Fibrinogen Binding to Human Blood Platelets: Effect of γ Chain Carboxyterminal Structure and Length

By Ellinor I. B. Peerschke, Charles W. Francis, and Victor J. Marder

Recent evidence suggests that fibrinogen binding to platelets is mediated by the 12 carboxyterminal amino acid residues of the γ chain. Because human plasma fibrinogen γ chains differ in mol wt and carboxyterminal amino acid sequence, we examined the effect of such γ chain heterogeneity on platelet-fibrinogen interactions, using two fibrinogens of distinct composition, separated by ion exchange chromatography. One fibrinogen possessed only γ chains of mol wt 50,000 (F500), the predominant γ chain species found in plasma. The other fibrinogen possessed equal amounts of γ chains with mol wt 50,000 and 57,500 (F757.5γ50), with the longer γ chain (γ75.5) possessing an amino acid extension at the carboxyterminal end. The latter fibrinogen was 50% less effective than F500 in supporting ADP-induced platelet aggregation at concentrations of 0.1 to 2 mg/mL. Scatchard analysis revealed no difference in the binding affinities of the two fibrinogens to ADP-treated platelets, but the amount of F757.5 that was bound to platelets at saturation was only 50% of that of F500. Fibrinogen receptors that remained unoccupied in the presence of saturating concentrations of F757.5, however, could be occupied by fresh F500. Excess unlabeled F500 displaced both radiolabeled fibrinogens from activated platelets, and both fibrinogens bound to the same platelet receptor, as judged by the inhibition of binding to stimulated platelets by a monoclonal antibody directed against the glycoprotein (GP) IIb/IIIa complex. Furthermore, an intact GPIIb/IIIa complex was required for these reactions, since platelets incubated with EDTA at 37 °C at alkaline pH failed to aggregate and bound neither fibrinogen in response to ADP following recalcification. Approximately 50% of each fibrinogen bound irreversibly to platelets after one hour and failed to dissociate in the presence of 10 mmol/L of EDTA or excess unlabeled F500. The data demonstrate that heterodimeric F757.5 binds less well to platelets and supports platelet aggregation only half as well as homodimeric F500. These results support prior conclusions that the carboxyterminal portion of the γ chain is important in platelet-fibrinogen interactions, and suggest that the 20 amino acid, hydrophobic γ chain carboxyterminal extension of F757.5 may sterically hinder the interaction of this fibrinogen with platelet receptors.

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Removal of von Willebrand factor. Prior to chromatography, the fibrinogen preparation at a concentration of 1 mg/mL contained 15 U/mL of von Willebrand factor (vWF) by Laurell electroimmunoassay,19 whereas F500 contained no detectable vWF, and F757.5 contained 0.10 U/mL. For some experiments, the vWF was removed by immunoadsorption. Protein A-Sepharose (Pharmacia) was swollen and incubated at 25 °C for 30 minutes with anti-vWF antiserum (Calbiochem, San Diego) in a ratio of 180 mg Protein/mL of sodium chloride, 0.05 mol/L of Tris-hydrochloric acid buffer, pH 7.6 containing 10 U/mL aprotinin (Trasyol, Mobay Chemical Co, NY), 0.02 mol/L of e-aminoacetocaproic acid (EACA), and 0.02% sodium azide.

SDS-PAGE. Labeled and unlabeled F500 and F757.5 were reduced and examined by SDS-PAGE21 using 7% gels. Fibrinogen associated with platelets following platelet stimulation with 10 mmol/L of ADP was analyzed by SDS-PAGE after gel-filtered platelet pellets (GFP) were centrifuged through silicone oil and after solubilizing the platelet pellets in buffer containing one part 3.3% SDS, 6 mmol/L of N-ethylmaleimide, and one part 1% SDS, 12.5 mmol/L Tris-hydrochloric acid, 20% glycerol, 0.05% bromophenol blue, pH 6.8. All gels were stained with Coomassie Brilliant Blue and were dried. Platelet-bound radiolabeled fibrinogen was visualized on autoradiograms prepared using Kodak X-OMAT AR film (Eastman Kodak Co, Rochester, NY).

