Published in final edited form as:

Nano Life. 2013 ; 3(1): 1340005-1–1340005-11. doi:10.1142/S1793984413400059.

PROBING $\alpha\text{IIb}\beta 3$: LIGAND INTERACTIONS BY DYNAMIC FORCE SPECTROSCOPY AND SURFACE PLASMON RESONANCE

SAMRAT DUTTA*, DAVID A. HORITA†, ROY R. HANTGAN#†,‡,§,||, and MARTIN GUTHOLD#*,§,||

*Department of Physics, Wake Forest University 1834 Wake Forest Road, Winston-Salem, NC 27106, USA

† Department of Biochemistry Wake Forest University School of Medicine Medical Center Boulevard Winston-Salem, NC 27157-1016, USA

These authors contributed equally to this work.

Abstract

The interaction between platelet integrin $\text{IIb } 3$ and fibrin(ogen) plays a key role in blood clot formation and stability. Integrin antagonists, a class of pharmaceuticals used to prevent and treat cardiovascular disease, are designed to competitively interfere with this process. However, the energetics of the integrin–drug binding are not fully understood, potentially hampering further development of this class of pharmaceuticals. We integrated dynamic force spectroscopy (DFS) and surface plasmon resonance (SPR) to probe the energetics of complex formation between $\text{IIb } 3$ and cHarGD, a cyclic peptide integrin antagonist. Analysis of $\text{IIb } 3$:cHarGD DFS rupture force data at pulling rates of 14 000 pN/s, 42 000 pN/s and 70 000 pN/s yielded $k_{\text{off}} = 0.02\text{--}0.09 \text{ s}^{-1}$, a dissociation energy barrier $\Delta G_d^\ddagger = 22\text{--}29 \text{ kJ/mol}$, and a potential well width $x_{-1} = 0.5\text{--}0.8 \text{ nm}$. SPR kinetic data yielded an association rate constant $k_{\text{on}} = 7 \times 10^3 \text{ L/mol}\cdot\text{s}$ and a dissociation rate constant $k_{\text{off}} = 10^{-2} \text{ s}^{-1}$, followed by a slower stabilization step ($\sim 400 \text{ s}$). Both DFS and SPR detected minimal interactions between $\text{IIb } 3$ and cHarGA demonstrating a key role for electrostatic interactions between the ligand aspartate and the integrin metal ion-dependent adhesion site (MIDAS). Our work provides new insights into the energy landscape of $\text{IIb } 3$'s interactions with pharmacological and physiological ligands.

Keywords

Surface plasmon resonance; dynamic force spectroscopy; atomic force microscopy; receptor: ligand binding; association/dissociation kinetics

1. Introduction

The major structural component of a hemostatic blood clot or pathological thrombus is an aggregate of platelets embedded in a fibrin mesh.¹ The key interaction between platelets and the fibrin mesh is the bond between the $\text{IIb } 3$ integrin receptor anchored in the platelet cell membrane and the KQAGDV sequence on fibrin.^{2–4} There are tens of thousands of $\text{IIb } 3$ integrins per platelet (diameter $\sim 2 \mu\text{m}$),⁴ many of which can simultaneously bind to the

numerous fibrin molecules (length, 45 nm) in a stretch of fibrin fiber that is attached to a platelet. Disrupting these noncovalent interactions with *integrin antagonists* has proven effective in treating cardiovascular disease but, as outlined below and recently reviewed by Cox *et al.*,³ much remains to be learned about the mechanisms and limitations of this class of pharmaceuticals.

The $\alpha\text{IIb}\beta_3$ integrin is a 226 kDa heterodimer stabilized by noncovalent, divalent cation-dependent interactions between the large extracellular regions of two elongated polypeptide chains, each with a single-transmembrane segment and a small cytoplasmic domain.² $\alpha\text{IIb}\beta_3$ is a regulated receptor, one that does not bind macromolecular adhesive proteins until activated by intracellular signals under physiological conditions or *in vitro* by priming ligands.⁵⁻⁷ Debate continues about the mechanisms that regulate the transition from resting to maximally activated extended receptor,⁸⁻¹⁰ but structural data with RGD peptides¹¹ and pharmaceuticals¹² bound to $\alpha\text{IIb}\beta_3$'s ectodomain indicate that the initial events in ligand recognition are mediated by subtle changes in electrostatic interactions in $\alpha\text{IIb}\beta_3$'s A-domain.

Fibrinogen is a 340 kDa heterodimer, comprised of three pairs of disulfide-bonded polypeptide chains, A α , B α and β .¹ Thrombin cleavage at the amino termini of the A α and B α chains releases fibrinopeptides A and B, thus forming ($\alpha\text{A}\alpha\text{B}\beta$)₂ fibrin monomers that readily polymerize to protofibrils. The protofibrils assemble laterally and longitudinally to form a 3D network of clotted fibrin.^{13,14} While fibrinogen has two distinct RGD sequences on its β chains that could mediate integrin recognition, the KQAGDV sites at the ends of its α -chains are primarily responsible for $\alpha\text{IIb}\beta_3$ binding.¹⁵⁻¹⁹ Upon binding to $\alpha\text{IIb}\beta_3$, the α -chain's intrinsically disordered carboxy-terminus apparently forms a hair-pin turn that enables complementary charged contacts at the $\alpha\text{IIb}\beta_3$ subunit interface.¹¹

Integrin antagonists, such as eptifibatide (Integrilin) and tirofiban (Aggrastat), that block the interactions between fibrin(ogen) and platelet $\alpha\text{IIb}\beta_3$ receptors have reduced mortality from cardiovascular disease.²⁰⁻²³ Unfortunately, these $\alpha\text{IIb}\beta_3$ competitive inhibitors carry small risks for hemorrhage and thrombocytopenia²⁴⁻²⁶ and large prothrombotic risks if administered without the anticoagulant heparin.²² Efforts to develop orally active integrin antagonists presented even bigger challenges—three Phase III clinical trials were stopped when safety committees noted *excess mortality* in the treated groups.²⁷⁻²⁹ A better understanding of the molecular mechanisms responsible for the strength and stability of $\alpha\text{IIb}\beta_3$: ligand interactions may aid the development of the next generation of safer and more effective integrin antagonists.

