Internalization of Bound Fibrinogen Modulates Platelet Aggregation

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In agonist-stimulated platelets, the integrin α₃β₃ (glycoprotein Ib-IIIa) is converted from an inactive to an active fibrinogen receptor, thereby mediating platelet aggregation. With time after agonist addition, at least two events occur: fibrinogen becomes irreversibly bound to the platelet and, when stirring is delayed, platelets lose the ability to aggregate despite the presence of maximally bound fibrinogen. Because we previously identified an actively internalized pool of α₃β₃ in platelets, we explored the possibility that both of these events might result from the internalization of fibrinogen bound to active α₃β₃. Under conditions of irreversible fibrinogen binding, fluorescence microscopy showed that biotinylated fibrinogen is rapidly internalized by activated platelets to a surface-inaccessible, intracellular pool. Flow cytometric analysis showed that the observed loss in accessibility to extracellular probes immediately precedes a loss in ability of the platelets to aggregate. Moreover, prevention of irreversible fibrinogen binding results in a prevention of internalization and a retention of aggregation capacity. Thus, the internalization of fibrinogen from the activated platelet surface appears to contribute not only to the irreversible phase of fibrinogen binding, but also to the downregulation of platelet adhesiveness. Fibrinogen internalization is therefore likely to represent a fundamental regulatory mechanism that modulates platelet function.

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Central to the maintenance of normal hemostasis is the ability of platelets to aggregate to form a platelet plug, thus preventing blood loss. Aggregation is mediated largely by the binding of the adhesive protein, fibrinogen, to the integrin α₃β₃ (glycoprotein Ib-IIIa) on the platelet surface. When platelets are stimulated by agonists, α₃β₃ is converted from an inactive conformation that does not bind soluble fibrinogen to an active conformation that is binding-competent. With time after exposure to a stimulus, platelets lose the capacity to aggregate if initiation of stirring is delayed. Additionally, with time, platelet-bound fibrinogen becomes progressively nonadhesive, irreversibly bound. Detailed analyses by Peerschke have suggested that this loss in aggregation capacity, which correlates with the loss of fibrinogen accessibility to certain antifibrinogen antibodies, is due to a qualitative change of surface-bound fibrinogen related to the development of irreversible fibrinogen binding.

In the present study, we investigated the localization of irreversibly bound fibrinogen. We observed an extensive, rapid intracellular accumulation of fibrinogen in unstirred, ADP-activated platelets. Sequestration of fibrinogen to an intracellular pool may explain, in part, its irreversible association with platelets, because this fibrinogen would be protected from displacement by inhibitors of fibrinogen binding to α₃β₃. Moreover, we observed that the time course of fibrinogen uptake by activated platelets immediately precedes a loss in their ability to aggregate upon stirring, whereas inhibition of fibrinogen uptake correlates with retention of aggregation capacity. Thus, the present study provides evidence that fibrinogen internalization by activated platelets contributes to the return of stimulated platelets to a nonadhesive state.

Materials and Methods

Chemicals and reagents. Bovine serum albumin (BSA; once recrystallized, essentially globulin free), prostaglandin I₂ (PGI₂), plasmin, and poly-L-lysine were purchased from Sigma Chemical Co (St Louis, MO). For binding studies, BSA (Pentax, Fraction V) was purchased from Miles Laboratories (Kankakee, IL). Parafomaldehyde was obtained from Polysciences (Warrington, PA). Sepharose CL-2B and PD-10 columns were obtained from Pharmacia, Inc (Piscataway, NJ). Triton X-100 was purchased from J.T. Baker Chemicals (Phillipsburg, NJ). Apyrase was the generous gift of Dr R.L. Kinlough-Rathbone (McMaster University, West Hamilton, Ontario, Canada). The apyrase stock had an ADPase activity of 7.14 x 10⁻⁴ mol ADP hydrolyzed/min/mL and an ATPase activity of 5.55 x 10⁻¹⁵ mol ATP hydrolyzed/min/mL. Other chemicals, unless indicated, were of reagent grade.

Platelet preparation. Whole blood was obtained from healthy, aspirin-free volunteers. The first 1 mL of blood was discarded and the remainder was drawn into a solution of 37°C acid citrate dextrose (1.4 mL/8.6 mL blood), containing 150 mmol/L PG1₂ (Sigma). Platelet-rich plasma (PRP) was isolated by centrifugation at 180g for 20 minutes. In studies using gel-filtered platelets, PRP obtained from 20 mL of whole blood was applied to a 2.5 x 10 cm column of Sepharose CL-2B (Pharmacia) pre-equilibrated at room temperature (RT) with Ca²⁺/Mg²⁺-free Tyrode’s-HEPES buffer (138 mmol/L NaCl, 12 mmol/L NaHCO₃, 10 mmol/L KCl, 5.5 mmol/L glucose, 0.36 mmol/L Na,HPO₄, 0.35% BSA, and 10 mmol/L HEPES, pH 7.4), and 1.5-mL fractions were collected into tubes containing 15 μL of a 1:100 dilution of the apyrase stock. The platelet concentration was adjusted to 2 to 4 x 10¹²/mL. Platelets were tested for their ADP-dependent aggregation response before each experiment. Platelets in the presence of 1.4 mg/mL fibrinogen, 2 mmol/L CaCl₂, and 1 mmol/L MgCl₂ were stimulated with ADP (70 μmol/L) under stirring conditions (1,200 rpm) and aggregation was monitored on a platelet aggregometer (Chrono-log, Haverton, PA).

