

# $\alpha$ I**IIb** $\beta$ 3 binding to a fibrinogen fragment lacking the $\gamma$ -chain dodecapeptide is activation dependent and EDTA inducible

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## Key Points

- Activation of  $\alpha$ I**IIb** $\beta$ 3 is required for its ancillary site interactions with fibrinogen fragment D lacking the  $\gamma$ -chain dodecapeptide ('D98').
- EDTA can paradoxically induce normal  $\alpha$ I**IIb** $\beta$ 3 to interact with fibrinogen fragment 'D98.'

Platelet integrin receptor  $\alpha$ I**IIb** $\beta$ 3 supports platelet aggregation by binding fibrinogen. The interaction between the fibrinogen C-terminal  $\gamma$ -chain peptide composed of residues  $\gamma$ -404-411 (GAKQAGDV) and the Arg-Gly-Asp (RGD) binding pocket on  $\alpha$ I**IIb** $\beta$ 3 is required for fibrinogen-mediated platelet aggregation, but data suggest that other ancillary binding sites on both fibrinogen and  $\alpha$ I**IIb** $\beta$ 3 may lead to higher-affinity fibrinogen binding and clot retraction. To identify additional sites, we analyzed the ability of platelets and cells expressing normal and mutant  $\alpha$ I**IIb** $\beta$ 3 to adhere to an immobilized fibrinogen plasmin fragment that lacks intact  $\gamma$ -404-411 ('D98'). We found the following: (1) Activated, but not unactivated, platelets adhere well to immobilized 'D98.' (2) Cells expressing constitutively active  $\alpha$ I**IIb** $\beta$ 3 mutants, but not cells expressing normal  $\alpha$ I**IIb** $\beta$ 3 or  $\alpha$ V $\beta$ 3, adhere well to 'D98.' (3) Monoclonal antibodies 10E5 and 7E3 inhibit the adhesion to 'D98' of activated platelets and cells expressing constitutively active  $\alpha$ I**IIb** $\beta$ 3, as do small-molecule inhibitors that bind to the RGD pocket. (4) EDTA paradoxically induces normal  $\alpha$ I**IIb** $\beta$ 3 to interact with 'D98.' Because molecular modeling and molecular dynamics simulations suggested that the  $\alpha$ I**IIb** L151-D159 helix may contribute to the interaction with 'D98,' we studied an  $\alpha$ I**IIb** $\beta$ 3 mutant in which the  $\alpha$ I**IIb** 148-166 loop was swapped with the corresponding  $\alpha$ V loop; it failed to bind to fibrinogen or 'D98.' Our data support a model in which conformational changes in  $\alpha$ I**IIb** $\beta$ 3 and/or fibrinogen after platelet activation and the interaction between  $\gamma$ -404-411 and the RGD binding pocket make new ancillary sites available that support higher-affinity fibrinogen binding and clot retraction.

## Introduction

Platelets play a major role in both thrombosis and hemostasis.  $\alpha$ I**IIb** $\beta$ 3 is a platelet- and megakaryocyte-specific integrin that mediates adhesion of platelets to ligands and is required for platelet aggregation and clot retraction.<sup>1,2</sup> Several ligands for  $\alpha$ I**IIb** $\beta$ 3, including von Willebrand factor (VWF), vitronectin, and fibronectin, contain an Arg-Gly-Asp (RGD) motif that interacts with a pocket on the receptor headpiece composed of contributions by both  $\alpha$ I**IIb** and  $\beta$ 3.<sup>2,3</sup> Fibrinogen contributes to platelet aggregation in vitro and thrombus formation in vivo.<sup>4,5</sup> It interacts with the RGD pocket on  $\alpha$ I**IIb** $\beta$ 3 through the last 8 residues ( $\gamma$ -404-411) in its unstructured C-terminal  $\gamma$ -chain dodecapeptide (HHLGGAKQAGDV;  $\gamma$ -12) rather than either of its 2 RGD motifs.<sup>6-10</sup> Ligand binding to  $\alpha$ I**IIb** $\beta$ 3 initiates a major conformational change in the receptor resulting in the receptor adopting a high-affinity conformation.<sup>11</sup>

Although the interaction between the fibrinogen  $\gamma$ -chain and the RGD pocket is necessary for fibrinogen binding to  $\alpha$ I**IIb** $\beta$ 3, it may not be sufficient because of the following: (1) Biochemical and biophysical

studies show fibrinogen binding is a time-dependent multistep process leading to higher-affinity and lack of reversibility.<sup>9,10,12-22</sup> (2) When reversibly dissociated, both  $\alpha$ IIb and  $\beta$ 3 can bind to immobilized fibrinogen.<sup>16</sup> (3) Platelets can adhere to fibrinogen fragments lacking  $\gamma$ -404-411,<sup>23,24</sup> but it is unclear whether the platelets need to be activated in order to bind. (4) Mutations of  $\alpha$ IIb at a distance from the RGD pocket, in particular at the  $\alpha$ IIb cap domain,<sup>25,26</sup> impair fibrinogen binding, as do monoclonal antibodies (mAb's) that bind in that region. For example, mAb 10E5, which binds to the  $\alpha$ IIb cap domain,<sup>11</sup> is a potent inhibitor of fibrinogen binding<sup>27</sup> even though it does not alter the RGD pocket. Similarly, mutations in the  $\beta$ 3 specificity determining loop<sup>28</sup> can interfere with fibrinogen binding. (5) Binding of fibrinogen to  $\alpha$ IIb $\beta$ 3 results in changes in the conformation of both  $\alpha$ IIb and  $\beta$ 3 as determined by the binding of mAb's specific for ligand-induced binding sites (LIBS),<sup>29-31</sup> potentially exposing additional sites. (6) Binding of fibrinogen to  $\alpha$ IIb $\beta$ 3 induces changes in the conformation of fibrinogen, thus also potentially exposing new sites.<sup>32-34</sup>

There may also be ancillary binding sites for the interaction of fibrin with  $\alpha$ IIb $\beta$ 3 because of the following: (1)  $\alpha$ IIb $\beta$ 3 is required for clot retraction, but clot retraction is essentially normal with fibrinogen lacking the  $\gamma$ -408-411 sequence.<sup>35,36</sup> (2) EDTA eliminates fibrinogen binding to the RGD pocket in  $\alpha$ IIb $\beta$ 3 but does not impair clot retraction.<sup>37</sup> (3) The conversion of fibrinogen to fibrin exposes new epitopes for mAb's and thus potentially new interaction sites.<sup>38</sup> (4) Binding of fibrin to  $\alpha$ IIb $\beta$ 3 has different physicochemical properties than binding to fibrinogen.<sup>39</sup>

Identifying ancillary binding sites for fibrinogen and/or fibrin on  $\alpha$ IIb $\beta$ 3 would provide a more comprehensive understanding of fibrinogen binding to platelets. Such sites may furnish novel therapeutic targets to prevent platelet thrombus formation. This is important because current small-molecule  $\alpha$ IIb $\beta$ 3 antagonists act as partial agonists and, under certain experimental conditions, can prime the receptor to bind fibrinogen by inducing the  $\beta$ 3 subunit to adopt high-affinity ligand-binding conformations.<sup>22,40-42</sup> These conformational changes have been hypothesized to contribute to the development of thrombocytopenia in ~0.5% to 1% of patients as a result of exposing epitopes for which some people have preformed antibodies,<sup>43</sup> and they may limit the efficacy of the current agents. Because the ancillary sites on  $\alpha$ IIb $\beta$ 3 may be different for fibrinogen and VWF, it may be possible to develop ligand-specific antagonists with potential therapeutic advantages, if, for example, selectively blocking fibrinogen binding prevents thrombus formation while preserving hemostasis mediated by VWF binding to  $\alpha$ IIb $\beta$ 3.

Regions of fibrinogen in addition to  $\gamma$ -404-411 and regions of  $\alpha$ IIb $\beta$ 3 in addition to the RGD binding pocket have been reported to affect ligand binding,<sup>25,38,44,45</sup> but there has been no detailed description of how any ancillary site on fibrinogen interacts with any ancillary region on  $\alpha$ IIb $\beta$ 3. Specifically, potential ancillary sites have been identified in the fibrinogen  $\gamma$ -module ( $\gamma$ -148-411), including  $\gamma$ -316-322 and  $\gamma$ -370-381.<sup>44-47</sup> Such sites are difficult to study using intact fibrinogen because of the confounding effect of the interaction of  $\alpha$ IIb $\beta$ 3 with  $\gamma$ -404-411. As a result, to identify additional sites of interaction between fibrinogen and  $\alpha$ IIb $\beta$ 3, we studied the adhesion of platelets and HEK293 cells expressing either normal  $\alpha$ IIb $\beta$ 3 or mutant  $\alpha$ IIb $\beta$ 3 to fibrinogen and plasmin fragments of fibrinogen that either contain or lack intact  $\gamma$ -404-411 (D100 and 'D98,' respectively), in the presence or absence of EDTA. The mutations studied were designed to (1) activate the receptor, (2) disrupt the metal ion dependent adhesion site (MIDAS) ( $\beta$ 3 D119A), and (3) alter the interaction of 'D98' with  $\alpha$ IIb

based on inferences from a molecular model of the  $\alpha$ IIb $\beta$ 3 headpiece-fibrinogen  $\gamma$ -module complex.