We focused on the cyclic peptide cHarGD (Cyclo (S,S)-L-Lysyl-L-Tyrosyl-Glycyl-L-Cystinyl-L-Homoarginyl-Glycyl-L-Aspartyl-L-Tryptophanyl-L-Prolyl-L-Cystine) because it is structurally and functionally similar to both the integrin antagonist eptifibatide³⁰⁻³³ and the KQAGDV integrin-recognition sequence on fibrinogen's α module.^{11,34} cHarGD binds avidly to the $\alpha\text{IIb}\beta_3$ receptor and effectively blocks platelet–fibrinogen interactions.³³ It is well suited to both dynamic force spectroscopy (DFS) and surface plasmon resonance (SPR) measurements since the lysine on its flexible linker³² enables covalent attachment to biosensors and AFM tips without disturbing its zwitterionic integrin-targeting sequence.

DFS is a single-molecule approach, in which the rupture forces of single-ligand–receptor bonds are determined as a function of the loading rate; kinetic and transition state properties can then be extracted from an analysis of the rupture force data.³⁵ Force is a particularly relevant parameter for the $\alpha\text{IIb}\beta_3$ –receptor interaction since it is continually exposed to the forces exerted by blood flow. Moreover, integrins in general play an important role in mechanotransduction—communicating cellular signals via mechanical means such as an applied force.^{36,37} DFS data have traditionally been analyzed using the Bell model³⁸; the

peak of the rupture force distributions is plotted as a function of loading rate to determine the critical rupture distance, x_{-1} , and the zero-force dissociation rate constant, k_{off} . Dudko, Hummer and Szabo recently pioneered a new analysis, termed *DHS formalism*, in which the lifetime (F) of molecular interactions is extracted from DFS data and plotted as a function of the applied force, F .³⁹⁻⁴¹ In addition to the dissociation rate constant, k_{off} , and potential well width, x_{-1} , obtained in Bell's analysis, it also provides the approximate shape of the potential well and the activation energy, $\Delta G_d^\ddagger$.

SPR is a bulk approach in which binding and dissociation rates of a ligand–receptor interaction are determined from the change in mass (SPR) as a receptor binds to a surface-bound ligand. It allows the determination of kinetic and thermodynamic parameters for macromolecular recognition processes,^{11,42-44} as we have done for IIB 3's interactions with macromolecular ligands.^{19,45,46}

DFS and SPR provide complementary kinetic information. DFS gives kinetic parameters as a function of force, whereas SPR gives kinetic parameters as a function of temperature and concentration, the traditional biochemical approach.

2. Results

DFS Data Analysis—Rupture Force Determination

DFS data obtained by lowering a cHarGD-functionalized tip onto an integrin-coated surface and retracting it at different pulling rates are presented in Fig. 1. A typical force curve with six distinct ruptures is shown in the inset of Fig. 1(b). Tip retraction proceeds from *right to left* in this insert plot; the tip breaks free of the surface after the 137 pN rupture. Multiple ruptures are expected since the surface is coated with many proteins and the AFM probe is functionalized with many ligands. Force curves with multiple ruptures are typical for this experimental set-up.^{47,48} The overall z -movement of the piezo (abscissa) from the first to the last rupture event of the force curve in the inset of Fig. 1(b) is about 90 nm, over twice the linker length, L ($L = 36$ nm). This can be explained by the observation that the micelles, into which the integrins are embedded, cause the surface to be rough,⁴⁹ and thus some bonds will form with ligands on the side of the AFM tip, and others will form with ligands on the tip apex.⁴⁷ In such a scenario, maximum z -piezo movement between ruptures can be as much as twice the linker length, plus the vertical distance between linkers on the tip.

The histograms for integrin:cHarGD rupture forces at the different loading rates show one primary rupture force peak for each loading rate [see Figs. 1(a) – 1(c)]. Less defined, additional, smaller peaks appear at force values that are approximately integer multiples of the force value of the primary peak. These distributions also indicate multiple integrin:cHarGD interaction between the AFM tip and the surface. The bonds sometimes ruptured simultaneously resulting in higher rupture forces or sequentially resulting in multiple ruptures. Fitting a Gaussian to each major peak yielded average rupture forces of 77 pN, 86 pN and 88 pN for the three loading rates. Rupture force data <30 pN were excluded from analysis because they were similar to the noise in the force curves.

The specificity of these rupture events was confirmed by a competition experiment and an experiment with an inactive compound. Force data from the competition experiment, in which integrin was pre-incubated with excess cHarGD, showed much lower rupture forces and very few multiple integrin:cHarGD rupture events [see Fig. 1(c), inset], confirming the specificity of the integrin:cHarGD interaction. The rupture force distribution with biologically inactive cHarGA on the tip exhibited a peak at ~30 pN, near the noise level (data not shown).

