FUNCTIONAL AND STRUCTURAL CORRELATIONS OF INDIVIDUAL 
\(\alpha\text{IIb}\beta3\) MOLECULES

Running Title: Structure-Function of \(\alpha\text{IIb}\beta3\) Molecules

Rustem I. Litvinov\(^1\), Chandrasekaran Nagaswami\(^1\), Gaston Vilaire\(^2\), Henry Shuman\(^3\), 
Joel S. Bennett\(^2\), and John W. Weisel\(^1\)‡

From the \(^1\)Department of Cell and Developmental Biology, \(^3\)Department of Physiology, and the 
\(^2\)Hematology-Oncology Division of the Department of Medicine, University of Pennsylvania 
School of Medicine, Philadelphia, PA 19104-6058, USA

‡To whom correspondence should be addressed: Department of Cell and Developmental 
Biology, University of Pennsylvania School of Medicine, 421 Curie Blvd., 1054 BRB II/III, 
Philadelphia, PA 19104-6058, USA. Tel.: 215-898-3573; Fax: 215-898-9871; Email: 
weisel@cellbio.med.upenn.edu

Regular manuscript

Grant Funding: Supported by grants HL57407, HL30954, HL40387, and HL62250 from the 
National Institutes of Health.

Scientific Category: Hemostasis, Thrombosis, and Vascular Biology

Abstract: 199 words. Total Text: 4,891 words.
Abstract

The divalent cation Mn\(^{2+}\) and the reducing agent dithiothreitol directly shift integrins from their inactive to their active states. We used transmission electron microscopy and laser tweezers-based force spectroscopy to determine whether structural rearrangements induced by these agents in the integrin \(\alpha{IIb}\beta3\) correlate with its ability to bind fibrinogen. Mn\(^{2+}\) increased the probability of specific fibrinogen-\(\alpha{IIb}\beta3\) interactions nearly 20-fold in platelets and both Mn\(^{2+}\) and dithiothreitol increased the probability more than 2-fold using purified proteins. Of three \(\alpha{IIb}\beta3\) conformations - closed with stalks touching, open with stalks separated, and globular without visible stalks - Mn\(^{2+}\) and dithiothreitol induced a significant increase in the proportion of open structures, as well as structural changes in the \(\alpha{IIb}\beta3\) headpiece. Mn\(^{2+}\) also increased the number of complexes between fibrinogen and purified \(\alpha{IIb}\beta3\) molecules, all of which were in the open conformation. Finally, Mn\(^{2+}\) induced the formation of \(\alpha{IIb}\beta3\) clusters that resulted from interactions exclusively involving the distal ends of the stalks. These results indicate that there is a direct correlation between \(\alpha{IIb}\beta3\) activation and the overall conformation of the molecule. Further, they are consistent with the presence of a linked equilibrium between single inactive and single active \(\alpha{IIb}\beta3\) molecules and active \(\alpha{IIb}\beta3\) clusters.
Introduction

The activity of circulating platelets is tightly regulated to prevent the spontaneous formation of platelet aggregates\(^1\). Thus, circulating platelets are inactive until they adhere to exposed subendothelial matrix or are stimulated by soluble agonists such as ADP and thrombin. Each activating event is associated with a change in platelet shape, reorganization of the platelet cytoskeleton, secretion of platelet granules, and an increase in the affinity of the integrin \(\alpha IIb\beta 3\) for soluble ligands such as fibrinogen and von Willebrand factor. The latter is responsible for platelet aggregation when the macromolecular ligands bind to activated \(\alpha IIb\beta 3\) and bridge adjacent platelets\(^2\).

Although the activation state of \(\alpha IIb\beta 3\) is normally regulated by agonist-generated “inside-out” signaling\(^1\), \(\alpha IIb\beta 3\) can also be activated experimentally by perturbing the conformation of its extracellular domain using Mn\(^{2+}\) ions\(^3,4\) or the reducing agent dithiothreitol (DTT)\(^5,6\). Thus, in platelets, Mn\(^{2+}\) has been reported to activate \(\alpha v\beta 3\) and \(\alpha IIb\beta 3\), thereby promoting their interaction with ligands such as fibrinogen, von Willebrand factor, vitronectin, and osteopontin, mimicking the consequences of conventional inside-out signaling\(^3,4\). Mn\(^{2+}\) also stabilizes platelet-fibrinogen interactions\(^7\). Moreover, in experiments using purified integrins, Mn\(^{2+}\) affects the binding kinetics, affinity, and specificity towards synthetic and natural ligands\(^8\text{--}11\). Mn\(^{2+}\)-induced changes in integrin function have been attributed to specific conformational rearrangements in the integrin ectodomain\(^12\), a suggestion supported by electron microscope studies showing that Mn\(^{2+}\) promotes the opening of integrin molecules into extended structures\(^13\text{--}15\).
Millimolar concentrations of DTT also induce platelet aggregation by directly stimulating ligand binding to $\alpha_{IIb}\beta3$. How DTT activates $\alpha_{IIb}\beta3$ is unclear. $\beta3$ has been reported to contain an extracellular redox site that is associated with the presence of 2 unpaired cysteines in inactive $\alpha_{IIb}\beta3$ and 6 unpaired cysteines following exposure of $\alpha_{IIb}\beta3$ to DTT. However, the identity of the putatively unpaired cysteines in either inactive or active forms of $\alpha_{IIb}\beta3$ has not been determined and it has been proposed that $\alpha_{IIb}\beta3$ activation by DTT may involve disulfide bond rearrangement of the originally unpaired cysteines, as well as overall bond reduction.

