

The Platelet Integrin α IIb β 3 Differentially Interacts with Fibrin Versus Fibrinogen*

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Fibrinogen binding to the integrin α IIb β 3 mediates platelet aggregation and spreading on fibrinogen-coated surfaces. However, *in vivo* α IIb β 3 activation and fibrinogen conversion to fibrin occur simultaneously, although the relative contributions of fibrinogen versus fibrin to α IIb β 3-mediated platelet functions are unknown. Here, we compared the interaction of α IIb β 3 with fibrin and fibrinogen to explore their differential effects. A microscopic bead coated with fibrinogen or monomeric fibrin produced by treating the immobilized fibrinogen with thrombin was captured by a laser beam and repeatedly brought into contact with surface-attached purified α IIb β 3. When α IIb β 3-ligand complexes were detected, the rupture forces were measured and displayed as force histograms. Monomeric fibrin displayed a higher probability of interacting with α IIb β 3 and a greater binding strength. α IIb β 3-fibrin interactions were also less sensitive to inhibition by abciximab and eptifibatid. Both fibrinogen- and fibrin- α IIb β 3 interactions were partially inhibited by RGD peptides, suggesting the existence of common RGD-containing binding motifs. This assumption was supported using the fibrin variants α D97E or α D574E with mutated RGD motifs. Fibrin made from a fibrinogen γ' / γ' variant lacking the γ C α IIb β 3-binding motif was more reactive with α IIb β 3 than the parent fibrinogen. These results demonstrate that fibrin is more reactive with α IIb β 3 than fibrinogen. Fibrin is also less sensitive to α IIb β 3 inhibitors, suggesting that fibrin and fibrinogen have distinct binding requirements. In particular, the maintenance of α IIb β 3 binding activity in the absence of the γ C-dodecapeptide and the α -chain RGD sequences suggests that the α IIb β 3-binding sites in fibrin are not confined to its known γ -chain and RGD motifs.

The interactions of the platelet integrin α IIb β 3 with fibrinogen and fibrin are essential for hemostasis and thrombosis.

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Thus, fibrinogen binding to activated α IIb β 3 mediates platelet aggregation and platelet spreading on a fibrinogen-containing matrix (1). However, individual activated α IIb β 3 molecules also attach to fibrin fibers (2) such that aggregated platelets are major components of hemostatic blood clots and thrombi (3). Further, the interaction of α IIb β 3 with fibrin is involved in platelet-driven clot contraction (4, 5). *In vivo*, blood clotting is catalyzed by thrombin, which simultaneously induces fibrinogen binding to α IIb β 3 and the conversion of fibrinogen to fibrin. However, the relative importance of fibrinogen versus monomeric and polymerized fibrin as α IIb β 3 ligands remains unclear (6).

Fibrinogen binds to α IIb β 3 on agonist-stimulated platelets with a K_d of \sim 100 nM, nearly 100-fold less than the concentration of fibrinogen in plasma, implying that the fibrinogen binding site on α IIb β 3 is immediately occupied when platelets are activated in a plasma environment (7, 8). Fibrinogen contains several sequence motifs that can potentially mediate its interaction with α IIb β 3. Residues located at the C terminus of the fibrinogen γ chain (residues 400–411) are necessary for fibrinogen binding to platelets (8, 9). Human fibrinogen also contains two common Arg-Gly-Asp (RGD) integrin recognition motifs in its A α chain (residues A α 95–97 and A α 572–574). However, deletion of these motifs does not impair the ability of fibrinogen to support platelet aggregation that is mediated by the KQAGDV sequence located at the C terminus of the fibrinogen γ chain (10, 11). Nonetheless, RGD-containing peptides inhibit α IIb β 3 function *in vitro* and are clinically effective antagonists of α IIb β 3 *in vivo* (1), presumably because they can compete with the γ chain motif for fibrinogen binding to α IIb β 3 (12).

Because of the experimental challenges involved in measuring the interaction of platelets with fibrin, most research has focused on the role of fibrinogen in platelet adhesion and aggregation. Thus, much less is known about the former interaction. However, differences in the ability of α IIb β 3 antagonists to inhibit clot contraction versus platelet aggregation suggest that the interactions of α IIb β 3 with fibrinogen and fibrin are different (13–18). The sites at which α IIb β 3 interacts with fibrin and fibrinogen also appear to be substantially different. Thus, whereas fibrinogen lacking the C-terminal γ chain residues is unable to support platelet aggregation, its ability to support clot contraction is unaffected (19, 20). Moreover, substitution of each RGD motif in the fibrinogen α chain with RGE has no effect on clot contraction (21), and although clot contraction is somewhat delayed when the RGD and C-terminal γ chain motifs are mutated, it is eventually indistinguishable from that

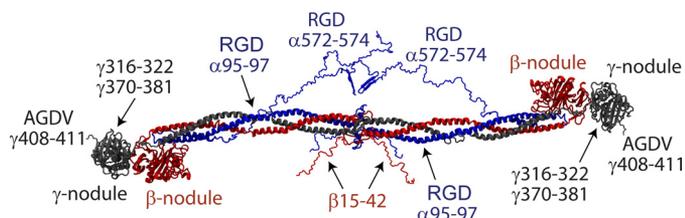


FIGURE 1. Diagram of the fibrinogen molecule with the A α (blue), B β (red), and γ (green) chains indicated. Putative α IIb β 3 binding sites are denoted with the color of the corresponding fibrinogen chain. The location of the sequences A α 1–26, B β 1–57, γ 395–411, and A α 221–610 were added computationally to the fibrinogen crystal structure (Protein Data Bank code 3GHG).

