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Dissecting Clot Retraction and Platelet Aggregation

CLOT RETRACTION DOES NOT REQUIRE AN INTACT FIBRINOGEN γ CHAIN C TERMINUS*

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Fibrinogen mediates the processes of platelet aggregation and clot retraction. Previous studies have demonstrated that fibrinogen binding to the platelet receptor $\alpha_{IIb}\beta_3$ requires the C-terminal residues of the fibrinogen γ chain. We made a recombinant human fibrinogen that lacks the γ chain C-terminal four residues (AGDV). As expected this fibrinogen did not support platelet aggregation. Unexpectedly, this variant did support clot retraction that was indistinguishable from retraction with normal recombinant or plasma fibrinogen. These results suggest that the site on fibrinogen that is required for platelet aggregation differs from the site on fibrin that is required for clot retraction.

The processes of platelet aggregation and clot retraction are mediated by fibrinogen *in vitro* and play a role in maintaining hemostasis *in vivo*. Fibrinogen is a large (340 kDa) plasma protein consisting of two sets of A α , B β , and γ chains. The protein is arranged in a symmetrical fashion with two lateral D domains and a central E domain (1). Previous work employing electron microscopy (2), synthetic peptides (3), and recombinant proteins (4, 5) has implicated the D domain, in particular residues γ 408–411 (AGDV), as the site on fibrinogen that interacts with platelets. This interaction involves the platelet integrin $\alpha_{IIb}\beta_3$, a heterodimeric transmembrane receptor. Antibodies and peptides that prevent fibrinogen binding to $\alpha_{IIb}\beta_3$ also prevent platelet aggregation and clot retraction (6–8). Together, these findings predict that the $\alpha_{IIb}\beta_3$ recognition site on fibrinogen (*i.e.* residues γ 408–411) must be present and accessible for aggregation or clot retraction to occur. Two studies support this prediction with respect to platelet aggregation (4, 5), but both have limitations. In one (4), recombinant γ chains lacking residues 408–411 were unable to support platelet aggregation; however, this variant γ chain was studied outside the context of the entire molecule. In the other (5), recombinant fibrinogen with a 20-amino acid insert in place of these four residues was unable to support platelet aggregation. However, the lack of aggregation cannot be directly attributed

to the loss of the AGDV residues, as the additional 20 amino acids may sterically impair aggregation. Moreover, neither study explored the effects that these alterations may have had on clot retraction.

We report that an intact recombinant fibrinogen lacking only residues γ 408–411 does not support aggregation, consistent with previous work (4, 5), but unexpectedly supports clot retraction to the same extent as normal recombinant and plasma fibrinogen. These findings suggest that other domains in fibrinogen participate in clot retraction.

MATERIALS AND METHODS

Construction of Expression Vectors—pMLP-A α , pMLP-B β , and pMLP- γ encoding the A α , B β , and γ chains of human fibrinogen have been described previously (9, 10). The vector pMLP- γ 407 was constructed from pMLP- γ and p647, described in Hettasch *et al.* (4). The C-terminal nucleotides from the *Escherichia coli* expression vector p647(γ 407), which includes a stop codon at position 408, were removed as a *Hind*III to *Bst*XI fragment. The corresponding sites on pMLP- γ were cleaved with *Not*I and *Bst*XI. The *Not*I and *Hind*III sites were blunt ended with T4 polymerase. The fragment from p647 was ligated into the cleaved vector to give pMLP- γ 407. The resulting mammalian expression vector was sequenced using an Applied Biosystems automated sequencer (Foster City, CA). Restriction digests and general cloning procedures have been described (9).

Cell Culture—CHO¹ transfections and growth conditions have been described (9). Normal recombinant fibrinogen, the predominant form found in human plasma, was secreted from CHO cells containing the pMLP-A α , pMLP-B β , and pMLP- γ vectors. Cell line CHO- γ 407 was constructed by transfecting pMLP- γ 407 into CHO cells harboring pMLP-A α and pMLP-B β . Transfected cells were selected in medium containing G418 (Life Technologies, Inc.), a neomycin analog, and histidinol (Aldrich). Fibrinogen production was monitored by enzyme-linked immunosorbent assay as described previously (9). Serum-free medium was pooled and stored at -20°C until the fibrinogen was purified.

