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Thrombin-Induced Changes in Platelet Membrane Glycoproteins Ib, IX, and IIb-IIIa Complex

By Alan D. Michelson and Marc R. Barnard

Platelet membrane glycoprotein Ib (GPIb) and the GPIIb-IIIa complex have central roles in the interaction of platelets with the plasma coagulation system, damaged vessel walls, and other platelets. We investigated the effects of thrombin on these glycoproteins. Monoclonal antibodies were used to assess platelet surface glycoproteins by flow cytometry, total platelet glycoprotein content by immunoassay, and glycoproteins released from platelets, also by immunoassay. Five new observations were made with regard to thrombin-induced changes in platelet membrane glycoproteins: (*a*) The marked decrease in platelet surface binding of antibodies directed at GPIb was not confined to antibodies directed at the von Willebrand factor binding site. (*b*) There was a marked decrease in platelet surface binding of an antibody directed at GPIX, with maintenance of the 1:1 ratio of platelet surface binding of antibodies directed at GPIb and GPIX. (*c*) Changes in platelet surface binding of antibodies were not restricted to a distinct subpopulation of platelets. (*d*) There was no associated platelet release of glycocalicin (a proteolytic fragment of GPIb). (*e*) There was no associated platelet release of the GPIIb-IIIa complex. These thrombin-induced changes may be important in modulating the reactivity of platelets with the damaged vessel wall and with each other.
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PLATELET MEMBRANE GLYCOPROTEIN (GP)Ib and the GPIIb-IIIa complex have central roles in the interaction of platelets with the plasma coagulation system, with damaged blood vessel walls, and with other platelets.[1] First, the glycocalicin portion of the α chain of GPIb contains a binding site(s) for thrombin.[2,3] Second, platelet adhesion to damaged blood vessel walls is mediated by the binding of the adhesive glycoprotein von Willebrand factor (vWf) to the glycocalicin portion of the α chain of platelet membrane GPIb.[1,4,5] Third, platelet aggregation is mediated by the activation-dependent binding of several adhesive glycoproteins, including fibrinogen, fibronectin, and vWf, to the platelet membrane GPIIb-IIIa complex.[1,6-8]

Thrombin-induced changes in GPIb and the GPIIb-IIIa complex may be important in modulating the reactivity of platelets with the damaged vessel wall and with other platelets. George et al[9] recently demonstrated that thrombin activation of platelets results in decreased binding of a monoclonal antibody directed at the vWf binding site on GPIb. A number of investigators have observed a thrombin-induced increase in the platelet surface expression of the GPIIb-IIIa complex.[9-12]

In unactivated platelets, platelet membrane GPIX is bound to platelet surface GPIb in a 1:1 ratio (the GPIb-IX complex).[13,14] However, the function of GPIX is unknown. In addition to their platelet surface pools, GPIb and the GPIIb-IIIa complex are present in intraplatelet pools.[10,11,15]

In the present study, a library of monoclonal antibodies was used to investigate thrombin-induced changes in GPIb, GPIX, and the GPIIb-IIIa complex. Different pools of these glycoproteins were assessed by the following techniques: (*a*) platelet surface glycoproteins by flow cytometry,[16,17] (*b*) glycoproteins released from platelets by enzyme-linked immunosorbent assay (ELISA),[5,18] and (*c*) total platelet glycoprotein content by ELISA of Triton X-100 platelet lysates.

