Characterization of the γ Chain Platelet Binding Site on Fibrinogen Fragment D

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Glycoprotein (GP) IIb/IIIa on adenosine diphosphate (ADP)-activated human platelets interacts with specific sites on the fibrinogen molecule leading to aggregation. We characterized the platelet-binding site on the γ chains of fibrinogen using plasminic fragments DγA and Dγ′. Fragment DγA, which contains the carboxy terminal γA400-411 platelet-binding sequence (HHLGGAKQAGDV), was 70-fold more active than the synthetic γA400-411 peptide in inhibiting ADP-induced platelet aggregation. Fragment DγA inhibited fibrinogen binding and also bound directly to ADP-activated platelets. The Kd values determined for fibrinogen and fragment DγA binding were 0.55 μmol/L and 1.2 μmol/L, respectively. In contrast, fragment Dγ′, which differs from fragment DγA with respect to its γ chain sequence from position 408 to the COOH-terminus at position 427, did not inhibit platelet aggregation or fibrinogen binding, and did not bind directly to the platelet surface. Denaturation of fragment DγA with guanidine-HCl caused a loss of inhibitory activity in platelet aggregation assays. These data indicate that the native conformation of the γ chain platelet-binding site on fibrinogen is important for optimal binding to GPIIb/IIIa.

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aminomethyl (DEAE)-cellulose chromatography (DE-52 resin; Whatman, Hillsboro, OR) using the Pharmacia FPLC system (Phar- 
macia, Piscataway, NJ).

D fragments were prepared from plasmin digests of peak 1 or peak 2 fibrinogen at 3 to 4 mg/mL in 50 mmol/L tris(hydroxy-
ethyl)aminomethane (Tris)-HCl, 150 mmol/L NaCl, 10 mmol/L 
CaCl₂, pH 8.6 buffer that had been digested for 40 minutes at 37°C. 
At this time all fibrinogen had been digested to core D and E 
fragments. Fragment DY₈ and fragment DY' were isolated from 
digests of peak 1 and peak 2 fibrinogen, respectively, using 
DEAE-cellulose chromatography. Owing to its more negatively 
charged γ chain, fragment DY' was well separated from fragment 
DY₈. The D fragments were concentrated by Amicon ultrafiltration 
(Amicon, Danvers, MA). By contrast, fragment DY₈ preparations 
concentrated by ammonium sulfate precipitation lost consid-
erable capacity to interact with platelets and had a circular 
dichroism (CD) spectrum similar to guanidine-denatured fragment 
DY₈ (data not shown). The purity and intactness of fragments were 
determined using a Packard Multi-Prias 4 gamma counter (Pack-
ard Instrument Co, Downers Grove, IL). Specific binding was 
determined by subtracting values for total ligand binding quanti-
ted in platelet pellets from nonspecific binding in the presence of 
either excess unlabeled ligand or the absence of ADP. Background 
values were obtained in the absence of ADP.

Peptides. Synthetic peptides corresponding to γ₁₄₀₀-₄₁₁ and 
γ₁₄₀₀-₄₂₇ were obtained from both Bachem, Inc (Torrance, CA) 
and The Peptide Synthesis laboratory of The Blood Center of 
Southeastern Wisconsin. The peptides from Bachem were synthe-
sized using T-BOC chemistry followed by HF cleavage and reverse-
phase high performance liquid chromatography (HPLC). The 
peptides from the Blood Center were prepared on a Milligen 9050 
automated peptide synthesizer (Millipore, Bedford, MA) using the 
F-moc polyamide continuous flow method.²⁸ Peptides from both 
sources were purified to greater than 97% purity by HPLC in 
a single peak. Analysis of peptides from Bachem included peptide 
sequencing and Fast Atom bombardment mass spectral analysis, 
and the peptides from the Blood Center were verified by amino 
acid composition. The total peptide preparations, each containing 
10 mg of peptide, were dissolved in platelet resuspension buffer 
without BSA to a final concentration of 5 mmol/L. Peptides from 
both sources behaved identically.

