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Research Article

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Identification of the Thrombin Receptor on Human Platelets by Chemical Crosslinking

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Abstract

To identify the molecular site of thrombin binding to the platelet membrane, we covalently linked ^{125}I-thrombin to platelets by using the bifunctional chemical cross-linking agents disuccinimidyl suberate and dithio*bis*(succinimidyl propionate). The proteins cross-linked to ^{125}I-thrombin by this method were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and followed by autoradiography. Two radiolabeled thrombin complexes were identified, a major species of M_r ~ 200,000 and a minor one of M_r ~ 400,000. Hirudin prevented the formation of both complexes. The radioactivity of the ~200,000-M_r complex was always 7–10-fold greater than the radioactivity of the ~400,000-M_r complex regardless of the thrombin concentration to which the platelets were exposed (0.1–29 nM). Although ^{125}I-thrombin complexes generated with thrombasthenic platelets (lacking glycoprotein IIb/IIIa) were indistinguishable from normal, no complexes appeared when Bernard-Soulier platelets (lacking glycoprotein Ib [GPIb]) were used. Complex formation was blocked by rabbit antiglycocalicin antiserum, but not by the monoclonal antibody 6D1, which is directed against the site on GPIb where von Willebrand factor (vWf) binds in the presence of ristocetin. Although cross-linking studies suggested that vWf might partially inhibit thrombin binding to platelets, this was not confirmed by equilibrium binding studies in the presence of vWf and ristocetin. The data suggest, therefore, that at all thrombin concentrations binding occurs at the same membrane site, despite evidence from equilibrium studies for high and low affinity classes of receptors, and that the ~400,000-M_r complex is simply a dimer of the ~200,000-M_r species. We conclude that the membrane site to which thrombin binds is the glycocalicin portion of platelet GPIb at a site remote from the point of ristocetin-dependent vWf binding.

Introduction

Alpha thrombin is an important and potent activator of platelet function (1). Although the mechanisms of its action are not completely understood, thrombin appears to bind to specific sites on the platelet surface. Tollefsen et al. (2) defined two classes of binding sites by equilibrium binding studies. One class is characterized by a relatively low surface density (~500 sites/cell) and by binding at thrombin concentrations corresponding to the threshold at which physiological responses occur (K_d ~ 0.2 nM). The other class is present in greater numbers (~50,000 sites/cell) but has a lower affinity for thrombin (K_d ~ 30 nM) and uncertain physiologic significance.

Whether these classes of binding sites represent different membrane structures or negative cooperativity within a single structure has never been clarified, nor have any of the membrane structures involved been identified with certainty. Nevertheless, several reports have implicated the membrane glycoprotein Ib (GPIb)[1] as the thrombin receptor. Purified GPIb and its derivative glycocalicin competitively inhibit thrombin binding to both classes of sites (3, 4). Yet, Fab fragments of anti-GPIb antisera are reported to have minimal effect on platelet aggregation induced by thrombin (5). Platelets from patients with Bernard-Soulier syndrome, which lack GPIb, bind decreased amounts of thrombin (6). However, these platelets are known to have other membrane abnormalities as well (7).

We have attempted to identify the platelet receptor for thrombin by chemically cross-linking ^{125}I-thrombin to intact platelets with bifunctional cross-linking agents and analyzing the radioactive complexes with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). We have used normal platelets, Bernard-Soulier platelets, and platelets from patients with Glanzmann's thrombasthenia, which lack the membrane glycoprotein IIb/IIIa (GPIIb/IIIa) complex (8). In addition, we have studied thrombin binding to normal platelets in the presence of several antibodies specific for certain membrane components and in the presence of von Willebrand factor (vWf) and ristocetin. In some instances, the cross-linking studies have been supplemented with binding studies under equilibrium conditions. We have obtained evidence that the platelet has only one binding site for thrombin, and that this is located on GPIb at a point remote from where vWf binds in the presence of ristocetin.

Methods

Human blood was obtained from healthy donors, patients with Glanzmann's thrombasthenia, and patients with the Bernard-Soulier syndrome. All subjects were informed about the scope of the study. Venipuncture was performed using a 19-gauge needle. Blood was collected into 0.1 volumes of 0.13 M sodium citrate, 10 mM Na_2EDTA, in polypropylene tubes. Platelet-rich plasma (PRP) was prepared from normal and throm-

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1. *Abbreviations used in this paper:* DFP, diisopropylfluorophosphate; DSP, dithio*bis*(succinimidyl propionate); DSS, disuccinimidyl suberate; DTT, dithiothreitol; GPIb, glycoprotein Ib; GPIIb/IIIa, glycoprotein IIb/IIIa; NEM, *N'*-ethylmaleimide; PRP, platelet-rich plasma; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; vWf, von Willebrand factor.

basthenic blood by centrifugation at 500 *g* for 10 min at 24°C. Platelets were isolated from this PRP on an arabino-galactan gradient as previously described (9). Bernard-Soulier PRP was produced by allowing the whole blood to sediment at room temperture for 3 h. Platelets were collected from the Bernard-Soulier PRP by washing away the plasma with saline-phosphate–EDTA buffer (0.15 M NaCl, 10 mM sodium phosphate, and 10 mM Na_2EDTA, pH 7.4) containing 1% bovine serum albumin (BSA). All platelets were suspended in this buffer, and in some experiments 6 mM *N'*-ethylmaleimide (NEM) and 1 mM leupeptin were included.

