Glycoprotein Ib (GPIb)-Dependent and GPIb-Independent Pathways of Thrombin-Induced Platelet Activation

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In this study, the question of whether glycoprotein Ib (GPIb) mediates both high and moderate affinity pathways of α-thrombin–induced platelet activation was examined. Flow cytometric studies, using a panel of monoclonal antibodies (MoAbs), showed that Serratia marcescens protease treatment removed greater than 97% of the glyocalcin portion of GPIb but did not affect the changes in the expression of GPI or GMP-140 that were induced by high concentrations of α-thrombin (10 nmol/L). However, Serratia treatment almost completely abolished the increase in platelet surface GMP-140 induced by low concentrations of α-thrombin (0.5 nmol/L) and diminished the downregulation of platelet surface GPI by 60.3% ± 5.8% (mean ± SEM, n = 3). When present in 20-fold molar excess, an MoAb directed against the α-thrombin/von Willebrand factor (vWF) binding domains of GPIb completely blocked the ristocetin-dependent binding of vWF to platelets but inhibited only to about 50% the binding of α-thrombin and the activation-dependent binding of vWF. In platelets treated with Serratia marcescens protease to remove GPIb, a concentration of this MoAb 16,000-fold in excess of the maximum possible remaining copies of GPIb failed to inhibit platelet activation by α-thrombin. These studies demonstrate that activation of intact platelets by α-thrombin proceeds by both GPIb-dependent and GPIb-independent mechanisms.

The nature of the initial interactions by which platelets are activated by α-thrombin is not fully understood although numerous putative thrombin receptors have been proposed. The most extensive evidence indicates that glycoprotein Ib (GPIb) functions as a high-affinity α-thrombin receptor (kd 0.3 nmol/L) in this interaction: (1) glyocalcin, the hydrolytic product of GPIb produced by the actions of platelet calpain, functions as a competitive inhibitor of the binding of α-thrombin to platelets; (2) there are concomitant decreases in high-affinity α-thrombin binding and GPIb content in Bernard-Soulier platelets, and in platelets from patients with myeloproliferative disorders; (3) platelets treated with chymotrypsin, elastase, or Serratia marcescens protease lack both GPIb and high-affinity binding sites for α-thrombin; and (4) α-thrombin can be chemically cross-linked to GPIb in intact platelets.

In the resting platelet, GPIb is tightly complexed with GPIX in a 1:1 stoichiometric ratio, but the function of GPIX is obscure. A marked downregulation of the platelet surface expression of both GPIb and GPIX in response to α-thrombin has recently been demonstrated. The mechanism of this downregulation of platelet surface GPI remains unknown, but it may be mediated by GPIb given that: (1) an α-thrombin binding site is present in the glyocalcin portion of GPIb, (2) α-thrombin is tightly complexed to GPIb, and (3) GPIb, unlike GPIX, is bound to actin-binding protein. Platelets also have a moderate-affinity receptor (kd 11 nmol/L) that functions within the physiologically relevant range of α-thrombin concentrations. Although the nature of this receptor had not been defined, it appeared possible that it could also be GPIb given that isolated GPIb and glyocalcin can bind thrombin with both high and moderate affinities similar to those seen in its binding to intact platelets. Moreover, about 1,000 copies of GPIb could remain on the surface of platelets that have been treated with Serratia marcescens protease to remove GPIb. Because only 50 GPIb molecules are required to fully activate platelets, these observations suggested that GPIb might be mediating both of these α-thrombin activation pathways.

In this study, we have used flow cytometry and biochemical methods to evaluate the possible contribution of GPIb to α-thrombin–induced platelet responses mediated through the moderate affinity pathway. These studies have involved the use of low concentrations of α-thrombin that would activate mainly the high-affinity pathway in intact platelets, the use of monoclonal antibodies (MoAbs), particularly the TM60 antibody directed against the thrombin-binding domain in GPIb to block the access of α-thrombin to the high-affinity receptor, and the use of Serratia marcescens protease to remove the high-affinity receptor. By these means we have demonstrated that the moderate-affinity pathway of platelet activation by α-thrombin is independent of GPIb.