The ability of Mn$^{2+}$ and DTT to enhance integrin function, as well as perturb the conformation of integrin ectodomains, provides an opportunity to test the hypothesis that there is an equilibrium between inactive and active integrin activation states that is consequence of a reversible structural rearrangement of the entire integrin molecule. To address this hypothesis, we used transmission electron microscopy to probe for structural differences between $\alpha_{IIb}\beta3$ in the presence of Ca$^{2+}$, Mn$^{2+}$, and DTT and laser tweezers-based force spectroscopy to measure the fibrinogen-binding function of the integrin in the presence of each at the single molecule level. We found that both Mn$^{2+}$ and DTT increase the probability of specific interactions between $\alpha_{IIb}\beta3$ and fibrinogen, but do so without changing the average yield strength of fibrinogen binding. Both agents also induce a change in the shape of the $\alpha_{IIb}\beta3$ headpiece, shift $\alpha_{IIb}\beta3$ from a closed conformation with stalks touching to an open conformation with stalks separated, and stimulate the formation of clusters of the open $\alpha_{IIb}\beta3$ conformer. These results indicate that there is a direct correlation between the activation of $\alpha_{IIb}\beta3$ and the overall conformation of the molecule. Further, they are consistent with the presence of a linked equilibrium between inactive and active $\alpha_{IIb}\beta3$ molecules and $\alpha_{IIb}\beta3$ clusters.
Methods

**Laser Tweezers Measurements** - Using laser tweezers to measure integrin function on platelets has been described previously in detail. Briefly, 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 to measure the strength of fibrinogen binding to human platelets or purified αIIbβ3 in the presence of either Ca$^{2+}$ or Mn$^{2+}$. For these measurements, human fibrinogen (American Diagnostica, Inc.) 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. Before use, the fibrinogen-coated beads were disaggregated by mild sonication and used at a concentration of $\approx 10^7$/ml. For studies using purified human αIIbβ3, the purified integrin (Enzyme Research Laboratories, Inc.) at a concentration of 1 mg/ml in 0.01 M HEPES buffer, pH 7.4, containing 60 mM octyl-glucoside was bound covalently to polyacrylamide-coated 1.4 µm silica pedestals using glutaraldehyde as previously described. Prior to immobilization, the αIIbβ3 was pre-incubated with either 1 mM CaCl$_2$, 1 mM MnCl$_2$, or 5 mM DTT/1 mM CaCl$_2$ in the same binding buffer at 37°C for 30 min. Interactions between fibrinogen and purified αIIbβ3 were studied in 0.1 M HEPES buffer, pH 7.4, containing 2 mg/ml bovine serum albumin, 0.1% Triton X-100, and either 1 mM CaCl$_2$, 1 mM MnCl$_2$, or 5 mM DTT/1 mM CaCl$_2$. To measure fibrinogen binding to αIIbβ3 on living platelets, an individual platelet was trapped from a suspension of gel-filtered human platelets containing $\approx 5 \times 10^6$ platelets/ml and $\approx 10^5$/ml fibrinogen-coated beads and manually attached to a 5 µm diameter silica pedestal coated with polylysine. All experiments with unstimulated platelets 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.

To measure the rupture force between fibrinogen and either purified αIIbβ3 or αIIbβ3 on platelets, a fibrinogen-coated bead, trapped by the laser light, was brought to a distance of 2-3 μm from the αIIbβ3-coated pedestal or immobilized platelet. After oscillation of the bead was initiated at 5 Hz or 50 Hz with 0.8 μm peak-to-peak amplitude, the bead was brought into contact with the platelet or the pedestal by micromanipulation using a keyboard-controlled piezoelectric stage. Data collection was initiated at the first contact between the bead and the platelet or pedestal. Rupture forces following repeated contacts between the platelet or the pedestal and the bead were collected for periods of several seconds to one minute and were displayed as normalized force histograms for each experimental condition. Because only a small percentage of contact/detachment cycles result in effective receptor-ligand binding/unbinding, data from 10 to 22 experiments, representing 3x10³-10⁵ individual measurements, were combined. Individual forces measured during each contact-detachment cycle were collected into 10 pN-wide bins. The number of events in each bin was plotted against the average force for that bin after normalizing for the total number of interaction cycles. The percentage of events in a particular force range (bin) represents the probability of rupture events at that tension. Optical artifacts observed with or without trapped latex beads produce signals that appeared as forces below 10 pN. Accordingly, rupture forces in this range were not considered when the data were analyzed.

**Transmission electron microscopy** - Rotary-shadowed samples were prepared using a modification of standard procedures by spraying a dilute solution of molecules in a volatile buffer (0.05 M ammonium formate) and glycerol (30-50%) onto freshly-cleaved mica and...
shadowing with tungsten in a vacuum evaporator (Denton Vacuum Co., Cherry Hill, NJ) 21. All specimens were examined in a FEI/Philips 400 electron microscope (Philips Electronic Instruments Co., Mahwah, NJ), operating at 80 kV and at a magnification of 60,000x. The molecular dimensions for approximately 100 individual images from the various groups of αIIbβ3 molecules were measured after scanning negative prints using Adobe Photoshop 7.0.1. Measurements were made with a Photoshop tool calibrated to have 1.6 nm resolution. Molecular dimensions of the digitized images were corrected for a shell of tungsten by subtracting 1 nm on each side of a measured molecule.
Results

Interaction of Mn\(^{2+}\)-treated platelets with fibrinogen – When probed by laser tweezers-based force spectroscopy, the vast majority of the interactions between surface-bound fibrinogen and unstimulated platelets in the presence of Ca\(^{2+}\) were characterized by rupture forces that ranged from 10 pN to several tens of pN. Moreover, the probability of detecting these rupture forces decreased exponentially as the rupture force increased (Fig. 1A). However, the same platelets incubated with 1 mM MnCl\(_2\) for 5 minutes at 25°C were highly reactive with fibrinogen-coated beads, producing a peak in the histogram of rupture forces that ranged from 60 to 110 pN (Fig. 1B). Thus, the cumulative probability of detecting rupture forces >60 pN, which we previously found to be specific for fibrinogen binding to \(\alpha\)IIb\(\beta\)3, increased nearly 20-fold. To confirm that the Mn\(^{2+}\)-induced peak of rupture forces resulted from fibrinogen binding to \(\alpha\)IIb\(\beta\)3, measurements were repeated in the presence of either of the \(\alpha\)IIb\(\beta\)3 antagonists, tirofiban or abciximab. As shown in Figs. 1C and 1D, each antagonist abrogated the effect of Mn\(^{2+}\) and returned the force histograms to that of unstimulated platelets incubated with Ca\(^{2+}\).