Purification of Recombinant Fibrinogen—Recombinant fibrinogen was purified from CHO serum-free medium as described (10). Briefly, fibrinogen was precipitated from the medium with 40% $(\text{NH}_4)_2\text{SO}_4$, and the precipitate was resuspended and purified by protamine-Sepharose chromatography. Purification was monitored by electrophoresis on 8% polyacrylamide gels by the method of Laemmli (11). Western blot analysis was performed as described (9) with a polyclonal antibody that reacts with fibrinogen and its degradation products (DAKO Corporation, Carpinteria, CA) and 4A5, a monoclonal antibody specific for the C terminus of the γ chain (12). Monoclonal antibody 4A5 was the generous gift of Dr. Gary Matsueda, Bristol-Myers Squibb Pharmaceuticals (Princeton, NJ). Plasma fibrinogen was purchased from Calbiochem (La Jolla, CA).

Isolation of Human Platelets—Human blood (60 ml) from healthy volunteers who had abstained from aspirin for 10 days was collected into polypropylene tubes containing 1:7 acid/citrate/dextrose anticoagulant. Platelet-rich plasma was obtained by centrifugation at $200 \times g$ for 25 min at ambient temperature. Prostaglandin E₁ (Sigma) was added to a final concentration of 20 nM. The platelet-rich plasma was then centrifuged at $800 \times g$ for 25 min, and the platelet pellet was resuspended in Tyrode's buffer, pH 7.2 (10 mM Hepes, 135 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 5.5 mM glucose, 2% bovine serum albumin (fraction V, Miles Pentex)). The platelets were applied to a Sepharose CL-2B column (Sigma) equilibrated with Tyrode's buffer and were eluted with the same buffer. The platelets were counted using a Coulter counter (Hialeah, FL) and adjusted to a final concentration of 2×10^8 ml⁻¹ in Tyrode's buffer with 1 mM Ca²⁺ and 2 mM Mg²⁺.

Platelet Aggregation Assay—Platelet aggregation in Tyrode's buffer was monitored with a platelet aggregometer (Chrono-Log). Fibrinogen (final concentration, 250 nM) was added to 0.4-ml platelet suspensions (2×10^8 platelets/ml) stirred at 37°C . Aggregation was monitored by

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¹ The abbreviation used is: CHO, Chinese hamster ovary.

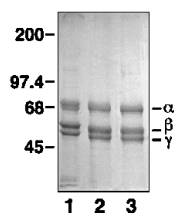


FIG. 1. Polyacrylamide gel electrophoresis on 8% gels run under reduced conditions according to the method of Laemmli (11) and developed with Coomassie Brilliant Blue R-250. Lanes: 1, plasma fibrinogen; 2, normal recombinant fibrinogen; 3, recombinant γ 407 fibrinogen.

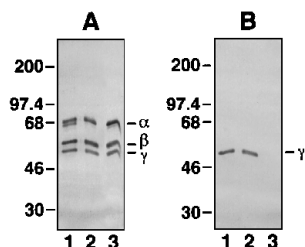


FIG. 2. Western blot analysis of recombinant fibrinogen under reduced conditions. An 8% polyacrylamide gel, run as in Fig. 1, was blotted onto a nitrocellulose membrane and developed as in Binnie *et al.* (9). Lanes: 1, plasma fibrinogen; 2, normal recombinant fibrinogen; 3, recombinant γ 407 fibrinogen. Primary antibodies were: A, rabbit polyclonal antiserum reactive with all three fibrinogen chains; B, 4A5, a monoclonal antibody specific for the C terminus of the γ chain (10).

the percent change in light transmission following activation with 10 μ M ADP (Sigma). Platelet aggregation in the absence of added fibrinogen or ADP was negligible.

