Platelets Have More Than One Binding Site for von Willebrand Factor

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ABSTRACT The binding of ^{125}I-von Willebrand factor (^{125}I-vWF) to platelets stimulated by thrombin, ADP, and a combination of ADP + epinephrine (EPI) is specific, saturable, and reversible. Active platelet metabolism and divalent cations are required for binding induced by these stimuli, but not by ristocetin, suggesting the existence of different mechanisms involved in the vWF-platelet interaction. A monoclonal antibody directed against an epitope of membrane glycoprotein (GP) Ib had no effect on the binding of ^{125}I-vWF to normal platelets stimulated by thrombin or a combination of ADP + EPI, but completely blocked ristocetin-induced binding. Binding induced by thrombin to GPIb-blocked platelets was specific. Moreover, thrombin-induced binding of ^{125}I-vWF was increased, rather than decreased, in two patients with the Bernard-Soulier syndrome whose platelets lacked GPIb. Conversely, monoclonal antibodies directed against the GPIIb/IIIa complex had no effect on ristocetin-induced binding of ^{125}I-vWF to normal platelets, but blocked thrombin- and ADP + EPI-induced binding. To exclude effects mediated by the platelet Fc receptor, a monoclonal IgG directed against an epitope present on human B cells and monocytes, but not expressed on resting or stimulated platelets, was used. It did not affect ^{125}I-vWF binding induced by any of the stimuli. These studies show that platelets have more than one binding site for vWF, and that they may be exposed by different stimuli.

INTRODUCTION

von Willebrand factor (vWF)[1] is a large multimeric glycoprotein that circulates in blood complexed with the Factor VIII procoagulant activity protein (1). It plays an essential role in platelet function as shown by the prolonged bleeding time in von Willebrand disease (1). Specific binding sites for vWF are induced on the platelet membrane by the antibiotic ristocetin (2), a nonphysiologic agent, as well as by thrombin (3, 4) and ADP (5). Platelet membrane glycoprotein (GP) Ib is considered to function as the surface receptor for vWF (6). In the Bernard-Soulier syndrome, a congenital bleeding disorder, platelets lack GPIb (7) and the ristocetin-induced binding of vWF is decreased (8). On the other hand, in Glanzmann thrombasthenia, another congenital platelet abnormality, there is a marked decrease of the membrane GP complex IIb/IIIa but normal content of GPIb (9). The ristocetin-induced binding of vWF to thrombasthenic platelets has been reported to be normal (8). In contrast, we have recently shown that binding of vWF to thrombasthenic platelets stimulated by thrombin is severely deficient (4). Therefore, we postulated that different sites are involved in

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[1] *Abbreviations used in this paper:* ADG, albumin density-gradient; VIII:C, Factor VIII procoagulant activity; EPI, epinephrine; GP, platelet membrane glycoprotein; PMSF, phenylmethylsulfonyl fluoride; PRP, platelet-rich plasma; vWF, von Willebrand factor.

the binding of vWF induced by different stimuli (4). In this report we provide evidence to confirm that hypothesis.

METHODS

Patients and controls. Two patients fulfilled all the accepted criteria for the diagnosis of the Bernard-Soulier syndrome. They have been extensively characterized in a previous report (10). Blood from the two patients was drawn by Dr. Margaret Johnson at Wilmington Medical Center, Delaware, and shipped overnight to Milwaukee, where experiments with Bernard-Soulier platelets were performed. Blood from a normal control was drawn at the same time and shipped similarly. All other experiments were performed in Milan, using fresh blood from normal volunteers. All subjects were aware of the experimental nature of these studies and gave their informed consent, according to the Declaration of Helsinki. They reported no intake of any drug for the week preceding the studies.

Preparation of washed platelets. Blood was drawn through 19-gauge needles into polypropylene syringes as one part of acid/citrate/dextrose to five parts of blood and immediately transferred into polypropylene tubes. Platelet-rich plasma (PRP) of the Bernard-Soulier patients and the normal control studied at the same time was separated from whole blood by layering on top of a solution made of two parts of dextran (4%, average $M_r \sim 500{,}000$; Sigma Chemical Co., St. Louis, MO) and one part of sodium metrizoate (32.8% wt/vol aqueous solution; Sigma Chemical Co.). Erythrocytes settled out in ~60–90 min, and the PRP remaining on top of the dextran-sodium metrizoate solution was carefully removed. Only the upper two-thirds were used to minimize leukocyte contamination. PRP for all other experiments was obtained by three successive differential centrifugation steps at 1,200 *g* for 60 s. Each time PRP was removed and the blood recentrifuged without mixing. Platelets were washed free of plasma constituents by one or both of two methods, the albumin density-gradient (ADG) technique of Walsh et al. (11), or the gel-filtration technique described by Marguerie et al. (12). The final platelet suspensions were all in modified calcium-free Tyrode buffer, containing 137 mM NaCl, 2 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 2.9 mM KCl, 5.5 mM glucose, 10 mM Hepes, pH 7.35, and 20 mg/ml bovine serum albumin (Fraction V, Calbiochem-Behring Corp., American Hoechst Corp., San Diego, CA). When experiments of serotonin release were performed, PRP was labeled before washing by incubating 20 ml with 0.5 μCi of [^{14}C]serotonin (5-hydroxy[2-^{14}C]tryptamine creatinine sulfate; Radiochemical Centre, Amersham, UK) for 40 min at 37°C. At the end of the washing procedure all platelet preparations responded with aggregation when stirred in the aggregometer in the presence of 8 μM ADP and fibrinogen (3.3 μM). However, ADG-washed platelets usually gave reversible aggregation to ADP and were unresponsive to epinephrine (EPI). On the contrary, gel-filtered platelets always gave irreversible aggregation to ADP, and responded to 20 μM EPI in the presence of fibrinogen (3.3 μM) with a typical two-wave aggregation. Contamination of plasma vWF in all washed platelet preparations was below 5×10^{-1} U/dl (1 dl of a normal plasma pool contains 1×10^2 U) as measured by immunoradiometric assay (13). No agglutination of washed platelets occurred in response to the addition of ristocetin (1.5 mg/ml) unless vWF was added to the suspension.

Preparation of apyrase. Apyrase was prepared by the method of Molnar and Lorand (14). It was finally dissolved in 0.15 M NaCl, and stored at −20°C until used. At a concentration of 9 μl/ml, it caused 93% conversion of 9 μM ADP to AMP and adenosine in 2 min at 37°C. Creatine phosphate and creatine phosphokinase were obtained from Sigma Chemical Co.

