Quantitative Analysis of Platelet \( \alpha_v \beta_3 \) Binding to Osteopontin Using Laser Tweezers

Rustem I. Litvinov, Gaston Vilaire, Henry Shuman, Joel S. Bennett and John W. Weisel

Department of Cell and Developmental Biology, Department of Physiology, and the Hematology-Oncology Division of the Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6058, USA

Running Title: Bimolecular integrin-osteopontin binding in platelets

‡To whom correspondence should be addressed: Hematology-Oncology Division, University of Pennsylvania School of Medicine, 421 Curie Blvd., BRB II/III, Rm 914, Philadelphia, PA 19104-6058, USA. Tel.: 215-573-3280; Fax: 215-573-3079; Email: bennetts@mail.med.upenn.edu
Summary

To determine whether platelet adhesion to surfaces coated with the matrix protein osteopontin requires an agonist-induced increase in the affinity of the integrin $\alpha v \beta 3$ for this ligand, we used laser tweezers to measure the rupture force between single $\alpha v \beta 3$ molecules on the platelet surface and osteopontin-coated beads. Virtually all platelets stimulated with 10 $\mu$M ADP bound strongly to osteopontin, producing rupture forces as great as 100 pN with a peak at 45-50 pN. By contrast, 90% of unstimulated, resting non-reactive platelets bound weakly to osteopontin, with rupture forces rarely exceeding 30-35 pN. However, $\approx$10% of unstimulated platelets, resting reactive platelets, exhibited rupture force distributions similar to stimulated platelets. Moreover, ADP stimulation resulted in a 12-fold increase in the probability of detecting rupture forces $>30$ pN compared to resting non-reactive platelets. Pre-incubating stimulated platelets with the inhibitory prostaglandin PGE$_1$, a cyclic RGD peptide, the monoclonal antibody abciximab, or the $\alpha v \beta 3$-specific cyclic peptide XJ735 returned force histograms to those of non-reactive platelets. These experiments demonstrate that ADP-stimulation increases the strength of the interaction between platelet $\alpha v \beta 3$ and osteopontin. Further, they indicate that platelet adhesion to osteopontin-coated surfaces requires an agonist-induced exposure of $\alpha v \beta 3$ binding sites for this ligand.

Abbreviations: OPN – osteopontin, PGE$_1$ – prostaglandin E$_1$, VN – vitronectin.
Integrin-mediated cell adhesion plays a fundamental role in processes as diverse as wound repair, cancer cell metastasis, organogenesis, implantation, and thrombosis (1). The ability of integrins to interact with ligands can be regulated, to a greater or lesser extent, by cellular metabolism, a process known as “inside-out signaling” (2). However, it is often not possible to determine experimentally whether inside-out signaling augments cell adhesion by increasing integrin avidity for ligands via integrin clustering or whether it actually increases the affinity of integrins for ligands. Agonist-induced changes in integrin avidity are thought to be largely responsible for the increase in \( \beta 2\)- and \( \beta 4\)-mediated adhesion of lymphocytes to ICAM-1 and VCAM-1, respectively, although agonist-stimulated changes in the affinity of these integrins for their ligands have also been reported (3,4). On the other hand, the ability of the platelet integrin \( \alpha IIb\beta 3\) to support platelet aggregation is due to an agonist-induced increase in the affinity of \( \alpha IIb\beta 3\) for soluble ligands (5).

Laser tweezers are an optical system in which external forces applied to a particle trapped by a laser can be accurately measured because the angular deflection of the laser beam is directly proportional to the lateral force applied to the particle. Laser tweezers are sensitive and accurate at the lower end of the force spectrum (0-150 pN) (6) and therefore are a suitable system with which to study integrin-ligand interactions (7). Moreover, because laser tweezers can measure the force of interaction between individual receptor-ligand pairs (7), they can potentially differentiate between agonist-stimulated changes in integrin avidity versus changes in integrin affinity. To use laser tweezers for this purpose, a ligand-coated bead can be trapped by the laser and repeatedly brought into contact with an integrin-coated pedestal or an immobilized cell so that the forces required to separate the two can be measured and displayed as “rupture force’ histograms.

