Effect of P to A Mutation of the N-Terminal Residue Adjacent to the Rgd Motif on Rhodostomin: Importance of Dynamics in Integrin Recognition

Jia-Hau Shiu1,9, Chiu-Yueh Chen1,9, Yi-Chun Chen1, Yao-Tsung Chang1, Yung-Sheng Chang2, Chun-Hao Huang1, Woei-Jer Chuang1,2*

1 Department of Biochemistry and Molecular Biology, Institute of Basic Medical Sciences, National Cheng Kung University College of Medicine, Tainan, Taiwan, 2 Institute of Biopharmaceutical Sciences, National Cheng Kung University College of Medicine, Tainan, Taiwan

Abstract

Rhodostomin (Rho) is an RGD protein that specifically inhibits integrins. We found that Rho mutants with the P48A mutation 4.4–11.5 times more actively inhibited integrin α5β1. Structural analysis showed that they have a similar 3D conformation for the RGD loop. Docking analysis also showed no difference between their interactions with integrin α5β1. However, the backbone dynamics of RGD residues were different. The values of the R2 relaxation parameter for Rho residues R49 and D51 were 39% and 54% higher than those of the P48A mutant, which caused differences in S2, Rho, and τc. The S2 values of the P48A mutant residues R49, G50, and D51 were 29%, 14%, and 28% lower than those of Rho. The Rho values of RGD residues R49 and D51 were 0.91 s⁻¹ and 1.42 s⁻¹; however, no Rho was found for those of the P48A mutant. The τc values of Rho residues R49 and D51 were 9.5 and 5.1 times lower than those of P48A mutant. Mutational study showed that integrin α5β1 prefers its ligands to contain (G/A)RGD but not PRGD sequences for binding. These results demonstrate that the N-terminal proline residue adjacent to the RGD motif affect its function and dynamics, which suggests that the dynamic properties of the RGD motif may be important in Rho’s interaction with integrin α5β1.

Introduction

The tripeptide sequence Arg-Gly-Asp (RGD) is the consensus sequence of many adhesive proteins, such as fibronectin, fibrinogen, vitronectin, and von Willebrand factor [1,2,3]. In mammals, 18 α and 8 β subunits assemble into 24 integrins. The RGD sequence is recognized by half of the 24 known integrins, whereas alternative short peptide sequences are recognized by other integrins [4]. In addition to adhesive proteins, the RGD sequence is found in many proteins, including dendroaspin [5], decorsin [6], savignyngrin [7], streptopain [8], γ-bungarotoxin [9], human herpesvirus 8 envelope glycoprotein B [10], and disintegrins [11]. Disintegrins are the peptides found in snake venoms of the viper family and mainly inhibit the functions of α1- and β3-associated integrins. They were first identified as inhibitors of integrin αIIbβ3 and were subsequently shown to bind with high affinity to other integrins and to block the interaction of integrins with RGD-containing proteins. They contain 47–84 amino acids with 4–7 disulfide bonds [17,18,19,20,21]. The RGD motif is located at the apex of a 5–11 residue loop, between two β strands of the protein, protruding 10–17 Å from the protein core [13]. The R and D sidechains in a flexible loop do not interact but nearly oppose each other by 180°.

Many studies have shown that the residues flanking the RGD motif of RGD-containing proteins affect their binding specificities and affinities on integrins [7,10,22,23,24,25]. For example, disintegrins with an ARGDW sequence have a higher affinity for binding with the integrin αIIbβ3, whereas disintegrins with an ARGDN sequence preferentially bind with integrins αvβ3 and α5β1 [24]. The amino acid sequences of the RGD loop from RIPRDPGM to TAVRDPGP of rhodostomin (Rho), resulting in a 196-fold decrease in inhibiting integrin αIIbβ3 [9]. Replacement of the N-terminal alanine with the proline of the RGD motif of elagantin, a disintegrin, with an ARGDN sequence preferentially bind with integrins αvβ3 and α5β1 [24]. The amino acid sequences of the RGD loop from RIPRDPGM to TAVRDPGP of rhodostomin (Rho), resulting in a 196-fold decrease in inhibiting integrin αIIbβ3 [9]. Replacement of the N-terminal alanine with the proline of the RGD motif of elagantin, a disintegrin, with an ARGDN sequence preferentially bind with integrins αvβ3 and α5β1 [24].
motif on the dynamics of disintegrin and the structure-activity relationships of RGD-containing proteins. Rho is obtained from Calloselasma rhodostoma venom and belongs to the family of disintegrins [26,27,28]. It consists of 68 amino acids, including 12 residues of cysteine and a PRGDPMP sequence at positions 40–53. We previously showed that Rho expressed in Pichia pastoris (P. pastoris) has the same function and structure as native protein [29]. In the present study, we expressed Rho P48A mutants and determined their activities in inhibiting the integrins αIIbβ3, αvβ3, and α5β1. We also used nuclear magnetic resonance (NMR) spectroscopy to compare 3D structures and backbone dynamics.