Highly purified alpha thrombin, ~3,000 U/mg, was provided by Dr. John Fenton, New York State Dept. of Health, Albany, NY. The thrombin was iodinated (Na ^{125}I) using agarose-bound lactoperoxidase as previously described (10). Iodination did not alter the fibrinogen clotting activity of the thrombin (11). ^{125}I-thrombin was treated with diisopropylfluorophosphate (DFP) by the method of Tollefson et al. (2).

Equilibrium binding studies. Isolated platelets were suspended in Tyrode's buffer (0.14 M NaCl, 3 mM KCl, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 2 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 7.35) with 5% BSA to give a final concentration of 4.8×10^8/ml normal platelets and 2.4×10^8/ml Bernard-Soulier platelets. Binding experiments were performed at room temperature by mixing 0.2 ml of the platelet suspensions with 0.2 ml of ^{125}I-thrombin (final concentration, 0.004–8.0 U/ml) in polyethylene tubes. The total radioactivity (cpm) of each sample was measured in an automatic gamma counter (Tracor Analytic, model 1185). The samples were incubated for 30 min. We (unpublished data) and others (2) have previously shown that binding equilibrium is reached within 10–15 min. After incubation the tubes were centrifuged at 2,000 *g* for 8 min, and the supernatants aspirated without disturbing the platelet pellets. The radioactivity of the tubes was remeasured after aspiration to obtain bound ^{125}I-thrombin. Unbound (supernatant) ^{125}I-thrombin was calculated as total minus bound cpm. Nonspecifically bound thrombin was measured by including hirudin 20 U/ml in the thrombin-platelet mixtures. Radioactivity measurements were converted to units of thrombin concentration by determining the specific activity of the labeled thrombin (3.3×10^5 cpm/U).

Equilibrium binding of ^{125}I-thrombin in the presence of vWf and ristocetin or antiglycocalicin antiserum. Platelets (2×10^8/ml) were suspended in saline-phosphate–EDTA buffer containing 1% BSA, 2–100 μg/ml vWf, and ristocetin (0, 1, or 2 mg/ml). 0.2 ml of the platelet suspension was mixed with 0.2 ml of 20 nM ^{125}I-thrombin for 15 min before the cells were isolated by centrifugation. In other experiments, the platelet-thrombin mixtures contained 20% (vol/vol) rabbit antiglycocalicin antiserum obtained from Dr. N. O. Solum (12), University of Oslo, Norway, or an equivalent volume of rabbit preimmune serum with or without hirudin. The concentrations of bound and free thrombin were determined as described above.

Cross-linking protocol. Platelets (10^9/ml) were incubated for 15 min at room temperature with ^{125}I-thrombin (0.1–100 nM). In our initial studies, this incubation was in saline-phosphate–EDTA, but in other experiments the protease inhibitors leupeptin and NEM were also added as a precaution. However, the presence of NEM and leupeptin did not alter the results obtained with SDS-PAGE. In some experiments inhibitors of thrombin or other agents were added to the platelets before thrombin or a cross-linking agent was added. Hirudin (20 U/ml), purified human vWf, ristocetin, the murine monoclonal antibodies 6Dl and 10E5 (gifts from Dr. Barry Coller, State University of New York at Stony Brook, NY), and the rabbit polyclonal antiserum against glycocalicin (from Dr. Solum) were tested. The vWf was purified as previously described (13). The monoclonal antibody 6D1 is directed against the site on GPIb which vWf binds in the presence of ristocetin (14). The antibody 10E5 reacts with the GPIIb/IIIa complex where fibrinogen binds (15).

After incubation, the platelets were rinsed once with saline-phosphate–EDTA and resuspended in the same buffer. The bifunctional cross-linking agents disuccinimidyl suberate (DSS) (16) or dithio*bis*(succinimidyl propionate) (DSP) (17) were freshly prepared. The formation of the radiolabeled cross-linked complexes was followed for 20 s to 60 min by analysis with SDS-PAGE and autoradiography. Although complexes appeared by 40 s and their radioactive intensity stabilized by 80 s, the cross-linking reaction was typically allowed to proceed for 15 min at ice-bath temperature before quenching with 10 or more volumes of ice-cold 10 mM Tris HCl, 1 mM EDTA (pH 7.4 for DSS; pH 8.5 for DSP). This mixture was centrifuged at 2,000 *g* for 30 min at 25°C. The pellet was dissolved in 3.3% SDS with heating at 100°C for 5–10 min. The sample was then centrifuged at 20,000 *g* for 60 min at 25°C. The supernatant was mixed with sample buffer (0.0625 M Tris HCl, 1% SDS, pH 6.8) for electrophoresis.

