> showed reduced inhibitory efficacy
Linker Region of the Disintegrin Elegantin

A

FIGURE 8. The elegantin linker domain harbors intrinsic \( \alpha_\beta_1 \)-integrin inhibitory activity. A comparative measurement of the association phase of elegantin-kistrin chimeras for CHO \( \alpha_5 \) cell suspensions. CHO \( \alpha_5 \) cells (1 \times 10^6/ml) suspended in Ca\(^{2+}\)/Mg\(^{2+}\)-supplemented buffers were incubated with a 100 nM recombinant fluorescein isothiocyanate-conjugated disintegrin for various time intervals. The reaction was stopped, and the level of cell bound disintegrin was determined by fluorescent-activated cell sorting analysis (see "Experimental Procedures"). Background levels of bound ligand were measured by inclusion of 10 mM EDTA to the reaction mixture. The results are expressed as percent ligand bound taking equilibrium cell bound values as 100%. Symbols are as for Fig. 6 (n = 3). Inset, an expanded abscissa displaying ligand association during over 2 min. B and C, CHO \( \alpha_5 \) cells (1 \times 10^5/ml) were preincubated with varying concentrations of the synthetic peptides as shown for 30 min prior to addition to microtitre wells coated with either Fn III\(_{6-10}\) PHSRN (B) or Fn III\(_{6-10}\) SPSDN (C). After 1 h at room temperature, adherent cells were quantified by colorimetric measurement of endogenous acid phosphatase. Background cell adhesion was determined by incubation of a sample of the cell suspension in the presence of 10 mM EDTA. Results are expressed as percent inhibition. All data points were performed in quadruplicate \( \pm \) S.E. Data shown are for a representative experiment (n = 3).

toward CHO \( \alpha_5 \) cell adhesion on Fn comparable to the inhibitory efficacy of Pro\(^{50}\) chimeras (kistrin RGD motif). This phenomenon, therefore, is not related to the recognition of RGD motif by the \( \alpha_5 \beta_1 \) complex as with the case of the presence of Pro\(^{50}\) in the RGD motif of the elegantin-kistrin chimeras (Fig. 4). Rather, replacement of the linker domain of elegantin with the corresponding sequence of kistrin reduced the inhibitory efficacy of the disintegrin toward CHO \( \alpha_5 \) cell adhesion on Fn III\(_{6-10}\) PHSRN as apposed to Fn III\(_{6-10}\) SPSDN indicating that the loss of inhibitory function is dependent upon an active Fn synergy site. It is also indirectly supported by the observation that a potent snake venom antagonist, dendroaspin (Ala\(^{42}\), Asn\(^{46}\)), containing the elegantin RGD motif but lacking a linker domain sequence, showed weak inhibitory activity under the same experimental conditions using Fn III\(_{6-10}\) PHSRN as a substrate but was a strong inhibitor of CHO \( \alpha_5 \) adhesion on Fn III\(_{6-10}\) SPSDN. Finally, a cyclic peptide corresponding to the linker region of elegantin inhibited CHO \( \alpha_5 \) cell adhesion upon Fn III\(_{6-10}\) PHSRN and Fn III\(_{6-10}\) SPSDN with comparable potency to the linear RGDS peptide, thus demonstrating intrinsic integrin inhibitory activity.