Interaction of Mn\(^{2+}\)-treated \(\alpha\)IIb\(\beta\)3 preparations with fibrinogen – To verify that the increase in rupture force we observed between platelets and fibrinogen-coated beads in the presence of Mn\(^{2+}\) resulted from an increase in the affinity of individual \(\alpha\)IIb\(\beta\)3 molecules and was independent of possible Mn\(^{2+}\)-induced changes in the platelet membrane, we measured rupture forces between fibrinogen-coated beads and purified \(\alpha\)IIb\(\beta\)3 that had been pre-incubated with 1 mM Mn\(^{2+}\) or 1 mM Ca\(^{2+}\). Consistent with our previously reported laser tweezers measurements using purified \(\alpha\)IIb\(\beta\)3, we found that the cumulative probability of detecting rupture forces >60 pN was 2.1%
in the presence of 1 mM Ca\textsuperscript{2+}, indicating that some of the purified αIIbβ3 was in an active conformation (Fig. 2A). Others have observed that approximately 10% of the αIIbβ3 isolated from platelets is in an active conformation \textsuperscript{22}. Nonetheless, as shown in Fig. 2B, the Mn\textsuperscript{2+}-treated preparations were much more reactive with fibrinogen-coated surfaces, such that the cumulative probability of detecting rupture forces >60 pN increased to 4.5%. The ability of the αIIbβ3 antagonists tirofiban and abciximab to decrease this probability to 0.4% confirmed that rupture forces >60 pN resulted from fibrinogen bound to αIIbβ3 (Figs. 2C and 2D). It is noteworthy that despite the presence of Mn\textsuperscript{2+}, the yield strength of αIIbβ3-fibrinogen binding was not changed substantially, whereas the cumulative probability of specific rupture forces >60 pN increased more than 2-fold. Thus, these results are consistent with the hypothesis that Mn\textsuperscript{2+} binding directly induces an increase in the affinity of individual αIIbβ3 molecules for fibrinogen.

\textit{Mn\textsuperscript{2+}-induced changes in the conformation of αIIbβ3} – Purified αIIbβ3 molecules from the same preparation used for the laser tweezers experiments were visualized by transmission electron microscopy after rotary shadowing with tungsten. Previous electron microscopy studies indicated that αIIbβ3 molecules are composed of a dense “headpiece” and two “stalks”, one from each subunit of the heterodimer \textsuperscript{23}. The headpiece, composed of the amino-terminal portions of the extracellular domains of αIIb and β3, contains the ligand-binding site of the integrin, whereas the stalks contain the transmembrane and cytoplasmic domains of each subunit. From the observations of more than two thousand individual images, we were able to distribute αIIbβ3 molecules into three groups. Group 1 consists of relatively compact molecules in which the stalks appear to touch (“closed” images) (Fig. 3A). Group 2 consists of extended molecules
in which the stalks are separated (“open” images) (Fig. 3B). Group 3 consists of globular heads in which the stalks are not visualized.

Although each group was present regardless whether αIIbβ3 had been incubated with Ca²⁺ or Mn²⁺, the distribution was significantly different. As shown in Table 1, there was a large increase in the proportion of open (Group 2) structures in the presence of Mn²⁺ (60±4% in the presence of 1 mM Mn²⁺ versus 11±4% in presence of Ca²⁺, p < 0.001), with corresponding decreases in the proportion of Group 1 (24±3% in the presence of 1 mM Mn²⁺ versus 51±12% in presence of Ca²⁺, p < 0.001) and Group 3 structures (17±3% in the presence of 1 mM Mn²⁺ versus 38±10% in presence of Ca²⁺, p < 0.001).

We also compared the molecular dimensions of individual αIIbβ3 molecules in the presence of Ca²⁺ or Mn²⁺ using digitized images of the electron micrographs. In the presence of Ca²⁺, there were no significant differences in the dimensions of the αIIbβ3 headpiece when the molecule was in either the open or closed conformation (Table 2). Moreover, the distance separating the αIIb and β3 stalks of open molecules did not change when αIIbβ3 was bound to fibrinogen (13.4±4.1 nm vs. 12.6±2.7 nm, respectively). The latter measurements suggest that stalk separation of ≈13 nm is sufficient to identify active αIIbβ3 molecules. By contrast, in the presence of Mn²⁺, there was an increase in both the length (10.9±1.5 nm vs. 12.6±1.5 nm, p < 10⁻⁸) and width (7.3±1.5 nm vs. 8.4±1.1 nm, p< 10⁻⁴) of the αIIbβ3 headpiece when αIIbβ3 shifted from a closed to an open conformation. In addition, the distance separating the stalks of open αIIbβ3 was significantly greater in the presence of Mn²⁺ than in the presence of Ca²⁺ (17.8±3.6 nm vs. 13.7±4.1 nm, p < 10⁻⁸). These measurements indicate that not only does Mn²⁺ shift
$\alpha$IIb$\beta$3 from a closed to an open conformation by inducing the separation of $\alpha$IIb and $\beta$3 stalks, but it also alters the overall size and shape of the headpiece.

