Networking in the Hemostatic System
INTEGRIN αIIbβ3 BINDS PROTHROMBIN AND INFLUENCES ITS ACTIVATION*

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Prothrombin activation is a pivotal event in thrombosis and hemostasis because thrombin can mediate fibrin formation and can activate and aggregate platelets. Platelet aggregation depends upon the binding of adhesive proteins to integrin αIIbβ3 on the platelet surface. In the present study, a novel interface between the blood coagulation system and platelets is demonstrated by showing that 1) prothrombin binds to αIIbβ3, and 2) this interaction accelerates prothrombin activation. Prothrombin bound to purified αIIbβ3 in a specific, saturable, and divalent cation-dependent manner. This interaction was inhibited by certain monoclonal antibodies to αIIbβ3, by the αIIbβ3 ligands fibrinogen and RGD peptides, but not by thrombin or unrelated proteins. Prothrombin also interacted with αIIbβ3 on resting and stimulated platelets as demonstrated by soluble ligand binding and platelet adhesion assays. Activation of prothrombin by Factor Xa alone or Factor Xa-Va was accelerated by αIIbβ3, and this enhancement was blocked by a monoclonal antibody that inhibited prothrombin binding to the receptor. Taken together, these data identify a previously unrecognized linkage between platelets and the blood coagulation system that may have a significant regulatory consequence.

The blood coagulation cascade and platelets collaborate to mediate thrombus formation and maintain homeostasis. Both arms of the hemostatic system must be functional to prevent excessive blood loss at sites of vascular injury. Linkages between the platelet and coagulation pathways are multifaceted and necessary to achieve efficient thrombus formation. Several examples of such networking are relevant to the present study. Thrombin, formed in the blood coagulation system, converts fibrinogen to fibrin, the major protein component of the hemostatic plug, and thrombin also is a major physiological and potent activator of platelets. Fibrinogen not only forms fibrin but also aggregates platelets by engaging its receptor, integrin αIIbβ3. Platelets influence thrombin generation by providing a surface for assembly of the prothrombinase complex, consisting of the zymogen, prothrombin, Factor Xa, Factor Va, and phospholipid.

Recently, Reverter et al. (1) identified a previously unrecognized linkage between the blood coagulation system and platelets by showing that certain antiplatelet agents could inhibit thrombin generation. These agents included a chimeric monoclonal antibody (mAb)17E3, which binds to αIIbβ3, and αIIbβ3 blocks ligand binding to this receptor and is currently being used as an antithrombotic drug in humans (2, 3), and peptides containing an Arg-Gly-Asp (RGD) sequence that interact with αIIbβ3 (4) and inhibit the binding of adhesive proteins to the receptor (5–7). Moreover, thrombin generation in the presence of platelets from patients with Glanzmann’s thrombasthenia, a congenital deficiency of αIIbβ3, was also reduced compared with normal platelets (1, 8). These observations suggest that αIIbβ3 can influence prothrombin activation, but the molecular mechanism for this effect was undetermined.

In the present study, we provide a potential basis for the influence of αIIbβ3 on thrombin formation. Noting that prothrombin contains an RGD sequence within its catalytic domain (9), we considered whether the zymogen could be a ligand for the receptor. A specific and saturable interaction of the zymogen with thrombin and platelet receptors was demonstrated by soluble ligand binding and platelet adhesion assays. Activation of prothrombin by Factor Xa alone or Factor Xa-Va was accelerated by αIIbβ3, and this enhancement was blocked by a monoclonal antibody that inhibited prothrombin binding to the receptor. Taken together, these data identify a previously unrecognized linkage between platelets and the blood coagulation system that may have a significant regulatory consequence.

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Experimental Procedures

Peptides, Proteins, and Antibodies—GRGDSP peptide was prepared as described (10). The cyclic RGD peptide, KYG(cys)-GRGDWP(cys-cys), was synthesized by the same method and cyclized with potassium ferricyanide (11). The peptides were purified to homogeneity by high pressure liquid chromatography using a C18 Vydac column and characterized by amino acid composition.