## Methods

### Fibrinogen, fibrinogen fragments, and mAb's

Human fibrinogen was obtained from Enzyme Research Labs, and fibrinogen fragments D100 and 'D98' were obtained from Haematologic Technologies. The latter were prepared as described in the supplemental Materials and Methods. Because our fragment resembles to a great extent the D98 fragment described by Lishko et al<sup>48</sup> (which was the fragment used in the study by Podolnikova et al<sup>24</sup>), most importantly for the current study in not having an intact  $\gamma$ -404-411 peptide, but may differ in minor aspects, we have chosen to name our fragment 'D98.' The mAb's 10E5 (anti- $\alpha$ IIb $\beta$ 3),<sup>11,27</sup> 7E3 (anti- $\alpha$ IIb $\beta$ 3 +  $\alpha$ V $\beta$ 3, which binds in the region between the specificity loop and  $\alpha$ 1 helix of  $\beta$ 3),<sup>49,50</sup> and 7E9 (anti-C-terminal  $\gamma$ -12 peptide)<sup>12</sup> were described previously. The anti-LIBS mAb AP5<sup>51</sup> and the anti- $\alpha$ V $\beta$ 3 mAb LM609<sup>52,53</sup> were generously provided by Peter Newman and David Cheresh, respectively.

### Molecular modeling of the $\alpha$ IIb $\beta$ 3 headpiece-fibrinogen $\gamma$ -module complex

The crystal structures of the  $\alpha$ IIb $\beta$ 3 headpiece- $\gamma$ -12 peptide complex (PDB ID: 2VDO), containing the last 7 residues of the bound dodecapeptide ( $\gamma$ -405-411), and the fibrinogen C-terminal  $\gamma$ -module (PDB ID: 1FIC) ( $\gamma$ -144-392) were used to create a model of the complex formed by the C-terminal  $\gamma$ -module of fibrinogen and  $\alpha$ IIb $\beta$ 3 head domain. Molecular docking was performed using ClusPro,<sup>54</sup> Haddock,<sup>55</sup> and SwarmDock<sup>56</sup> using 3 separate sets of restraints as described in supplemental Materials and Methods and supplemental Figure 1. The 55 structures exhibiting at least 6 ligand-receptor contacts between fibrinogen residues  $\gamma$ -316-322 or  $\gamma$ -370-381 and mAb 10E5 binding site residues on  $\alpha$ IIb, as well as suitable conformations of the loop linking residues  $\gamma$ -144-392 of the  $\gamma$ -module and  $\gamma$ -405-411 in the  $\alpha$ IIb $\beta$ 3 RGD binding pocket were clustered in 13 groups. Representative structures of these clusters were subjected to molecular dynamics (MD) simulations as described in the supplemental Materials and Methods.

### Generation of mutants and stable cell lines

HEK293 cells expressing normal human  $\alpha$ IIb $\beta$ 3 were previously described,<sup>57,58</sup> and the  $\alpha$ IIbF992A/F993A (FF),  $\beta$ 3N339S,  $\beta$ 3 $\Delta$ 717,  $\beta$ 3D119A, and  $\alpha$ IIb(FF) $\beta$ 3D119A mutants, as well as the  $\alpha$ V 144-154/ $\alpha$ IIb 148-166 loop swap mutant [ $\alpha$ IIb( $\alpha$ V)], were generated using the QuikChange XL Site-directed Mutagenesis kit as described in supplemental Materials and Methods.

### Platelet and cell adhesion assay

The platelet and HEK293 cell adhesion assays to immobilized fibrinogen and fibrinogen fragments D100 and 'D98' were performed as previously described<sup>42</sup> with minor modifications described in the supplemental Materials and Methods.

### Mass spectrometry (MS) analysis of fibrinogen fragments D100 and 'D98'

MS of trypsin-treated fibrinogen fragments D100 and 'D98' was performed by liquid chromatography (LC)-MS/MS (Ultimate 3000 nano-HPLC system coupled to a Q-Exactive Plus mass spectrometer, Thermo Scientific) with details described in the supplemental Materials and Methods.

### Statistics

Repeated measures analysis of variance and paired Student *t* test were used to calculate the statistical significance and the *P* values for adhesion.

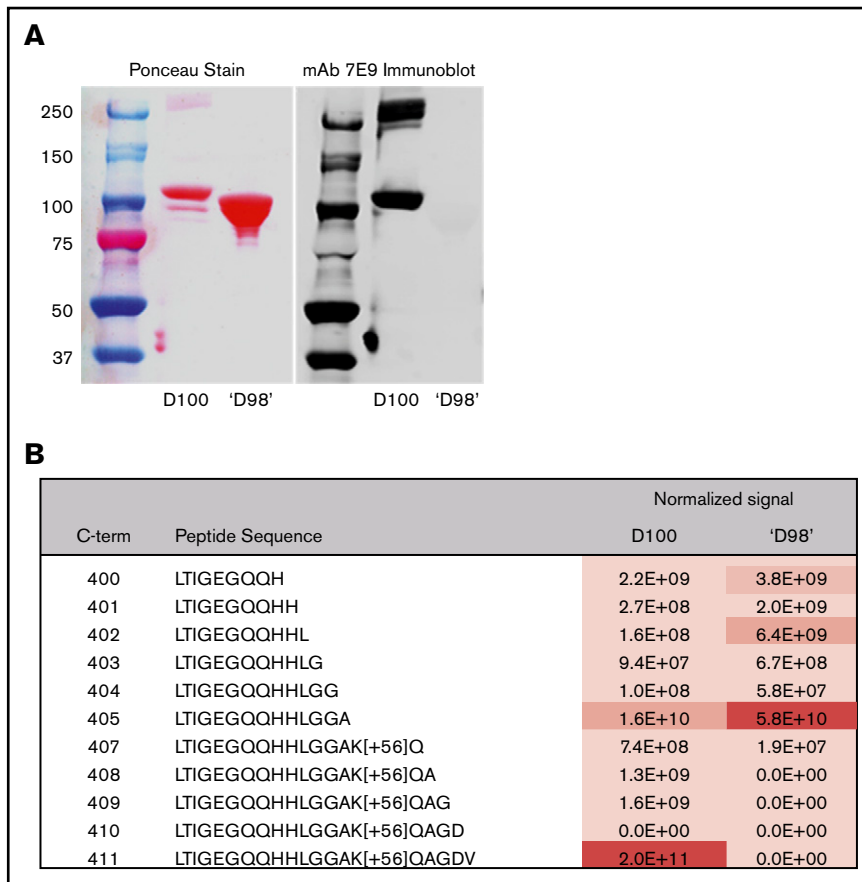
## Results

### Fibrinogen fragment 'D98,' unlike D100, lacks the C-terminal $\gamma$ -chain dodecapeptide

The 'D98' fragment contains the majority of the D domain, but unlike fragment D100, does not contain an intact C-terminal  $\gamma$ -12

**Figure 1. Fibrinogen fragment D100, but not fragment 'D98,' is recognized by an antibody to the fibrinogen C-terminal  $\gamma$ -chain dodecapeptide ( $\gamma$ -12); the C-termini of D100 and 'D98' are primarily  $\gamma$ -411 and  $\gamma$ -405, respectively.**

(A) Fragment D100 and fragment 'D98' were electrophoresed in a 10% acrylamide gel. Separated proteins were then transferred to a nitrocellulose membrane and stained with Ponceau (left). After washing away the stain, the membrane was immunoblotted with mAb 7E9, which reacts with the fibrinogen  $\gamma$ -12 peptide. (B) MS-based quantitation of C-terminal peptides. D100 and 'D98' gel bands were treated with propionic anhydride, which modifies primary amines, and then digested with trypsin. Propionic anhydride-modified residues are marked with [+56]. C-terminal peptides of D100 and 'D98' were measured by parallel reaction monitoring<sup>71</sup> and signals extracted and analyzed by Skyline.<sup>72</sup> Signals normalized to the amount of fibrinogen C-terminal  $\gamma$ -chain in the 2 samples are shown for each peptide. For the D100 sample, the most abundant peptide measured was the C-terminal  $\gamma$ -chain peptide  $\gamma$ -392-411 (LTIGEGQQHHLGGAK[+56]QAGDV) containing the intact  $\gamma$ -12 peptide. No signal for this peptide was identified in the 'D98' sample; rather, the most abundant signal was assigned to LTIGEGQQHHLGGGA, which ends at  $\gamma$ -405. See supplemental Table 2 for additional validation of C termini, assignment of N termini, and peptides used for normalization.



dodecapeptide. Immunoblot analysis using mAb 7E9 indicated the absence of the intact  $\gamma$ -12 peptide in 'D98' (Figure 1A). LC-MS/MS analysis of D100 confirmed a possible N terminus of A63 (supplemental Table 2) and the dominance of the C-terminal peptide containing intact  $\gamma$ -12 (Figure 1B), consistent with the report by Lishko et al.<sup>48</sup> In sharp contrast, LC-MS/MS analysis of 2 closely separated 'D98' bands showed that the most abundant C-terminal peptide detected ended at A405, with the C-terminal peptide containing intact  $\gamma$ -12 being virtually undetectable (Figure 1B). Lishko et al reported that the N terminus of their D98 fragment was S86, whereas we have identified 1 peptide with an N terminus at M89 in our 'D98,' and we infer the presence of at least 1 additional peptide with an N terminus somewhere between P76 and K88. (supplemental Table 2).