MATERIALS AND METHODS

Antibodies. Table 1 lists the eight murine monoclonal antibodies used in this study. We used three different antibodies (6D1, WM23, and 3G6) directed against the glycocalicin portion of the α chain of platelet membrane GPIb. Antibody 6D1 is directed at or very near the vWf binding site on GPIb,[4] whereas WM23 and 3G6 are not directed against the vWf binding site (Berndt et al[13] and B. Adelman, personal communication). WM23 has been characterized as being directed against the macroglycopeptide portion of glycocalicin rather than the terminal protein portion that contains the vWf and 6D1 binding sites (M.C. Berndt, personal communication). After complete platelet solubilization with sodium dodecyl sulfate, polyacrylamide gel electrophoresis and Western blotting, both 3G6 (B. Adelman, personal communication) and WM23 (M.C. Berndt, personal communication) recognize only a single band (GPIb) in both reduced and unreduced gels. FMC25 is directed against platelet membrane GPIX.[13,19] Antibody 10E5 is directed against the platelet membrane GPIIb-IIIa complex.[20] WM18 is also directed against the platelet membrane GPIIb-IIIa complex but at a different epitope to 10E5 because WM18 has little effect on platelet aggregation (M.C. Berndt, personal communication), whereas 10E5 inhibits platelet aggregation induced by adenosine diphosphate (ADP), epinephrine, and collagen.[20] As a marker of platelet activation, we used KC4, a monoclonal antibody directed against the platelet activation–dependent granule–external membrane (PADGEM) protein.[21,22] PADGEM protein is a component of the α granule membrane of resting platelets that is expressed on the platelet plasma membrane during platelet activation.[22] PADGEM protein is distinct from the surface glycoproteins of resting platelets but identical to GMP-140, the antigen recognized by monoclonal antibody S12.[22,23] As a control in the flow cytometric assay, we used OX6, a monoclonal antibody directed against rat lymphocyte Ia antigen.[24]

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Table 1. Monoclonal Antibodies Used in This Study

Antibody	Antigen	Source*
6D1	Human platelet membrane GPIb (vWf binding site on glycocalicin)	Dr Barry Coller
WM23	Human platelet membrane GPIb (macroglycopeptide portion of glycocalicin; not the vWf binding site)	Dr Michael Berndt
3G6	Human platelet membrane GPIb (glycocalicin; not the vWf binding site)	Dr Burt Adelman
FMC25	Human platelet membrane GPIX	Dr Michael Berndt
10E5	Human platelet membrane GPIIb-IIIa complex (inhibits platelet aggregation induced by ADP, epinephrine, collagen)	Dr Barry Coller
WM18	Human platelet membrane GPIIb-IIIa complex (little effect on platelet aggregation)	Dr Michael Berndt
KC4	Human PADGEM protein	Dr Bruce Furie
OX6	Rat lymphocyte Ia antigen	Dr Bruce Woda

*For references, see Materials and Methods.

Thrombin activation of washed platelets. Blood was drawn by venipuncture from healthy adult volunteers who had not ingested aspirin within the previous ten 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) and 50 ng/mL (final concentration) prostaglandin E_1 (PGE_1, Sigma Chemical Co, St Louis). After resting for one hour at 22°C, the blood was centrifuged at 150 g for 15 minutes at 22°C and the platelet-rich plasma removed. The platelets were washed twice by centrifugation at 2,000 g for ten minutes at 22°C and resuspension in Tyrode's buffer (138 mmol/L NaCl, 29 mmol/L KCl, 12 mmol/L $NaHCO_3$, 0.4 mmol/L $NaHPO_4$, 0.1% glucose, 0.35% bovine serum albumin, pH 6.5) with 50 ng/mL PGE_1. After the second wash, 5×10^5 platelets/μL were suspended in Tyrode's buffer, pH 7.3, with 50 ng/mL PGE_1 and either 5 mmol/L EDTA or 2 mmol/L $CaCl_2$. Purified human α-thrombin (generously provided by Dr John Fenton II, New York Department of Health, Albany) at concentrations between 6.4×10^{-5}, and 1.0 U/10^8 platelets was added to the sample, which was then gently inverted once. In control assays performed in parallel, buffer only (no thrombin) was added. The samples were incubated undisturbed (to prevent platelet aggregation) for ten minutes at 37°C. To stop the reaction, hirudin (Sigma), 2.0 U/10^8 platelets, was added to all samples, including those with no thrombin. After gentle inversion, the samples were incubated at 22°C for five minutes. Each sample was then divided into two separate aliquots. The first aliquot was fixed at 22°C for 30 minutes with an equal volume of 2% formaldehyde in Tyrode's buffer, pH 7.3, with either 5 mmol/L EDTA or 2 mmol/L $CaCl_2$. These samples were then washed twice in Tris-buffered saline (10 mmol/L Tris-HCl, 0.15 mol/L NaCl, pH 7.8) by centrifugation at 2,000 g for ten minutes at 22°C, resuspended at 50,000 platelets/μL in Tris-buffered saline, and assayed by flow cytometry for platelet surface glycoproteins (see the next section). The second aliquot of thrombin-activated and control platelets was centrifuged at 2,000 g for ten minutes at 22°C. The supernatants were removed for ELISA determination of platelet release of glycocalicin (a proteolytic fragment of GPIb) and the GPIIb-IIIa complex (see later). The platelet pellets were lysed in Tyrode's buffer containing 1% Triton X-100 (Sigma), 50ng/mL PGE_1, and either 5 mmol/L EDTA or 2 mmol/L $CaCl_2$. Before being assayed, the supernatants and lysates were stored at −80°C for up to seven days. Before assay by ELISA for the total platelet content of GPIb and the GPIIb-IIIa complex (see later), the lysates were thawed and, to remove any cellular debris, centrifuged at 22°C for four minutes at 8,000 g (conditions that result in only a small degree of sedimentation of actin filament–associated GPIb[25]).

