Dynamics and functional differences between dendroaspin and rhodostomin: Insights into protein scaffolds in integrin recognition

Chun-Ho Cheng, Yi-Chun Chen, Jia-Hau Shiu, Yao-Tsung Chang, Yung-Sheng Chang, Chun-Hau Huang, Chiu-Yueh Chen, and Woei-Jer Chuang*

Department of Biochemistry and Institute of Basic Medical Sciences, National Cheng Kung University
College of Medicine, Tainan 701, Taiwan

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Abstract: Dendroaspin (Den) and rhodostomin (Rho) are snake venom proteins containing a PRGDMP motif. Although Den and Rho have different 3D structures, they are highly potent integrin inhibitors. To study their structure, function, and dynamics relationships, we expressed Den and Rho in *Pichia pastoris*. The recombinant Den and Rho inhibited platelet aggregation with the K_i values of 149.8 and 83.2 nM. Cell adhesion analysis showed that Den was 3.7 times less active than Rho when inhibiting the integrin $\alpha IIb\beta 3$ and 2.5 times less active when inhibiting the integrin $\alpha v\beta 3$. In contrast, Den and Rho were similarly active when inhibiting the integrin $\alpha 5\beta 1$ with the IC_{50} values of 239.8 and 256.8 nM. NMR analysis showed that recombinant Den and Rho have different 3D conformations for their arginyl-glycyl-aspartic acid (RGD) motif. However, the comparison with Rho showed that the docking of Den into integrin $\alpha v\beta 3$ resulted in a similar number of contacts. Analysis of the dynamic properties of the RGD loop in Den and Rho showed that they also had different dynamic properties. These results demonstrate that protein scaffolds affect the function, structure, and dynamics of their RGD motif.

Keywords: dendroaspin; dynamics; disintegrin; disulfide bond; folding; rhodostomin; *Pichia pastoris*

Introduction

Venom toxins are small proteins with many disulfide bonds. They have multiple functions directed toward

a variety of molecular targets, including a diversity of receptors, enzymes, and ion channels.^{1,2} It is known that toxins with a similar fold can have different functions. In contrast, toxins with unrelated folds can also have similar functions.^{3–5} Many toxins are obtained from a variety of species, such as snakes, ticks, and leeches, and contain an arginyl-glycyl-aspartic acid (RGD) motif with different protein scaffolds.^{6–11} They specifically inhibit the integrin-binding function and are potent integrin antagonists. Many of these proteins have potential as therapeutic agents that can directly target integrins. Structural and functional studies of RGD-containing toxins suggest that the inhibitory potency of these proteins lies in subtle positional requirements of the RGD motif at the apex of a flexible loop, a structural feature for binding to integrins.¹² However, little is

Abbreviations: Den, dendroaspin; HSQC, heteronuclear single quantum correlation; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; *P. pastoris*, *Pichia pastoris*; RGD, arginyl-glycyl-aspartic acid; Rho, rhodostomin; TOCSY, total correlated spectroscopy.

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*Correspondence to: Woei-Jer Chuang, Department of Biochemistry and Institute of Basic Medical Sciences, National Cheng Kung University College of Medicine, Tainan 701, Taiwan. E-mail: wjcnmr@mail.ncku.edu.tw

known about the effect of protein scaffolds on the structure–function–dynamics relationships of RGD-containing proteins. Therefore, we selected two protein scaffolds with a PRGDMP motif to study the effect.

Dendroaspin (Den) is a snake venom protein isolated from *Dendroaspis jamesoni kaimosae* and is a short-chain neurotoxin homolog from the venom of Elapidae snakes with weak neurotoxicity.¹³ Den consists of 59 amino acids that include eight cysteine residues and a PRGDMP sequence at positions 42–47.¹⁴ Unlike neurotoxins, it contains an RGD motif and is a potent inhibitor of platelet aggregation. Den inhibited adenosine diphosphate (ADP)-stimulated platelets in a saturable manner with the inhibition constant of 172 nM.¹³ Rhodostomin (Rho) is obtained from *Calloselasma rhodostoma* venom and belongs to the family of disintegrins.¹⁵ It consists of 68 amino acids, which includes 12 residues of cysteine and a PRGDMP sequence at positions 48–53. We previously showed¹⁶ that Rho expressed in *Pichia pastoris* has the same function and structure as native protein. Rho binds to unstimulated and ADP-stimulated platelets in a saturable manner with the dissociation constants of 76 and 74 nM, respectively.¹⁷ It is 2.3 times more active than Den.

To compare the structure, function, and dynamics relationships of Den and Rho, it is essential to express them with the correct fold and high yield. Expression of three-fingered toxins with the correct fold has been obtained; however, the yield is less than 1 mg/L, or refolding of the protein is required.^{18–22} Many reports^{2,16,23} have shown that highly disulfide-bonded proteins can be expressed in *P. pastoris* with high yields. In the present study, we expressed a three-fingered toxin, Den, in *P. pastoris* with high yield and compared its backbone dynamics with those of Rho. This comparative study on the function, structure, and dynamics of Den and Rho serves as a basis for insight into the structure–function–dynamics relationships of integrin antagonists with different scaffolds.

Results

Expression, purification, and characterization of Den and Rho

Den and Rho were expressed in *P. pastoris* X-33 strain using the pPICZαA vector. Recombinant Den and Rho expressed in *P. pastoris* were purified to homogeneity using Ni²⁺-chelating chromatography and C18 reversed-phase HPLC. Based on SDS–polyacrylamide gel electrophoresis, proteins produced in *P. pastoris* were homogenous (Supporting Information Fig. 1). The yields of Den and Rho produced in *P. pastoris* were 8–15 and 12–20 mg/L, respectively. In addition, the yield of ¹⁵N-labeled Den and Rho produced in *P. pastoris* was 5–10 and 14–17 mg/L.

Figure 1. 2D ¹H–¹⁵N HSQC spectra of recombinant Den at pH 4. The protein concentration was 1 mM. Correlation peaks are labeled according to residues type and sequence number. The resonances of the side chains were labeled with “s” sign, and the peaks connected by lines correspond to Gln and Asn side-chain NH₂ group.