MATERIALS AND METHODS

Materials. Ristocetin was obtained from Lundbeck (Copenhagen, Denmark), collagen from Hormon Chemie (Munich, Germany), hirudin (1,000 U/mg), bovine serum albumin (BSA) (essentially fatty acid free prepared from fraction V), human fibrinogen (fraction I), galactose oxidase (Daectylum dendroides, Type V: 200 to 600 units/mg), and potato apyrase (grade I) from Sigma Chemical Co (St Louis, MO), human plasma cryoprecipitate from Midoriijj Co (Tokyo, Japan), Protein A-Sepharose CL-4B, Sephacryl S-200, PD-10 columns (Sephadex G-25M), and DEAE-

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S. pneumoniae neuraminidase from Seikagaku Fine Chemicals Co (Tokyo, Japan). Iodine (I2) and sodium borohydride (H2) were purchased from NEN-Dupont (Boston, MA). The other reagents were analytical grade.

Mouse gamma globulin was from Cappel Laboratories (Cochranville, PA). Sheep antiserum against human platelet membranes was prepared by Bethyl Laboratories (Beaumont, TX), and purified by ammonium sulfate precipitation (10% to 30% saturation) and fractionation on DEAE-Sephadex-chromatography with 40 mmol/L phosphate buffer (pH 7.7) containing 25 mmol/L NaCl. The breakthrough fraction was pooled for further use.

Human α-thrombin (~2,900 U/mg) was kindly supplied by Dr John W. Fenton II or was prepared from a human prothrombin concentrate.29 Bovine α-thrombin was obtained from Warner-Lambert Co (Morris Plains, NJ) and was further purified to a specific activity of greater than 2,000 U/mg.30 von Willebrand factor (vWF) was purified from human plasma cryoprecipitate.31 α-Thrombin and vWF were iodinated by the chloramine-T method to give specific radioactivities of greater than 2,000 U/mg.31,32 von Willebrand factor (vWF) was purified from human plasma cryoprecipitate.31 α-Thrombin and vWF were iodinated by the chloramine-T method33 to give specific radioactivities of 16,160 dpm/unit and 3,480 dpm/μg, respectively.

Serrata marcescens protease was generously supplied by Dr Herbert A. Cooper, The University of North Carolina at Chapel Hill, and Dr Arnold Kreger, Wake Forest University. It was reconstituted in 50 mmol/L Tris, pH 7.4, to a concentration of 0.5 mg/mL and stored at ~70°C.

**MoAbs.** The MoAbs TM60 (IgG2a) was used in both the biochemical and flow cytometric studies. TM60 inhibits both thrombin- and ristocetin-induced platelet aggregation34 and binds to a domain in the terminal 45-Kd peptide of GPIb in which the thrombin and vWF binding sites overlap.24,25 TM60 was produced as previously described23 and purified from culture medium by adsorption on Protein A-Sepharose CL-4B. Antibody concentration was determined spectrophotometrically by assuming A 1% = 280 = 14.0. For the biochemical studies, purified antibody was iodinated by the chloramine-T method35 and free iodine was removed by gel filtration on PD-10 columns (Pharmacia Fine Chemicals). The specific radioactivity of iodinated TM60 was 77,300 dpm/μg.

Other MoAbs used in the flow cytometric studies were: S12 directed against GMP-140,36 also referred to as PADGEM protein,37 which is a component of the α-granule membrane and is a very precise marker of platelet activation because it is only expressed on the platelet surface after secretion;6 6D12 and AK35 directed against the vWF receptor domain and the macroglycoprotein domain, respectively, of glycopocalcin; FMC25 directed against GPⅡbX5-8; AK1 directed against the GPIb-IX complex; and 10E5 directed at, or very near, the fibrinogen receptor on the platelet membrane GPIb-IIIa complex.48 All these antibodies are of the IgG class. Antibodies 6D1, AK3, FMC25, AK1, and TM60 were labeled with fluorescein isothiocyanate (FITC)49 while antibodies S12 and 10E5 were biotinylated.47 The biotinylated and fluoresceinated antibodies appeared to function normally in that their binding to platelets was saturable at antibody concentrations similar to their respective unlabelled antibodies.