Flow cytometric assessment of platelet surface glycoproteins. Fixed, washed thrombin-activated and control platelets (50,000/μL) were prepared as described earlier and incubated for 20 minutes at 22°C with a saturating concentration of one of five monoclonal antibodies (6D1, WM23, 3G6, FMC25, or 10E5) to assess platelet surface glycoproteins. In parallel experiments, aliquots of these platelet suspensions were incubated with either monoclonal antibody KC4 or monoclonal antibody OX6 to assess platelet activation and background binding, respectively. As described by George et al[9] the platelet surface GPIIb-IIIa complex was assessed on platelets activated in a Ca^{++}-containing buffer, whereas the platelet surface GPIb-IX complex was assessed on platelets activated in an EDTA-containing buffer. Thus, the platelet samples that had been prepared in the presence of 2 mmol/L $CaCl_2$ (see earlier) were incubated with either 10E5 or OX6, whereas the platelet samples that had been prepared in the presence of 5 mmol/L EDTA were incubated with either 6D1, WM23, 3G6, FMC25, or OX6. Platelet surface expression of PADGEM protein was assessed by antibody KC4 on platelets prepared in the Ca^{++}-containing buffer. All platelet samples were then washed twice in Tris-buffered saline by centrifugation at 2,000 g for ten minutes at 22°C and incubated for 20 minutes at 22°C with a saturating concentration of fluorescein isothiocyanate—labeled goat antimouse IgG antibody (Cooper Biomedical, Malvern, PA). The platelets were then washed twice, resuspended in Tris-buffered saline, and analyzed in a FACS 440 (Becton Dickinson, Mountain View, CA). For each sample, the fluorescence signal from 10,000 individual cells was measured. Forward–light scatter profiles indicated that there was neither platelet clumping nor detectable contamination by other cell types in any of the samples. Histograms were gated for platelets by their characteristic forward and orthogonal light scatter, thereby excluding any possible contribution from other cells or debris. To compare results in linear form, data obtained from fluorescence channels in a logarithmic mode were converted to their linear equivalents (relative specific fluorescence) as previously described.[17]

ELISA determination of platelet release of glycocalicin and the GPIIb-IIIa complex. Supernatants obtained from centrifugation of thrombin-activated and control platelets were assessed for their glycocalicin content by an ELISA using monoclonal antibody 6D1 as previously described.[5,18] In parallel assays, 6D1 was replaced by either monoclonal antibody 10E5 or monoclonal antibody WM18. As described earlier, the supernatants used for assays with 6D1 were EDTA-containing, whereas the supernatants used for assays with 10E5 and WM18 were Ca^{++}-containing.

ELISA determination of total platelet content of GPIb and the GPIIb-IIIa complex. Lysates obtained after Triton X-100 solubilization of thrombin-activated and control platelets were assessed in the ELISA for their ability to inhibit monoclonal antibody 6D1 binding to platelet surface GPIb. This assay measures the total number of GPIb receptors for vWf because 6D1 binds with equal avidity to GPIb and glycocalicin.[5] The assay was validated by demonstrating a comparable total number of GPIb molecules per platelet in experiments in which the monoclonal antibody to GPIb was changed from 6D1 to WM23 or 3G6 and in other experiments in which, rather than Triton X-100 platelet lysates, the aqueous phase of Triton X-114–solubilized platelets[26] was assessed (A.D. Michelson, M.R. Barnard, E. Carroll: unpublished observations). Serial dilutions of the lysates obtained after Triton X-100 solubilization of thrombin-activated and control platelets were also assessed in the ELISA for their ability to inhibit the binding of either monoclonal antibody 10E5 or monoclonal antibody WM18 to the platelet surface. As described earlier, the lysates used for assays with 6D1