Previously, we used laser tweezers to measure rupture forces between the platelet integrin \( \alpha IIb\beta 3\) and its principal ligand fibrinogen (8). We found that measured rupture forces could be segregated into three classes: first, rupture forces of 10 pN or less were contacts during which no
binding occurred or were optical artifacts; second, rupture forces of 10-60 pN that represented non-specific interactions between the fibrinogen-coated beads and the ⅡbⅢ-coated pedestal; and third, rupture forces ranging from 60-150 pN and a peak yield strength of 80-100 pN that resulted from specific binding of fibrinogen to ⅡbⅢ. Moreover, because we observed only one well-defined peak of specific ⅡbⅢ-fibrinogen rupture forces, rather than a series of peaks that was a multiple of a single rupture force, we concluded that the peak represented the interaction of individual ⅡbⅢ and fibrinogen molecules. In addition, rupture events nearly all occurred as a single step, also consistent with the probability that each one represented the rupture of a single ligand-receptor pair.

Platelets express a second Ⅲ integrin, ⅤⅢ. Although there are 50- to 500-fold fewer copies of ⅤⅢ compared to ⅡbⅢ on platelets (9,10), ⅤⅢ mediates the adhesion of platelets to the matrix proteins osteopontin (OPN) and vitronectin (VN), at least in vitro, and like ⅡbⅢ, does so only following platelet stimulation (11-13). There are two possible parallel explanations for the increase in ⅤⅢ-mediated platelet adhesion to OPN and VN following platelet stimulation. First, platelet stimulation could increase the avidity of ⅤⅢ for its ligands by inducing ⅤⅢ clustering (14). Second, platelet stimulation could increase the affinity of ⅤⅢ for OPN and VN by inducing a conformational change in ⅤⅢ, similar to the way in which platelet stimulation enables ⅡbⅢ to bind soluble ligands (5). In support of the second possibility, we reported that incubating platelets with the reducing agent dithiothreitol or with the divalent cation Mn$^{2+}$ induces platelet adhesion to OPN, indicating that ⅤⅢ-mediated platelet adhesion can be induced by perturbing the conformation of extracellular domain of ⅤⅢ (13). On the other hand, Pampori et al., using a monovalent ligand-mimetic antibody that selectively binds to the activated form of ⅤⅢ (15), were unable to detected antibody binding to resting or thrombin-stimulated platelets.
Here we use laser tweezers to examine the consequences of agonist stimulation on the activity of \( \alpha_v\beta_3 \) on platelets at the level of single molecules. We found that platelet stimulation specifically increases the force of interaction between individual \( \alpha_v\beta_3 \) and OPN molecules. Thus, our data demonstrate that the mechanism responsible for platelet adhesion to OPN-coated surfaces requires an agonist-induced exposure of \( \alpha_v\beta_3 \) binding sites for this ligand.
Experimental Procedures

Reagents and Materials – The RGD-containing peptide XJ735 (16) and recombinant human OPN were gifts of Dr. Shaker Mousa (Dupont Pharmaceuticals). Plain silica microspheres and COOH-modified latex beads were obtained from Bangs Laboratories, Inc. (Carmel, IN). Sigma (St. Louis, MO) supplied N,N'-methylene-bis-acrylamide, bovine serum albumin (BSA) (≥99%), poly-L-lysine hydrobromide (m.w. >300,000), 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDAC), prostaglandin E₁ (PGE₁), ammonium persulfate (SigmaUltra), and 2-morpholinoethanesulfonic acid (MES). Acrylamide and N, N, N’, N’-tetramethylethylenediamine (TEMED) were purchased from BioRad (Hercules, CA). Glutaraldehyde was obtained from EMS (Fort Washington, PA). Abciximab (ReoPro™) was purchased from Eli Lilly (Indianapolis, IN). All other reagents were analytical grade.