Analysis of DFS data

Rupture force histograms were analyzed using the DHS method⁴¹ by transforming them into force-dependent lifetimes, $\tau(F)$,

$$\tau(F) = \frac{\int_F^\infty p(F') dF'}{p(F) \dot{F}(F)}, \quad (1)$$

where $p(F)$ is the normalized rupture force probability distribution; and $\dot{F}$ is the loading rate dF/dt . By converting the integral into a sum over F , the formula can be applied to the rupture force distributions,

$$\tau\left(F_0 + \left(k - \frac{1}{2}\right) \Delta F\right) = \frac{\left(h_k/2 + \sum_{j=k+1}^N h_j\right) \Delta F}{h_k \dot{F}\left(F_0 + \left(k - \frac{1}{2}\right) \Delta F\right)}. \quad (2)$$

Here, a rupture force histogram contains N bins of width ΔF that start at F_0 and end at $F_N = F_0 + N \Delta F$. We used $F_0 = 36.5$ pN and $\Delta F = 7$ pN. The number of counts in the k th bin is C_k , resulting in height $h_k = C_k / (N_{\text{tot}} \Delta F)$, where N_{tot} is the total number of counts; $k = 1, 2, \dots$ runs the number of bins. Use of a long linker provides cHarGD with the necessary mobility to bind to IIb 3 but decreases the effective pulling rate. The effective pulling rate, $\dot{F}$, as a function of pulling force, F , and the linker properties, was calculated via

$$\dot{F}(F) = \nu \left(\frac{1}{\gamma} + \frac{2\beta L l_p (1 + \beta L l_p)}{3 + 5\beta F l_p + 8(\beta F l_p)^{5/2}} \right)^{-1}, \quad (3)$$

where γ is the spring constant of the AFM cantilever; ν the pulling speed; and L and l_p are the contour length and persistence length of the flexible linker. We used $l_p = 0.38$ nm and $L = 36$ nm for the 3400MW PEG linker.^{50,51}

This analysis results in the data points of the $\tau(F)$ versus F plot shown in Figs. 1(d) – 1(f). The data points are then fit with

$$\tau(F) = \tau_0 \cdot \left(1 - \frac{\nu F x_{-1}}{\Delta G_d^\ddagger}\right)^{1-1/\nu} \cdot e^{-\beta \Delta G_d^\ddagger} \left[1 - \left(1 - \frac{\nu F x_{-1}}{\Delta G_d^\ddagger}\right)^{1/\nu}\right]. \quad (4)$$

In this expression, x_{-1} is the distance to the transition state; τ_0 is the lifetime at zero force (inverse of dissociation constant, k_{off}); and $\Delta G_d^\ddagger$ is the apparent free energy of activation for dissociation of IIb 3:cHarGD in the absence of an external force. The scaling factor specifies the nature of the underlying free energy profile; $\nu = 1/2$ corresponds to a harmonic well with a cusp-like barrier ($U_0(x) = \Delta G_d^\ddagger \cdot (x/x_{-1})^2$ for $x < x_{-1}$, and $-\infty$ for $x > x_{-1}$); $\nu = 2/3$ corresponds to a potential that contains linear and cubic terms ($U_0(x) = 1.5 \cdot \Delta G_d^\ddagger \cdot (x/x_{-1}) - 2 \cdot \Delta G_d^\ddagger \cdot (x/x_{-1})^3$ for $x < x_{-1}$, and $-\infty$ for $x > x_{-1}$); $\nu = 1$ recapitulates Bell's formula. Both $\nu = 1/2$ and $\nu = 2/3$ fit well, resulting in a dissociation constant, $k_{-1} = 0.02$ – 0.09 s⁻¹, a rupture distance, $x_{-1} = 0.5$ – 0.8 nm, and an activation energy barrier, $\Delta G_d^\ddagger = 22$ – 29 KJ/mol. Table 1 lists specific values for each fit.

Equation (4) fits well in the force regime below ~100 pN but deviates at larger values, because Eq. (4) no longer applies above a critical force, F_C , at which the activation barrier becomes very deformed or ceases to exist; i.e., $\nu \cdot F_C \cdot x_{-1} \sim \Delta G_d^\ddagger$.⁴¹

SPR Measurements of Integrin α Ib β 3: cHarGD Binding

Our goal was to determine the rate, affinity and specificity of the interactions between α Ib β 3 and cHarGD. Increasing concentrations of α Ib β 3, isolated in a resting yet responsive conformation in octyl glucoside micelles⁴⁵ were delivered to a biosensor surface derivatized with either cHarGD or a biologically inactive analog, cHarGA. SPR data showed time- and concentration-dependent increases in RU when α Ib β 3 interacted with cHarGD [see Fig. 2(a), solid symbols]. In contrast, only baseline signals were observed with cHarGA (open symbols). Attempts to fit the cHarGD binding data to a single-site kinetic model⁵² proved problematic, yielding large deviations between the biphasic association and dissociation profiles and the fitted lines. We previously demonstrated by analytical ultracentrifugation, circular dichroism, fluorescence spectroscopy and electron microscopy that ligand-induced conformational changes characterize α Ib β 3 interactions with cHarGD.³³ Recognizing that cHarGD binding perturbs α Ib β 3's resting conformation,³³ we focused on a ligand-induced conformational change model that several investigators have successfully applied to analyze multiphasic SPR kinetic data.⁵³⁻⁵⁷

SPR Data Analysis — Kinetics

Data obtained with α Ib β 3 binding to cHarGD were fit to a reversible two-state bimolecular interaction model to determine the forward (k_{a1} , L/mol-s) and reverse (k_{d1} , s⁻¹) rate constants for the initial binding step and the forward (k_{a2} , s⁻¹) and reverse (k_{d2} , s⁻¹) rate constants for the subsequent stabilizing conformational change:

