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 (Fγ10), 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 (Fγ10,γ57.5), with the longer γ chain (γ57.5) possessing an amino acid extension at the carboxyterminal end. The latter fibrinogen was 50% less effective than Fγ10 in supporting ADP-induced platelet aggregation at concentrations of .01 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 Fγ10,γ57.5 that was bound to platelets at saturation was only 50% that of Fγ10.

Fibrinogen receptors that remained unoccupied in the presence of saturating concentrations of Fγ10,γ57.5, however, could be occupied by fresh Fγ10. Excess unlabeled Fγ10 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/IIa complex. Furthermore, an intact GP Iib/IIa 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 Fγ10. The data demonstrate that heterodimeric Fγ10,γ57.5 binds less well to platelets and supports platelet aggregation only half as well as homodimeric Fγ10. 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 Fγ10,γ57.5 may sterically hinder the interaction of this fibrinogen with platelet receptors.

HUMAN plasma fibrinogen γ chains vary not only with respect to charge and sialic acid content, but also to mol wt. Two γ chain variants have been identified which differ not only in mol wt as shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) but also in their carboxyterminal amino acid sequence. Most plasma fibrinogen γ chains and all of the γ chains found in platelet fibrinogen are of mol wt 50,000 (γ50). In contrast, γ chains of mol wt 57,500 (γ57.5), also known as γ' chains, constitute ~5% of plasma fibrinogen γ chains and are absent from platelet fibrinogen preparations. The increase in mol wt of γ57.5 chains is due to an additional carboxyterminal amino acid sequence beginning four residues prior to the normal γ chain termination. An intermediate length γ chain variant, mol wt 55,000, has also been described and constitutes ~1.5% of total plasma fibrinogen γ chains.

The carboxyterminal region of the fibrinogen γ chain is rich in functional sites, for example, for calcium binding, factor XIIIa-catalyzed fibrin crosslinking, and platelet-fibrinogen interactions. Because structural variations may influence these functional properties, we compared the ability of fibrinogen possessing only γ50 chains (Fγ10) with fibrinogen possessing equal amounts of γ50 and γ57.5 chains (Fγ10,γ57.5) to bind to platelets and to support ADP-induced aggregation.

MATERIALS AND METHODS

Fibrinogen fractionation. Fibrinogen (Grade L, Kabi AB, Stockholm, Sweden) was chromatographed on a diethylaminoethane (DEAE) Sephacel column (Pharmacia Fine Chemicals, Piscataway, NJ) using a combined pH and ionic strength gradient. Fractions containing fibrinogen with only γ50 chains or with both γ50 and γ57.5 chains were pooled, concentrated by precipitation at 50% saturated ammonium sulfate, and dialyzed against 0.15 mol/L 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 ε-aminocaproic acid (EACA), and 0.02% sodium azide.

SDS-PAGE. Labeled and unlabeled Fγ10 and Fγ10,γ57.5 were reduced and examined by SDS-PAGE using 7% gels. Fibrinogen associated with platelets following platelet stimulation with 10 mmol/L of ADP was analyzed by SDS-PAGE after gel-filtered platelets (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).

Removal of von Willebrand factor. Prior to chromatography, the fibrinogen preparation at a concentration of 1 mg/mL contained 0.15 U/mL of von Willebrand factor (vWF) by Laurell electrophromunoassay, whereas Fγ10 contained no detectable vWF, and Fγ10,γ57.5 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 antisemur (Calbiochem, San Diego) in a ratio of 180 mg Protein A-Sepharose/mL of fibrinogen.