SDS gel electrophoresis and autoradiography. Samples were heated in sample buffer with or without reducing agent (10 mM dithiothreitol [DTT]) at 100°C for 5 min before loading onto a 1.5-mm thick slab gel. The Laemmli system of electrophoresis buffers was used (18). The separating gels were composed of 5% acrylamide, 10% acrylamide, or a composite of 0.5% agarose and 2.25% acrylamide. Stacking gels of 3% acrylamide were used with the 5% gels. After electrophoresis the gels were stained with Coomassie Blue, dried and exposed to X-Omat AR film (Eastman Kodak Co., Rochester, NY) at −70°C. On occasion gels were sliced at 2-mm intervals, and the radioactivity of the individual slices was determined.

Reagents. DSS and DSP were purchased from Pierce Chemical Co. (Rockford, IL). Litex agarose was purchased from Accurate Chemical and Scientific Corp. (Denver, CO), and acrylamide and *bis*acrylamide were purchased from Bio-Rad Laboratories (Richmond, CA). Na ^{125}I was from Amersham Corp. (Arlington Heights, IL). Ristocetin was purchased from Lundbeck (Copenhagen, Denmark). NEM was obtained from Aldrich Chemical Co. (Milwaukee, WI). Leupeptin, lactoperoxidase, DFP, and hirudin were from Sigma Chemical Co. (St. Louis, MO). All other chemicals used in this study were of reagent grade.

Results

Addition of the cross-linking agent DSP to preincubated mixtures of ^{125}I-thrombin and platelets resulted in the appearance of three bands of radioactivity on 5% SDS-PAGE (Fig. 1). Identical results were obtained when ^{125}I-DFP–thrombin was substituted. A control study performed with ^{125}I-thrombin and DSS but without platelets showed only an ^{125}I band corresponding to monomeric thrombin in 5% gels. Another control that included ^{125}I-thrombin

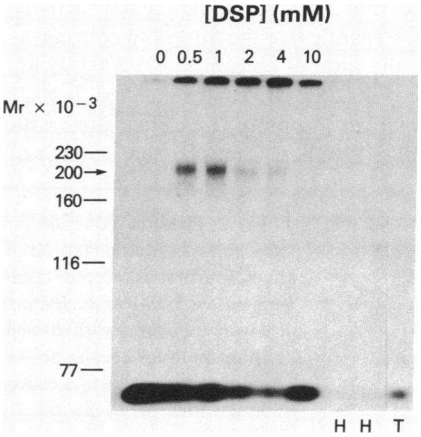

Figure 1. Autoradiograph of SDS-PAGE slab gel showing the effect of various concentrations of DSP (0–10 mM) on ^{125}I-thrombin–platelet cross-linking. The samples in the lanes marked H were cross-linked with 1 mM DSP in the presence of hirudin (10 U/ml, 20 U/ml). The lane marked T shows ^{125}I-thrombin electrophoresed without platelets or cross-linking agent. The migration distances of the molecular weight standards are indicated on the left side of the figure. The standard proteins included reduced vWf (M_r 230,000), intact IgG (M_r 160,000), β-galactosidase (M_r 116,000), and transferrin (M_r 77,000). The arrow on the left denotes the cross-linked complex (M_r ~ 200,000).

and platelets but no cross-linking agent revealed only an ^{125}I-band that also co-migrated with free thrombin (not shown). These bands seen in the controls corresponded to the most rapidly migrating band of radioactivity in the cross-linked samples. Furthermore, when the cross-linked samples (formed with the thio-cleavable agent DSP) were treated with a reducing agent (DTT), the two bands of higher M_r disappeared, while the band at M_r ~ 38,000 remained. This confirmed that the two bands of higher M_r were in fact due to covalent cross-linking by DSP, and that the band at M_r 38,000 was not a cross-linked species (Fig. 2).

The same pattern of radioactive bands was present in unreduced samples cross-linked with the noncleavable agent DSS (Fig. 2). In contrast, under reducing conditions, the middle band with M_r ~ 200,000 migrated slightly further to a position corresponding to M_r ~ 180,000. With 10% SDS-PAGE, only two autoradiographic bands appeared, one at the origin (M_r > 180,000) and one at M_r ~ 38,000, co-migrating with free ^{125}I-thrombin (data not shown).

The intensity of the unreduced middle band on 5% SDS-PAGE was maximal with 0.5 mM and 1 mM DSP (Fig. 1). At higher concentrations of DSP, less of the cross-linked material could be dissolved in SDS. When 10 mM DSP was used, only 38% of the radiolabeled material remained in the supernatant of samples centrifuged at 20,000 *g* for 60 min, whereas with 1 mM DSP, 90% of the radioactivity remained in the supernatant, and in the absence of DSP 94% remained soluble. Therefore, 1 mM DSP was used in subsequent experiments.

With an initial thrombin concentration of 20 nM and a platelet concentration of 10^9/ml, ~2.5% of the total thrombin in an individual sample became bound to the platelets. With 1 mM DSP, ~15% of this platelet-bound ^{125}I-thrombin migrated with the ~200,000-M_r complex, as quantitated by measuring the radioactivity of 2-mm gel slices.

