Interaction of Integrin $\alpha_{IIb}\beta_3$ with Multiple Fibrinogen Domains during Platelet Adhesion*

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We have investigated how modulation of integrin $\alpha_{IIb}\beta_3$ function influences the mechanisms that initiate platelet thrombus formation onto surface-bound fibrinogen and isolated fibrinogen domains. Under stationary conditions and with full activation of platelets blocked by prostaglandin E$_1$, the carboxyl-terminal $\gamma_{400-411}$ sequence is necessary for establishing initial contact with the immobilized substrate. Molecules containing a single copy of this sequence, like the plasmin-generated fibrinogen fragment D, support platelet spreading, but the resulting attachment to the surface is loose and disrupted by minimal peeling force. In contrast, platelets adhere firmly to intact fibrinogen under the same conditions, suggesting that recognition of contact sites outside a single D domain can secure the firm interaction not supported by a single $\gamma_{400-411}$ sequence. If platelets are activated, the $\gamma_{400-411}$ sequence is no longer necessary to initiate the adhesion process but becomes sufficient, even as a single copy, to mediate stable surface attachment in the absence of shear stress. Under conditions of flow, however, intact fibrinogen but not fragment D can support adhesion, regardless of whether platelets have the potential to become activated or not. These results indicate the functional relevance of multiple fibrinogen domains during the initial stages of the platelet adhesion process.

Fibrinogen is required for normal hemostasis not only as the precursor of fibrin but also to mediate platelet thrombus formation (1). With respect to the latter role, fibrinogen can support both platelet-surface and platelet-platelet interactions, i.e. platelet adhesion and aggregation, respectively (2, 3), by binding to the glycoprotein IIb-IIIa receptor (integrin $\alpha_{IIb}\beta_3$) (4–6). These two functions occur in sequence at the onset of hemostasis and, when deranged in pathological conditions, may cause vascular occlusion. Platelet adhesion and aggregation are influenced by changes in the recognition specificity of $\alpha_{IIb}\beta_3$, which, as present on the membrane of nonactivated platelets, serves as a specific receptor for surface-bound fibrinogen (7, 8) but, after activation, acquires the ability to interact with other immobilized adhesive proteins, particularly von Willebrand factor (8). Moreover, $\alpha_{IIb}\beta_3$ activation is required for the binding of soluble fibrinogen and von Willebrand factor (9, 10) leading to aggregation (3, 11).

Fibrinogen contains three putative platelet interaction sites, namely the sequence Arg-Gly-Asp-Phe (RGDF) at $\alpha$-$405–98$, the sequence Arg-Gly-Asp-Ser (RGDS) at $\alpha$-$572–575$, and the dodecapeptide sequence His-His-Leu-Gly-Gly-Ala-Lys-Asp-Ser-Gly-Val (HHLGKQAGDV) at $\gamma_{400-411}$ (12, 13). Small synthetic peptides reproducing each of the three sequences have been shown to bind to $\alpha_{IIb}\beta_3$ regardless of its state of activation (14, 15). It is not yet known, however, how these different sites, and possibly others (16), contribute to the complex adhesive functions of the intact fibrinogen macromolecule. Evidence obtained with recombinant mutants indicates a predominant role for the carboxyl-terminal $\gamma$ chain dodecapeptidase in the binding of soluble fibrinogen to activated platelets and thus in mediating aggregation (17, 18). Moreover, it has been postulated that the presence of two $\gamma$ chain carboxyl-terminal domains in the dimeric fibrinogen molecule may influence the adhesion of nonstimulated platelets when the ligand is immobilized onto a surface (19). To elucidate mechanisms important in the initiation of platelet response to vascular injury, we have evaluated the ability of intact fibrinogen and isolated fibrinogen fragments to interact with platelets and support their attachment to a surface. The results obtained indicate that multiple sites are responsible for the adhesive potential of fibrinogen depending on the state of $\alpha_{IIb}\beta_3$ activation. Modulation of the interaction with distinct domains in an appropriate substrate may be an example applicable to the activity of different integrins involved with the adhesive functions of vascular cells exposed to flowing blood.