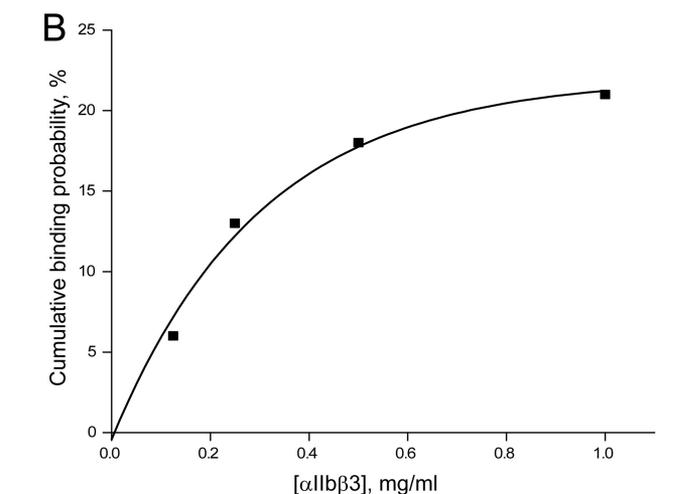
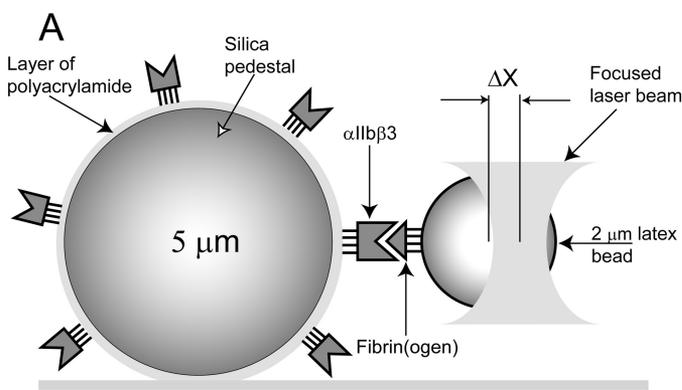
mediated by intact fibrin. These observations suggest that a site or sites in addition to the RGD and C-terminal γ chain motifs participate in the interaction of platelets with fibrin during thrombus growth and clot contraction. Candidate sites proposed previously include residues 15–42 in the β chain (22), as well as residues 316–322 (23, 24) and 370–381 (25, 26) in the γ chain (Fig. 1).

Because the interactions of platelets with fibrin appear to be important for the generation of a hemostatic plug or thrombus that is able to withstand the hemodynamic stresses present in circulating blood, mechanistic insights into α IIb β 3-fibrin interactions may provide new avenues for the development of novel treatment modalities for the thrombotic complications of cardiovascular disease and ischemic stroke. Here, we have tested the hypotheses that α IIb β 3-mediated platelet interactions with fibrin may be stronger than those with fibrinogen because of broader specificity, faster kinetics, and higher affinity and result from the exposure of cryptic α IIb β 3 binding sites when fibrinogen is converted to fibrin.

Experimental Procedures

Production of Recombinant Fibrinogens—Recombinant human fibrinogens were expressed in transfected BHK cells as described previously (27). Briefly, recombinant fibrinogens were isolated from the conditioned medium of BHK cells that were transfected with expression vectors encoding the A α , B β , and γ chain cDNAs. Purified recombinant fibrinogens were isolated in a two-column procedure using protamine-agarose and GPRPC-agarose affinity chromatography.

Model System to Study the Interaction of α IIb β 3 with Fibrinogen and Fibrin— α IIb β 3 interactions with human fibrinogen and fibrin were studied at the single-molecule level using a biophysical methodology that we have developed called optical trap-based force spectroscopy (28–31). In this method, each of two contacting surfaces is coated with one type of interacting molecule. Here, purified human α IIb β 3 was immobilized on micron-size stationary silica beads, whereas fibrinogen or monomeric fibrin was bound to freely moving latex beads. Under visual microscopic control, a bead coated with either fibrinogen or monomeric fibrin was trapped in a fluid chamber by a focused laser beam and moved in an oscillatory manner so that it tapped a stationary α IIb β 3-coated pedestal anchored to the bottom surface of the flow chamber (Fig. 2A). When the immobilized fibrinogen or fibrin monomer on the bead interacted with α IIb β 3 on the pedestal, tension was produced when the



bead was displaced from the laser focus until the α IIb β 3-fibrinogen or α IIb β 3-fibrin bond ruptured. The applied force was then displayed as a signal proportional to the strength of α IIb β 3-fibrinogen or α IIb β 3-fibrin binding (29, 31). Such signals were on the order of piconewtons and quantitatively characterize the interactions of α IIb β 3 with fibrin and fibrinogen at the nanomechanical molecular level.

Coating Surfaces with α IIb β 3—Purified human α IIb β 3 (Abcam) at 1 mg/ml in 0.055 M borate buffer, pH 8.5, containing 60 mM *n*-octyl- β -D-glucoside and 3 mM CaCl₂ was activated with 1 mM MnCl₂ for 30 min at 37 °C. The activated α IIb β 3 was bound covalently to 5- μ m spherical silica pedestals anchored to the bottom of a chamber as previously described (29, 31). Briefly, pedestals coated with a thin layer of polyacrylamide were activated with 10% glutaraldehyde (30 min, 37 °C), after which the activated α IIb β 3 (1 mg/ml in the activation mixture) was immobilized for 2 h at 4 °C. The chamber was then washed with 20 volumes of the same buffer to remove noncovalently adsorbed protein; blocked with 2 mg/ml BSA in 0.055 M borate buffer, pH 8.5, containing 150

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mM NaCl, 3 mM CaCl₂ for 30 min at 4 °C; and equilibrated with 20 volumes of cold (4 °C) 0.1 M HEPES buffer, pH 7.4, containing 150 mM NaCl, 3 mM CaCl₂, 1 mM MnCl₂, 2 mg/ml BSA, and 0.1% (v/v) Triton X-100 for 30 min at 4 °C before rupture force measurements were performed. The α Ib β 3 coating concentration (1 mg/ml) at which the cumulative probability of fibrinogen binding reached saturation was determined experimentally (Fig. 2B).

Coating Beads with Fibrinogen and Monomeric Fibrin—Purified human fibrinogen (Hyphen Biomed, Neuville-sur-Oise, France) and recombinant human fibrinogen variants were prepared as previously described (27) and were covalently attached to 1.75- μ m carboxylate-modified latex beads (Bangs Laboratories, Fishers, IN) using *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride as the cross-linking agent. As the first step in the procedure, carboxyl groups on the beads were activated by mixing 1 ml of 0.1 M MES buffer, pH 5.2, 20 μ l of a 10% bead suspension, and 3 mg of dry carbodiimide, followed by constant rotation for 15 min at 4 °C. Next, the beads were centrifuged, washed once with binding buffer (0.055 M borate buffer, pH 8.5), and resuspended in 1 ml of 20 μ g/ml fibrinogen in the binding buffer containing 150 mM NaCl. After 30 min of incubation with stirring at room temperature, the beads were sedimented and resuspended in a blocking solution (2 mg/ml BSA in the binding buffer). Before each experiment, fibrinogen-coated beads were freshly prepared and mildly sonicated to disrupt aggregates. To generate beads coated with monomeric fibrin, the fibrinogen-coated beads were treated with human α -thrombin (1 unit/ml, 37 °C, 1 h) followed by centrifugation and resuspension in 0.1 M HEPES buffer, pH 7.4, containing 150 mM NaCl, 3 mM CaCl₂, 1 mM MnCl₂, 2 mg/ml BSA, and 0.1% (v/v) Triton X-100. This treatment has been previously shown to generate biologically active fibrin molecules on a surface (33, 34).