Laser Tweezer Measurements - We used a custom-built laser tweezers setup assembled from a Nikon Diaphot 300 inverted microscope, 100x 1.3NA Fluor lens and a Spectra Physics FCBar Nd:YAG laser (8) to measure the strength of OPN binding to unstimulated and agonist-stimulated human platelets. For these measurements, recombinant human OPN, prepared as described previously (17), was covalently bound to 0.93 μm carboxylate-modified latex beads using EDAC as a cross-linking agent in a two-step procedure described in the TechNote #205 issued by Bangs Laboratories, Inc. and previously used to bind fibrinogen. The density of OPN on the bead surface, measured using I¹²⁵-labeled OPN, was 29,500 ± 3,200 molecules per μm².

To measure OPN binding to platelets, an individual platelet was trapped from a suspension of gel-filtered human platelets containing ≈5 x10⁶ platelets/ml and ≈10⁵/ml OPN-coated beads and manually attached to a 5 μm diameter silica pedestal coated with polylysine. All the experiments were performed in a 4 mM HEPES gel-filtration buffer, pH 7.4, containing 135 mM NaCl, 2.7 mM KCl, 5.6 mM glucose, 1 mM CaCl₂, 3.3 mM NaH₂PO₄, and 0.35 mg/ml bovine serum albumin. An OPN-coated bead, trapped by the laser light, was then brought to a distance of 2-3
m from the immobilized platelet. After oscillation of the OPN-coated bead was initiated at 50 Hz with 0.8 m peak-to-peak amplitude, the bead was brought into contact with the platelet by micromanipulation using a keyboard-controlled piezoelectric stage. Data collection was initiated at the first contact between the bead and the platelet. Rupture forces following repeated contacts between the platelet and the bead were collected for periods of several seconds to one minute and were displayed as normalized force histograms for each experimental condition.

The studies presented here resulted from measurements using 376 individual platelets and a total of 650,000 contact cycles between OPN-coated beads and platelets. Thus, there were approximately 1700 contacts per platelet. Individual forces measured during each contact-detachment cycle were collected into 5 pN bins. The number of events in each bin was plotted against the average force for that bin after normalizing by the total number of interaction cycles. The percentage of events in a particular force range (bin) represented the probability of rupture events at that force, with 100% being the total of all contact cycles, including binding and non-binding events. The results from individual experiments were averaged so that each histogram shown represents from $10^4$ to $10^5$ contacts. Signals corresponding to ruptures forces $\leq 10$ pN could be recorded even in the absence of a trapped latex bead, indicating that such signals represent contacts during which no binding occurred or optical artifacts (8). Accordingly, rupture forces in this range were considered non-binding events when data were analyzed.
Results

Interaction of OPN with Unstimulated Platelets – Initially, we studied the interaction of freshly isolated unstimulated gel-filtered platelets with OPN-coated beads. When observed by light microscopy, these platelets had an oval or discoid shape with rare short protrusions. Individual platelets were easily trapped by a focused laser beam and gently brought into contact with a polylysine-coated surface to which they immediately attached without spreading. As shown in Fig. 1, approximately 90% of the attached platelets interacted weakly with the OPN-coated beads, exhibiting rupture forces in the range of 10 pN to 60. Moreover, the rupture forces segregated into two populations – a predominant population with a range of 10-30 pN and a cumulative probability of ≈ 1% of total contacts and a smaller population with rupture forces ranging from 30 to 60 pN and a cumulative probability of ≈ 0.1%. In contrast, ≈ 10% of the platelets interacted strongly with the beads, exhibiting rupture forces of up to 100 pN and with a broad peak at 40-55 pN. To verify the specificity of the interaction of OPN with the latter group of platelets, rupture forces were measured in the presence of a cyclic RGD-peptide (cRGD), a competitive inhibitor of OPN binding to αvβ3. As shown in Fig. 2, cRGD decreased the probability of rupture events at all forces, but the probability of detecting rupture forces >30 pN decreased dramatically, such that the resulting rupture force histograms resembled those of the less reactive platelets shown in Fig. 1. This result suggests that the more reactive platelets had been inadvertently activated during the gel-filtration procedure or when they were attached to the polylysine-coated pedestal.