EXPERIMENTAL PROCEDURES

Purification of Adhesive Proteins—Fibrinogen was purified from blood collected in acid/citrate/dextrose anticoagulant containing 0.1 M $\epsilon$-aminocaproic acid, using the glycine precipitation method (20) as previously reported (21). Fragments D and E were prepared by digestion of purified fibrinogen with plasmin in 20 mM Hepes/150 mM NaCl, pH 7.4 (7). After purification, the fibrinogen fragments were characterized using specific monoclonal antibodies reacting with the different putative adhesion sites in fibrinogen as described previously in detail (7, 22).

Monoclonal Antibodies—The anti-fibrinogen monoclonal antibody used for these studies, LJ-$\gamma$698 (IgM), was generated using as immunogen a synthetic peptide corresponding to residues 400–411 of the fibrinogen $\gamma$ chain and has been shown to recognize an epitope located in the D domain of the molecule including the carboxyl-terminal region of the $\gamma$ chain (7, 23). Two complex specific anti-$\alpha_{IIb}\beta_3$ antibodies (IgG) were utilized; their preparation and characterization has already been reported (8, 21). LJ-$\gamma$CP8 inhibits the interaction of soluble ligands, including fibrinogen and von Willebrand factor, with activated platelets as well as the adhesion of nonactivated platelets to immobilized fibrinogen; LJ-$\gamma$P4, in contrast, has no appreciable inhibitory effect on the ligand-binding function of the receptor. The two antibodies do not

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Platelet Adhesion Assay and Scanning Electron Microscopy—The stationary adhesion assay was performed essentially as described previously (24) and was used to "activate" \(\alpha_{IIb}\beta_{3}\). This antibody interacts with an epitope modulated by ligand occupancy of the receptor and located in the amino-terminal region of \(\beta_{3}\) (24); binding of the antibody, in turn, leads to the ligand interaction with \(\alpha_{IIb}\beta_{3}\) when platelet activation is inhibited (24). IgG and IgM antibodies were purified as previously reported (7) and stored in 20 mM Hepes/150 mM NaCl, pH 7.4, at -70°C until used.

Platelet Interaction with Adhesive Domains of Immobilized Fibrinogen—First we evaluated the extent to which intact fibrinogen and two plasmin-derived fragments containing known \(\alpha_{IIb}\beta_{3}\)-binding sites support platelet adhesion. Sequence-specific antibodies were used to demonstrate the presence of the carboxy-terminal dodecapeptide sequence at \(A^{288-311}\) in fragment D and of the RGDF sequence at \(A^{95-98}\) in fragment E (7). Examination by scanning electron microscopy revealed that platelets in the plasma milieu became firmly attached to surfaces coated with fibrinogen or fragment D, showing multiple pseudopodia, extensive spreading and aggregates consisting of platelets interacting mainly with other adhering platelets rather than with the surface (Fig. 1, upper row). These platelets appeared activated, in agreement with the findings in a previous study demonstrating that platelets attached to immobilized fibrinogen can bind the activation-dependent anti-\(\alpha_{IIb}\beta_{3}\) monoclonal antibody PAC1 (8). In contrast, platelets treated with PGE\(_1\) to inhibit activation were attached to intact fibrinogen but not to fragment D and, in accordance with previous data (7, 8), exhibited various stages of spreading but essentially no tendency to aggregate (Fig. 1, lower row).

RESULTS AND DISCUSSION

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Extensive survey of fragment E-coated surfaces revealed no platelets attached under any conditions (Fig. 1).