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Following stimulation with 10 Mmol/L ADP, the supernatant was removed after centrifugation. The buffer was changed to $0.15 \text{ mol/L of sodium chloride, } 0.05 \text{ mol/L of Tri-hydrochloric acid, } \text{pH 7.6, containing } 0.02 \text{ mol/L of EACA, } 10 \text{ U/mL of aprotinin and } 0.02\% \text{ sodium azide by chromatography on a column of Sepharose G-100 (Pharmacia). The final preparation contained no detectable vWF by Laurell immunonanassy.}^{23} \text{ No change in the migration of bands as determined by SDS-PAGE was noted following this procedure.}

**Fibrinogen characterization.** The γ chain composition of F$^{150}$ and F$^{150,312}$ was confirmed by SDS-PAGE. The γ chains of F$^{150}$ migrated as a single band with mol wt 50,000, whereas the γ chains of F$^{150,312}$ were equally distributed among bands of mol wt 50,000 and mol wt 57,500 (Fig 1). The αa chain degradation of both F$^{150}$ and F$^{150,312}$ was comparable, and as shown previously, insufficient to influence fibrinogen binding or platelet aggregation.

**Fibrinogen iodination.** Purified F$^{150}$ and F$^{150,312}$ were labeled with $^{125}\text{T}$ using chloramine. Following iodination, 99% of the radioactivity associated with fibrinogen preparations precipitated in 10% trichloroacetic acid, and 94% to 98% was chottable 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 mol/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 fibrinogen lacking elongated γ chains. Platelet-rich plasma was obtained by centrifugation (280 g, 15 minutes). GFP were prepared as described previously. EDTA-treated and control, Ca-EDTA-treated GFP were prepared as described by Zucker and Grant.²⁶

**Fibrinogen binding.** Platelets stimulated with 10 μmol/L of ADP were incubated with increasing concentrations of radiolabeled fibrinogen for two minutes, after which platelet-bound fibrinogen was separated from free fibrinogen by centrifugation of platelets through silicone oil.²⁵ The results were analyzed using a Hewlett Packard (Corvallis, OR) HP-85 curve-fitting program according to the method of Scatchard²⁷ and as recommended by Klotz.²⁸ The interaction between ADP-treated GFP and radiolabeled fibrinogen was also evaluated at intervals during the first 60 minutes, and fibrinogen dissociation was measured after addition of EDTA (10 mmol/L final concentration) or excess unlabeled F$^{150}$ (10 mg/mL).

Displacement of platelet-bound radiolabeled fibrinogen by unlabeled F$^{150}$ was studied as follows. ADP-stimulated platelets were first incubated with radiolabeled fibrinogen. After two minutes, a tenfold excess of F$^{150}$ was added, and the amount of radioactivity remaining with platelets after 2, 15 and 30 minutes was quantified. Competition of unlabeled fibrinogen with radiolabeled F$^{150}$ for platelet receptors was studied by measuring the binding of trace amounts of radiolabeled F$^{150}$ to ADP-stimulated platelets in the presence of 0.05, 0.15, and 0.50 mg/mL of unlabeled F$^{150}$ and F$^{150,31}$. In other studies, platelets were incubated for two minutes with near-saturating concentrations of unlabeled F$^{150}$ or F$^{150,31}$ (0.8 to 1.0 mg/mL), and the binding of $^{125}\text{T-F}^{150}$ added subsequently to achieve a final concentration of 1.0 mg/mL, was quantified.

The platelet membrane binding sites for F$^{150}$ and F$^{150,31}$ were investigated by incubating GFP (five minutes, 22 °C) with a monoclonal antibody specific for the GPIIb/IIIa complex (10E5, 20 μg/mL final concentration) (a generous gift from Dr Barry Coller, SUNY, Stony Brook).²⁹ Thirty binding of radiolabeled F$^{150}$ and F$^{150,31}$ was evaluated two minutes after platelet stimulation with 20 μmol/L.

**Platelet aggregation.** Platelet aggregation studies were performed using a dual-channel aggregometer (Chrono-Log Corp, Havertown, Pa). GFP (0.45 mL) were stimulated with 10 μmol/L of ADP (Sigma Chemical Co, St Louis) in the presence of either 50 μL F$^{150}$ or F$^{150,31}$ diluted with 0.15 mol/L of sodium chloride to achieve fibrinogen concentrations ranging from 0.01 to 2.0 mg/mL in the final platelet suspensions. The buffer used for dialysis of the fractionated fibrinogen preparations had no effect on the slope or extent of platelet aggregation.