Interaction of OPN with ADP-stimulated Platelets – Platelet activation is required to induce platelet adhesion to OPN-coated surfaces (11-13). Based on previous measurements of fibrinogen binding to αIIbβ3 (8), the results shown in Figs. 1 and 2 suggest that rupture forces between OPN and αvβ3 <30 pN may be non-specific, whereas forces >30 pN may correspond to OPN binding to an activated conformation of αvβ3. To test this possibility, 10 µM ADP was
added to the platelet suspension immediately before it was studied. As shown in Fig. 3, ADP stimulation substantially affected the interaction of platelets with the OPN-coupled beads. After stimulation, virtually all of the platelets studied had a peak of rupture force at 45-50 pN, indicating that OPN bound strongly to all platelets, compared to only 10% of the unstimulated cells. Moreover, this peak of yield force was far more pronounced than that of the reactive unstimulated cells. Overall, there was a 12-fold increase in the probability of the forces >30 pN when the ADP-stimulated platelets were compared to unstimulated reactive platelets, as well as a less pronounced change in smaller non-specific forces. It should be noted that previous experiments using a variety of proteins not known to interact specifically with platelets indicate that rupture forces < 30 pN represent non-specific interactions (8). In contrast, rupture forces with a peak at 45-50 pN, induced by ADP, correspond to specific OPN-\( \alpha \text{v}\beta 3 \) binding.

To confirm that the 45-50 pN peak of rupture stimulated by ADP represents a specific interaction between \( \alpha \text{v}\beta 3 \) and OPN, the measurements were repeated using known inhibitors of OPN binding to \( \alpha \text{v}\beta 3 \). Competitive inhibitors of OPN binding to \( \alpha \text{v}\beta 3 \) - cRGD; XJ735, a cyclic RGD-based peptide specific for \( \alpha \text{v}\beta 3 \) (16); and abciximab, a murine-human chimeric Fab fragment of the monoclonal antibody 7E3 that binds to both \( \alpha \text{v}\beta 3 \) and \( \alpha \text{IIb}\beta 3 \) (18) - reduced the probability of high yield force interactions by 3- to 6-fold (Fig. 4) without affecting the lower force non-specific interactions. In addition, PGE\textsubscript{1}, a prostaglandin that functions as a global inhibitor of platelet reactivity by increasing the platelet concentration of cAMP, returned the force histogram of ADP-stimulated platelets to that of resting non-reactive platelets.

It is also noteworthy that whereas the pattern of rupture forces exhibited by the inadvertently stimulated platelets (Fig. 1b) was essentially the same as that of ADP-stimulated platelets, the probability of encountering rupture forces >30 pN was substantially decreased. This indicates that there were fewer activated \( \alpha \text{v}\beta 3 \) molecules per platelet and suggests that these platelets were only partially activated as compared to platelets exposed to 10 \( \mu \text{M} \) ADP.
Because the results shown in Fig. 4 imply that rupture forces $>30$ pN represent specific interactions between OPN and $\alpha v 3$ on agonist-stimulated platelets, we next compared the probability of interaction and the average yield strength of the 3 groups of platelets studied. This comparison, shown in Fig. 5, demonstrates that the probability of specific interactions between OPN and $\alpha v 3$, i.e., interactions with rupture forces $>30$ pN, is directly proportional to the degree of platelet stimulation. Nonetheless, the average yield strength remained constant at a value of 45-50 pN, suggesting that like $\alpha IIb 3$, $\alpha v 3$ resides on the platelet surface in either an inactive or an active state and that platelet stimulation results in a shift to a greater proportion of active, ligand binding, complexes.
Discussion