Purification of vWF. For a typical preparation, 20–30 bags of human plasma cryoprecipitate prepared by the method of Pool et al. (15) were resuspended at 37°C in 200 ml of a buffer consisting of 20 mM Tris, 20 mM sodium citrate (tribasic), 20 mM ε-aminocaproic acid, pH 7, containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The redissolved cryoprecipitate was then adsorbed with $Al(OH)_3$ (16). The further purification steps were performed according to Newman et al. (17), as modified by Switzer and McKee (18). Gel filtration was performed on a 5 × 80-cm column of Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with a buffer consisting of 20 mM imidazole, 100 mM NaCl, 10 mM sodium citrate (tribasic), 20 mM EACA, pH 6.5, and containing PMSF as indicated. vWF was present in the void volume protein peak along with Factor VIII procoagulant activity (VIII:C). The fractions corresponding to the ascending part of the peak were pooled and concentrated by dialysis against polyethylene glycol (average M_r = 40,000). The vWF preparations used in these experiments had a protein concentration of between 0.56 and 0.78 mg/ml and a specific activity of 116–128 U of ristocetin cofactor activity, 117–135 U of Factor VIII-related antigen, and 47–52 U of VIII:C/mg of protein. The purity of the final product was assessed by electrophoresis in 5% acrylamide-0.25% bisacrylamide disc gels, containing sodium dodecyl sulfate (SDS), after reduction of the protein by dithiothreitol (19). Some preparations of purified vWF were also treated with rabbit anti-human fibrinogen IgG coupled to Sepharose 4B-CL beads to remove trace contamination of fibrinogen. The procedure used was that recently described by Fujimoto et al. (3). The antiserum used was obtained from Beheringwerke (Scopitto, Italy). Fibrinogen contamination was evaluated by specific radioimmunoassay (kindly performed by Dr. Edward F. Plow, Scripps Clinic and Research Foundation).

Radioiodination of purified vWF. This was accomplished with ^{125}I by the method of Fraker and Speck (20) to a specific activity of between 0.48 and 0.89 mCi/mg. The preparations of ^{125}I-vWF were characterized by electrophoretic analysis in SDS agarose gels as described (21).

Preparation of monoclonal antibodies. BALB/c female mice were given a primary intraperitoneal immunization with 1×10^9 washed platelets (albumin was omitted from the platelet suspension in this case) in 0.5 ml of Freund's complete adjuvant. Three subsequent intraperitoneal immunizations with 1×10^9 washed platelets suspended in 0.9% saline were given at 2-wk intervals. 1 wk after the last injection, the mice were given 2×10^9 platelets intravenously. 4 d later the mice were killed and their spleen cells used for fusion. Hybridization was carried out using 2.5×10^8 spleen cells mixed at a 1:1 ratio with P3-x-63Ag8-653 (nonsecretor) myeloma cells, according to the method of Oi and Herzenburg (22), using 50% polyethylene glycol 1500. Positive hybridomas were selected using partially purified platelet membrane glycoproteins (GPIIb/IIIa complex and GPIb), as well as a microtiter screening assay for the inhibition of ristocetin-induced platelet aggregation (23). Selected hybrids were cloned by limiting dilution. Clones were grown to sufficient density and 5×10^5 cells injected intraperitoneally into "Pristane primed" BALB/c × DBA/2 Fl mice. Mice were tapped for ascites fluid usually after 2–3 wk. Monoclonal IgG were prepared from the ascites fluid by three sequential 50% ammonium sulfate precipitations, followed by dialysis against

potassium phosphate buffer (0.01 M, pH 7.4) and affinity purification using Protein A Sepharose (Pharmacia Fine Chemicals). Elution from Protein A was achieved using a Tris-glycine buffer, pH 2.6. Purified IgG were finally dialyzed against 0.05 M Tris–HCl buffer, 0.1 M NaCl, pH 7.4.

Characterization of monoclonal antibodies. The specificity of monoclonal antibodies to platelet membrane glycoproteins was assessed after radiolabeling with ^{125}I by the chloramine-T method (24). The radiolabeled antibodies were then used in the second dimension of crossed immunoelectrophoresis mixed with unlabeled rabbit antiplatelet antisera (25). Triton X-100-solubilized platelets were electrophoresed in the first dimension. The immunologic reactivity of the monoclonal antibodies was substantiated by identification of the platelet membrane glycoprotein immunoprecipitate patterns (26). Alternatively, platelet membranes were radiolabeled with ^{125}I by the lactoperoxidase method (9), solubilized with Nonidet-P40, and incubated with the monoclonal antibodies. Antibody-bound membrane proteins were precipitated with Protein A Sepharose, electrophoresed in SDS polyacylamide gels and identified by autoradiography. Monoclonal antibody AP1 reacted with GPIb and was an IgG1. AP2 and B59.2 reacted with GPIIb/IIIa and were IgG1 and IgG2a respectively. The control monoclonal antibody (B33.1) was an IgG2a anti-HLA-DR common determinant and did not react with platelets (27). AP1 and AP2 were prepared by one of us (Dr. Montgomery in collaboration with T. J. Kunicki, Blood Center of S. E. Wisconsin, Milwaukee), whereas B59.2 and B33.1 were obtained through the courtesy of Dr. Bice Perussia and Dr. Giorgio Trinchieri, the Wistar Institute, Philadelphia, PA.

Binding experiments. In all binding experiments platelets were at a final concentration of 10^8/ml. Incubation with varying concentrations of ^{125}I-vWF and the appropriate stimulus was performed at room temperature (20°–25°C) and under nonstirring conditions, for the indicated period of time, in 1 ml polypropylene tubes. Each stimulus was added from a 10× stock solution freshly prepared and kept in ice until used. In parallel mixtures, nonspecific binding was determined in the presence of a 50-fold excess of unlabeled vWF. At the end of the incubation period, 50 μl of the platelet suspension (in duplicate for each experimental point) was layered onto 400 μl of 20% sucrose in the modified Tyrode buffer, using 1-ml conical polypropylene tubes. The samples were centrifuged for 4 min at 13,000 *g* at room temperature in an Eppendorf microcentrifuge (Eppendorf, Hamburg, FRG), the tips of the tubes containing the sedimented platelets were cut with a scalpel, and the platelet-associated radioactivity was measured. In experiments where the effect of monoclonal antibodies on ^{125}I-vWF binding was evaluated, monoclonal IgG was added to the experimental mixtures at the indicated final concentrations immediately before addition of radioligand and stimulus. In some experiments, platelets were preincubated with the monoclonal IgG for 10–60 min at room temperature before addition of the other reagents. Analysis of bound ligand was performed after counting the platelet pellets by lysing the platelets with 30 μl of a buffer consisting of 10 mM Tris–HCl and 1 mM EDTA, pH 8, containing 5% SDS. The samples were incubated for 20 min at 60°C and extracted ^{125}I-vWF was analyzed by SDS agarose electrophoresis (21). More than 80% of bound radioactivity could be accounted for in the supernatant of lysed platelets. The line best fitted to the experimental points of each binding curve was obtained by means of a nonlinear fit program utilizing a Hewlett-Packard model 85 desk-top computer (Hewlett-Packard Co., Palo Alto, CA).