The quality of the fit can be judged by the close correspondence between the solid lines and the data in Fig. 2(a) as well as the tightly distributed residuals. This data set yielded $k_{a1} = 7.14 \pm 0.04 \times 10^3$ L/mol-s and $k_{d1} = 1.24 \pm 0.01 \times 10^{-2}$ s⁻¹ at 25°C. The observation that k_d exhibited minimal temperature dependence suggests that the SPR-probed lowest energy dissociation path⁵⁸ for the cHarGD: α Ib β 3 complex is dominated by an entropic barrier,⁴⁴ as we have previously reported for fibrinogen, the integrin's primary physiological ligand.¹⁹ The kinetic parameters for the conformational change step were $k_{a2} = 1.87 \pm 0.01 \times 10^{-3}$ s⁻¹ and $k_{d2} = 6.84 \pm 0.02 \times 10^{-4}$ s⁻¹. The overall K_d for the reaction was 4.68 ± 10^{-7} mol/L.

Comparable SPR traces were obtained for the α Ib β 3:cHarGD system at 37 °C [see Fig. 2(b)]; there the most pronounced change was a 30% reduction in k_{a1} . SPR data obtained at 15, 20, 25, 30 and 37 °C confirmed that the rate constant for the initial binding step decreased modestly at increased temperatures while k_{d1} was nearly constant.

3. Discussion

DFS measurements of cHarGD: α Ib β 3 interactions yield dissociation rate constants in the range $k_{\text{off}} \sim 0.02\text{--}0.09$ s⁻¹. The resultant $t_{\text{off}} \sim 26 \pm 15$ s is comparable in magnitude to the initial dissociation step measured by SPR, and in reasonable agreement given the necessary extrapolation to the zero-force dissociation rate in DFS, and considering that dissociation is force-induced in DFS and temperature-induced in SPR. Indeed, Berquand *et al.* reported k_{off} rates for an antibody:antigen system determined by SPR and DFS that differ by a factor of 3.⁵⁹ Morfill *et al.* found that for some pairs of antibody:peptide complexes, the dissociation

rates obtained from DFS and SPR agree within a factor of two, suggesting that thermal dissociation (SPR) and force-induced dissociation (DFS) follow the same dissociation paths.⁵⁸ Moreno *et al.* found a good correlation between DFS unbinding forces and SPR dissociation rates.⁶⁰ However, for other pairs, the dissociation rates differ by a factor of 10, indicating different dissociation paths. Residence times may also contribute to the difference in dissociation rate since, in DFS experiments, the tip-bound ligand contacts the $\alpha_{IIb} \beta_3$ receptor for less than 30 s, which may be too short to complete the stabilization steps detected by SPR.

Using the DHS analysis for the DFS data also allows extraction of the shape of the energy landscape.⁴⁰ Both $\Delta G^\ddagger = 1/2$ (harmonic potential) and $\Delta G^\ddagger = 2/3$ (linear/cubic potential) gave good fits of the data to Eq. (4) (see Table 1). As noted by Dudko and colleagues, agreement between the two different barriers validates the analysis method.⁴⁰

Fitting the data to Eq. (4) yielded an activation energy of 22–29 kJ/mol. Simulations of the dependence of k_{-1} on $\Delta G_d^\ddagger$ suggested a somewhat wider range of 25–37 kJ/mol (see Fig. 3). In Fig. 3, we also included the SPR data point for the off-rate, $k_{off} = 1.24 \pm 0.01 \times 10^{-2} \text{ s}^{-1}$ at 25°C. It can be seen that it is a bit higher (slower off-rate), than the extrapolated zero-force off-rate from the DFS analysis. As discussed above, these differences might be because the applied force in DFS perturbs the protein, causing a less-than-ideal binding geometry, or forces the ligand into a different dissociation path. However, both DFS and SPR detected minimal interactions between $\alpha_{IIb} \beta_3$ and cHarGA demonstrating a key role for electrostatic interactions between the ligand aspartate and the integrin metal ion-dependent adhesion site (MIDAS) (see Fig. 4).^{11,12}

Other researchers have used DFS to study $\alpha_{IIb} \beta_3$'s interactions with other ligands. Lee and Marchant used AFM-based DFS at high loading rates and a Bell analysis³⁸ to find dissociation rates and potential widths of $k_{off} \sim 1.5 \text{ s}^{-1}$, $x_{-1} \sim 1.0 \text{ nm}$ and $k_{off} \sim 48 \text{ s}^{-1}$, $x_{-1} \sim 1.1 \text{ nm}$ for the $\alpha_{IIb} \beta_3$:RGDSPA and $\alpha_{IIb} \beta_3$:HHLGGAKQAGDV interactions, respectively.^{48,61} We obtained a comparable value of $x_{-1} = 0.6 \text{ nm}$ for $\alpha_{IIb} \beta_3$:cHarGD dissociation. However, our DFS data yielded 50-fold slower dissociation rates with cHarGD, a cyclic peptide that binds platelet $\alpha_{IIb} \beta_3$ receptors 300-fold more tightly than the linear RGDSPA peptide^{33,62} that Lee and Marchant used. Thus affinity alone can probably explain these differences in dissociation rates. The even faster ~20 ms half-life they reported for HHLGGAKQAGDV reflects the flexible peptide's weaker affinity for $\alpha_{IIb} \beta_3$.¹⁵