It is often not possible to determine experimentally whether integrin-mediated cell adhesion is due to the increased avidity that accompanies integrin clustering or is due to an increase in integrin affinity for adhesive ligands. Because laser tweezers can measure the force of interaction between individual receptor-ligand pairs (7), they have the potential to differentiate between these possibilities. We reported previously that the integrin $\alpha_v\beta_3$ mediates the adhesion of agonist-stimulated, but not unstimulated, platelets to surfaces coated with OPN (11). However, whether platelet adhesion is due exclusively to the integrin-clustering that accompanies ligand binding or results from an agonist-induced increase in $\alpha_v\beta_3$ affinity for OPN was not clear before the results presented in this paper. Although we have found that perturbing the conformation of the extracellular portion of $\alpha_v\beta_3$ with either dithiothreitol or Mn$^{2+}$ also induces platelet adhesion to OPN (13), suggesting that a conformational change in $\alpha_v\beta_3$ is sufficient to support adhesion, the inability to detect binding of the ligand-mimetic $\alpha_v\beta_3$ antibody WOW-1 to thrombin-stimulated platelets (15) suggests that this might not be the case. Accordingly, we applied laser tweezers technology to this question, much as we had used the technology to study the interaction of fibrinogen to platelet $\alpha$IIb$\beta_3$ (8). Our results demonstrate directly that ADP-stimulation increases the force of interaction between individual platelet $\alpha_v\beta_3$ and OPN molecules. Thus, our data imply that the mechanism responsible for platelet adhesion to OPN-coated surfaces requires an agonist-induced increase in the affinity of $\alpha_v\beta_3$ for this ligand.

The validity of this conclusion is based on our ability to determine first, which laser tweezers signals correspond to specific interactions between activated $\alpha_v\beta_3$ and OPN and second, whether the specific signals result from the interaction of single $\alpha_v\beta_3$ and OPN molecules.

The strength of non-specific protein-protein interactions, measured using laser tweezers or atomic force microscopy, can be as large as several tens of piconewtons, partially overlapping...
forces produced by specific receptor-ligand interactions (19,20). To discriminate between specific and non-specific interactions, we performed experiments using quiescent unstimulated platelets and established inhibitors of OPN binding to \( \alpha v 3 \). Although the vast majority of quiescent platelets were non-reactive with OPN-coated beads, such that rupture forces decreased exponentially from 10 pN and the probability of rupture forces \( > 30 \) pN was negligible, a small proportion of these platelets exhibited ruptures forces as great as those detected following platelet stimulation with ADP. Partial activation of platelets by handling is a well known phenomenon. Because our experiments determine the reactivity of individual platelets, they will detect platelets that had been inadvertently stimulated during preparation. Nevertheless, such platelets were useful in studying OPN-platelet interactions because the frequency of their specific interactions with OPN was lower than that of ADP-activated platelets. Thus, they can be regarded as partially activated, regardless of the nature of the activating stimulus. Furthermore, pre-incubating non-stimulated and ADP-stimulated platelets with the inhibitory prostaglandin PGE\(_1\) prevented the appearance of rupture forces \( > 30 \) pN, implying that rupture forces below 30 pN represent the non-specific background. Similarly, stimulating platelets with ADP in the presence of the chimeric mAb Fab fragment abciximab or either of the cyclic peptides cRGD or XJ735 prevented the appearance of rupture forces \( > 30 \) pN, indicating that rupture forces \( > 30 \) pN correspond to the specific binding of OPN to \( \alpha v 3 \).

The likelihood that the rupture forces we measured correspond to the interaction between single \( \alpha v 3 \) and OPN molecules is based on three arguments (7). First, histograms of rupture forces that result from multiple interactions should appear as a series of quantized peaks that are multiples of a single value of force and have probabilities inversely proportional to the number of bonds (21,22). Apart from the non-specific interactions discussed above, we observed only a single well-defined peak in the force histograms, suggesting that this peak represents individual ligand-receptor bonds. Second, the proportion of touching cycles in the laser tweezers apparatus that result in the formation and rupture of \( \alpha v 3 \)-OPN bonds is largely dependent on surface
density of each (20,23). The surface density of OPN was ≈1 molecule per ~35 nm², but how many retained the conformation and spatial orientation compatible with binding to vβ3 is unknown, as is the density of vβ3 on the platelet surface. Accordingly, the only available parameter is the actual frequency of specific binding events, each displayed as a force signal >30 pN. Even for ADP-activated platelets, which had the greatest frequency of specific binding events, the probability of specific interactions was always less than 5%. If we were to assume that the rupture force peak of 40-45 pN that we observed with a 5% probability represents the interactions of two ligand-receptor pairs, then single interactions at 20-25 pN would have a hypothetical probability of 22%, while the observed probability of rupture forces of this magnitude was actually 0.3%, implying that the 40-45 pN peak represents single ligand-receptor interactions. Similarly, there is no peak observed at 90-100 pN because, with 5% probability of single ligand-receptor pairs, the probability of two ligand-receptor pairs is 0.25%. Lastly, if multiple bonds were formed during a contact between a platelet and an OPN-coated bead, it is unlikely that all of the bonds would ruptured simultaneously; rather, it is probable that they would be ruptured sequentially such that multiple steps should have been observed during bond breakage. However, the rupture events we observed all occurred as a single step with a resolution time of 0.5 ms.