Far ultraviolet circular dichroism (CD) measurements. CD spec-
tra were recorded on a JASCO J500A spectropolarimeter (Easton, 
MA), interfaced to an IBM-compatible computer. Nondenatured 
D fragment preparations were dialyzed extensively against 50 
mmol/L sodium phosphate, pH 8.6 before analysis. Fragment DY₈ 
was denatured by dialysis against 200 mmol/L Tris-HCl, 5 mol/L 
guanidine-HCl, pH 8.6, followed by 50 mmol/L sodium phosphate, 
pH 8.6. Samples were transferred to 0.2-mm path length cylindrical 
quartz cells, and were scanned at a speed of 5 nm/min at a gain of 
20 mdeg/full scale, and a 2 second time constant. The solvent 
background obtained under identical experimental conditions was 
subtracted from protein CD data. Conversion of CD data to mean 
residue ellipticity assumed a mean residue molecular weight of 
115.²⁹ The estimation of protein secondary structure distribution 
was performed using CONTIN software³⁰ on a Digital Equipment 
Corporation VAX computer at Marquette University (Milwaukee, 
WI).

RESULTS

Fragments DY₈, and DY', or the γ₁₄₀₀-₄₁₁ and γ₁₄₀₀-₄₂₇ 
peptides, were tested over a range of concentrations for their 
effect on ADP-activated, fibrinogen-mediated aggrega-
tion of washed human platelets (Fig 1). Fragment DY₈ (Fig 
1A) and γ₁₄₀₀-₄₁₁ (Fig 1B) were effective inhibitors of 
aggregation, in contrast to fragment DY' and γ₁₄₀₀-₄₂₇, 
which did not inhibit significantly. The concentration re-
quired for 50% inhibition (IC₅₀) of the rate of platelet 
aggregation by fragment DY₈ in the presence of 0.44 
mmol/L fibrinogen was 1.76 μmol/L; for γ₁₄₀₀-₄₂₇, it was 
130 μmol/L.

We showed a correlation between the native conformation 
of fragment DY₈ and its ability to inhibit platelet aggregation. 
Nondenatured fragment DY₈, and guanidine-
denatured fragment DY₈ were examined for secondary 
structural changes by far ultraviolet CD spectropolarimer-
ometry (Fig 2, inset). A comparison of the CD spectra showed an 
increase in random structure for denatured fragment DY₈ 
concomitant with a decrease in α-helix and β-sheet struc-
ture. The CD spectrum of nondenatured fragment DY₈ was 
essentially the same as that of nondenatured fragment DY₈.

When DY₈ preparations were tested for inhibitory activity in 
platelet aggregation assays, denatured fragment DY₈ did 
not inhibit at 3 μmol/L (the limit of solubility), whereas
fibrinogen was 0.55 ± 0.05 μmol/L, with 78,900 ± 900 molecules bound/platelet at saturation. The kd for fragment DγA was 1.2 ± 0.11 μmol/L, with 48,500 ± 2,100 molecules bound/platelet.

**DISCUSSION**

We have studied the interaction between fragments DγA or Dγ', and ADP-activated platelets as a means of characterizing the γ chain platelet-binding site on fibrinogen in its native configuration. Experiments using fragment Dγ' and the γ'400-427 peptide have further addressed the identification of the primary sequence of amino acids that comprise the γ chain platelet-binding site. Studies by Kloczewiak et al.,15,16 who first identified the platelet-binding sequence as extending from amino acids 400-411 on the γA chain, showed that the sequence from 407 to 411 was more effective as an inhibitor of platelet-fibrinogen interactions than that from 400 to 406. Furthermore, fibrinogen fragment D containing this sequence was shown to compete by inhibiting fibrinogen-supported platelet aggregation. Plow et al24 discussed the importance of the γA408-411 sequence (AGDV) as a potential adhesive sequence analogous to RGDV and the necessity of the DV sequence for platelet GPIIb/IIIa interaction. In this context, the AGDV sequence is replaced in the γ' chain variant by a different sequence.12 Peak 2 fibrinogen molecules that have one γA and one γ' chain only bind 50% as well to ADP-activated platelets as do peak 1 fibrinogen molecules having two γA chains.25-27 Our experiments showed that monovalent fragment Dγ' neither inhibited ADP-induced platelet aggregation (Fig 1), fibrinogen binding to ADP-activated platelets (Fig 3), nor did it bind directly to the platelet surface (Fig 4). In contrast, fragment DγA was highly reactive in each experiment. Not only do these experiments directly confirm the important contribution by γA408-411 to the formation of the γA chain platelet-binding site, but they also provide strong evidence that fragment DγA contains no other significant platelet-binding site.

