Independent Modulation of von Willebrand Factor and Fibrinogen Binding to the Platelet Membrane Glycoprotein IIb/IIIa Complex as Demonstrated by Monoclonal Antibody

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Abstract

In this study we have used two new monoclonal antibodies, designated LJP5 and LJP9, as well as a previously described one, AP2, all specific for the platelet membrane glycoprotein (GP)IIb/IIIa complex. None of them reacted with dissociated GPIIb or GPIIIa. The monovalent Fab fragment of both LJP5 and LJP9 bound to unstimulated platelets in a saturable manner, but binding was markedly decreased after platelets had been incubated at 37°C in the absence of added extracellular calcium. The binding of LJP9 was not affected by AP2, but was blocked by excess LJP5. On the contrary, the binding of LJP5 was blocked in the presence of both AP2 and LJP9. Thus, these antibodies bound to distinct epitopes of GPIIb/IIIa. At saturation, the binding to unstimulated platelets was between 2.41 and 10.9 $\times$ 10^4 molecules/platelet for LJP5 and between 3.47 and 9.1 $\times$ 10^4 molecules/platelet for LJP9 (range of 11 and 10 experiments, respectively). Binding increased up to 50% after thrombin stimulation. The estimated association constant, K_a, was 2.7 $\times$ 10^7 M^{-1} for LJP5 and 3.85 $\times$ 10^7 M^{-1} for LJP9. Both LJP5 and LJP9 partially inhibited the association of $^{45}Ca^{2+}$ with the surface of unstimulated platelets. Moreover, both antibodies blocked the binding of von Willebrand factor (vWF) to stimulated platelets, whereas only LJP9, but not LJP5, blocked fibrinogen binding. LJP9 was also a potent inhibitor of platelet aggregation, whereas LJP5 was without effect in this regard. The results of the present study demonstrate that independent modulation of vWF and fibrinogen binding to stimulated platelets can be attained with monoclonal antibodies directed against distinct epitopes of GPIIb/IIIa.

Introduction

The multimeric glycoprotein von Willebrand factor (vWF)[1] plays an essential role in the formation of a normal platelet plug at sites of vascular injury, but the exact molecular mechanisms underlying this function have yet to be unraveled (1). Its specific binding to the platelet membrane glycoprotein (GP)Ib can be experimentally demonstrated using ristocetin (2, 3) or after desialylation of the molecule (4). More recently, it has been shown that thrombin, as well as other platelet agonists, also induce the binding of vWF to platelets (5, 6). In this case, however, binding occurs at site(s) localized on the GPIIb/IIIa complex (2, 7).

Fibrinogen is also essential for normal platelet aggregation and is thought to function by interacting with a specific receptor on GPIIb/IIIa (8, 9). Fibrinogen can inhibit vWF binding to stimulated platelets (10–13), and the reverse also applies (10). Moreover, synthetic peptides representing the carboxyl terminal of the γ chain of fibrinogen, and much smaller than the parent molecule, can block the binding of both fibrinogen and vWF to GPIIb/IIIa (10, 14), thus suggesting that the site involved is very similar, if not identical, for both ligands. Similar conclusions have been reached based on experiments with monoclonal antibodies that inhibited the binding of both fibrinogen and vWF, as well as fibronectin and thrombospondin, to thrombin-stimulated platelets (15). Hence, the concept has been developed that a common binding site on GPIIb/IIIa may mediate the interaction of a family of adhesive glycoproteins with stimulated platelets.

In order to test the latter hypothesis, we have undertaken the characterization of a group of monoclonal antibodies directed against GPIIb/IIIa to see whether they all behaved similarly in inhibiting the binding of vWF and fibrinogen to platelets. For these studies, the smallest possible immunological probe was used, namely the monovalent Fab fragment derived from purified monoclonal IgG. We have obtained evidence that blocking distinct epitopes of the GPIIb/IIIa complex has distinct effects on the binding of vWF and fibrinogen to stimulated platelets.