Fibrinogen labeling. Fibrinogen was isolated from fresh human plasma by the method of Kazal et al with a gelatin-Sepharose chromatography step to remove contaminating fibronectin and was dialyzed into Ca²⁺/Mg²⁺-free Tyrode’s buffer lacking BSA. Fibrinogen was labeled with [¹²⁵I] (1 mCi), using chloramine T as described; with NHS-biotin (Pierce, Rockford, IL); or with both. For labeling...
with biotin, fibrinogen was first dialed into a buffer of 0.1 mol/L NaHCO₃ and 0.1 mol/L NaCl, pH 8.2. The concentration of fibrinogen was adjusted to 1.5 mg/ml and sulfo-NHS-biotin was added dry at 0.2 mg/ml, of solution. The solution was mixed on an orbital mixer (Adams Nutator) for 30 minutes at RT. Uncoupled biotin was separated from biotinylated-fibrinogen on a PD-10 column (Pharmacia) blocked with BSA and pre-equilibrated in Ca²⁺/Mg²⁺-free Tyrode's-HEPES buffer lacking BSA. The extent of biotinylation was determined by Method I, as described by the manufacturer. Acetone precipitation of fibrinogen showed that 17 to 25 mol of biotin was present per 1 mol of fibrinogen. The concentration of biotinylated fibrinogen was determined on Coomassie-blue stained sodium dodecyl sulfate (SDS) gels by densitometry, relative to fibrinogen standards. For flow cytometry studies, fibrinogen was conjugated with fluorescein isothiocyanate (FITC) using FITC-celite as previously described.¹⁵ Fibrinogen (labeled and unlabeled) was centrifuged for 10 minutes in a microfuge (Haake Buchler Instruments, Inc, Saddle Brook, NJ) at 10,500g before use. In two to three experiments that directly compared the irreversible binding properties of ¹²⁵I-biotinylated fibrinogen to ¹²⁵I-fibrinogen, we found that 39.5% and 68.8% of ¹²⁵I-biotinylated fibrinogen versus 39.2% and 62.2% of ¹²⁵I-fibrinogen became irreversibly bound at 10 minutes and 60 minutes, respectively. These data confirm previous observations that biotinylated fibrinogen retains properties of the native molecule.¹⁶

Fibrinogen binding. Binding of ¹²⁵I-fibrinogen to platelets was performed in a manner similar to that described by Marguerie and Plow.¹⁶ Briefly, gel-filtered platelets (5 × 10⁷/mL final concentration) were added last to an incubation mixture containing ¹²⁵I-fibrinogen (0.1 to 0.35 mg/mL) in a Tyrode's buffer that included 2.5 mmol/L CaCl₂, 1.25 mmol/L MgCl₂, and 0.6% BSA with ADP (70 μmol/L), and the platelets were incubated at RT without stirring for various times as indicated in the figure legends. Total platelet bound fibrinogen was quantified by centrifuging the platelets (40,500g for 10 minutes in a microfuge (Haake Buchler Instruments, Inc, Saddle Brook, NJ) at 10,500g before use. In two to three experiments that directly compared the irreversible binding properties of ¹²⁵I-biotinylated fibrinogen to ¹²⁵I-fibrinogen, we found that 39.5% and 68.8% of ¹²⁵I-biotinylated fibrinogen versus 39.2% and 62.2% of ¹²⁵I-fibrinogen became irreversibly bound at 10 minutes and 60 minutes, respectively. These data confirm previous observations that biotinylated fibrinogen retains properties of the native molecule.¹⁶

In double-label experiments, platelets incubated with biotinylated fibrinogen and MoAb15 (an anti-β₁ antibody that does not inhibit fibrinogen binding)²⁹ were stimulated with 10 μmol/L thrombin receptor agonist peptide (TRP) corresponding to the tethered ligand sequence SFLLRNPNDKYEPF for 30 minutes at RT in the absence of stirring and allowed to internalize fibrinogen. After uptake, samples were centrifuged through sucrose and resuspended in BSA-free Tyrode's-HEPES buffer with 1 mmol/L CaCl₂. Samples were subsequently treated with buffer or 2% NaN₃ for 15 minutes at RT, washed, and then exposed to either buffer or 0.5 U/mL plasmin for 30 minutes at RT. After fixation, platelets were permeabilized with lysophosphatidylcholine and counterstained with rhodamine avidin.

Flow cytometry. Unstimred, washed platelets were stimulated with TRP (100 μmol/L) at 22°C in the presence of FITC-fibrinogen (1.47 μmol/L). At a variety of time points, samples were rapidly diluted 1:10 with 4°C buffer and immediately placed on ice to halt further redistribution of α₂β₁-FTC-fibrinogen complexes. Samples were analyzed on a Becton Dickinson FACStar analyzer (Becton Dickinson, San Jose, CA) formatted for single-color analysis. The fluorescence channel was set at logarithmic gain and was calibrated with 2-μm Calibrite Beads (Becton Dickinson). Acquisition was gated using a lower limit threshold that excluded background scatter. Acquired values reflect the mean fluorescence intensity for 10,000 events. Values acquired reflect the mean fluorescence intensity for 10,000 events. After analyses for log green fluorescence, rabbit anti-fluorescein was added to each tube, and the same samples were reanalyzed for residual unquenched fluorescence. Because the cells were diluted in ice-cold buffer and maintained at 4°C before FACs analysis, access of the quenching antibody is limited to extracellular/surface-associated fluorescein. We therefore have interpreted protection from quenching by anti-fluorescein as evidence of internalization.