A third band of radioactivity remained at the top of 5% gels (Fig. 1). In more porous slab gels, however, this band diminished and a faint band appeared with M_r ~ 400,000 (Fig. 3). On occasion, such slabs were cut into 2-mm slices and the radioactivity of the slices quantitated. Regardless of the thrombin concentration used (0.1–20 nM), the ratio of cpm in the ~200,000-M_r band to the cpm in the ~400,000-M_r band remained 7–10:1. When platelets, unexposed to thrombin, were incubated with DSP and separated by SDS-PAGE, Coomassie Blue staining of the gels revealed a protein pattern similar to that of non-cross-linked platelets, but with a large amount of material remaining at the origin (Fig. 4).

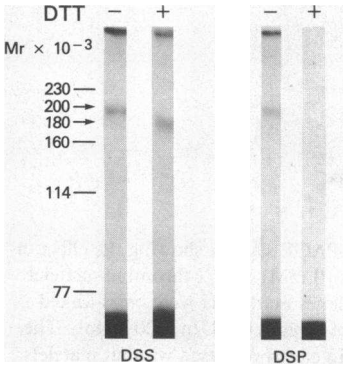

Figure 2. Effect of reduction on the electrophoretic migration of the ^{125}I-thrombin receptor complex on 5% SDS-PAGE. Platelets were incubated with 20 nM ^{125}I-thrombin for 15 min at room temperature before cross-linking with 1 mM DSS or DSP. Lanes of samples reduced with 10 mM DTT are marked +. Arrows show the ~200,000-M_r complex before reduction and the ~180,000-M_r species after reduction when the non-cleavable agent DSS is used. In contrast, when DSP (thio-cleavable) is employed, the radiolabeled band at M_r ~200,000 is absent in the presence of DTT (i.e., the ^{125}I-thrombin is cleaved from the complex). Molecular weight standards were the same as in Fig. 1.

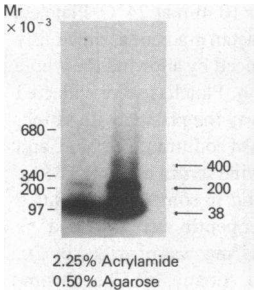

Figure 3. Autoradiograph of ^{125}I-thrombin platelet complexes electrophoresed in a slab gel of 0.5% agarose and 2.25% acrylamide. Platelets were incubated with 5 nM (*left* lane) and 20 nM (*right* lane) ^{125}I-thrombin. The migration distances of the molecular weight standards are indicated on the left side of the figure. The standard proteins included monomeric and dimeric fibrinogen (M_r 340,000 and 680,000), myosin H-chain (M_r 200,000), and phophorylase B (M_r 97,000). Three autoradiographic bands with M_r ~400,000, ~200,000, and ~38,000 (dye front) are noted. Free ^{125}I-thrombin co-migrates with the band at M_r ~ 38,000.

When hirudin was added to the thrombin-platelet mixtures, subsequent cross-linking with DSP produced no M_r 200,000 ^{125}I-thrombin complex. A faint band was present on 5% SDS-PAGE, corresponding to free thrombin nonspecifically associated with the platelets, and a small amount of radioactivity remained at the top of the gel (Fig. 1, lane *H* and Fig. 9, lane *3*). Unlabeled thrombin competed with ^{125}I-thrombin for binding to platelets, as shown in Fig. 5. At a molar ratio of labeled to unlabeled thrombin of 1:1, there was a slight diminution in the appearance of the ~200,000-M_r band, and at ratios of 1:100 and 1:500 the radioactive ~200,000-M_r band was not seen.

When platelets from a patient with the Bernard-Soulier syndrome (lacking GPIb) were substituted for normal platelets in the cross-linking studies, no radioactive bands appeared at M_r ~ 200,000 or ~400,000 in gels of 0.5% agarose and 2.25% acrylamide (Fig. 6). To confirm this apparent lack of thrombin binding to the Bernard-Soulier platelets, an equilibrium binding study was performed. A Scatchard plot of the data is shown in Fig. 7. For comparison, a parallel study with normal platelets is also presented. The only binding demonstrable to the Bernard-Soulier platelets is represented by a horizontal line (bound/free = 2.3±0.45 ml/10^{10} cells, mean±SD), which approximates the asymptote approached by the hyperbolic plot of the data for the normal platelets. The intercept of this line also coincides with the constant bound/free ratio observed in the presence of hirudin (2.6±0.40 ml/10^{10} cells, mean±SD) with free thrombin concentrations of 0.01–6 U/ml. By these criteria, the thrombin associated with the Bernard-Soulier platelets represents only nonspecific (unsaturable) binding.

SDS-PAGE of the platelets from a patient with Glanzmann's

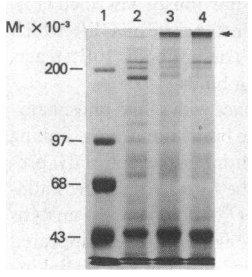

Figure 4. Coomassie Blue stain of 5% SDS-PAGE slab gel of control platelets (lane *2*) and platelets treated with 1 mM DSS (lane *3*) or 1 mM DSP (lane *4*). Note the excessive amount of protein remaining at the top of the lanes containing the cross-linked samples. The molecular weight standards (lane *1*) included myosin H-chain (M_r 200,000), phosphorylase B (M_r 97,000), bovine serum albumin (M_r 68,000), ovalbumin (M_r 43,000), alpha chymotrypsin (M_r 25,700), and β-lactoglobulin (M_r 18,400).