Measurements of α Ib β 3-Fibrin(ogen) Rupture Forces, Data Processing, and Analysis—To measure the rupture force between α Ib β 3 and fibrinogen or fibrin, a suspension of fibrinogen- or fibrin monomer-coated beads was injected into a microscopic flow chamber with α Ib β 3-coated pedestals attached to the bottom. A fibrinogen- or fibrin-coated bead, trapped by the laser light, was brought to a distance of 2–3 μ m from the integrin-coated pedestal. After oscillation of the bead was initiated, the bead was brought into contact with the pedestal by micromanipulation using a keyboard-controlled piezoelectric stage. Data collection was initiated at the first contact between the bead and the pedestal. Rupture forces following repeated contacts between the pedestal and the bead were collected for several minutes and were displayed as normalized force histograms for each experimental condition. Because only a small percentage of contact/detachment cycles result in effective α Ib β 3-fibrinogen or α Ib β 3-fibrin monomer binding/unbinding, data from more than 10 different bead-pedestal pairs, representing from 10³ to 1.5 \times 10⁴ individual measurements, were combined for each experimental condition. 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 per-

centage 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 appear as forces below 10 pN (31). Accordingly, rupture forces in this range were not considered when these data were analyzed. Measurements were performed in 0.1 M HEPES buffer, pH 7.4, containing 150 mM NaCl, 3 mM CaCl₂, 2 mg/ml BSA, and 0.1% (v/v) Triton X-100. Fibrinogen and fibrin monomer-coated bead were oscillated in a triangular waveform at 10 Hz with the pulling velocity of 24 μ m/s, corresponding to a loading rate of \sim 2,600 pN/s with a trap stiffness of 0.11 \pm 0.03 pN/nm as computed from the bandwidth of Brownian motion. Contact duration between interacting surfaces was 25 \pm 1 ms. Force signals were collected at 2000 scans per second (0.5-ms time resolution).

Results

Interactions of α Ib β 3 with Immobilized Fibrinogen versus Fibrin—The interactions of surface-attached α Ib β 3 with immobilized fibrinogen and monomeric fibrin were studied in parallel. Control α Ib β 3-fibrinogen interactions manifested characteristic bimodal rupture force histograms with rupture forces of up to 140 pN (Fig. 3A), similar to those we previously observed (31). This rupture force distribution consists of the sum of exponentially decreasing weak to moderate (20–60 pN) forces, and a Gaussian-like distribution of strong (>60 pN) rupture forces peaking at 70–80 pN. The two clusters of rupture forces represent at least two types of single-molecule α Ib β 3-fibrinogen interactions that differ in kinetics, loading rate dependence, and susceptibility to inhibition or activation (29). Thus, fibrinogen-reactive α Ib β 3 exists in at least two interconvertible conformational states that form α Ib β 3-fibrinogen complexes having distinct affinities and discrete binding and unbinding kinetics (28, 30).

The rupture force spectrum for α Ib β 3 bound to monomeric fibrin was remarkably different from α Ib β 3 bound to fibrinogen, although it also had two observable binding regimes. The major difference was a significant 3-fold higher cumulative binding probability throughout the entire force range (Fig. 3B and Table 1). This difference was not due to a difference in the surface density of fibrinogen and fibrin monomer because the fibrin monomer was produced *in situ* by treating fibrinogen-coated beads with thrombin so that the surface densities of fibrinogen and monomeric fibrin were essentially identical. Moreover, because the monomeric fibrin was covalently attached to the bead surface, substantial fibrin oligomerization was not possible. Further, because the pedestals were saturated with α Ib β 3 (Fig. 2B), the difference in the probability of specific binding under standard experimental conditions was determined primarily by the intrinsic reactivity (on rates) of fibrinogen and fibrin toward α Ib β 3. The possibility that physically absorbed thrombin was responsible for the higher non-specific surface reactivity was excluded in control experiments where fibrinogen-coated beads were treated with thermally or D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone-inactivated thrombin without a change in reactivity toward α Ib β 3-coated pedestals (not shown). The average cumulative binding probability of >30% and the overall rupture force profile with