Bovine serum albumin (BSA; fraction V, crystalline) was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Human prothrombin, purchased from Alexis Corp (San Diego, CA), was more 99% pure as assessed by SDS-PAGE (12). The preparations used contained only one major Coomassie Blue staining band, and this protein reacted with mAb to prothrombin (Biodiesing International, Kennebunk, ME) in Western blots. Human thrombin with a specific activity of >1000 NIH units/mg protein was from Alexis Corp. Factor Xa with specific activity of 1029 plasma equivalent units/mg protein and Spectrozyme TH, the chromogenic substrate used to measure the amidolytic activity of thrombin, were purchased from American Diagnostica Inc. (Greenwich, CT). mAb 7E3 (13) was kindly provided by Dr. Barry S. Coller, (Mt. Sinai School of Medicine, New York, NY); mAb CRC64 was described previously (14); mAb 4F10 was from Dr. Virgil Woods (University of California, San Diego, CA); and mAb LM609 was from Dr. David Chereh, Scripps Research Institute (La Jolla, CA). FITC goat antimouse IgG was purchased from Zymed Laboratories (South San Francisco, CA).

Purification of Proteins—Fibrinogen was purified from fresh human plasma by differential ethanol precipitation (15). αIIbβ3 was purified...
from octylglucoside extracts of human platelets by affinity chromatography using a KYGDRDP-Sepharose column followed by elution with excess RGD-containing peptide (4). The preparations used exhibited only two major bands corresponding to the α_{1b}β_{3} and subunits and did not react with mAb LM609, indicative of negligible α_{1b}β_{3} contamination. Further treatment of platelets with thrombin was accomplished by treating with thrombin after post-coating with BSA, prothrombin was added at concentrations of 20–50 mg/ml. mAb 7E3 (20 μg/ml) was used to inhibit prothrombin binding to the receptor, and nonspecific mouse IgG at the same concentration served as control. After selected incubation times in the presence of 1 mM Ca^{2+} to allow binding to the immobilized receptor, prothrombin activation was initiated by addition of 2 μg/ml Factor Xa or a mixture of 2 μg/ml Factor Xa and 2 μg/ml Factor Va. Thrombin generation was assayed at 37 °C by measuring the amidolytic activity of thrombin in samples removed after 12 min for Factor Xa-induced activation or 6 min for Factors Va-Va-induced activation. These samples (10 μl) were added to the prewarmed (37 °C) thrombin chromogenic substrate, Spectrozyme TH (100 μl), at a final concentration of 0.5 μl in 0.1 M NaCl, 0.05 μl Tri/HCl, 0.05% BSA (w/v), 20 mM EDTA, pH 7.9. The release of free chromophore (p-nitroanilide-diacetate) from substrate was monitored as a function of time at 405 nm in a Molecular Devices microplate reader. The means of V_{max} obtained using the Softmax program were used to calculate the thrombin concentration. Thrombin of a known specific activity was used as a calibrator. The means ± S.D. of quadruplicate measurements are expressed as the molar concentration of thrombin generated or as the percentage of increase above samples added to wells with no receptor but otherwise treated in an identical fashion.

Thrombin generation was also quantified in the following manner. Prothrombin was incubated in wells coated with α_{1b}β_{3} in the presence of mAb 7E3 (20 μg/ml) or the same concentration of mouse IgG. After 3 h of incubation, Spectrozyme TH was added to each well to a final concentration of 25 μM, and then prothrombin activation was initiated by addition of 2 μg/ml Factor Xa. The changes of absorbance were monitored using Molecular Devices microplate reader.