The binding mechanism of RGD ligands to integrin complexes has been the subject of intense study over the last decade. The biological significance of the role of the RGD IBM was established predominantly through biochemical and functional approaches, which have been consolidated and refined through the application of structural methods to study integrin-ligand binding. However, biochemical studies have also demonstrated the importance of additional ligand structures that support or “synergize” with the RGD \( \beta\)-loop. The most intensely studied synergy domain is the region present in the III\(_{\alpha}\) module of Fn with its core residues mapped as corresponding to the sequence PHSRN (18–21). These studies demonstrated the pivotal role for the Fn synergy region in promoting \( \alpha_\beta_1 \)-dependent cell adhesion. The significance of the Fn synergy sequence to the biological activity of Fn has been confirmed by several additional studies. For example, Sechler et al. (29) showed that the synergy site in Fn was essential for the formation of an \( \alpha_\beta_1 \)-driven accumulation of an Fn fibrillar matrix. Mesendoderm extension was also shown to be dependent on the activity of the Fn III\(_{\alpha}\) synergy site during Xenopus gastrulation (30). The synergy interaction of Fn with \( \alpha_\beta_1 \) is also thought to promote nodule initiation during bone morphogenesis (31). The latter finding was supported by the recent observation that co-localization of the RGD and PHSRN peptide sequences within a synthetic matrix enhanced osteoblast adhesion, spreading, and focal adhesion formation compared with RGD alone (32). Whereas the important role of the Fn synergy region is well established through biochemical and functional studies, the mechanism of its action in the ligand binding process remains controversial. Mould et al. (26) showed that, in a structural model of a molecular complex of the truncated head region of Fn III\(_{6-10}\) PHSRN (8), a model based on comparative measurement of the association phase of elegantin-kistrin chimeras for CHO \( \alpha_5 \) cell suspensions, the binding mechanism of RGD ligands to integrin complexes has been the subject of intense study over the last decade. The biological significance of the role of the RGD IBM was established predominantly through biochemical and functional approaches, which have been consolidated and refined through the application of structural methods to study integrin-ligand binding. However, biochemical studies have also demonstrated the importance of additional ligand structures that support or “synergize” with the RGD \( \beta\)-loop. The most intensely studied synergy domain is the region present in the III\(_{\alpha}\) module of Fn with its core residues mapped as corresponding to the sequence PHSRN (18–21). These studies demonstrated the pivotal role for the Fn synergy region in promoting \( \alpha_\beta_1 \)-dependent cell adhesion. The significance of the Fn synergy sequence to the biological activity of Fn has been confirmed by several additional studies. For example, Sechler et al. (29) showed that the synergy site in Fn was essential for the formation of an \( \alpha_\beta_1 \)-driven accumulation of an Fn fibrillar matrix. Mesendoderm extension was also shown to be dependent on the activity of the Fn III\(_{\alpha}\) synergy site during Xenopus gastrulation (30). The synergy interaction of Fn with \( \alpha_\beta_1 \) is also thought to promote nodule initiation during bone morphogenesis (31). The latter finding was supported by the recent observation that co-localization of the RGD and PHSRN peptide sequences within a synthetic matrix enhanced osteoblast adhesion, spreading, and focal adhesion formation compared with RGD alone (32).
Linker Region of the Disintegrin Elegantin

**α₅β₁** with Fn III₁–₁₀ obtained by x-ray scattering, the III₉ domain interacts with the β-propeller domain of the α₅-subunit, consistent with previous work, which mapped the synergy interaction site to propeller blades 2 and 3. Furthermore, substitution of residues Tyr²⁰⁴ → Ala and Ile²¹⁰ → Ala located in a loop within blade 3 reduced the affinity of the interaction between **α₅ β₁**, and Fn III₉–₁₀ by 5- and 200-fold, respectively, while having little affect upon the interaction of the integrin with Fn III₆–₁₀ SPSDLN. These observations support a model in which the Fn synergy site forms part an extended contact surface between the integrin α₅β₁ and Fn III₉–₁₀. Support for this notion was extended using electron microscopy to study a complex comprising the entire ectodomain of the integrin α₅β₁ in complex with Fn III₇–₁₀. In this molecular complex, the Fn synergy region was also positioned in close proximity to the surface of the α₅-subunit β-propeller domain (33). However, Takagi et al. (34) observed that in complexes of truncated α₅β₁ with Fn 7–10, no extensive contact between Fn III₉ and the α₅-subunit was present. These workers suggested that the synergy site in Fn promotes cell adhesion by promoting the association phase of the ligand binding process either by optimization of the RGD loop conformation or by electrostatic steering.

The subtle relationship between the RGD and synergy sites of Fn for acquiring full α₅β₁-dependent adhesive activity have been demonstrated. Altroof and co-workers (35) showed that targeted mutagenesis of residues within Fn III₉, which effect domain stability, and at the interface between III₉ and III₁₀ that alter the angle of interdomain tilt, abrogate α₅β₁-dependent adhesion to levels those observed for Fn synergy domain mutants (36).

