Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 2068–2071, March 1982
Medical Sciences

γ and α chains of human fibrinogen possess sites reactive with human platelet receptors

(platelet aggregation/adhesion/antibody probe)

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Communicated by Grant W. Liddle, December 28, 1981

ABSTRACT **Fibrinogen, a clottable plasma protein, agglutinates both prokaryotic cells (e.g., staphylococci) and eukaryotic cell fragments (e.g., platelets) through interaction with specific receptors. To identify the region of the fibrinogen molecule responsible for its interaction with human platelets, we prepared polypeptide chain subunits (α, β, and γ) of human fibrinogen by reduction and carboxymethylation. A mixture of the chains induced aggregation (clumping) of human platelets separated from plasma proteins and treated with ADP. When individual chains of fibrinogen were tested, γ-chain multimers caused platelet aggregation at a molar concentration comparable with that of intact human fibrinogen. The β chain remained inactive, and the α chain was 1/4th to 1/5th as reactive as the γ chain. Monospecific antibody fragments against the γ chain inhibited binding of ^{125}I-labeled fibrinogen to the human platelet receptor and blocked aggregation of platelets induced by ADP in the presence of fibrinogen or γ-chain multimers. These results indicate that the γ chain of human fibrinogen bears the main site for interaction with the platelet receptor.**

Adhesion of platelets to the inner surface of an injured blood vessel wall and to each other plays a fundamental role in hemostasis and thrombosis. In normal hemostasis, adhering platelets seal off the ruptured vessel wall by forming a hemostatic plug that prevents excessive loss of blood (1). As is well known, the interaction of platelets with the vessel wall after injury requires plasma glycoproteins such as factor VIII/von Willebrand factor and fibrinogen. In congenital deficiencies of either of these glycoproteins, a hemorrhagic diathesis is noted in which the capillary bleeding time is prolonged (2, 3).

The essential role of fibrinogen in the adhesion and aggregation of platelets has gained considerable interest since the recent demonstration that fibrinogen binds to specific receptor sites on platelets. This binding occurs only when platelets are stimulated with certain agonists, such as ADP, epinephrine, and thrombin (4–7). Thus, the two central aspects of the platelet–fibrinogen interaction in adhesion and aggregation phenomena are (*i*) the nature of the platelet receptor for fibrinogen and (*ii*) localization of the binding region on fibrinogen that reacts with the platelet receptor. An overall structure for fibrinogen, which is comprised of three pairs of polypeptide chains (α, β, and γ) arranged in three general domains (one central E and two symmetrically positioned terminal D), has been described in terms of its covalent structure (8). Localization of the region on the fibrinogen molecule responsible for interaction with the platelet receptor remains unknown; this article reports a study designed to establish which structural subunit of fibrinogen participates in its interaction with receptors on human platelets.

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MATERIAL AND METHODS

Preparation of Human Fibrinogen, its Chains, and Fragments. Human fibrinogen was prepared from blood plasma (San Diego Bank, San Diego, CA) by cold ethanol precipitation (9). Alternatively, commercially available human fibrinogen (grade L, Kabi, Stockholm, Sweden) was used in some experiments. Before use, the low-solubility fraction of commercial fibrinogen was prepared by ammonium sulfate precipitation (10) and factor VIII contamination was removed by gel filtration on Sepharose 2B, resulting in a small peak of high-molecular-weight material appearing in the void volume, which was discarded. Polypeptide chains of fibrinogen were purified as described (11, 12). Rabbits were immunized against purified human fibrinogen and isolated fibrinogen chains, as described (13). IgG fractions were prepared by precipitation with ammonium sulfate and chromatography on Sephadex G-150 and then were passed through affinity columns made of fibrinogen or individual chains and Sepharose 4B. Antibody $F(ab)_2$ fragments were prepared by pepsin digestion according to the general procedure of Nisonoff (14) and Fab fragments were prepared according to Porter's method (15) as described elsewhere (13).