Our experiments have directly addressed the subject of conformation as an important characteristic of the γA chain platelet-binding site. Fragment DγA was over 70-fold more effective as an inhibitor of platelet aggregation than the synthetic peptide γA400-411 (Fig 1). The affinity of fragment DγA for ADP-activated platelets (kd = 1.2 μmol/L; Fig 4) was 27.5 times greater than that determined for the synthetic peptide by Andrieux et al.18 (kd = 33 μmol/L). Because fragment DγA contains no additional platelet reactive sites other than the γA400-411 sequence, we attributed the different reactivities to the more well-defined conformation assumed by the sequence in the fragment compared with the peptide. In our experiments, we were able to correlate the reduction of α-helix and β-sheet structure in guanidinedenatured fragment DγA with a decreased ability to inhibit platelet aggregation (Fig 2). Marguerie et al.15 first reported the ability of fragment D1 to inhibit ADP-activated platelet aggregation, but they required a 1,000-fold molar excess to achieve 50% inhibition (compared with a fourfold molar excess in our present

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**Fig 1.** The effect of fragment DγA, fragment Dγ', γA400-411, and γ'400-427 on ADP-activated platelet aggregation. Platelet aggregation was measured using an aggregometer and was initiated by the addition of 0.44 μmol/L fibrinogen and 20 μmol/L ADP, in the presence or absence of fibrinogen derived D fragments and γ chain peptides. The rate and extent of aggregation is indicated by open and closed symbols, respectively. (A) Fragment DγA compared with fragment Dγ'; (շ) rate, fragment DγA; (כ) extent, fragment DγA; (צ) rate, fragment Dγ'; (ד) extent, fragment Dγ'; (ה) rate, γA peptide; (ו) extent, γA peptide; (ז) rate, γ' peptide; (ח) extent, γ' peptide.

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non-denatured fragment DγA retained significant inhibitory activity (Figs 1 and 2).

We examined the dose-dependent ability of fragment DγA to interfere with fibrinogen binding to ADP-activated platelets in comparison with fragment Dγ' (Fig 3). Fragment DγA interfered with the binding of fibrinogen to platelets and exhibited an IC₅₀ of 2.5 μmol/L in the presence of 0.44 μmol/L fibrinogen. Fragment Dγ' had no measurable effect.

When we assessed the direct binding of 125I-fibrinogen, 125I-fragment DγA, and 125I-fragment Dγ' to ADP-activated, washed platelets, specific and saturable binding of both fibrinogen and fragment DγA to platelets was shown. Fragment Dγ' did not bind (Fig 4). Scatchard analyses for fibrinogen and fragment DγA binding provided dissociation constants (kd) and the number of molecules of each ligand bound/platelet at saturation (Fig 4, inset). The kd for
Denaturation of their fragment during its isolation may explain the disparity between these results. Cierniakiewski et al.\textsuperscript{40,41} reported that the COOH-terminus of the $\gamma$ chain assumes a more constrained conformation in both native fibrinogen and fragment $D_1$ than it does in a small peptide.

The use of native, monovalent fibrinogen fragments has enabled us to provide the best approximation to date of the physiologic interaction between the GPIIb/IIIa receptor on activated platelets and the $\gamma_A$400-411 platelet-binding ligand. The $k_d$ of fibrinogen for ADP-activated platelets represents a measure of the average affinity for platelet GPIIb/IIIa of multiple ligands present on whole fibrinogen. Using fragment $D_{\gamma A}$, we were able to characterize the affinity of a single monovalent ligand in a native state. The relatively small magnitude of difference between $k_d$ values for fibrinogen and fragment $D_{\gamma A}$ indicates, as previously suggested,\textsuperscript{10,15,16} that the $\gamma_A$ chain site is a strong contributor to the interaction between fibrinogen and platelet GPIIb/IIIa. The slightly lower $k_d$ for fragment $D_{\gamma A}$ may reflect (1) a slight relaxation of conformation of the $\gamma_A$ chain site in fragment $D_{\gamma A}$ compared with that in fibrinogen, (2) the presence of only one $\gamma_A$ chain per molecule of fragment $D_{\gamma A}$ versus two $\gamma_A$ chains per molecule of peak 1 fibrinogen, or (3) the absence of Aa chain platelet-reactive sites on fragment $D_{\gamma A}$. Additional experiments using native fibrinogen or Fragment $D_{\gamma A}$: (H) Fibrinogen; (0) fragment $D_{\gamma A}$; (△) fragment $D_{\gamma A}$. Inset, Scatchard plots for fibrinogen and fragment $D_{\gamma A}$ binding were derived from specific binding isotherms using the ENZFITTER program. (H) Fibrinogen; (0) fragment $D_{\gamma A}$.
fibrinogen fragments containing isolated Aα chain binding sites and normally circulating, high-solubility derivatives of fibrinogen having truncated Aα chains will enable us to fully characterize the molecular events leading to the formation of the primary hemostatic plug.

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Characterization of the gamma chain platelet binding site on fibrinogen fragment D

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