The precise interaction site for the elegantin linker domain on the integrin α₅β₁ was not identified in the present work. However, our data suggest that the site of interaction is located within amino acids 1–232 of the α₅-subunit, a region that has been previously identified as harboring structural elements conferring α₅β₁ receptor specificity and the putative contact region for the Fn synergy site (26). Furthermore, our data suggest that the linker domain of elegantin does not promote the association phase of the binding mechanism but rather slows the interaction considerably. This observation suggests that binding of elegantin to the α₅β₁ integrin involves a multi-contact-multistep interaction mechanism giving rise to greater inhibitory specificity toward the physiological ligand Fn.

The precise mechanism by which the linker domain of elegantin down-regulates α₅β₁-dependent adhesion remains unknown and requires further investigation. However, our data suggest that the linker region in elegantin, a highly basic domain, negatively regulates the synergy site interaction on the integrin α₅β₁ either directly or allosterically. Under the experimental conditions employed in this study, both the linker domain peptide P₁ and the RGDS peptide were comparable inhibitors of CHO α₅ cell adhesion on either Fn III₆–₁₀ PSHRN or Fn III₆–₁₀ SPSDLN substrates, although significantly greater concentrations of peptide were required to block adhesion on Fn III₆–₁₀ PSHRN. Because the linker domain cyclic peptide blocked CHO α₅ adhesion on Fn III₆–₁₀ SPSDLN, it appears to show a capacity to negatively modulate the interaction of the α₅β₁ interaction with the RGD loop of Fn III₁₀. The potency of P₁ as an integrin inhibitory molecule is striking considering that it plays an ancillary or synergistic role to the RGD β-loop. Furthermore, it is highly unlikely that P₁ assumes the native, extended conformation of the linker domain observed for the analogous region of the related disintegrin flavovirdin and as predicted in our model of elegantin as a consequence of the interdomain disulfide and hydrogen bonding that is present in the parent disintegrin (Fig. 1).

Structural studies have shown that the RGD tri-peptide binds to a pocket at the interface of the α₅- and β₁-subunits of the integrin heterodimer within the globular head region (7, 8). Binding of the RGD sequence and chemical mimetics invariably involves coordination of the metal ion-dependent adhesion site Mg²⁺ ion by a carboxyl group adjacent to a basic moiety or amino acid. It is highly unlikely, therefore, that the linker domain peptide (CRFKKKTIC) behaves as a RGD mimetic due to the absence of a free carboxyl group in the peptide. However, because the linker peptide P₁ was able to inhibit CHO α₅ cell adhesion on Fn III₆–₁₀ SPSDLN, a solely RGD-dependent event, competition between the two adhesive peptides appears to occur. Our favored model, therefore, is that the linker domain most likely interacts with the α₅-subunit and acts as an inhibitor of Fn binding by interfering with the synergy site interaction directly and RGD interaction site allosterically. However, it is possible that the linker peptide binds to a site on the α₅-subunit that blocks both Fn synergy and RGD interactions simultaneously by interfering, in the latter case, with the predicted hydrogen bonding between the guanidinium moiety of arginine and an acidic residue in the α₅-subunit. In support of the view that peptide P₁ binds to the α₅-subunit, we observed that the pattern of inhibition of CHO α₅/α₅ cells was essentially similar to CHO α₅ cells. Furthermore, Mould et al. (37) showed that mutations in the predicted region of the α₅-subunit reduced the affinity of α₅β₁ for Fn III₆–₁₀ SPSDLN, suggesting that an allosteric relationship may exist between the synergy and RGD interaction sites on the α₅β₁ complex. Through construction of a molecular model of the α₅-subunit built upon the crystal structure of the α₅-subunit, we have identified a highly acidic face in the α₅-subunit β-propeller domain located at the interface with the β₁-subunit that could potentially form electrostatic bonds with the highly basic linker domain of elegantin (data not shown). Further studies are needed to identify the precise location of the elegantin linker domain interaction site on the α₅β₁ complex, which would facilitate the design of experiments to delineate the distinctive mechanisms of action of the linker region and RGD sequence.

The present work has identified a domain within elegantin with intrinsic integrin α₅β₁ inhibitory activity. This widens the number of disintegrin-derived sequences known to interact and regulate integrin function. Previous work using the barbourin IBM as a template for the construction of therapeutic antagonists of the integrin α₅β₁ highlighted the importance of disintegrins as model integrin ligands (10). The discovery of the biological activity of the elegantin linker domain could, therefore, offer further insight for the development of novel integrin antagonists.
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