Besides altering the size and configuration of individual $\alpha$IIb$\beta$3 molecules, Mn$^{2+}$ increased their tendency to oligomerize. Whereas oligomers were uncommon in the presence of Ca$^{2+}$, 38±10% of the open forms of $\alpha$IIb$\beta$3 in the presence of Mn$^{2+}$ consisted of dimers, trimers, and higher order oligomers (Fig. 4, A-C). Moreover, as shown in Fig. 4D, the distribution of monomers, dimers, trimers, and higher order oligomers could be fit to an exponential function (Fig. 4D), as previously described for equilibria involving molecules that self-assemble into oligomers such as actin\textsuperscript{24,25}, hemoglobin S\textsuperscript{26}, and prions\textsuperscript{27}. It is also noteworthy that the oligomers resulted from interactions that exclusively involved the distal ends of the stalks and that oligomers composed of three or five molecules did not form closed rosettes as one would expect if the stalks underwent homomeric interactions.

\textit{Mn$^{2+}$-induced complex formation between $\alpha$IIb$\beta$3 and fibrinogen.} Previously, we observed that $\alpha$IIb$\beta$3 bound to fibrinogen tended to have an open conformation with separated stalks\textsuperscript{23}. To quantify these observations and to measure the distance separating the stalks when $\alpha$IIb$\beta$3 was bound to fibrinogen, we mixed fibrinogen with $\alpha$IIb$\beta$3 at different molar ratios in the presence of 1 mM Mn$^{2+}$ and incubated at 37°C for 30 min before the mixture was sprayed onto mica and rotary-shadowed with tungsten. As illustrated by Fig 5A, fibrinogen displayed its typical trinodular structure with two lateral D nodules and a central E nodule and $\alpha$IIb$\beta$3 complexes were present in the three different conformations described above. Although the majority of individual fibrinogen and $\alpha$IIb$\beta$3 molecules were separated from each other, a minor fraction formed bimolecular and trimolecular complexes of two possible stoichiometric ratios, either 1:1
or 1:2 of αIIbβ3 to fibrinogen (Figs. 5B-D). As we reported previously,$^{23}$ αIIbβ3 was always spatially oriented so that its headpiece was attached to the end of a fibrinogen molecule and the stalks of two integrins attached to one fibrinogen were oriented in opposite directions. The orientation was such that the entire complex had a two-fold axis of symmetry through the center of the fibrinogen, i.e. a rotation of 180° about this axis brings one αIIbβ3 into the other one. All of the αIIbβ3 molecules interacting with fibrinogen were in the open conformation, implying that this conformation represents its activated state.

Because αIIbβ3 was in excess, quantitative analysis of complex formation was based on the relative proportion of fibrinogen molecules participating in the complexes versus those remaining free. Comparison of the fractions of free fibrinogen molecules in the presence and in the absence of Mn$^{2+}$ clearly showed the promoting effect of manganese ions on fibrinogen binding to αIIbβ3. Thus, in the absence of Mn$^{2+}$, 23±8% of the fibrinogen molecules were bound to αIIbβ3. In the presence of Mn$^{2+}$, the percentage increased significantly to 66±6%. Further, we found that the distance separating the stalks of open αIIbβ3 increased from 17.8±3.6 nm in the presence of Mn$^{2+}$ alone to 19.9±4.7 nm (p = 10$^{-3}$) when Mn$^{2+}$-treated αIIbβ3 was bound to fibrinogen (Table 2).

**DTT-induced changes in the activity and conformation of αIIbβ3.** αIIbβ3 on platelets and transfected tissue culture cells is activated by incubating the cells with DTT.$^{5,6,28}$ Therefore, to determine whether the changes in the conformation of αIIbβ3 induced by Mn$^{2+}$ are unique to this cation, we repeated the measurements described above using DTT as the stimulus for αIIbβ3 activation. First, we used laser tweezers to measure rupture forces between fibrinogen-coated beads and purified αIIbβ3 that had been pre-incubated with 5 mM DTT for 30 min in the
presence of 1 mM Ca\textsuperscript{2+}. Similar to Mn\textsuperscript{2+}, DTT increased the cumulative probability of detecting rupture forces >60 pN in the presence of Ca\textsuperscript{2+} from 1.9 ± 0.5% to 3.2 ± 0.6% (Table 3). Moreover, there was no difference between the average yield strength of fibrinogen binding to \( \alpha\text{IIb}\beta3 \) in the presence of Mn\textsuperscript{2+} or DTT, suggesting that the \( \alpha\text{IIb}\beta3 \) activation state was similar under both sets of conditions.

DTT-treated \( \alpha\text{IIb}\beta3 \) molecules were then visualized by transmission electron microscopy after rotary shadowing with tungsten. Like Mn\textsuperscript{2+}, DTT treatment resulted in a nearly 3-fold increase in the number of \( \alpha\text{IIb}\beta3 \) molecules in the open, rather than closed, conformation and in the formation of \( \alpha\text{IIb}\beta3 \) clusters. Further, DTT induced changes in the molecular dimensions of individual \( \alpha\text{IIb}\beta3 \) molecules that were similar to the changes induced by Mn\textsuperscript{2+}. Thus, the distance separating the \( \text{IIb} \) and \( \beta3 \) stalks of open molecules significantly increased from 13.1±2.8 nm in the presence of Ca\textsuperscript{2+} to 18.1±3.9 nm in the presence of DTT and Ca\textsuperscript{2+} (\( p = 4.8 \times 10^{-23} \)) (Table 4). Moreover, there were significant increases in both the length (11.9±1.9 nm vs. 13.4±2.0 nm, \( p < 10^{-6} \)) and width (8.7±1.4 nm vs. 9.2±1.1 nm, \( p < 0.034 \)) when \( \alpha\text{IIb}\beta3 \) was treated with DTT. Thus, these measurements confirm that agents that activate \( \alpha\text{IIb}\beta3 \) by perturbing its extracellular domain induce separation of the \( \alpha\text{IIb} \) and \( \beta3 \) stalks. They also indicate that these agents can alter the overall size and shape of the \( \alpha\text{IIb}\beta3 \) headpiece as well.
Discussion