**Fibrinogen binding.** Platelets incubated with 10 μmol/L of ADP were incubated with increasing concentrations of radiolabeled fibrinogen for two minutes, after which platelet-bound fibrinogen was separated from free fibrinogen by centrifugation of platelets through silicone oil. The results were analyzed using a Hewlett Packard (Corvallis, OR) HP-85 curve-fitting program according to the method of Scatchard and as recommended by Klotz. The interaction between ADP-treated GFP and radiolabeled fibrinogen was also evaluated at intervals during the first 60 minutes, and fibrinogen dissociation was measured after addition of EDTA (10 mmol/L final concentration) or excess unlabeled F$^{150}$ (10 mg/mL).

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**Table 1. ADP-Induced Platelet Aggregation Supported by Increasing Concentrations of F$^{150}$ and F$^{150,31}$.**

<table>
<thead>
<tr>
<th>Fibrinogen (μg/mL)</th>
<th>Platelet Aggregation (Initial Slope)* in the Presence of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F$^{150}$</td>
</tr>
<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 show in Fig 3 to illustrate the markedly decreased aggregation of ADP-treated platelets in the presence of F20575. (A) and F50 (B).

**RESULTS**

**Aggregation cofactor activity.** The extent of ADP-induced primary aggregation supported by F50,575 was ~50% that of F0 at all fibrinogen concentrations tested (0.01 to 2.0 mg/mL) (Fig 2). The rate of platelet aggregation was similarly diminished (Table 1). A representative aggregometer tracing of GFP stimulated with 10 mmol/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 F50,575.

**Binding to ADP-treated GFP.** Both fibrinogens bound to platelets in a specific and saturable manner (Fig 4), but 50% less F50,575 than F50 bound at saturation. Platelet receptors that remained unoccupied in the presence of F50,575, however, were accessible to F50 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 unlabelled F50 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 unlabelled F50 or 10 mmol/L of EDTA after 60 minutes of binding.

Both unlabeled fibrinogen preparations competed with trace amounts of labeled F50 for platelet receptors (Table 4), although F50,575 competed less effectively. Approximately 1.5-fold more labeled F50 bound to platelets in the presence of 0.05 mg/mL of F50,575 than in the presence of F50. This ratio increased to 2.0 at concentrations of unlabeled fibrinogens (0.5 mg/mL) that approached saturation of the platelet receptors. These observations are consistent with the overall 50% decrease in F50,575 binding to stimulated platelets as compared to F50 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 (Fig 7). ADP-treated

**Table 2. Binding of 125I-F50 or 125I-F50,575 to ADP-Treated Platelets Preincubated With Near-Saturating Concentrations of Unlabeled F50 and F50,575**

<table>
<thead>
<tr>
<th>Fibrinogen Bounda</th>
<th>Preincubation Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>F50</td>
<td>F50,575</td>
</tr>
<tr>
<td>Isotonic saline</td>
<td>31,176 ± 12,365</td>
</tr>
<tr>
<td>F50,575</td>
<td>19,911 ± 8,147</td>
</tr>
<tr>
<td>F50</td>
<td>544 ± 611</td>
</tr>
</tbody>
</table>

Gel-filtered platelets were preincubated in the presence of saline, 1.0 mg/mL of unlabeled F50,575 or F50 and 10 mmol/L of ADP. After two minutes, 125I-labeled F50, or F50,575 (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 10⁵ platelets, mean ± SD, n = 3.