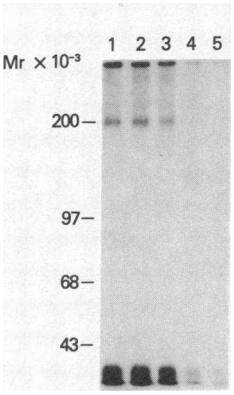

Figure 5. Effect of unlabeled thrombin on the formation of the M_r ~200,000 complex of ^{125}I-thrombin and platelet protein. ^{125}I-thrombin (20 mM) was mixed with native thrombin in a ratio of unlabeled to labeled of 1:1 (lane *2*), 10:1 (lane *3*), 500:1 (lane *4*), or 1,000:1 (lane *5*) before being added to the platelets and cross-linked with 1 mM DSP. No unlabeled thrombin was added to the ^{125}I-thrombin used in the sample in lane *1*. The ^{125}I-thrombin was effectively displaced from the thrombin-platelet complexes by unlabeled thrombin.

thrombasthenia incubated with thrombin and cross-linking agents is shown in Fig. 8. Despite the lack of the GPIIb/IIIa complex in these platelets (8), the radioactive bands derived by thrombin cross-linking were indistinguishable from those seen with normal platelets.

In an attempt to identify the submolecular components of the membrane site(s) involved in thrombin binding, we studied the effect of two monoclonal and one polyclonal antibody against platelet glycoproteins. Neither the monoclonal antibody, 6D1, which is directed against the ristocetin-dependent vWf binding site on GPIb, nor the monoclonal antibody, 10E5, which is directed against the fibrinogen binding site on GPIIb/IIIa, altered the appearance of the crosslinked bands appearing on 5% gels. However, the polyclonal antibody against glycocalicin completely inhibited the formation of the ^{125}I-thrombin–platelet complexes (Fig. 9). Preimmune serum had no discernible effect. The inhibition by antiglycocalicin was confirmed by measuring the equilibrium binding of 20 nM thrombin to platelets in the presence of the antibody and in the presence of preimmune serum with and without hirudin. Even though the concentration of antiserum was less in these experiments than in the cross-linking studies (20 vs. 50%, vol/vol), ~80% of the specific binding at equilibrium was inhibited by the antibody.

Incubating platelets with ^{125}I-thrombin in the presence of vWf and ristocetin appeared to reduce the intensity of the ~200,000-M_r band by ~20% (Fig. 10), whereas vWf alone had no apparent effect. However, equilibrium binding studies with 20 nM thrombin in the presence of ristocetin (1 or 2 mg/ml) and vWf (2–100 μg/ml) failed to demonstrate any inhibition (Fig. 11).

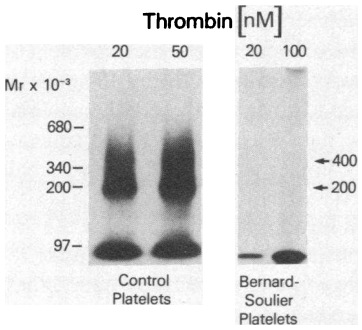

Figure 6. Analysis of the ^{125}I-thrombin–platelet complexes of normal (*left*) and Bernard-Soulier (*right*) platelets in an SDS-gel of 0.5% agarose and 2.25% acrylamide. Normal platelets were incubated with 20 and 50 nM ^{125}I-thrombin, while the Bernard-Soulier patient's platelets were incubated with 20 nM and 100 nM ^{125}I-thrombin. Cross-linking was performed with 1 mM DSS. Molecular weight standards were the same as in Fig. 3. The arrows indicate radiolabeled species of M_r ~400,000, ~200,000, and ~38,000 (dye front). In contrast to normal platelets, the platelets from the Bernard-Soulier patient show only a band that co-migrates with free thrombin.