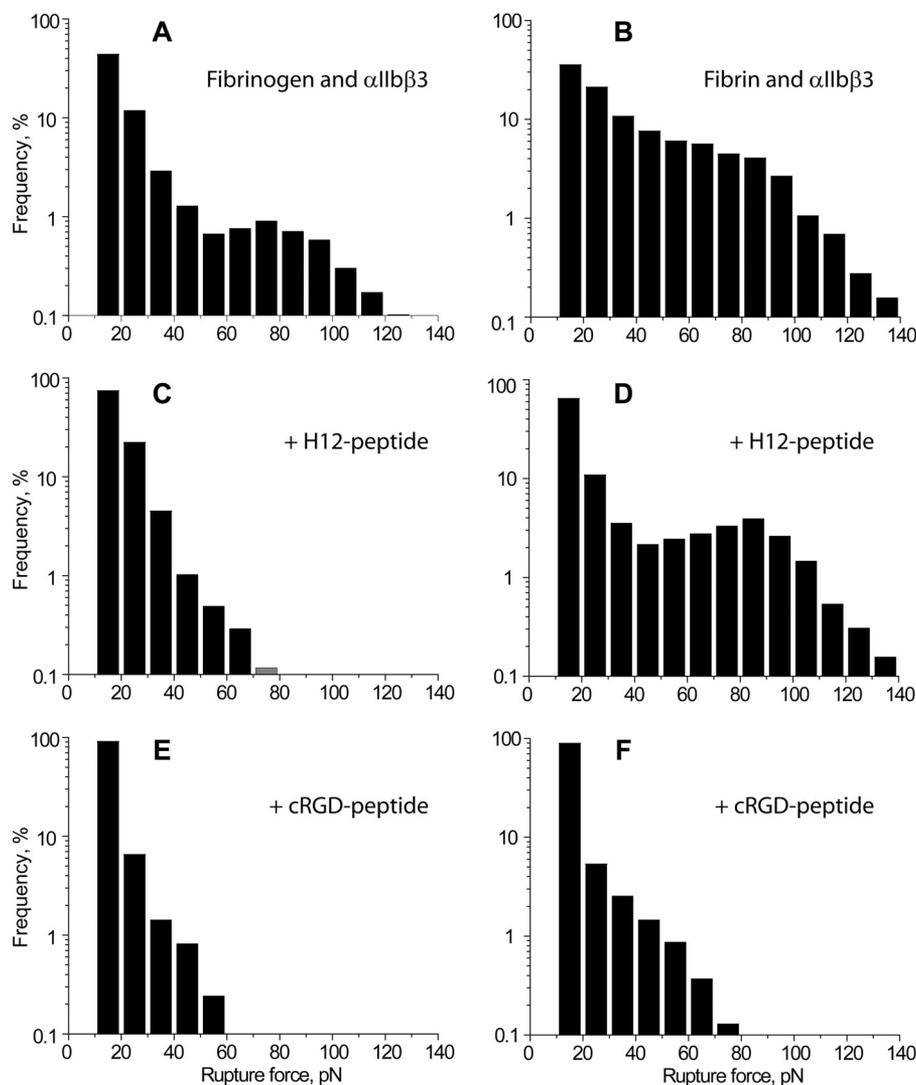


FIGURE 3. Rupture force spectra for α IIb β 3 binding to either wild-type fibrinogen or wild-type fibrin in the absence and presence of inhibitors. A, α IIb β 3 binding to immobilized wild-type fibrinogen. B, α IIb β 3 binding to immobilized wild-type fibrin monomer. Fibrin monomer was produced by treating fibrinogen-coated latex beads with thrombin. C–F, binding interactions shown in A and B repeated in the presence of 1 mM γ C-dodecapeptide (H12) (C and D) or 1 mM cyclic RGD (cRGD) peptide (E and F). As described under “Experimental Procedures,” data were collected from not less than 10 pedestal bead pairs collected in three or more experiments. The histograms shown in the figure resulted from 13,580 contacts in A; 14,356 contacts in B; 6,334 contacts in C; 10,367 contacts in D; 9,564 contacts in E, and 7,867 contacts in F.

TABLE 1

Cumulative binding probability of fibrinogen (Fg) and fibrin (Fn) with the integrin α IIb β 3 in the absence and presence of H12 and the cyclic RGD peptide (cRGD)

All the differences between Fg and Fn in the rows, as well as between H12 and cRGD in the columns, are statistically significant ($p < 0.01$).

Fibrinogen (purified)	Cumulative binding probability	Normalized extent of inhibition	Corresponding fibrin	Cumulative binding probability	Normalized extent of inhibition
	%	%		%	%
Fg	11.5 \pm 4.4		Fn	33.3 \pm 10.3	
Fg + H12	4.5 \pm 1.1	61 \pm 15	Fn + H12	23.3 \pm 7.1	30 \pm 9
Fg + cRGD	2.5 \pm 0.7	78 \pm 22	Fn + cRGD	14.4 \pm 5.2	57 \pm 20

barely discernible force peaks suggest that despite the absence of polymerization on the interacting surface, a substantial portion of the α IIb β 3-fibrin interactions were multimolecular. This was likely due to the high α IIb β 3 binding activity of fibrin monomers and perhaps to the incidental formation of short oligomers between primarily clustered/aggregated fibrin molecules as well. The absence of distinct force peaks precludes determining the binding strength for bimolecular α IIb β 3-fibrin interactions. Moreover, it is likely that strong multiple fibrin-

α IIb β 3 interactions were near or beyond the upper limit of forces measurable by the optical trap (\sim 130–140 pN) and therefore were missing or distorted in the rupture force histograms. Indirect confirmation of this assumption comes from the observation that beads coated with fibrin, unlike those that were coated with fibrinogen, were often irreversibly attached to α IIb β 3-coated pedestals shortly after they were brought in contact, implying that their binding strength exceeded the power of the optical trap.

Distinct Fibrin- α IIb β 3 and Fibrinogen- α IIb β 3 Interactions

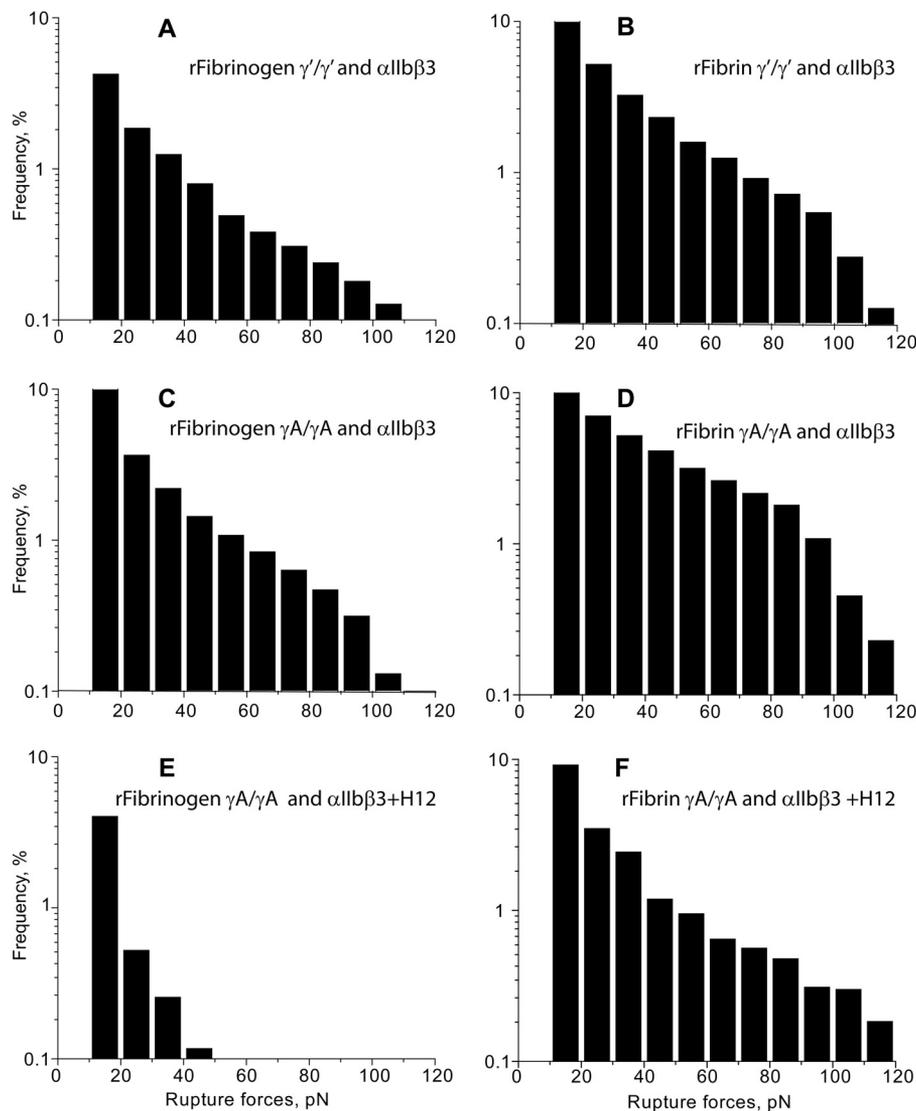


FIGURE 4. *A–D*, rupture force spectra for α IIb β 3 binding to recombinant fibrinogen or fibrin containing either two γ' chains (*A* and *B*) or two γ A chains (*C* and *D*). Fibrin monomers were generated as described in Fig. 3. *E* and *F*, binding interactions shown in *C* and *D* in the presence of 1 mM H12. The data were collected as described under “Experimental Procedures” and in the legend to Fig. 3. The histograms shown in the figure were generated from 11,356 contacts in *A*; 12,563 contacts in *B*; 6,334 contacts in *C*; 9,367 contacts in *D*; 6,856 contacts in *E*; and 9,564 contacts in *F*.