To calculate the percentage of surface-associated FITC-fibrinogen, logarithmic FACs data, in arbitrary fluorescence units, were converted to linear values while being collected. The amount of quenched fluorescence was then calculated as the difference between total fluorescence and the residual unquenched fluorescence present.
after addition of antifluorescein antibody (see below). This value divided by the quenching efficiency yielded an estimation of the amount of surface-associated fluorescence. The percentage of surface-associated fluorescence was determined as the ratio of surface to total fluorescence. Quenching efficiency was established for each FITC-fibrinogen preparation by evaluating the ability of a standard addition of antifluorescein to quench FITC-fibrinogen added at $t = 0$ to resting platelets as well as the ability of antifluorescein to quench FITC-fibrinogen bound to stimulated, fixed platelets.

The percentage of surface-associated FITC-fibrinogen was calculated as follows: (1) Total – Unquenched = Amount Quenched; (2) Amount Quenched $\div$ Quenching Efficiency = Amount on Surface. The quenching efficiency was calculated as follows: (3) (Amount on Surface $\div$ Total) $\times$ 100 = Percent on Surface.

RESULTS

Contribution of fibrinogen internalization to irreversible fibrinogen binding. We previously identified a mobile pool of $\alpha_{IIa}, \beta_3$ that appears to be internalized in resting platelets via the formation of endocytic vesicles. These results led us to evaluate the localization of fibrinogen bound to $\alpha_{IIa}, \beta_3$ on activated platelets. We activated platelets with ADP in the presence of $^{125}$I-fibrinogen, followed by the addition of excess EDTA or control buffer. As shown in Fig 1, the proportion of irreversibly bound fibrinogen increased with time so that, within 1 hour, a majority of the bound fibrinogen was not displaced by EDTA, in agreement with previous studies.

The cellular localization of the irreversibly bound fibrinogen was determined by fluorescence microscopy. For localization studies, biotinylated fibrinogen, which exhibits irreversible binding properties similar to that of $^{125}$I-fibrinogen (see the Materials and Methods), was used to distinguish it from endogenous $\alpha$-granule fibrinogen. To localize bound fibrinogen by immunofluorescence microscopy, conditions of total and irreversible binding established in

![Fig 1. Time course of specific and irreversible fibrinogen binding. Gel-filtered platelets in the presence of $^{125}$I-fibrinogen (320 nmol/L) were activated with ADP (70 $\mu$mol/L) in the absence of stirring for the times indicated. Irreversible binding was defined as the amount of binding that remained after an additional 30 minutes of incubation of some samples with EDTA (12 nmol/L). Nonspecific binding was determined from samples in which EDTA was added at time 0. Data shown are specific binding (total – nonspecific) and are the average of three samples from four experiments $\pm$ SEM.](image)

![Fig 2. Time course of fluorescent localization of biotinylated fibrinogen. Gel-filtered platelets (2.9 $\times$ 10$^8$/4 mL) were incubated with biotinylated fibrinogen (430 nmol/L) and activated with ADP (70 $\mu$mol/L) for 0, 1, 10, or 60 minutes under unstirred conditions. The platelets were subsequently sedimented through sucrose, fixed, permeabilized or left intact, and stained with rhodamine-avidin. Similar cell densities are present in each panel as verified by phase contrast microscopy (data not shown). Fluorescent staining observed in (c), (e), and (g) is believed to represent unintentionally permeabilized platelets. Original magnification $\times$ 1,500.](image)
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similar to that observed for total biotinylated fibrinogen binding (Fig 3b v Fig 2h). When the binding of biotinylated fibrinogen was initiated in the presence of a 90-fold excess of unmodified fibrinogen, no surface or intraplatelet staining was observed (Fig 3c and d), further confirming the specificity of the observed staining. Moreover, platelets incubated with free biotin remained unlabeled, showing that the observed staining pattern did not result from nonspecific uptake of biotin (Fig 3e and f). Finally, proteins from platelets with irreversibly bound biotinylated fibrinogen were electrophoresed on reduced SDS-polyacrylamide gels, transferred onto nitrocellulose, and probed with HRP-avidin. These analyses showed no apparent difference in electrophoretic mobility between unbound biotinylated fibrinogen and platelet-associated biotinylated fibrinogen, confirming that the biotinylated fibrinogen was not proteolytically modified (data not shown). These data raise the possibility that irreversible fibrinogen binding may occur as a result of fibrinogen sequestration to antibodies, inhibitory peptides, or EDTA.