These findings indicate that the carboxyl terminus of the \(\gamma\) chain in fragment D can support stable adhesion mediated by \(\alpha_{IIb}\beta_{3}\) only when platelets have the potential to become activated. This is not when they are kept in the "resting" state and that the RGDF sequence in fragment E is not active under the same conditions. Thus, isolated fragments D and E (Fig. 1), as well as a combination of the two immobilized together on a surface (not shown here), cannot function like intact fibrinogen in supporting adhesion. This suggests that additional sequences not present in these two fragments are involved in the process and/or that only the native molecule can present multiple adhesive sites in the appropriate conformation for interacting with platelets. As shown below, however, this does not rule out the occurrence of specific interactions with single domains resulting in loose and/or transient attachment that cannot be detected in standard assays because it is disrupted during the washing steps performed to reduce "nonspecific" background.

These interpretations rely on the notion that platelets maintained in their plasma environment with intact davalent cation concentrations should be minimally altered by uncontrolled stimulation. Other investigators, however, have found that they behave like washed platelets stimulated by the combination of epinephrine and ADP, whereas nonstimulated washed platelets behave like PGE\(_1\)-treated platelets in plasma (19). It is debatable whether any ex vivo study is compatible with the absence of stimulation, because the unavoidable manipulations necessary to remove platelets from the circulation may be sufficient to cause functional perturbation. Moreover, the def-
initiation of whether circulating platelets are truly resting is essentially arbitrary. For example, platelets in plasma never exposed to exogenous agonists may act like stimulated washed platelets because of the presence of small quantities of activating substances, like ADP, released from erythrocytes or platelets themselves. Alternatively, nonstimulated washed platelets may appear to function like PGE1-treated platelets because they are rendered refractory to weak stimuli by activation during isolation procedures. In spite of these problems, our results are in agreement with the previous study (19), demonstrating that platelets can adhere firmly to fragment D, containing a single \( \gamma \) chain carboxyl-terminal domain, only when they become activated and that nonstimulated platelets adhere only to substrates, like intact fibrinogen, with two such domains. In view of these findings, additional experiments were designed to evaluate whether the \( \gamma \) chain carboxyl-terminal sequences are necessary and sufficient to support the adhesive functions of fibrinogen and whether platelet activation can influence their interaction with \( \alpha_{IIb} \beta_3 \).

**Time Course of Platelet Adhesion to Immobilized Fibrinogen and Fragment D: Role of the \( \gamma \) Chain Carboxyl Terminus and Effect of Platelet Activation—Platelets in their plasma environment were firmly attached to fibrinogen already after 20 min of incubation and reached nearly maximal adhesion by 30 min, a time at which stable attachment to fragment D was still negligible; however, comparable adhesion to the two substrates was observed at 60 min (Fig. 2). In several experiments, platelets were exposed to the immobilized substrates for up to 80 min, but the results obtained were essentially as seen at 60 min. Of note, no adhesion to fragment E occurred under the same conditions (six experiments; not shown). Adhesion of untreated platelets to both fibrinogen and fragment D was completely inhibited at all time points tested by the anti-\( \alpha_{IIb} \beta_3 \) monoclonal antibody LJ-CP8, confirming that the process is strictly dependent on the function of this receptor (Fig. 2). On the other hand, an antibody against the \( \gamma \) chain carboxyl terminus, LJ-Z69/8, could completely block adhesion to fragment D but was only partially effective in blocking adhesion to fibrinogen at later time points (Fig. 2).

Platelets treated with PGE\(_1\) to inhibit the response to activating stimuli still adhered well to intact fibrinogen but, unlike
untreated platelets, their interaction was completely inhibited by the anti-\(\gamma\) chain dodecapeptide antibody, as well as by the anti-\(a_{IIb}\beta_3\) antibody, regardless of the length of incubation (Fig. 3). Moreover, PGE\(_1\)-treated platelets could not attach firmly to fragment D, even after the first time interval of 20 min (Fig. 3). In marked contrast to these results, platelets in plasma supplemented with PGE\(_1\) were used instead of untreated platelets. The results represent the means ± S.E. for nine separate experiments, each performed in duplicate.