Role of the γ A/ γ' Chain Polymorphism in the Interaction of α IIb β 3 with Fibrinogen and Fibrin—It is generally accepted that α IIb β 3-fibrinogen interactions occur via the C-terminal γ chains and that neither of the two A α chain RGD motifs is required (1). To directly address the role of the γ chain sequences in the interaction of α IIb β 3 with fibrin, we performed force spectroscopy measurements with recombinant homodimeric γ A/ γ A and γ'/γ' fibrinogen and fibrin. The latter proteins are alternatively spliced variants in which the C terminus of the fibrinogen γ chain, the physiologic α IIb β 3 binding site on fibrinogen, is altered by adding new amino acids from 408 to 427 (35), thereby impairing the binding of soluble fibrinogen to α IIb β 3 (36). Although γ'/γ' fibrinogen remained reactive with α IIb β 3, its overall reactivity was \approx 2-fold less than that of γ A/ γ A fibrinogen (Fig. 4, *A* and *C*, and Table 2). Replacing γ A chains with γ' chains also attenuated rupture forces >60 pN and partially reduced weaker interactions as well. That fibrinogen γ'/γ' maintained its ability to interact with α IIb β 3 implies

that the C terminus of the γ chain is not the only motif involved in the interaction of immobilized fibrinogen with α IIb β 3. The differences in the rupture force spectra between Figs. 3*A* and 4*C* reflect differences in the reactivity of plasma-purified *versus* recombinant fibrinogen with α IIb β 3, the latter displaying lower reactivity.

γ A/ γ A fibrin and γ'/γ' fibrin, each derived from the respective fibrinogen, exhibited greater binding probabilities and stronger rupture forces compared with the parental fibrinogen molecules. However, the average cumulative binding probability of γ'/γ' fibrin was \approx 40% less than γ A/ γ A fibrin (Fig. 4, *B* and *D*, and Table 2), indicating that the C-terminal γ A sequence 408–411 is involved in the interaction of α IIb β 3 with monomeric fibrin as well as fibrinogen. Further, a dodecapeptide corresponding to the C terminus of the γ -chain inhibited the interaction of α IIb β 3 with γ A/ γ A fibrin by \approx 60%, whereas under the same conditions it suppressed fibrinogen- α IIb β 3 interactions by 90% (Fig. 4, *E* and *F*, and Table 2). These results indicate

TABLE 2

Cumulative binding probability of the corresponding recombinant fibrinogen (rFg) and fibrin (rFn) variants with the integrin α IIb β 3 in the absence and presence of H12

All the differences between rFg and rFn in the rows are statistically significant ($p < 0.01$). The differences between rFg γ A/ γ A or rFn γ A/ γ A and the corresponding variants γ' / γ' , (A) α D574E, and (A) α D97E, as well as the effects of H12, are also statistically significant ($p < 0.01$).

Recombinant fibrinogen variants	Cumulative binding probability	Normalized percentage of baseline	Corresponding fibrin variants	Cumulative binding probability	Normalized percentage of baseline
rFg γ A/ γ A	10.8 \pm 3.4	%	rFn γ A/ γ A	28.5 \pm 11.2	%
rFg γ A/ γ A + H12	1.1 \pm 0.4	90 \pm 33	rFn γ A/ γ A + H12	10.5 \pm 2.2	63 \pm 13
rFg γ' / γ'	5.7 \pm 1.8	47 \pm 15	rFn γ' / γ'	16.2 \pm 5.0	43 \pm 13
rFg A α D574E	7.5 \pm 2.3	31 \pm 9	rFn α D574E	15.6 \pm 8.2	45 \pm 23
rFg A α D574E + H12	2.9 \pm 0.8	73 \pm 20	rFn α D574E + H12	10.4 \pm 5.8	64 \pm 36
rFg A α D97E	6.7 \pm 1.9	38 \pm 11	rFn α D97E	15.8 \pm 7.5	45 \pm 21
rFg A α D97E + H12	1.7 \pm 0.5	84 \pm 25	rFn α D97E + H12	9.6 \pm 3.4	66 \pm 23

that the C-terminal γ A sequence 400–411 mediates the interaction of α IIb β 3 with both immobilized fibrinogen and fibrin; however, the relative contribution of this sequence is less substantial for fibrin- α IIb β 3 rather than for fibrinogen- α IIb β 3 interactions.

Role of the (A) α Chain RGD Motifs in the Interaction of α IIb β 3 with Fibrinogen and Fibrin—The interaction of soluble fibrinogen with α IIb β 3 is mediated by the C terminus of the fibrinogen γ chain (10, 11). Nonetheless, soluble fibrinogen binding to α IIb β 3 is inhibited by RGD-containing peptides (1) whose binding site on α IIb β 3 overlaps with that of peptides corresponding to C-terminal γ chain residues (12). It is also noteworthy that the affinity of RGD-containing peptides for α IIb β 3 is greater than that of γ chain peptides (8, 9, 37). Because the fibrin(ogen) (A) α chain contains two RGD motifs at residues 95–98 and 572–575, it is possible that the residual interaction of immobilized γ' / γ' fibrinogen and γ' / γ' fibrin with α IIb β 3 may be mediated by one or both of these otherwise cryptic (A) α chain RGD motifs exposed when fibrinogen is immobilized on a surface or when fibrinogen is converted to fibrin (38). To address this possibility, we tested the consequences of mutating the RGDF motif at A α 95–98 and the RGD motif at A α 572–575 to RGEF and RGEF, respectively.

The effects of mutating either RGD motif in intact immobilized fibrinogen were qualitatively and quantitatively similar, implying that neither motif preferentially interacts with α IIb β 3 (Figs. 5 and 6 and Table 2). In comparison with control recombinant γ A/ γ A fibrinogen (Fig. 4A), A α D97E and A α D574E fibrinogens were 69 and 62% less reactive toward α IIb β 3, respectively (Figs. 5A and 6A and Table 2). Thus, both motifs can support the interaction of immobilized fibrinogen with α IIb β 3. Likewise, mutating the RGD motifs in monomeric fibrin reduced its interaction with α IIb β 3 by 55% (Figs. 4B, 5B, and 6B and Table 2). Nonetheless, like wild-type fibrin, the cumulative probability of α IIb β 3 binding to mutated monomeric γ A/ γ A fibrin remained \approx 2-fold greater than the cumulative probability of α IIb β 3 binding to mutated fibrinogen (Figs. 5, A and B, and 6, A and B, and Table 2).