**Preparation of washed platelets for binding studies.** Blood from healthy volunteers (80 mL) was drawn into a syringe containing one-tenth vol of 3.8% citric acid. Platelet-rich plasma was prepared by centrifugation at 200g for 15 minutes and acidified to pH 6.5 by an addition of 1 mol/L citric acid. Platelets were isolated by centrifugation at 1,200g for 15 minutes and suspended in calcium-free modified Tyrode’s buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 0.42 mmol/L NaH2PO4, 12.5 mmol/L NaHCO3, 2 mmol/L MgCl2, 5.5 mmol/L dextrose, 0.35% BSA, pH 6.5), containing 25 U/mL of heparin and 3 U/mL of apyrase.40 After incubation at 37°C for 10 minutes, platelets were collected by centrifugation at 1,200g for 10 minutes and resuspended in pH 7.4 Tyrode’s buffer containing 1 mmol/L Ca2+ and Mg2+.

**Binding sites for TM60.** Binding studies were performed by the technique previously described.44 Briefly, platelets (100 μL; 3 × 1010/μL) were placed in microtiter wells of Millipore plates type HA (Millipore Corp, Bedford, MA) and incubated with various concentrations of 125I-TM60 (0 to 60 nmol/L) for 120 minutes at 37°C. Following filtration, platelets retained on the plate were washed three times with Tris-buffered saline (TBS; 10 mmol/L Tris.HCl, pH 7.6, 0.15 mol/L NaCl; 200 μL) and were counted by a gamma counter (Aloka, Tokyo, Japan). Nonspecific binding was determined in the presence of 100-fold excess of mouse gamma globulin.

**Binding of vWF.** Washed platelets were resuspended in Tyrode’s buffer to give 3 × 1010 platelets/μL. Platelet suspensions (700 μL) were preincubated with 65 μg/mL of TM60 or TBS as a control for 30 minutes at room temperature, followed by the addition of 29.5 μg of 125I-vWF and 1.4 mg/mL of ristocetin. After a further 15 minutes of incubation, the platelet suspensions were placed over a 10% sucrose solution and centrifuged at 10,000g for 5 minutes at 4°C to separate the platelets from free vWF in the supernatant. Nonspecific binding was determined in the presence of a 50-fold excess of unlabeled vWF. Radioactivity associated with the platelets was measured by a gamma counter.

**Binding of α-thrombin.** α-Thrombin binding experiments were performed using fixed platelets as described by Jung et al.44 Fixed platelets were washed twice with TBS and resuspended in the same buffer to 3 × 1010 platelets/μL. Platelet suspensions (200 μL) were preincubated with TM60 (50 μL) for 30 minutes at 20°C before incubation with increasing concentrations of iodinated α-thrombin for 20 minutes at room temperature (20 to 25°C). Following this procedure the platelet suspension was layered on 10% sucrose and centrifuged to separate the bound and unbound α-thrombin.

**Serrata protease treatment of platelets.** The method of Harmon and Jameson was used. Lyophilized, purified Serrata marcescens protease was reconstituted in 50 mmol/L Tris, pH 7.4, to a concentration of 0.7 mg/mL and stored at ~80°C. For flow cytometry studies, blood was drawn by venepuncture from healthy adult volunteers who had not ingested aspirin within the previous 10 days. The blood was drawn directly into a syringe containing 1/7 vol of acid-citrate-dextrose (85 mmol/L trisodium citrate, 71 mmol/L citric acid, 111 mmol/L dextrose, pH 4.5), resulting in a final pH of 6.5. The blood was centrifuged (150g for 15 minutes at 22°C) and the platelet-rich plasma removed. Platelets were separated from plasma by centrifugation (1,800g for 10 minutes at 22°C) and gentle resuspension in citrate-albumin buffer (11 mmol/L glucose, 128 mmol/L sodium chloride, 4.3 mmol/L monobasic sodium phosphate, 7.5 mmol/L dibasic sodium phosphate, 4.8 mmol/L sodium citrate, 2.4 mmol/L citric acid, 0.35% BSA, pH 6.5). The platelets were washed three times with the citrate-albumin buffer and resuspended at a concentration of 1.0 × 1010/μL in modified Tyrode’s buffer, pH 7.4 containing 1 mmol/L Mg2+. Aliquots of the platelet suspension were incubated (30 minutes at 20°C) with or without purified Serrata protease at final concentrations between 0.1 and 2.5 μg/mL. After addition of an equal volume of the citrate-albumin wash buffer, the samples were centrifuged (2,000g for 4 minutes at 22°C). The supernatants were stored at ~80°C until analysis by enzyme-linked immunosorbent assay (ELISA) for glycopocalcin content (see below). The platelets were resuspended in modified Tyrode’s buffer supplemented with either 5 mmol/L EDTA or 2 mmol/L CaCl2, pH 7.4.