Integrins can be activated in vitro by cleavage with specific proteases, stabilization of their activated states using monoclonal antibodies, exposure to reducing agents such as DTT, and incubation with the divalent cation Mn\textsuperscript{2+}. How each of these treatments alters integrin activation states is not entirely clear. For example, although Ca\textsuperscript{2+} or Mg\textsuperscript{2+} are required for ligand binding to integrins, neither by themselves activate integrins, whereas Mn\textsuperscript{2+}, at millimolar concentrations, induces integrin-mediated cell adhesion, binding of isolated integrins to immobilized ligands, and binding of soluble ligands to integrins on cell surfaces.

The crystal structure of the extracellular portion of the integrin αvβ3 revealed that it contains eight divalent cation-binding sites. Four sites were located in the β-propeller domain of αv, one at the α subunit genu (knee), and three in the β3 βA domain. The number of divalent cations bound to the βA domain appears to be directly related to the presence or absence of ligand. Thus, in the absence of ligand, only the cation binding site in the βA ADMIDAS motif is occupied, whereas in the presence of Mn\textsuperscript{2+} and a cyclic RGD ligand, each of the remaining two βA domain binding sites contain a cation. One of these sites is located in the βA MIDAS (metal ion-dependent adhesion site) and Mn\textsuperscript{2+} at this site contacts one of the ligand Asp carboxylate oxygens. A second Mn\textsuperscript{2+} is located 6 Å away from the MIDAS at a site designated as the ligand-induced metal binding site (LIMBS), but does not interact with ligand. Although it has been postulated that Mn\textsuperscript{2+} affects integrin activation states by antagonizing inhibitory effects of Ca\textsuperscript{2+}, analysis of the crystal structure of the αvβ3 extracellular domain suggests that by occupying sites in the MIDAS and LIMBS motifs of the βA domain, Mn\textsuperscript{2+} stabilizes its ligand-occupied conformation.
It is currently thought that integrins such as $\alpha$IIb$\beta$3 reside on cell surfaces in a thermodynamic equilibrium between inactive and active conformations that can be perturbed by altering the relative position of an integrin $\alpha$ and $\beta$ stalks $^{13,34}$. An essential element of this hypothesis is that the equilibrium can also be perturbed by altering the conformation of the integrin extracellular domain. To test this premise, we used laser tweezers-based force spectroscopy and electron microscopy to correlate the functional and ultrastructural consequences of exposing the platelet integrin $\alpha$IIb$\beta$3 to either Mn$^{2+}$ or DTT. Laser tweezers are an optical system in which external forces applied to a spherical 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 and are sensitive and accurate at the lower end of the force spectrum (0-150 pN) $^{39,40}$. Previously, we found that specific binding of fibrinogen to $\alpha$IIb$\beta$3 resulted in rupture forces ranging from 60-150 pN and an average yield strength of 80-100 pN $^{18}$. Because the specific rupture forces occurred as a single well-defined peak, they likely represent the interaction of individual $\alpha$IIb$\beta$3 and fibrinogen molecules. Using living platelets and isolated $\alpha$IIb$\beta$3 molecules, we found that Mn$^{2+}$ and DTT increased the affinity of $\alpha$IIb$\beta$3 for fibrinogen and that the rupture forces between fibrinogen and Mn$^{2+}$- or DTT-stimulated $\alpha$IIb$\beta$3 were essentially the same as those we measured previously using ADP- and thrombin-related activation peptide (TRAP)-stimulated platelets $^{18}$. Thus, like physiologic platelet agonists, Mn$^{2+}$ and DTT shift $\alpha$IIb$\beta$3 from an inactive to an active conformation and do so in the absence of $\alpha$IIb$\beta$3 clustering. However, unlike Mn$^{2+}$-and DTT-activated $\alpha$4$\beta$1 that was found to have an affinity for VCAM-1 or a ligand peptide intermediate between its inactive and fully active state $^{41-43}$, we detected only two $\alpha$IIb$\beta$3 activation states. Thus, we found essentially no difference in the spectrum of rupture forces between fibrinogen and $\alpha$IIb$\beta$3 regardless whether we measured it in the presence of Ca$^{2+}$,
Mn$^{2+}$, or DTT or whether platelets were stimulated with ADP or TRAP. One might conclude erroneously that there are intermediate activation states when large ensembles of $\alpha$IIb$\beta$3 molecules are studied because time-averaged mixtures of low- and high-affinity $\alpha$IIb$\beta$3 molecules are being measured. Moreover, because the activation of integrin by Mn$^{2+}$ is presumably reversible$^{12}$, it is reasonable to assume that there is an equilibrium between the closed and open forms that is shifted toward the open form by Mn$^{2+}$. Thus, it is not necessary to hypothesize an intermediate conformer since such a conformer can be attributed to the mixture of two forms of the integrin.