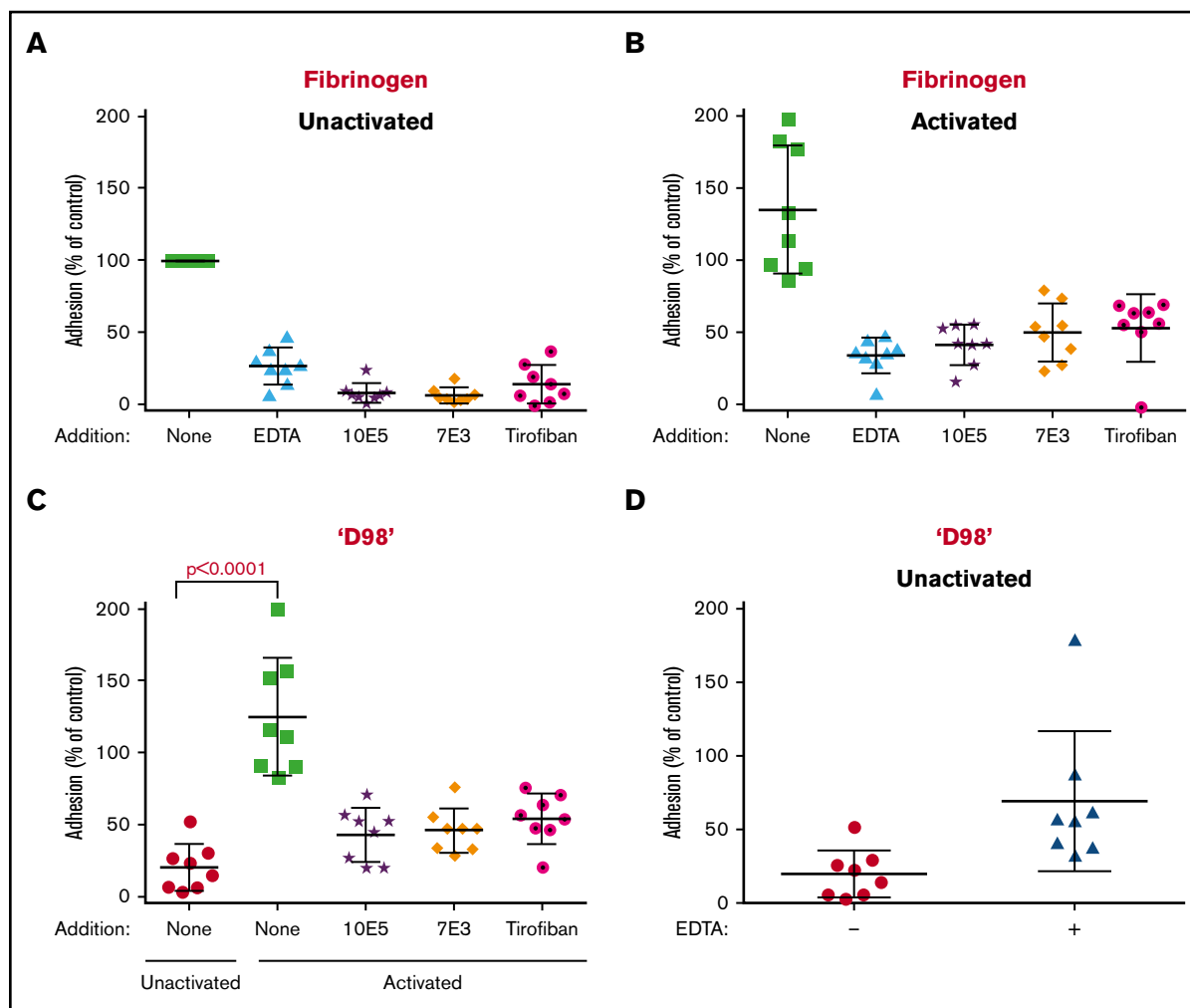
**Unactivated and activated platelets adhere to microtiter wells coated with fibrinogen, whereas only activated platelets adhere well to wells coated with 'D98'**

Platelets from healthy donors adhered to microtiter plates coated with 10  $\mu$ g/mL fibrinogen, and the adhesion was inhibited by EDTA (10 mM), mAb's 10E5 and 7E3 (both 20  $\mu$ g/mL), and tirofiban (1  $\mu$ M) (Figure 2A). Activating the platelets by adding a thrombin receptor activating peptide (TRAP; 10  $\mu$ M) resulted in ~35% overall increase in adhesion (Figure 2B). EDTA, the mAb's 10E5 and 7E3, and tirofiban all inhibited the adhesion, but not to the same extent as with unactivated platelets.

In sharp contrast, unactivated platelets bound much less well to immobilized 'D98' than to fibrinogen, whereas TRAP-activated platelets adhered to 'D98' better than unactivated platelets adhered to fibrinogen, and nearly as well as activated platelets bound to fibrinogen (Figure 2C). The mAb's 10E5 and 7E3 decreased adhesion of activated platelets to 'D98' by 66% and 63%, respectively, and tirofiban decreased adhesion by 57% (all  $P \leq .001$ ). The combination of adenosine 5'-diphosphate (ADP) + epinephrine as a platelet activator increased platelet adhesion to 'D98' (from  $944 \pm 363$  to  $3952 \pm 1760$  arbitrary fluorescence intensity units (AFU); supplemental Figure 2), but the increase was at the borderline of statistical significance ( $P = .06$ ). Paradoxically, EDTA increased the adhesion of unactivated platelets to immobilized 'D98' in all donors, with the variable increases averaging 3.5-fold ( $P = .02$ ) (Figure 2D).

**Cells expressing normal  $\alpha$ IIb $\beta$ 3 adhere to fibrinogen and D100 but do not adhere to immobilized 'D98'; cells expressing constitutively active  $\alpha$ IIb $\beta$ 3 adhere to 'D98'**

HEK293 cells expressing normal  $\alpha$ IIb $\beta$ 3 ( $\alpha$ IIb $\beta$ 3-HEK) adhered to fibrinogen and D100 but did not adhere to immobilized 'D98' (Figure 3A, left panel). In contrast, HEK293 cells expressing  $\alpha$ IIb $\beta$ 3 containing activating mutations in the cytoplasmic domain of  $\alpha$ IIb [ $\alpha$ IIb(FF) $\beta$ 3-HEK] bound to 'D98,' and HEK293 cells expressing  $\alpha$ IIb $\beta$ 3 with other activating mutations ( $\beta$ 3N339S and  $\beta$ 3 $\Delta$ 717) also supported adhesion to 'D98' (Figure 3A, right



**Figure 2. Both unactivated and activated platelets adhere to immobilized fibrinogen, whereas only activated platelets adhere to fibrinogen fragment ‘D98.’**

Calcein-labeled washed platelets ( $2 \times 10^9/\mu\text{L}$ ;  $50 \mu\text{L}$ ) were tested either before (unactivated) or after activation with  $10 \mu\text{M}$  TRAP for adhesion to fibrinogen or ‘D98’ immobilized in microtiter wells by coating at  $10 \mu\text{g}/\text{mL}$ . Platelets were incubated for 1 hour at  $22^\circ\text{C}$  in the presence or absence of EDTA ( $10 \text{ mM}$ ), 10E5 ( $20 \mu\text{g}/\text{mL}$ ), 7E3 ( $20 \mu\text{g}/\text{mL}$ ), or tirofiban ( $1 \mu\text{M}$ ), followed by washing of unbound platelets and analysis of calcein fluorescence. The adhesion of unactivated platelets to fibrinogen was defined as 100% adhesion, and all values were normalized to this value in each of 8 separate experiments. (A) EDTA, 10E5, 7E3, and tirofiban inhibited adhesion of unactivated platelets to fibrinogen by 73%, 92%, 94%, and 86%, respectively ( $P < .0001$  for all). (B) Activated platelets adhered to immobilized fibrinogen as well or better than unactivated platelets; EDTA, 10E5, 7E3, and tirofiban inhibited adhesion by 75%, 70%, 63%, and 63%, respectively ( $P < .001$  for all). (C) Unactivated platelets adhered poorly to ‘D98’ (80% less adhesion compared with unactivated platelet adhesion to fibrinogen), whereas activated platelets adhered much better (25% more adhesion compared with unactivated platelet adhesion to fibrinogen). Adhesion was inhibited by 10E5, 7E3, and tirofiban by 66%, 63%, and 57%, respectively ( $P < .01$  for all). (D) Adhesion of unactivated platelets to ‘D98’ was increased by treating the platelets with  $10 \text{ mM}$  EDTA ( $P = .02$ ). Data reported as mean  $\pm$  standard deviation (SD).

panel). In contrast, HEK293 cells expressing  $\alpha\text{V}\beta 3$  showed no adhesion to ‘D98.’