**Intact**

**Permeable**

![Figure 3](image)

**Fig 3.** Irreversible binding and negative controls. To localize irreversibly bound fibrinogen, gel-filtered platelets were stimulated with 70 μmol/L ADP for 60 minutes without stirring in the presence of biotinylated fibrinogen (430 nmol/L). Samples were subsequently incubated with EDTA (12 mmol/L) for 30 minutes to remove reversibly bound fibrinogen and were processed and stained as in Fig 2. Incubation conditions in (a) and (b) consisted of incubation with biotinylated fibrinogen followed by EDTA. Incubation conditions in (c) and (d) consisted of preincubation with 90-fold excess unlabeled fibrinogen followed by biotinylated fibrinogen. Incubation conditions in (e) and (f) consisted of incubation with free biotin. Residual punctate staining observed in (a) is believed to represent unintentionally permeabilized cells. Original magnification × 1,500.

the re-expression of internalized fibrinogen on the platelet surface. To test this possibility, gel-filtered platelets augmented with 200 nmol/L biotinylated fibrinogen were activated with 20 μmol/L ADP for 30 minutes at RT to stimulate fibrinogen binding and internalization. After an additional incubation in the presence or absence of plasmin (0.5 U/mL) for 30 minutes at RT, platelets were fixed, permeabilized, and counterstained with rhodamine avidin. In the absence of plasmin, the typical staining pattern for internalized biotinylated fibrinogen was observed (Fig 4a). Treatment of parallel samples with plasmin before fixation essentially eliminated the previously observed intracellular staining (Fig 4b). However, metabolic inactivation of biotinylated fibrinogen but before plasmin treatment, resulted in a retention of intracellular biotinylated fibrinogen (Fig 4d). These data suggest that the ability of plasmin to cleave irreversibly bound/internalized fibrinogen in live (metabolically active) cells most likely stems from the secondary ability of the enzyme to induce the redistribution of fibrinogen and/or αIIbβ3-fibrinogen complexes to the platelet surface, where fibrinogen becomes accessible for digestion.

**Internalization of fibrinogen bound to αIIbβ3 correlates with loss of platelet aggregation.** We next examined the possibility that rapid intracellular sequestration of fibrinogen in activated platelets could explain the observation that platelets stimulated with an agonist before initiation of stirring exhibit a time-dependent loss in their aggregation response despite the presence of maximally bound fibrinogen. To investigate whether internalization of bound fibrinogen is responsible for the decreased ability of agonist-stimulated platelets to aggregate when initiation of stirring is delayed, we first established a time course for loss of the aggregation response. Platelets were stimulated with a thrombin receptor agonist peptide (TRP) corresponding to the tethered ligand sequence (SFLLRNPNKDVYEPF). TRP (100 μmol/L) was added to unstirred platelets, after which stirring was initiated at varying times and the aggregation response was recorded. As illustrated in Fig 5a, the aggregation response was lost with time, confirming that TRP elicits a similar response to that reported for ADP, epinephrine, and thrombin. The observed failure of platelets to aggregate to TRP was not due to a lack of platelet viability with prolonged in vitro incubation times because subsequent stimulation of the same platelets with a different agonist (ADP) elicited an aggregation response (Fig 5b). A different agonist (ADP) was used to assess viability because a second stimulation by TRP in the presence of stirring failed to elicit an aggregation response, as expected, due to desensitization of the thrombin receptor (Fig 4b).

To investigate a possible relationship between the time-dependent loss of the aggregation response and internalization of bound fibrinogen in unstirred, stimulated platelets, we used flow cytometry (FACS). For these studies, fibrinogen was directly conjugated with FITC. Previous detailed studies by Hantgan have shown that FITC labeling of fibrinogen does not alter its clottability or fibrin assembly function or the rate, extent and specificity of binding to platelets. In preliminary studies, FITC-fibrinogen (0.79
μmol/L) was incubated with citrated PRP for 30 minutes at 22°C in the presence or absence of TRP and examined by FACS to confirm the specificity of binding. As shown in Fig 6, FITC-fibrinogen underwent activation-dependent binding to platelets in response to 100 μmol/L TRP. Similar results were obtained after stimulation with either ADP (70 μmol/L) or phorbol myristate acetate (PMA; 20 μmol/L). Additionally, preincubation with either EDTA (12 mmol/L) or GRGDSP (1 mmol/L) resulted in 95% and 80% inhibition of FITC-fibrinogen binding, respectively.

Having shown specific FITC-fibrinogen binding, we next performed a time course of stimulation of FITC-fibrinogen binding and FACS analyses of FITC-fibrinogen distribution in unstirred platelets. Because FACS lasers readily penetrate intact cells and thus excite both surface and intracellular FITC-fibrinogen, our experimental strategy relied on the use of a rabbit antifluorescein IgG to rapidly quench surface/extracellular fluorescence, thereby distinguishing between surface and internalized FITC-fibrinogen. In these studies, unstirred, washed platelets were stimulated with TRP (100 μmol/L) at 22°C in the presence of FITC-fibrinogen (1.47 μmol/L). At the indicated time points, samples were rapidly diluted 1:100 with 4°C buffer and immediately placed on ice to halt further redistribution of αIIbβ3-FITC-fibrinogen complexes. After FACS analyses for fluorescence fluorescence, rabbit antifluorescein was added to each tube and the same samples were reanalyzed for residual unquenched fluorescence. Because αIIbβ3 internalization is inhibited at 4°C,17.22 access of the quenching antibody is limited to surface-associated fluorescein. As expected, FITC-fibrinogen bound to unstirred TRP-activated platelets with time (Fig 7, O). Despite fibrinogen binding to these platelets, parallel samples preincubated with TRP for similar time points before the initiation of stirring showed a time-dependent loss of the aggregation response (○). These data suggest that the loss of aggregation response does not result from the dissociation of bound fibrinogen. Because we have hypothesized that internalization of bound fibrinogen modifies the platelet aggregation response, we determined the relative amount of FITC-fibrinogen present on the platelet surface. A plot of the percentage of surface-associated, antibody-accessible FITC-fibrinogen (calculated as described in the Materials and Methods) showed a loss of FITC-fibrinogen from the platelet surface that preceded the observed loss of aggregation (A). These data suggest that, 60 minutes after the initiation of stimulation in the absence of stirring, fibrinogen is maximally bound, yet platelets fail to aggregate once stirring is initiated. The demonstration that the loss of FITC-fibrinogen from the platelet surface is followed by a loss of aggregation capacity suggests that internalization of fibrinogen bound to αIIbβ3 serves to modulate platelet function.