Mass spectrometry was used to determine their molecular weights. The experimental molecular weights of Den and Rho produced in *P. pastoris* were 7844.4 and 8417.4, which were in excellent agreement with the calculated values of 7844.8 and 8417.1, respectively. The values were calculated by assuming that all cysteines formed disulfide bonds, which indicated the formation of four disulfide bonds in Den and six disulfide bonds in Rho.

Inhibition of platelet aggregation

The recombinant Den expressed in *P. pastoris* inhibited platelet aggregation with a K_I of 149.8 ± 13.0 nM, which was consistent with the reported value of 172.0 ± 22.0 nM (Table I).¹³ The recombinant Rho expressed in *P. pastoris* inhibited platelet aggregation with a K_I of 83.2 ± 10.4 nM, which was as potent as native Rho (Table I). Recombinant Den was 1.8 times less active than recombinant Rho in inhibiting platelet aggregation. These results showed that different scaffolds had little effect on their interactions with integrin α IIB β 3 of the platelets.

Inhibition of cell adhesion to fibrinogen and fibronectin

Inhibition of cell-expressing integrins α IIB β 3, α v β 3, and α 5 β 1 to their ligands by Den and Rho was examined. The adhesions of CHO-expressing α IIB β 3 and α v β 3 to immobilized fibrinogen are integrins α IIB β 3- and α v β 3-dependent, respectively.²⁴ They were consistent with the results of different integrin function-blocking mAbs.^{25,26} In addition, the adhesion of K562 cells to fibronectin (FN) in the presence of 500 μ M Mn²⁺ was shown to be predominantly α 5 β 1-dependent.²⁷ Their inhibitory constants are summarized in Table I. Den and Rho inhibited the

Table I. Summary of the Inhibition of Platelet Aggregation and Cell Adhesion by Den and Rho

Protein	Platelet	IC ₅₀ (nM)			
		$\alpha_{IIb}\beta_3$ /Fg	$\alpha_v\beta_3$ /Fg	$\alpha_5\beta_1$ /Fn	
DEN	149.8 ±13.0	77.4 ±20.3	32.5 ±9.4	239.8 ±41.9	
Rho	83.2 ±10.4	21.0 ±11.2	13.0 ±5.7	256.8 ±87.5	

adhesion of CHO cells that expressed integrin $\alpha_{IIb}\beta_3$ to immobilized fibrinogen with the IC₅₀ values of 77.4 and 21.0 nM. Similar to the result of the platelet aggregation assay, Den was 3.7 times less active in inhibiting the expression of the integrin $\alpha_{IIb}\beta_3$ than was Rho. Den and Rho inhibited CHO cells that expressed $\alpha_v\beta_3$ from adhering to immobilized fibrinogen with the IC₅₀ values of 32.5 and 13.0 nM. Den and Rho inhibited K562 cells from adhering to immobilized FN with the IC₅₀ values of 239.8 and 256.8 nM, respectively. In contrast to their similar inhibitory activity of integrin $\alpha_5\beta_1$, Den exhibited 3.7 and 2.5 times less active in inhibiting the expression of the integrins $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ than was Rho.

Structure determination

NMR spectroscopy was used to examine the folding of Den. Because there are ¹H chemical shifts of Den at pH 4, we first measured 2D and 3D NMR spectra of Den under the same conditions (Figs. 1 and 2 and Supporting Information Figs. 2 and 3).^{28,29} ¹H-¹⁵N HSQC spectra of recombinant Den at pH 4 showed that the NH chemical shifts were consistent with the reported values, which indicated that recombinant Den had the correct fold (Fig. 1). Only the H^N chemical shift of I2 ($\Delta\delta = -0.67$ ppm) downshifts more than 0.5 ppm because of six extra histidines at the N-terminus. Although the chemical shifts of the recombinant Den expressed in *P. pastoris* were consistent with those of native Den, it is still necessary to identify its disulfide pairings and secondary structures. Therefore, we performed NOESY experiments of Den at pH 2, 4, and 5.5 in 100% D₂O to determine the four disulfide bonds of Den. Their pairings can be determined by searching H^β to H^β, H^β to H^α, and H^α to H^α NOEs between different cysteines. Specifically, the NOEs between H^β and H^β of different cysteines can provide 98% uniqueness.³⁰ NOESY spectra of Den at pH 4 were used to examine the NOE patterns of the disulfide bridges (Supporting Information Fig. 2). All four cysteine pairs of 3–22, 17–37, 39–51, and 52–57 were found from their H^β/H^β and H^β H^α NOE patterns in the spectra. NMR analysis of the secondary structures of recombinant Den showed that it exhibited the double- and triple-stranded antiparallel β -sheets and three loops as do native proteins. The formation of double- and triple-stranded antiparallel β -sheets was characterized by

the H^α-H^α, H^α-H^N, and H^N-H^N NOE patterns of the connecting strands, the slowly exchanging amide protons, and the downfield-shifted α protons. Strip plots of ¹⁵N-edited NOESY of Den at pH 4 clearly showed the NOEs between H^α of C22 to H^N of G38, H^α of K24 to H^N of G36, H^α of N25 to H^N of Y50, H^α of R35 to H^N of N25, H^α of C37 to H^N of Y23, H^N of Y23 to H^N of G36, H^N of K24 to H^N of Y50, and H^N of N25 to H^N of R34, which indicated the formation of triple-stranded antiparallel β sheets (Supporting Information Fig. 3). NOEs of W27 to I32 and M46, as well as a slow *H/D* exchange rate for the side-chain amino protons of N58, were found as native proteins, which suggested that they maintain their tertiary fold (Fig. 3). Based on our NMR studies, the recombinant Den produced in *P. pastoris* has the same three-fingered fold as native proteins.²⁹

The solution structure of Den was determined using NMR spectroscopy and the hybrid distance geometry-dynamical simulated annealing method. ¹H-¹⁵N HSQC spectra of recombinant Den were recorded at pH 4, 4.8, and 5.2. Upon raising the pH from 4.0 to 5.2, many of the amide proton resonances decreased in intensity, and the R43, G44, and D45 residues showed similar NOE patterns. Therefore, NMR spectra at pH 4 were chosen for structure determination. NMR assignment of Den was obtained by analyzing standard 2D homonuclear and 3D heteronuclear NMR data. The 3D structure of Den was calculated using 773 experimentally derived restraints with an average of 13.1 restraints per residue (Table II). The 20 best structures of Den from 100 initial structures are shown in Figure 3.