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A-Sepharose to .3 mL of antiserum. Following centrifugation, the Sepharose was washed with buffer (.1 mmol/L of Tris-hydrochloric acid, pH 8.3 containing 10% NP40, 10% deoxycholate, 5 mg/mL of ovalbumin and .2 mol/L of disodium EDTA). An aliquot of .2 mL of washed, suspended Sepharose was then added to 0.8 mL of fibrinogen sample and incubated at 25 ºC for 30 minutes, and the supernatant was removed after centrifugation. The buffer was changed to .15 mol/L of sodium chloride, .05 mol/L of Tris-hydrochloric acid, pH 7.6, containing .02 mol/L of EACA, 10 U/mL of aprotinin and .02% sodium azide by chromatography on a column of Sepharose G-100 (Pharmacia). The final preparation contained no detectable vWF by Laurell electroimmunonassay.23 No change in the migration of bands as determined by SDS-PAGE was noted following this procedure.

**Fibrinogen characterization.** The γ chain composition of FІβ0 and FІβ0,575 was confirmed by SDS-PAGE. The γ chains of FІβ0 migrated as a single band with mol wt 50,000, whereas the γ chains of FІβ0,575 were equally distributed among bands of mol wt 50,000 and mol wt 57,500 (Fig 1). The Aα chain degradation of both FІβ0 and FІβ0,575 was comparable, and as shown previously,24 insufficient to influence fibrinogen binding or platelet aggregation.

**Fibrinogen iodination.** Purified FІβ0 and FІβ0,575 were labeled with 125I using chloramine T.25 Following iodination, 99% of the radioactivity associated with fibrinogen preparations precipitated in 10% trichloroacetic acid, and 94% to 98% was clotted by purified human thrombin (1 U/mL) (a generous gift from Dr John J. Fenton, NY State Department of Health, Albany, NY).

**Platelet preparation.** Blood was collected from volunteers after obtaining informed consent according to the Declaration of Helsinki. It was anticoagulated with 0.1 vol 3.2% sodium citrate in the presence of 0.05 vol 1 mmol/L of acetyl salicylic acid. In some studies, aspirin was omitted. Unless specified, experiments were performed using aspirin-treated platelets to prevent the release of platelet membrane-bound fibrinogen lacking elongated γ chains.25 Platelet-rich plasma was obtained by centrifugation (280 g, 15 minutes). GFP were prepared as described previously.25 EDTA-treated and control, Ca-EDTA-treated GFP were prepared as described by Zucker and Grant.26

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**Table 1. ADP-Induced Platelet Aggregation Supported by Increasing Concentrations of FІβ0 and FІβ0,575**

<table>
<thead>
<tr>
<th>Concentration (μg/mL)</th>
<th>Platelet Aggregation (Initial Slope)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>30 ± 10</td>
</tr>
<tr>
<td>50</td>
<td>45 ± 8</td>
</tr>
<tr>
<td>100</td>
<td>72 ± 11</td>
</tr>
<tr>
<td>300</td>
<td>75 ± 9</td>
</tr>
<tr>
<td>1,000</td>
<td>76 ± 7</td>
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</tbody>
</table>

*Light transmission units per minute, mean ± SD, n = 4.
of ADP. Platelets incubated with 10 mmol/L of EDTA for 15 minutes at 37 °C and pH 8.1 were also evaluated for their ability to bind F¹⁰⁸ and F(polyGlycine) following recalcification and stimulation with 10 µmol/L of ADP. This EDTA treatment causes an irreversible loss of platelet aggregability[8] that is related to alterations of the GPIIb/IIIa complex. Platelets incubated with the combination of calcium (10 mmol/L) and EDTA (10 mmol/L) served as controls.

RESULTS

Aggregation cofactor activity. The extent of ADP-induced primary aggregation supported by F¹⁰⁸[30,31] was ~50% that of F¹⁰⁸ at all fibrinogen concentrations tested (0.01 to 2.0 µmol/mL) (Fig 2). The rate of platelet aggregation was similarly diminished (Table 1). A representative aggregometer tracing of GFP stimulated with 10 µmol/L of ADP in the presence of 0.2 mg/mL of each fibrinogen is shown in Fig 3 to illustrate the markedly decreased aggregation of ADP-treated platelets in the presence of F³⁶⁵.

Binding to ADP-treated GFP. Both fibrinogens bound to platelets in a specific and saturable manner (Fig 4), but 50% less F¹⁰⁸ than F³⁶⁵ bound at saturation. Platelet receptors that remained unoccupied in the presence of F³⁶⁵[30], however, were accessible to F¹⁰⁸ added subsequently (Table 2). Equilibrium binding was attained one minute after incubation of ADP-treated GFP with the radiolabeled fibrinogen preparations (Table 3). Virtually all of both labeled fibrinogens could be displaced from ADP-treated platelets by excess unlabeled F¹⁰⁸ one minute after binding had occurred (Fig 5, Table 3). Approximately half of each radiolabeled fibrinogen remained associated with platelets in the presence of 10 mg/mL of unlabeled F¹⁰⁸ or 10 µmol/L of EDTA after 60 minutes of binding.

