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 plasmic 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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METHODS AND MATERIALS

Reagents. Bovine serum albumin (BSA)-RIA grade, fraction V; prostaglandin E1 (PGE1); apyrase-grade III from potatoes; adenosine-5'-diphosphate (ADP)-grade X from equine muscle were purchased from Sigma Chemical Co (St Louis, MO). Tris-HCl was purchased from Aldrich Chemical Co, Inc (Milwaukee, WI). N-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Trasylol was purchased from Mobay Chemical Corp (New York, NY). NaCl25 was purchased from Dupont-New England Nuclear (Boston, MA).

Preparation of purified human fibrinogen and fragments. Human fibrinogen fraction I-2 was isolated from pooled plasma according to the procedure of Mosesson and Sherry.22 Band I fibrinogen with intact Aa chains was isolated from fraction I-2 by precipitation with 16% saturated ammonium sulfate,23 dissolved in 0.3 mol/L NaCl, and stored at −70°C until use. Fibrinogen preparations were routinely assayed for coagulability24 and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).25 Peak 1 fibrinogen, containing two γA chains, and peak 2 fibrinogen containing one γA chain and one γ' chain,11,16 were isolated by diethyl-acetic acid COOH-terminal sequence from amino acids 408-427,12,14,22-24 Available evidence suggests that the γ' sequence does not contain a platelet-binding site.25-27

Participation of Aa chains in platelet-fibrinogen interactions have also been reported,25,26,27 Arg-Gly-Asp (RGD), the shortest sequence that can support cell attachment,28 is found as RGDF at Aa 95-98 and as RGDS at Aa 572-575 in fibrinogen.31 Synthetic peptides containing RGDS or RGDF, and antibodies against either peptide, inhibit the interaction between fibrinogen and platelets.12,13,52-55 RGDS and γA400-411 interact directly with GPIIb/IIIa, most likely through mutually exclusive binding sites on the receptor complex.16,21,36-38 Furthermore, peptide derivatives of RGDS and HHLGGAKQAGDV have been directly crosslinked to GPIIb/IIIa,36,38 thus permitting identification of specific regions on GPIIb/IIIa to which these peptides are bound.36,39

Synthetic peptides and antibodies17,21,32,39 have been useful for characterizing the location of specific sites on fibrinogen that interact with platelets. Some studies have shown that fibrinogen fragment D1 (containing the γ chain platelet-binding sequence) inhibits fibrinogen-supported platelet aggregation.15,16 However, the interaction between GPIIb/IIIa on intact platelets and specific native platelet reactive sites on fibrinogen has not been evaluated. To address this question, we have undertaken studies designed to evaluate platelet-reactive sites arranged in more native configurations. The present report describes the characterization of the γA chain platelet-binding site within plasmic fibrinogen fragment DγA. This fragment contains the γA400-411 platelet-binding sequence, does not contain either Aa RGD sequence, and has been shown to retain much or all of its native conformation.30,31 Fragment DγA binding to platelets, and the inhibition of ADP-induced platelet aggregation were assessed and compared with that of fragment Dγ' containing the variant γ' COOH-terminal sequence.

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Submitted June 4, 1991; accepted January 22, 1992.

Supported by American Heart Association, Wisconsin Affiliate Grant No. FA-26, American Heart Association Grant No. 900998, and NHLBI Grants No. HL-28444 and HL-47000.

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0006-4971/92/7910-0022$3.00/0


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aminomethane (Tris)-HCl, 150 mmol/L NaCl, 10 mmol/L 
man, Hillsboro, OR) using the Pharmacia FPLC system (Phar-
DEAE-cellulose chromatography. Owing to its more negatively 
At this time all fibrinogen had been digested to core D and E 
monitored by disulfide-reduced and nonreduced SDS-PAGE on 
fragments. Fragment D\textsubscript{Y}A and fragment D\textsubscript{Y}' were isolated from 
charges concentrated by ammonium sulfate precipitation lost consid-
sulfate precipitation lost considerable 
by contrast, fragment D\textsubscript{Y}A preparations 
and had a circular 
whereas 