**RESULTS AND DISCUSSION**

**Direct Binding of Prothrombin to α_{1b}β_{3}**—In view of the evidence linking thrombin generation and α_{1b}β_{3} on platelets (1, 8), we sought to determine whether prothrombin could bind directly to the receptor. For these analyses, prothrombin was radiolabeled to a specific activity of 5 × 10^{10} cpm/μg. When characterized by SDS-PAGE, the radiolabeled prothrombin migrated as a single major band with the same mobility as nonlabeled prothrombin (Fig. 1B). The direct interaction of 125I-prothrombin with purified α_{1b}β_{3} from platelets was then evaluated by immobilizing the receptor on microtiter plate wells, a system utilized in numerous studies of this integrin (16, 17, 21, 22). As shown in Fig. 1A, a saturable interaction was observed. At the highest concentration of 125I-prothrombin added (200 μg/ml), a 40-fold excess of nonlabeled prothrombin inhibited binding by ≥85%. By comparison, 125I-prothrombin binding to immobilized BSA was minimal and similar to that observed with α_{1b}β_{3}-coated plates in the presence of excess nonlabeled prothrombin. The stoichiometry of the maximal number of prothrombin molecules specifically bound to α_{1b}β_{3} defined by subtracting the binding to the BSA-coated plates, to the number of immobilized α_{1b}β_{3} receptors, quantitated with 125I-CRC64, an α_{1b}β_{3}-specific mAb (14), was 1.22 (molar ratio), suggesting that each α_{1b}β_{3} is capable of binding a single prothrombin molecule.

Two separate approaches verified that authentic prothrombin bound to α_{1b}β_{3}. First, the radioactivity bound and eluted from the immobilized receptor had the same mobility as the added prothrombin on SDS-PAGE (Fig. 1B). Moreover, the eluted material reacted with a mAb to prothrombin (Fig. 1B). Second, potential artifacts due to radioidination were excluded by detecting the binding of prothrombin to α_{1b}β_{3} in an enzyme-linked immunosorbent assay format with a prothrombin mAb. The absorbance developed with the prothrombin mAb buffer, and adherent cells were quantitated by counting the bound radioactivity in a β-counter. Nonspecific platelet adhesion was defined as the radioactivity bound to wells coated only with BSA, and this value was subtracted in the reported results.

**Thrombin Generation Assays**—α_{1b}β_{3} was immobilized onto 96-well microtiter plate wells, thrombin activation was accomplished by treating with thrombin after post-coating with BSA, prothrombin was added at concentrations of 20–50 mg/ml. mAb 7E3 (20 μg/ml) was used to inhibit prothrombin binding to the receptor, and nonspecific mouse IgG at the same concentration served as control. After selected incubation times in the presence of 1 mM Ca^{2+} to allow binding to the immobilized receptor, prothrombin activation was initiated by addition of 2 μg/ml Factor Xa or a mixture of 2 μg/ml Factor Xa and 2 μg/ml Factor Va. Thrombin generation was assayed at 37 °C by measuring the amidolytic activity of thrombin in samples removed after 12 min for Factor Xa-induced activation or 6 min for Factors Va-Va-induced activation. These samples (10 μl) were added to the prewarmed (37 °C) thrombin chromogenic substrate, Spectrozyme TH (100 μl), at a final concentration of 0.5 μl in 0.1 M NaCl, 0.05 μl Tri/HCl, 0.05% BSA (w/v), 20 mM EDTA, pH 7.9. The release of free chromophore (p-nitroanilide-diacetate) from substrate was monitored as a function of time at 405 nm in a Molecular Devices microplate reader. The means of V_{max} obtained using the Softmax program were used to calculate the thrombin concentration. Thrombin of a known specific activity was used as a calibrator. The means ± S.D. of quadruplicate measurements are expressed as the molar concentration of thrombin generated or as the percentage of increase above samples added to wells with no receptor but otherwise treated in an identical fashion.

Prothrombin was added in Buffer A containing 2 mg/ml BSA and the selected divalent cations (1 mM Ca^{2+} unless otherwise specified). After incubation for selected times (150–180 min) at 37 °C, wells were washed four or five times with Buffer A, and bound prothrombin was quantitated by counting the bound radioactivity in a γ-counter. The bound prothrombin was eluted and analyzed by SDS-PAGE, autoradiography, and Western blotting using prothrombin specific mAbs. No difference between nonlabeled prothrombin, labeled prothrombin, and eluted prothrombin was found. In some experiments, α_{1b}β_{3}-coated wells were preincubated with 20 μg/ml mAbs, peptides, or proteins prior to the addition of 125I-prothrombin. Nonspecific binding was measured in the presence of a 40-fold excess of unlabeled ligand. Prothrombin binding to wells, coated by BSA, served as an additional negative control. Data were determined as the means of triplicate or quadruplicate measurements at each experimental point.