Both unlabeled fibrinogen preparations competed with trace amounts of labeled F¹⁰⁸ for platelet receptors (Table 4), although F³⁶⁵[30] competed less effectively. Approximately 1.5-fold more labeled F³⁶⁵ bound to platelets in the presence of 0.05 mg/mL of F³⁶⁵ than in the presence of F¹⁰⁸. This ratio increased to 2.0 at concentrations of unlabeled fibrinogens (0.5 µmol/mL) that approached saturation of the platelet receptors. These observations are consistent with the overall 50% decrease in F³⁶⁵[30] binding to stimulated platelets as compared to F¹⁰⁸ binding. Both fibrinogens bound to the same platelet receptor, the GPIIb/IIIa complex, as demonstrated by the complete inhibition of fibrinogen binding after preincubating platelets with an anti-GPIIb/IIIa monoclonal antibody (10E5) (Table 5). In addition, neither fibrinogen bound to platelets that had lost their ability to aggregate following prolonged exposure to EDTA[28,31] (Table 5).

Scatchard analyses of fibrinogen binding to ADP-treated platelets yielded upwardly concave plots (Fig 6). The apparent dissociation constants for high-affinity and low-affinity binding of the two fibrinogen preparations derived according to the methods of Rosenthal[22] and Feldman[33] were not significantly different: 0.124 ± 0.06 µmol/mL and 0.105 ± 0.05 µmol/mL, respectively, for high-affinity F¹⁰⁸ and F³⁶⁵ binding to platelets, and 2.08 ± 1.0 µmol/mL and 1.98 ± 0.98 µmol/mL for low-affinity binding. The amount of F³⁶⁵[30] bound to platelets at saturation appeared to be 50% less than the amount of F¹⁰⁸ bound. This was confirmed by plotting fibrinogen binding against the log of the fibrinogen concentration, as recommended by Klotz[28] (Fig 7). ADP-treated

Table 2. Binding of ¹²⁵I-F¹⁰⁸ or ¹²⁵I-F³⁶⁵ to ADP-Treated Platelets Preincubated With Near-Saturating Concentrations of Unlabeled F¹⁰⁸ and F³⁶⁵

<table>
<thead>
<tr>
<th>Preincubation Media</th>
<th>F¹⁰⁸</th>
<th>F³⁶⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotonic saline</td>
<td>31,176 ± 12,365</td>
<td>10,235 ± 4,124</td>
</tr>
<tr>
<td>F¹⁰⁸</td>
<td>19,911 ± 8,147</td>
<td>605 ± 312</td>
</tr>
<tr>
<td>F³⁶⁵</td>
<td>544 ± 611</td>
<td>282 ± 416</td>
</tr>
</tbody>
</table>

Gel-filtered platelets were preincubated in the presence of saline, 1.0 mg/mL of unlabeled F²⁰⁵[27] or F³⁶⁵ and 10 µmol/L of ADP. After two minutes, ¹²⁵I-labeled F¹⁰⁸, or F³⁶⁵ (1.0 mg/mL, final concentration) were added, and their binding was quantified after an additional two minutes by centrifugation of platelets through silicone oil.

* Molecules per 10⁶ platelets, mean ± SD, n = 3.

Table 3. Binding of ¹²⁵I-F¹⁰⁸ or ¹²⁵I-F³⁶⁵ to ADP-Stimulated Platelets as a Function of Time

<table>
<thead>
<tr>
<th>Radiolabeled Fibrinogen Bound (% of Maximum)</th>
<th>by Addition of</th>
<th>Unlabeled F¹⁰⁸ (10 mg/mL)</th>
<th>EDTA (10 µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
<td>p¹⁰⁸</td>
<td>p³⁶⁵</td>
<td>p¹⁰⁸</td>
</tr>
<tr>
<td>1</td>
<td>98 ± 2</td>
<td>100 ± 1</td>
<td>97 ± 5</td>
</tr>
<tr>
<td>2</td>
<td>100 ± 4</td>
<td>97 ± 3</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>5</td>
<td>100 ± 3</td>
<td>100 ± 4</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>15</td>
<td>97 ± 1</td>
<td>98 ± 4</td>
<td>97 ± 1</td>
</tr>
<tr>
<td>30</td>
<td>99 ± 4</td>
<td>99 ± 4</td>
<td>99 ± 4</td>
</tr>
<tr>
<td>60</td>
<td>100 ± 2</td>
<td>99 ± 5</td>
<td>49 ± 12</td>
</tr>
</tbody>
</table>

Binding experiments of radiolabeled fibrinogen were performed three times, with results indicated as mean ± SD. Fibrinogen displacement was measured five minutes after the addition of unlabeled fibrinogen or EDTA.