Figure 2. Amide strip plots of Den and Rho. A: Amide strips from R43 to M46 of Den and (B) from R49 to M52 of Rho at pH 6.0. The dNN (*i*, *i* + 1) and d α N (*i*, *i* + 1) NOE connectivities are shown.

Figure 3. Solution structures of Den. Stereoview of 20 lowest-energy NMR structures of Den were shown. The β -sheet secondary structure is shown in light blue.

The backbone RMSD value of Den was 1.25 ± 0.44 Å, and the backbone RMSD values of Den for β -sheet regions (3–22, 17–37, 39–51, and 52–57) were 0.48 ± 0.10 Å. Based on Ramachandran analysis, all dihedral angles of Den were in the allowed region. A summary of the restraints and structural statistics is presented in Table II. Overall, the tertiary structure of Den has an elongated and asymmetric shape and consists of three two-stranded antiparallel β -sheets with many tight turns and loops.

Structural difference between the RGD motif of Den and Rho

Because Den and Rho have different 3D scaffolds, we compared only their 3D conformations of the RGD loop that is the binding site for integrins. Ten of 20 of the Den and Rho structures were selected to align the nine-residue RGD loop, and the RMS deviations of the nine-residue backbone atoms of Den and Rho were 0.356 and 0.551 Å, respectively [Fig. 4(A,B)]. The RGD loop conformations of Den and Rho were found to have similar conformations forming a type I turn; however, there were differences in their backbone conformation (Fig. 4). This is consistent with the NOE amide strips from R43 to M46 of Den (Fig. 2).

No structural differences between the integrin $\alpha\beta3$ complexes of Den and Rho

The docking of Den into integrin $\alpha\beta3$ was used to identify its integrin-interacting residues, because they were most active in inhibiting integrin $\alpha\beta3$. The distance and the hydrogen bond restraints were derived from the X-ray structure of integrin $\alpha\beta3$ complexed with a cyclic RGD pentapeptide (PDB code 1L5G), and eight key interactions were found between integrin $\alpha\beta3$ and the R and D residues.³¹ Using these restraints, we docked Den into integrin $\alpha\beta3$. A comparison with Rho showed that the docking of Den into integrin $\alpha\beta3$ resulted in a similar number of contacts (Supporting Information Table SI). The key contacts—seven hydrogen bonds and

two salt bridges between the R and D residues of the RGD motif and integrin $\alpha5\beta1$ —were the same.

Dynamics analysis of Den

^1H - ^{15}N -correlated NMR spectroscopy was used to measure ^{15}N R_1 , ^{15}N R_2 , and ^1H - ^{15}N NOE parameters for Den. The optimized value of τ_m for Den was determined to be 5.2 ns. This value was smaller than that estimated from the trimmed mean R_2/R_1 ratios, which was 3.6 ns. However, the values of the other parameters agreed with the values of long neurotoxin III, toxin α , and cardiotoxin (CTX), which were 4.6, 3.7, and 4.8 ns. Because Den is an axially symmetric ellipsoid, the axially symmetric model was used for the analysis. Starting from the initial estimates of τ_m , $D_{\parallel}/D_{\perp}$, θ and Ψ , we analyzed the ^{15}N relaxation data by using axially symmetric models

Table II. Summary of Structural Restraints and Statistics for Den

Restraints used in the structure calculation	
<i>Distance and dihedral angle restraints</i>	
Intraresidue	69
Sequential	105
Medium range	249
Long range	271
Hydrogen bonds	21
Dihedral angles	58
Total	773
Energy statistics	
<i>X-PLOR energy (kcal mol⁻¹)</i>	
E_{NOE}	63.39 ± 6.11
E_{vdw}	3.40 ± 0.85
Geometric statistics	
<i>Deviations from idealized geometry</i>	
All backbone atoms (Å)	1.25 ± 0.44
Backbone atoms (2–5, 13–17, 21–26, 34–38, and 49–54) (Å)	0.48 ± 0.10
All heavy atoms (Å)	2.03 ± 0.43
Heavy atoms (2–5, 13–17, 21–26, 34–38, and 49–54) (Å)	1.21 ± 0.22
<i>Ramachandran analysis</i>	
Most favored regions (%)	74.5
Additionally allowed regions (%)	25.5
Generously allowed regions (%)	0.0

Figure 4. Structural alignment of the RGD loop. Structural alignments of the nine-residue RGD loop containing RI[P/A]RGDMPD sequences of (A) Den and (B) Rho. Ten of 20 Den and Rho structures were aligned, and the RMS deviations of the nine-residue backbone atoms of Den and Rho were 0.356 and 0.551 Å, respectively. The side chains of R, D, and others are shown in blue, red, and green, respectively. C: Superimposition of the nine-residue RGD loop of the average structure of Den and Rho. The side chains of R and D are shown in light blue and light red, respectively.

for rotational diffusion tensor. The obtained diffusion tensor of Den was asymmetric with $D_{\parallel}/D_{\perp} = 1.259$, which was higher than the reported values of 1.12

for long neurotoxin III and 1.06 for toxin α . The RGD-containing loop III, particularly the G44 residue, and the N-terminal region with low-NOE values showed distinctive backbone flexibility, which is consistent with the NMR structure of Den. The R_1 and R_2 values were very similar throughout the sequence, except that the residues G44, D45, and M46 in the loop III had lower NOE values, the G38 residue had a higher R_2 value, and the residues R1, F40, and G44 had lower R_1 values (Fig. 5 and Supporting Information Fig. S4).