Materials and Methods

Expression of Rho and Its Mutants in P. pastoris and Purification

The expression of Rho and eleven mutants (P48A, M52W, P48A/M52W, M52N, P48A/M52N, M52G/P53W, P48A/M52G/P53W, M52D/P53L, P48A/M52D/P53L, M52D/P33M, and P48A/M52D/P33M) in P. pastoris was accomplished by following protocols previously described [27,28]. The expression kit and the yeast transfer vector, pPICZαA, were purchased from Invitrogen. The wild-type construct was used to produce the pPICZαC in 100 mL of yeast nitrogen base (YNB) medium (1% yeast extract, 2% peptone, and 2% dextrose) with 0.05% 15NH4Cl) in 100 mM of potassium phosphate buffer with pH 7.0 containing 1% ammonium sulphate and amino acids, 2% dextrose, and 0.1% (w/v) NaN3. Methanol (1% w/v) was added once every 12 h to induce protein expression for 2 days. The 15N-labelled proteins were produced as follows: 100 μL of cell stock grew at 30°C in 100 mL of yeast nitrogen base (YNB) medium (1% yeast extract, 2% peptone, and 2% dextrose) containing 100 μg/mL of Zeocin for 48 h. The cells were then transferred into 900 mL of YNB medium. After another 48 h, the cells were collected by centrifugation and grown in 1 L of minimal methanol medium (1.34% YNB with ammonium sulphate without amino acids and 4×10⁻⁵ M biotin). Methanol (1% w/v) was added once every 12 h to induce protein expression for 2 days.

The supernatant was collected by centrifugation and dialyzed against 10 L of H2O and once against 5 L of binding buffer twice against 10 L of H2O and once against 5 L of binding buffer until it was used.

Fibronectin Purification

Fibronectin was purified from citrated human plasma using gelatin-Sepharose 4B affinity chromatography as previously described [29]. One hundred milliliters of human plasma was centrifuged at 5000 rpm for 30 min and then filtered through Whatman filter paper. The filtrate was applied to a pre-equilibrated gelatin-Sepharose resin with phosphate-buffered saline (PBS: 10 mM phosphate buffer, 0.15 M NaCl [pH 7.4]) at pH 7 containing 5 mM EDTA, 0.05% (w/v) NaN3, and 1 mM benzamidine. The resin was washed with 1 M NaCl and 1 mM benzamidine at pH 7, and fibronectin was eluted using 1 M urea and 1 mM benzamidine at pH 7. The fractions were dialyzed three times against 4 L of PBS buffer at pH 7.4 and concentrated using Amicon with a 10-kDa cutoff membrane. The yields of fibronectin were 15–20 mg, and the purification of human fibronectin was greater than 95% as determined using SDS-PAGE. Purified fibronectin was stored at −70°C until it was used.

Mass Spectrometric Measurements

The molecular weights of Rho mutant proteins were confirmed using an API 365 triple-quadrupole mass spectrometer equipped with a TurboSpray source (PE-Sciex, Thornhill, Canada). Protein samples (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, USA) 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 API 365 mass spectrometer.

Platelet Aggregation Assay

Venous blood (9 parts) from healthy donors who had not received any medication for at least two weeks were collected in 3.8% sodium citrate (1 part). Blood was centrifuged at 70×g for 10 min to obtain platelet-rich plasma (PRP) and allowed to stand for 5 min. Then, PRP was collected. Platelet-poor plasma (PPP) was prepared from the remaining blood by centrifuging it at 800×g for 10 min. The PPP platelet count was measured on a haematology analyzer, and the platelets were diluted to 250,000 platelets/μL. A solution of PRP (190 μL) and either Rho or PBS buffer (10 μL) was incubated for 5 min in a Hema Trace 601 aggregometer at 37°C. Ten microlitres of 200 mM ADP was added to monitor the response of platelet aggregation by light transmission.

Cell Adhesion Assay

A cell adhesion assay was done using protocols previously described [29]. Ninety-six-well microtitre plates (Costar, Corning, USA) were coated with 100 μL of PBS buffer containing 200 μg/ml fibronectin or 25 μg/mL fibronectin, and incubated overnight at 4°C. Non-specific protein binding sites were blocked by incubating each well with 200 μL of heat-denatured 1% bovine serum albumin (BSA) (Calbiochem) at room temperature for 1.5 h. The heat-denatured BSA was discarded and each well was washed twice with 200 μL of PBS.

Chinese hamster ovary (CHO) cells that expressed the integrins αβ3/β3 (CHO-αβ3) and αIIbβ3 (CHO-αIIbβ3) were kindly provided by Dr. Y. Takada (Scripps Research Institute) and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) medium [29]. Human erythroblasts K562 cells were purchased from ATCC and cultured in Roswell Park Memorial Institute (RPMI)-1640 medium containing 5% foetal calf serum. 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) at pH 7.35 containing 1 mM MgSO4, 2 mM CaCl2, and 500 μM MnCl2. CHO and human erythroblasts K562 cells were diluted to 3 and 2.5×105 cells/mL, respectively, and 100 μL of the cells were used for the assay. Rho and its mutants (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 non-adhered cells were removed by washing them twice with 200 μL of PBS. After the non-adhered 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. Colorization was done by adding 150 μL of colorizing solution (50% alcohol and 0.1% acetic acid). The resulting absorbance was read at 600 nm and the readings were correlated with the number of adhering cells. Inhibition was defined as

\[
\%\text{ inhibition} = 100 - \frac{OD_{600}(\text{Rho protein} - \text{treated sample})}{OD_{600}(\text{untreated sample})} \times 100
\]

The reported IC₅₀ values are the average of at least three separate experiments.

Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR experiments were done at 27°C on a Bruker Avance 600- and 700-MHz spectrometer equipped with pulse field gradients and xz-gradient triple-resonance probes. In these experiments, samples were dissolved in 10% D₂O/90% H₂O or 100% D₂O at a concentration of 3 mM; pH was adjusted with 100 mM KOD to 6.0. 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 Rho and its P48A mutant was used for the 2D ¹H-¹⁵N HSQC, 3D ¹H-¹⁵N edited-TOCSY-, and NOESY-HSQC experiments. Mixing times of 30–90 ms and 60–130 ms were used for TOCSY and NOESY experiments, respectively. The centre frequencies of double resonance experiments were 4.75 ppm (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 liquid NH₃. The nitrogen chemical shift was determined from the 3JNH coupling constants. For ¹H-¹⁵N relaxation rate constants, R₁ and R₂, were obtained from exponential fits of the peak height data using least-squares fit software SigmaPlot (Jandel Scientific). The reported R₁ 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 ¹⁵N relaxation rate constants, R₁ and R₂, and the ¹H-¹⁵N steady state NOE values were analyzed using the FastModelFree program [33]. 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:

\[
J(\omega) = \frac{2}{5} \left[ \frac{S^2 \tau_{\text{m}}}{1 + (\omega \tau_{\text{m}})^2} + \frac{S^2 - S_z^2}{1 + (\omega \tau_{\text{m}})^2} \right],
\]

Structure Calculations

Structures were calculated using the program X-PLOR with the hybrid distance geometry-dynamical simulated annealing method [30]. NOEY cross-peak intensities—categorized into strong, medium, weak, and very weak—were converted into distance restraints. The S-S covalent bonds were deleted and introduced as pseudo-NOE distances with the S-S distances constrained to the upper limit of 3.0 Å and 2.2 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 J₃N₄H₅ coupling constants. For J₃N₄H₅ values less than 5 Hz, ϕ values were restricted from −30° to −90°, and for J₃N₄H₅ values greater than 10 Hz, ϕ values were restricted from 0° to 170°. Two restrictions were used for each NH-CO backbone hydrogen bond with δSδO restricted to 2.4–3.3 Å and dHδO 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 0.05 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 twenty 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 [31,32].

| Integrin/Cell | IC₅₀ (nM) | IC₅₀Rho/IC₅₀P48A |
|---------------|----------|-----------------|
| α₁β₃/Platelet | 83.2 ± 10.4 | 110.3 ± 14.1 | 0.75 |
| α₁β₃/CHO | 21.0 ± 11.2 | 31.6 ± 7.2 | 0.66 |
| α₁β₃/CHO | 13.0 ± 5.7 | 15.8 ± 3.3 | 0.82 |
| α₁β₃/K562 | 256.8 ± 27.5 | 590.0 ± 18.4 | 4.35 |

*The IC₅₀ values are the average value of 3–5 experiments.

*Inhibition of ADP-induced platelet aggregation by Rho proteins was measured in human PRP.

*Inhibition of integrin α₁β₃-expressing CHO cell adhesion to immobilized fibrinogen by Rho proteins.

*Inhibition of integrin α₁β₃-expressing CHO cell adhesion to immobilized fibrinectin by Rho proteins.
where $\frac{1}{\tau} = \frac{1}{\tau_m} + \frac{1}{\tau_g}$ and $S^2 = S_m^2 + S_g^2$, $\tau_m$ is the overall rotational correlation time of the molecule, $\tau_g$ is the effective correlation time for the motions on the slower of the two time scale, $S_m^2$ is the square of the generalized order parameter, and $S_g^2$ and $S_e^2$ are the squares of order parameters for the motion on the slow and fast time scale, respectively [34].

Molecular Docking

The dockings of Rho and its P48A mutant to integrin α5β1 were done using the docking program HADDOCK 2.1 with hydrogen bond and distance restraints [35]. The structure of integrin α5β1 was modelled using the program MODELLER [36] with integrin αβ3 (PDB code 1L5G) as the template, and the starting structures of Rho and its P48A mutant were their average minimized NMR structures. The interaction restraints were derived from the X-ray structure of integrin αβ3 in complex with a cyclic pentapeptide (c(-RGDf[NMe]V-)) using the software iMolTalk [37]. 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, G50, and D51 residues and integrin α5β1 were 30, 7, and 39, respectively. They were the contacts between the R49 residue and the residues L214 and S216 of integrin α5, between the G50 residue and the residues S121, Y122, S123, F187, Q189, and D227 of integrin β1, and between the D51 residue and the residues S121, Y122, S123, G212, N213, and E218 and Mn$^{2+}$ of the MIDAS (metal-ion-dependent adhesion site) of β1. Additional 0.5 Å distance was added to the upper and lower limits in the direct 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 inter-molecular energies were refined by semi-flexible simulated annealing in the second stage. Both the side-chains and the backbone atoms of the residues 46–54 were defined as flexible and allowed the residues to move in a semi-rigid-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 by clustering based on the pairwise RMSD differences. The 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.

**Table 2.** Summary of inhibition of K562 cell adhesion to fibronectin by Rho and its mutants.

| P-type Protein$^a$ | $^{\text{a}}$IC50 (nM) | A-type Protein$^b$ | $^{\text{b}}$IC50 (nM) | $^{\text{a}}$IC50 (nM)/$^{\text{b}}$IC50 (nM) |
|-------------------|------------------|-------------------|------------------|------------------|
| P                 | R G D M P         | A                 | R G D M P        | 256.8±87.5       |
|                   |                   |                   |                  |                  |
| P                 | R G D W P         | A                 | R G D W P       | 403.5±43.9       |
|                   |                   |                   |                  |                  |
| P                 | R G D N P         | A                 | R G D N P       | 357.0±80.6       |
|                   |                   |                   |                  |                  |
| P                 | R G D G W         | A                 | R G D G W       | 1238.4±632.9     |
|                   |                   |                   |                  |                  |
| P                 | R G D D L         | A                 | R G D D L       | 3017.0±801.5     |
|                   |                   |                   |                  |                  |
| P                 | R G D D M         | A                 | R G D D M       | 4047.3±1784.3    |

$^a$P-type proteins contain a P residue at the 48 position.

$^b$A-type proteins contain a A residue at the 48 position.