**Platelet studies.** Venous blood drawn into acid-citrate dextrose 
acantoagulant was obtained from normal volunteers who had given 
prepared from platelet-rich 
by differential centrifugation in the presence of 
and PGE\textsubscript{1}, essentially as described by Kiniough-
Platelet suspensions were adjusted to 3 
aggregation was monitored using a Chronolog Model 560 whole 
was denatured by dialysis against 200 mmol/L Tris-HCl, 5 mol/L 
fragment D\textsubscript{Y}' was well separated from fragment 
results were purified to greater than 97% purity by HPLC in a 
automated peptide synthesizer (Millipore, Bedford, MA) using the 
from both Bachem, Inc (Torrance, CA) and The Peptide Synthesis laboratory of The Blood Center of 
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FIBRINOGEN γ CHAIN PLATELET BINDING

Fig 1. The effect of fragment DyA, fragment Dy', 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 DyA compared with fragment Dy'; (○) rate, fragment DyA; (□) extent, fragment DyA; (●) rate, fragment Dy'; (●) extent, fragment Dy'; (●) rate, fragment Dy; (●) extent, fragment Dy. (B) Peptide γA400-411 compared with γ'400-427: (○) rate, γA peptide; (□) rate, γ' peptide; (△) rate, γ' chain peptide; (▲) extent, γ chain peptide; (●) extent, γ' chain peptide.

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

We examined the dose-dependent ability of fragment DyA to interfere with fibrinogen binding to ADP-activated platelets in comparison with fragment Dy' (Fig 3). Fragment DyA 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 Dy' had no measurable effect.

When we assessed the direct binding of 125I-fibrinogen, 125I-fragment DyA, and 125I-fragment Dy' to ADP-activated, washed platelets, specific and saturable binding of both fibrinogen and fragment DyA to platelets was shown. Fragment Dy' did not bind (Fig 4). Scatchard analyses for fibrinogen and fragment DyA binding provided dissociation constants (kd) and the number of molecules of each ligand bound/platelet at saturation (Fig 4, inset). The kd for fibrinogen was 0.55 ± 0.05 μmol/L, with 78,900 ± 900 molecules bound/platelet at saturation. The kd for fragment DyA was 1.2 ± 0.11 μmol/L, with 48,500 ± 2,100 molecules bound/platelet.

DISCUSSION

We have studied the interaction between fragments DyA or Dy', and ADP-activated platelets as a means of characterizing the γ chain platelet-binding site on fibrinogen in its native configuration. Experiments using fragment Dy' 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 al.54 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 Dy' 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 DyA 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 DyA 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 DyA was over 70-fold more effective as an inhibitor of platelet aggregation than the synthetic peptide γA400-411 (Fig 1). The affinity of fragment DyA for ADP-activated platelets (kd = 1.2 μmol/L; Fig 4) was 27.5 times greater than that determined for the synthetic peptide by Andréux et al.26 (kd = 33 μmol/L). Because fragment DyA 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 guanidine-denatured fragment DyA with a decreased ability to inhibit platelet aggregation (Fig 2). Marguerie et al.55 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
Denaturation of their fragment during its isolation may explain the disparity between these results. Cierniowski et al. reported that the COOH-terminus of the γ chain assumes a more constrained conformation in both native fibrinogen and fragment D1 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 \( \gamma_{A400-411} \) 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, that the \( \gamma \) 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 \( \gamma \) chain site in fragment \( D_{\gamma A} \) compared with that in fibrinogen, (2) the presence of only one \( \gamma \) chain per molecule of fragment \( D_{\gamma A} \) versus two \( \gamma \) chains per molecule of peak 1 fibrinogen, or (3) the absence of \( \alpha \) chain platelet-reactive sites on fragment \( D_{\gamma A} \). Additional experiments using native fibrinogen or fragment D [ [pmol/L] ]

\[ % \text{Fibrinogen Bound} \]

\[ \text{Fibrinogen or Fragment D [ [pmol/L] ]} \]

\[ \text{Pmol Bound/10^6 Platelets} \]

\[ \text{Fibrinogen or Fragment D [ [pmol/L] ]} \]

\[ \text{Scatchard plots for fibrinogen and fragment } D_{\gamma A} \text{ binding were derived from specific binding isotherms using the ENZFITTER program.} \]

\[ \text{Fibrinogen; } \text{(O) fragment } D_{\gamma A}; \text{ (m) fragment } D_{\gamma A} \text{ binding were derived from specific binding isotherms using the ENZFITTER program.} \]

\[ \text{Fibrinogen; } \text{(O) fragment } D_{\gamma A}; \text{ (m) fragment } D_{\gamma A} \text{ binding were derived from specific binding isotherms using the ENZFITTER program.} \]
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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