**Binding of Prothrombin to Washed Platelets**—Platelets, isolated by differential centrifugation followed by gel filtration (18), were suspended at 1 × 10^{10}/ml in modified Tyrode’s buffer, containing 0.1% BSA, 1 mM CaCl_{2} or 10 mM EDTA. Platelets were preincubated with mAb 7E3 (20 μg/ml), nonimmune immunoglobulins (20 μg/ml), or fibrinogen (1 μg/ml) for 5 min, before 125I-prothrombin was then added at selected concentrations. After 1.5 h at room temperature, platelet-bound ligand was separated by centrifugation through 20% sucrose for 25 min at 22 °C in Beckman microfuge, and the cell-bound radioactivity was measured in a γ-counter. For some experiments, the platelets were activated with phorbol myristate acetate (PMA) at 400 nM or with ADP at 10 μM for 15 min. Thrombin was used to activate platelets at 0.1 unit/ml, and 3 mM PPACK was added 10 min later.

**Flow Cytometry**—Isolated platelets were suspended at 1 × 10^{10}/ml in modified Tyrode’s buffer containing 0.1% BSA, 1 mM CaCl_{2}, 3 μg/ml prostaglandin I_{2}, and 2 mM PPACK (Buffer B) and incubated with prothrombin (100 μg/ml), cyclic RGD peptide (50 μM), or with no addi-
positions for 60 min at room temperature. LIBS 1 mAb (19) or control mouse IgG was added to a final concentration of 50 μg/ml. After 30 min, the platelets were washed by centrifugation in Buffer B, incubated with FITC-goat anti-mouse IgG on ice for 20 min, and then analyzed by flow cytometry. Flow cytometry was performed using a FACScan instrument (Becton Dickinson, San Jose, CA); 10,000 events were recorded; and the data were analyzed using the CellQuest software program (version 1.2).

**Phosphatidylinositol Analyses**—Platelets were labeled in platelets, each platelet with 3^{2}Cr (specific activity 100 cpm/100 platelets) described (20). The radiolabeled platelets were then isolated as described above and suspended at 5 × 10^{10}/ml in modified Tyrode’s buffer containing 0.2% BSA and 1 mM CaCl_{2}. The platelet suspension (100 μl) was added into microtiter wells, which had been coated with prothrombin at a concentration of 10 μg/ml and post-coated with BSA (30 mg/ml). After 90 min at 37 °C, the wells were washed four or five times with Tyrode’s buffer, and adherent cells were quantitated by counting the bound radioactivity in a β-counter. Nonspecific platelet adhesion was defined as the radioactivity bound to wells coated only with BSA, and this value was subtracted in the reported results.
rate experiments. B and 125I-prothrombin (both stained with Coomassie); the radioactivity means excess of nonlabeled prothrombin (\(\bullet\)) also is shown. The data are means \(\pm\) S.D. of quadruplicates and are representative of three separate experiments. B, SDS-PAGE analysis of nonlabeled prothrombin and 125I-prothrombin (both stained with Coomassie); the radioactivity bound to \(\alpha_{IIb}\beta_3\)-coated wells and eluted with SDS (autoradiography); and a Western blot of the prothrombin eluted from the \(\alpha_{IIb}\beta_3\)-coated wells.

was 1.352 with \(\alpha_{IIb}\beta_3\)-coated wells compared with 0.124 when the prothrombin was added to BSA-coated wells.