The mutated positions are shown in boldface.

doi:10.1371/journal.pone.0028833.t002

**Table 3.** Summary of structural restraints and statistics for Rho P48A mutant.

| Restraints used in the structure calculation |
|--------------------------------------------|
| Distance and dihedral angle restraints      |
| Intra-residue                               | 145 |
| Sequential                                  | 111 |
| Medium range                                | 333 |
| Long range                                  | 387 |
| Hydrogen bonds                              | 9   |
| Dihedral angles                             | 57  |
| Total                                       | 1048|

| Energy statistics                           |
|---------------------------------------------|
| X-PLOR energy (kcal mol$^{-1}$)             |
| $E_{\text{XPLOR}}$                         | 23.38±6.91 |
| $E_{\text{vdw}}$                           | 11.49±2.99  |

| Geometric statistics                        |
|---------------------------------------------|
| Deviations from idealized geometry          |
| All backbone atoms (Å)                      | 1.04±0.23 |
| Backbone atoms (13–14, 20–21, 33–34, 37–38, 43–45, and 55–57) (Å) | 0.38±0.10 |
| All heavy atoms (Å)                         | 1.62±0.25  |
| Heavy atoms (13–14, 20–21, 33–34, 37–38, 43–45, and 55–57) (Å) | 0.87±0.12 |

| Ramachandran analysis                      |
|---------------------------------------------|
| Most favored regions (%)                    | 75.2 |
| Additionally allowed regions (%)            | 23.6 |
| Generously allowed regions (%)              | 1.2 |

doi:10.1371/journal.pone.0028833.t003

**Figure 1.** Structures of Rho P48A mutant. Stereo views of the 20 lowest-energy NMR structures of the Rho P48A mutant.
doi:10.1371/journal.pone.0028833.g001
Protein Data Bank Accession Number and Assignment
The coordinates of 20 calculated structures of P48A mutant were deposited in the Protein Data Bank under accession number 2PJI.

Ethics Statements
The ethics approval for our study was approved by an independent ethics committee of National Cheng Kung University. The collection of human serum was followed the guidelines and regulation. We have obtained written informed consent from all participants involved in this study.

Results
Protein Expression and Purification of Rho and its Mutants
Rho and eleven mutants were expressed with pPICZαA vector in P. pastoris X33 strain. Rho and its P48A, M52W, P48A/M52W, M52N, P48A/M52N, M52G/P53W, P48A/M52G/P53W, M52D/P53L, P48A/M52D/P53L, M52D/P53M, and P48A/M52D/P53M mutants produced in P. pastoris were purified to homogeneity, according to SDS-polyacrylamide gel electrophoresis (data not shown), using Ni²⁺-chelating chromatography and C18 reversed-phase HPLC. The yields of Rho and its mutants were 10–25 mg/L. Mass spectrometry showed that the experimental molecular weights deviated less than 1 Da when compared with the theoretical values, which had been calculated by assuming that all cysteines form disulfide bonds, and would result in the formation of six disulfide bonds in Rho and its mutants (Table S1 and Figure S1).

Inhibition of Platelet Aggregation
Rho produced in P. pastoris inhibited platelet aggregation with a KI of 83.2±10.4 nM (Table 1), which is as potent as native Rho [21]. The mutation of P48 to A in Rho caused only a 1.3-fold decrease in activity in the inhibition of platelet aggregation with a KI of 110.3±14.1 nM. These results showed that the P48 residue in the RGD loop of Rho has little effect on the interaction between Rho and platelet integrin αIIbβ3.

Inhibition of Cell Adhesion to Fibrinogen and Fibronectin
The adhesion of CHO cells that express αIIbβ3 and αvβ3 to immobilized fibrinogen is dependent on the affinity states of the αIIbβ3 and αvβ3 integrins, respectively [38]. These assays used mAbs that blocked the functions of αIIbβ3 and αvβ3 [25,39]. In addition, the adhesion of K562 cells to fibronectin in the presence of 500 μM of Mn²⁺ is predominantly α5β1-dependent [25]. Rho and its P48A mutant inhibited the adhesion of CHO cells that express the integrin αIIbβ3 to immobilized fibrinogen with the IC₅₀ values of 21±11.2 and 31.6±7.2 nM, respectively (Table 1).

Figure 2. 2D ¹H-¹⁵N HSQC spectra of Rho (black) and its P48A mutant (red) at pH 6.0. Correlation peaks are labelled according to residue type and sequence number. The peaks connected by lines correspond to Gln and Asn side chain NH₂ groups. The peaks denoted by the suffix “s” (e.g., Q60s, N11s) are the side-chain resonances of Asn, Gln, and Arg.

doi:10.1371/journal.pone.0028833.g002
This result was consistent with their activity in inhibiting ADP-induced platelet aggregation. Similarly, Rho and its P48A mutant inhibited the adhesion of CHO cells that express the integrin αvβ3 to immobilized fibrinogen with the IC50 values of 13.2 ± 5.7 and 15.8 ± 3.3 nM, respectively. Rho and its P48A mutant inhibited K562-cell adhesion to immobilized fibronectin with the IC50 values of 256.8 ± 87.5 and 39.0 ± 28.4 nM, respectively. In contrast to their inhibition of integrins αIIbβ3 and αvβ3, P48A mutant showed a 4.4-fold increase in its inhibition of integrin αvβ1. These results showed that the N-terminal alanine residue adjacent to the RGD motif increased its inhibition of integrin αvβ1, but not of integrins αIIbβ3 and αvβ3.

We also expressed a series of Rho proteins, M52W, P48A/M52W, M52N, P48A/M52N, M52G/P53W, P48A/M52G/P53W, M52D/P53L, P48A/M52D/P53L, M52D/P53M, and P48A/M52D/P53M mutants to confirm the effect of the N-terminal alanine of the RGD motif on inhibiting integrin αvβ1. The results were consistent with that of P48A mutant, and the mutant proteins containing a P48A mutation showed a 4.4–11.5-fold increase in the inhibition of integrin αvβ1 (Table 2). These results suggest that RGD proteins with the RGD motif containing an N-terminal proline residue may weaken their binding to integrin αvβ1.