Platelet release studies. For serotonin release studies, 5 μM imipramine (Geigy S.p.A., Milan, Italy) was added to washed platelets labeled with [^{14}C]serotonin (see above). Release of [^{14}C]serotonin was assessed after any indicated incubation time period by determining in a liquid scintillation spectrometer (Packard Instrument S.p.A., Milan, Italy) the radioactivity of 5 μl of the supernatant obtained after spinning out the platelets at 13,000 *g* for 1 min in the microcentrifuge. Release was expressed as percentage of the total content measured in the same volume of platelet suspension after lysis with 1% Triton X-100.

RESULTS

Characterization of purified vWF. The purified vWF used in these experiments appeared homogeneous on SDS polyacrylamide gel electrophoresis under reducing conditions (Fig. 1). Possible fibrinogen contamination was evaluated by radioimmunoassay and was below the detection limit of the assay. Fibrinogen contamination was, therefore, <5 μg/mg of protein in the purified vWF preparations (<0.5% on a wt/wt basis).

Characterization of the binding assay. To evaluate the efficacy of the method used to separate platelet-bound from free ligand, ^{125}I-vWF (8×10^5 cpm) was layered onto 20% sucrose in modified Tyrode buffer and centrifuged as described. In the absence of platelets, <0.5% of the counts added was recovered in the tube tip. Under the same conditions, platelet recovery in the tube tip was >90% as measured by quantitation of ^{14}C-labeled platelets. No release of [^{14}C]serotonin occurred during the centrifugation step.

Time course experiments. The time course of the association of ^{125}I-vWF to platelets stimulated with thrombin (0.25 U/ml), ADP (20 μM), and a combination of ADP + EPI (20 μM each) is shown in Fig. 2. In the presence of thrombin, platelets bound approximately two and four times more ^{125}I-vWF than with ADP + EPI or ADP alone, respectively. No binding was observed with EPI alone, at concentrations up to 100 μM. The binding to unstimulated platelets was <10, 17, and 35% of the total binding to thrombin-, ADP + EPI-, and ADP-stimulated platelets, respectively. The binding to unstimulated platelets was not time dependent, whereas the binding induced by the different stimuli reached a plateau at between 30 and 40 min (Fig. 2). The binding to unstimulated platelets corresponded to that observed with each one of the stimuli when a 50-fold excess (wt/wt) of unlabeled vWF was added to the platelet suspension before ^{125}I-vWF. Therefore, nonspecific binding was the same with all stimuli, and accounted for only a minor fraction of the total ^{125}I-vWF bound to stimulated platelets.

When ristocetin (1.5 mg/ml final concentration) was used as a stimulus, the results obtained were comparable to those previously reported by others (2).

Specific binding induced by thrombin, ADP, or ADP + EPI was blocked by 5 mM EDTA, and did not occur

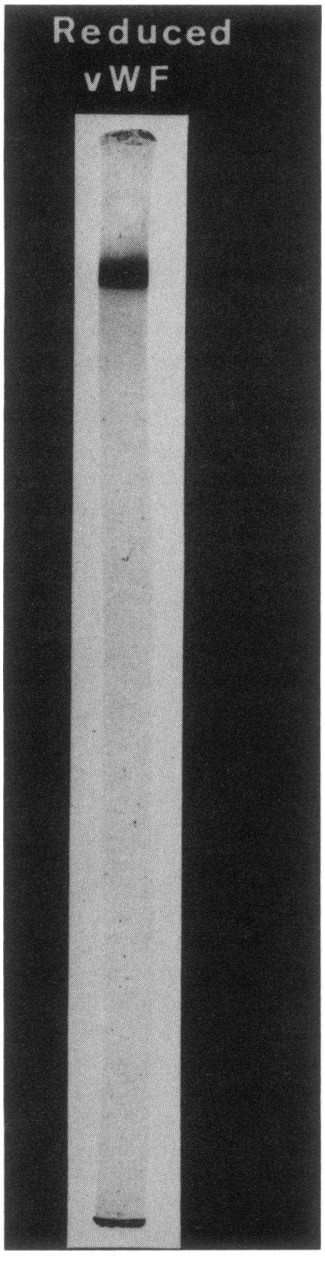

FIGURE 1 Electrophoretic analysis of purified vWF. The sample from a purified vWF preparation was analyzed after reduction with 50 mM dithiothreitol at 56°C for 4 h in the presence of 1% SDS. Approximately 40 μg of protein was applied to a 5% acrylamide-0.25% bis-acrylamide disc gel (see reference 19 for details on electrophoretic conditions). The gel was stained with Coomassie Blue R250. Cathode at the top.

with formalin-fixed platelets. However, binding induced by ristocetin was observed using formalin-fixed platelets and in the presence of EDTA.

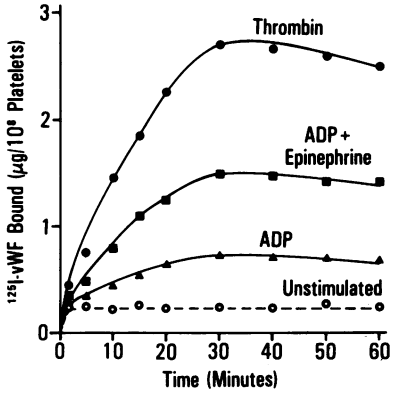

FIGURE 2 Time course of the association of ^{125}I-vWF with platelets. Washed platelets at a final concentration of 10^8/ml were mixed with 5.6 μg/ml ^{125}I-vWF at room temperature (20°–25°C) and stimulated with 0.25 U/ml thrombin, 20 μM ADP, or 20 μM ADP + 20 μM EPI for the indicated period of time. In unstimulated platelets, Tyrode buffer was used instead of the stimulus. Phase separation of bound from free ligand was achieved by centrifugation at 13,000 *g* for 4 min through 20% sucrose in modified Tyrode buffer. Total binding values are reported without subtraction of nonspecific binding. Note that binding to unstimulated platelets was not time dependent.