Using laser tweezers, we found that the average adhesion strength (the mean value of rupture forces > 30 pN) for OPN-vβ3 interactions was 47 ± 7 pN and Lehenkari and Horton, using atomic force microscopy to measure the binding force between OPN and constitutively active osteoclast vβ3 at a similar loading rate, reported a binding force of 50 ± 2 pN (24). However, the average adhesion strength of fibrinogen binding to IIbβ3 on ADP- and thrombin-stimulated platelets was nearly 2-fold greater at 80-90 pN (8). Although vβ3 and IIbβ3 are homologous proteins (25) whose mechanisms of activation are likely similar (13), sites at which OPN and fibrinogen bind to these integrins undoubtedly differ. Thus, whereas fibrinogen binds to IIbβ3
via the carboxyl-terminus of its $\alpha$ chain (26), OPN binding to $\alpha v \beta 3$ is mediated by its RGD motif (27), accounting, at least in part, for the measured differences in OPN and fibrinogen binding.

The activation state of $\alpha v \beta 3$ has been also studied in cells other than platelets. Filardo and Cheresh reported that truncating the $\alpha v$ cytoplasmic tail before the motif PPQEE impairs the ability of M21-L melanoma cells to adhere to vitronectin-coated surfaces, but has no effect on their interaction with adenovirus penton base protein (28), suggesting that $\alpha v \beta 3$ may exist in several ligand binding conformations. Similarly, Boettiger et al., using a spinning disc assay to measure the strength of cell adhesion, observed that whereas the $\alpha v \beta 3$-mediated adhesion of K562 cells to fibronectin is a single-stage process that is independent of cellular metabolism, adhesion to vitronectin is a multistage process that is enhanced by intracellular signals (29). By contrast, Felding-Habermann et al. reported that $\alpha v \beta 3$ is present in either of two functional states on human breast cancer cells that affected their metastatic potential, although the factors responsible for the shift from one state to the other were not established (30). We have found that $\alpha v \beta 3$ on platelets is also present in two activation states that are regulated by platelet stimulation (11). In previous studies of fibrinogen binding to platelet $\alpha IIb \beta 3$ using laser tweezers, we found that agonist concentration affected the probability of encountering an activated $\alpha IIb \beta 3$ molecule, but had no effect on the strength of fibrinogen binding (8). Similarly, in the current work, we found that the strength of OPN binding to $\alpha v \beta 3$ was unaffected by the degree of platelet stimulation, implying that the function of platelet agonists is to shift $\alpha v \beta 3$ from its inactive to active state.
Acknowledgements

We thank Dr. Shaker Mousa for generous gifts of the peptide XJ735 and recombinant human OPN. This work was supported by grants HL57407, HL30954 and HL62250 from the National Institutes of Health.
References