### Effect of mAb’s, $\alpha\text{IIb}\beta 3$ antagonists, and soluble fibrinogen and ‘D98’ on adhesion of $\alpha\text{IIb}(\text{FF})\beta 3$ -HEK to ‘D98’

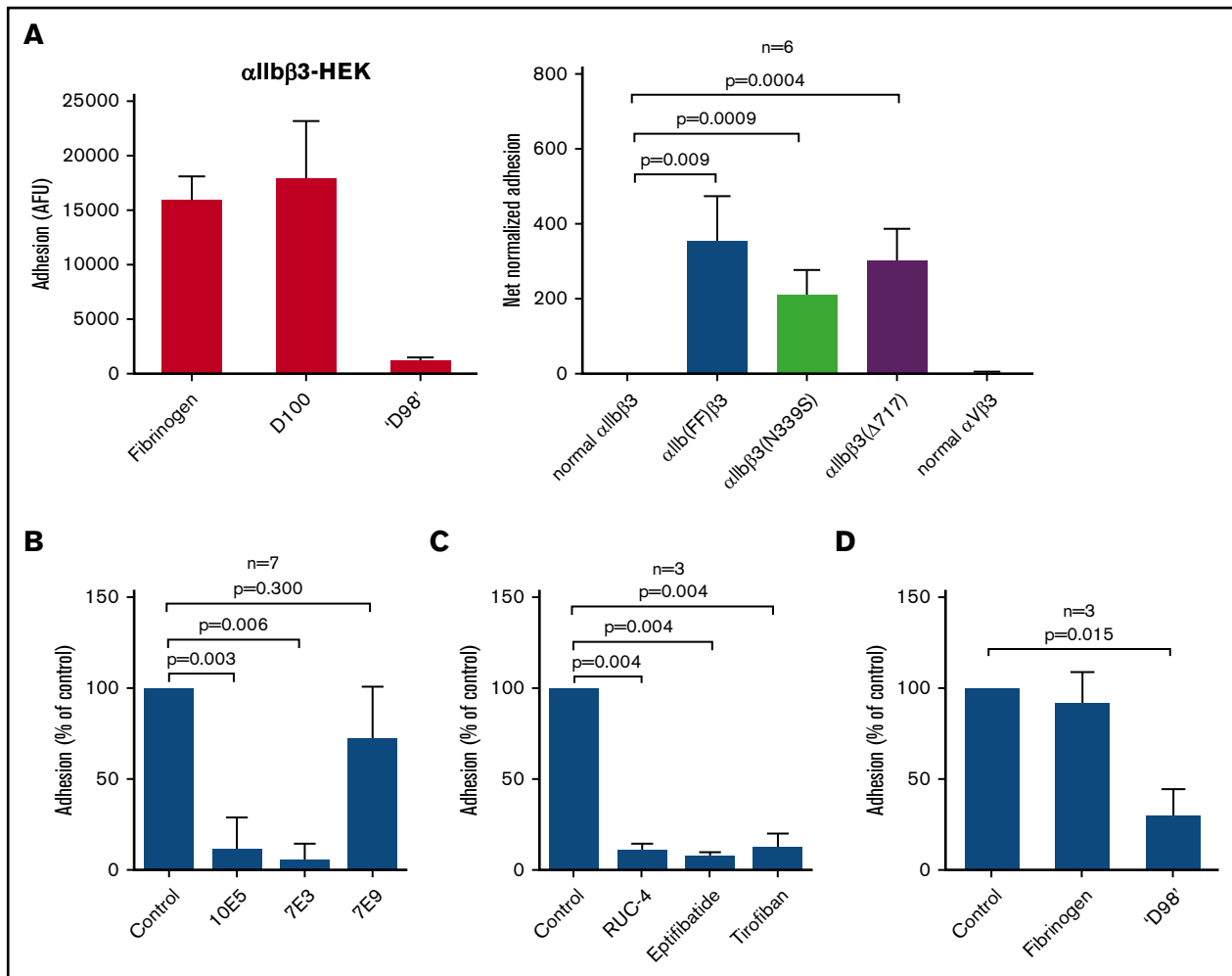
The adhesion of  $\alpha\text{IIb}(\text{FF})\beta 3$ -HEK to immobilized ‘D98’ was inhibited by mAb’s 10E5 and 7E3 (Figure 3B). The adhesion was not, however, significantly inhibited by mAb 7E9. In addition, cells treated with either of the RGD-based small-molecule inhibitors of  $\alpha\text{IIb}\beta 3$ , tirofiban ( $10 \mu\text{M}$ ) or eptifibatid ( $100 \mu\text{M}$ ), or the novel inhibitor RUC-4 ( $5 \mu\text{M}$ ), which does not bind to the MIDAS  $\text{Mg}^{2+}$ ,<sup>59</sup> adhered less well compared with untreated cells (Figure 3C). The adhesion of

$\alpha\text{IIb}(\text{FF})\beta 3$ -HEK to ‘D98’ was inhibited by soluble ‘D98’ ( $1.0 \text{ mg}/\text{mL}$ ), but not by soluble fibrinogen ( $1.5 \text{ mg}/\text{mL}$ ) (Figure 3D).

### Paradoxical effect of EDTA in enhancing adhesion of $\alpha\text{IIb}\beta 3$ -HEK293 to ‘D98’

EDTA ( $10 \text{ mM}$ ) inhibited the adhesion of  $\alpha\text{IIb}\beta 3$ -HEK cells to immobilized fibrinogen in the presence of  $2 \text{ mM Ca}^{2+}/1 \text{ mM Mg}^{2+}$  (Figure 4A, left panel). Surprisingly,  $10 \text{ mM}$  EDTA enhanced the adhesion of  $\alpha\text{IIb}\beta 3$ -HEK to immobilized ‘D98’ nearly 25-fold (Figure 4A, right panel). EDTA did not inhibit the adhesion of  $\alpha\text{IIb}\beta 3$ -HEK cells to D100 (Figure 4B).

Preincubating fibrinogen or ‘D98’ with EDTA did not inhibit adhesion of  $\alpha\text{IIb}\beta 3$ -HEK cells to fibrinogen or enhance adhesion



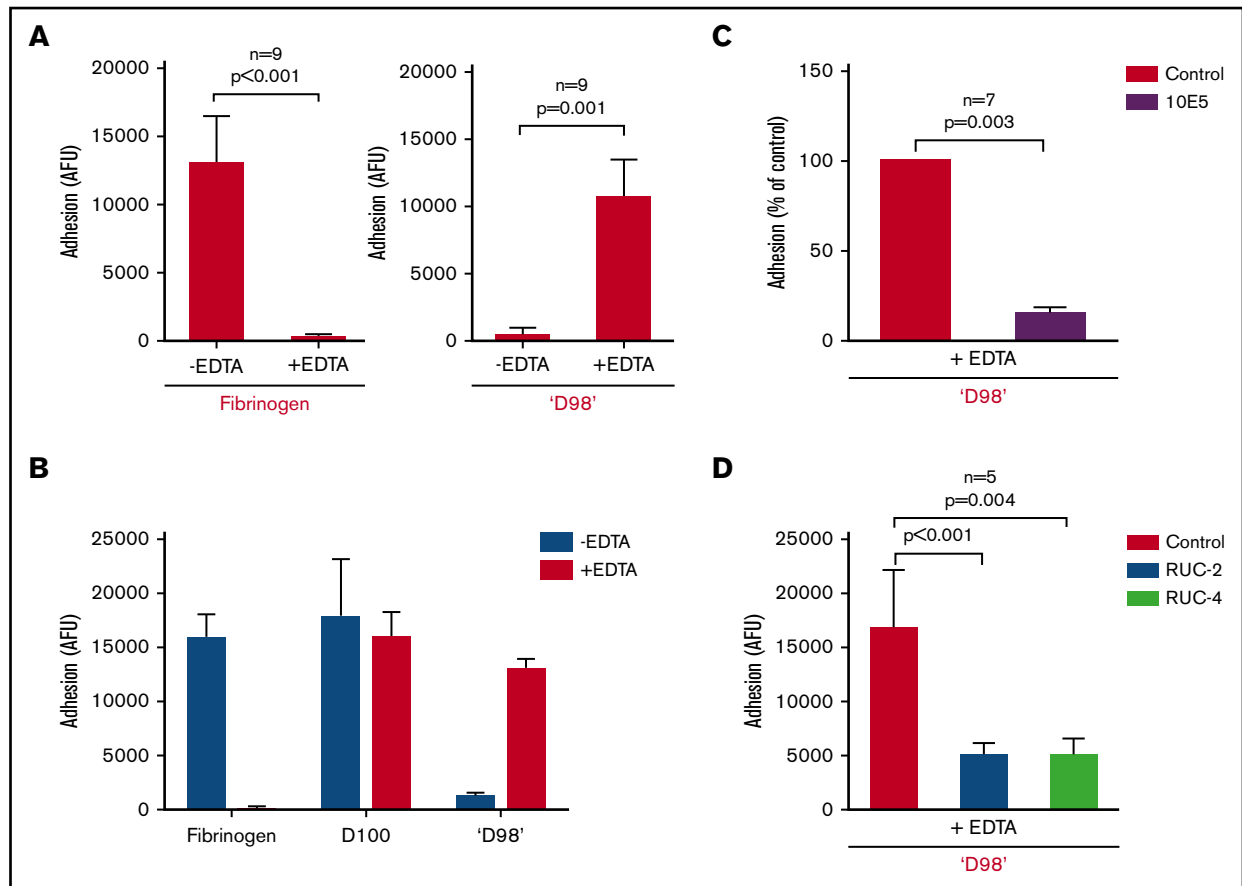
**Figure 3. HEK293 cells expressing normal  $\alpha$ IIb $\beta$ 3 adhere to fibrinogen and D100 but do not adhere to immobilized fibrinogen fragment 'D98,' whereas adhesion does occur with HEK293 cells expressing constitutively active  $\alpha$ IIb $\beta$ 3 mutants; adhesion of  $\alpha$ IIb(FF) $\beta$ 3-HEK cells to immobilized 'D98' is inhibited by function blocking antibodies to  $\alpha$ IIb $\beta$ 3, small-molecules inhibitors of  $\alpha$ IIb $\beta$ 3, and 'D98,' but not by an antibody to the fibrinogen  $\gamma$ -12 peptide or soluble fibrinogen.**