Affinity of labeled and unlabeled vWF. The affinities of unlabeled and radiolabeled vWF for thrombin-stimulated platelets were comparable (Fig. 3). This was determined by measuring the binding of ^{125}I-vWF in mixtures in which the final concentration of the ligand was kept constant but the proportion of labeled and unlabeled ligand was varied. A linear relationship was found between the percentage of ^{125}I-vWF in the mixtures and the amount bound (Fig. 3).

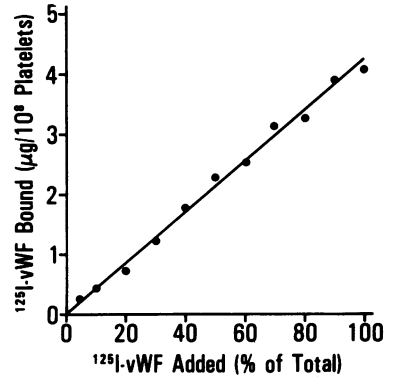

FIGURE 3 Relative binding of ^{125}I-vWF and unlabeled vWF to thrombin-stimulated platelets. The total vWF concentration in the mixtures was kept constant (12 μg/ml), but the proportion of labeled and unlabeled vWF was varied as indicated. Platelets were stimulated with 0.25 U/ml of thrombin and binding was measured after incubation for 30 min at room temperature.

Effect of agonist concentration on ^{125}I-vWF binding and platelet release reaction. The relationship between the dose of each stimulus required to support ^{125}I-vWF binding and induce platelet release of [^{14}C]-serotonin was investigated using thrombin-, ADP-, or ADP + EPI-stimulated platelets (Fig. 4). When thrombin was used, specific binding occurred in parallel with the release reaction. Thrombin concentrations as low as 0.0125 U/ml were effective in this regard. A plateau in the thrombin dose-response curve was observed at 0.5 U/ml, with no further increase in binding at concentrations as high as 2 U/ml (Fig. 4). Thrombin stimulation of platelets followed by thrombin neutralization with a fourfold (U/U) excess of hirudin (obtained from Pentapharma, Basel, Switzerland) before addition of the radioligand resulted in binding similar to that observed with thrombin and radioligand added simultaneously to platelets. Therefore, the binding observed was not related to any action of thrombin on the ligand, but rather to thrombin stimulation of platelets. Addition of an ADP scavenger (apyrase at a concentration of 9 μl/ml; see Methods) to the mixture before thrombin caused a decrease of specific binding to ~30–40% of the binding observed in the absence of the ADP scavenger (Fig. 4). Similar results were obtained with the addition of creatine phosphate (7.5 mM) and creatine phosphokinase (12 U/ml) instead of apyrase. At variance with thrombin, ADP- or ADP + EPI-induced binding was independent of the platelet release reaction. The latter was not observed with these two stimuli under the experimental conditions used (Fig. 4). The dose-dependency of ristocetin-induced binding was comparable to that previously reported by others (2).

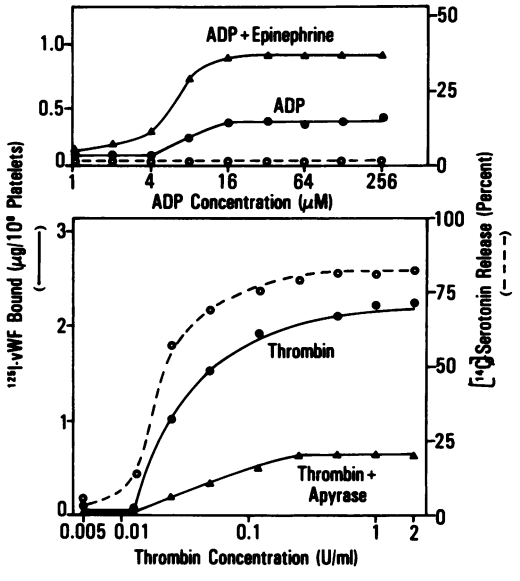

FIGURE 4 ^{125}I-vWF binding and serotonin release as a function of stimulus concentration. Platelets were labeled with [^{14}C]serotonin as indicated in the Method section, and then washed. vWF was added at a concentration of 5.6 μg/ml to 10^8 platelets/ml, followed by the appropriate concentration of stimulus (lower panel: thrombin; upper panel: ADP) taken from a 10× stock solution. In the case of ADP + EPI combination, ADP concentration was varied as indicated, and EPI was 20 μM in all mixtures. Unlabeled vWF was added to mixtures where serotonin release was measured, and ^{125}I-vWF to mixtures where binding was measured. Binding and release were measured after a 30-min incubation at room temperature. Release was expressed as percentage of total [^{14}C] serotonin content. Binding values shown represent specific binding, obtained after subtracting from total measured the nonspecific binding observed in the presence of a 50-fold excess of unlabeled vWF. In the experiments performed to evaluate the effect of apyrase on thrombin-induced binding (lower panel), apyrase was added at a concentration of 9 μl/ml (Methods) 5 min before the addition of thrombin.

Specificity of binding. Competition experiments were performed to demonstrate the specificity of the association of ^{125}I-vWF to thrombin-stimulated platelets. As shown in Table I, human fibronectin, transferrin, and IgM at >100-fold excess (wt/wt) were not effective in displacing the binding of ^{125}I-vWF significantly. In addition, the serum from three patients with severe von Willebrand disease (Factor VIII-related antigen in plasma $< 1 \times 10^{-4}$ U/ml) had a minimal effect on binding of ^{125}I-vWF, whereas serum from three normal individuals had an inhibitory effect (Table I).

Dissociation of bound ^{125}I-vWF from platelets. The capacity of unlabeled vWF to displace ^{125}I-vWF bound to thrombin-stimulated platelets was analyzed. Displacement of bound ^{125}I-vWF was dependent upon the time of addition of unlabeled vWF (Fig. 5). When a 50-fold excess (wt/wt) of the latter was added before

TABLE I
Specificity of the Binding of ^{125}I-vWF to Thrombin-stimulated Platelets

Competing component	Final concentration in the mixtures	Percent inhibition of ^{125}I-vWF binding
Transferrin	2 mg/ml	12
IgM	1 mg/ml	9
Fibronectin	0.5 mg/ml	14
Severe vWd serum	75%	13–18
Normal serum	75%	58–61
Unlabeled vWF	0.26 mg/ml	88

The final concentrations of the other components in the mixtures were: ^{125}I-vWF, 2.6 μg/ml; platelets, 1×10^8/ml; thrombin, 0.25 U/ml. In the experiments with serum, samples from three different normals or patients with severe von Willebrand disease were used, and the results indicate the range of values observed. The percent inhibition was calculated relative to total binding of ^{125}I-vWF. Note that in the case of normal serum, if one assumes an average vWF concentration of 10 μg/ml, then unlabeled vWF is at an approximate threefold excess over ^{125}I-vWF.