These data also suggest that as much as 50% of fibrinogen bound to αIIbβ3 can be removed from the platelet surface before the extent of platelet aggregation is significantly affected. This finding is in keeping with aggregation data obtained from studies of platelets from patients with type I Glanzmann’s thrombasthenia, an inherited disorder in which homozygotes with little or no αIIbβ3 show minimal or absent platelet aggregation. However, heterozygotes, which express only 50% to 60% of the normal amounts of αIIbβ3, exhibit normal aggregation responses.11.12 Similarly, it has been proposed that 50%, but not 10%, of the normal levels of αIIbβ3 is required for platelet plug formation and other platelet-associated hemostatic responses.12 Thus, these studies and the present work suggest that platelets have an excess of αIIbβ3 that is well above the amount required to support full aggregation and that there is a threshold level of fibrinogen-bound αIIbβ3 that must be removed from the platelet surface before affecting the extent of aggregation.

To evaluate whether internalization of bound fibrinogen in unstirred, stimulated platelets results in a net loss of αIIbβ3 from the surface, gel-filtered platelets augmented with unlabeled fibrinogen were activated with 10 μmol/L ADP for various times at RT to allow fibrinogen binding and internal...
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Fig 5. (a) Time-course of loss of aggregation response. In the absence of stirring, washed platelets augmented with 1.47 μmol/L fibrinogen were incubated with thrombin receptor peptide (100 μmol/ L TRP; double arrows) for 5, 20, and 60 minutes, at which time stirring was initiated. Maximal aggregation was determined in parallel control samples in which TRP was added immediately before the initiation of stirring (single arrow, 0 time point sample). (b) Effect of ADP on platelets desensitized with TRP. Unstirred platelets were incubated in the presence of 100 μmol/L TRP for 30 minutes (trace B). After initiation of stirring at t = 0, platelets were exposed to a second dose of TRP (100 μmol/L) at t = 4.25 minutes and a subsequent dose of ADP (70 μmol/L) at t = 6.25 minutes. The submaximal response to ADP may result from partial desensitization of the ADP receptor by TRP-stimulated secretion of ADP. In control samples (trace A), TRP was added immediately before the initiation of stirring (single arrow). Similar results were obtained when unstirred platelets were first stimulated with ADP and subsequently stimulated with TRP in the presence of stirring (data not shown).

Fig 6. Activation-dependent binding of FITC-fibrinogen. Fluorescence histograms of resting (REST) overlaid with TRP-stimulated (TRP) platelets in plasma.

Fig 7. Comparison of FITC-fibrinogen binding and surface expression with aggregation. Unstirred, washed platelets augmented with FITC-fibrinogen were stimulated with TRP at 22°C for a variety of times, after which samples were rapidly diluted 1:10 with 4°C buffer and immediately placed on ice to halt further redistribution of αDβ3-FITC-fibrinogen complexes. After analyses for fluorescein fluorescence, rabbit anti-fluorescein was added to each tube, and the same samples were reanalyzed for residual/unquenched fluorescence. Curves correspond to (1) a time course of total FITC-fibrinogen binding to unstirred, TRP-activated platelets C; and (2) a time course of the percentage of surface-associated αDβ3-FITC-fibrinogen complexes. Calculated as described in the Methods. It should be noted that, at t = 0, less than 10% binding of FITC-fibrinogen was observed, all of which represents surface bound fibrinogen. Parallel unstirred samples were stimulated with TRP for a variety of times at 22°C in an aggregometer, after which stirring was initiated and the aggregation response was recorded. The third curve reflects a time course of percent of total platelet aggregation response after initiation of stirring e.
Fig 8. Quantitation of surface $\alpha_{IIb}\beta_3$ molecules under conditions of agonist-induced fibrinogen uptake. Gel-filtered platelets were incubated for increasing periods of time with 10 $\mu$mol/L ADP in the presence of 50 nmol/L fibrinogen at RT. Internalization was inhibited by addition of ice-cold buffer at the indicated time points and the total number of $\alpha_{IIb}\beta_3$ molecules/platelet was determined by direct binding of MoAb $^{125}$I-AP-3 (50 nmol/L for 1 hour at 4°C with or without a 16-fold excess of unlabeled AP-3 to determine nonspecific binding. Specific binding of $^{125}$I-AP-3 (total - nonspecific) is reported as a function of the incubation time of the platelets with ADP and fibrinogen at RT.