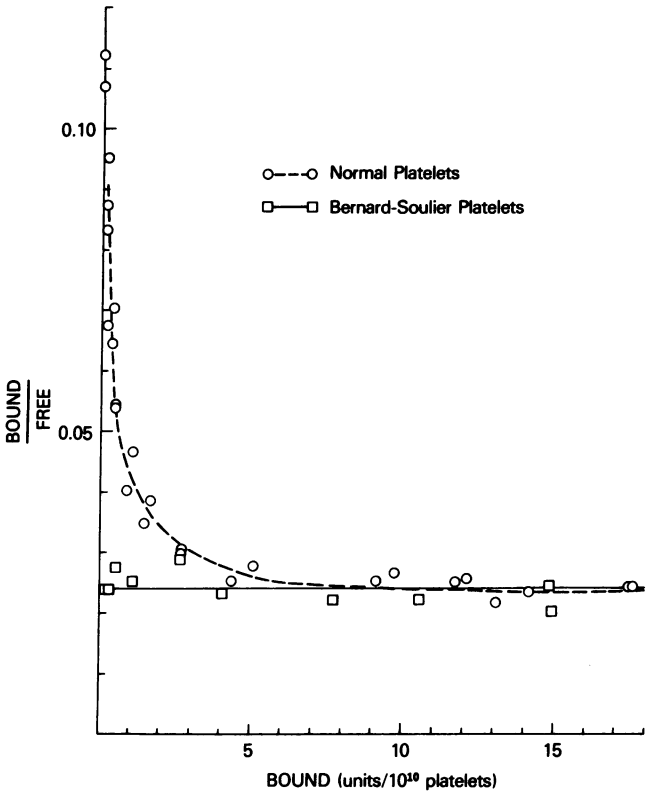

Figure 7. Scatchard plot representing the binding of thrombin to normal and Bernard-Soulier platelets. The horizontal line through the Bernard-Soulier points approximates the asymptote of the hyperbolic curve depicting the data for the normal platelets. The vertical intercept of this line (bound/free ~ 2.3 ml/10^{10} cells) agrees well with the bound/free ratio measured in the presence of hirudin (2.6±0.40 ml/10^{10} cells, mean±SD). The binding experiments were performed at 25°C.

Discussion

We have studied the binding of alpha thrombin to the platelet surface by cross-linking radiolabeled thrombin to platelets with the bifunctional agents DSP and DSS and by measuring the

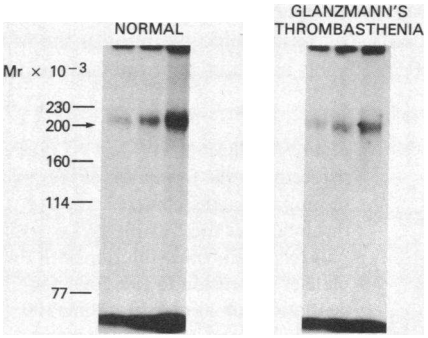

Figure 8. Autoradiograph of normal platelets and platelets from a patient with Glanzmann's thrombasthenia, exposed to (left to right) 10, 20, and 100 nM ^{125}I-thrombin and cross-linked with 1 mM DSS. The samples were unreduced. The migration distance corresponding to M_r ~ 200,000 is indicated by an arrow. ^{125}I-thrombin cross-linked complexes of the thrombasthenic patient's platelets are indistinguishable from those formed with normal platelets. Molecular weight standards were the same as shown in Fig. 1.

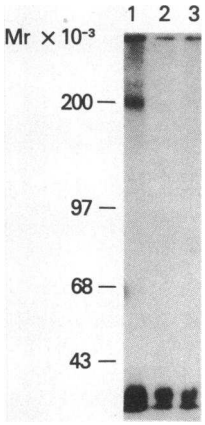

Figure 9. Effect of rabbit antiglycocalicin antiserum on the formation of ^{125}I-thrombin–platelet complexes. ^{125}I-thrombin was mixed with preimmune rabbit serum (lane *1*), antiglycocalicin antiserum (lane *2*), or hirudin (lane *3*) before dilution with platelets to give final incubation mixtures of 50% (vol/vol) rabbit serum, 20 nM thrombin, and 20 U/ml hirudin. Crosslinking was performed with 1 mM DSS. Shown is an autoradiograph of a 5% SDS-PAGE slab of these samples. The antiglycocalicin antibody, like hirudin, blocked the formation of the M_r ~ 200,000 complex. Molecular weight standards included myosin H-chain (M_r 200,000), phosphorylase B (M_r 97,000), bovine serum albumin (M_r 68,000), and ovalbumin (M_r 43,000).

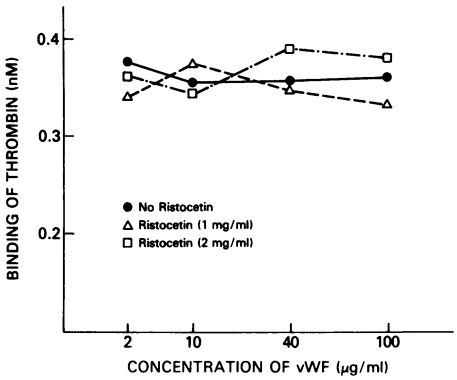

Figure 11. Effect of vWf and ristocetin on the binding of ^{125}I-thrombin to platelets. Various concentrations of vWf (2–10 μg/ml) were incubated with the platelets with or without ristocetin at room temperature for 15 min before the addition of 20 nM ^{125}I-thrombin. Specific binding of ^{125}I-thrombin was measured as described in the text: (●), without ristocetin; (△), in the presence of 1 mg/ml of ristocetin; (□), in the presence of 2 mg/ml of ristocetin. Purified vWf in the presence or absence of ristocetin had no effect on the binding of thrombin to platelets.

association of thrombin with platelets under equilibrium conditions. As we and others have shown, in the presence of the leech peptide hirudin, the only thrombin binding that occurs is unsaturable, and is equivalent to the nonspecific binding calculated from the limiting value of bound/free on a Scatchard plot (Fig. 7) (19, 20). When hirudin was added to ^{125}I-thrombin before mixing with platelets and exposure to a cross-linking agent, the only autoradiographic bands appearing after electrophoretic separation (SDS-PAGE) were very faint, at the top of the gels (to be discussed below) and at M_r ~ 38,000, corresponding to a small amount of free thrombin (Figs. 1 and 9). Therefore, the ^{125}I bands we observed on SDS-PAGE of samples prepared without hirudin are interpreted to have resulted from the reaction of thrombin with a specific membrane binding site.