(A) HEK293 cells ( $2 \times 10^3$  cells/ $\mu$ L; 50  $\mu$ L) expressing normal  $\alpha$ IIb $\beta$ 3 were labeled with calcein and added to microtiter wells precoated with fibrinogen, D100, or 'D98' (10  $\mu$ g/mL coating concentration) for 1 hour at 22°C (left). After washing, the fluorescence of adherent cells was measured.  $\alpha$ IIb $\beta$ 3-HEK cells adhered to immobilized fibrinogen and D100 but did not adhere to 'D98.' Data reported as mean  $\pm$  SD. HEK293 cells expressing normal  $\alpha$ IIb $\beta$ 3, normal  $\alpha$ V $\beta$ 3, or constitutively active  $\alpha$ IIb $\beta$ 3 mutants were allowed to adhere to 'D98' (right). Whereas the cells expressing normal  $\alpha$ IIb $\beta$ 3 or  $\alpha$ V $\beta$ 3 did not adhere, HEK293 cells expressing any 1 of the 3 activating mutations [ $\alpha$ IIb(FF),  $\beta$ 3N339S, or  $\beta$ 3 $\Delta$ 717] demonstrated substantial adhesion ( $n = 6$ ;  $P < .01$  compared with normal  $\alpha$ IIb $\beta$ 3). Data reported as mean  $\pm$  SD. Net normalized adhesion is adhesion value (AFU) divided by the expression level (minus background) of each cell line. Expression levels were measured by mAb 10E5 binding and expressed as geometric mean fluorescence intensity (GMFI): normal  $\alpha$ IIb $\beta$ 3  $69 \pm 40$  AFU;  $\alpha$ IIb(FF) $\beta$ 3  $31 \pm 16$  AFU;  $\alpha$ IIb $\beta$ 3(N339S)  $58 \pm 17$  AFU;  $\alpha$ IIb $\beta$ 3( $\Delta$ 717)  $86 \pm 40$  AFU; normal  $\alpha$ V $\beta$ 3 (using mAb LM609)  $187 \pm 24$  AFU. (B) Adhesion of HEK293 cells expressing the  $\alpha$ IIb(FF) $\beta$ 3 mutant to 'D98' was tested in the presence of mAb's 10E5 (anti- $\alpha$ IIb; 20  $\mu$ g/mL), 7E3 (anti- $\beta$ 3; 20  $\mu$ g/mL), and mAb 7E9 (anti-fibrinogen  $\gamma$ -12; 20  $\mu$ g/mL). 10E5 and 7E3 both inhibited adhesion ( $n = 7$ ;  $P < .01$  for each), whereas 7E9 did not ( $P = .30$ ). (C) Adhesion of  $\alpha$ IIb(FF) $\beta$ 3-HEK cells to immobilized 'D98' was also inhibited by the small-molecule inhibitors of  $\alpha$ IIb $\beta$ 3 RUC-4 (5  $\mu$ M), eptifibatid (100  $\mu$ M), and tirofiban (10  $\mu$ M) ( $n = 3$ ;  $P < .005$  for each). (D) Soluble fibrinogen (1.5 mg/mL) did not inhibit the adhesion of  $\alpha$ IIb(FF) $\beta$ 3-HEK cells to 'D98' ( $n = 3$ ;  $P = .50$ ), but soluble 'D98' (1.0 mg/mL) did inhibit the adhesion ( $n = 3$ ;  $P = .02$ ). Data reported as mean  $\pm$  SD.

of  $\alpha$ IIb $\beta$ 3-HEK cells to 'D98,' whereas pretreating the  $\alpha$ IIb $\beta$ 3-HEK cells with EDTA inhibited adhesion to fibrinogen and enhanced adhesion to 'D98' (supplemental Figure 3).

EDTA (3 mM) was sufficient to block adhesion to fibrinogen, and 3 to 4 mM EDTA was required to enhance adhesion to 'D98.' The effect of EDTA was rapid (control = 403 AFU; 0 minutes = 9667 AFU; 5 minutes = 13 112 AFU; 60 minutes = 11 981 AFU; averages of 2 experiments).

EDTA-induced adhesion of  $\alpha$ IIb $\beta$ 3-HEK to 'D98' was inhibited by mAb 10E5 (Figure 4C). The effect of mAb 7E3 could not be tested because EDTA dramatically reduces the binding of mAb 7E3. LM609 (anti- $\alpha$ V $\beta$ 3) did not inhibit EDTA-induced adhesion to 'D98' (data not shown). The effects of eptifibatid and tirofiban could not be tested because both antagonists bind to the MIDAS Mg<sup>2+</sup> ion. Both RUC-2 and RUC-4 inhibited EDTA-induced adhesion of  $\alpha$ IIb $\beta$ 3-HEK to 'D98' (Figure 4D).



**Figure 4. Paradoxical effect of EDTA in enhancing adhesion of  $\alpha\text{IIb}\beta\text{3}$ -HEK cells to fibrinogen fragment 'D98'.** (A) HEK293 cells expressing normal  $\alpha\text{IIb}\beta\text{3}$  ( $2 \times 10^3/\mu\text{L}$ ;  $50 \mu\text{L}$ ) were added to microtiter wells precoated with fibrinogen ( $10 \mu\text{g}/\text{mL}$  coating concentration) for 1 hour at  $22^\circ\text{C}$  in the absence and presence of EDTA ( $10 \text{ mM}$ ) (left). EDTA dramatically inhibited adhesion ( $n = 9$ ;  $P < .001$ ). Normal  $\alpha\text{IIb}\beta\text{3}$ -HEK cells ( $2 \times 10^3/\mu\text{L}$ ;  $50 \mu\text{L}$ ) were added to microtiter wells precoated with 'D98' ( $10 \mu\text{g}/\text{mL}$  coating concentration) for 1 hour at  $22^\circ\text{C}$  in the absence and presence of EDTA ( $10 \text{ mM}$ ) (right). The EDTA dramatically increased adhesion ( $n = 9$ ;  $P = .001$ ). (B) Normal  $\alpha\text{IIb}\beta\text{3}$ -HEK cells bound to D100 in the absence and presence of EDTA. Conditions as per panel A with D100 coated at  $10 \mu\text{g}/\text{mL}$  ( $n = 3$ ). (C) The mAb 10E5 ( $20 \mu\text{g}/\text{mL}$ ) inhibited EDTA-induced adhesion of  $\alpha\text{IIb}\beta\text{3}$ -HEK cells to 'D98' ( $n = 7$ ;  $P = .003$ ). (D) Small-molecule inhibitors of  $\alpha\text{IIb}\beta\text{3}$  RUC-2 ( $10 \mu\text{M}$ ) and RUC-4 ( $5 \mu\text{M}$ ) inhibited the EDTA-induced adhesion of  $\alpha\text{IIb}\beta\text{3}$ -HEK to 'D98' when added after the EDTA ( $n = 5$ ;  $P < .005$ ). Similar results, not shown, were obtained when RUC-2 or RUC-4 were added before EDTA ( $n = 5$ ;  $P < .005$ ). Data reported as mean  $\pm$  SD.

### Role of MIDAS residue D119A in EDTA-induced adhesion vs FF-activated adhesion to 'D98'

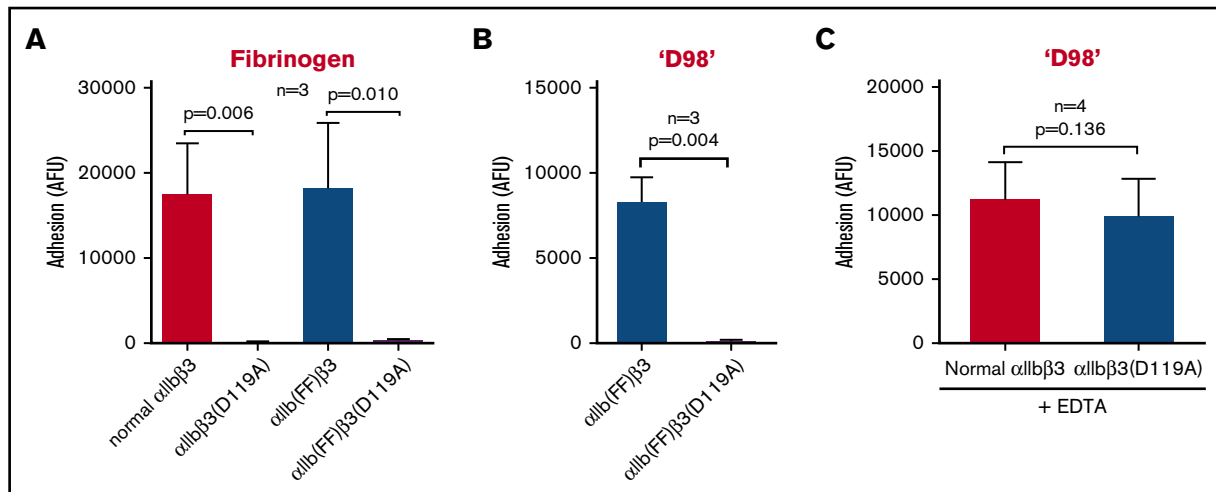
$\alpha\text{IIb}\beta\text{3}$ -HEK cells containing the  $\beta\text{3}$  D119A mutation did not adhere to fibrinogen, and adding the  $\beta\text{3}$  D119A mutation to  $\alpha\text{IIb}(\text{FF})\beta\text{3}$ -HEK led to loss of adhesion to both fibrinogen and 'D98' (Figure 5A-B). Of note, the D119A mutation did not affect EDTA-induced adhesion to 'D98' (Figure 5C).