1. Bennett, J. S. (1996) *Trends Cardiovasc.Med.* **6**, 31-37

2. Shattil, S. J., Kashiwagi, H., and Pampori, N. (1998) *Blood* **91**, 2645-2657

3. Lub, M., van Kooyk, Y., and Figdor, C. G. (1995) *Immunol Today* **16**, 479-483

4. Stewart, M., and Hogg, N. (1996) *J Cell Biochem* **61**, 554-561

5. Shattil, S. J., Hoxie, J. A., Cunningham, M., and Brass, L. F. (1985) *J. Biol. Chem.* **260**, 11107-11114

6. Visscher, K., and Block, S. M. (1998) *Methods Enzymol* **298**, 460-489

7. Weisel, J. W., Shuman, H., and Litvinov, R. I. (2003) *Curr Opin Struct Biol* **13**, 227-235

8. Litvinov, R. I., Shuman, H., Bennett, J. S., and Weisel, J. W. (2002) *Proc Natl Acad Sci U S A* **99**, 7426-7431

9. Lawler, J., and Hynes, R. O. (1989) *Blood* **74**, 2022-2027

10. Coller, B. S., Cheresh, D. A., Asch, E., and Seligsohn, U. (1991) *Blood* **77**, 75-83

11. Bennett, J. S., Chan, C., Vilaire, G., Mousa, S. A., and DeGrado, W. F. (1997) *J. Biol. Chem.* **272**, 8137-8140

12. Helluin, O., Chan, C., Vilaire, G., Mousa, S., DeGrado, W. F., and Bennett, J. S. (2000) *J Biol Chem* **275**, 18337-18343

13. Paul, B. Z. S., Vilaire, G., Kunapuli, S. P., and Bennett, J. S. (2003) *J. Thrombos. Haemostas.* **1**, 814-820
14. Bazzoni, B., and Hemler, M. E. (1998) *Trends Biochem Sci* **23**, 30-34

15. Pampori, N., Hato, T., Stupack, D. G., Aidoudi, S., Cheresh, D. A., Nemerow, G. R., and Shattil, S. J. (1999) *J. Biol. Chem.* **274**, 21609-21616

16. Bach, A. C. I., Espina, J. R., Jackson, S. A., Stouten, P. F. W., Duke, J. L., Mousa, S. A., and DeGrado, W. F. (1996) *J. Am. Chem. Soc.* **118**, 293-294

17. Bennett, J. S., Chan, C., Vilaire, G., Mousa, S. A., and DeGrado, W. F. (1997) *J Biol Chem* **272**, 8137-8140

18. Coller, B. S. (1985) *J. Clin. Invest.* **76**, 101-108

19. Willemsen, O. H., Snel, M. M., van der Werf, K. O., de Grooth, B. G., Greve, J., Hinterdorfer, P., Gruber, H. J., Schindler, H., van Kooyk, Y., and Figdor, C. G. (1998) *Biophys J* **75**, 2220-2228

20. Stout, A. L. (2001) *Biophys J* **80**, 2976-2986

21. Allen, S., Davies, J., Davies, M. C., Dawkes, A. C., Roberts, C. J., Tendler, S. J., and Williams, P. M. (1999) *Biochem J* **341** (Pt 1), 173-178

22. Lee, I. I., and Marchant, R. E. (2000) *Colloids Surf B Biointerfaces* **19**, 357-365

23. Goldsmith, H. L., McIntosh, F. A., Shahin, J., and Frojmovic, M. M. (2000) *Biophys J* **78**, 1195-1206

24. Lehenkari, P. P., and Horton, M. A. (1999) *Biochem Biophys Res Commun* **259**, 645-650

25. Fitzgerald, L. A., Poncz, M., Steiner, B., Rall, S. C., Jr., Bennett, J. S., and Phillips, D. R. (1987) *Biochemistry* **26**, 8158-8165
26. Basani, R. B., D'Andrea, G., Mitra, N., Vilaire, G., Richberg, M., Kowalska, M. A., Bennett, J. S., and Poncz, M. (2001) *J Biol Chem* **276**, 13975-13981

27. Helluin, O., Chan, C., Vilaire, G., Mousa, S., DeGrado, W. F., and Bennett, J. S. (2000) *J Biol Chem* **275**, 18337-18343

28. Filardo, E. J., and Cheresh, D. A. (1994) *J Biol Chem* **269**, 4641-4647

29. Boettiger, D., Lynch, L., Blystone, S., and Huber, F. (2001) *J Biol Chem* **276**, 31684-31690

30. Felding-Habermann, B., O'Toole, T. E., Smith, J. W., Fransvea, E., Ruggeri, Z. M., Ginsberg, M. H., Hughes, P. E., Pampori, N., Shattil, S. J., Saven, A., and Mueller, B. M. (2001) *Proc Natl Acad Sci U S A* **98**, 1853-1858
Figure Legends