Using a constant-force laser trap, Litvinov *et al.* investigated the dissociation of the $\alpha_{IIb} \beta_3$:fibrinogen bond, the natural bond with which cHarGD competes.⁶³ Interestingly, the zero-force lifetime for the $\alpha_{IIb} \beta_3$:fibrinogen bond (~15 s) and the $\alpha_{IIb} \beta_3$:cHarGD bond (11–42 s) are the same order of magnitude. However, the force-dependent lifetime decreases much faster for the “artificial” $\alpha_{IIb} \beta_3$:cHarGD bond than the natural $\alpha_{IIb} \beta_3$:fibrinogen bond, with increasing force. For example, at 50 pN force the lifetime of the $\alpha_{IIb} \beta_3$:cHarGD bond is 0.05 s, as compared to 0.8 s for the $\alpha_{IIb} \beta_3$:fibrinogen bond; that is, the natural bond is more stable under an applied force. Using laser tweezers, Sun *et al.* determined a rupture force (peak of Gaussian rupture force distribution) of 57 pN for the interaction of $\alpha_{IIb} \beta_3$ with the cyclic peptide *cyclo*(Arg-Gly-Asp-D-Phe-Lys) at a loading rate of 2000 pN/s.⁶⁴ Their peptide is structurally similar to the peptide we used; the lower rupture force value can be attributed to the lower loading rate, and is thus, most likely, consistent with our values.

4. Conclusions

When designing drugs to interfere with platelet integrin–ligand bonds it should be kept in mind that when a fibrin fiber is bound to a platelet, or when platelet–platelet interactions are

mediated by fibrin, that there are numerous such interactions in parallel.⁶⁵ Consequently, when only one of these bonds is broken, the whole complex will remain intact since the other parallel bonds are still intact.⁶⁶ In essence, multiple, parallel, weak, short lifetime bonds result in an overall very strong, long lifetime bond, as originally recognized by Bell.³⁸ Indeed, our SPR data demonstrate that cHarGD dissociates from $\alpha\text{IIb}\beta 3$ some 40-fold faster than does echistatin, a 5 kDa disintegrin with an RGD integrin-targeting sequence,⁴⁵ and 130-fold faster than fibrinogen, $\alpha\text{IIb}\beta 3$'s primary physiological ligand.¹⁹ These observations suggest that additional contacts, beyond those illustrated in Fig. 4, stabilize the interactions of these protein ligands with the $\alpha\text{IIb}\beta 3$ integrin. Thus, better drugs to prevent clot formation may require multivalency and/or longer lifetimes under an applied force, the natural environment in blood flow.

5. Materials and Methods

Integrin $\alpha\text{IIb}\beta 3$ Purification

Milligram quantities of highly purified $\alpha\text{IIb}\beta 3$ were isolated from outdated human blood platelets (Blood Bank, NCBH, Winston-Salem, NC) as described.⁴⁵ Monodisperse $\alpha\text{IIb}\beta 3$ was obtained by size-exclusion chromatography at 4°C on a 1.6×40 cm Sephacryl S-300 column equilibrated in a pH 7.4 buffer containing 13 mM NaCl, 10 mM HEPES, 30 mM n-octyl- β -D-glucopyranoside and 1 mM CaCl_2 /1 mM MgCl_2 (HSCM-OG). Peak fractions were pressure concentrated in an Amicon device with a PLHK cellulose membrane and 100 kDa retention. $\alpha\text{IIb}\beta 3$ concentrations were determined spectrally.⁴⁵

Synthetic Integrin Ligands

Cyclo(S,S)-L-Lysyl-L-Tyrosyl-Glycyl-L-Cystinyl-L-Homoarginyl-Glycyl-L-Aspartyl-L-Tryptophanyl-L-Prolyl-L-Cystine (cHarGD) was synthesized, purified and characterized by amino acid analysis and mass spectrometry by the Protein Analysis Core Laboratory of the Comprehensive Cancer Center of Wake Forest University (Winston-Salem, NC) using previously described protocols.³³ Cyclo(S,S)-L-Lysyl-L-Tyrosyl-Glycyl-L-Cystinyl-L-Homoarginyl-Glycyl-L-Alanyl-L-Tryptophanyl-L-Prolyl-L-Cystine (cHarGA) was synthesized, purified by HPLC and characterized by UV spectroscopy and mass spectrometry by AnaSpec, Inc. (San Jose, CA).

Dynamic Force Spectroscopy

Sample Preparation: $\alpha\text{IIb}\beta 3$ in HSCM-OG (50 μl at 22 $\mu\text{g/ml}$), embedded in micelles, was deposited on a freshly cleaved muscovite mica surface (Paramount Corporation, NY, NY), allowed to sit for two minutes and gently rinsed with HSCM-OG for 15 s. AFM imaging demonstrated a sparse monolayer of adherent integrins. Force spectroscopy experiments were done in 100 μl HSCM without detergent.

DFS Measurements: Force spectroscopy was performed with a Topometrix Explorer (Veeco Instruments, Woodbury, NY) interfaced with a nano-Manipulator (3rd Tech, Chapel Hill, NC). Triangular silicon-nitride AFM tips (cantilever spring constant = 0.14 N/m) were functionalized with cHarGD peptide using a 3400 MW, COOH-terminated, flexible PEG linker (Novascan Technologies, Inc., Ames, IA). This functionalization results in tens of cHarGD molecules attached to the tip apex; several can come into contact with the surface during a single force curve. The nanoManipulator controlled movement of the AFM tip along the z -axis. Force curves were collected at random spots on a large substrate area ($100 \times 100 \mu\text{m}$) at loading rates of 14 000 pN/s, 42 000 pN/s and 70 000 pN/s. Loading rates were then corrected for the flexible PEG linking cHarGD to the tip [see Eq. (3), results]. The value of the rupture force, I , as measured in nA by the AFM, was converted to nN via $F = (1/S) \cdot I$, where S is the sensitivity of the AFM set-up (typical value, $S = 0.14$ nA/nm).