Typical of the binding of adhesive ligands to \(\alpha_{IIb}\beta_3\), the interaction of prothrombin with the receptor was cation-dependent; Ca\(^2+\) and Mn\(^2+\) supported binding, and EDTA blocked the interaction (Fig. 2A). Three different mAbs to \(\alpha_{IIb}\beta_3\), 7E3, CRC64, and 10E5, which are known to block the binding of adhesive ligands to the receptor (13, 14, 23), inhibited prothrombin binding to \(\alpha_{IIb}\beta_3\) (Fig. 2B). Fibrinogen, a major physiological ligand of \(\alpha_{IIb}\beta_3\), also inhibited prothrombin binding, but an unrelated protein, transferrin, did not (Fig. 2B). Known peptide ligands of \(\alpha_{IIb}\beta_3\), GRGDSP, a potent cyclic RGD peptide (11) and the fibrinogen \(\gamma\)-chain peptide H12 (not shown) were effective inhibitors of prothrombin binding, whereas a control peptide had no effect (Fig. 2B). Both prothrombin and plasminogen contain kringle domains, but plasminogen did not inhibit prothrombin binding (Fig. 2C). In addition, thrombin did not produce significant inhibition of prothrombin binding. Taken together, this inhibitory profile is similar to that of fibrinogen and other adhesive protein ligands that bind to \(\alpha_{IIb}\beta_3\) via an “RGD recognition specificity” (22).

Prothrombin Binding to Platelets—The capacity of \(\alpha_{IIb}\beta_3\) on intact platelets to interact with prothrombin was evaluated. As shown in Fig. 3A, 125I-prothrombin exhibited dose-dependent binding to washed human platelets; maximal binding was observed at the physiological concentration of 100 \(\mu\)g/ml. This binding was cation-dependent; it was supported by Ca\(^2+\) and inhibited by EDTA (Fig. 3A). The involvement of \(\alpha_{IIb}\beta_3\) in the interaction was demonstrated by the capacity of mAb 7E3 to inhibit binding. The extent of inhibition by the mAb was \(-50\%\) (Fig. 3B). Stimulation of platelets with 400 nm phorbol myristate acetate (Fig. 3B) or 10 \(\mu\)M ADP (not shown) did not enhance prothrombin binding to the platelets. Thrombin stimulation of the platelets did substantially increase 125I-prothrombin binding to platelets, but the increase was insensitive to \(\alpha_{IIb}\beta_3\) mAbs and is likely to reflect prothrombin interaction with other platelet constituents, such as newly exposed phospholipids (24, 25). Verifying the functional state of the platelet preparations used in these analyses, 125I-fibrinogen did not bind to the resting platelet preparations but did

![Fig. 1. Prothrombin binding to purified \(\alpha_{IIb}\beta_3\). A, binding isotherms were constructed by incubating increasing concentrations of 125I-prothrombin to microtiter wells, coated with purified \(\alpha_{IIb}\beta_3\) (\(\bullet\)) or BSA (○). After 180 min at 37 °C, wells were washed, and bound prothrombin was quantitated by counting the bound radioactivity in a \(\gamma\)-counter. Binding to the \(\alpha_{IIb}\beta_3\)-coated plates in the presence >40-fold excess of nonlabeled prothrombin (\(\bullet\)) also is shown. The data are means \(\pm\) S.D. of quadruplicates and are representative of three separate experiments. B, SDS-PAGE analysis of nonlabeled prothrombin and 125I-prothrombin (both stained with Coomassie); the radioactivity bound to \(\alpha_{IIb}\beta_3\)-coated wells and eluted with SDS (autoradiography); and a Western blot of the prothrombin eluted from the \(\alpha_{IIb}\beta_3\)-coated wells.](image1)