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were EDTA-containing, whereas the lysates used for assays with 10E5 and WM18 were Ca^{++}-containing. Control assays in which platelets were replaced by buffer only and then treated with 1% Triton X-100 resulted in the detection of no GPIb (n = 3) or GPIIb-IIIa complex (n = 3), thereby demonstrating that the dilutions of Triton used did not interfere with the ELISA.

Platelet counts. Platelet counting was performed on a Coulter Model ZBI (Coulter Electronics, Hialeah, FL).

RESULTS

A flow cytometric method was used to demonstrate the effect of thrombin on platelet surface glycoproteins. As shown in Fig 1, thrombin resulted in a marked reduction in the platelet surface binding of each of three monoclonal antibodies (6D1, WM23, and 3G6) directed against different epitopes on GPIb (Table 1). In addition, thrombin resulted in a similar reduction in the platelet surface binding of a monoclonal antibody (FMC25) directed against GPIX (Fig 1). In contrast, thrombin resulted in a marked increase in the platelet surface binding of a monoclonal antibody (10E5) directed against the GPIIb-IIIa complex (Fig 1). Parallel assays using monoclonal antibody KC4 (directed against PADGEM protein) demonstrated that the thrombin-induced changes in the platelet surface binding of monoclonal antibodies directed against GPIb, GPIX, and the GPIIb-IIIa complex were associated with platelet activation (Fig 1).

As determined by relative specific fluorescence, in unactivated platelets the ratio of platelet surface binding of 6D1:FMC25:10E5 (monoclonal antibodies directed at GPIb, GPIX, and the GPIIb-IIIa complex, respectively) was 1.0:0.9:2.0 (means of five separate experiments). These ratios are comparable to the ratio of platelet surface GPIb:GPIX:GPIIb-IIIa complex reported by other investigators using radioligand binding methods.[1,13,14] In maximally activated platelets (thrombin, 1.0 U/10^8 platelets), the ratio of platelet surface binding of 6D1:FMC25:10E5 was 1.0:0.9:29.1 (means of five separate experiments). Similarly, despite the thrombin-induced changes, there was maintenance of the approximately 1:1 ratio of the platelet surface binding of 6D1:FMC25 at all concentrations of thrombin tested (Figs 1 and 2).

The flow cytometric method of analyzing platelet surface glycoproteins is able to detect distinct subpopulations of platelets because each platelet is analyzed individually.[16,17] However, the thrombin-induced changes in binding of monoclonal antibodies to platelet surface GPIb, GPIX, and the GPIIb-IIIa complex were not restricted to a distinct subpopulation of platelets, irrespective of whether the thrombin concentration resulted in partial or complete activation of