Fig 3. A representative aggregometer tracing of platelets stimulated with 10 µmol/L of ADP in the presence of 0.2 mg/mL of F¹⁰⁸ (A) and F³⁶⁵ (B).

Fig 4. Binding of F¹⁰⁸ (+) and F³⁶⁵ (Ø) to aspirin-treated platelets stimulated with 10 µmol/L of ADP as a function of increasing fibrinogen concentrations.
DNA

z

z

a

Fig 7. Analysis of F ( +) and F ( ) binding as recommended by Klotz.28

Table 4. Binding of Radiolabeled F to ADP-Stimulated Platelets in the Presence of Unlabeled Fibrinogens

<table>
<thead>
<tr>
<th>Unlabeled Fibrinogen (mg/mL)</th>
<th>Radiolabeled F ( %) Binding</th>
<th>Radiolabeled F ( %) Binding</th>
<th>Ratio (B/A)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>in the Presence of Unlabeled Fibrinogen</td>
<td>in the Presence of Unlabeled Fibrinogen</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(A)</td>
<td>(B)</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>30 ± 3</td>
<td>46 ± 4</td>
<td>1.5</td>
</tr>
<tr>
<td>0.15</td>
<td>24 ± 1</td>
<td>40 ± 1</td>
<td>1.7</td>
</tr>
<tr>
<td>0.50</td>
<td>19 ± 2</td>
<td>38 ± 1</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Binding of radiolabeled fibrinogen was compared to binding in the absence of unlabeled fibrinogen; data are recorded as mean ± SD of five experiments.

Table 5. Effect of Monoclonal Antibody 10E5, EDTA, or Ca-EDTA on the Binding of F and F to ADP-Stimulated Platelets

<table>
<thead>
<tr>
<th>Platelets Incubated With</th>
<th>F ( % of Control)</th>
<th>F ( % of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10E5 (20 µg/mL)</td>
<td>0 ± 2</td>
<td>0 ± 3</td>
</tr>
<tr>
<td>EDTA (10 mM/L) (37 °C, pH 8.1)</td>
<td>5 ± 9</td>
<td>5 ± 11</td>
</tr>
<tr>
<td>Ca-EDTA (37 °C, pH 8.1)</td>
<td>98 ± 10</td>
<td>105 ± 11</td>
</tr>
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</table>

*Results expressed as mean ± SD of three experiments.

and unlabeled F and F free of vWF showed that platelet binding of the labeled and unlabeled fibrinogens was indistinguishable.

SDS-PAGE of platelet-bound fibrinogens. Because F consists of approximately equal amounts of y50 and y57.5 chains, studies were performed to examine the mol wt distribution of the y chains of platelet-associated F and F. As shown in Fig 1, the y chain distribution of platelet-associated F was the same as that of the F starting material. Electrophoresis of platelet-bound F and the F starting material served as controls.

DISCUSSION

The predominant y chain species of normal human fibrinogen has a relative mol wt of ~50,000 (y50), whereas higher mol wt variants have been described recently with mol wt of 55,000 (y55) and 57,500 (y57.5). The increase in mol wt of the y57.5 chain is due to an elongated carboxyterminal amino acid sequence in which the carboxyterminal four amino acids of the y50 chain are replaced by a negatively charged 20 amino acid sequence. Because the y chains of human fibrinogen appear to possess the major determinants supporting platelet-fibrinogen interactions, studies were conducted to compare the ability of a fibrinogen preparation containing only mol wt 50,000 y chains (F) and a fibrinogen preparation possessing approximately equal amounts of y chains of mol wt 50,000 and mol wt 57,500 (F) to bind to platelets and support ADP-induced aggregation. The chromatographic elution profile of these fibrinogen preparations and of their constituent chains indicate that F is a heterodimer composed of molecules with one y50 and one y57.5 chain.