Methods

Monoclonal antibody and Fab fragment preparation. Monoclonal antibodies against human platelet membrane glycoproteins were produced by immunizing BALB/c mice with intraperitoneal injections of washed platelet suspensions. These were prepared by gel filtration of platelet-rich plasma through Sepharose CL 2B (Pharmacia Fine Chemicals, Piscataway, NJ) as previously described (2), the only difference being that no albumin was used in the eluting buffer. The first injection consisted of 10^8 platelets in Freund's complete adjuvant. Two boosters of 10^8 and 1.5 $\times$ 10^8 platelets, respectively, in Freund's incomplete adjuvant were given at 1-wk intervals. A final booster of 3 $\times$ 10^8 platelets without adjuvant was given 4 d before fusion. Mouse spleen cells were fused with mouse plasmacytoma cells at a ratio of 6:1. The two new antibodies described in this report, designated LJP5 and LJP9, were derived from separate fusions. LJP5 was obtained using P3X63-Ag 8.653 plasmacytoma cells, and LJP9 using NS-1 cells. Fusion and growth of hybridomas were performed using standard technology, as previously described (16). Positive hybridomas producing anti-platelet antibodies were selected by

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1. *Abbreviations used in this paper:* ACD, acid/citrate/dextrose; GP, platelet membrane glycoprotein; vWF, von Willebrand factor.

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enzyme-linked immunoadsorbent assay (17). This assay detected mouse IgG reacting with washed platelets bound to plastic microtiter wells by means of Poly-L-lysine hydrobromide, M_r = 150,000–300,000 (Sigma Chemical Co., St. Louis, MO). Positive hybridomas were subcloned twice by limiting cell dilution (18). Monoclonal antibody was then produced in mouse ascites fluid as described (16). The reference anti-GPIIb/IIIa monoclonal antibody, AP2, was prepared as previously described (19).

Purified IgG was obtained from ascites fluid as follows. AP2 (IgG1) and LJP9 (IgG2b) were isolated by binding to Protein A-Sepharose (Sigma Chemical Co.) followed by elution at pH 6 and 4.5, respectively (20). LJP5 (IgG1) did not bind to Protein A-Sepharose with a sufficiently high affinity, and was purified on DEAE Affi-Gel Blue (Bio-Rad Laboratories, Richmond, CA) (21). Subclass specificity of the IgG molecules was defined by Outcherlony immunodiffusion (22) against the appropriate antisera. Fab fragments were prepared from the purified IgG. Note that the terminology Fab has been used, for the sake of simplicity, even when IgG (as for LJP5 and LJP9) was digested with pepsin, in which case the monovalent fragment derived is more appropriately called Fab'. Fab refers properly to monovalent fragment obtained by papain digestion (as for AP2). LJP5 was digested to $F(ab')_2$ with 10% (wt/wt) pepsin (Sigma Chemical Co.) in a 0.116 M acetate buffer, pH 3.8, containing 0.05 M NaCl, for 5 h at 37°C (23, 24). The optimum conditions for digestion (pH, time of incubation, pepsin concentration) were determined experimentally in each case. After dialysis in 0.02 M Tris, 0.15 M NaCl buffer, pH 7.4, reduction to Fab was achieved with 0.01 M cysteine for 60 min at 37°C, followed by S-carboxymethylation with 0.03 M iodoacetamide for 60 min at room temperature (22–25°C). LJP9 was digested similarly to LJP5, but the pH used was 4.5 to avoid excessive digestion of the IgG. Monovalent Fab could be isolated directly from the digestion mixture by high-pressure molecular sieve chromatography (Waters Associates, Milford, MA) using two columns (dimensions 60 × 2.15 cm, Bio-Rad) mounted in series, one packed with TSK gel G-4000 SW and the other with G-2500 SW (Bio-Rad). The other main product isolated from the digestion mixture was Fab/c, as well as undigested IgG, in accordance with the previously reported fragmentation pattern of mouse IgG2b (24). AP2 (IgG1) was too susceptible to pepsin digestion for Fab to be obtained in satisfactory quantities. Therefore, this IgG was digested with 5% papain (wt/wt) (Sigma) in 0.15 M phosphate buffer, pH 7, for 90 min at 37°C (25). The mixture contained also 0.01 M cysteine and 0.002 M EDTA. The reaction was terminated by addition of 0.05 M iodoacetamide for 60 min at room temperature. This achieved, at the same time, S-carboxymethylation of Fab. Undigested Fc was separated from the mixture by passage through a Protein A-Sepharose column to which Fab did not bind. All Fab preparations were finally concentrated to between 1.5 and 4 mg/ml by ultrafiltration and extensively dialyzed against 0.02 M Tris, 0.15 M NaCl buffer, pH 7.4. Polyacrylamide gel electrophoresis (10% acrylamide with 5% cross-linking) in the presence of sodium dodecyl sulfate (SDS) (26) was used to assess the purity of each Fab preparation. Protein concentration was measured by the method of Lowry et al. (27), using bovine serum albumin (Sigma Chemical Co.) and bovine IgG (Miles Scientific, Naperville, IL) as standards, or by light absorption at 280 mm assuming $E^{1\%}_{280}$ = 14.2.