### Cells expressing $\alpha\text{V}\beta\text{3}$ bind to immobilized fibrinogen, but not immobilized 'D98,' even when treated with EDTA

HEK293 cells expressing normal  $\alpha\text{V}\beta\text{3}$  adhered to immobilized fibrinogen at levels similar to those of HEK293 cells expressing normal  $\alpha\text{IIb}\beta\text{3}$ , and EDTA nearly eliminated the adhesion (supplemental Figure 4A).  $\alpha\text{V}\beta\text{3}$ -HEK cells did not adhere to 'D98,' and adding EDTA did not increase their adhesion (supplemental Figure 4B).

### Molecular modeling of the $\alpha\text{IIb}\beta\text{3}$ headpiece-fibrinogen $\gamma$ -module complex

The representative structure of the most populated cluster (cluster 1) of the  $\alpha\text{IIb}\beta\text{3}$  headpiece-fibrinogen  $\gamma$ -module complexes deviated substantially from the initial conformation during MD simulations, whereas the representative structure of the second largest cluster (cluster 2) remained reasonably stable (supplemental Figure 5). Figure 6 shows the relaxed, representative structure of this cluster at the end of a 230 ns MD simulation. Direct polar interactions that involve 2 residues (E157 and N158) of the  $\alpha\text{IIb}$  helix segment L151-D159 are seen in this structure. Although only residue E157 was included in the modeling restraints used in series 1, both E157 and N158 were included in the restraints used in series 2 and 3 (see supplemental Materials and Methods). Specifically, these direct polar interactions are between  $\alpha\text{IIb}$  E157 and fibrinogen K356 side chains, between  $\alpha\text{IIb}$  E157 backbone and fibrinogen R375 side chain, and between  $\alpha\text{IIb}$  N158 side chain and fibrinogen



**Figure 5. Mutating MIDAS residue D119A eliminates binding of normal  $\alpha$ IIb $\beta$ 3-HEK and  $\alpha$ IIb(FF) $\beta$ 3-HEK cells to immobilized fibrinogen and binding of  $\alpha$ IIb(FF) $\beta$ 3-HEK cells to 'D98,' but does affect adhesion of normal  $\alpha$ IIb $\beta$ 3-HEK cells to 'D98' in the presence of EDTA.** HEK293 cells ( $2 \times 10^3/\mu\text{L}$ ;  $50 \mu\text{L}$ ) expressing normal  $\alpha$ IIb $\beta$ 3, the  $\alpha$ IIb $\beta$ 3 constitutively active mutant  $\alpha$ IIb(FF) $\beta$ 3, the  $\alpha$ IIb $\beta$ 3(D119A) MIDAS-disrupting mutant, or the combined  $\alpha$ IIb(FF) $\beta$ 3(D119A) mutant were labeled with calcein ( $10 \mu\text{M}$ ) and then added to microtiter wells precoated with fibrinogen or 'D98' (each at  $10 \mu\text{g}/\text{mL}$  coating concentration) for 1 hour at  $22^\circ\text{C}$  in the absence and presence of EDTA ( $10 \text{ mM}$ ). The fluorescent signal of adherent cells was measured after washing away the nonadherent cells. (A) The normal  $\alpha$ IIb $\beta$ 3-HEK and  $\alpha$ IIb(FF) $\beta$ 3-HEK cells adhered to fibrinogen, whereas the  $\alpha$ IIb $\beta$ 3(D119A)-HEK MIDAS mutant and the combined  $\alpha$ IIb(FF) $\beta$ 3(D119A)-HEK mutant did not adhere to fibrinogen ( $n = 3$ ;  $P \leq .01$ ). (B)  $\alpha$ IIb(FF) $\beta$ 3-HEK cells adhered to 'D98,' whereas the same cells with the MIDAS-disrupting mutation  $\beta$ 3(D119A), did not adhere ( $n = 3$ ;  $P = .004$ ). (C) EDTA treatment ( $10 \text{ mM}$ ) of  $\alpha$ IIb $\beta$ 3(D119A)-HEK cells increased their adhesion to 'D98' to the level of normal  $\alpha$ IIb $\beta$ 3-HEK cells ( $n = 4$ ;  $P > .05$ ). Data reported as mean  $\pm$  SD. Expression levels for each cell line were measured based on mAb 7E3 binding and are expressed as GMFI: normal  $\alpha$ IIb $\beta$ 3  $53 \pm 26$  AFU;  $\alpha$ IIb $\beta$ 3(D119A)  $36 \pm 11$  AFU;  $\alpha$ IIb(FF) $\beta$ 3  $25 \pm 5$  AFU;  $\alpha$ IIb(FF) $\beta$ 3(D119A)  $27 \pm 2$  AFU.

R375 backbone. Water-mediated interactions are also seen between  $\alpha$ IIb D159 and fibrinogen Q407 side chains, and between  $\alpha$ IIb E157 and fibrinogen W376 side chains. Heavy atoms of 4 residues on the  $\alpha$ IIb helix, Leu151, Arg153, Ile154, and Asn158, are within  $5 \text{ \AA}$  from 10E5 in the 10E5- $\alpha$ IIb $\beta$ 3 headpiece crystal structure 2VDO (supplemental Figure 6), suggesting that our model is consistent with competitive binding between fibrinogen/'D98' and 10E5. Of note, both R375 and W376 are contained in the  $\gamma$ -370-381 peptide identified by Podolnikova et al,<sup>45</sup> and the  $\alpha$ IIb helix segment L151-D159 was contained in 1 of the 3 peptides that reacted most strongly with radiolabeled D-dimer reported by Podolnikova et al.<sup>38</sup> Three out of the 13 clusters included structures in which residues contained in the  $\gamma$ -316-322 region interact with  $\alpha$ IIb, but because there were only 2 structures in each of these clusters, they were not pursued further.

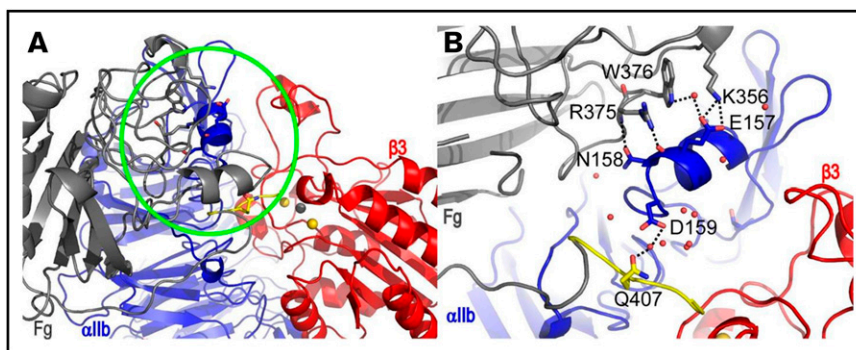
### Swapping the $\alpha$ IIb 148-166 loop for the comparable $\alpha$ V $\beta$ 3 loop leads to loss of adhesion to both fibrinogen and 'D98,' and EDTA does not enhance adhesion to 'D98'

To assess the role of the  $\alpha$ IIb helix segment L151-D159 in binding to 'D98,' we prepared HEK293 cells expressing  $\alpha$ IIb $\beta$ 3 in which the  $\alpha$ V 144-154 loop was swapped for the  $\alpha$ IIb 148-166 loop [ $\alpha$ IIb( $\alpha$ V) $\beta$ 3-HEK] (supplemental Figure 4C). These cells did not adhere to immobilized fibrinogen, but pretreatment with  $5 \text{ mM}$  dithiothreitol, a reducing agent that activates platelet  $\alpha$ IIb $\beta$ 3,<sup>60</sup> dramatically increased their adhesion (supplemental Figure 4A). They also did not adhere to 'D98' even in the presence of EDTA, and adding dithiothreitol did not affect the adhesion (supplemental Figure 4B).