The lifetime versus force data were fitted with Kaleidagraph 3.51 (Synergy Software, Reading, PA).

Specificity Controls: The specificity of the integrin:cHarGD interaction was tested with excess cHarGD in solution as a competitive inhibitor. First, force curves on immobilized integrin were collected, as described above. The buffer on the surface was then carefully wicked off and replaced with 100 μ L of 200 nM cHarGD in HSCM to pre-occupy the ligand binding pocket³³ and a new set of force curves was collected. Experiments were also performed with AFM tips functionalized with cHarGA, a biologically inactive peptide³⁰ and immobilized IIB 3.

SPR Biosensor Preparations: CM-5 biosensor chips (Biacore, Inc., Piscataway, NJ) were activated for amine coupling by reaction with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide in a Biacore T100 instrument. Sequential aliquots of cHarGD or cHarGA, diluted to 250 μ g/mL in 0.1 M sodium acetate buffer, pH 5.0, were delivered to the sample chamber, yielding immobilization densities of 54–87 response units (RU); 0.4–0.7 mM immobilized ligand.⁴² Residual active groups were blocked with ethanolamine.

SPR Spectroscopy: Interactions between immobilized ligands and the IIB 3 integrin were measured in the Biacore T100. Signals from both the reference and sample channels were collected at a rate of 10 Hz. Reagents were maintained at $25.0 \pm 0.1^\circ\text{C}$ in the sample compartment; experiments were performed at 15, 20, 25, 30, 35 and 37°C with control to $\pm 0.01^\circ\text{C}$ in the analysis chamber. Two start-up cycles were performed in which HSCM-OG buffer was delivered, followed by a two-step regeneration cycle: 20 mM EDTA in HEPES-buffered saline pH 7.4 with 30 mM octyl glucoside (HS-OG) for 180 s then 5 M NaCl in HS-OG for 180 s. During the binding steps, increasing concentrations (0–4500 nM) of IIB 3 integrin were delivered at 30 μ L/min for 700 s. Dissociation of the integrin:ligand complexes was monitored for 1600 s, as HSCM-OG buffer flowed over the biosensor surface at 30 μ L/min. Residual bound integrin was displaced by the regeneration cycle. The biosensor was then equilibrated for 300 s with HSCM-OG prior to delivery of the next integrin aliquot. The sensorgram data (sample–reference signals) were further corrected by subtracting the time-dependent profiles obtained with a buffer blank. These doubly corrected signals⁴³ were then fit globally by nonlinear regression (Biacore Evaluation software), first, to a single-site binding model, then to a ligand-induced conformational change model. In each case, the quality of the fit was judged by the residuals and the reduced χ^2 value.⁴⁵

Acknowledgments

Thanks to Mary C. Stahle for the isolation and characterization of the IIB 3 integrin preparations used in this work as well as her skilled technical assistance with the Biacore T100. Thanks to Julie Edelson, Ph.D., Office of Research and Sponsored Programs, Wake Forest University, for her editorial insights. Thanks to the NIH Research Resource Grant P41 EB002025 for technical support. Supported by Grant-in-Aid 0855257E from the American Heart Association, Mid-Atlantic Affiliate (to R. R.H.), Institutional Development Grant 2006-IDG-1004 from the North Carolina Biotechnology Center (to R.R.H.), Grant CMMI-0646627 from the National Science Foundation (to M.G.), the Wake Forest University Cross-Campus Collaborative Research Fund (to R.R.H and M.G.).

Reference

1. Hantgan, RR.; Lord, ST. Fibrinogen structure and physiology, in Hemostasis and Thrombosis. In: Colman, RW.; Marder, VJ.; Clowes, AW.; George, JN.; Goldhaber, SZ., editors. Basic Principles and Clinical Practice. Lippincott Williams and Wilkins; Philadelphia: 2006. p. 285-316.
2. Askari JA, Buckley PA, Mould AP, et al. J. Cell Sci. 2009; 122:165. [PubMed: 19118208]
3. Cox D, Brennan M, Moran N. Nat. Rev. Drug Discov. 2010; 9:804. [PubMed: 20885411]