Preparation of Human Platelets. Platelets were isolated from human blood drawn freshly from healthy volunteers who were fasting on the morning of blood collection and had abstained from taking aspirin or other medication during the preceding 10 days. Platelets were immediately separated from plasma proteins by stepwise albumin gradient centrifugation and Sepharose 2B gel filtration as described (16). Platelets were suspended in Hepes, pH 7.35/0.1% dextrose/0.35% albumin and used within 2 to 3 hr after blood collection. The platelet preparation was adjusted to a final count of 1×10^8/0.5 ml.

Platelet Aggregation. Aggregation of platelets was measured photometrically in a Payton dual channel aggregometer (Payton Associates, Buffalo, NY), according to Born's method (17). Results were recorded as maximal light transmission (T_{max}), slope value, or both (18).

Binding of ^{125}I-Labeled Fibrinogen to Human Platelets. This was done at room temperature without stirring, as described (7).

RESULTS AND DISCUSSION

Localization of Platelet-Reactive Site on Fibrinogen Chains. Our initial studies were designed to establish which polypeptide chain subunit of human fibrinogen bears the site(s) for interaction with the platelet receptor. Aggregation of platelets induced by ADP requires fibrinogen (19–22) and is concomitant with its binding to the platelet receptor (4, 5, 23). Therefore, our basic test system used ADP-induced platelet aggregation.

Abbreviations: T_{max}, maximal light transmission; CM, carboxymethyl.

Human platelets were separated from plasma fibrinogen, as verified by lack of a response to ADP and absence of detectable plasma fibrinogen in the supernatant of sedimented platelets using the staphylococcal clumping test (24). Under such conditions, addition of fibrinogen (1.4 mg/ml, 3.7 μM) resulted in platelet aggregation (Fig. 1). When carboxymethyl (CM)-fibrinogen composed of a mixture of separated subunits of fibrinogen dispersed in ammonium bicarbonate (pH 7.8) was tested, aggregation was somewhat less than with intact fibrinogen. However, isolated γ chains (7.5 μM) produced aggregation equivalent to that of intact fibrinogen. α chains in an equimolar concentration produced a weaker effect, and β chains were inactive. Detailed values for platelet aggregating activity of fibrinogen subunits in the presence and absence of ADP is given in Table 1.

The effect of isolated γ chains was concentration dependent in terms of aggregation parameters. The full aggregating effect was obtained with 120 μg of γ chain (4.5 μM) per 10^8 platelets and a definite aggregating effect was still observed with as little as 40 μg of γ chain (1.5 μM) per 10^8 platelets (Fig. 2). α chain gave visible aggregation at 270 μg (7.5 μM) per 10^8 platelets but, at <216 μg (6.2 μM) did not show detectable evidence of platelet aggregation (results not shown).

The β-chain multimers were inactive toward platelets. This means that, in spite of a significant homology between β and γ chains (25, 26), the latter possess a highly chain-specific region interacting with platelets. The most significant difference between β and γ chains is that the β chain lacks the carboxy-terminal segment containing crosslinking sites present in the γ chain (27). Lack of reactivity by the β chain also indicates lack of contamination of this chain by γ chain, which makes such a possibility even less likely in the case of α chain.

Because the platelet-aggregating activity of CM-fibrinogen was somewhat less than that of the isolated γ chain, we examined the possibility of interference by the β chain. Indeed, an equimolar mixture of γ and β chains showed decreased aggregating activity as compared with that of the γ chain alone (Table 1). Thus, random reassociation of the γ chain in the presence of β chains diminishes the reactivity of the γ-chain multimers with platelet receptor. This may explain the relatively weaker reactivity of the mixture of α, β, and γ chains in the CM-fibrinogen preparation.