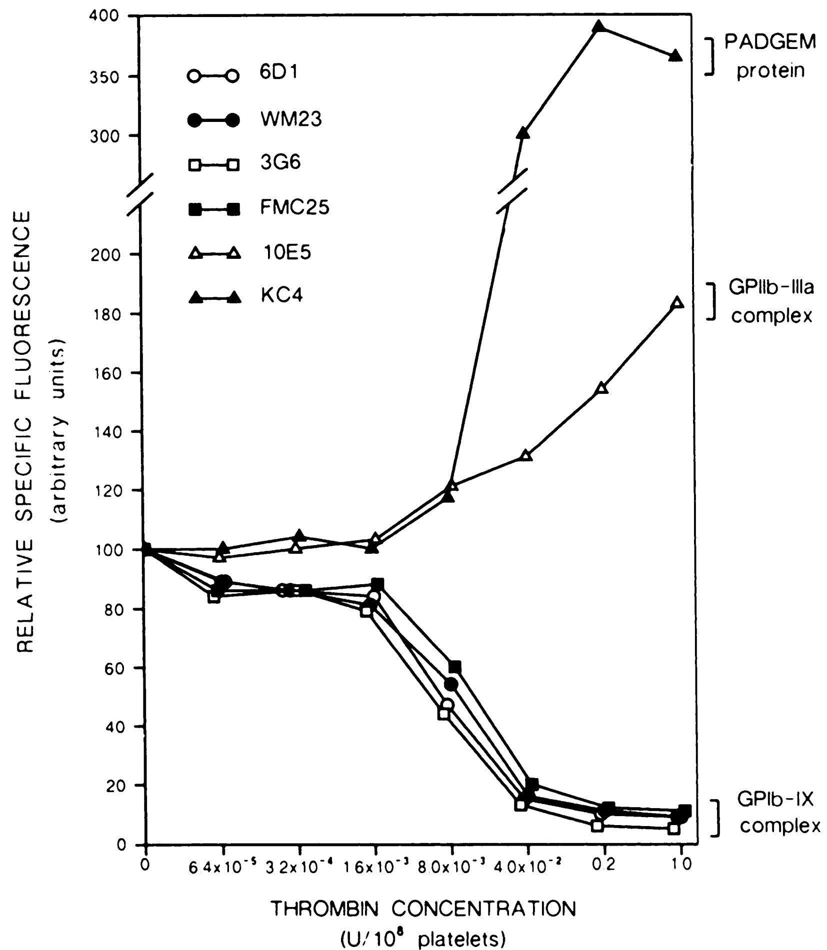

Fig 1. Effect of thrombin on the binding of monoclonal antibodies to the platelet surface as determined by flow cytometry. Platelets were incubated with monoclonal antibodies directed against either GPIb (6D1, WM23, and 3G6), GPIX (FMC25), the GPIIb-IIIa complex (10E5), or PADGEM protein (KC4). All samples were then incubated with fluoresceinated goat antimouse antibody. For each antigen, the relative specific fluorescence of control platelets was arbitrarily assigned 100 units.

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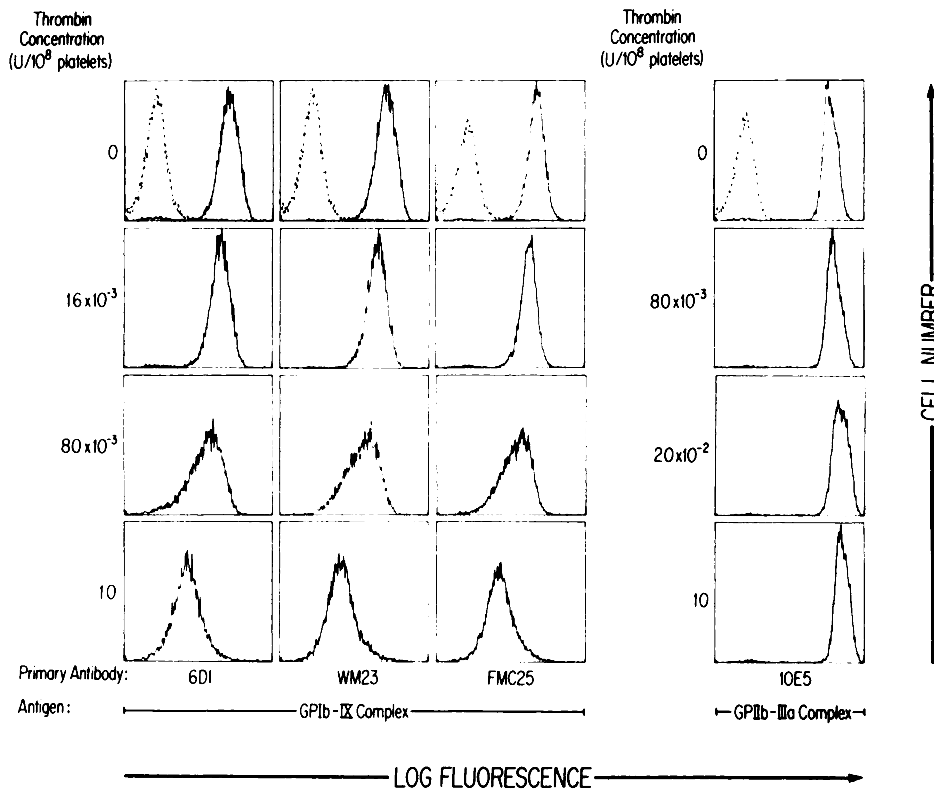