**Structure Determination**

The solution structure of Rho and the 3D structure of the P48A mutant were determined using NMR spectroscopy and the hybrid distance geometry-dynamical simulated annealing method [27]. NMR spectra were recorded at pH 6.0. NMR assignment of the P48A mutant was obtained by analyzing standard 2D homonuclear and 3D heteronuclear NMR data (data not shown). We also did NOESY experiments at pH 6.0 in 100% D2O to determine their six disulfide bonds. Their pairings were identified using CβH to CβH, CβH to CβH, and CαH to CαH NOEs between different cysteines [28]. Our analysis showed that the disulfide pairings (C4–C19, C6–C14, C13–C36, C27–C33, C32–C57, and C45–C64) of Rho and its P48A mutant followed the flavidin-type but not albolabrin-type pattern [18]. The secondary structures of the P48A mutant consisted of three short regions of two-stranded antiparallel β-sheets (residues 14–16 and 22–19, 32–34 and 37–39, and 43–45 and 55–57). The formations of three two-stranded antiparallel β-sheets were characterized by the CαH-CαH, CαH-

Figure 3. A structural comparison of Rho and its P48A mutant. (A) Superimpositions of the 3D structures of Rho (2PJF, cyan) and its P48A mutant (2PJI, light purple). Structural alignments of (B) the nine-residue RGD loop containing RI[P/A]RGDMPD sequences of Rho and (C) its P48A mutant. Ten of twenty Rho and its P48A mutant structures were aligned; the RMS deviations of the nine-residue backbone atoms of Rho and its P48A mutant were 0.551 and 0.475 Å, respectively. The side chains of A48, R49, D51, and others are shown in purple, blue, red, and green, respectively.

doi:10.1371/journal.pone.0028833.g003
NH, and NH-NH NOE patterns of the connecting strands, the slowly exchanging amide protons, and the downfield-shifted $\alpha$ protons. Three short regions of antiparallel $\beta$-sheet structures have been observed by the NOE pattern analysis (Figure S2).

The 3D Structure of the P48A mutant was calculated using 1048 experimentally derived restraints with an average of 15.4 restraints per residue (Table 3). The 20 best structures of the P48A mutant from 100 initial structures are shown in Figure 1. The backbone RMSD value of the P48A mutant is 1.04±0.23 Å, and the backbone RMSD values of the P48A mutant for three $\beta$-sheet regions (13–14, 20–21, 33–34, 37–38, 43–45, and 53–57) was 0.39±0.10 Å. Based on a Ramachandran analysis, all dihedral angles of the P48A mutant were in the allowed region. A summary of the restraints and structural statistics is presented in Table 3. Overall, the tertiary structure of the P48A mutant has an elongated and asymmetric shape and consists of three two-stranded antiparallel $\beta$-sheets with many tight turns and loops.

**No Structural Difference between Rho and its P48A Mutant**

The superimposition of the $^{15}$N-HSQC spectra of Rho and its P48A mutant is shown in Figure 2. The chemical difference was calculated using the formula: $\Delta \delta = \sqrt{(0.2(\Delta \alpha)^2 + (\Delta \beta)^2)}$. Chemical shift differences larger than 0.3 ppm were observed only for the residues close to mutation sites for R46, I47, R49, G50, D51, and D55, which were 0.52, 0.42, 0.57, 0.49, 0.91, and 0.38 ppm. Although there were chemical shifts of these residues, the amide strips from I47 to M52 of $^{15}$N-edited NOESY of Rho and its P48A mutant exhibited similar NOE patterns (Figure S3). Superimposing 3D structures of Rho and its P48A mutant showed that there was no difference in their overall structures (Figure 3A). Their RGDM motif, the binding sites for integrin, had a similar conformation: a type-I turn. Ten out of twenty of Rho and its P48A mutant structures were selected to align the nine-residue RGD loop, and the RMS deviations of the nine-residue backbone atoms of Rho and its P48A mutant were 0.55 and 0.40 Å, respectively (Figures 3B and 3C). This structural analysis showed that their RGD loops have the same backbone conformation.

**No structural differences between the integrin $\alpha_5\beta_1$ complexes of Rho and the P48A mutant**

The dockings of Rho and the P48A mutant into integrin $\alpha_5\beta_1$ were used to identify their integrin-interacting residues. The models of the integrin $\alpha_5\beta_1$ complexes were built using HADDOCK 2.1 software [35]. The distance and hydrogen bond restraints were derived from the X-ray structure of integrin $\alpha_5\beta_3$ complexed with a cyclic RGD pentapeptide (PDB code 1L5G), and eight key interactions were found between integrin $\alpha_5\beta_3$ and the R and D residues [37]. According to sequence and structure alignments between integrins $\alpha_5\beta_3$ and $\alpha_5\beta_1$, we identified the corresponding residues of integrin $\alpha_5\beta_1$. For example, the R residue of the cyclic peptide forms a bidentate salt-bridge hydrogen bond with the D218 residue $\beta_1$ subunit, and its corresponding responding residue of the $\alpha_5$ subunit was the D227 residue. The carboxylate oxygen of the D residue of the cyclic peptide forms hydrogen bonds with the residues S121 and S123 of the $\beta_3$ subunit, and the corresponding residues of the $\beta_1$ subunit were residues S121 and S123. The other carboxyl oxygen of the D residue of the cyclic peptide forms a hydrogen bond with the Y122 residue $\beta_1$ subunit, and their corresponding residues of integrin $\alpha_5\beta_1$ were Y122 and N213 (Figure 4). Using these restraints, we docked Rho and the P48A mutant to integrin $\alpha_5\beta_1$, which, the analysis showed, resulted in the same number of contacts (Table S2). The key contacts—seven hydrogen bonds and two salt bridges between the R and D residues of the RGD motif and integrin $\alpha_5\beta_1$—were the same.
The total number of interactions between the R49 and D51 residues and integrin α5β1 were 41 and 49, respectively. In contrast, the P48 residue of Rho and the A48 residue of P48A mutant did not interact with integrin α5β1. This is consistent with our reported structures that both the A and P residues were located in the interior of the RGD loop (Figure 3C).