The binding sites for RGD-containing peptides and the γ chain dodecapeptide on α IIb β 3 overlap (12). Consequently, RGD and dodecapeptide binding to α IIb β 3 is mutually exclusive (39). Accordingly, we posited that free dodecapeptide should further reduce the reactivity of fibrinogen and fibrin with mutated RGD motifs toward α IIb β 3. As shown in Figs. 5C and 6C and Table 2, binding of A α D574E- and A α D97E-fibrin-

ogen to α IIb β 3 was reduced by 73 and 84%, respectively, by the presence of 1 mM dodecapeptide. Moreover, as would be predicted from data shown in Table 1, the inhibitory effect of the free dodecapeptide on mutant fibrin- α IIb β 3 interactions was significantly less pronounced than in the corresponding mutant fibrinogens (64% for α D574E and 66% for α D97E, $p < 0.01$) (Figs. 5D and 6D and Table 2). Thus, these results indicate that both (A) α chain RGD motifs play a role in the interaction of α IIb β 3 with immobilized fibrinogen and fibrin. However, because 1 mM γ C-dodecapeptide² did not completely abrogate the interaction of α IIb β 3 with α D97E and α D574E fibrinogen or fibrin, it is likely that additional α IIb β 3-binding sites are exposed when fibrinogen and monomeric fibrin are immobilized.

Differential Susceptibility of α IIb β 3-Fibrinogen and α IIb β 3-Fibrin Interactions to Inhibitors—Besides being quantitatively different, fibrinogen- α IIb β 3 and fibrin- α IIb β 3 interactions respond differently to well characterized α IIb β 3-fibrinogen antagonists. α IIb β 3 binding to fibrinogen-coated beads was highly sensitive to competitive inhibition by the γ C-dodecapeptide (>60% inhibition), whereas α IIb β 3 binding to fibrin monomer-coated were inhibited by only 30% (Fig. 3C and Table 1). Similarly, a cyclic RGD peptide more effectively inhibited the interaction of α IIb β 3 with fibrinogen (\sim 80%) than with fibrin monomer (\sim 60%, $p < 0.01$) (Fig. 3E and Table 1). Further, it is apparent from inspection of the rupture force histograms that both inhibitors completely abrogated α IIb β 3-fibrinogen interactions with rupture forces >60 pN, whereas the weaker force signals were only partially reduced. By contrast, α IIb β 3-fibrin interactions were essentially unaffected by the γ C-dodecapeptide at rupture forces >60 pN (Fig. 3D) and substantially less affected by cyclic RGD (Fig. 3F), consistent with a readily apparent difference in α IIb β 3 binding to fibrinogen and fibrin.

To determine whether these observed differences were exclusive to peptides derived from the fibrinogen molecule, we repeated experiments using the α IIb β 3 antagonists abciximab and eptifibatide. Both inhibitors suppressed α IIb β 3 binding to fibrinogen and fibrin in a dose-dependent manner, but α IIb β 3-fibrin interactions were less sensitive compared with α IIb β 3-fibrinogen interactions. The concentration dependences of the inhibitory effects of abciximab and eptifibatide on the α IIb β 3-

²The abbreviations used are: γ C, C-terminal portion of the fibrinogen and fibrin γ chain; H12, γ chain dodecapeptide.

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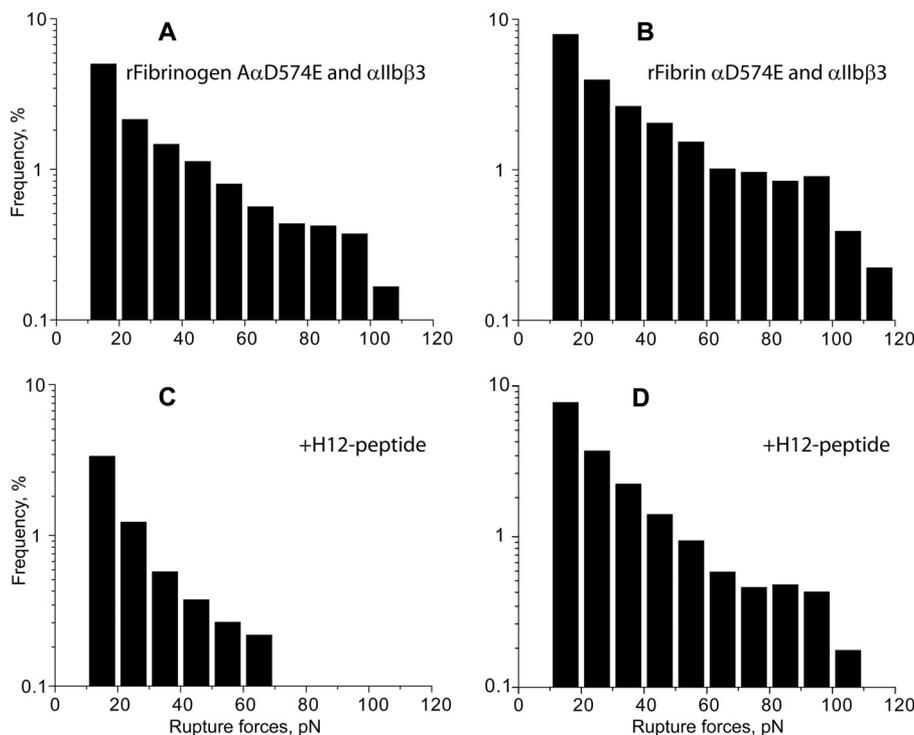


FIGURE 5. Rupture force spectra for α Ib β 3 binding to recombinant fibrinogen and fibrin in which the RGDS motif at (A) α 572–575 was mutated to RGES [(A) α D574E]. A, α Ib β 3 binding to A α D574E fibrinogen. B, α Ib β 3 binding to A α D574E fibrin. C and D, α Ib β 3 binding to (A) α D574E fibrinogen and fibrin in the presence of 1 mM H12. The data were collected as described under “Experimental Procedures” and in the legend to Fig. 3. The histograms shown in the figure were generated from 10,223 contacts in A; 11,445 contacts in B; 7,357 contacts in C; and 8,289 contacts in D.