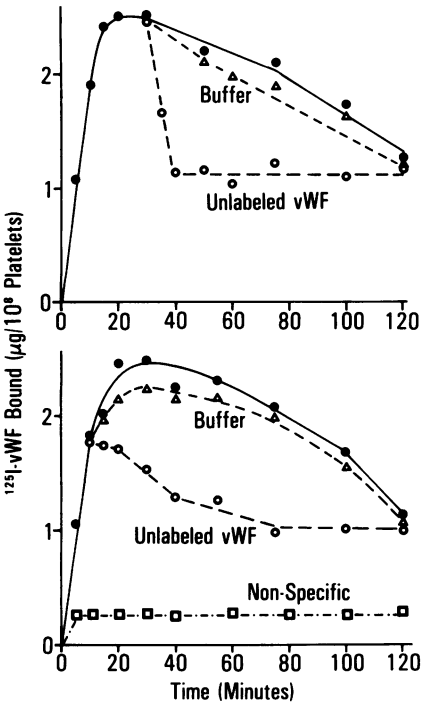

FIGURE 5 Displacement of platelet-bound ^{125}I-vWF by dilution or addition of a 50-fold excess of unlabeled vWF. Platelets (10^8/ml) were resuspended in modified Tyrode buffer, and mixed with 5.6 μg/ml ^{125}I-vWF and 0.25 U/ml thrombin. Samples were taken at various times to measure bound ^{125}I-vWF. After incubation for 10 min (lower panel) or 30 min (upper panel) at room temperature, unlabeled vWF was added at a 50-fold excess (○). At the same time, parallel incubation mixtures were diluted with the same amount of modified Tyrode buffer (△). A third series of mixture was left undiluted as a control (●). Samples were taken from each mixture at various times after these additions and bound ^{125}I-vWF was determined. Nonspecific binding was measured in the presence of a 50-fold excess of unlabeled vWF added before the addition of thrombin (shown in the lower panel) (□). Note that nonspecific binding was not time dependent.

the binding of ^{125}I-vWF had reached maximal levels, no further increase in binding of the radiolabeled ligand occurred during continued incubation. On the contrary, a progressive dissociation of the bound radioligand was observed, but was incomplete. Apparently irreversible binding corresponded to ~30–50% of maximal binding (Fig. 5). When excess unlabeled vWF was added to the mixtures after maximal binding had been reached, a more rapid dissociation of the bound radioligand was observed, but was again incomplete (Fig. 5). Appropriate controls were included to take into account the dilution effect of the addition of unlabeled vWF (Fig. 5). In control mixtures incubated in the absence of excess unlabeled vWF, a slow, spontaneous dissociation of bound ^{125}I-vWF followed the plateau of binding. Even in this case, apparently irreversible binding corresponded to 30–50% of maximal binding (Fig. 5). This apparently irreversible binding was not related to internalization of vWF molecules, since washing of platelets in the presence of EDTA after maximal binding had been reached resulted in removal of 99% of the radioactivity previously associated to platelets.

Saturation of binding. The binding of ^{125}I-vWF to thrombin-stimulated platelets (Fig. 6), as well as ADP- or ADP + EPI-stimulated platelets (data not shown), was saturable. This was evident after subtraction of nonspecific from total measured binding in mixtures that had been incubated with increasing concentrations of ^{125}I-vWF and constant platelet number for 30 min. This incubation time was selected on the basis of the results shown in Figs. 2 and 5. Although binding of ^{125}I-vWF was not strictly at equilibrium, a relatively stable plateau of binding was always observed between ~20 and 40 min of incubation. Nonspecific binding increased linearly with increasing concentrations of ^{125}I-vWF and was therefore nonsaturable (Fig. 6). Nonspecific binding was the same at any concentration of any of the stimuli used, when measured in the presence of a 50-fold excess of unlabeled vWF, and corresponded to the binding to unstimulated platelets.

Effect of monoclonal antibodies on the binding of ^{125}I-vWF. Different monoclonal antibodies directed against specific platelet membrane glycoproteins were evaluated for their effect on the platelet binding of ^{125}I-

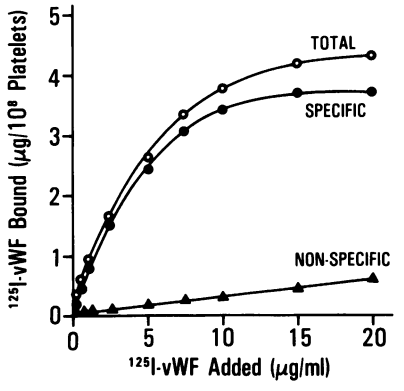

FIGURE 6 Binding of ^{125}I-vWF to thrombin-stimulated platelets as a function of ^{125}I-vWF concentration. Washed platelets (10^8/ml) were mixed with increasing concentrations of ^{125}I-vWF and stimulated with 0.5 U/ml of thrombin. After incubation for 30 min at room temperature, the amount of ^{125}I-vWF bound to platelets was measured. Nonspecific binding was measured in parallel mixtures where a 50-fold excess of unlabeled vWF was added before the addition of thrombin. The nonspecific binding measured in this way was identical to that observed when platelets were incubated with ^{125}I-vWF alone in the absence of thrombin stimulation. Specific binding was calculated by subtracting nonspecific from total measured binding.

vWF induced by different stimuli. To exclude possible nonspecific effects, a monoclonal antibody (B33.1) directed against an epitope expressed on B cells and monocytes, but not on unstimulated or stimulated platelets, was first tested. The ^{125}I-vWF binding to thrombin-stimulated platelets in the presence of 435 μg/ml of this antibody was identical to that observed when monoclonal IgG was substituted for by Tyrode buffer. In preliminary experiments it was also demonstrated that ^{125}I-labeled monoclonal IgG prepared from antibody AP1 (anti-GPIb) bound to thrombin-stimulated platelets in a manner identical to that observed with unstimulated platelets. This demonstrated that possible membrane changes induced by thrombin did not affect the reactivity of membrane glycoproteins with their specific monoclonal antibodies. The addition of 2 or 5 mM Ca^{2+} to the experimental mixtures did not change the binding of ^{125}I-vWF to thrombin- or ADP + EPI-stimulated platelets, nor did it affect the results observed in the presence of the monoclonal antibodies. Preincubation of platelets with the monoclonal IgG for up to 60 min gave results similar to those observed when the antibody was added to the experimental mixture just before ^{125}I-vWF and the appropriate stimulus.