Fig 2. Effect of different concentrations of thrombin on the binding of monoclonal antibodies to the platelet surface as determined by flow cytometry. The solid lines represent platelets activated with the indicated concentrations of thrombin and then incubated with the indicated primary monoclonal antibody. The dashed lines represent platelets incubated with an irrelevant primary monoclonal antibody (background). All samples were then incubated with a fluoresceinated goat antimouse antibody. For each panel, the x-axis represents log fluorescence, and the y-axis represents cell number.

platelets (Fig 2). This is demonstrated in Fig 2 by the fact that, for all antibodies tested, increasing concentrations of thrombin resulted in a gradual shift of a single peak (to the left for the GPIb-IX complex, to the right for the GPIIb-IIIa complex).

For both control and maximally thrombin-activated platelets, the total platelet GPIb content and glycocalicin released from platelets were determined by ELISA using antibody 6D1. As shown in the upper portion of Table 2, the total platelet content of GPIb did not change significantly after activation with thrombin, 1.0 U/10^8 platelets. As shown in the lower portion of Table 2, the glycocalicin released from platelets was, as expected, very low in the control and did not rise appreciably in the thrombin-activated platelets.

For both control and maximally thrombin-activated platelets, the total platelet content and platelet release of the GPIIb-IIIa complex were determined by ELISA using antibody 10E5. As shown in Fig 3, the total platelet content of the GPIIb-IIIa complex did not change significantly after activation with thrombin, 1.0 U/10^8 platelets. Likewise, the platelet release of the GPIIb-IIIa complex was very low in the control and did not rise significantly in the thrombin-activated platelets (Fig 3). In parallel assays using antibody WM18, there was similarly no thrombin-induced change in the total platelet content of the GPIIb-IIIa complex and no release of the GPIIb-IIIa complex from platelets (data not shown).

Table 2. Effect of Thrombin (1.0 U/10^8 Platelets) on the Total Platelet GPIb Content and Release of Glycocalicin as Determined by ELISA Using Monoclonal Antibody 6D1

Parameter	Percentage of Total GPIb Content of Control Platelets (Mean ± SEM, n = 6)
Total platelet GPIb content*	
Control platelets	100.0 ± 18.4
Thrombin-activated platelets	97.4 ± 18.7
Glycocalicin released from platelets†	
Control platelets	0.3 ± 0.05
Thrombin-activated platelets	0.9 ± 0.1

*Determined from 1% Triton X-100 lysates of platelets.
†Determined from supernatants.

DISCUSSION

In this study, the effects of thrombin on platelet membrane GPIb, GPIX, and the GPIIb-IIIa complex were investigated. Five new observations were made.

First, the thrombin-induced change in the glycocalicin portion of the α chain of platelet surface GPIb was not confined to its vWf binding site. In addition to the previously reported[9] thrombin-induced marked decrease in binding of a monoclonal antibody directed at the vWf binding site on GPIb, we observed a comparable marked decrease in the binding of two other monoclonal antibodies directed at different epitopes on the glycocalicin portion of the α chain of GPIb. The thrombin-induced decrease in binding of monoclonal antibodies directed at GPIb was not due to occupation of the antibody binding site by thrombin bound to its receptor on GPIb[2,3] because (*a*) three monoclonal antibodies (6D1, WM23, and 3G6) directed against different epitopes on

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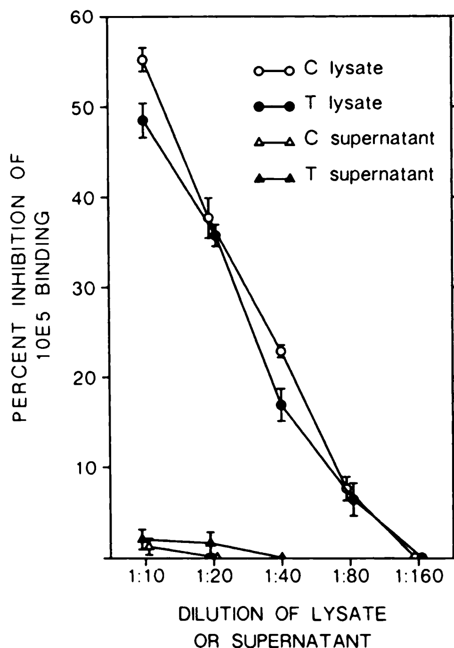