**Dynamics Differences between Rho and its P48A Mutant**

1H-15N correlated NMR spectroscopy was used to measure 15N R1, 15N R2, and 1H-15N NOE parameters for Rho and its P48A mutant. They were measured at 600 MHz and 700 MHz 1H to confirm the results. The NOE and R1 relaxation parameters of Rho and its P48A mutant were similar throughout the sequence (Figure 5 and Figure S4). The major differences between Rho and the P48A mutant were found in the R2 relaxation parameters of the R49 and D51 residues. Their R2 values, measured at 600 MHz 1H, were 8.32±0.13 s⁻¹ and 10.02±0.10 s⁻¹ for Rho and 5.98±0.10 s⁻¹ and 6.50±0.02 s⁻¹ for the P48A mutant. They were consistent with the results measured at 700 MHz 1H, and the R2 values for the Rho residues R49 and D51 were 39% and 54% higher than those of the P48A mutant (Table S3). The square of the generalized order parameter, S², the effective internal correlation time, τe, and a conformational exchange broadening parameter, R ex, for each backbone amide NH vector were determined using model-free formalism to compare their differences [34]. The optimized τm values of the P48A mutant was determined to be 6.38 ns, and its obtained diffusion tensor was fully asymmetric with D///D⊥ = 1.25. They were very similar to the reported values of Rho and the measurement at 700 MHz 1H [27].

The optimized values of S², τe, and R ex of Rho and its P48A mutant were obtained from the measurements of 600 and 700 MHz 1H (Figure 6, Figure S5, Table 4, and Table S3). The observable S² differences between Rho and its P48A mutant were found only in the RGD residues: the S² values of the R49, G50, and D51 residues of the P48A mutant were 29%, 14%, and 28% lower than those of Rho. Their differences in RGD motif can be seen more clearly in the plots shown in Figures 7A and 7B. Furthermore, the major differences were found in τe and in the R ex of R49 and D51: the R ex values of R49 and D51 residues of Rho were 0.91 s⁻¹ and 1.42 s⁻¹, but we found no R ex values for the P48A mutant residues. The τe values of R49 and D51 of Rho were 0.11 ns and 0.19 ns, and those of the P48A mutant were 1.04 ns and 0.98 (Table 4). The τe values of R49 and D51 residues of Rho were 9.5 and 5.1 times higher than those of the P48A mutant. The τe difference in the RGD motif between Rho and the P48A mutant can be seen clearly in the structural plot shown in Figures 7C and 7D. These results suggest that the flexibility and

---

**Figure 5.** (A) A comparison of the relaxation parameters of Rho (■) and its P48A mutant (□). 15N-R1 with error. (B) 15N-R2 with error. (C) 1H-15N steady-state NOE with error. These experiments were done using 600-MHz NMR. Gaps indicate the proline residues. The β-sheet secondary structure is shown in the top panel.

doi:10.1371/journal.pone.0028833.g005
fast motion of the R49 and D51 residues on the ps/ns time scale may be important for binding integrin α5β1. It has been shown that the RGD loop and the C-terminal region of echistatin exhibit concerted motions [17]. In contrast, these regions of Rho did not exhibit concerted motions because no long-range NOEs between their backbone atoms were identified (Figure S2).

Effect of N-terminal Residue Adjacent to the RGD Motif on the Inhibition of Integrin α5β1
To study the effect of the N-terminal residue of the RGD motif on the inhibition of integrin α5β1, we produced P48G, P48Y, P48F, P48W, P48L, and P48I mutants in P. pastoris. Compared with the P48A mutant, their inhibition of integrin α5β1 was 1.6, 5.3, 4.8, 3.3, 4.8, and 4.8 times lower (Table 5). The P48A and P48G mutants exhibited highest inhibitory activity. In contrast, the N-terminal residue containing the large hydrophobic amino acids was less inhibitory. These results suggest that proteins containing N-terminal alanine and glycine residues adjacent to the RGD motif may increase their binding to integrin α5β1.

Discussion
The residues flanking the RGD motif of RGD-containing proteins affect their binding specificities and affinities on integrins [7,10,22,23,24,25,40]. In the present study, we showed that the replacement of N-terminal proline with alanine or glycine adjacent to the RGD motif in Rho increased their binding affinity to integrin α5β1; similar results were found with Rho P48A mutants having different C-terminal residues adjacent to the RGD motif. No structural differences between Rho and its P48A mutant or between their integrin α5β1 complexes were found. The only difference was found in the backbone dynamics of the RGD residues. The proline-to-alanine mutation increased the flexibility of the RGD residues and the fast motion of the R and D residues on the ps/ns timescale. These results showed that the N-terminal proline residue adjacent to the RGD motif of Rho affects its function and dynamics, but does not affect 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 integrin α5β1.