In three separate experiments performed with different platelet and ^{125}I-vWF preparations, the specific binding induced by thrombin and ADP + EPI was not significantly affected by the anti-GPIb antibody (AP1) at a concentration that completely blocked ristocetin-induced binding. In contrast, one of the anti-GPIIb/IIIa antibodies (B59.2) inhibited thrombin- and ADP + EPI-induced binding by 48–62 and 58–63%, respectively. This inhibitory effect on thrombin-induced binding was present at all thrombin concentrations tested, from 0.05 to 0.5 U/ml. The other anti-GPIIb/IIIa antibody (AP2) was tested only for its effect on thrombin-induced binding, and it was found to cause >80% inhibition. When binding studies were performed with purified vWF that had been passed through an antifibrinogen column, the same results were obtained. Both antibodies to GPIIb/IIIa had no significant effect on ristocetin-induced binding of ^{125}I-vWF (experiments with AP2 were performed by D. Pidard, R. R. Montgomery, and T. J. Kunicki, personal communication). The results of one such experiment are shown in Fig. 7. The monoclonal IgG concentration that gave maximal inhibition was determined in dose-response studies. Higher concentrations than those here reported (Fig. 7) did not increase the extent of inhibition. Control binding isotherms were obtained in the presence of the control monoclonal IgG (B33.1) at a concentration well in excess of that used with any specific monoclonal IgG (Fig. 7). In some experiments, control and specific IgG, or anti-GIb and anti-GPIIb/IIIa IgG, were added together in the same mixtures.

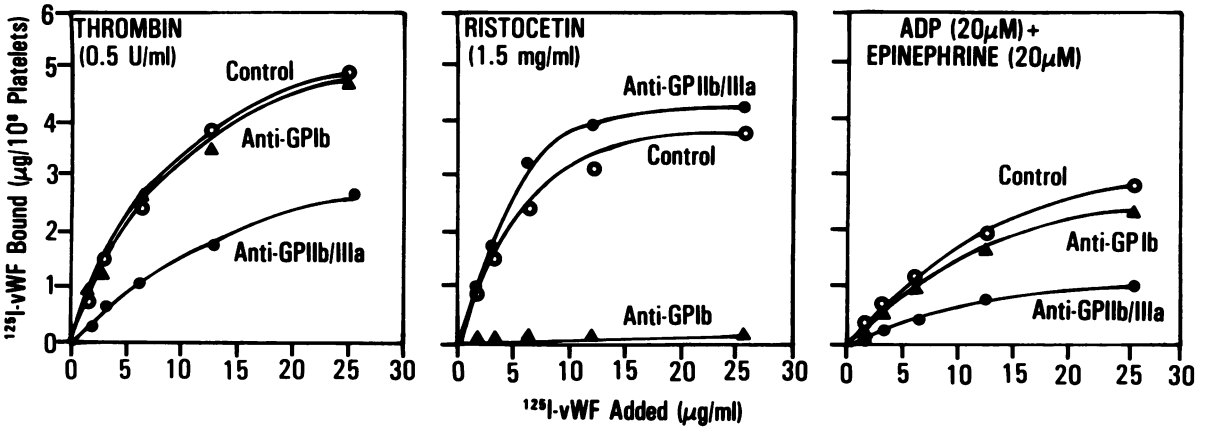

FIGURE 7 The effect of monoclonal antibodies against platelet membrane GPIb or IIb/IIIa on the specific binding of ^{125}I-vWF induced by thrombin (*left* panel), ristocetin (*middle* panel), or a combination of ADP + EPI (*right* panel). Platelets (10^8/ml) were mixed with ^{125}I-vWF (as indicated on the abscissa) and monoclonal IgG in the following concentrations: control (B33.1; anti-HLA-DR common determinant) = 435 μg/ml; anti-GPIb (AP1) = 11.6 μg/ml; anti-GP IIb/IIIa (B59.2) = 298 μg/ml. The appropriate agonist (thrombin, 0.5 U/ml; ristocetin 1.5 mg/ml; ADP + EPI, 20 μM each) was then added, and after incubation for 30 min at room temperature, binding was determined. All experimental points are the average of duplicates. Nonspecific binding was always <15% of total, and was subtracted from data shown. The binding isotherms each represent the best fitted line for all experimental points obtained by computer analysis. Note that the difference in thrombin-induced binding, as compared to Fig. 6, is within the range for different normal platelets (see reference 4).

Ristocetin-induced binding of ^{125}I-vWF to platelets was inhibited only when anti-GPIb antibody was present, and thrombin-induced binding when anti-GPIIb/IIIa antibody was present, irrespective of the other monoclonal IgG added.

Binding to Bernard-Soulier platelets. In the two related patients with the Bernard-Soulier syndrome studied, specific thrombin-induced binding of ^{125}I-vWF to ADG-washed platelets was increased, rather than decreased, when compared with normal platelets prepared under identical conditions (Fig. 8). Preliminary studies had shown that these platelets isolated in a similar manner failed to agglutinate to and bind vWF in response to ristocetin.

Characterization of bound ^{125}I-vWF. Analysis by SDS agarose electrophoresis of the bound ligand extracted from the platelet pellets after phase separation showed that all multimers of ^{125}I-vWF bound to platelets stimulated with the three different agonists (Fig. 9). Moreover, decreased thrombin- and ADP + EPI-induced binding in the presence of anti-GPIIb/IIIa antibody was represented by a parallel decrease of all multimers bound and not a specific subset of them (Fig. 9).

DISCUSSION

Specific binding sites for vWF exist on human platelets and are expressed by different platelet agonists (2–5). In this study we demonstrate that the ristocetin-induced binding of vWF (2) involves different sites than the thrombin- (3, 4), ADP- (5), or ADP + EPI-induced binding.

The concept that ristocetin induces specific binding sites for vWF on platelets is generally accepted. Therefore, our purpose for these studies was to verify that our binding assay with ristocetin could reproduce the results already reported in the literature (2), as indeed it did. The demonstration that thrombin (3, 4) and ADP (5) also induce binding of vWF to platelets is more recent. Therefore, we thought it appropriate to characterize extensively the binding assay performed with these agonists in order to demonstrate that the site induced fulfills the criteria for a specific binding site. The conclusion that this is the case is supported by the demonstration that the binding of vWF induced by thrombin, ADP, and ADP + EPI is time dependent, specific, and saturable. Specificity of binding is indicated by (*a*) the capacity of unlabeled vWF to inhibit competitively the binding of ^{125}I-vWF with the same apparent affinity; (*b*) the failure of other proteins, and notable all the proteins in severe von Willebrand disease serum, to compete for binding; and (*c*) the identification of bound material as a multimeric protein with the typical structure of plasma vWF (21).