When normal platelets were cross-linked to ^{125}I-thrombin in the absence of hirudin, a consistent finding was a species with M_r ~ 200,000, compatible in size with a bimolecular complex of one thrombin (M_r ~ 38,000) and one GPIb (M_r ~ 165,000). In 5% gels, ^{125}I-bands were also detected at the dye front and at the origin (M_r > 350,000). The possibility of complexes of M_r lower than 200,000 was excluded by 10% SDS-PAGE, in which the only bands visualized were at the top of the gel and at M_r ~ 38,000 (uncross-linked thrombin).

In gels of 0.5% agarose and 2.25% acrylamide, a minor cross-linked species of M_r ~ 400,000 was also evident (Fig. 3). Although it was not always well separated from the band at M_r ~ 200,000, the absence of any radiolabeled material in the M_r range 200,000–350,000 in 5% gels indicated that the faint band in the more porous system represented a distinct molecular complex rather than a group of complexes of M_r 200,000–400,000. Since there are no recognized membrane proteins sufficiently large to produce a bimolecular unit of this size with thrombin, the M_r ~ 400,000 band must be either (*a*) a dimer of the ~200,000-M_r complex, (*b*) a heteromultimer of the ~200,000-M_r complex and ~5 additional ^{125}I-thrombin molecules, or (*c*) multiple ^{125}I-thrombin molecules linked to a membrane protein or group of proteins other than that in the ~200,000-M_r band. The latter two possibilities are unlikely both because they would require multiple cross-linking reactions within a single structure and because there is no evidence of the expected family of less cross-linked components of such a conglomerate. Therefore, the most reasonable explanation for the ~400,000-M_r complex is that it is a dimer of the ~200,000-M_r species. This implies that the thrombin-receptor units in the intact platelet membrane are in close proximity, since the bridging distance of DSP is only 12 A. This possibility has previously been raised by Ganguly and Gould (21), who suggested that thrombin stimulation may lead to clustering of the thrombin receptors.

In addition to the cross-linked complexes with M_r ~ 200,000 and ~400,000, most of the polyacrylamide gels retained a variable amount of material at the origin of the lanes, regardless of the porosity of the slab matrix. Although the ~400,000-M_r complex would not be expected to enter the 5% gels, the material remaining at the top of these slabs was frequently too concentrated to be explained by this complex alone. Furthermore, small amounts of material remained at the origin of the 0.5% agarose, 2.25% acrylamide slabs. Even hirudin, which prevented the formation of cross-linked complexes, did not always eliminate retention of radioactivity at the top of the electrophoretic lanes. Similar observations have been commonly made by others using cross-linking agents (16, 17, 22). The phenomenon is generally attributed to entrapment of radiolabeled protein in an extensively cross-linked matrix of high molecular weight and limited solubility in SDS. This explanation is consistent with our observation that only 38% of the radioactivity of a thrombin-platelet sample

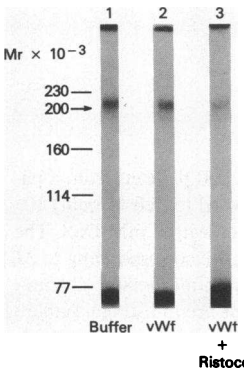

Figure 10. Effect of vWf or vWf plus ristocetin on the formation of ^{125}I-thrombin–platelet complexes. ^{125}I-thrombin was mixed with vWf or vWf plus ristocetin before dilution with the platelet suspension to give final concentrations of 20 nM thrombin, 100 μg/ml vWf, and 1 mg/ml ristocetin. The apparent reduction in the autoradiographic intensity of the M_r ~ 200,000 formed in the presence of vWf plus ristocetin was confirmed by slicing the lanes and measuring the cpm/2 mm. By this method the ~200,000-M_r band in the vWf-ristocetin lane was reduced by 22%.

remained in the supernatant after solubilization with SDS if cross-linking was accomplished with 10 mM DSP, whereas 90% remained in the supernatant if the DSP was only 1 mM. Also, when platelet proteins (unexposed to thrombin) were separated on SDS-PAGE and stained with Coomassie Blue, previous treatment of the sample with cross-linking agents was associated with marked retention of material at the electrophoretic origin, consistent with a highly cross-linked matrix of platelet protein unrelated to thrombin. Therefore, we feel that the radioactivity remaining at the origin of the electrophoretic separations of the ^{125}I-thrombin–platelet samples includes nonspecifically trapped material and not covalently bound thrombin-platelet complexes of $M_r > 400{,}000$.

Our observations, therefore, favor a single binding site for thrombin to the platelet membrane, although equilibrium studies have previously demonstrated two classes of receptors and the curvilinear configuration of our own Scatchard analysis of normal thrombin binding is consistent with this interpretation (Fig. 7). The possibility that we have selectively lost a class of cross-linked complexes during our sample preparation for SDS-PAGE appears unlikely, since ~90% of the ^{125}I-thrombin originally bound to the platelets was solubilized in SDS for electrophoresis. Furthermore, the results of the equilibrium studies are as compatible with a single binding site that manifests negative cooperativity as they are with the presence of two independent binding sites.