![Fig. 2. Characterization of prothrombin binding to purified \(\alpha_{IIb}\beta_3\). A, effect of divalent cations. 125I-Prothrombin (25 mg/ml) was incubated for 180 min in \(\alpha_{IIb}\beta_3\)-coated wells in the presence of 1 mM Ca\(^{2+}\), 1 mM Mn\(^{2+}\), or 10 mM EDTA. B and C, specificity of prothrombin binding to \(\alpha_{IIb}\beta_3\). 125I-Prothrombin (25 mg/ml) was added to microtiter wells coated with \(\alpha_{IIb}\beta_3\) and preincubated 15 min with anti-\(\alpha_{IIb}\beta_3\) mAbs, 7E3, CRC64, and 10E5 or control IgG (20 \(\mu\)g/ml each); GRGDSP or a control peptide (SRYKKGD) (100 \(\mu\)g/ml each); fibrinogen or an unrelated protein (transferrin) (1 \(\mu\)g/ml); or a 30-fold molar excess of plasminogen, prothrombin, or thrombin (inhibited with PPACK). Incubations with 125I-prothrombin were in the presence of 1 mM Ca\(^{2+}\) for 150 min at 37 °C. In A, B, and C, bound prothrombin was quantitated by counting the bound radioactivity in a \(\gamma\)-counter. Values represent the means \(\pm\) S.D. of three to five representative experiments. The binding of prothrombin to \(\alpha_{IIb}\beta_3\) in the presence of 1 mM Ca\(^{2+}\) and control IgG was assigned a value of 100%.](image2)
bind to the platelets stimulated with the various agonists. Moreover, as shown in Fig. 3B, fibrinogen did not inhibit $^{125}$I-prothrombin binding to the nonstimulated platelets, whereas it inhibited $^{125}$I-prothrombin binding to the stimulated cells by $\sim 50\%$.

The direct binding of prothrombin to $\alpha_{IIb}\beta_3$ on intact platelets was further verified by the demonstration that this ligand-induced expression of LIBS. LIBS are not expressed by $\alpha_{IIb}\beta_3$ when the receptor is unoccupied but are evoked when it is occupied by a ligand with an RGD recognition specificity (19, 26). As shown by fluorescence-activated cell sorter analyses (Fig. 3C), prothrombin induced expression of the epitope recognized by the prototypic LIBS mAb LIBS I. The mean fluorescence intensity of the platelets increased from 17.5 (arbitrary units) in the absence of prothrombin to 40.5 in the presence of prothrombin (100 $\mu$g/ml). A saturating concentration (50 $\mu$m) of the cyclic RGD peptide induced a fluorescence signal of 79.6.

Recognition of prothrombin by $\alpha_{IIb}\beta_3$ on platelets also was demonstrable in adhesion assays. Platelets, radiolabeled with $^{51}$Cr, adhered specifically to immobilized prothrombin (4.3-fold increase compare with immobilized BSA). mAbs to $\alpha_{IIb}\beta_3$ and GRGDSP inhibited adhesion to the immobilized ligand (Fig. 3C). These data indicate that platelets adhere to prothrombin in an $\alpha_{IIb}\beta_3$-dependent manner.

**Effect of Prothrombin Binding to $\alpha_{IIb}\beta_3$ on Thrombin Generation**—A functional consequence of prothrombin binding to $\alpha_{IIb}\beta_3$ was demonstrated with respect to its activation to thrombin. Initial rates of thrombin generation were measured in $\alpha_{IIb}\beta_3$-coated plates in the presence of 7E3 or nonimmune mouse IgG. The results of the typical experiment are shown in Fig. 4. mAb 7E3, which inhibits prothrombin binding to $\alpha_{IIb}\beta_3$ (Fig. 2B), reduced the rate of thrombin generation by 25–30%.

As summarized in Table I, blockade of prothrombin binding to $\alpha_{IIb}\beta_3$ by 7E3 reduced thrombin generation induced by Factor Xa or by Factor Xa-Factor Va to a similar extent. Accelerated prothrombin activation by $\alpha_{IIb}\beta_3$ was also noted when the chromogenic substrate was added directly to the $\alpha_{IIb}\beta_3$-coated wells (not shown).