## Discussion

Our studies indicate the following: (1) Activated, but not unactivated, platelets adhere well to immobilized 'D98.' (2) Cells expressing constitutively active  $\alpha$ IIb $\beta$ 3 mutants, but not cells expressing normal  $\alpha$ IIb $\beta$ 3, adhere well to immobilized 'D98.' (3) EDTA treatment increases the adhesion of platelets and cells expressing normal  $\alpha$ IIb $\beta$ 3 to 'D98.' (4) The mAb's 10E5 and 7E3 inhibit the adhesion to 'D98' of activated platelets and cells expressing a constitutively active  $\alpha$ IIb $\beta$ 3, as do the small-molecule inhibitors that bind to the RGD pocket. (5) The mAb 10E5 and the small-molecule inhibitors RUC-2 and RUC-4, which do not require the presence of a MIDAS metal ion for binding (as do the other small-molecule  $\alpha$ IIb $\beta$ 3 antagonists based on the RGD sequence), inhibited the adhesion of cells expressing normal  $\alpha$ IIb $\beta$ 3 to 'D98' in the presence of EDTA. (6) Cells expressing  $\alpha$ V $\beta$ 3 adhere to fibrinogen, but not 'D98,' even in the presence of EDTA. (7) Cells expressing an  $\alpha$ IIb $\beta$ 3 mutant in which the  $\alpha$ IIb 148-166 loop was swapped with the corresponding  $\alpha$ V loop failed to bind to fibrinogen or 'D98' in the absence or presence of EDTA. Our finding that cells expressing normal  $\alpha$ IIb $\beta$ 3 could bind to D100 in the absence or presence of EDTA, whereas they only adhered to fibrinogen in the absence of EDTA, and only bound to 'D98' in the presence of EDTA, supports there being 2 separate mechanisms of binding, one involving the interaction of  $\gamma$ -404-411 (in fibrinogen and D100) with the  $\alpha$ IIb $\beta$ 3 RGD binding pocket, and another involving a site on D100 and 'D98' (but cryptic in fibrinogen) that interacts with a site on  $\alpha$ IIb $\beta$ 3 exposed by receptor activation or induced by EDTA.

These data provide new insights into the mechanism of adhesion to 'D98,' with potential implications for localizing ancillary binding sites on  $\alpha$ IIb $\beta$ 3 and fibrinogen. In an elegant series of studies, Zamarron et al and Podolnikova et al identified the role of the  $\gamma$ -365-383 region of fibrinogen through studies with mAb 2G5, raised against the fibrinogen



**Figure 6.  $\alpha$ IIb $\beta$ 3 headpiece-fibrinogen  $\gamma$ -module complex showing interactions between residues on the  $\alpha$ IIb helix segment L151-D159 and fibrinogen  $\gamma$ -module.**

(A) Snapshot at 230 ns from MD simulations of a representative structure of cluster 2 showing a relaxed model of fibrinogen binding to  $\alpha$ IIb $\beta$ 3. Fibrinogen  $\gamma$ -module,  $\alpha$ IIb, and  $\beta$ 3 are colored in gray, blue, and red, respectively. The last 7 amino acids of fibrinogen are colored in yellow. The important contact region identified is highlighted with a green circle. (B) A zoom-in view of the contacts in the 230 ns snapshot is shown from a slightly different perspective and with fibrinogen residue 291-306 removed for clarity. Direct and water-mediated hydrogen bonds are indicated with dotted lines.

D-D fragment, and peptides from this region.<sup>24,32</sup> They showed that mAb 2G5 (1) binds to  $\gamma$ -373-385, (2) inhibits platelet aggregation and clot retraction, (3) reacts with fibrinogen bound to  $\alpha$ IIb $\beta$ 3 or immobilized on a microtiter well, (4) does not bind soluble fibrinogen, and (5) does not inhibit fibrinogen binding to activated platelets.<sup>24,32,45</sup> Moreover, a peptide containing the sequence  $\gamma$ -365-383 (P3) supported  $\alpha$ IIb $\beta$ 3-mediated cell adhesion but did not inhibit the binding of soluble fibrinogen to activated platelets. The adhesion of platelets to P3 was inhibited by mAb 7E3 (anti- $\alpha$ IIb $\beta$ 3 + anti- $\alpha$ V $\beta$ 3) and a mAb to  $\alpha$ 5 $\beta$ 1 but not a mAb to  $\alpha$ V $\beta$ 3. They also identified 8 separate negatively charged sites on the  $\alpha$ IIb  $\beta$ -propeller as potential binding sites for the positively charged  $\gamma$ -370-381 peptide.<sup>38</sup>

Podolnikova et al also studied the impact of P3 on platelet adhesion to D98.<sup>24</sup> Using gel-filtered, calcein-labeled platelets in the presence of 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, they found that adhesion of unactivated platelets to D98 was ~45% of the adhesion to fibrinogen fragment D100, which contains an intact  $\gamma$ -12 peptide. Activating platelets with a combination of 10  $\mu$ M ADP and epinephrine increased adhesion to D100 by 1.7-fold but had no impact on adhesion to D98. They concluded that adhesion to D98 was not activation dependent. They went on to show that the P3 peptide inhibited adhesion of unactivated platelets to D98. They concluded that the P3 peptide region of fibrinogen is cryptic on soluble fibrinogen and becomes exposed upon fibrinogen binding to platelets or fibrin formation, where it can mediate additional interactions with unactivated  $\alpha$ IIb $\beta$ 3 that are important in platelet aggregation.

In contrast to their results, we found that unactivated platelet adhesion to 'D98' was only ~20% of the adhesion of unactivated platelets to fibrinogen and that activating platelets with TRAP dramatically increased adhesion to 'D98,' reaching just modestly less than the adhesion of activated platelets to fibrinogen. To try to reconcile our findings with those of Podolnikova et al, we also tested the combination of ADP + epinephrine (which they used) in our assay and found that although it increased adhesion to 'D98,' the effect was not statistically significant ( $P = .06$ ). Thus, we conclude that adhesion to 'D98' requires potent activation of  $\alpha$ IIb $\beta$ 3.

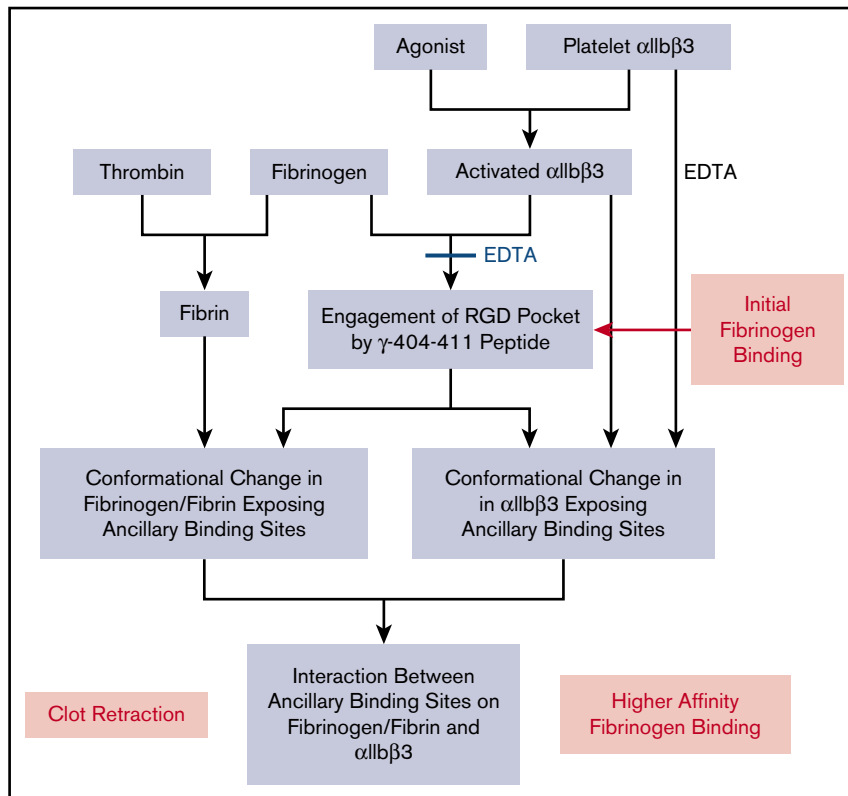
We confirmed the difference between unactivated and activated  $\alpha$ IIb $\beta$ 3 in mediating adhesion to 'D98' using HEK293 cells expressing

mutations in  $\alpha$ IIb or  $\beta$ 3 that induce constitutive fibrinogen binding. These data suggest that the ancillary binding site(s) on  $\alpha$ IIb $\beta$ 3 for 'D98' is cryptic on unactivated  $\alpha$ IIb $\beta$ 3 and is exposed with activation by strong agonists or activating mutations. As with platelets, this behavior contrasts sharply with adhesion to intact immobilized fibrinogen, which does not require platelet activation.<sup>1,27</sup>

Despite the absence of intact  $\gamma$ -12 peptide on 'D98,' the small-molecule  $\alpha$ IIb $\beta$ 3 antagonists that bind to the RGD pocket inhibited the adhesion of both platelets and HEK293 cells expressing activated  $\alpha$ IIb $\beta$ 3 to 'D98.' Similarly, adding the  $\beta$ 3 D119A mutation, which is known to alter the MIDAS and eliminate ligand binding to the constitutively active  $\alpha$ IIb(FF) $\beta$ 3 receptor, led to loss of binding to 'D98.' Collectively, these data suggest either that the RGD pocket can bind both the  $\gamma$ -404-411 peptide and the ancillary fibrinogen site(s) or that altering the RGD pocket allosterically affects the ancillary site(s). Of note, the mAb 10E5, which binds to the  $\alpha$ IIb cap domain and inhibits fibrinogen binding and platelet aggregation,<sup>11</sup> also inhibited the adhesion to 'D98,' raising the possibility that the ancillary site(s) is in the cap domain, which is unique to  $\alpha$ IIb.<sup>61</sup> Collectively, our data are similar to those of Peerschke and Galanakis who studied the adhesion of platelets to the D60 plasmin degradation product of fibrinogen, which they confirmed lacked the RGD and intact  $\gamma$ -12 sequences.<sup>23</sup> They found that adhesion to D60 required platelet activation with either ADP or thrombin and that the adhesion could be inhibited by RGD peptides and mAb 10E5.