Staining for biotinylated fibrinogen observed in Fig 9b and d, despite excess extracellular biotinylated fibrinogen, suggests that $\alpha_{IIb}\beta_3$, returning to the surface is either unactivated or deactivated. Although the lack of staining could be due to occupancy of $\alpha_{IIb}\beta_3$ by endogenous (unlabeled) fibrinogen, the fact that these platelets fail to aggregate once stirring is initiated (data not shown) makes this interpretation less likely. Thus, cycling of bound fibrinogen from the platelet surface to an intracellular pool is apparently responsible for the loss of aggregation response in unstirred, stimulated platelets as well as the development of irreversible fibrinogen binding.

Based on these results, we reasoned that agents that fail to support the development of irreversible binding should similarly fail to stimulate internalization of bound fibrinogen. Moreover, we predicted that, under these conditions, bound fibrinogen would remain on the platelet surface, where it would be available to participate in cell-cell contact after the initiation of stirring, thereby preventing the loss of aggregation response observed in unstirred, stimulated platelets. To test these hypotheses, delayed stirring experiments were repeated in the presence or absence of Zn$^{2+}$, because previous studies have shown that stimulation of platelets with ADP or thrombin in the presence of Zn$^{2+}$ fails to support the development of irreversible fibrinogen binding. As shown in Fig 10, unstirred platelets stimulated with TRP (100 $\mu$mol/L) exhibited aggregation, but failed to aggregate when stirring was initiated at the end of the incubation period. The presence of Zn$^{2+}$ prevented aggregation in these platelets as well as the development of irreversible fibrinogen binding.

Fig 10. Effect of Zn$^{2+}$ on unstirred, TRP-stimulated platelets. In the absence of stirring, gel-filtered platelets augmented with fibrinogen (430 nmol/L) were incubated with TRP (100 $\mu$mol/L, double arrows) in the presence (upper trace) or absence (lower trace) of ZnCl$_2$ (0.6 mmol/L) for 30 minutes, at which time stirring was initiated and the aggregation response was recorded.

Fig 9. Immunofluorescent localization of internalized $\alpha_{IIb}\beta_3$ and fibrinogen in activated, unstirred platelets. Platelets incubated with biotinylated fibrinogen (Biotin-Fbg) and MoAb15 (a monoclonal anti-$\beta_3$ antibody that does not inhibit fibrinogen binding) were stimulated with 100 $\mu$mol/L TRP for 30 minutes at RT in the absence of stirring and allowed to internalize fibrinogen. After uptake, samples were fixed and either left intact or permeabilized with lysophosphatidylcholine and counterstained with rhodamine avidin and fluorescein goat anti-mouse IgG. Arrows highlight colocalization of internalized $\alpha_{IIb}\beta_3$ and fibrinogen staining. Original magnification $\times$ 1,500.
aggregate once stirring is initiated (lower trace). In contrast, TRP-stimulated platelets (Fig 10, upper trace).

In parallel samples, we investigated the effect of Zn$^{2+}$ on the ability of unstirred, TRP-stimulated platelets to internalize biotinylated fibrinogen using immunofluorescence microscopy. In these studies, unstirred, gel-filtered platelets augmented with biotinylated fibrinogen (430 nmol/L) were stimulated with TRP (100 µmol/L) in the presence or absence of ZnCl$_2$ (0.6 mmol/L) for 30 minutes, at which time samples were fixed and either left intact or permeabilized with Triton X-100 and subsequently stained with rhodamine-avidin. In the absence of Zn$^{2+}$, intact platelets showed no detectable surface staining for biotinylated fibrinogen (Fig 11a), whereas permeabilized cells showed extensive intracellular staining for biotinylated fibrinogen (Fig 11b). In contrast, inclusion of Zn$^{2+}$ in the incubation mixture resulted in the appearance of surface labeling of intact cells for biotinylated fibrinogen (Fig 11c). Permeabilized platelets also show a rim staining pattern (Fig 11d) suggesting that biotinylated fibrinogen was largely excluded from the cell interior.

Taken collectively, these results suggest that the ability of platelets to internalize fibrinogen-bound $\alpha_{IIb}\beta_3$ downregulates platelet aggregation. Conversely, the inhibition of internalization apparently results in the maintenance of aggregation capacity. Thus, these results suggest that internalization of fibrinogen-bound $\alpha_{IIb}\beta_3$ represents a fundamental regulatory mechanism that modulates platelet function.

DISCUSSION

In the present study we have observed that bound fibrinogen is rapidly internalized by agonist-stimulated platelets. In addition, fibrinogen internalization correlates with (1) the development of irreversible fibrinogen binding to stimulated, unstirred platelets and (2) a loss in the ability of these platelets to aggregate on initiation of stirring, despite the presence of maximally bound fibrinogen. Thus, internalization of fibrinogen by activated platelets is likely to contribute to or account for both of these events. Based on these results, we propose that fibrinogen redistribution via internalization represents a regulatory mechanism enabling platelets to rapidly clear reactive, surface-associated ligand.