The platelets were treated with Serrata protease by a slightly modified procedure in larger scale preparations when required for biochemical studies.

**Flow cytometric analysis of platelet surface GP.** Fixed, washed platelets were incubated (20 minutes at 22°C) with a saturating
concentration of one of the biotinylated or FITC-labeled MoAbs, washed (1,800g for 10 minutes at 22°C), and resuspended in phosphate-buffered saline (PBS) before flow cytometric analysis. In the assays with a biotinylated antibody, there was an additional incubation (20 minutes at 22°C) with phycoerythrin-streptavidin (Jackson ImmunoResearch, West Grove, PA), followed by a further wash in PBS. Samples were analyzed in an EPICS Profile flow cytometer (Coulter Cytometry, Hialeah, FL) or a FACS 440 (Becton Dickinson FAC Systems, Mountain View, CA). The fluorescence of FITC and phycoerythrin were detected using 525-nm and 575-nm band pass filters, respectively. Antibody binding was determined by analyzing 10,000 individual platelets for FITC or phycoerythrin fluorescence. To compare results in linear form, data obtained from fluorescence channels in a logarithmic mode were converted to their linear equivalents. Background binding obtained from parallel assays with purified FITC-labeled or biotinylated mouse IgG (Calbiochem) was subtracted from each sample. Using MoAbs double-labeled with 125I and biotin, Shattil et al10 and Johnston et al6 have previously demonstrated a direct linear relationship between fluorescence and the number of antibody binding sites per platelet as determined by 125I-labeled and fluorescent-labeled antibody.

To study the effects of α-thrombin activation, platelets in modified Tyrode’s buffer were incubated (10 minutes at 37°C) with or without purified human α-thrombin at final concentrations of 0, 0.5, or 10 nmol/L. The samples were then fixed with 1% formaldehyde (30 minutes at 22°C), washed twice with PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 1.5 mmol/L KH2PO4, 8.1 mmol/L Na2HPO4, pH 7.4) by centrifugation (1,800g for 10 minutes at 22°C), resuspended at 50,000 platelets/μL in PBS, and analyzed by flow cytometry. As originally described by George et al,22 the GPIb-IX complex was analyzed on platelets thrombin-activated in EDTA-containing Tyrode’s buffer, whereas the GPIIB-IIIa complex and GMP-140 were analyzed on platelets α-thrombin-activated in Ca2+-containing Tyrode’s buffer.

Crossed immunoelectrophoresis (CIE). CIE was performed using Serratia-treated platelets that had been washed with a modified citrate wash buffer22 and then resuspended (1.5 × 10/μL) in Tris-EDTA buffer, pH 7.4 (8.6 mmol/L Tris, 96.5 mmol/L NaCl, 85.7 mmol/L dextrose, 1 mmol/L EDTA). To detect the binding of invidual α-thrombin to membrane components in experiments with Serratia-proteolysed platelets, 10 μL of Triton X-100 soluble fraction of platelets (4 × 107/μL) were incubated with 10 μL of α-thrombin (3.8 × 105 dpm/μL) and run against sheep anti-whole platelet antibody in the second dimension. The samples were electrophoresed at 100 V for 30 minutes in the first dimension and at 50 V for 15 hours in the second dimension.

Serotonin release. For serotonin release studies, platelets were subjected to a citrate wash procedure27 before being incubated with 35 mmol/L 1H-serotonin (5-hydroxy[1,2-H(N)] tryptamine creatinine sulfate; 30.4 Ci/mmol; NEN-Dupont). A preliminary experiment was performed to determine the dose-response curves for the maximum release of 1H-serotonin by various α-thrombin concentrations to determine the concentration causing 50% to 80% release into intact and Serratia-treated platelets. In the second set of experiments, the two platelet preparations of thrombin X-100 soluble fractions of platelets (4 × 107/μL) were incubated with TCA (10 μL of α-thrombin (3.8 × 105 dpm/μL) and run against sheep anti-whole platelet antibody in the second dimension. The samples were electrophoresed at 100 V for 30 minutes in the first dimension and at 50 V for 15 hours in the second dimension.