Our docking and molecular dynamics studies suggested that the  $\alpha$ IIb cap 3 insert, which includes the  $\alpha$ -helical residues E157, N158, and D159, participates in interacting with fibrinogen residues K356, R375, W376, and Q407. Of note, R375 and W376 are contained in the P3 peptide, and the  $\alpha$ IIb residues E157-D159 are contained in 1 of the 8 regions Podolnikova et al identified as potential binding sites for P3.<sup>38</sup> Moreover, 4 residues on the  $\alpha$ IIb L151-D159 helix (Leu151, Arg153, Ile154, and Asn158) are within 5 Å heavy atom distance to 10E5, suggesting that this helix is involved in 10E5 binding. In addition, Podolnikova et al reported that a peptide composed of  $\alpha$ IIb residues 153-162 inhibited platelet adhesion to D98 by 33% and inhibited clot retraction with a 50% inhibitory concentration (IC<sub>50</sub>) of 200  $\mu$ M, but only inhibited platelet adhesion to fibrinogen by 10%.<sup>38</sup> They also showed an ~65% reduction in the adhesion to D98 of HEK293 cells





**Figure 7. Working model of the multistep process of fibrinogen binding and the platelet-fibrin interaction contributing to clot retraction.** Activation of platelet  $\alpha\text{IIb}\beta\text{3}$  with an agonist results in  $\alpha\text{IIb}\beta\text{3}$  being able to bind fibrinogen by the engagement of the  $\gamma\text{-404-411}$  peptide in the  $\alpha\text{IIb}\beta\text{3}$  binding pocket. EDTA can prevent the binding of fibrinogen and reverse the binding if added rapidly after the addition of fibrinogen. The binding of fibrinogen leads to conformational changes in fibrinogen that expose 1 or more ancillary binding sites. Platelet  $\alpha\text{IIb}\beta\text{3}$  activation alone is sufficient to expose ancillary binding site(s), but there may be an additional contribution from fibrinogen engaging the  $\alpha\text{IIb}\beta\text{3}$  binding pocket. The ancillary binding site(s) on fibrinogen and  $\alpha\text{IIb}\beta\text{3}$  then interact, resulting in higher-affinity fibrinogen binding, which under certain experimental conditions may appear irreversible over the time course of the experiments. A similar mechanism may contribute to clot retraction, with fibrin formation initiating conformational changes in fibrinogen that mimic, at least in part, those induced by fibrinogen binding to  $\alpha\text{IIb}\beta\text{3}$ . Although data vary, the weight of evidence supports the need for activated platelets to participate in clot retraction. Paradoxically, EDTA treatment appears to initiate conformational changes in  $\alpha\text{IIb}\beta\text{3}$  akin to those produced by activation and ligand binding as judged by the ability of cells expressing normal  $\alpha\text{IIb}\beta\text{3}$  to bind to ‘D98’ in the presence of EDTA.

expressing a mutant  $\alpha\text{IIb}\beta\text{3}$  containing  $\alpha\text{IIb E157A/D159S/W162F}$ .<sup>38</sup> The  $\alpha\text{IIb}$  helix segment L151-D159 is absent in  $\alpha\text{V}$ , and so we prepared a hybrid receptor in which the  $\alpha\text{V 144-154}$  loop was swapped for the  $\alpha\text{IIb 148-166}$  loop. This led to the loss of cell adhesion to both ‘D98’ and fibrinogen. Although this is consistent with a role for this region in forming an ancillary binding site, this experiment cannot differentiate between loss of the residues implicated in binding fibrinogen by the molecular docking and MD simulation studies and other changes produced by the swap (eg, shortening of the loop). Of note, Kamata et al did not find a reduction in fibrinogen binding to  $\alpha\text{IIb}\beta\text{3}$  expressed on CHO cells with single alanine substitutions of E157, N158, or D159 when activated by mAb PT25-2.<sup>25</sup>

The paradoxical effect of EDTA in enhancing the adhesion of unactivated platelets and cells expressing normal  $\alpha\text{IIb}\beta\text{3}$  to ‘D98’ was unexpected because EDTA is commonly used to inhibit integrin-mediated ligand binding, presumably by chelating the MIDAS divalent cation. In fact, one operational definition of “irreversibly” bound fibrinogen is the inability of EDTA to elute fibrinogen from  $\alpha\text{IIb}\beta\text{3}$ .<sup>62</sup> The EDTA-induced adhesion of cells expressing normal  $\alpha\text{IIb}\beta\text{3}$  to ‘D98’ was also partially inhibited by mAb 10E5 and the small-molecule antagonist RUC-4, both of which

inhibit the adhesion of  $\alpha\text{IIb(FF)}\beta\text{3-HEK}$  cells to ‘D98.’ The MIDAS-disrupting D119A mutation, however, had no effect on EDTA-induced adhesion of cells expressing normal  $\alpha\text{IIb}\beta\text{3}$  to ‘D98,’ unlike its effect on adhesion of  $\alpha\text{IIb(FF)}\beta\text{3-HEK}$  cells. Thus, it is possible that EDTA treatment exposes a different site(s) for ‘D98’ than the one exposed by receptor activation, or that EDTA treatment exposes the same site and stabilizes it from allosteric alteration by the D119A mutation. Peerschke and Galanakis did not study the effect of EDTA alone on adhesion of platelets to D60, but they reported that EDTA was less effective in inhibiting platelet adhesion to D60 than to fibrinogen.<sup>23</sup> Thus, our findings have implications for using EDTA to probe the reversibility of fibrinogen binding.

EDTA has a number of effects on  $\alpha\text{IIb}\beta\text{3}$ . At 22°C and neutral pH, it inhibits ligand binding to  $\alpha\text{IIb}\beta\text{3}$  without dissociating the complex, whereas at higher temperature and pH it irreversibly dissociates the complex.<sup>37,63-66</sup> It also exposes the epitopes for several LIBS antibodies, including the AP5 epitope on  $\beta\text{3}$ <sup>51</sup> and the PMI-1 epitope on  $\alpha\text{IIb}$ ,<sup>67</sup> indicating that it induces conformational changes akin to those produced by ligand binding. Moreover, the platelets of patients with Glanzmann thrombasthenia because of a D119Y mutation bound PMI-1 in the absence of EDTA, linking disruption of the MIDAS with allosteric

modifications in  $\alpha$ IIb.<sup>67,68</sup> Although EDTA prevents fibrinogen binding to  $\alpha$ IIb $\beta$ 3, it does not inhibit  $\alpha$ IIb $\beta$ 3-mediated clot retraction,<sup>37</sup> which is consistent with the finding that clot retraction can be supported by fibrinogen molecules lacking  $\gamma$ -408-411 and both RGD sequences.<sup>35,36</sup> Other data that potentially link our results to the mechanism of clot retraction include the observations that both the P3 peptide and mAb 10E5 inhibit clot retraction.<sup>24,27</sup> Nonetheless, studies with the D-dimer fibrin fragment showed interactions with 16 separate  $\alpha$ IIb peptide regions, 10 of which correspond to regions implicated in fibrinogen binding, but only 3 of which correspond to regions implicated in P3 binding.<sup>38</sup>

In conclusion, our data are consistent with a model in which activation of platelet  $\alpha$ IIb $\beta$ 3 initially results in fibrinogen binding by  $\gamma$ -404-411 engaging the RGD binding pocket (Figure 7). This binding can be reversed by EDTA or excess fibrinogen.<sup>62</sup> Bound fibrinogen undergoes conformational changes as reported by a number of different mAb's (mAb 2G5 [anti-FG-RIBS1; anti- $\gamma$ -373-385],<sup>32</sup> mAb 9F9<sup>69</sup> [anti- $\gamma$ -112-119],<sup>33</sup> and mAb 155B16 [anti-A $\alpha$ -95-98]),<sup>33</sup> and 1 or more of these conformational changes could expose ancillary binding sites. The ancillary binding site(s) on fibrinogen and activated  $\alpha$ IIb $\beta$ 3 then interact, resulting in strengthening of the interaction. A similar process may also contribute to platelet-fibrin interactions that support clot retraction, with fibrin formation initiating conformational changes in fibrinogen that can expose new sites that interact with activated  $\alpha$ IIb $\beta$ 3.<sup>70</sup> Finally, we found a paradoxical effect of EDTA in inducing a conformational change in  $\alpha$ IIb $\beta$ 3 that supports adhesion to 'D98,' providing a tool to better understand the conformational changes underlying the exposure of ancillary binding sites for fibrinogen on  $\alpha$ IIb $\beta$ 3 and raising cautions about the use of this reagent to define the reversibility of fibrinogen binding.

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## Authorship

Contribution: H.Z. designed and performed research, analyzed data, and wrote the manuscript; Y.S. designed and performed the molecular docking and molecular dynamics studies and wrote the molecular modeling sections; J.L. designed and performed experiments and helped analyze the data; G.A.D. performed the 'D98' characterization experiments and analyzed data; J.P.F. and H.M. performed the MS experiment and analyzed the MS data; M.F. designed and supervised the molecular docking and molecular dynamics studies and wrote the molecular modeling sections; and B.S.C. designed research, analyzed data, and wrote the manuscript.

Conflict-of-interest disclosure: B.S.C. has royalty interests in abciximab (Centocor) through the Research Foundation of the State University of New York and the VerifyNow Assays (Accumetrics) through the Icahn School of Medicine at Mount Sinai; he is also a consultant to Scholar Rock. The remaining authors declare no competing financial interests.

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