The square of the generalized order parameter (S^2), the effective internal correlation time (τ_e), and a conformational exchange broadening parameter (R_{ex}) for each backbone amide NH vector were determined using the model-free formalism (Fig. 6 and Supporting Information Fig. S5).^{32,33} The average S^2 values of Den overall and the β -sheet were 0.84 and 0.90, respectively. Compared to the rest of the Den backbone, only the S^2 values of the residue R1 were lower than that of G48. Most residues of Den exhibited extensive flexibility with fast motion on the picosecond per nanosecond time scale. The N-terminal region was the most flexible region with an average S^2 value of 0.26; however, the C-terminal was very rigid with an average S^2 value of 0.90. The average S^2 values of loops I and II were 0.87 and 0.81, and loop III, the flexible RGD loop, had an average S^2 value of 0.69. The average S^2 values of N-terminus and loops II and III were lower than the overall average value, which suggested that these regions were flexible. The tips of loop I (L7 and T9), loop II (N31, I32, I33, and R34), and loop III (D45, and M46) in Den exhibited flexible motions on the picosecond per nanosecond timescale.

Comparison of the dynamic properties of Den and other three-fingered toxins

Backbone dynamics of two three-fingered toxins, toxin α , and CTX were determined using NMR spectroscopy.^{34,35} A comparison of the backbone dynamics of these two toxins with that of Den showed that they were different from their average S^2 values for different regions (Table III). It is consistent that the average S^2 values of the three-fingered toxins' β -sheet were higher, and those of loops II and III and of the N-terminal region were lower than the overall average values. In contrast, the average S^2 values of loop I and the C-terminal region were diverse and depended on individual three-fingered toxins. For example, the average S^2 values of loop I and the C-terminal region of Den and toxin α were higher than their overall average value. In contrast, those of CTX were lower than its overall average value. Interestingly, their target-interacting loops had lower S^2 values. For example, loop III of Den, the integrin-binding loop, had the lowest average S^2 value. Loop II of toxin α and CTX also had the lowest average S^2 values.

Figure 5. The relaxation parameters of Den. A: ^{15}N R_1 with error. B: ^{15}N R_2 with error. C: ^1H - ^{15}N steady-state NOE with error. Gaps indicate the proline residues, and the β -sheet secondary structure is shown.

Comparison of the dynamic properties of Den, Rho, and RGD-containing proteins

The backbone dynamics of many RGD-containing peptides and proteins, including Rho, echistatin, FN, and tenascin (TN), were determined using NMR spectroscopy.^{36,35,37,38} A summary of these values of the residues R, G, D, and M/S is presented in Table IV. Although both Den and Rho have a PRGDMP amino acid sequence in the loop, the backbone dynamics of this sequence are very different from those of the RGD motif, except that their M residues exhibited very similar backbone dynamics. The S^2 values of the R, G, D, and M residues in the RGD loop of Den were 0.82, 0.47, 0.64, and 0.63, and in

the RGD loop of Rho were 0.75, 0.63, 0.78, and 0.66. The S^2 values of the Rho R and D residues were close to its overall average value. In contrast, the S^2 values of Den D residue were lower than its overall average value, and the value of the G residue was 0.47, which indicated that the RGD loop of Den was more flexible than that of Rho.

In comparison with the backbone dynamics of other RGD-containing proteins, the Den and Rho relaxation parameters were very diverse. For example, the ranges of the S^2 order parameters of R, G, D, and M/S residues were 0.46–0.86, 0.39–0.83, 0.46–0.78, and 0.44–0.78. The values of the τ_e and R_{ex} parameters were also very diverse, and limited

Figure 6. The model-free parameters of Den. Generalized order parameters (A) S^2 , (B) τ_m , and (C) R_{ex} . Gaps indicate the proline residues, and the β -sheet secondary structure is shown. Only some fitting models resulted in the R_{ex} and τ_e terms.

Table III. Average Order Parameter Values of Three-Fingered Toxins

Proteins	Average S^2						
	Overall	β -Sheets	N-terminal region	Loop I	Loop II	Loop III	C-terminal region
DEN ^a	0.84	0.90	0.26	0.87	0.81	0.69	0.90
Toxin α^b	0.80	0.83	ND ^d	0.84	0.70	0.75	0.84
CTX ^c	0.80	0.84	0.72	0.78	0.77	0.79	0.76

^a N-terminal region (1), β -sheets (2–5, 13–17, 21–26, 34–38, and 49–54), loop I (6–12), loop II (27–33), loop III (39–48), and C-terminal region (55–59) of DEN are determined.

^b N-terminal region (1), β -sheets (2–4, 14–16, 23–29, 34–40, and 49–54), loop I (5–13), loop II (30–33), loop III (41–48), and C-terminal region (55–61) of toxin α are determined.

^c N-terminal region (1), β -sheets (2–4, 11–13, 20–25, 34–39, and 50–54), loop I (5–10), loop II (26–33), loop III (40–49), and C-terminal region (55–60) of CTX are determined.

^d ND, not determined.

correlations were found from these parameters. However, it is consistent that their RGD loop was flexible and that the degree of flexibility depended on the proteins. For example, the RGD loops of Ech and FN were more flexible than that of TN (Table IV). Most of the G residue exhibited fast motion on the picosecond per nanosecond time scale, and the D residue exhibited slow motion on the microsecond per millisecond time scale and fast motion on the picosecond per nanosecond time scale. These dynamic properties may be important for their binding to integrins.

Discussion

The structure and dynamics relationships of the RGD motif of RGD-containing proteins affect their binding specificities and affinities on integrins.^{35–38} In the present study, we showed that Den and Rho

have different tertiary folds with similar 3D conformation for the RGD motif. However, their ability to inhibit the integrins $\alpha v\beta 3$, $\alpha 5\beta 1$, and $\alpha IIb\beta 3$ was different and to inhibit the integrin $\alpha v\beta 3$ was significantly different. In contrast, docking analysis showed no interaction differences between their RGD motif and the integrin $\alpha v\beta 3$. A difference was found, however, in the backbone dynamics of their RGD residues. These results showed that protein scaffolds affect their function and dynamics but not the conformation of the RGD motif, which suggests that the flexibility and the motions of the RGD residues may be important for their interaction with integrins.