ELISA for supernatant glycocalcin. Samples stored at −80°C (see above) were thawed, centrifuged (8,000g for 4 minutes at 22°C), and the supernatants diluted in Tris-buffered saline (10 mmol/L Tris-HCl, pH 7.6, 0.015 mol/L NaCl, 0.05% Tween 20, 1% BSA with 200 μg/mL leupeptin and 4 mmol/L phenylmethylsulfonfyl fluoride). Assays for glycocalcin content were then performed by ELISA with either antibody 6D1 or antibody AK3 as previously described.

RESULTS

Flow cytometry. Treatment of washed platelets for 30 minutes at 22°C with increasing concentrations of Serratia protease resulted in a progressive reduction in the binding to the platelet surface of three GPIb-specific MoAbs TM60, 6D1, and AK3 (Fig 1). TM60 and 6D1 are directed against the N-terminal peptide segment,3,6 and AK3 against the macroglycopeptide segment1,8,9 of the glycocalcin portion of GPIb. At a Serratia protease concentration of 2.5 μg/mL, the decreases in binding were 99.8% ± 0.1% (mean ± SEM; n = 6), 99.3% ± 0.1% (n = 12), and 97.0% ± 0.5% (n = 6) for TM60, 6D1, and AK3, respectively. As determined by ELISA with MoAbs 6D1 and AK3 (data not shown), there was a reciprocal increase in the appearance of the proteolytic fragment glycocalcin in the supernatant. Taken together, these data demonstrate that Serratia protease at 2.5 μg/mL causes virtually complete cleavage of the glycocalcin portion of platelet surface GPIb within 30 minutes.

In contrast, MoAb reactivity with platelet surface GPIIX (MoAb FMC 25) and the GPIIb-IIIa complex (MoAb 10E5) was not reduced by Serratia protease treatment (Fig 1). Given that the binding of 10E5 is complex-specific, these results indicate that Serratia protease did not cause dissociation of the GPIIb-IIIa complex. GPIIX and the residual GPIb fragment also appeared to remain complexed after Serratia protease treatment, as judged by the continued binding of the complex-specific MoAb AK1 (Fig 1). Thus, Serratia protease selectively cleaves the glycocalcin complex.

![Fig 1. Effect of Serratia protease on platelet surface glycoproteins. In Figs 1, 2, and 3, washed platelets were incubated (30 minutes at 22°C) with or without Serratia protease, fixed, incubated with a saturating concentration of a fluorescent-labeled MoAb and analyzed by flow cytometry. For antibodies 6D1, TM60, AK3, FMC25, AK1, and 10E5, the fluorescence intensity of non-Serratia protease-treated platelets was assigned 100 units. For antibody S12, the fluorescence intensity of maximally activated (α-thrombin 10 nmol/L) non-Serratia protease-treated platelets was assigned 100 units (see Fig 2). Glycocalcin release from the platelet surface by Serratia protease was measured by ELISA using 6D1. Data are mean ± SEM, n = 3.](www.bloodjournal.org)
cin portion of platelet surface GPIb without reducing the platelet surface expression of GPIX or the complexing of residual GPIb with GPIX.

A number of investigators have previously shown that Serratia protease does not cause platelet activation as measured by serotonin secretion. In the present experiments, Serratia protease induced no significant increase in the exposure of GMP-140 as measured by the activation-dependent MoAb S12 (Fig 1). Antibodies against GPIX (FMC25), the GPIb-IX complex (AK1), and against the GPIIb-IIIa complex (10E5) exhibited slight increases in binding following Serratia protease treatment (Fig 1), possibly because of increased antibody access to surface epitopes as a result of the removal of the glyocalicin portion of platelet surface GPIb. Platelet activation would not account for these increases, because it results in decreases in platelet surface binding of FMC25 and AK1 (see below).