The possibility that there are only two $\alpha$IIb$\beta$3 activation states is supported by the electron microscope images of single $\alpha$IIb$\beta$3 molecules. Although we detected three basic $\alpha$IIb$\beta$3 structures: open, closed, and globular, it is likely that the latter two are related because a comparable fraction of each was converted to the open form by Mn$^{2+}$ or DTT. Similar images were obtained in earlier electron microscope studies of $\alpha$IIb$\beta$3$^{23}$ and $\alpha$5$\beta$1$^{13,36}$, regardless of the molecular staining technique. These structures were present in the absence of Mn$^{2+}$ or DTT, but each agent converted most of the $\alpha$IIb$\beta$3 molecules to the open form. In electron microscope images of negatively-stained $\alpha$v$\beta$3, Takagi et al. observed inactive molecules that had a bent conformation$^{13}$, similar to the bent conformation of $\alpha$v$\beta$3 in crystals$^{38}$, and that $\alpha$v$\beta$3 was both extended and active in the presence of Mn$^{2+}$. We did not observe bent forms of $\alpha$IIb$\beta$3, even though our $\alpha$IIb$\beta$3 preparations clearly contained inactive and active molecules. However, Takagi et al. studied recombinant $\alpha$v$\beta$3 molecules containing a carboxyl-terminal clasp, whereas we studied $\alpha$IIb$\beta$3 molecules isolated from platelets. Accordingly, our studies and those of Takagi et al. may not be comparable.
Takagi et al. also observed that the Stokes (hydrodynamic) radius of Mn\(^{2+}\)-treated \(\alpha\nu\beta3\) in the absence of ligand was intermediate between that of \(\alpha\nu\beta3\) in presence of Ca\(^{2+}\) and that of \(\alpha\nu\beta3\) complexed with an RGD-containing ligand \(^{13}\). On the other hand, Mould et al. observed no gross differences in the conformation of the \(\alpha5\beta1\) headpiece in the presence of Ca\(^{2+}\) and Mn\(^{2+}\) by solution x-ray scattering, but their data were also consistent with an opening of the headpiece that involved an outward movement of the \(\beta1\) hybrid domain and downward swing of the \(\alpha7\) helix in the presence of Mn\(^{2+}\) \(^{44}\). We did not detect a difference in the dimensions of the closed and open conformations of \(\alpha\mathrm{IIb}\beta3\) or in the distance separating the \(\alpha\mathrm{IIb}\) and \(\beta3\) stalks of open and fibrinogen-bound \(\alpha\mathrm{IIb}\beta3\) in the presence of Ca\(^{2+}\). Thus, these dimensions are at least sufficient to identify an active conformation of \(\alpha\mathrm{IIb}\beta3\). Moreover, like Takagi et al. \(^{13}\), we found an increase in the size of the \(\alpha\mathrm{IIb}\beta3\) headpiece and a further increase in the distance separating the \(\alpha\mathrm{IIb}\) and \(\beta3\) stalks in the presence of Mn\(^{2+}\) and DTT. Nonetheless, X-ray crystallography revealed no changes in the structure of the extracellular portion of \(\alpha\nu\beta3\) when the crystals were soaked with buffer containing MnCl\(_2\) \(^{14}\). There are at least two possibilities to reconcile the difference between these results. First, contact forces in pre-existing crystals may prevent the structural change normally induced by Mn\(^{2+}\). Second, transmembrane and cytoplasmic segments absent in the crystal of the ectodomain may be critical to integrin activation since they were shown to have a major impact on the ligand-binding activity and the shape of the integrin \(^{45}\).

We found a clear correlation between the ability of \(\alpha\mathrm{IIb}\beta3\) to bind fibrinogen and the presence of open \(\alpha\mathrm{IIb}\beta3\) molecules. Thus, all \(\alpha\mathrm{IIb}\beta3\) molecules bound to fibrinogen, whether in the presence of Ca\(^{2+}\) or Mn\(^{2+}\) had separated stalks. Moreover, we found that fibrinogen binding to \(\alpha\mathrm{IIb}\beta3\) was enhanced by Mn\(^{2+}\) and DTT in parallel with the increased fraction of open \(\alpha\mathrm{IIb}\beta3\)
conformers. Because there is an equilibrium involving the inactive and active conformations of \( \alpha{IIb\beta3} \), these observations suggest that the differential effects of \( \text{Ca}^{2+} \) and \( \text{Mn}^{2+} \) on the \( \alpha{IIb\beta3} \) activation state are a function of the ability of each cation to stabilize one conformational state or the other. In the presence of \( \text{Ca}^{2+} \), the probability of encountering an active open \( \alpha{IIb\beta3} \) conformation was approximately 10%, but in the presence of \( \text{Mn}^{2+} \), the probability increased to \( \approx 60\% \). It is also noteworthy that as would be expected in a chemical equilibrium, active and inactive molecules coexisted in the presence of either cation. Thus, \( \text{Mn}^{2+} \) appears to induce \( \alpha{IIb\beta3} \) activity by stabilizing the active conformation of the \( \alpha{IIb\beta3} \) headpiece, thereby shifting the chemical equilibrium in the active direction and transmitting the conformational change to the stalks. The changes in the dimensions of the \( \alpha{IIb\beta3} \) headpiece are likely a consequence of the differences in size and electronegativity of \( \text{Ca}^{2+} \) and \( \text{Mn}^{2+} \) ions and of the additional \( \text{Mn}^{2+} \) bound to the headpiece in presence of ligand.