4. Shattil SJ, Kashiwagi H, Pampori N. Blood. 1998; 91:2645. [PubMed: 9531572]
5. Phillips DR, Charo IF, Scarborough RM. Cell. 1991; 65:359. [PubMed: 2018971]
6. Humphries MJ, McEwan PA, Barton SJ, et al. Trends Biochem. Sci. 2003; 28:313. [PubMed: 12826403]
7. Collier BS, Shattil SJ. Blood. 2008; 112:3011. [PubMed: 18840725]
8. Arnaout MA, Mahalingam B, Xiong JP. Ann. Rev. Cell Dev. Biol. 2005; 21:381. [PubMed: 16212500]
9. Luo BH, Carman CV, Springer TA. Ann. Rev. Immunol. 2007; 25:619. [PubMed: 17201681]
10. Rocco M, Rosano C, Weisel JW, et al. Structure. 2008; 16:954. [PubMed: 18547527]
11. Springer TA, Zhu JH, Xiao T. J. Cell Biol. 2008; 182:791. [PubMed: 18710925]
12. Xiao T, Takagi J, Collier BS, et al. Nature. 2004; 432:59. [PubMed: 15378069]
13. Hantgan RR, Hermans J. J. Biol. Chem. 1979; 254:11272. [PubMed: 500644]
14. Weisel J. Biophys. J. 1986; 50:1079. [PubMed: 3801570]
15. Hawiger J. Semin. Hematol. 1995; 32:99. [PubMed: 7652584]
16. Farrell DH, Thiagarajan P. J. Biol. Chem. 1994; 269:226. [PubMed: 8276798]
17. Farrell DH, Thiagarajan P, Chung DW, et al. Proc. Nat. Acad. Sci. USA. 1992; 89:10729. [PubMed: 1438269]
18. Rooney MM, Parise LV, Lord ST. J. Biol. Chem. 1996; 271:8553. [PubMed: 8621481]
19. Hantgan RR, Stahle MC, Lord ST. Biochemistry. 2010; 49:9217. [PubMed: 20828133]
20. Karvouni E, Katrakis DG, Ioannidis JPA. J. Am. Coll. Cardiol. 2003; 41:26. [PubMed: 12570940]
21. Kong DF, Hasselblad V, Harrington RA, et al. Am. J. Cardiol. 2003; 92:651. [PubMed: 12972100]
22. Bhatt DL, Topol EJ. Jama-J. Am. Med. Assoc. 2000; 284:1549.
23. Linco® AM, Cali® RM, Topol EJ. J. Am. Coll. Cardiol. 2000; 35:1103. [PubMed: 10758948]
24. Atar S, Cannon CP, Murphy SA, et al. Am. Heart J. 2006; 151:976. [PubMed: 16644315]
25. Investigators PP. New Eng. J. Med. 1998; 338:1488. [PubMed: 9599103]
26. Investigators PS. New Engl. J. Med. 1998; 338:1498. [PubMed: 9599104]
27. Heeschen C, Hamm CW. Lancet. 2000; 355:330. [PubMed: 10665546]
28. Quinn MJ, Plow EF, Topol EJ. Circulation. 2002; 106:379. [PubMed: 12119257]
29. Chew DP, Bhatt DL, Sapp S, et al. Circulation. 2001; 103:201. [PubMed: 11208677]
30. Scarborough RM, Naughton MA, Teng W, et al. J. Biol. Chem. 1993; 268:1066. [PubMed: 8419315]
31. Phillips DR, Scarborough RM. Am. J. Cardiol. 1997; 80:B11.
32. Cierniewski CS, Byzova T, Papierak M, et al. J. Biol. Chem. 1999; 274:16923. [PubMed: 10358039]
33. Hantgan RR, Lyles DS, Mallett TC, et al. J. Biol. Chem. 2003; 278:3417. [PubMed: 12426312]
34. Hantgan RR, Rocco M, Nagaswami C, et al. Protein Sci. 2001; 10:1614. [PubMed: 11468358]
35. Guthold M, Superfine R, Taylor R. Biomed. Microdevices. 2001; 3:9.
36. Schwartz MA. Cold Spring Harbor Perspect. Biol. 2010; 2:a005066.
37. Roca-Cusachs P, Iskratsch T, Sheetz MP. J. Cell Sci. 2012; 125:3025. [PubMed: 22797926]
38. Bell G. Science. 1978; 200:616.
39. Dudko OK, Filippov AE, Klafter J, et al. Proc. Nat. Acad. Sci. USA. 2003; 100:11378. [PubMed: 13679588]
40. Dudko OK, Hummer G, Szabo A. Phys. Rev. Lett. 2006; 96:108101. [PubMed: 16605793]
41. Dudko OK, Hummer G, Szabo A. Proc. Nat. Acad. Sci. USA. 2008; 105:15755. [PubMed: 18852468]
42. Schuck P. Ann. Rev. Biophys. Biomol. Struct. 1997; 26:541. [PubMed: 9241429]
43. Day YSN, Baird CL, Rich RL, et al. Protein Sci. 2002; 11:1017. [PubMed: 11967359]
44. Winzor DJ, Jackson CM. Anal. Biochem. 2005; 337:289.
45. Hantgan RR, Stahle MC, Horita DA. Biochemistry. 2008; 47:2884. [PubMed: 18237139]
46. Hantgan RR, Stahle MC. Biochemistry. 2009; 48:8355. [PubMed: 19640007]

47. Schonherr H, Beulen MWJ, Bugler J, et al. J. Am. Chem. Soc. 2000; 122:4963.
48. Lee I, Marchant RE. Ultramicroscopy. 2003; 97:341. [PubMed: 12801687]
49. Konidala P, He LZ, Niemeyer B. J. Mol. Graph. Model. 2006; 25:77. [PubMed: 16386443]
50. Lee H, Scherer NF, Messersmith PB. Proc. Nat. Acad. Sci. USA. 2006; 103:12999. [PubMed: 16920796]
51. Stroh C, Wang H, Bash R, et al. Proc. Nat. Acad. Sci. USA. 2004; 101:12503. [PubMed: 15314231]
52. Schuck P. Curr. Opin. Biotechnol. 1997; 8:498. [PubMed: 9265731]
53. De Crescenzo G, Grothe S, Lortie R, et al. Biochemistry. 2000; 39:9466. [PubMed: 10924142]
54. Hancock MA, Spencer CA, Koschinskym ML. Biochemistry. 2004; 43:12237. [PubMed: 15379562]
55. Walsh STR. Biochemistry. 2010; 49:8766. [PubMed: 20815339]
56. Honjo E, Watanabe K, Tsukamoto T. J. Biochem. 2002; 131:267. [PubMed: 11820942]
57. Herschhorn A, Oz-Gleenberg I, Hizi A. Biochem. J. 2008; 412:163. [PubMed: 18260826]
58. Morfill J, Neumann J, Blank K, et al. J. Mol. Biol. 2008; 381:1253. [PubMed: 18619976]
59. Berquand A, Xia N, Castner DG, et al. Langmuir. 2005; 21:5517. [PubMed: 15924483]
60. Moreno N, Chevalier M, Ronzon F, et al. J. Mol. Recognit. 2011; 24:1105. [PubMed: 22038817]
61. Lee I, Marchant RE. Surf. Sci. 2001; 491:433.
62. Hantgan RR, Paumi C, Rocco M, et al. Biochemistry. 1999; 38:14461. [PubMed: 10545168]
63. Litvinov RI, Barsegov V, Schissler AJ, et al. Biophys. J. 2011; 100:165.
64. Sun JEP, Vranic J, Composto RJ, et al. Integr. Biol. 2012; 4:84.
65. Hantgan RR, Nichols WL, Ruggeri ZM. Blood. 1990; 75:889. [PubMed: 2302458]
66. Braaten JV, Jerome WG, Hantgan RR. Blood. 1994; 83:982. [PubMed: 8111066]

Fig. 1.