In marked contrast to these results, platelets in plasma stimulated by the addition of exogenous epinephrine attached firmly to fragment D even after the first time interval of 20 min; the interaction was completely inhibited by the monoclonal antibodies LJ-Z69/8 against the \(\gamma\) chain carboxyl terminus and LJ-CP8 against \(a_{IIb}\beta_3\) (Fig. 4). The same platelets adhered well to fibrinogen, but in this case the interaction was still completely inhibited by LJ-CP8 but only minimally affected by LJ-Z69/8 (Fig. 4).

These findings support the concept that the \(\gamma\) chain carboxyl terminus is the only \(a_{IIb}\beta_3\) interactive site in fragment D but cannot mediate irreversible attachment unless platelets are activated. The \(\gamma^{100-411}\) sequence is clearly necessary for initiating platelet adhesion to fibrinogen when full activation is blocked and under these conditions two copies of it, as opposed to the single one in fragment D, may support irreversible attachment, as previously suggested (19). Alternatively, the distinct properties of intact fibrinogen may indicate functional co-operation between this sequence and one or more additional sites present in fibrinogen but not in fragment D. When platelets can become activated, the \(\gamma\) chain carboxyl-terminal sequence is no longer strictly required to initiate or mediate adhesion to intact fibrinogen, although it retains its essential role in fragment D. This is in agreement with the results of previous studies demonstrating that several domains in fibrinogen can interact with activated \(a_{IIb}\beta_3\) (7).

Our findings show that untreated platelets (not inhibited by PGE\(_1\)) exposed for a sufficiently long time to immobilized fibrinogen or fragment D in the absence of any added stimulus, exhibit adhesive properties similar to those of platelets activated by exogenous epinephrine. Indeed, in assays like the one described here performed in the absence of flow, membrane contacts developing when platelets sediment onto the surface over time can induce the release reaction (27), leading to the local availability of agonists like ADP and thromboxane A\(_2\) (28), as well as favor guanylate cyclase activation and signal transduction (29); thus platelets become activated. Of note, in experiments not reported here, we found that agitation preventing platelet sedimentation during the assay effectively inhibited adhesion to fragment D but not to fibrinogen, in agreement with all the other results indicating that activation is essential for stable attachment to the former but not the latter. It appears, therefore, that the relatively slow time course of adhesion to fragment D exhibited by untreated platelets is a reflection of the slow process of activation upon sedimentation, not the consequence of other undefined properties of this substrate. In agreement with this hypothesis, platelet attachment to fragment D occurred more rapidly after activation with exogenous epinephrine. In the latter situation, as well as in the case of adhesion to fibrinogen, the time course of the process may reflect the rate at which platelets sediment and can interact with the surface more than the generation of stimuli deriving from close contact. Along similar lines, the fact that the anti-\(\gamma\) chain antibody had no effect on adhesion to fibrinogen of platelets stimulated with epinephrine (Fig. 4), whereas it inhibited partially but significantly that of untreated platelets (Fig. 2), also seems to indicate that activation upon sedimentation is a slow process involving platelets in a nonsynchronous manner; thus only platelets that are not yet activated in the well would fail to adhere to fibrinogen in the presence of the antibody, as shown in Fig. 3 for those treated with PGE\(_1\).

**Mechanisms of Platelet Adhesion to Fibrinogen**

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**Fig. 3.** Time course of adhesion of PGE\(_1\)-treated platelets to fibrinogen and fragment D: effect of a monoclonal antibody against the \(\gamma\) chain carboxyl terminus. The experiment was performed as described in the legend to Fig. 2 with the only exception being that platelets in plasma supplemented with PGE\(_1\) were used instead of untreated platelets. The results represent the means ± S.E. for nine separate experiments, each performed in duplicate.