Preparation of washed platelet suspensions. Washed platelets for aggregation and binding studies were prepared from blood drawn through 19-gauge needles into polypropylene syringes and transferred into polypropylene tubes containing 1/6th final volume of acid/citrate/dextrose (ACD), pH 4.5. Platelet-rich plasma was obtained as previously described (2). Platelets were routinely washed free of plasma constituents by the albumin density-gradient technique of Walsh et al. (28), with minor modifications. These consisted in the addition of apyrase (grade III, Sigma) to the platelet-rich plasma (5 U/ml based on 5'-ATPase activity) and to the buffer used in the two subsequent washes (1 and 0.2 U/ml, respectively). The platelets were finally resuspended in Tyrode's buffer, pH 7.4, and counted before use (2). Platelet suspensions devoid of divalent cations were obtained as described by Marguerie et al. (29). The calcium content of the buffers used for the latter procedure was <5 μM, as determined by absorption spectrophotometry.

Surface labeling of platelet membrane glycoproteins. Blood was drawn in ACD and platelet-rich plasma prepared as described (2). The platelets were then pelleted at 2,000 *g* for 8 min at room temperature, in polypropylene tubes, and subsequently resuspended in a buffer composed of 0.01 M Tris, 0.002 M EDTA, 0.15 M NaCl, pH 7.4 (Tris-EDTA). ACD at a final concentration of 15% (vol/vol) was also added. The platelet suspension was pelleted once more, and the platelets were then resuspended in Tris-EDTA at a count of 1×10^9/ml. 1 mCi of carrier-free Na ^{125}I (Amersham Corp., Arlington Heights, IL) was added to each ml of the platelet suspension, followed by 10 μl of 2.5 nM lactoperoxidase (Sigma Chemical Co.). The mixture was kept in agitation with gentle stirring. Five successive 10-μl aliquots of 0.003 M H_2O_2, diluted in the same Tris-EDTA buffer, were added at 10-s intervals, and the reaction was then terminated by diluting the mixture with 9 vol of Tris-EDTA buffer. The labeled platelets were washed twice in Tris-EDTA containing ACD, and finally resuspended in a buffer composed of 0.05 M Tris, 0.15 M NaCl, pH 7.4, containing 200 μg/ml leupeptin (Chemicon Cal-Med, El Segundo, CA), 0.01 M EDTA and 0.005 M *N*-ethylmaleimide (Sigma Chemical Co.). The platelet count was adjusted to 4×10^9/ml, and lysis was achieved by addition of 1% (vol/vol) Triton X-100 (Eastman Kodak Co., Rochester, NY) in 30 min at 4°C with constant agitation. The mixture was then centrifuged at 100,000 *g* for 30 min at 4°C, and the solubilized membrane glycoproteins remaining in the supernatant were aliquoted and stored at −70°C until used.

Immunoisolation of platelet membrane glycoproteins. This was performed following a technique recently described (30). In brief, polyvinyl chloride microtiter plates (Dynatech Laboratories, Alexandria, VA) were coated with 10 μg of affinity-purified rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories, Avondale, PA) dissolved in phosphate buffer, pH 7.2, as indicated (30). After overnight incubation at 4°C, the plates were washed and the monoclonal antibody to be tested (usually culture fluid supernatant or ascites) was added to several wells (usually four), followed by incubation for 4 h at room temperature. After washing, blocking of unreacted sites on the plastic, and additional washing, 100 μl of radiolabeled platelet membrane proteins, prepared as described above, was added to each well and incubated for 4 h at room temperature. The plates were then washed four times with phosphate buffer containing 0.5% Triton and 0.1% SDS, after which 50 μl of hot (boiling) 0.01 M phosphate buffer, pH 7.0, containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.05% bromophenol blue was added to each well. After incubation for 5 min, the samples from wells containing the same monoclonal antibody were pooled, boiled for an additional 5 min, and then analyzed in SDS-polyacrylamide gel electrophoresis.