The presence of the RGD amino acid sequence is not sufficient for its biological activity. Studies on identifying the criteria for biologically active RGD motif were well characterized.⁴² The RGD motif, the

Table IV. Relaxation Data and Dynamic Parameters of RGD-Containing Proteins

	R_1 (s ⁻¹)	R_2 (s ⁻¹)	NOE	S^2	τ_e (ns)	R_{ex} (s ⁻¹)	IC ₅₀ (nM)
R43 ^{Den^a}	1.95 ± 0.06	6.36 ± 0.17	0.69 ± 0.01	0.82 ± 0.02			32.5 ± 9.4 ^a
R49 ^{Rho^b}	1.69 ± 0.08	8.32 ± 0.13	0.48 ± 0.01	0.75 ± 0.01	0.11 ± 0.04	1.39 ± 0.15	13.0 ± 5.7 ^b
R78 ^{FN10^c}				0.46	3.25		835 ± 287 ^e
R76 ^{TN3^c}				0.86			30 ^f
G44 ^{Den^a}	1.36 ± 0.05	3.63 ± 0.03	0.03 ± 0.01	0.47 ± 0.02	0.09 ± 0.01		32.5 ± 9.4 ^a
G50 ^{Rho^b}	1.55 ± 0.02	5.09 ± 0.03	0.32 ± 0.02	0.63 ± 0.01	0.81 ± 0.03		13.0 ± 5.7 ^b
G79 ^{FN10^c}				0.39	2.75		835 ± 287 ^e
G77 ^{TN3^c}				0.83			30 ^f
G25 ^{Ech^d}	2.44 ± 1.27	9.06 ± 1.58	0.35 ± 0.02	0.50 ± 0.02	1.19 ± 0.05	4.44 ± 0.15	20.7 ± 8.0 ^g
D45 ^{Den^a}	1.61 ± 0.05	5.17 ± 0.02	0.39 ± 0.01	0.64 ± 0.03	0.08 ± 0.03		32.5 ± 9.4 ^a
D51 ^{Rho^b}	1.72 ± 0.03	10.03 ± 0.02	0.50 ± 0.02	0.78 ± 0.01	0.11 ± 0.01	2.94 ± 0.16	13.0 ± 5.7 ^b
D80 ^{FN10^c}				0.52	2.40		835 ± 287 ^e
D78 ^{TN3^c}				0.77		1.32	30 ^f
D26 ^{Ech^d}	2.86 ± 1.01	5.88 ± 1.40	0.46 ± 0.02	0.46 ± 0.02	1.67 ± 0.08	1.17 ± 0.13	20.7 ± 8.0 ^g
M46 ^{Den^a}	1.57 ± 0.06	4.99 ± 0.27	0.35 ± 0.01	0.63 ± 0.02	0.08 ± 0.01		32.5 ± 9.4 ^a
M52 ^{Rho^b}	1.51 ± 0.05	6.08 ± 0.01	0.44 ± 0.01	0.66 ± 0.01	0.07 ± 0.01		13.0 ± 5.7 ^b
S81 ^{FN10^c}				0.44	2.41		835 ± 287 ^e
M79 ^{TN3^c}				0.78			30 ^f

^a Dendroaspin from this study.

^b Dynamic properties and IC₅₀ of rhodotomin are reported by Chen et al.³⁸

^c Dynamic properties of type III domain of fibronectin and tenascin are reported by Carr et al.³⁷

^d Dynamic properties of echistatin are reported by Chen et al.³⁵

^e IC₅₀ of fibronectin is reported by Belvisi et al.³⁹

^f IC₅₀ of tenascin is reported by Chung and Erickson.⁴⁰

^g IC₅₀ of echistatin is reported by Chen et al.⁴¹

important binding site for integrin, lies in a mobile loop ranging from 5 to 11 residues joining two strands of β sheet.¹² The RGD motif is located at the apex of a long loop, between two β strands of the protein, protruding 10–17 Å from the protein core.^{6–12} The flexibility and solvent exposure of the RGD loop are responsible for the recognition and fitting the interface between integrin α and β subunits. Studies^{35,37,38} also show that the dynamic properties of the RGD motif may be important in its interaction with integrins. It is known that the residues flanking the RGD motif of RGD-containing proteins affect their binding specificities and affinities on integrins.^{27,36} Although Den and Rho have the same PRGDMP sequence in their RGD loop, they exhibited diverse specificity in inhibiting integrins. These results suggest that not only the flanking residues but also protein scaffold can affect the backbone dynamics of the RGD motif.

The synergy between structure and dynamics is essential to the function of integrin complexes.^{6–12} Many studies have compared the binding affinity of RGD-containing peptide with their 3D conformations and backbone dynamics.^{27,36,38,43} As shown in Table IV, the residues of the RGD loop of these proteins exhibited extensive flexibility on fast motion on the picosecond per nanosecond time scale. In general, proteins and peptides with a flexible RGD loop exhibited lower binding affinity and interacted with many integrins. However, proteins and peptides with a specific RGD conformation exhibited higher binding affinity and interacted with specific integrins. This is consistent with our results that the RGD loop of the tenth-type III domain of FN (FN10) is more flexible than those of Den, Rho, and Ech, and FN10 exhibited lowest integrin-binding affinity (Table IV). The flexibility of the RGD loop in FN supports its recognition to diverse integrins. In contrast, the more rigid RGD loop in TN reflects their specificity for particular integrins.³⁷ Analyses of the results indicate that the dynamic properties and 3D conformation of the RGD motif affect their specificity and binding affinity to integrins. However, backbone dynamics of individual RGD residues of these proteins were not correlated with their functions. For example, the S^2 value for the RGD residues in Den and Rho was different: the S^2 value of the R residue in Den was larger than Rho; and the S^2 values of the G and D residues in Den were smaller than those of Rho. The correlation of structure and dynamics of the RGD-containing proteins with function is required for further investigation.