**Fig. 4.** Time course of adhesion of epinephrine-treated platelets to fibrinogen and fragment D. The experiment was performed as described in the legend to Fig. 2 with the only exception being that platelets in plasma were treated with epinephrine (final concentration, 20 \(\mu M\)) for 10 min before addition to the substrate-coated wells. The results represent the means ± S.E. for nine separate experiments, each performed in duplicate. Statistical evaluation confirmed that the inhibitory effect of the antibody LJ-Z69/8 was not significant at later time points in the case of adhesion to fibrinogen (Student's t distribution of control versus treated groups: \(p < 0.025\) at 20 min; \(p > 0.5\) at 60 min) but remained highly significant in the case of adhesion to fragment D (\(p < 0.001\) at all time points tested).
binding to it and induce the ability to interact with soluble ligands (30), the typical feature of activated α_{Ib}β_{3} (31). In this case, platelets in plasma treated with AP5 became firmly attached to both intact fibrinogen and fragment D whether in the presence or in the absence of PGE_{1} (Fig. 5). Such a finding supports the concept that the modulation of α_{Ib}β_{3} function, usually thought of as activation, can explain the different adhesive properties of platelets that can respond to stimulation as compared with those treated with PGE_{1} that cannot become fully activated. Of note, soluble fibrinogen binding that is expected to occur following treatment with AP5 (24) or epinephrine (32) did not interfere with platelet adhesion to surface-bound substrates, perhaps because the latter interaction with α_{Ib}β_{3} is immediately irreversible as opposed to the initially reversible binding of soluble ligands (33).

Because all the experiments reported to this point were performed in the presence of plasma proteins, it is possible that unidentified molecules interacting differently with immobilized fragment D or fibrinogen modify the surface to which platelets are exposed and influence the process of adhesion. To rule out this out, we performed additional studies, to be described in detail elsewhere,\(^2\) using heterologous cells expressing recombinant α_{Ib}β_{3}. In this case, in the absence of any plasma protein, the monoclonal antibody AP5 in the presence of Mn\(^{2+}\) could induce the cells to mimic the function of "stimulated" platelets (adhesion to both fibrinogen and fragment D), whereas cells treated with the antibody but without Mn\(^{2+}\) behaved like resting platelets (more prominent adhesion to fibrinogen), demonstrating that specific differences in the receptor-substrate interaction are responsible for the findings observed.

Immobile Fragment D Can Interact with PGE_{1}-treated Platelets—The results of the experiments reported to this point suggest that a single copy of the γ chain dodecapeptide sequence may interact with nonactivated α_{Ib}β_{3} and mediate responses necessary to initiate platelet adhesion, albeit not sufficient to support irreversible attachment without α_{Ib}β_{3} activation. In order to examine this hypothesis, platelets treated with PGE_{1} were exposed to immobilized fragment D for an appropriate time and then fixed after a single gentle washing step, instead of the usual four, to preserve the stability of weak interactions with the surface; the specimens were then processed and analyzed by scanning electron microscopy. Under these conditions, extensively spread platelets could be visualized on both fibrinogen and fragment D but not on fragment E or on a bovine serum albumin-coated control surface, where platelets retained the nonspread morphology typically seen with nonactivated platelets (Fig. 6, upper row). Examination at low magnification demonstrated that the occurrence of spreading involved a majority of the platelets interacting with fragment D (Fig. 6, lower row). The anti-α_{Ib}β_{3} monoclonal antibody, LJ-CP8, abolished spreading (not shown). These findings prove that the γ\(^{400–411}\) sequence, even when present in a single copy as in fragment D, is recognized by α_{Ib}β_{3} on platelets that cannot become fully activated.

A Mechanistic Interpretation of Platelet Adhesion to Fibrinogen in the Absence of Flow—The results of the stationary adhesion assays reported here support a schematic model for the mechanism of platelet adhesion to immobilized fibrinogen involving the interaction of multiple domains of the substrate with α_{Ib}β_{3}. The latter appears as the necessary receptor participating in the process and possibly the only one, because the other platelet receptor that can bind fibrinogen, α_{IIb}β_{3} (34, 35), is present in limited copy number on platelets (36, 37) and presumably has a negligible functional role. With regard to fibrin-

\(^2\) I. Stuiver, T. E. O'Toole, T. Kunicki, M. H. Ginsberg, and Z. M. Ruggeri, manuscript in preparation.