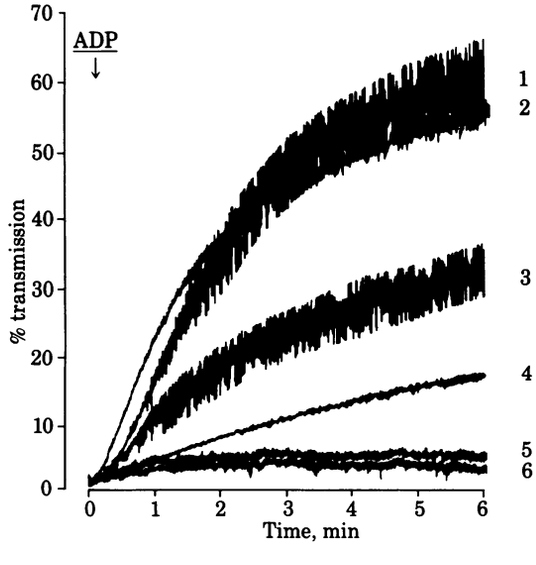

FIG. 1. Human platelet aggregation induced by ADP (5 μM) in the presence of fibrinogen (3.7 μM), CM-fibrinogen (3.7 μM), and isolated α, β, and γ chains (7.5 μM). For details, see legend to Table 1. Curves: 1, γ chain; 2, fibrinogen; 3, CM-fibrinogen; 4, α chain; 5, β chain; 6, buffer.

Table 1. Platelet reactivity of human fibrinogen, reduced CM-fibrinogen, and isolated α, β, and γ chains

System	Platelet aggregation	
	T_{max}	Slope
Platelets/fibrinogen/buffer	0	0
Platelets/fibrinogen/ADP	57	22
Platelets/CM-fibrinogen/ADP	28	9
Platelets/α chain/buffer	0	0
Platelets/α chain/ADP	25	7
Platelets/β chain/buffer	0	0
Platelets/β chain/ADP	0	0
Platelets/γ chain/buffer	6	0.5
Platelets/γ chain/ADP	55	26
Platelets/γ/β chain/ADP	40	18

Human platelets (1×10^8/0.5 ml) were separated from plasma proteins and mixed with fibrinogen, reduced CM-fibrinogen, or isolated fibrinogen chain, and then ADP was added to 5 μM. The mixture was stirred in an aggregometer cuvette at 37°C and aggregation was measured as T_{max}, which represents the maximal light transmission, and as slope, which was obtained by drawing a tangent to the steepest part of the curve and calculating the change per min (18). Fibrinogen was used at 3.7 μM and α, β, and γ chains were used at 7.5 μM.

Structural Features of Fibrinogen γ Chains Required for Platelet Aggregation. Since γ chains are poorly soluble in aqueous solution, the preparation used was in the form of spontaneously formed multimers dispersed in 50 mM ammonium bicarbonate (pH 7.8). The multimeric structure of the γ-chain material was confirmed by gel filtration on Sephadex G-100. γ chains were present in the void volume, indicating that the size of the multimers was $\geq$ 100,000 daltons, or at least twice as large as the monomeric γ chain (47,000 daltons). α and β chains demonstrated a similar pattern of self-association, since they also appeared in the void volume of the Sephadex G-100 column. Although we do not know how the isolated chains of fibrinogen self-associate in aqueous solution and whether the extent of this self-association of the γ chain differs from that of the α and β chains, available evidence from gel filtration indicates that all three chains form similar multimers. Thus, the effect of the γ chain is not due solely to multimer formation but is related to the presence of the chain-specific region recognizing platelet receptor.

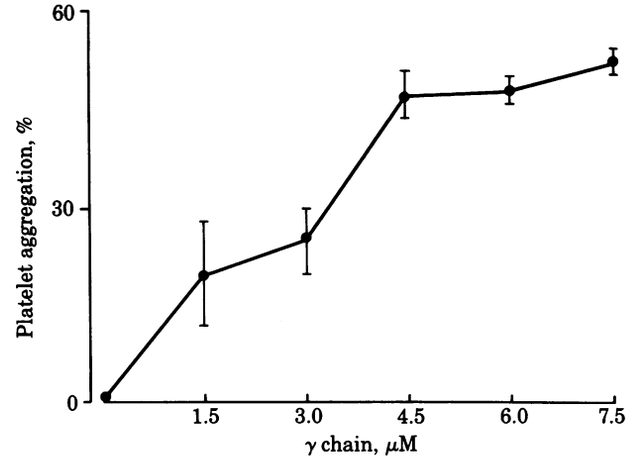

FIG. 2. Effect of γ-chain concentration on platelet aggregation in the presence of ADP. Results represent mean ± SEM. For details, see legend to Table 1.