The binding of a panel of MoAbs to control platelets and platelets treated with Serratia protease (2.5 μg/mL, 30 minutes) was assessed by flow cytometry following treatment (10 minutes at 37°C) with buffer only, with 0.5 nmol/L human α-thrombin or with 10 nmol/L α-thrombin. These two α-thrombin concentrations were selected because 0.5 nmol/L α-thrombin resulted in partial, and 10 nmol/L α-thrombin resulted in maximal, activation-dependent changes in the binding to control platelets of all MoAbs assessed (Fig 2). These α-thrombin concentrations correspond to the observed kds for high- and moderate-affinity binding to platelets. When control platelets were treated with either concentration of α-thrombin there were marked increases in the platelet surface binding of an MoAb (S12) directed against GMP-140 and marked decreases in the binding of MoAbs directed against the GPIb-IX complex (FMC25, AK1, 6D1, and AK3) (Fig 2), as previously reported.

When Serratia-modified platelets were treated with 0.5 nmol/L α-thrombin the activation-dependent increase in S12 binding seen in control platelets was not observed and the activation-dependent decreases seen in the binding of MoAbs directed against the GPIb-IX complex (FMC25 and AK1) were reduced by 60.9% ± 5.6% (n = 3, P = .041) and 60.2% ± 0.5% (n = 3, P = .043), respectively (Fig 2). However, when Serratia-modified platelets were treated with the higher concentration of α-thrombin (10 nmol/L) the binding of the antibodies S12 (GMP-140-specific), FMC25 (GPIX-specific), and AK1 (GPIb-IX complex-specific) were similar to those seen following α-thrombin treatment of control platelets. It should also be noted that there was no increase in the surface expression of GPIb in Serratia-modified platelets following treatment at either thrombin concentration (Fig 2).

Histograms obtained from a representative flow cytometry experiment with antibody S12 are shown in Fig 3. Irrespective of whether high-dose or low-dose α-thrombin was used, distinct subpopulations of platelets did not appear in experiments with S12 (Fig 3) or the other antibodies studied (data not shown).

CIE. The differing effects of low concentrations of α-thrombin on intact and Serratia-treated platelets was
supported by CIE (Fig 4). When intact platelets were treated with 0.5 nmol/L of bovine \(^{125}\)I-\(\alpha\)-thrombin and then subjected to CIE, a similar pattern of immunoprecipitation arcs was seen both by Coomassie staining (Fig 4a) and by autoradiography (Fig 4b). Following Serratia treatment, the arc corresponding to GP Ib was not detectable by Coomassie blue staining (Fig 4c) and essentially no radiolabeled arcs were detectable by autoradiography (Fig 4d).

**Inhibition of secretion by TM60.** To evaluate the contribution to platelet activation of surface components other than GP Ib, platelets were treated with Serratia marcescens protease under conditions such that less than 3% of GP Ib remained as detected by flow cytometry (see above) or by densitometric analysis of periodate-labeled platelets following gel electrophoresis.\(^13\)

Preliminary dose-response studies (Fig 5, insert) showed that the concentrations of human \(\alpha\)-thrombin required for half maximal release of serotonin were approximately 0.5 nmol/L with intact platelets and 5 nmol/L with Serratia-proteolyzed platelets. These two concentrations of \(\alpha\)-thrombin were then used to induce secretion from intact and Serratia-treated platelets, respectively, in the presence of increasing concentrations of TM60 (Fig 5). Values were normalized to compensate for the differing sensitivities to \(\alpha\)-thrombin of platelets from the six different donors studied. With intact platelets, increasing concentrations of TM60 caused a progressive inhibition of release of serotonin induced by \(\alpha\)-thrombin and release was reduced to 20% of control values at a TM60 concentration of 90 \(\mu\)g/mL (Fig 5) corresponding to a 36-fold molar excess of TM60 over GP Ib. With Serratia-treated platelets, no inhibition of secretion induced by \(\alpha\)-thrombin was detectable at TM60 concentrations as high as 1,200 \(\mu\)g/mL. Because a minimum of 97% of GP Ib has been cleaved by Serratia treatment, this concentration of TM60 constitutes a 16,000-fold excess of the maximum possible remaining amount of GP Ib. Furthermore, aggregation of Serratia-treated platelets at an \(\alpha\)-thrombin concentration as high as 2.5 nmol/L was not inhibited by TM60 at 106 \(\mu\)g/mL corresponding to a 1,400-fold molar excess of TM60 over GP Ib (data not shown).