Proline residue is the only common imino acid in proteins with a bulky pyrrolidine ring that restricts the conformational range of its adjacent residues. The lack of a proton on the imino nitrogen of proline blocks the hydrogen-bond formation required for α-helix and β-sheet secondary structures, and thus disrupts the propagation of neighbouring secondary structures. Therefore, it is commonly found in the turn and loop structures of proteins [41,42]. The occurrence of a proline residue in a protein sequence often has a strong influence on the protein’s stability, structure, and function [42,43,44,45]. The effects of proline residue are diverse, and they depend on their neighbouring residues and structural contexts [42,43,44,45]. For example, the replacement of proline on lysozyme and lambda repressor caused protein instability [42,44]. In contrast, the replacement of...

Figure 6. A comparison of the model-free parameters of Rho (■) and its P48A mutant (□). (A) The square of the generalized order parameter S². (B) The effective internal correlation time τₑ. (C) The conformational exchange broadening parameter Rₑx. Gaps indicate the proline residues. The β-sheet secondary structure is shown in the top panel.

doi:10.1371/journal.pone.0028833.g006
proline on the $\alpha$-subunit of tryptophan synthase and staphylococcal nuclease increased their protein stability [46,47,48]. In some cases, the effect of proline replacement on protein stability can be only marginal [49,50]. The proline residue is also important in many structural elements, such as an N-terminal cap residue in the $\alpha$-helix, a terminal residue in the $\alpha$-helix, and a corner residue in the $\beta$-turn structure. Our findings are an example of a role for N-terminal amino acid adjacent to the RGD motif in determining the activity of RGD proteins. We found that the mutation of proline to alanine or glycine on Rho increased their binding affinity to integrin $\alpha_5\beta_1$. The N-terminal proline residue adjacent to the RGD motif may provide an unfavourable environment for inhibiting integrin $\alpha_5\beta_1$. The analyses of the primary sequences of disintegrins showed that >98% of their RGD loops have an $\text{A}RGD$ amino acid sequence [26]. The RGD loop sequences of natural integrin $\alpha_5\beta_1$ ligands, such as fibronectin, osteopontin, and thrombospondins, are $\text{G}RGDS$, $\text{G}RGDS$, and $\text{G}RGD(A/I)$ [1]. These results suggest

|       | $R_1 (s^{-1})$ | $R_2 (s^{-1})$ | NOE  | $S^2$   | $\tau_e (ns)$ | $R_{ex} (s^{-1})$ |
|-------|----------------|----------------|------|---------|---------------|------------------|
| A48P48Aa | 1.68±0.07     | 7.65±0.13     | 0.64±0.01 | 0.74±0.02 | 1.04±0.14     |                  |
| R49WTb  | 1.69±0.08     | 8.32±0.13     | 0.48±0.01 | 0.75±0.01 | 0.11±0.01     | 0.91±0.26        |
| R49P48Aa | 1.63±0.04     | 5.98±0.01     | 0.42±0.01 | 0.53±0.02 | 1.04±0.06     |                  |
| R78P48Aa | 0.46          | 3.25          |      |         |               |                  |
| G50WTb  | 1.55±0.02     | 5.09±0.03     | 0.32±0.02 | 0.50±0.03 | 0.84±0.08     |                  |
| G50P48Aa | 1.51±0.02     | 4.90±0.01     | 0.28±0.01 | 0.43±0.02 | 0.93±0.06     |                  |
| G79P48Aa | 0.39          | 2.75          |      |         |               |                  |
| D51WTb  | 1.72±0.03     | 10.03±0.02    | 0.50±0.02 | 0.85±0.01 | 0.19±0.03     | 1.42±0.37        |
| D51P48Aa | 1.68±0.02     | 6.50±0.02     | 0.47±0.02 | 0.61±0.02 | 0.98±0.09     |                  |
| D80P48Aa | 0.52          | 2.40          |      |         |               |                  |

*Rhodostomin P48A mutant from this study.
Rhodostomin from Chen et al. [27].
Dynamic properties of fibronectin type III domain reported by Carr et al. [52].
doi:10.1371/journal.pone.0028833.t004

Figure 7. Representations of the dynamic properties of Rho and its P48A mutant. A sausage representation of the backbone dynamics of (A) Rho and (B) its P48A mutant. The diameter of the sausage is inversely proportional to the generalized order parameter of the corresponding residue. The side chains of P48/A48, R49, and D51 are shown. The magnitudes of the generalized order parameter are blue, white, and red. A representation of the effective internal correlation times of (C) Rho and (D) its P48A mutant. The residues containing the effective internal correlation times ($\tau_e$)>500 ps, >50 ps, and <50 ps are shown in dark pink, light pink, and black. The residues without relaxation data and the fitting model are shown in white.

doi:10.1371/journal.pone.0028833.g007
that integrin α5β1 prefers its ligands to contain (G/A)RGD but not PRGD sequences for binding.

The synergy between structure and dynamics is essential to the function of biological complexes. We found that the effect of the N-terminal proline residue of the RGD motif in Rho on reducing its binding affinity to integrin α5β1 may be due to its effect on the dynamic properties of the RGD motif. The proline effect is commonly attributed to the limitation of its backbone entropy. The cis-trans isomerisation of the proline peptide bond is also responsible for the activities of many proteins and peptides [51]. Proline isomerization can induce conformational heterogeneity and control the binding and function of globular proteins. In the present study, we found that the proline residue of P48A mutants did not exhibit cis-trans isomerisation and had only a trans conformation. The P48A mutation caused a decrease in the R2 values of the R and D residues, which resulted in a decrease of the rigidity, the disappearance of conformational exchange, and the increase of fast motion on the ps-ns timescale of the RGD motif. The RGD motif, the integrin-binding site, of P48A mutant is clearly more flexible on the ps-ns timescale than on that of Rho, as indicated by both the dramatically lower-order parameters and much larger effective internal correlation times. This effect may facilitate the P48A mutants to interact with integrin α5β1. This is consistent with the dynamic features of the RGD motif in fibroconnectin, which preferentially binds to integrin α5β1 [32]. The conformational freedom of the RGD loop in P40A mutant results in high flexibility and solvent exposure of this loop, which may be responsible for its fast recognition and fitting to integrin α5β1 [17,34,53,54]. However, a detailed investigation of the individual integrin α5β1 ligands may be required.