In these kinetic analyses, only a small fraction ($\sim 0.2\%$; Fig. 1A) of the total prothrombin is directly bound to the receptor when the activators are added. To achieve the observed 25% receptor-mediated acceleration requires a several hundred-fold enhancement in the rate of activation of bound as compared to activity was measured in a $\gamma$-counter. Values are the means $\pm$ S.D. of three representative experiments. B, effect of mAb 7E3 and fibrinogen on prothrombin binding to resting or PMA-stimulated platelets. Prior to adding $^{125}$I-prothrombin at 30 $\mu$g/ml, platelets were preincubated without any agents, with 7E3 (20 $\mu$g/ml), or with fibrinogen (1 $\mu$m) in the presence of 1 mM CaCl$_2$ (open bars). Platelets were stimulated with 400 nM PMA for 15 min and then preincubated with or without fibrinogen (1 $\mu$m), and prothrombin at a final concentration of 30 $\mu$g/ml was added to each sample (solid bars). Data are the means $\pm$ S.D. of four representative experiments. C, induction of LIBS by prothrombin binding to platelets. Isolated platelets were incubated without ligands (lines 1 and 2), with prothrombin (100 $\mu$g/ml, line 3) or with cyclic RGD peptide (50 $\mu$m, line 4) for 60 min at room temperature. LIBS I (lines 2, 3, and 4) or control mouse IgG (line 1) was added at 50 $\mu$g/ml for 30 min. After washing, the platelets were incubated with FITC-labeled goat antimouse IgG, and the platelets were analyzed by flow cytometry. D, platelet adhesion to immobilized prothrombin. Platelets were labeled by $^{51}$Cr, gel-filtered, and suspended in Tyrode’s buffer containing 0.1% BSA and 1 mM Ca$^{2+}$ at 5 $\times$ 10$^7$/ml. The platelet suspension (100 $\mu$l) was added to microtiter wells coated with prothrombin (10 $\mu$g/ml) or with BSA and incubated for 120 min in the presence of control IgG, mAb 7E3, and mAb CRC64 (20 $\mu$g/ml each) or GRGDSP or control peptide (100 $\mu$m each). Specific adhesion to prothrombin was calculated by subtracting the number of platelets adherent to BSA (in the absence or the presence of inhibitors) from the number of platelets adherent to prothrombin.
RGD-dependent cell adhesion (31), whereas thrombin must be denatured to exhibit such activity (32). Mutational analyses of the RGD sequence in prothrombin offers an approach to test this hypothesis. However, because the RGD sequence resides proximal to the active site of thrombin, the enzymatic potential of such prothrombin mutants may be compromised.

Several corollaries arise from this hypothesis. First, depending upon the exposure of the RGD sequence, certain prothrombin activation intermediates may bind well to α₁β₃ and others may not. Such differential interactions with α₁β₃ may influence the pathways of prothrombin activation (33). Second, because several integrins recognize RGD sequences (34), prothrombin binding may not be restricted to α₁β₃. Our preliminary studies indicate that prothrombin also can bind to α₁β₃. This particular interaction could have a significant impact on prothrombin activation on the surface of the endothelium. Third, a pathway in which a zymogen, in this case prothrombin, binds to an integrin and is released to the local cell surface environment upon activation, in this case thrombin, to potentially react with other receptors may be a common mechanism for control of protease activity. There is another example of an integrin that can bind a zymogen and amplify its activation. This scenario applies to Factor X, which binds to α₂β₃ (Mac-1, CD11b/CD18) on monocytic/myeloid cells and supports the cell-associated formation of factor Xa (35, 36).

In the study of Reverter et al. (1), the acceleration of prothrombin activation by α₁β₃ on platelets was measured in fibrinogen-depleted plasma, i.e. in the absence of an abundant ligand for α₁β₃ binding. Because fibrinogen only binds with high affinity to activated α₁β₃, we anticipated that fibrinogen would be an effective competitor of prothrombin binding to activated but not to nonstimulated platelets, and the data supported this prediction. These observations raise the possibility that the circulating platelet may be pre-armed with a small amount of prothrombin bound to α₁β₃. This bound prothrombin might serve as a primary target for initial activation by the Factor Xa-Va complex, which assembles on the platelet surface. As thrombin is formed, it is released from α₁β₃ and can stimulate the platelet to amplify the cell’s prothrombin binding capacity by exposing new phospholipid binding sites. Because the α₁β₃ becomes activated as a result of platelet stimulation, whether unoccupied or occupied by residual prothrombin, it would bind fibrinogen or other available ligands to support the adhesive functions of the platelet. Thus, prothrombin binding to α₁β₃ may play an important physiological role in the early events of thrombus formation. Consistent with this postulated functional role, the RGD sequence is evolutionarily conserved (37).

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