Clot Retraction Assay—Clot retraction experiments were performed following the procedure of Tuszyński *et al.* (7). Gel-filtered platelets (2×10^8 platelets/ml) were preincubated at 37 $^{\circ}$ C for 10 min with 300 nM fibrinogen to give a final volume of 0.5 ml. Human α -thrombin, a generous gift of Dr. Frank Church, University of North Carolina (Chapel Hill, NC), was added to a final concentration of 0.5 unit/ml. The clots formed without stirring in <5 min and were incubated at 37 $^{\circ}$ C for 15 min. The tubes were transferred to ice and photographed 1 h later. An alternative method of measuring retraction involved a modified version of the Chen *et al.* procedure (8). Clot length and width were measured with a ruler every 2 min following the addition of thrombin and were used to calculate clot area. Control experiments were performed in the absence of platelets or the absence of added fibrinogen. Addition of thrombin to platelet suspensions without added fibrinogen did not result in clot formation, and addition of thrombin to fibrinogen solutions in the absence of platelets resulted in a fibrin clot that did not retract.

RESULTS AND DISCUSSION

We characterized the purified recombinant proteins by SDS-polyacrylamide gel electrophoresis and Western blots. SDS-polyacrylamide gel electrophoresis run under reducing conditions (Fig. 1) demonstrated that all three chains were present in both the normal recombinant fibrinogen and the γ 407 variant. Electrophoresis under non-reducing conditions showed that the chains assembled into a protein with a molecular mass of 340 kDa (data not shown). Western blot analysis with a polyclonal antibody (Fig. 2A) confirmed the presence of all three chains in both recombinant proteins. A duplicate blot developed with monoclonal antibody 4A5 (Fig. 2B), a monoclonal antibody specific for the C terminus of the γ chain, showed immunoreactivity with both plasma and normal recombinant fibrinogen but not with the γ 407 variant. Thus, the C-terminal epitope for 4A5 has been perturbed in the γ 407 variant. This result is consistent with the lack of γ chain residues 408–411, as predicted from the DNA sequence data of the γ chain expression plasmid.

We tested the recombinant proteins in platelet aggregation

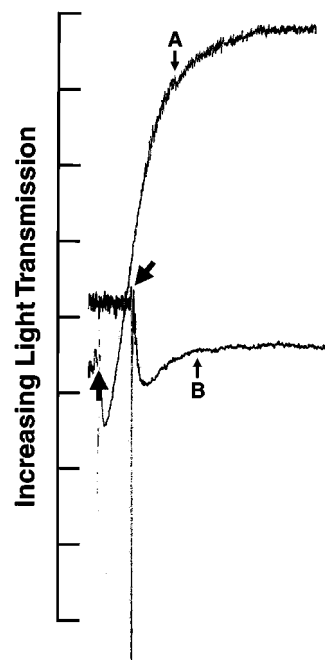


FIG. 3. ADP-induced aggregation of platelets. Platelets (2×10^8 platelets/ml) were preincubated at 37 $^{\circ}$ C with 250 nM fibrinogen prior to activation with 10 μ M ADP (indicated by arrows). Aggregation of stirred solutions was measured as the increase in light transmission. Curve A is for normal recombinant; curve B is for recombinant γ 407 fibrinogen.

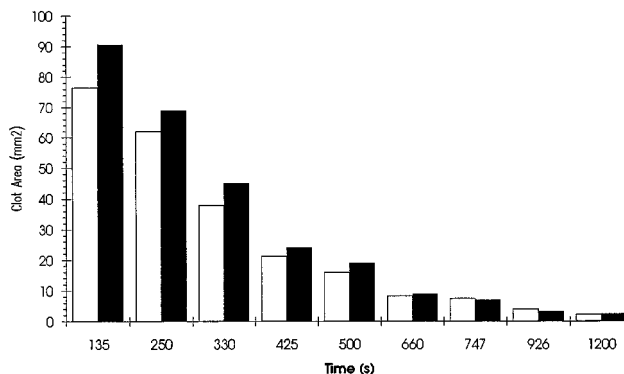


FIG. 4. Time course for clot retraction. Platelets (2×10^8 platelets/ml) were preincubated with 300 nM fibrinogen (to give a final volume of 0.5 ml) at 37 $^{\circ}$ C for 10 min prior to the addition of 0.5 unit/ml human α -thrombin. The clots were incubated at 37 $^{\circ}$ C, and length and width were measured in mm at the indicated times. The figure presents the average clot area ($n = 2$). White bars represent normal recombinant fibrinogen, and black bars represent γ 407 fibrinogen.

assays (Fig. 3). Platelets were activated upon addition of 10 μ M ADP, as indicated by the initial decrease in light transmission due to platelet shape change (13). Following activation, platelet samples in the presence of normal recombinant fibrinogen exhibited a dramatic increase in light transmission due to the formation of aggregates (14). In contrast, platelets in the presence of γ 407 changed shape but did not aggregate. Similar results were obtained with the addition of ADP to platelets in the absence of added fibrinogen (data not shown).