In conclusion, we found that the N-terminal proline residue adjacent to the RGD motif affects the function and dynamic properties of the RGD motif, which shows that the dynamic properties of the RGD motif in RGD-containing proteins may be important for integrin recognition. Our functional analysis also showed that the integrin α5β1 ligands prefer to have N-terminal residue that contains either glycine or alanine amino acid. These results provide important dynamic information for designing potent RGD mimetics and serve as the basis for exploring the structure and the functional relationships of RGD-binding integrins and their ligands.

Supporting Information

Table 5. Summary of inhibition of K562 cell adhesion to fibronectin by Rho and its mutants.

| Protein | IC50 (nM) |
|---------|-----------|
| P       | 231.4     |
| A       | 59.0      |
| G       | 92.2      |
| Y       | 310.6     |
| F       | 282.1     |
| W       | 193.2     |
| L       | 283.4     |
| I       | 281.7     |

doi:10.1371/journal.pone.0028833.t005

References

1. Vale R, Kreis T (1999) Guidebook to the Extracellular Matrix, Anchor and Adhesion Proteins. Oxford: New York: Oxford University Press.
2. Hohenester E, Engel J (2002) Domain structure and organisation in extracellular matrix proteins. Matrix Biol 21: 113–128.
3. Valonakis I, Campbell ID (2007) Extracellular matrix from atomic resolution to ultrastructure. Curr Opin Cell Biol 19: 578–583.
4. Ruoslahti E (1996) RGD and other recognition sequences for integrins. Annu Rev Cell Dev Biol 12: 697–715.
Williams JA, Lu X, Rahman S, Keating C, Kakkar V (1993) Dendroaspin: a potent integrin receptor inhibitor from the venom of Dendroaspis viridis and D. jamesoni. Biochem Soc Trans 21: 735.

Seymour JL, Henzel WJ, Nevins B, Studis JT, Lazarus RA (1990) Decorin. A potent glycoprotein IIIa-IIb antagonist and platelet aggregation inhibitor from the leech Macrobodella decora. J Biol Chem 265: 10143–10147.

Mans BJ, Louw AI, Neitz AW (2002) Savigmyrin, a platelet aggregation inhibitor from the soft tick Ornithodoros savignyi, presents the RGDC integrin recognition motif on the Kunitz-BPTI fold. J Biol Chem 277: 21371–21378.

Chen CY, Luo SC, Kuo CF, Lin YS, Wu JJ, et al. (2004) Structure and function of the integrin alphaIIbbeta3 tail. J Biol Chem 279: 15169–15175.

Wu JJ, Kuo CF, Chen CY, Chang IJ, Lin YS, et al. (2003) Maturation processing and characterization of streptopain. J Biol Chem 278: 17336–17343.

Shiu JH, Chen CY, Chang LS, Chen YC, Chen YC, et al. (2004) Solution structure of the integrin alphaIIbbeta3 tail. J Mol Biol 340: 527–540.

Rahman S, Aitken A, Flynn G, Formstone C, Savidge GF (1998) Modulation of the interaction of the integrin alphaIIbbeta3 with its ligand fibronectin by the RGD-containing loop. J Mol Biol 281: 247–257.

McLane MA, Marcinkiewicz C, Vijay-Kumar S, Wierzchicka-Patynowski I, Niewiarowski S (1998) Viper venom disintegrins and related molecules. Proc Soc Exp Biol Med 219: 109–119.

Niewiarowski S, McLane MA, Kleczewiak M, Stewart GJ (1994) Disintegrins and other naturally occurring antagonists of platelet fibrinogen receptors. Semin Hematol 31: 289–300.

Blobel CP, White JM (1992) Structure, function and evolutionary relationship of proteins containing a disintegrin domain. Curr Opin Cell Biol 4: 760–765.

Calvete JJ (2005) Structure-function correlations of snake venom disintegrins. J Mol Biol 348: 439–459.

Blobel CP, White JM (1992) Structure, function and evolutionary relationship of proteins containing a disintegrin domain. Curr Opin Cell Biol 4: 760–765.

Niewiarowski S, McLane MA, Kleczewiak M, Stewart GJ (1994) Disintegrins and other naturally occurring antagonists of platelet fibrinogen receptors. Semin Hematol 31: 289–300.

Blobel CP, White JM (1992) Structure, function and evolutionary relationship of proteins containing a disintegrin domain. Curr Opin Cell Biol 4: 760–765.

Calvete JJ (2005) Structure-function correlations of snake venom disintegrins. J Mol Biol 348: 439–459.

Niewiarowski S, McLane MA, Kleczewiak M, Stewart GJ (1994) Disintegrins and other naturally occurring antagonists of platelet fibrinogen receptors. Semin Hematol 31: 289–300.

Blobel CP, White JM (1992) Structure, function and evolutionary relationship of proteins containing a disintegrin domain. Curr Opin Cell Biol 4: 760–765.

Calvete JJ (2005) Structure-function correlations of snake venom disintegrins. J Mol Biol 348: 439–459.

Niewiarowski S, McLane MA, Kleczewiak M, Stewart GJ (1994) Disintegrins and other naturally occurring antagonists of platelet fibrinogen receptors. Semin Hematol 31: 289–300.