**Table 3. Fibrinogen Binding to ADP-Stimulated Platelets as a Function of Time**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>p50</th>
<th>p50,575</th>
<th>Unlabeled F50 Bound (10 mg/mL)</th>
<th>EDTA (10 mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>98 ± 2</td>
<td>100 ± 1</td>
<td>97 ± 5</td>
<td>94 ± 7</td>
</tr>
<tr>
<td>2</td>
<td>100 ± 4</td>
<td>97 ± 3</td>
<td>90 ± 0</td>
<td>96 ± 3</td>
</tr>
<tr>
<td>5</td>
<td>99 ± 3</td>
<td>100 ± 2</td>
<td>99 ± 0</td>
<td>98 ± 2</td>
</tr>
<tr>
<td>15</td>
<td>98 ± 4</td>
<td>99 ± 3</td>
<td>99 ± 0</td>
<td>99 ± 2</td>
</tr>
<tr>
<td>30</td>
<td>99 ± 4</td>
<td>99 ± 3</td>
<td>99 ± 0</td>
<td>99 ± 2</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.
platelets bound 53,741 ± 15,653 (mean ± SD) molecules of F\(^{\gamma 50}\) and 28,563 ± 13,419 molecules of F\(^{\gamma 50.57.5}\) at saturation. Similar results were obtained with non-aspirin-treated platelets (Table 6), although the overall fibrinogen binding was increased as reported by others.\(^{34}\)

Although vWF was present in unfractionated fibrinogen and F\(^{\gamma 50.57.5}\), and it can bind to stimulated platelets,\(^{33,36}\) little binds in the presence of fibrinogen.\(^{37,38}\) To confirm this, studies were performed using fibrinogen preparations free of vWF, and it can bind to stimulated platelets,\(^{33,36}\) little binds in the presence of fibrinogen.\(^{37,38}\) To confirm this, studies were performed using fibrinogen preparations free of vWF (data not shown). ADP-induced platelet aggregation was not measurably affected by the presence or absence of vWF, and the binding of labeled F\(^{\gamma 50}\) or F\(^{\gamma 50.57.5}\) with contaminating vWF could be completely inhibited by a 100-fold excess of unlabeled, vWF-free F\(^{\gamma 50}\) or F\(^{\gamma 50.57.5}\), respectively. Moreover, isotope dilution experiments using labeled F\(^{\gamma 50}\) and F\(^{\gamma 50.57.5}\) containing trace amounts of contaminating vWF and unlabeled F\(^{\gamma 50}\) and F\(^{\gamma 50.57.5}\) free of vWF showed that platelet binding of the labeled and unlabeled fibrinogens was indistinguishable.

**DISCUSSION**

The predominant \(\gamma\) chain species of normal human fibrinogen has a relative mol wt of ~50,000 (\(\gamma 50\)), whereas higher mol wt variants have been described recently with mol wt of 55,000 (\(\gamma 55\)) and 57,500 (\(\gamma 57.5\)). The increase in mol wt of the \(\gamma 57.5\) chain is due to an elongated carboxyterminal amino acid sequence in which the carboxyterminal four amino acids of the \(\gamma 50\) chain are replaced by a negatively charged 20 amino acid sequence.\(^9\) Because the \(\gamma\) chains of human fibrinogen appear to possess the major determinants supporting platelet-fibrinogen interactions,\(^{39}\) studies were conducted to compare the ability of a fibrinogen preparation containing only mol wt 50,000 \(\gamma\) chains (F\(^{\gamma 50}\)) and a fibrinogen preparation possessing approximately equal amounts of \(\gamma\) chains of mol wt 50,000 and mol wt 57,500 (F\(^{\gamma 50.57.5}\)) 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\(^{\gamma 50.57.5}\) is a heterodimer composed of molecules with one \(\gamma 50\) and one \(\gamma 57.5\) chain.\(^{10,11}\)

Compared to F\(^{\gamma 50}\), F\(^{\gamma 50.57.5}\) 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\(^{39}\)
indicating that fibrinogen which possessed approximately equal amounts of mol wt 50,000 and elongated mol wt 57,500 γ chains was only 50% as effective in supporting ADP-induced platelet aggregation at low concentrations (64 μg/mL) and bound less well to platelets than fibrinogen which contained two γ 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 F²⁰⁵⁷⁵ and its decreased binding to exposed platelet membrane receptors over a range of fibrinogen concentrations from 0.01 to 2.0 mg/mL. More-
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Fibrinogen binding to human blood platelets: effect of gamma chain carboxyterminal structure and length

EI Peerschke, CW Francis and VJ Marder