**Fig. 6. Interaction of nonactivated PGE_{1}-treated platelets with fragment D.** Platelets in plasma supplemented with PGE_{1} were allowed to attach to immobilized fibrinogen or fibrinogen fragments under the conditions described in the legend to Fig. 2. However, in order to determine the morphology of any loosely attached platelets that would otherwise be detached by the four washing steps described for the experiment presented in Fig. 2, at the end of the 60-min incubation, surface-associated platelets were fixed and processed for scanning electron microscopy after a single washing step. Upper row, high magnification; bar, 5 μm. Lower row, low magnification; bar, 30 μm. Note extensive platelet spreading on fibrinogen (A) and fragment D (B) but not on fragment E (C) where residual platelets retain a nonspread morphology. Compare the results shown here for fragment D with those shown in Fig. 2 (lower row) performed under identical conditions except for the number of washing steps.

**Fig. 5. Effect of α_{IIb}β_{3} activation with the monoclonal antibody, AP5, on platelet adhesion to fibrinogen and fragment D.** The experiment was performed as described in the legend to Fig. 2, except that platelets in plasma with or without the addition of PGE_{1} were treated with the activating monoclonal antibody AP5 (Fab; final concentration, 50 μg/ml) for 20 min before addition to the substrate-coated wells for 60 min.
The synergistic effect of the initial contact with a thrombogenic surface is always subjected to adhesion to surface-bound fibrinogen. It can also occur after platelet stimulation with an exogenous agonist (Fig. 4). The difference between the former and the latter is probably one of extent of activation. In the case of a weak stimulus, like that developed when platelets sediment onto a surface, the synergistic effect of the initial contact with an appropriate fibrinogen domain, like the $\gamma^{400-411}$ sequence, is still required for adhesion to occur; therefore, nonstimulated but metabolically active platelets adhere selectively to fibrinogen and fragment D, as shown here, but not to other potential adhesive substrates like fibronectin or vitronectin (8). In the case of a stronger stimulus, for example the combination of ADP plus epinephrine (7), activated $\alpha_{IIb}\beta_3$ becomes less selective and mediates adhesion to other fibrinogen domains, like fragment E, and to other substrates (7). The latter processes take place under the same conditions required for soluble ligand binding to $\alpha_{IIb}\beta_3$, indicating that they depend on full activation of the receptor. Clearly, functional modulation of $\alpha_{IIb}\beta_3$ has a profound impact on the mechanism of platelet adhesion to surface-bound fibrinogen.

The multi-domain structure of fibrinogen is required to support platelet adhesion under flow conditions—platelet adhesion with a thrombogenic surface is always subjected to the effects of hemodynamic forces except in limited areas of the vascular system where stagnation may develop. The pathophysiological relevance of the multi-domain structure of fibrinogen with regard to the expression of adhesive function was highlighted by experiments performed with flowing blood at a wall shear rate of 50 s$^{-1}$, a relatively low level of shear stress chosen to allow visualization of weak interactions. Platelets with the full potential to become activated adhered irreversibly to intact fibrinogen and, after 5 min of perfusion, formed a homogeneous monolayer on the surface; in contrast, there was essentially no interaction with immobilized fragments D and E (Fig. 7), even though platelets treated in a similar manner adhere to fragment D under stationary conditions (Fig. 1). Identical results were obtained with PGE$_2$-treated platelets, indicating that the function of multiple domains in surface-bound fibrinogen may be important for the initiation of thrombus formation by supporting irreversible platelet attachment before full activation takes place. Subsequent activation of $\alpha_{IIb}\beta_3$ may then reinforce adhesion and mediate thrombus growth by allowing interacting with soluble adhesive ligands.

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