Toxins with unrelated folds can have similar functions. Three-fingered snake toxins show a wide spectrum of biological activities with a similar fold. In this study, we expressed Den in *P. pastoris* and used NMR spectroscopy to confirm its folding. Our analyses of the NOE patterns between different cys-

teines, the chemical shifts, and the secondary structures of Den indicate that the folding of Den expressed in *P. pastoris* was correct. The disulfide pairings of Den were in close spatial proximity, which is consistent with its reported disulfide pairings.^{13,14} The present study provides the first direct evidence that highly disulfide-bonded three-fingered toxins can be expressed in *P. pastoris* with the correct fold and be labeled with the ¹⁵N isotope. Comparing the backbone dynamics of Den with those of toxin α and CTX showed that they were different from their average S^2 values for different regions. In particular, the RGD loop of Den had the lowest average S^2 value, which is consistent with the RGD loops of Rho and FN being the most flexible region in these proteins. Although the relaxation parameters of the RGD residues in proteins with different scaffolds are diverse, it is evident that most of the G residue exhibited fast motion on the picosecond per nanosecond time scale, and D residue exhibited slow motion on the microsecond per millisecond time scale and fast motion on the picosecond per nanosecond time scale. These results suggest that not only conformation but also the dynamic properties of the RGD motif are important for its biological activity.

In conclusion, this is the first comparison of the relaxation parameters of the RGD loop of different protein scaffolds. We expressed Den, a three-fingered toxin containing four disulfide bonds, in a *P. pastoris* expression system. We found that Den produced in *P. pastoris* has the same function and structure as those in native protein. This study provides the direct evidence that highly disulfide-bonded three-fingered toxins can be expressed in *P. pastoris* with the correct fold. This evidence may serve as the basis for uncovering the species and tissue in which evolution has used this adhesive domain. Our study also suggests that the inhibitory potency and selectivity of RGD-containing proteins cannot be determined only by the sequence content of the RGD loop. This study may serve as the basis for exploring the structure, dynamics, and functions relationships of integrins and their ligands.

Materials and Methods

Expression and purification of Den and Rho in *P. pastoris*

The expression and purification of Den and Rho in *P. pastoris* were done following previously described protocols.^{16,44} Den and Rho expression in *P. pastoris* was achieved using yeast transfer vector (pPICZ α A; Invitrogen) and an expression kit (EasySelect version A; Invitrogen) with minor modifications to the manufacturer's instructions. The structural gene of Den was amplified using a polymerase chain reaction (PCR) with the sense primer:

5'-AATTGGAATTCCATCATCATCATCATCATGGTAAGGAATGTGACTGTTCTT-3', has *Eco* RI recognition and six histidine residues for facilitating purification. The antisense primer was 5'-CCGCGGCCGCGGTCACTGGTATCTTGGACAGTCAGC-3' with *Sac* II recognition and a TCA stop codon. The structural gene of Rho was amplified using PCR with the sense primer: 5'-AATTGAATTCCATCATCATCATCATCATAGAATTTGTTTTAATCATCAATCTTCTCAACCAAACTACT-3', which has *Eco* RI recognition and six histidine residues for facilitating purification. The antisense primer was 5'-AATTCCGCGGATTATTACAACTTCAGATTCACAACAAGACAACCTTAATACCTGGCTTAACAGTTGGACA-3' with *Sac* II recognition and a TAA stop codon. The structural gene of Rho was amplified using a PCR with the sense primer: 5'-AATTGAATTCCATCATCATCATCATCATAGAATTTGTTTTAATCATCAATCTTCTCAACCACA AACTACT-3', which has *Eco* RI recognition and six histidine residues for facilitating purification. The antisense primer was 5'-AATTCCGCGGATTATTACAACTTCAGATTCACAACAAGACAACCTTAATACCTGGCTTAACAGTTGGACA-3' with *Sac* II recognition and a TAA stop codon. The PCR product was purified and then cloned into the *Eco* RI and *Sac* II sites of pPICZαA. The recombinant plasmid was transformed into the *DH5α* strain, and the colony was selected using an agar plate with low-salt lateral binding (1% tryptone + 0.5% yeast extract + 0.5% NaCl + 1.5% agar [pH 7.5]) and 25 µg/mL of the antibiotic phleomycin D1 (Zeocin; Invitrogen). After the clone had been confirmed by sequencing the insert, 10 µg of plasmid was digested with *Sac* I to linearize the plasmid. The linearized construct was transformed into the *Pichia* strain X-33 using the heat-shock method, and the transformation was done with a kit (*Pichia* EasyComp; Invitrogen). The transformant was integrated at the AOX1 locus using single crossover, and the colony was selected using an agar plate with yeast peptone dextrose (1% yeast extract, 2% peptone, 2% glucose, and 2% agar) and 100 µg/mL of Zeocin. We picked the highest protein expression clone of Den and Rho from a number of clones with multiple copies of the Den gene insertion.

We produced ¹⁵N-labeled Den and Rho as follows: 100 µL of cell stock was grown at 30°C in 200 mL of ¹⁵N minimal medium [0.34% yeast nitrogen base (YNB) without ammonium sulfate and amino acids, 2% dextrose, 4 × 10⁻⁵% biotin, and 0.05% ¹⁵NH₄Cl] in 100 mM potassium phosphate buffer [pH 6] with 100 µg/mL of Zeocin for 48 h. The cells were then transferred into 800 mL of ¹⁵N minimal medium. After another 48 h, the cell densities reached OD₆₀₀ of 25–30. The cells were then centrifuged, collected, and grown in 1 L of ¹⁵N minimal methanol medium (0.34% YNB without ammonium sulfate and amino acids, 4 × 10⁻⁵% biotin, 1%

methanol, and 0.05% ¹⁵NH₄Cl). Once every 24 h, 1% methanol was added to induce protein expression for 2 days. The supernatant was collected using centrifugation and dialyzed twice against 4 L of H₂O. The final solution was loaded into a nickel-chelating column and eluted with a gradient of 200 mM imidazole. The recombinant Den and Rho produced in *P. pastoris* was further purified by reversed-phase C18 HPLC with a gradient of 15–20% acetonitrile. The purification of recombinant proteins was greater than 95% pure as judged using tricine–SDS–PAGE.