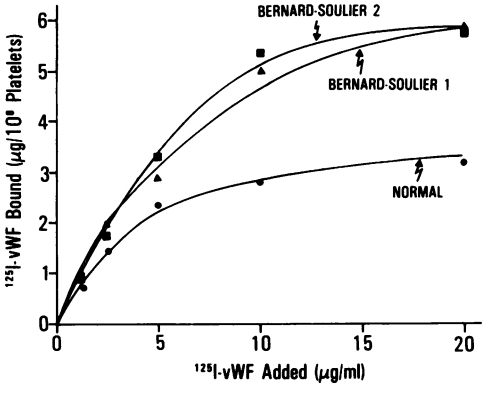

FIGURE 8 Specific binding of ^{125}I-vWF to thrombin (0.5 U/ml)-stimulated platelets in two Bernard-Soulier patients and a normal control. Experimental conditions are as described in the legend to Fig. 7, with the exception that Tyrode buffer was added instead of control monoclonal IgG. Results shown are not corrected for the increased volume of Bernard-Soulier platelets.

Fibrinogen is a likely contaminant of purified vWF preparations. We obtained direct evidence that this was not the case for the purified vWF used in these studies by demonstrating the absence of any measurable fibrinogen using a sensitive radioimmunoassay. Moreover, several observations provide indirect evidence that possible fibrinogen or fibronectin contamination cannot explain the binding observed with our purified vWF preparations. (*a*) EPI-stimulated platelets bind fibrinogen (28), but not fibronectin (29) nor vWF. ADP-stimulated platelets bind fibrinogen (12) and vWF, but not fibronectin (29). Thrombin is the only effective stimulus to induce platelet binding of fibronectin (29). (*b*) A combination of ADP + EPI increases the binding of vWF over that observed with ADP alone. Thrombin-induced binding, however, is more than double that observed with the combination of ADP + EPI. Thrombin-induced binding of fibrinogen, on the contrary, occurs at levels similar to those observed with ADP or ADP + EPI stimulation (30). (*c*) The stoichiometries of vWF and fibrinogen binding are clearly different. Half saturation of binding occurs at ~5 μg/ml of added vWF, as opposed to ~60 μg/ml of added fibrinogen (12, 28, 30). The fraction of added fibrinogen that binds to platelets corresponds to 2–3% at best (12, 28, 30), whereas it can be as high as 50% in the case of vWF binding. It is important to note that the parameters of vWF binding cannot be expressed in molar terms because of the heterogeneous nature of the molecule and the uncertainty about the molecular weight of the different multimers that interact with platelets (21).

When thrombin was used to induce vWF binding, the capacity to support specific binding and induce platelet secretion of serotonin were closely parallel

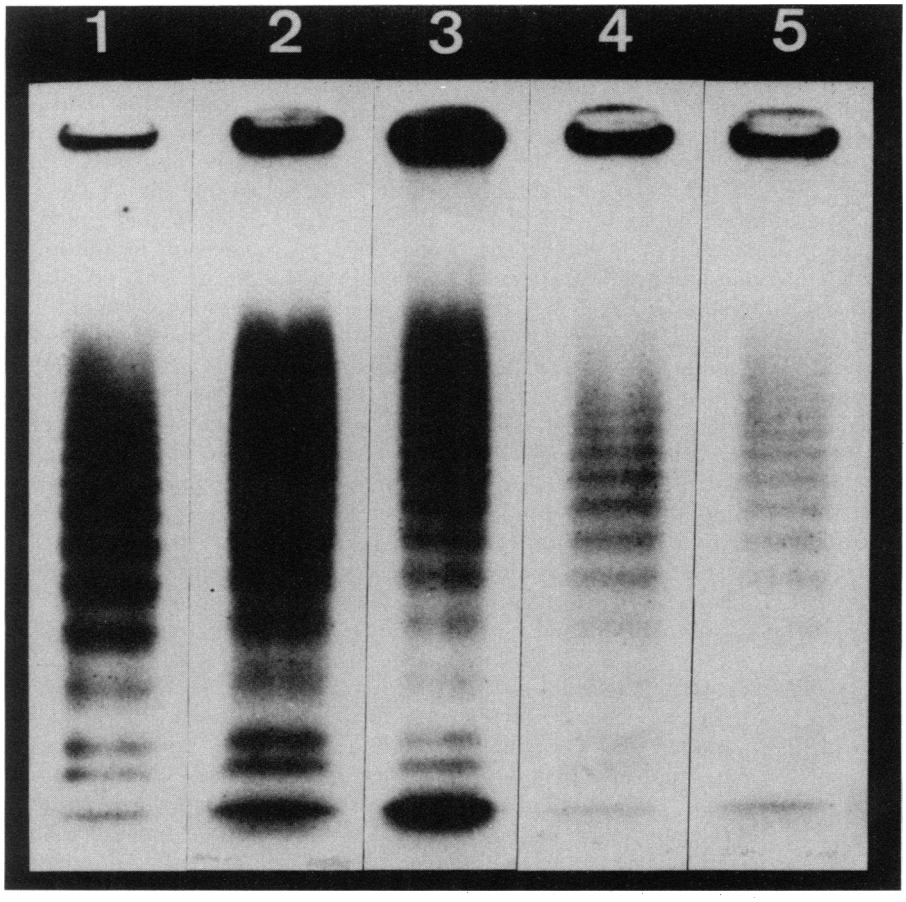

FIGURE 9 SDS agarose gel electrophoresis of free and bound ^{125}I-vWF. Lane 1, free ^{125}I-vWF used as ligand in the experimental mixtures; lanes 2 to 4, ^{125}I-vWF bound in the presence of thrombin (0.5 U/ml), ristocetin (1.5 mg/ml), and ADP + EPI (20 μM each), respectively, and extracted from the platelet pellets after phase separation; lane 5, ^{125}I-vWF bound in the presence of thrombin and an anti-GPIIb/IIIa monoclonal antibody (B59.2). The same amount of ^{125}I-vWF was added to all experimental mixtures. All bands detected in lane 1 correspond to multimers of vWF as they are observed in plasma after reaction with specific antibodies (see reference 21). The relative increase of the fastest moving band observed in bound ligand (lanes 2 and 3) was not a constant finding. It is possibly related to partial disaggregation of the larger multimers after platelet lysis.

events. On the contrary, when ADP or ADP + EPI were used as agonists, binding of vWF was independent of the platelet release reaction. In the presence of an ADP scavenger, thrombin-induced binding of vWF was reduced by approximately two-thirds. This suggests that endogenous ADP released from platelets is necessary for maximal expression of vWF binding sites induced by thrombin. A specific thrombin action must be postulated, however, as binding induced by exogenous ADP or the combination of ADP + EPI was always significantly less than that observed with thrombin. This action of thrombin must occur on platelets and not on the ligand, as the same results are observed when thrombin is incubated with platelets and then neutralized by hirudin before addition of the ligand. The thrombin concentration necessary to induce significant vWF binding to platelets was as low as 20–50 mU/ml. Since experiments were performed at low platelet concentration, in the absence of stirring, and at room temperature, the concentrations of thrombin may be higher than those actually required in vivo to induce vWF binding sites.