Dynamic force spectroscopy data on IIB 3:cHarGD binding. The left panel shows the distribution of rupture forces obtained at pulling rates of (a) $0.1 \mu\text{m/s}$ (14 000 pN/s); (b) $0.3 \mu\text{m/s}$ (42 000 pN/s); and (c) $0.5 \mu\text{m/s}$ (70 000 pN/s). The inset in (b) depicts a typical force curve with several ruptures. Including excess cHarGD in solution dramatically reduced the rupture forces (panel C inset), confirming specificity. Panels (d), (e) and (f) show the force dependence of life-time analysis obtained at pulling rates of (d) 14 000 pN/s, (e) 42 000 pN/s and (f) 70 000 pN/s. In all three curves, the slightly higher fit corresponds to 0.5 and the lower fit to 1.5 [see Eq. (4)]. Linker correction [see Eq. (3)] is included in fits of panels (d), (e) and (f).

Fig. 2.

Kinetics of IIb 3 binding to immobilized cHarGD measured by SPR. Increasing integrin concentrations (0, 100, 250, 500, 1050, 2000, 3000, 4500 nM) were delivered to the surface at 25°C (a) or 37°C (b) during a 700-s binding step. Dissociation was monitored for 1600 s as buffer was delivered to the chip. Solid lines were obtained by fitting the RU versus time profiles (solid symbols) to a ligand-induced conformational change binding model [see Eq. (5)]; residual plots above each panel. The 25°C panel includes data (open symbols) obtained with inactive peptide cHarGA; no significant RU changes were observed.

Fig. 3.

Effect of the activation energy, $\Delta G_d^\ddagger$ on Eq. (4).

$$\tau(F) = \tau_0 \cdot \left(1 - \frac{\nu F x_{-1}}{\Delta G_d^\ddagger}\right)^{1-1/\nu} \cdot e^{-\beta \Delta G_d^\ddagger} \left[1 - \left(1 - \frac{\nu F x_{-1}}{\Delta G_d^\ddagger}\right)^{1/\nu}\right].$$

In this simulation we used, a lifetime $\tau_0 = 27$ s, and a distance to the transition state, $x_{-1} = 0.68$ nm. It can be seen that the data fall within a range of $\Delta G_d^\ddagger$ from $10 k_B T$ to $15 k_B T$ (25–37 kJ/mol). The dashed plot with $\nu = 1$ indicates Bell's equation, $\tau(F) = \tau_0 \cdot e^{-\beta \Delta G_d^\ddagger - \nu F x_{-1}}$ with a life time of $\tau_0 = 27$ s. The solid diamond at the upper left corner denotes the SPR-determined 80 s τ_0 .

Fig. 4. DFS and SPR approaches to measuring integrin:ligand binding. (a) DFS measurements were performed using cHarGD's flexible linker to immobilize the 1200 Da ligand on an AFM tip, enabling reversible binding to the 225 kDa α IIb β_3 integrin adherent to a mica surface. (b) SPR was performed with the ligand, cHarGD immobilized on a biosensor and α IIb β_3 delivered in detergent micelles, a configuration designed to optimize the signal/noise ratio. (c) Schematic of the integrin headpiece that harbors a binding site for physiological, pharmacological and synthetic ligands at the α IIb β_3 interface. α IIb, blue; β_3 , red; octyl glucoside micelles, green; carbohydrates, gray. cHarGD polypeptide backbone, yellow; oxygen, red; nitrogen, blue. Ca^{2+} ions are green spheres, the MIDAS Mg^{2+} is an orange sphere. The key interactions between cHarGD and α IIb β_3 are the D7 CO_2^- with the Mg^{2+} ion, the D7 H^{N} with the α IIb D217 O, and the Har5 with β_3 D224 CO_2^- .

Table 1

Summary of data from DFS analysis of integrin:cHarGD interaction. β , parameter describing potential shape; τ_0 , lifetime of bond ($1/k_{\text{off}}$); x_{-1} , reaction coordinate distance to transition state, $\Delta G_d^\ddagger$, activation energy.

Loading rate (pN/s)		τ_0 (s)	x_{-1} (nm)	$\Delta G_d^\ddagger$ (KJ/mol)	R
14 000	1/2	29.7 ± 11.1	0.66 ± 0.06	24.4 ± 0.8	0.997
42 000	1/2	23.4 ± 27.9	0.68 ± 0.19	24.8 ± 2.7	0.975
70 000	1/2	41.8 ± 24.3	0.81 ± 0.10	28.9 ± 1.4	0.997
Average		31.6	0.71	26.0	
14 000	2/3	15.4 ± 4.0	0.52 ± 0.03	22.0 ± 1.0	0.996
42 000	2/3	11.1 ± 8.1	0.53 ± 0.10	22.4 ± 3.0	0.973
70 000	2/3	18.7 ± 7.0	0.64 ± 0.05	26.6 ± 1.7	0.996
Average		15.1	0.56	23.7	