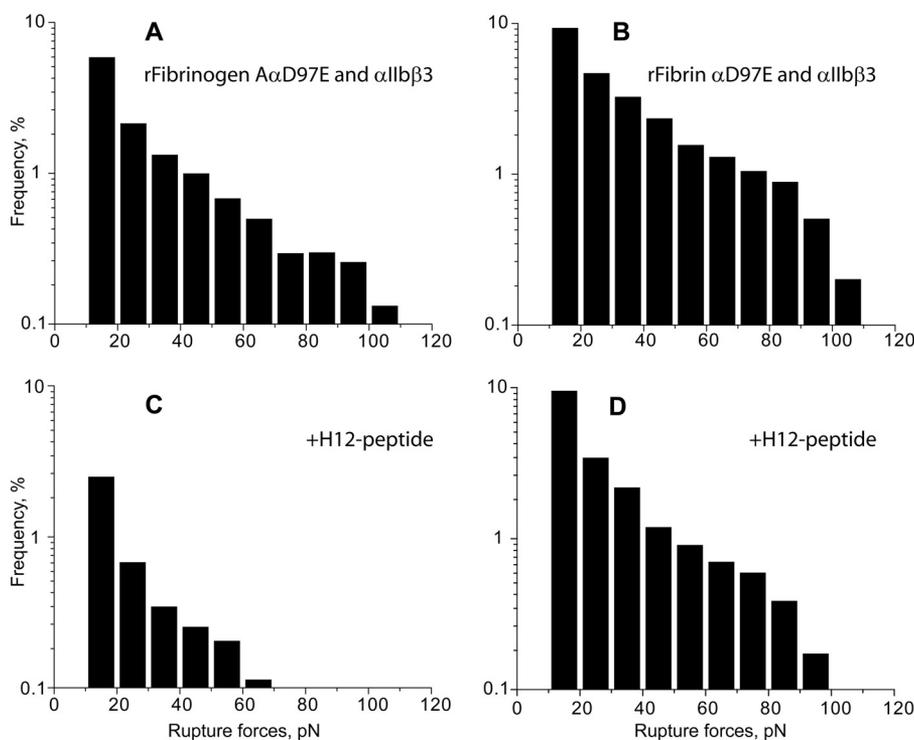


FIGURE 6. Rupture force spectra for α Ib β 3 binding to recombinant fibrinogen and fibrin in which the RGDF motif at (A) α 95–98 was mutated to RGEF [(A) α D97E]. A, α Ib β 3 binding to A α D97E fibrinogen. B, α Ib β 3 binding to α D97E fibrin. C and D, α Ib β 3 binding to (A) α D97E fibrinogen and fibrin in the presence of 1 mM H12. The data were collected as described under “Experimental Procedures” and in the legend to Fig. 3. The histograms shown in the figure were generated from 11,654 contacts in A; 11,114 contacts in B; 6,244 contacts in C; and 7,075 contacts in D.

fibrin and α Ib β 3-fibrinogen interactions were fitted with exponentials, revealing that α Ib β 3-fibrin interactions were 4.5-fold less susceptible to inhibition as judged from the slopes

(Fig. 7). Taken together, these results reiterate that the interaction of α Ib β 3 with fibrin is quantitatively and qualitatively different from its interaction with fibrinogen.

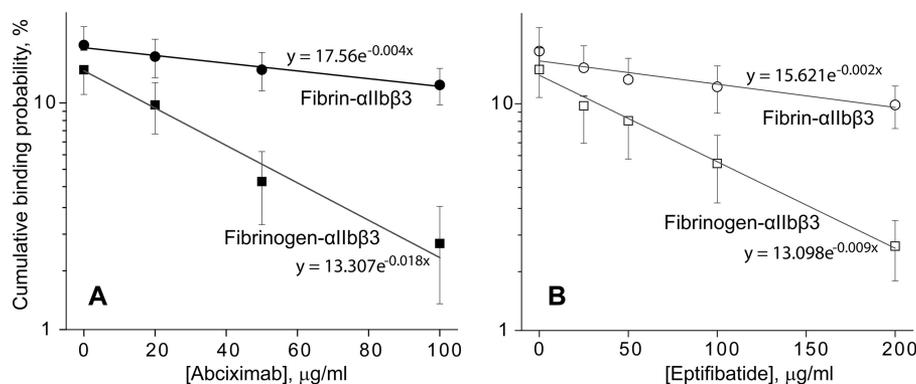


FIGURE 7. Cumulative probability of α IIB β 3-fibrinogen and α IIB β 3-fibrin binding plotted against the concentrations of abciximab (A) and eptifibatide (B) added to the reaction chamber. The plots were generated by fitting the experimental results to the exponential functions shown in the figure. The results shown are the means and standard deviations of three experiments.

Discussion

The existing paradigm is that fibrinogen is necessary and sufficient for platelet aggregation, although it is known that other ligands can also support platelet aggregation. However, clotting *in vivo* is more complex and various *in vitro* and *in vivo* models are beginning to clarify the differences. Our presumption is that *in vivo*, platelet aggregation may initially begin with fibrinogen binding to α IIB β 3 but quickly evolves to monomeric or oligomeric fibrin binding as fibrinogen is converted to fibrin by thrombin generated on or near the surface of activated platelets (Fig. 8). Moreover, platelet aggregation/adhesion and fibrin polymerization may be initiated simultaneously, in contrast to the traditional sequential view that fibrinogen-mediated platelet aggregation is followed by fibrin formation (40). In other words, even platelet-rich areas in a thrombus contain fibrin and much of the platelet aggregation and adhesion processes are mediated by fibrin, not fibrinogen. Thus, fibrin likely plays a major unappreciated role in platelet aggregation and adhesion *in vivo*, and it is important to understand how the interactions of platelets with fibrin differ from those with fibrinogen.

A number of reports in the literature indicate that the α IIB β 3 binding sites on fibrin may be substantially different from fibrinogen, suggesting that fibrinogen conversion to fibrin is accompanied by exposure of otherwise cryptic binding sites for α IIB β 3. An increase in the number of the α IIB β 3 binding sites and enlargement of the platelet interacting surface in a fibrin clot may lead to strong platelet adhesion on fibrin followed by conformational perturbations of the integrin (41, 42) and enhanced and/or selective outside-in signaling (43). In addition to the C-terminal portion of the fibrinogen γ chain (γ 400–411 including the binding spot γ 408–411 AGDV), a well established α IIB β 3 binding site, major candidates for sites that mediate fibrin binding α IIB β 3 are residues α 95–98 (RGDF) and α 572–575 (RGDS) located in the α chain because RGD sequences are the most general integrin binding motif. Nonetheless, additional α IIB β 3 binding sequences may be exposed when fibrinogen is converted to fibrin as well. Thus, platelet spreading on surfaces coated with fibrin lacking β chain residues 15–42 is substantially impaired (22). Further, antibodies against the γ 316–322 region inhibit clot retraction (23), and a site designated P3 at γ 370–383 has been identified that also

mediates fibrin clot retraction and binds α IIB β 3 (25, 26). Mutational analyses have confirmed that both γ 408–411 AGDV and P3 are required for the full adhesive activity of fibrin for platelets. It is noteworthy that P3 residues γ 373–383 are fibrin-specific and are exposed upon the transformation of fibrinogen to fibrin (32), whereas the AGDV sequence is exposed constitutively in both fibrin and fibrinogen.