Table 2. Binding of ^{125}I-labeled fibrinogen to human platelets and their aggregation in the presence of anti-γ-chain and antifibrinogen antibody fragments

Addition	Fibrinogen bound, ng per 10^8 platelets	Binding inhibition, %	Platelet aggregation, T_{max}	Aggregation inhibition, %
None	266	0	60	0
Anti-γ-chain Fab	106	60	0	100
Antifibrinogen Fab	32	88	10	89

^{125}I-Labeled fibrinogen (33 μg) was incubated with anti-γ-chain Fab (314 μg) for 30 min at 37°C and then tested in a steady-state binding system using 5 μM ADP to stimulate platelets (7). Percent inhibition was determined by reference to a system without added antibodies in which binding or aggregation were maximal. For platelet aggregation, fibrinogen (350 μg) was incubated with anti-γ-chain antibody Fab (430 μg) or antifibrinogen Fab (580 μg) for 30 min at 37°C and then added to platelets stimulated with 5 μM ADP.

Since fibrinogen has a dimeric structure and is composed of two γ chains in addition to pairs of α chains and β chains (28), aggregation of platelets by the γ chains would require dimers or larger multimers. Fortuitously, due to the poor solubility of the isolated γ chains in aqueous solution, we worked with dispersed multimers of this chain and fulfilled requirements for multivalent γ-chain arrangement. However, when the γ chains were separated twice on CM-cellulose and solubilized in 0.15 M sodium barbital buffer (pH 8.55) they retained a monomeric structure, eluting from a Sephadex G-100 column at a position corresponding to 3 times the void volume. This solution of monomeric chain did not aggregate ADP-treated platelets. A parallel control containing the same 1/10 vol of sodium barbital buffer (pH 8.55) did not inhibit platelet aggregation with fibrinogen and ADP.

Binding of ^{125}I-Labeled Fibrinogen to Human Platelets and their Aggregation is Inhibited by Anti-γ-Chain Antibody Fragments. Because aggregation of ADP-treated platelets in the presence of plasma fibrinogen is concomitant with its binding to a specific platelet receptor (4, 5, 23), the effect of anti-γ-chain antibody Fab fragments on the binding of ^{125}I-labeled fibrinogen to human platelets was next examined. The amount of bound ^{125}I-labeled fibrinogen was compared with a control from which antibody was omitted. The binding of ^{125}I-labeled fibrinogen to platelet receptor was 40% of the control value, and in a similar experiment, antibody fragments against the entire fibrinogen molecule reduced the binding to 12% of the control value (Table 2). Immunoinhibition of binding of fibrinogen by anti-γ-chain antibody was paralleled by complete inhibition of platelet aggregation induced by ADP in the presence of purified plasma fibrinogen. The inhibitory effect of anti-γ-chain antibody fragments on platelet aggregation induced by ADP in the presence of fibrinogen or in the presence of isolated γ chain is shown in Fig. 3. Taken together, these experiments indicate that antibody against γ chain recognizes and blocks this determinant, which is essential for binding of fibrinogen to platelet receptor and for platelet aggregation.

Contribution of the α Chain to Interaction of Fibrinogen Subunits with Human Platelets. Our results also show that the α chain of fibrinogen interacts with human platelets, although the interaction is significantly weaker than those of the γ chain or intact fibrinogen. On the basis of minimal molar concentration required to aggregate platelets, α-chain reactivity is 1/5th that of the γ chain, assuming that both chains self-associate in aqueous solution to the same extent. The role of the α chain in the interaction of human fibrinogen with human platelets previously has been postulated on the basis of the progressive loss of reactivity accompanying the degradation of the α-chain portion of fibrinogen (22, 23). Interpretation of these results also requires taking into account degradation of the γ chain during proteolysis with plasmin, as exemplified by the loss of 109 residues at the carboxy terminus (29).