Compared to F, F bound to half as many platelet receptors at saturation (0.8 to 1.0 mg/mL), and supported ADP-induced platelet aggregation less well. These observations are consistent with a recent study by Harfenist et al.
indicating that fibrinogen which possessed approximately equal amounts of mol wt 50,000 and elongated mol wt 57,500 \( \gamma \) chains was only 50% as effective in supporting ADP-induced platelet aggregation at low concentrations (64 \( \mu \)g/mL) and bound less well to platelets than fibrinogen which contained two \( \gamma \) chains of mol wt 50,000.

The present study extends these observations. A direct correlation has been established between the decreased platelet aggregation support of FT\(_{57.5}\) and its decreased binding to exposed platelet membrane receptors over a range of fibrinogen concentrations from 0.01 to 2.0 mg/mL. Moreover, the data suggest that platelet receptors remaining unoccupied in the presence of saturating concentrations of FT\(_{57.5}\) are accessible to FT\(_{50}\) fibrinogen.

Despite differences in the extent of fibrinogen receptor saturation, both FT\(_{50}\) and FT\(_{57.5}\) appear to bind to the same platelet receptor, the glycoprotein IIb/IIIa complex. The binding of both was completely inhibited by a monoclonal antibody (10E5) specific for this complex, and was markedly reduced after platelet incubation with EDTA at an alkaline pH, a process that appears to dissociate the GPIIb/IIIa complex. In addition, both FT\(_{50}\) and FT\(_{57.5}\) were shown to bind irreversibly to platelets after 60 minutes.

Both fibrinogens also demonstrated similar binding kinetics. The dissociation constants calculated for high-affinity binding of FT\(_{50}\) and FT\(_{57.5}\) were not significantly different. High-affinity binding constants, however, are generally too variable (0.06 to 0.18 \( \mu \)mol/L) to detect small (two- to threefold) differences in affinity with certainty. Although these differences could contribute to decreased FT\(_{57.5}\) binding, the observation that FT\(_{57.5}\) binding approached saturation and failed to increase even at concentrations exceeding saturating doses (> 1.0 mg/mL) suggests that differences in affinity alone are unlikely to be responsible.

High-affinity platelet recognition sites have been localized to the carboxyterminal pentadecapeptide of the fibrinogen \( \gamma \) chain, \( \gamma_{400-411} \). This segment is composed of highly hydrophilic amino acids, with the exception of valine, and lacks any predominant secondary structure. It possesses the donor and acceptor sites for factor XIII\(_a\) catalyzed cross-linking, and is susceptible to proteolysis by plasmin. It has also been shown to inhibit fibrinogen binding to stimulated platelets and, when covalently linked to albumin at sufficiently high density, supports platelet aggregation.

Although the smallest \( \gamma \) chain peptide possessing platelet recognition activity is the pentapeptide \( \gamma_{400-411} \), optimum activity apparently requires either the dodecapeptide \( \gamma_{400-411} \) or the decapetide \( \gamma_{402-411} \). Several amino acids have been implicated as contributing directly to platelet recognition. Modification of lysine 406, for example, with acetyl anhydride can abolish the inhibitory effect of dodecapeptide \( \gamma_{400-411} \) on fibrinogen binding. It has been proposed that this lysine residue participates in the formation of a salt bridge with valine 411 or asparagine 410, which may stabilize a \( \gamma \) chain loop fitting the platelet fibrinogen receptor. Controversy persists regarding the importance of histidine 400 and 401.

Thus, most of the amino acids that have been specifically implicated as contributing to platelet recognition, with the possible exception of valine 411, are located beyond the \( \gamma_{408-411} \) sequence replaced by the hydrophobic 20 amino acid tail in FT\(_{57.5}\). The data, however, demonstrate that the interaction of FT\(_{57.5}\) with platelets is markedly decreased. It is possible that loss of valine 411 destabilizes the fibrinogen binding site sufficiently to decrease interaction via the \( \gamma_{57.5} \) chain, whereas the hydrophobic extension of the \( \gamma \) chain provides sufficient steric hindrance to prevent saturation of platelet receptors.

The function of elongated \( \gamma \) chains is as yet unknown. Their low concentration in plasma and their decreased support of platelet aggregation may suggest a teleologic assistance. Their function in plasma and their decreased support of platelet aggregation may suggest a teleologic selectivity for a more functional \( \gamma \) chain. Fibrinogen secreted from platelets, for example, has been reported to lack elongated \( \gamma \) 57.5 chains, and to be more effective than plasma fibrinogen in supporting ADP-induced and epinephrine-induced aggregation. The low concentrations of elongated \( \gamma \) chains present in plasma, however, are unlikely to exert a significant effect on platelet aggregation in vivo.

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