Stimulated fibrinogen binding is critical in platelet aggregation, a process central to hemostasis and thrombosis. Moreover, platelet aggregation can be regulated at the level of $\alpha_{IIb}\beta_3$, activation or of fibrinogen binding to $\alpha_{IIb}\beta_3$. The work reported here suggests a third potential level of regulation of platelet aggregation, i.e., the internalization of platelet-bound fibrinogen. The observed inaccessibility of bound fibrinogen to rhodamine-avidin, except on membrane permeabilization, suggests that platelets redistribute bound fibrinogen to a membrane-bound intracellular pool. Moreover, the accumulation of fibrinogen into this inaccessible pool is time-dependent, as are the phenomena of irreversible fibrinogen binding and the progressive loss of aggregation capacity. Thus, whereas previous studies have suggested that the development of irreversible fibrinogen binding, loss of accessibility to antifibrinogen antibodies, and a related loss in aggregation capacity are due to a qualitative change in surface bound fibrinogen, the present studies provide an alternative interpretation, namely, that these phenomena could occur as the result of internalization of bound fibrinogen. However, it should be noted that our studies do not suggest that an irreversible interaction between platelet $\alpha_{IIb}\beta_3$ and fibrinogen cannot or does not occur. Indeed, we and others have identified conditions in which either purified $\alpha_{IIb}\beta_3$ and fibrinogen or $\alpha$, $\beta_3$ and vitronectin or fibronectin interact with one another in an apparently irreversible manner. Nonetheless, the present study emphasizes that interpretations of data involving fibrinogen binding to acti-
vated platelets must take into account the dynamic nature of \( \alpha_{\text{IIb}} \beta_3 \) receptor cycling.

One possibility raised by the present studies is that internalization of bound fibrinogen may occur as a consequence of receptor-mediated endocytosis. Such internalization would readily explain the loss of fibrinogen dissociability and accessibility. Conversely, Peerschke\textsuperscript{12,23} has interpreted the accessibility of irreversibly bound fibrinogen to digestion by plasmin as evidence of surface localization. However, plasmin is known to activate platelets by inducing \( \alpha \)-granule secretion and redistribution of \( \alpha_{\text{IIb}} \beta_3 \).\textsuperscript{2-28} Furthermore, we have shown that irreversibly bound fibrinogen is susceptible to plasmin digestion only in metabolically active cells. Taken collectively, these data suggest that the ability of plasmin to cleave irreversibly bound/internalized fibrinogen most likely stems from the secondary ability of the enzyme to induce the redistribution of fibrinogen and/or \( \alpha_{\text{IIb}} \beta_3 \)-fibrinogen complexes to the platelet surface, where fibrinogen becomes accessible for digestion. Perhaps more importantly, these studies suggest that internalized fibrinogen can be recycled back to the platelet surface in response to secondary stimulation.

In related studies, megakaryocytes have been shown to internalize fibrinogen in an \( \alpha_{\text{IIb}} \beta_3 \)-dependent manner, resulting in the transport of fibrinogen to secretory \( \alpha \)-granules.\textsuperscript{18,38-41} In addition, the administration of biotinylated fibrinogen to guinea pigs results in the appearance of labeled fibrinogen in the \( \alpha \)-granules of megakaryocytes and platelets.\textsuperscript{38,40} Similarly, in humans, administration of fibrinogen to an a fibrinogenemic patient resulted in the elevation of labeled fibrinogen in the \( \alpha \)-granules of megakaryocytes and platelets.\textsuperscript{38,40} The observation that platelets from patients with Glanzmann’s thrombasthenia, ie, platelets with little or no functional \( \alpha_{\text{IIb}} \beta_3 \), show a corresponding decrease or lack of platelet \( \alpha \)-granule fibrinogen additionally supports an \( \alpha_{\text{IIb}} \beta_3 \)-mediated pathway of endocytosis in platelets.\textsuperscript{42} Moreover, ultrastructural studies have showed that antibody-tagged \( \alpha_{\text{IIb}} \beta_3 \) redistributes to \( \alpha \)-granules of unstimulated platelets.\textsuperscript{43} Finally, recent studies in our laboratory using monoclonal anti-\( \alpha_{\text{IIb}} \beta_3 \) antibodies and a high-affinity RGD-containing ligand show that \( \alpha_{\text{IIb}} \beta_3 \) is internalized in resting platelets.\textsuperscript{44} Taken collectively, these studies and the present work strongly support the hypothesis that internalization of fibrinogen by platelets occurs via an \( \alpha_{\text{IIb}} \beta_3 \)-dependent endocytic mechanism.