Fibrinogen Chains and the Problem of Platelet Receptor. Until now, the problem of interaction between fibrinogen and human platelets was primarily addressed from the standpoint of the characteristics of platelet receptors, their number, affinity, and relationship to membrane glycoproteins (4–7, 23, 30). In this study, we have provided functional evidence that the γ chain bears a site(s) for reacting with the platelet receptor. In

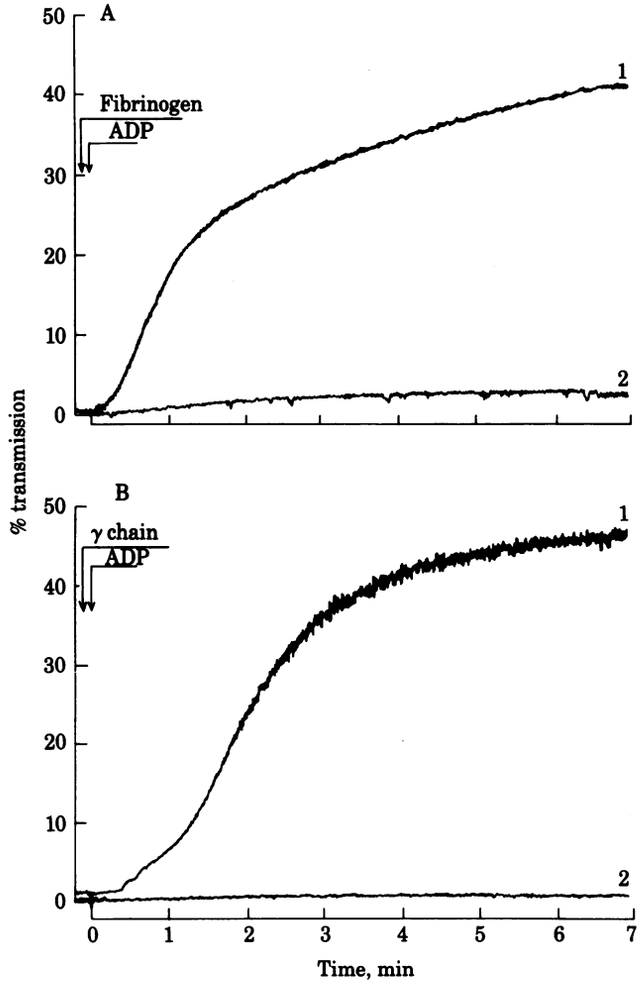

FIG. 3. Blocking effect of anti-γ-chain antibody F(ab)$_2$ fragments on interaction of 3.7 μM fibrinogen (*A*) and 7.5 μM γ chain (*B*) with human platelets in the presence of 5 μM ADP. Curves: 1, buffer only; 2, anti-γ-chain F(ab)$_2$ treated.

addition, the α chain also possesses a reactive, albeit less avid, site for platelets. These findings introduce a higher degree of complexity to the fibrinogen–receptor interaction than was previously thought. They raise the possibility that each chain interacts with a separate type of receptor on human platelets—i.e., a fibrinogen receptor of γ-chain specificity and a fibrinogen receptor of α-chain specificity. It has been reported recently that human platelets possess two classes of receptors for fibrinogen: high affinity with low capacity and low affinity with high capacity (23, 30). It is possible that γ-chain and α-chain binding regions on human fibrinogen can react with separate classes of receptors.

In summary, we have determined that a site reacting with platelet receptors for fibrinogen is localized on the γ chain. Another site of weaker reactivity is situated on the α chain. Thus, the γ chain bears not only the crosslinking site, important for stabilized fibrin formation (31), but also the main site(s) responsible for the interaction of fibrinogen with cell surfaces such as the staphylococcal clumping receptor (13) and the platelet membrane receptor.

We thank Marcia Riley for the preparation of fibrinogen and its chains used in this study. This work was supported by National Institutes of Health Grants HL-25,935, HL-25-107, HL-08,338, HE-12,759, HE-18,576, and GM-17,702.

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