Fig 3. Effect of thrombin (1.0 U/10^8 platelets) on the total platelet content and release of the GPIIb-IIIa complex as determined by ELISA using monoclonal antibody 10E5. The total platelet content of the GPIIb-IIIa complex was determined from 1% Triton X-100 lysates of control platelets (open circles, C lysate) and thrombin-activated platelets (closed circles, T lysate). Platelet release of the GPIIb-IIIa complex was determined from supernatants of control platelets (open triangles, C supernatant) and thrombin-activated platelets (closed triangles, T supernatant). Results are means ± SEM, n = 3.

GPIb produced similar results and (*b*) monoclonal antibody 6D1 does not interfere with the binding of thrombin to platelets.[2]

Second, we observed a thrombin-induced marked decrease in the binding of a monoclonal antibody directed at GPIX. Although the function of GPIX is presently unknown, our data suggest the possibility that GPIX has a role in platelet activation. It has previously been demonstrated in unactivated platelets that surface GPIb is bound to GPIX in a 1:1 ratio (the GPIb-IX complex).[13,14] The present data suggest that during the thrombin-induced changes in platelet surface GPIb and GPIX there is maintenance of this 1:1 ratio.

Third, the flow cytometric method enabled us to determine that the thrombin-induced changes in the binding of mononclonal antibodies to GPIb, GPIX, and the GPIIb-IIIa complex were not restricted to a distinct subpopulation of platelets. Radioligand binding studies such as those used by George et al[9] are unable to make this distinction because the results are averaged measurements of large numbers of platelets. In contrast, because each platelet is analyzed individually, flow cytometry is able to detect distinct subpopulations of platelets, as we have recently demonstrated in children with chronic myeloid leukemia.[17]

Fourth, despite the thrombin-induced marked decrease in binding of monoclonal antibody 6D1 to platelet surface GPIb, quantitative ELISA (performed in parallel and using the same monoclonal antibody) revealed no significant thrombin-induced release of glycocalicin from the platelet and no change in the total platelet content of GPIb. In view of our previous finding that proteolytic cleavage of glycocalicin does not interfere with its measurement by our ELISA,[18] the direct measurement of released glycocalicin in the present study demonstrated that the thrombin-induced marked decrease in the platelet binding of anti-GPIb monoclonal antibodies was not the result of cleavage of platelet surface GPIb. The present study together with previous studies by other investigators[27,28] suggests that platelet surface GPIb is still present on the platelet surface after thrombin activation but its accessibility to multiple probes is greatly decreased because of either (*a*) the marked clustering of GPIb molecules that occurs on the platelet surface after thrombin activation[29]; (*b*) the marked shape change that occurs in platelets during thrombin activation (less likely because of the associated increased accessibility of surface GPIIb-IIIa complex); or (*c*) a major conformational change in the GPIb-IX complex (also less likely because such a change would not be expected to result in the same decrease in binding of four monoclonal antibodies [6D1, WM23, 3G6, and FMC25] directed at different epitopes on the GPIb-IX complex and three of these antibodies [WM23, 3G6, and FMC25] recognize their epitope on Western blots, which suggests that they are relatively insensitive to conformational changes in the antigen).

Fifth, unlike α granule constituents such as platelet factor 4 and β-thromboglobulin,[30] the GPIIb-IIIa complex was not released from platelets during thrombin activation. Although the present investigation did not directly exclude the possibility that uncomplexed GPIIb and/or GPIIIa subunits were released from platelets during thrombin activation, the lack of change in the total platelet content of the GPIIb-IIIa complex, as determined by two different complex-specific monoclonal antibodies, did not support this view.

In conclusion, given the central roles of GPIb and the GPIIb-IIIa complex in platelet adhesion and aggregation,[1] the presently described thrombin-induced changes in these glycoproteins are likely to be important in modulating the reactivity of platelets with the damaged blood vessel wall and with other platelets.

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