**Effect of TM60 on \(\alpha\)-thrombin binding to platelets.** Preliminary studies demonstrated that the binding of TM60 to platelets was saturable at approximately 50 nmol/L (8.25 \(\mu\)g/mL) when 3 \(\times\) 10\(^5\) platelets/\(\mu\)L were used and that TM60 bound to \sim 28,000 sites/platelet with \(k_d\) 3.4 nmol/L (\(n = 5;\) data not shown). A fixed platelet suspension (6.0 \(\times\) \(10^6\)/mL) was incubated with TM60 (90 \(\mu\)g/mL) for 30 minutes before the addition of increasing concentrations of \(^{125}\)I-\(\alpha\)-thrombin; based on 30,000 GP Ib sites/platelet, this concentration of TM60 is 20-fold higher than that required to saturate all the GP Ib present in the platelet suspension. Under these conditions thrombin binding was inhibited by approximately 50% (Fig 6).

**Effect of TM60 on vWF binding.** In nonactivated platelets, vWF binds to GP Ib in the presence of ristocetin while...
in stimulated platelets vWF binds to GPIb-IIIa. In the present study, resting platelets bound less than 0.5 μg vWF/10^8 platelets (Fig 7, column 1) while in the presence of ristocetin (1.5 mg/10^8 platelets) the amount of vWF bound increased to ~5.6 μg/10^8 platelets (column 2). However, if TM60 (65 μg/mL) was added to the platelets before the addition of ristocetin there was no increase in the amount of vWF bound above that seen in resting platelets (column 3); that is, TM60 was able to inhibit completely the ristocetin-dependent binding of vWF to GPIb.

Activation of platelets with a low concentration of bovine α-thrombin (0.25 nmol/L) resulted in an increase in the binding of vWF from less than 0.5 μg/10^8 platelets in resting platelets to ~3.5 μg/10^8 platelets in α-thrombin–activated platelets in the absence of ristocetin (Fig 7, column 4) but this activation-dependent binding was reduced to about 1.5 μg if the platelets were incubated with TM60 (65 μg/mL) before the addition of α-thrombin (column 5); that is, in contradistinction to the complete inhibition of the ristocetin-dependent binding of vWF seen with TM60, the activation-dependent binding was inhibited only 50% by a 30-fold excess of TM60.

DISCUSSION

Two complementary approaches, biochemical and flow cytometric, have been used in the present work to identify the presence of GPIb-dependent and GPIb-independent mechanisms of α-thrombin-induced platelet activation. The flow cytometric technique showed that treatment of platelets with Serratia marcescens protease removed greater than 97% of the glyocalicin portion of GPIb as measured by the decrease in fluorescence seen with the MoAbs 6D1, AK3, and TM60. This value is similar to that previously obtained by scanning densitometry of autoradiographs from sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of platelets labeled by the periodate-borotritide procedure. Although glyocalicin was essentially completely cleaved from GPIb by Serratia protease, platelet surface GPIX and the complex between the residual GPIb moiety and GPIX appeared to remain intact as judged by the continued binding of the MoAbs FMC25 (GPIX-specific) and AK1 (GPIb-IX complex-specific). Furthermore, Serratia protease treatment did not result in the exposure of GMP-140 (MoAb S12), confirming previous reports that this enzyme does not cause platelet activation.

Treatment of intact platelets with 0.5 nmol/L α-thrombin, corresponding to the minimum amount that causes full aggregation and serotonin secretion in intact platelets, resulted in the expected increased expression of GMP-140 (MoAb S12) and decreases in the binding of MoAbs directed against the GPIb-IX complex (FMC25, AK1, 6D1, AK3). In contrast, treatment of Serratia-modified platelets with this low concentration of α-thrombin did not induce surface expression of GMP-140 and resulted in only modest decreases (about 40% of that seen in control platelets) in the binding of FMC25 and AK1, reflecting the decreased sensitivity of these platelets to activation by α-thrombin. However, at high α-thrombin concentrations (10 nmol/L) the flow cytometric changes observed in Serratia-treated platelets were identical with those seen in intact platelets. It may be noted that only a single population of partially activated platelets expressing GMP-140 was seen...
with control platelets activated with 0.5 nmol/L α-thrombin and that no evidence was obtained for distinct subpopulations of fully activated or nonactivated platelets (Fig 3, top panel).