Figure 1. Force histograms of the interactions of OPN-coated beads with two fractions of unstimulated platelets, non-reactive and reactive. Rupture forces between OPN-coated beads and single unstimulated gel-filtered platelets were collected as described in the “Experimental Procedures”. Individual forces measured during each contact-detachment cycle were collected in 5 pN bins. The number of events in each bin (force range) was then plotted against the average force for that bin after normalizing for the total number of interaction cycles. The percentage of events in a particular bin represented the probability of rupture events at that force. Forces below 10 pN were regarded as zero. Approximately 90% of the unstimulated platelets (white bars) exhibited only forces < 30 pN, whereas the remaining platelets (shaded bars) also exhibited a peak of rupture force of 30-60 pN.

Figure 2. Effect of a cyclic RGD peptide (cRGD) on the interaction of unstimulated reactive platelets with OPN-coated beads. Aliquots of a suspension of gel-filtered platelets were incubated in the presence (shaded bars) or absence (white bars) of 40 µM cRGD for 15 min at 25°C. Rupture forces between single platelets and OPN-coated beads were then measured as described for Fig. 1. In the presence of cRGD, there is a substantial decrease in the probability of rupture forces in the 30-60 pN range such that the histogram now resembles that of the unstimulated non-reactive platelets shown in Fig. 1.

Figure 3. Force histograms of the interaction of OPN-coated beads with ADP-stimulated platelets. Aliquots of a suspension of gel-filtered platelets were incubated in the presence (shaded bars) or absence (white bars) of 10 µM ADP before the interaction of single platelets with OPN-coated beads was measured using laser tweezers as described in Fig. 1.
Figure 4. Effect of specific αvβ3 antagonists on rupture forces between OPN-coated beads and αvβ3 on ADP-stimulated platelets. Aliquots of a suspension of gel-filtered platelets were incubated with ADP and various αvβ3 antagonists before the interaction of OPN-coated beads with single platelets was measured using laser tweezers as described in Fig. 1. A. 2 nM PGE1, B. 200 µg/ml abciximab, C. 0.1 mM XJ735, D. 40 µM cRGD. E. Bar graph derived from the histograms shown in A-D illustrating the reduction in probability of detecting rupture force > 30 pN in the presence of the various inhibitors.

Figure 5. Probability and average adhesion strength derived for the force histograms of unstimulated non-reactive, unstimulated reactive, and ADP-stimulated platelets with OPN-coated beads. A. Normalized histograms of rupture forces resulting from binding events using unstimulated non-reactive, unstimulated reactive, and ADP-stimulated platelets. B. The probability of specific rupture events between OPN and αvβ3 (rupture forces > 30 pN) was calculated from the histograms shown in panel A. C. The average adhesion strength for the specific interaction between OPN-coated beads and unstimulated non-reactive, unstimulated reactive, and ADP-stimulated platelets.
Figure 2

![Graph showing Probability vs Rupture forces, pN for With and Without cRGD]
Figure 4

A. Without PGE1
   - Without PGE1
   - With PGE1

B. Without abciximab
   - Without abciximab
   - With abciximab

C. Without XJ735
   - Without XJ735
   - With XJ735

D. Without cRGD
   - Without cRGD
   - With cRGD

E. Pos. Control, PGE1, Abciximab, XJ735, cRGD
   - Probability, %
   - Rupture forces, pN
Figure 5

A

Unstim. non-reactive platelets

Unstim. reactive platelets

Stimulated platelets

Rupture forces, pN

Probability, %

B

Unstim. non-reactive platelets

Unstim. reactive platelets

Stimulated platelets

Probability, %

C

Unstim. non-reactive platelets

Unstim. reactive platelets

Stimulated platelets

Average adhesion strength, pN

60

50

40

30

20

10

0