Fibrinogen mediates platelet aggregation by bridging integrin \( \alpha_{\text{IIb}} \beta_3 \) molecules on adjacent platelets.\textsuperscript{44} Because internalized fibrinogen is inaccessible to antibody (\( R_s = 40 \text{ Å}^2 \)) probes, it is almost certainly inaccessible to \( \alpha_{\text{IIb}} \beta_3 \) (\( R_s = 74 \text{ Å}^2 \)) on other platelets. Moreover, there is a close temporal coincidence between loss of platelet aggregation and loss of fibrinogen accessibility.\textsuperscript{12,23} In addition, when loss of fibrinogen accessibility is prevented by pretreatment with chymotrypsin, cytochalasin D,\textsuperscript{13} or Zn\textsuperscript{+2} (Figs 10 and 11), the time-dependent loss of platelet aggregation is also prevented. Thus, the internalization of fibrinogen reported here is likely to be involved in the downregulation of platelet aggregation. Furthermore, because stimulation of TRP desensitized platelets with ADP resulted in aggregation, these studies might also suggest that, after stimulation by a second, different agonist, newly internalized fibrinogen is re-expressed on the platelet surface, where it can participate in aggregate formation. This possibility is supported by the present studies with plasmin that show that internalized fibrinogen can be re-expressed on the platelet surface. Alternatively, subpopulations of \( \alpha_{\text{IIb}} \beta_3 \) receptors may be activated by stimulation with different agonists. These receptors may represent a previously unactivated subpopulation of \( \alpha_{\text{IIb}} \beta_3 \) and/or a previously activated subpopulation that has returned to the surface in an unactivated state. This possibility is supported by the demonstration that \( \alpha_{\text{IIb}} \beta_3 \)-fibrinogen complexes are rapidly internalized and apparently replaced on the surface with unoccupied and therefore either unactivated or deactivated \( \alpha_{\text{IIb}} \beta_3 \). Additional experiments are required to determine whether these represent alternative or concurrent events.

Our observation of fibrinogen internalization by activated platelets is consistent with an emerging model of functionally mobile integrin receptors. In recent studies on vascular integrins, it has been reported that \( \alpha_{\text{IIb}} \beta_3 \), \( \alpha_\beta_1 \), \( \alpha\beta_3 \), \( \alpha\beta_5 \), and \( \alpha\beta_2 \) (Mac-1) undergo endocytosis and cycling, whereas \( \alpha\beta_1 \), \( \alpha\beta_4 \), \( \alpha\beta_3 \), \( \alpha\beta_1 \), \( \alpha\beta_5 \), and \( \alpha\beta_2 \) (LFA-1) do so much more slowly.\textsuperscript{17,22,47-51} These studies suggest that the key determinant of whether an integrin cycles is an intrinsic property of the particular integrin rather than a particular cell type.\textsuperscript{48} Although the physiologic/pathologic significance of integrin cycling is not clearly understood, cycling of \( \alpha\beta_3 \) has been suggested to mediate cell migration and spreading\textsuperscript{47,50} and internalization of bacteria by nonphagocytic cells.\textsuperscript{52} In platelets, antibody- or RGD-peptide–tagged \( \alpha_{\text{IIb}} \beta_3 \) is actively internalized and cycles in resting cells.\textsuperscript{17,22} The present studies suggest that cycling of \( \alpha_{\text{IIb}} \beta_3 \) in activated platelets modulates the amount of surface-bound fibrinogen.

Although \( \alpha_{\text{IIb}} \beta_3 \) internalization occurs basally in resting platelets, fibrinogen internalization by \( \alpha_{\text{IIb}} \beta_3 \) would appear by necessity to require platelet activation, because a large body of evidence has established that soluble fibrinogen does not bind to \( \alpha_{\text{IIb}} \beta_3 \) on resting platelets.\textsuperscript{1} Although it has recently been suggested that \( \alpha_{\text{IIb}} \beta_3 \) on unstimulated platelets may bind and internalize soluble fibrinogen \textit{in vitro},\textsuperscript{40} our observation that resting platelets readily internalize \( \alpha_{\text{IIb}} \beta_3 \),\textsuperscript{2,22} but fail to internalize biotinylated fibrinogen (Fig 2b) argues against this possibility. Another possibility is that fibrinogen uptake might occur via fluid-phase pinocytosis without the requirement of binding. If fluid-phase pinocytosis contributed significantly to fibrinogen uptake, then conditions that allow \( \alpha_{\text{IIb}} \beta_3 \) internalization in platelets (eg, incubation with an RGD ligand\textsuperscript{47}) should also allow fibrinogen uptake. However, incubation of platelets with RGD peptides and\textsuperscript{125I}-fibrinogen completely inhibits any association of\textsuperscript{125I}-fibrinogen with platelets, thereby arguing against pinocytosis as an internalization mechanism.\textsuperscript{53} Thus, in the absence of pinocytosis, uptake of fibrinogen that occurs \textit{in vivo},\textsuperscript{18,38,34-55} most likely involves transient mild cycles of platelet activation that facilitate fibrinogen binding and uptake. The observation that in vivo, older platelets exhibit a loss of membrane as well as \( \alpha \)-granule contents,\textsuperscript{56} supports the concept of recurrent cycles of circulating platelet activa-
tion and recovery. Additionally, platelets have been shown to become activated by shear stress in vitro and flow systems and to recover from mild activation, as evidenced by their ability to undergo reversible activation or aggregation in vitro either spontaneously or more rapidly on exposure to naturally occurring platelet antagonists such as PG1 or nitric oxide. Consequently, fibrinogen uptake itself could aid in the process of platelet recovery from stimulation due to a rapid clearing of this adhesive protein from the platelet surface.

In conclusion, our results suggest that fibrinogen can be rapidly internalized by ADP-stimulated platelets to a nondissociable, antibody-inaccessible, intracellular pool. The correlation of internalization with a loss of aggregation response suggests that loss of aggregability of unstirred platelets is due to fibrinogen internalization. Thus, \( \alpha_{\text{IIb}} \beta_{3} \) internalization, which downregulates surface fibrinogen in circulating platelets appears to represent a novel antithrombotic mechanism.

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