It has been clearly established that Serratia-treated platelets show reduced sensitivity to α-thrombin.2,13,23 The present results indicate that, even in the absence of the glycocalicin portion of GPIb, sufficiently high concentrations of α-thrombin can induce maximal surface expression of GMP-140 and downregulation of the expression of GPIIX and the GPIb-IX complex. However, occupancy of only about 50 high-affinity α-thrombin sites is sufficient to fully activate intact platelets.24 Because Serratia-treated platelets may have as much as 3% residual intact GPIb molecules, whether measured by flow cytometry (present studies) or autoradiography,25 this would correspond to about 1,000 residual GPIb molecules capable of binding α-thrombin and is more than sufficient to induce full activation.

Conclusive evidence that this activation was not mediated by residual GPIb present in Serratia-treated platelets was obtained by adding a large molar excess of the MoAb TM60, which is directed against the α-thrombin binding domain of glycocalicin.22,28,29 Assuming 3% residual GPIb on Serratia-treated platelets, the present studies show that α-thrombin–induced serotonin release is not inhibited by a 16,000-fold molar excess of the MoAb. These results demonstrate that there is a second pathway of α-thrombin–induced platelet activation that is independent of GPIb.

These results were further supported by studies of the differential effects of TM60 on the binding of vWF to α-thrombin–activated platelets. While vWF binds to GPIb on unactivated platelets, activation results in the expression of vWF binding sites on GPIIb/IIIa.50,51 TM60 completely inhibits platelet aggregation induced by ristocetin/vWF and by low, but not high, concentrations of α-thrombin.27 The present work has shown that, while TM60 is able to completely inhibit the binding of vWF to unactivated platelets, it inhibits the binding of α-thrombin to unactivated platelets only to about 50%. These results demonstrate that unactivated platelets possess on their surface a binding site for α-thrombin that is distinct from the binding domain on the glycocalicin–derived 45-kDa peptide, whereas all of the binding of vWF to unactivated platelets is mediated through this site. Our observation that TM60 inhibited the binding of α-thrombin to platelets only to about 50% is in good agreement with the previous observation that complete removal of GPIb by treatment with Serratia protease removed only about 40% of the total α-thrombin–binding capacity of control platelets.23

It has been suggested that the high-affinity site (GPIb) is not a true receptor but merely serves to accelerate reactions that are occurring through another receptor that is essential for platelet activation.54,55 While this question cannot be answered in the absence of methods for specifically blocking this second site, the present results show that it is unrelated to GPIb.

The component mediating the GPIb-independent pathway of platelet activation by α-thrombin has so far not been identified. We (present studies), and others,7 have shown by CIE that α-thrombin binds to both GPIb and GPIIb/IIIa as well as to several other unidentified proteins. However, platelets treated with Serratia protease to remove GPIb showed no binding of α-thrombin to GPIIb/IIIa or other components by CIE. Given that the low concentrations (0.5 nmol/L) of α-thrombin used in these experiments does not result in activation of Serratia-treated platelets, these results suggest that the previously observed binding to these platelet glycoproteins is an activation-dependent phenomenon that does not occur in intact unstimulated platelets. The inability to detect an alternative thrombin–binding component in Serratia-treated platelets probably only indicates that the appropriate antibody is not present in the anti-whole platelet antibody used in these studies.

In the previously proposed model for metabolic pathways mediating α-thrombin–induced platelet activation,1 it was tentatively suggested that the moderate affinity receptor might be an uncomplexed form of GPIb.4,28 However, this model now requires modification because the present results demonstrate that the moderate affinity pathway of α-thrombin–induced platelet activation is mediated by a receptor immunologically unrelated to GPIb. The nature of this receptor is being actively sought.

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Glycoprotein Ib (GPIb)-dependent and GPIb-independent pathways of thrombin-induced platelet activation

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