Mass spectrometry measurements

The molecular weights of Den and Rho were confirmed using an API 365 triple quadrupole mass spectrometer equipped with a TurboIonSpray source (PE Sciex, Thornhill, Canada). Protein solutions (1–10 µM in 50–90% methanol or acetonitrile with 0.1% formic acid) were infused into the mass spectrometer using a syringe pump (Harvard Apparatus, South Natick, MA) at a flow rate of 12–20 µL/min to acquire full-scan mass spectra. The electrospray voltage at the spraying needle was optimized at 5000–5300 V. The molecular weights of proteins were calculated using computer software provided with the spectrometer.

Platelet aggregation assay

Venous blood (nine parts) from healthy donors who had not received any medication for at least 2 weeks was collected in 3.8% sodium citrate (one part). Blood was centrifuged at 150g for 10 min to obtain platelet-rich plasma (PRP) and allowed to stand for 5 min, after which PRP was collected. Platelet-poor plasma (PPP) was prepared from the remaining blood by centrifuging it at 2000g for 25 min. The PPP platelet count was measured on a hematology analyzer and diluted to 250,000 platelets/µL. A solution of 190 µL of PRP and 10 µL of either Rho or PBS buffer was incubated for 5 min using an aggregometer (Hema Tracer 601; Nikoh Bioscience, Tokyo) at 37°C. Ten microliters of 200 mM ADP was added to monitor the response of platelet aggregation using light transmission.

Cell adhesion assay

A cell adhesion assay was done using protocols previously described.^{27,36,43} Ninety-six-well microtiter plates (Costar; Corning) were coated with 100 µL of PBS buffer containing 200 µg/mL fibrinogen or 25 µg/mL FN and incubated overnight at 4°C. Nonspecific protein-binding sites were blocked by incubating each well with 200 µL of heat-denatured 1% BSA (Calbiochem) at room temperature for 1.5 h. The heat-denatured BSA was discarded, and each well was then washed twice with 200 µL of PBS.

CHO cells that expressed the integrins αvβ3 (CHO-αvβ3) and αIIbβ3 (CHO-αIIbβ3) were kindly

provided by Dr. Y. Takada (Scripps Research Institute) and maintained in DMEM.^{36,43} Human erythroleukemia K562 cells were purchased from ATCC and cultured in Roswell Park Memorial Institute (RPMI)-1640 medium containing 5% FCS. Harvested K562 cells were washed in PBS buffer containing 1 mM EDTA and resuspended in Tyrode's buffer (150 mM NaCl, 5 mM KCl, and 10 mM Hepes) [pH 7.35] containing 1 mM MgSO₄, 2 mM CaCl₂, and 500 μM MnCl₂. CHO and human erythroleukemia K562 cells were diluted to 3 and 2.5 × 10⁵ cells/mL, respectively, and 100 μL of the cells was used for the assay. Den and Rho (0.001–500 μM), which were used as inhibitors, were added to the cells and incubated at 37°C in a 5% CO₂ atmosphere for 15 min. The treated cells were then added to the coated plate and reacted at 37°C (5% CO₂) for 1 h. The reacting solution was then discarded, and nonadhered cells were removed by washing them twice with 200 μL of PBS. After the nonadhered cells had been removed by rinsing the wells with the same buffer, adhered cells were quantified using a crystal violet assay. The well was fixed with 100 μL of 10% formalin for 10 min and then dried. A solution of 50 μL of 0.05% crystal violet was added to the well at room temperature for 20 min. Each well was then washed four times with 200 μL of distilled water and dried. Coloring solution (150 μL of 50% alcohol and 0.1% acetic acid) was then added. The resulting absorbance was read at 600 nm, and the readings were correlated with the number of adhering cells. Inhibition was defined as % inhibition = 100 – [OD₆₀₀ (Rho protein-treated sample)/OD₆₀₀ (untreated sample)] × 100. The reported IC₅₀ values are the average of at least three separate experiments.

NMR spectroscopy

NMR experiments were done at 27°C on a spectrometer (Bruker Avance 600- and 700 MHz) equipped with pulse-field gradients and xyz-gradient triple-resonance probes. In these experiments, samples were dissolved in 10% D₂O/90% H₂O or 100% D₂O at a concentration of 3 mM; pHs were adjusted to 4.0 or 6.0 with 100 mM KOD. NMR spectra of Ech at pH 4 were performed for resonance assignment. The data were processed with Topspin Version 1.3 software and analyzed with Aurelia software. 2D NOESY, TOCSY, and DQF-COSY NMR spectra were recorded in the phase-sensitive absorption mode with quadrature detection in both F1 and F2 dimensions. A concentration of 2 mM ¹⁵N-labeled Den and Rho was used for the 2D ¹H-¹⁵N HSQC, 3D ¹H-¹⁵N edited-TOCSY, and NOESY-HSQC experiments. Mixing times of 30–90 and 60–150 ms were used for TOCSY and NOESY experiments, respectively. The center frequencies of double resonance experiments were 4.75 (¹H) and 118 ppm (¹⁵N). The observed ¹H chemical shifts were referenced with respect to the

H₂O or HOD signal, which was 4.754 ppm downfield from external sodium 3-trimethylsilylpropionate-2,2,3,3-d₄ in D₂O (0.0 ppm) at 300 K. The nitrogen chemical shift was referenced to external ¹⁵NH₄Cl (3 mM in 1M HCl) at 300 K, which is 24.93 ppm downfield from liquid NH₃.