Although \( \alpha{IIb\beta3} \) activation by DTT likely involves overall disulfide bond reduction, as well disulfide bond rearrangement \(^{16}\), the identity of the cysteines involved is not clear. Because \( \alpha{IIb\beta3} \) activation appears to involve changes in the conformation of \( \beta{A} \) and hybrid domains \(^{44,46-47}\), it would be logical to assume that the relevant cysteines are located in these domains, an assumption consistent with the changes in the dimensions of the \( \alpha{IIb\beta3} \) headpiece that we detect in the presence of DTT. Nonetheless, the free cysteines identified when \( \alpha{IIb\beta3} \) is exposed to mild reducing conditions are located in the epidermal growth factor-like repeats that constitute the \( \beta{3} \) stalk \(^{16,38}\), as is an activating Cys583→Tyr mutation \(^{17}\). Thus, it is likely that the perturbed disulfide bonds that are responsible for \( \alpha{IIb\beta3} \) activation by DTT remain to be identified.
Clusters of αIIbβ3 molecules have been observed on the surface of thrombin-stimulated platelets and αIIbβ3 clustering has been induced in vitro as well \(^{49,50,51}\). Using Mn\(^{2+}\)-activated αIIbβ3 and electron microscopy, we observed that in the absence of membrane or cytoskeletal constraints, isolated αIIbβ3 formed dimers, trimers, and higher order oligomers that involved the distal ends of αIIb and β3 stalks. Thus, these images indicate that directly perturbing the activation state of the αIIbβ3 extracellular domain also results in the formation of αIIbβ3 oligomers in the absence of ligand, as would be predicted from the equilibrium model of integrin regulation. It is also noteworthy that the trimers and pentamers were always open, as would be predicted if the ends of the stalks only undergo homo-oligomerization, i.e., the formation of α-α or β-β subunit oligomers. Although it is possible that this observation could be the result of steric interference, it is consistent with the hypothesis that homomeric associations involving transmembrane domains are associated with integrin activation and clustering \(^{45}\). It also suggests that a component of Mn\(^{2+}\)-induced modulation of integrin function in cell membranes may result in increased integrin avidity arising from Mn\(^{2+}\)-induced aggregation or clustering of integrin molecules.

In conclusion, we used laser tweezers and electron microscopy to demonstrate a direct correlation between conformational changes in individual αIIbβ3 molecules and their ligand-binding activity. Using Mn\(^{2+}\) and DTT as activating tools, we found that separation of the αIIb and β3 stalks is an integral part of the mechanism leading to the exposure of the fibrinogen binding site in the αIIbβ3 ectodomain. Moreover, because the open conformation induced by Mn\(^{2+}\) and DTT occurred in the absence of ligand binding, it likely represents a primary activating
event, perhaps mimicking the consequences of agonist-induced stimulation of \(\alpha_{IIb}\beta3\) in platelet membranes.
References


42. Chigaev A, Blenc AM, Braaten JV, et al. Real time analysis of the affinity regulation of alpha 4-integrin. The physiologically activated receptor is intermediate in affinity between resting and Mn(2+) or antibody activation. J Biol Chem. 2001;276:48670-48678.


Table 1: Analysis of transmission electron microscope images of purified αIIbβ3 molecules in the absence and in the presence of Mn$^{2+}$.

<table>
<thead>
<tr>
<th></th>
<th>Group 1: Closed structures</th>
<th>Group 2: Open structures</th>
<th>Group 3: Globular structures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without Mn$^{2+}$</td>
<td>51±12%</td>
<td>11±4%</td>
<td>38±10%</td>
</tr>
<tr>
<td>With Mn$^{2+}$</td>
<td>24±3%</td>
<td>60±4%</td>
<td>17±3%</td>
</tr>
</tbody>
</table>

All the differences are statistically significant at p<0.001.
Table 2: Molecular dimensions of individual αIIbβ3 molecules in the presence of Ca\(^{2+}\) or Mn\(^{2+}\).

<table>
<thead>
<tr>
<th></th>
<th>Head-Transverse Length (nm)</th>
<th>Head-Vertical Length (nm)</th>
<th>Stalk Separation (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(^{2+})/Closed (123)(^a)</td>
<td>11.2±1.5(^b)</td>
<td>8.0±1.5</td>
<td>-</td>
</tr>
<tr>
<td>Ca(^{2+})/Open (97)</td>
<td>12.1±1.8</td>
<td>8.0±1.6</td>
<td>13.7±4.1</td>
</tr>
<tr>
<td>Ca(^{2+})/Fibrinogen (66)</td>
<td>-</td>
<td>-</td>
<td>12.6±2.7</td>
</tr>
<tr>
<td>Mn(^{2+})/Closed (95)</td>
<td>10.9±1.5</td>
<td>7.3±1.5</td>
<td>-</td>
</tr>
<tr>
<td>Mn(^{2+})/Open (95)</td>
<td>12.6±1.5(^c)</td>
<td>8.4±1.1(^d)</td>
<td>17.8±3.6</td>
</tr>
<tr>
<td>Mn(^{2+})/Fibrinogen (71)</td>
<td>-</td>
<td>-</td>
<td>19.9±4.7(^e)</td>
</tr>
</tbody>
</table>

\(a\) (), Number of images measured

\(b\) Mean ±1 S.D.

\(c\) Mn\(^{2+}\)/Open vs. Mn\(^{2+}\)/Closed, p < 10\(^{-8}\)

\(d\) Mn\(^{2+}\)/Open vs. Mn\(^{2+}\)/Closed, p<10\(^{-4}\)

\(e\) Mn\(^{2+}\)/Fibrinogen vs. Mn\(^{2+}\)/Open, p = 10\(^{-3}\)
Table 3: Comparison of Mn$^{2+}$- and DTT-induced fibrinogen binding to αIIbβ3 measured using laser tweezers.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Average Yield Strength, pN</th>
<th>Cumulative Probability, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca$^{2+}$</td>
<td>82±15</td>
<td>2.1±0.6</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>88±14</td>
<td>4.5±0.8</td>
</tr>
<tr>
<td>No DTT, Ca$^{2+}$</td>
<td>89±9</td>
<td>1.9±0.5</td>
</tr>
<tr>
<td>DTT, Ca$^{2+}$</td>
<td>88±9</td>
<td>3.2±0.6</td>
</tr>
</tbody>
</table>
Table 4: Molecular dimensions of individual αIIbβ3 molecules in the presence of Ca$^{2+}$ or DTT.