Because it is methodologically difficult to do quantitative measurements of platelet binding to insoluble fibrin, conventional experimental approaches have been ineffective. Accordingly, our studies make use of a biophysical technology (optical trap-based force spectroscopy) to measure receptor-ligand binding and unbinding at the single-molecule level. Earlier, this technology enabled us to determine quantitative parameters of integrin-fibrinogen interactions at interfaces and to study their molecular mechanisms (28, 29, 31, 37). Because we can convert fibrinogen immobilized on a surface to fibrin monomer (33, 34), we applied this technology to obtain quantitative characteristics and to identify fibrin structures involved in α IIB β 3-fibrin interactions. By directly measuring the binding strength between individual fibrin and α IIB β 3 molecules, we found clear differences from the interaction of α IIB β 3 with fibrinogen. To identify sites by which fibrin interacts with α IIB β 3, we used two independent approaches. First, rupture force spectroscopy was performed in the absence and presence of specific α IIB β 3 antagonists (cyclic RGD and dodecapeptide γ 400–411, as well as the α IIB β 3 antagonists abciximab and eptifibatide). Second, we made use of recombinant fibrinogen and fibrin mutants lacking either the γ 408–411 AGDV motif [fibrin(ogen) γ' / γ'] or the RGD motifs α A α D97E and α A α D574E.

Our results can be summarized as follows. The effect of replacing the γ A with the γ' chains in fibrin was similar to competitive inhibition by free γ C-dodecapeptide, confirming involvement of the γ C integrin-binding site in fibrin binding to α IIB β 3. Interactions of fibrin γ' / γ' with α IIB β 3 were strongly inhibited by cyclic RGD, implying that the fibrin RGD motifs play a role as well. γ' / γ' fibrin was more reactive with α IIB β 3 than γ' / γ' fibrinogen, suggesting that cryptic integrin-binding sites, perhaps one or both α chain RGD motifs are exposed in fibrin and synergize with the γ C binding site. This assumption was supported using the fibrin mutants α D97E or α D574E, which were slightly less reactive than fibrin γ A/ γ A. Mainte-

Distinct Fibrin- α IIB β 3 and Fibrinogen- α IIB β 3 Interactions

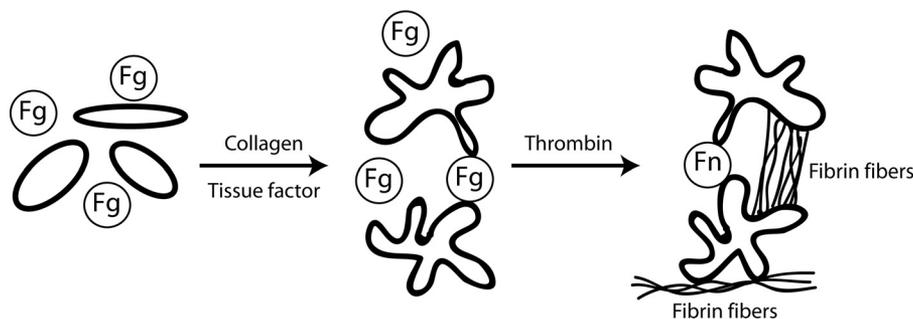


FIGURE 8. **Cartoon illustrating the potential role of fibrin in platelet aggregation.** Platelet aggregation initially begins with fibrinogen (Fg) binding to α IIB β 3 but quickly evolves into α IIB β 3 interaction with monomeric, oligomeric, or polymeric fibrin (Fn) as fibrinogen is converted to fibrin by thrombin generated at or near the surface of the activated platelets.

nance of the α IIB β 3 binding activity of fibrin γ'/γ' in cyclic RGD or mutation of the α RGD motifs confirms that the α IIB β 3 binding sites in fibrin are not confined to the γ C-terminal 400–411.

Our results also suggest the following general conclusions. First, the binding specificity of α IIB β 3 with fibrinogen depends on whether fibrinogen is soluble or surface-attached. At the surface, fibrinogen lacking the γ 408–411 AGDV site is still reactive with α IIB β 3, indicating exposure of additional α IIB β 3-binding sites upon immobilization. Second, binding interactions of surface-attached fibrin and fibrinogen with α IIB β 3 are clearly different, both qualitatively and quantitatively. Fibrin binds to α IIB β 3 with a higher frequency at the same surface density, implying a higher association constant. This quantitative difference between fibrin and fibrinogen also reflects differences in specificity. The relative α IIB β 3 binding activity of the variant fibrin γ'/γ' , lacking the γ C-terminal AGDV motif, suggests that this structure may not be a major integrin-binding site in fibrin. Third, the reduced interaction of fibrinogen and fibrin mutants lacking α chain RGD motifs implies that these motifs are involved in the interaction of α IIB β 3 with fibrin and immobilized fibrinogen as opposed to soluble fibrinogen. Binding to these motifs may even be preferred as we (37) and others (8, 9) have shown that the affinity of RGD for α IIB β 3 is greater than of AGDV. Fourth, the absence of RGD in the presence of free γ C-dodecapeptide did not completely abrogate the interactions of fibrin with α IIB β 3, suggesting the existence of additional sites in fibrin other than γ AGDV, α RGDE, and α RGDS. This point could be formally addressed by producing a compound fibrinogen mutant lacking the γ -chain C-terminal dodecapeptide and both α -chain RGD motifs. At this time, however, there are no laboratories producing and purifying such mutant fibrinogens. Thus, procuring this construct is not currently feasible. Fifth, α IIB β 3-fibrin interactions are less susceptible to α IIB β 3 antagonists than α IIB β 3-fibrinogen, suggesting that drugs that inhibit platelet interactions with fibrin, as well as fibrinogen, could be more effective antithrombotic agents.

The principal goal of this study was to improve our understanding of how platelets and α IIB β 3 participate in thrombosis and to aid in advancing our therapeutic capabilities for cardiovascular diseases. Our results may help to explain, in part, why the currently available α IIB β 3 antagonists are less effective than expected. Importantly, they provide a new target for development of more effective α IIB β 3 antagonists.

Author Contributions—R. I. L., J. W. W., and J. S. B. conceived and designed the research; D. H. F. and R. I. L. performed the experiments; and R. I. L., D. H. F., J. W. W., and J. S. B. analyzed the data and wrote the paper.

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The Platelet Integrin α I **β 3 Differentially Interacts with Fibrin *Versus* Fibrinogen**

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