Most of our experiments were performed with a thrombin concentration (20 nM), which should have been sufficient to saturate the high affinity receptor class (K_d 0.2 nM) and to bind extensively to the low affinity class (K_d 30 nM). To evaluate whether the minor ~400,000-M_r complex identified under these conditions might represent the low capacity, high affinity binding site, studies were repeated with thrombin concentrations as low as 0.1 nM, which should have highlighted binding to the high affinity receptor. However, the ~200,000-M_r complex remained dominant, approximately sevenfold as intense as the ~400,000-M_r complex, just as when the much higher thrombin concentrations were used. This is further evidence, therefore, that thrombin binds to the same site regardless of the concentration of thrombin in solution.

Although this conclusion agrees with the work of Tollefsen and Majerus (22), who found a single complex of M_r ~ 200,000 when crosslinking DFP-thrombin to platelets with glutaraldehyde, it is not supported by the results of Larsen and Simons (23), who used a photo-activatable thrombin analogue. When this analogue was blocked at its active site, it formed cross-linked complexes of M_r ~ 400,000, ~200,000, and ~46,000 with platelets at thrombin concentrations > 20 nM, whereas only the ~200,000-M_r complex was apparent at thrombin concentrations ≤ 2 nM. When the active site of the analogue was not blocked, the M_r of the 200,000 complex shifted to ~120,000. The differences between these observations and ours may be related to the fact that in the earlier studies thrombin binding occurred with the platelets suspended in a modified Tyrode's buffer without protease inhibitors. This may have allowed proteolysis of the platelet membrane or the thrombin-receptor complexes. Proteolysis appeared to be prevented in our study by the presence of EDTA or EDTA with leupeptin and NEM. Coller has shown that GPIb is susceptible to proteolysis, which can be inhibited by these agents (24). Harmon and Jamieson (25) have also recently published evidence for thrombin receptor heterogeneity, but their technique of radiation inactivation determines the size of functional units rather than the size of the specific protein to which thrombin binds.

Glanzmann's platelets, which lack GPIIb/IIIa, formed cross-linked complexes with ^{125}I-thrombin indistinguishable from those seen with normal platelets (Fig. 8). GPIIb/IIIa, therefore, appears not to contain the thrombin binding site. This conclusion is consistent with the lack of binding inhibition in the presence of the monoclonal antibody 10E5. Furthermore, it agrees with the observations of White, Workman, and Lundblad (26), who demonstrated normal thrombin binding to Glanzmann's platelet under equilibrium conditions.

On the other hand, our studies with Bernard-Soulier platelets, which neither generated the cross-linked thrombin complexes nor demonstrated any specific thrombin binding at equilibrium, strongly suggest that thrombin binds to GPIb. Nevertheless, because Bernard-Soulier platelets are known to have membrane abnormalities in addition to their absence of GPIb, other lines of evidence are required to assign the binding site to this glycoprotein (7). Such evidence is provided by the marked inhibition of thrombin binding in the presence of the polyclonal antiglycocalicin antiserum. By equilibrium binding methods, this antiserum eliminated ~80% of specific thrombin binding, and by the cross-linking technique the antiserum prevented formation of the ~200,000-M_r complex. These studies, therefore, localize the binding site to a particular domain of GPIb, the glycocalicin portion of the molecule. This localization is also supported by the behavior of the ~200,000-M_r complex under reducing conditions (Fig. 2). In the presence of DTT the M_r of the complex, formed with the noncleavable cross-linking agent, decreased by ~20,000, consistent with the loss expected by reductive cleavage of the beta (nonglycocalicin) portion of GPIb (M_r 23,000) and the A chain of thrombin (M_r ~ 4,600) (27, 28). Furthermore, this behavior of the reduced complex implies that the cross-linking is to the B chain of the thrombin molecule.

In an attempt to localize the thrombin binding site even more precisely, the cross-linking studies were performed in the presence of the monoclonal antibody 6D1, which inhibits the ristocetin-dependent binding of vWf to GPIb (14). This antibody had no apparent effect on formation of the ~200,000-M_r complex. Therefore, similar studies were performed to see whether vWf itself would inhibit thrombin binding in the presence of ristocetin. Although the cross-linking studies suggested that the vWf multimers, which are manyfold larger than the 6D1 antibody, might have a moderate inhibitory effect on thrombin binding (~20%), this impression was not confirmed by equilibrium binding methods. Kao et al. (29) found a similar lack of competition of vWf and thrombin for platelet binding. Therefore, it appears that thrombin binds at a site beyond the realm where steric hindrance from the very large vWf is operative.

In summary, our data are consistent with the conclusion that platelets bind thrombin specifically to a single species of membrane protein, GPIb, despite evidence from equilibrium studies that two functional classes of binding sites exist. Furthermore, this binding occurs between the B chain of the thrombin molecule and the glycocalicin portion of GPIb, at a point remote from where vWf binds in the presence of ristocetin.

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