<table>
<thead>
<tr>
<th></th>
<th>Head-Transverse Length (nm)</th>
<th>Head-Vertical Length (nm)</th>
<th>Stalk Separation (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca$^{2+}$/Closed (96)</td>
<td>11.6±1.8$^a$</td>
<td>9.0±1.5$^b$</td>
<td>-</td>
</tr>
<tr>
<td>Ca$^{2+}$/Open (93)</td>
<td>11.9±1.9$^c$</td>
<td>8.7±1.4$^d$</td>
<td>13.1±2.8$^e$</td>
</tr>
<tr>
<td>DTT/Closed (110)</td>
<td>12.4±1.5$^a$</td>
<td>8.7±1.2$^b$</td>
<td>-</td>
</tr>
<tr>
<td>DTT/Open (123)</td>
<td>13.4±2.0$^c$</td>
<td>9.2±1.1$^d$</td>
<td>18.1±3.9$^e$</td>
</tr>
</tbody>
</table>

$^a$ Ca$^{2+}$/Closed vs. DTT/Closed, p = 0.027974
$^b$ Ca$^{2+}$/Closed vs. DTT/Closed, p = 0.344849
$^c$ Ca$^{2+}$/Open vs. DTT/Open, p = 2.98 x 10$^{-7}$
$^d$ Ca$^{2+}$/Open vs. DTT/Open, p = 0.033581
$^e$ Ca$^{2+}$/Open vs. DTT/Open, p = 4.85 x 10$^{-23}$
Figure Legends

**Fig. 1. Force distribution histograms of fibrinogen binding to Mn$^{2+}$-activated platelets as measured using laser tweezers.** The histograms represent rupture forces $>60$ pN, previously shown to be specific fibrinogen binding to $\alpha$IIb$\beta$3 $^{18}$. The average yield strength in pN and the cumulative probability of specific fibrinogen binding (%) for each histogram are also shown. **A.** Unstimulated platelets. **B.** Platelets incubated with 1 mM Mn$^{2+}$ for 5 min at 25$^\circ$C. **C.** Platelets incubated with 1 mM Mn$^{2+}$ in the presence of 20 $\mu$M tirofiban. **D.** Platelets incubated with 1 mM Mn$^{2+}$ in the presence of 100 $\mu$g/ml abciximab.

**Fig. 2. Force distribution histograms of fibrinogen binding to purified surface-bound $\alpha$IIb$\beta$3 pre-incubated with Ca$^{2+}$ or Mn$^{2+}$.** In the experiments shown, fibrinogen-coated beads were oscillated at 5 Hz touching the $\alpha$IIb$\beta$3-coated pedestals repeatedly. The data represent rupture forces $>60$ pN, indicative of specific fibrinogen binding to $\alpha$IIb$\beta$3 $^{18}$. The average yield strength in pN and the cumulative probability of specific fibrinogen binding (%) for each histogram are also shown. **A.** Fibrinogen binding to $\alpha$IIb$\beta$3 immobilized in the presence of 1 mM Ca$^{2+}$. **B.** Fibrinogen binding to $\alpha$IIb$\beta$3 immobilized in the presence of 1 mM Mn$^{2+}$. **C.** Fibrinogen binding to $\alpha$IIb$\beta$3 treated with 1 mM Mn$^{2+}$ measured in the presence of 50 $\mu$M tirofiban. **D.** Fibrinogen binding to $\alpha$IIb$\beta$3 treated with 1 mM Mn$^{2+}$ measured in the presence of 100 $\mu$g/ml abciximab.

**Fig. 3. Transmission electron microscopy of purified $\alpha$IIb$\beta$3 in the presence of 1 mM Ca$^{2+}$ (A) or 1 mM Mn$^{2+}$ (B).** Individual $\alpha$IIb$\beta$3 molecules were visualized using transmission electron microscopy after rotary shadowing with tungsten. The images could by classified into
three groups, of which two are shown. A. Closed structures with the tips of the αIIb and β3 stalks touching that were obtained in the presence of 1 mM Ca$^{2+}$. B. Open structures with the αIIb and β3 stalks separated that were obtained in the presence of 1 mM Mn$^{2+}$. A minority of structures consisting of globular headpieces with no visible stalks were also observed in the presences of both Ca$^{2+}$ and Mn$^{2+}$.

**Fig. 4. Transmission electron microscopy of αIIbβ3 oligomers observed in the presence of Mn$^{2+}$.** A. Most frequently, dimers were observed with one-tail or two-tails touching. B. There were fewer integrin trimers. C. Tetramers and larger oligomers were rare. D. Size distribution of αIIbβ3 oligomers. The data were fit to an exponential function using Microsoft Excel.

**Fig. 5. Transmission electron microscopy of αIIbβ3-fibrinogen complexes formed in the presence of Mn$^{2+}$.** A. Separate αIIbβ3 and fibrinogen molecules. B. and C. Bimolecular and trimolecular complexes of αIIbβ3 and fibrinogen. It is noteworthy that the αIIbβ3 molecules involved in these complexes all were in the open conformation. D. Fibrinogen bound to an αIIbβ3 oligomer.
Figure 2

- **A**: Sample distribution with a peak at 82±15 pN, 2.1% probability.
- **B**: Highest peak at 88±14 pN, 4.5% probability.
- **C**: Sample distribution with a peak at 85±12 pN, 0.4% probability.
- **D**: Sample distribution with a peak at 83±14 pN, 0.4% probability.

Probability, %
Figure 4

A

B

C

D

\[ y = 3997.2e^{-1.1787x} \]
Figure 5
Functional and structural correlations of individual αIIbβ3 molecules

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