Structure calculations

Structures were calculated using the program X-PLOR with the hybrid distance geometry-dynamical simulated annealing method.⁴⁵ NOESY cross-peak intensities—categorized into strong, medium, weak, and very weak—were converted into distance constraints of 1.8–2.8, 1.8–3.6, 1.8–5.0, and 2.5–6.0 Å, respectively. Pseudoatom corrections were used for methylene, methyl, and aromatic protons, and an additional 0.5 Å was added to the upper-limit distances involving methyl protons. The dihedral angles ϕ were determined from the ³J_{NH α} coupling constants. For ³J_{NH α} values less than 5 Hz, ϕ values were restricted from –30° to –90°, and for ³J_{NH α} values greater than 10 Hz, ϕ values were restricted from –100° to –170°. Two restraints were used for each NH-CO backbone hydrogen bond with d_{N-O} restricted to 2.4–3.3 Å and d_{H-O} to 1.7–2.3 Å. A family of 100 structures was generated using NOE distance, dihedral angle, and hydrogen bond restraints. The S-S covalent bonds were deleted and reintroduced as pseudo-NOE distances with the S-S distances constrained to the upper limit of 2.1 Å. During the first phase of dynamics at 2000 K, the value of the force constant of the NOE term was kept constant at 50 kcal/mol^{–1} Å^{–2}. The repulsion term was gradually increased from 0.03 to 4.0 kcal/mol^{–1} Å^{–2} and the torsion angle term from 5 to 200 kcal/mol^{–1} rad^{–2}. The simulated annealing refinement consisted of a 9-ps cooling dynamic followed by 200 cycles of Powell minimization. The 20 lowest-energy structures were accepted based on violations of distance restraints less than 0.5 Å, dihedral angle restraints less than 5°, a van der Waals energy cutoff value of 35 kcal/mol, and an NOE energy cutoff value of 55 kcal/mol. The structure figures were prepared using the MOLMOL or the PyMOL program.^{46,47}

Measurements of NMR dynamics

Backbone dynamics of Den and Rho acquired at two spectrometers (600 and 700 MHz). The ¹⁵N-spin-lattice (R_1) and spin–spin (R_2) relaxation rate constants and steady-state ¹H-¹⁵N NOEs were measured from ¹H-detected ¹H-¹⁵N correlation spectra recorded with sensitivity-enhanced pulse sequences. A recycle delay of 6 s was used, and 128 complex T_1 increments of 32 scans were acquired. A series of 10 experiments with relaxation delays of 30, 100, 150, 300, 450, 600, 800, 1000, 1500, and 3000 ms were done to measure T_1 . A series of 10 experiments with relaxation delays of 18, 36, 48, 72, 90, 100, 120, 150,

300, and 500 ms were done to measure T_2 . The longitudinal and transverse relaxation rate constants, R_1 and R_2 , were obtained from exponential fits of the peak height data using least-squares fit software (SigmaPlot; Jandel Scientific). The reported R_i values are the mean values of two independent data sets. In the NOE experiment, two spectra—one with the NOE and one without—were collected. The NOE was calculated as the ratio of peak heights in spectra collected with and without NOE. The reported NOE value was the average value of three pairs of NOE experiments.

The heteronuclear ^{15}N relaxation rate constants, R_1 and R_2 , and the ^1H - ^{15}N steady-state NOE values were analyzed using the FastModelFree program.⁴⁸ In this approach, the overall and internal molecular motions were assumed to be independent, and the spectral density function for a molecule undergoing isotropic tumbling was calculated using the appropriate expression:

$$J(\omega) = 2/5[(S^2\tau_m/[1 + (\omega\tau_m)^2]) + (S_f^2 - S^2\tau/[1 + (\omega\tau)^2])],$$

where $1/\tau = 1/\tau_m + 1/\tau_e$ and $S^2 = S_s^2 S_f^2$, τ_m is the overall rotational correlation time of the molecule, τ_e is the effective correlation time for the motions on the slower of the two time scales, S^2 is the square of the generalized order parameter, and S_s^2 and S_f^2 are the squares of order parameters for the motion on the slow and fast time scales, respectively.³²

Molecular docking

The docking of Rho and Den to integrin $\alpha v\beta 3$ was done with the docking program HADDOCK 1.3 using hydrogen bond and distance restraints.⁵⁰ The starting structures for the docking were the average-minimized NMR structures of Rho and integrin $\alpha v\beta 3$ (PDB code 1L5G). The interaction restraints were derived from an X-ray structure of integrin $\alpha v\beta 3$ in complex with a cyclic pentapeptide {c[–RGDf(NMe)V–]} using the software iMol-talk.^{31,51} The defined distance threshold was 4 Å, and the interaction restraints between the RGD motif and integrin were used for calculation. The input restraints between the R49/R43, G50/G44, and D51/D45 residues, and integrin $\alpha v\beta 3$ were 34, 10, and 34, respectively. They were the contacts between the R49/R43 residue and the residues Asp150, Tyr178, Gln180, and Asp218 of integrin αv , between the G50/G44 residue and the residues R216 and A218 of $\beta 3$, and between the D51/D45 residue and the residues Ser121, Tyr122, Ser123, Arg214, Asn215, Arg216, Asp217, and Ala218 and Mn^{2+} of the MIDAS of $\beta 3$. An additional 0.5 Å distance was added to the upper and lower limits in interaction restraints. Using these restraints, the standard

HADDOCK protocol for protein docking was done with minor modifications. This protocol combines three stages of molecular dynamics calculations, including heating and cooling with a progressive increase of the flexibility at the binding interface. In the first stage, 500 conformations were calculated using a rigid-body docking protocol. The best 100 structures in terms of their intermolecular energies were refined using semiflexible simulated annealing in the second stage. Both the side chains and the backbone atoms of the residues 46–54 of Rho, 40–48 of Den, 149–152, 176–181, 211–216, and 217–219 of αv , and 120–124 and 212–221 of $\beta 3$ were defined as flexible, which allowed them to move in a semirigid-body docking protocol to search for conformational rearrangements. The resulting 100 structures with the lowest intermolecular energy values were refined with explicit water molecules in the last stage. The structures were classified using clustering based on the pairwise RMSD differences. Two clusters of structures were found by fitting them over the RGD residues with an average RMSD value <1.5 Å for the backbone atoms of all the amino acids in 100 integrin complexes.

Protein Data Bank accession numbers and NMR assignments

The co-ordinates of 20 calculated structures of Den and Rho have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>), under accession numbers 2LA1 and 2PJF, respectively. ^1H and ^{15}N resonances of Den and Rho have been deposited in the BioMagResBank databank under accession numbers BMRB-6915 and BMRB-5117.

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