We examined clot retraction to see if the region on fibrinogen required for aggregation was also required for this seemingly related process. The clot formed in the presence of γ 407 was indistinguishable from the clots formed with plasma or normal recombinant fibrinogen. Clot retraction was followed for 20 min at 37 $^{\circ}$ C. Retraction rates were similar for both normal recombinant and γ 407 fibrinogen (Fig. 4). The final contracted clots formed with normal recombinant, γ 407, or plasma fibrinogen

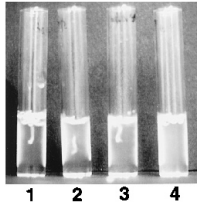


FIG. 5. **Photographs of the final retracted clots.** Platelets (2×10^8 platelets/ml) were preincubated with 300 nM fibrinogen (to give a final volume of 0.5 ml) at 37 °C for 10 min prior to the addition of 0.5 unit/ml human α -thrombin. The clots were incubated at 37 °C for 15 min and transferred to ice. The photograph was taken after 1 h. Tubes: 1, normal recombinant fibrinogen; 2, recombinant γ 407 fibrinogen; 3, plasma fibrinogen; 4, no added fibrinogen.

(Fig. 5) were also indistinguishable. When thrombin was added to platelet suspensions without added fibrinogen, there was no clot formation (Fig. 5). When thrombin was added to solutions of recombinant or plasma fibrinogen, fibrin clots formed but did not retract (data not shown). Thus, the residues AGDV on the γ chain of fibrinogen are apparently not required for clot retraction.

Because activation of platelets by thrombin results in the release of fibrinogen from the α -granules (15), it is possible that the platelet fibrinogen may contribute to the retraction of the clots. Therefore, we plan to perform clot retraction experiments using platelets from an afibrinogenemic patient, which will serve as a definitive control.

In the experiments discussed here, we used genetic engineering to produce a variant fibrinogen molecule to probe two seemingly related events. Consistent with previous results, removal of the C-terminal AGDV residues from the γ chain of fibrinogen abolished the ability of the protein to support platelet aggregation. Unexpectedly, deletion of these residues did not impair clot retraction. This finding apparently contradicts previous findings that implicate AGDV in binding to $\alpha_{IIb}\beta_3$ during clot retraction. We hypothesize that $\alpha_{IIb}\beta_3$ interacts with residues AGDV during aggregation and interacts with one or both of the consensus binding sequences for integrins (RGD) in the A α chain during clot retraction. It has been shown that the peptide RGDS can retard clot retraction (16). Using monoclonal antibodies, other investigators have shown that inaccessible sites on fibrinogen become accessible upon conversion to fibrin (17). Perhaps the RGD sites are not accessible in fibrin-

ogen but are exposed in the fibrin molecule. These regions on the A α chain may then interact with $\alpha_{IIb}\beta_3$ and mediate fibrin-platelet interactions, including clot retraction. Thus, the ligand site on fibrinogen that mediates aggregation may differ from the site on fibrin that mediates clot retraction. Yet, the platelet receptor for both processes is $\alpha_{IIb}\beta_3$ (7, 8, 18). We plan to test the hypothesized role of RGD in clot retraction by analysis of additional variant fibrinogens.

In summary, we find that the γ chain C-terminal residues AGDV are critical for platelet aggregation but not for clot retraction. These findings suggest a separate role for the consensus binding sequence RGD at either position 95–97 or 572–574 on the A α chain of fibrinogen. Finally, the γ 407 variant may also provide a means of differentiating between platelet signaling events involved in aggregation *versus* clot retraction.

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