The interaction of vWF with the thrombin-induced binding site is reversible. However, if unlabeled vWF was added after binding of ^{125}I-vWF had occurred, displacement was incomplete. Thus, a certain proportion of the bound molecules had adopted a stable interaction with platelets. It is interesting to observe that

the binding of vWF to the ristocetin-induced binding site is also only partially reversible (2). Similar results have also been observed for fibrinogen interaction with platelets (31). Another characteristic of vWF binding to the thrombin-induced site is its partial spontaneous reversibility upon prolonged incubation. Since the levels of cyclic AMP have been shown to be important in regulating vWF binding to thrombin-stimulated platelets (3), but not to ristocetin-treated platelets (32), our findings might be related to fluctuations of intraplatelet cyclic AMP following platelet stimulation.

After extensive characterization of the platelet vWF binding site induced by thrombin, ADP, and ADP + EPI, our purpose was to evaluate whether this binding site corresponded to that induced by ristocetin. The suggestion that this might not be the case derived from (*a*) the different metabolic and divalent cation requirements for expression and/or function of these binding sites, as observed during the course of these studies and by others (3); (*b*) our recent observation that thrombin-induced binding of vWF is deficient in Glanzmann thrombasthenia (4), whereas ristocetin-induced binding is normal (8).

There is ample evidence that GPIb functions as the platelet binding site for vWF in the presence of the antibiotic ristocetin (2, 6–8). Indeed, this conclusion is supported by the results here reported that demonstrate complete suppression of ristocetin-induced binding of vWF by a specific monoclonal antibody directed against GPIb. This monoclonal antibody (AP1) is different from that reported by Ruan et al. (33) that inhibited only 50% of ristocetin-induced vWF binding to fixed platelets. AP1 inhibits >98% of vWF platelet binding induced by ristocetin. Since both antibodies are monoclonal, the epitopes recognized on the GPIb molecule are probably different. AP1 appears to be directed against or very close to the specific epitope involved in vWF binding. Recently, Coller et al. (34) have also reported studies with a different monoclonal antibody to GPIb that completely blocks ristocetin-induced binding of vWF to platelets. It is evident, however, that GPIb, or at least the epitope recognized by the antibody used in these studies and essential for ristocetin-induced binding, is not involved as part of the vWF binding site exposed on platelets stimulated by thrombin or ADP + EPI. This conclusion is further substantiated by the finding that thrombin-induced binding was increased, rather than decreased, in two Bernard-Soulier patients whose platelets had been shown in previous studies to have extremely low levels of GPIb (10). Bernard-Soulier platelets adhere poorly to subendothelium (35) and are severely defective in their capacity of binding vWF multimers in the presence of ristocetin (8). Hence, it has been postulated that the vWF binding sites exposed by ristocetin are involved in subendothelium-platelet interaction mediated by vWF. Following this hypothesis, the thrombin-induced binding sites for vWF are likely to be less important in this interaction, as they are normally expressed on Bernard-Soulier platelets.

We have recently described the severely deficient thrombin-induced binding of vWF to thrombasthenic platelets and suggested that the GPIIb/IIIa complex may be a component of the binding site for vWF exposed on thrombin-stimulated platelets (4). Such an hypothesis is further supported by the studies here reported. In fact, blocking of GPIIb/IIIa by monoclonal antibodies results in reduced thrombin-induced as well as ADP + EPI-induced binding of vWF. These results suggest the existence of common mechanisms leading to the expression of vWF binding sites on platelets stimulated by these three physiological agonists, but not by ristocetin.

One of the anti-GPIIb/IIIa antibodies tested only partially blocked the platelet binding of vWF induced by thrombin, but the other was more effective in this regard. The less effective antibody might recognize an epitope of the GPIIb/IIIa complex not directly related to the binding site, and therefore interfere with vWF binding only by steric hindrance, whereas the other might recognize an epitope more closely related or correspondent to the vWF binding site.

Appropriate controls were performed to demonstrate that the effect of the monoclonal antibodies was on specific binding of vWF. This was shown by the fact that the addition of excess monoclonal IgG directed against an epitope not present on platelets had no effect on the results, thus ruling out the possibility of nonspecific effects related to interaction with the Fc receptor on platelets. Moreover, the vWF binding to thrombin-stimulated platelets in the presence of the anti-GPIb antibody corresponded to the binding that could be completely suppressed by excess unlabeled vWF. This, together with the demonstrated time-dependence and saturability, shows that thrombin-induced binding of vWF in the presence of blocked GPIb is specific. The possibility that the blocking effect of anti-GPIIb/IIIa antibodies was on the binding of contaminants present in the vWF preparations, rather than on the binding of vWF itself, is unlikely as shown by (*a*) the lack of any detectable contaminant, and notably fibrinogen, in the vWF preparations used; and (*b*) the direct demonstration that decreased binding of radiolabeled ligand in the presence of anti-GPIIb/IIIa antibodies was accompanied by a decrease in vWF multimers bound to platelets, as detected by SDS agarose electrophoresis and autoradiography after solubilization of >80% of bound ligand.

In conclusion, these studies, as well as the results previously obtained in Glanzmann thrombasthenia (4),

demonstrate that the platelet membrane GPIIb/IIIa complex, but not GPIb, is involved in the expression of vWF binding sites on thrombin- and ADP + EPI-stimulated platelets. Ristocetin-induced binding of vWF, on the contrary, is GPIb-dependent but GPIIb/IIIa-independent. Therefore, distinct binding sites for vWF are exposed on the platelet surface by different stimuli. Although the physiological significance of thrombin-induced binding of vWF to platelets is still unclear, the recent demonstration that thrombin increases the membrane expression of platelet-released vWF (36, 37) suggests a possible role for the GPIIb/IIIa-related binding site in this mechanism.

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