Crossed immunoelectrophoresis of platelet membrane glycoproteins. This was performed as previously described (31), using 100 μg of platelet lysate in the first dimension and running the second dimension against polyspecific rabbit anti-human platelet antibody (purified IgG). Purified IgG of the monoclonal antibody to be tested (50 μg) was incorporated in the intermediate gel of the second dimension. After electrophoresis, gels were washed twice and then incubated with at least 4×10^6 cpm (~80 μg) of ^{125}I-labeled affinity-purified rabbit anti-mouse IgG (Zymed Laboratories, Inc., San Francisco, CA). Following an 18-h incubation at ambient temperature in a humidified chamber, the gels were washed, dried and stained with Coomassie blue-R (Sigma Chemical Co.) as previously described (31). Autoradiography was accomplished by storing dried gels in the dark for 4–24 h between a Kodak X-Omat R film and a duPont Cronex Lightning Plus screen (E. I. du Pont de Nemours, Inc., Wilmington, DE), at ambient temperature.

Aggregation studies. Platelet-rich plasma for aggregation studies was prepared as described above, the only difference being that blood was anticoagulated with 0.11 M trisodium citrate in a proportion of 9:1. For these experiments, the platelet count in platelet-rich plasma or washed platelet suspensions was adjusted so that the final count in the experimental mixture was 2×10^8/ml. The effect of monoclonal Fab on platelet aggregation was tested by incubating the Fab and the platelets for 3 min at 37°C, without stirring, before transferring to the stirred cell of the aggregometer and adding the agonist. When washed platelets were used, purified fibrinogen (300 μg/ml, 8.8×10^{-7} M) and $CaCl_2$ (2 mM) were added to the mixture together with the antibody. Aggregation was re-

corded as increase in light transmission through the mixture using a Lumiaggregometer (Chrono Log Corporation, Havertown, PA) and a dual-channel recorder (Kipp and Zonen, Bohemia, NY).

Purification of fibrinogen and vWF. Fibrinogen was purified from blood collected into 1/10 volume of 0.11 M trisodium citrate anticoagulant, containing 1 M ϵ-aminocaproic acid and 0.365 mg/ml of Aprotonin (Sigma Chemical Co.), by means of precipitation with polyethylene glycol 1,000 (Sigma Chemical Co.) as previously described (32). Analysis of the purified fibrinogen by 7.5% polyacrylamide (5% cross-linking) slab gel electrophoresis in the presence of SDS and under reducing conditions showed the characteristic Aα, Bβ, and γ chains (33). Absence of vWF and fibronectin contamination was further established by immunoblotting (34) with monospecific antibodies. The purified fibrinogen was at least 92% clottable.

vWF was purified following methods previously described in detail (2, 35, 36). Analysis of the purified vWF by 5% polyacrylamide (5% cross-linking) slab gel electrophoresis in the presence of SDS and under reducing conditions demonstrated one major band of apparent M_r = 220,000. In particular, no fibrinogen was detected in the vWF preparations (2). Absence of fibrinogen and fibronectin contamination was further established by immunoblotting with monospecific antibodies. All preparations of purified vWF had >100 U of ristocetin cofactor activity and vWF antigen per milligram of protein, and had a multimeric structure like that of plasma vWF (2).

Radioiodination of proteins. Purified proteins were radiolabeled with carrier-free $Na^{125}I$ (Amersham Corp.) using Iodogen (Pierce Chemical Co., Rockford, IL), as described by Fraker and Speck (37). All radiolabeled proteins were analyzed by polyacrylamide gel electrophoresis and showed no structural alterations as compared to the unlabeled counterparts. Specific activity was between 0.4 and 1.2 mCi/mg of protein for all ligands. Radioactivity was over 90% precipitable in 10% trichloroacetic acid in all cases.

Binding studies. The binding of radiolabeled anti-GPIIb/IIIa Fab to platelets was tested with both platelet-rich plasma and washed suspensions. Fab and platelets (final count between 0.3 and 1 × 10^8/ml) were incubated for 30 min at 22–25°C, and platelets were then separated from the reaction mixture by layering duplicate aliquots of the latter onto 20% sucrose in a microcentrifuge tube (2). After centrifugation at 13,000 *g* for 4 min (Beckman Instruments, Inc., Fullerton, CA), the tip of the tubes containing the pelleted platelets was cut with a scalpel and the platelet-bound radioactivity counted in a gamma-scintillation counter (Packard Instrument Co., Inc., Downers Grove, IL). Nonspecific binding was determined in the presence of at least a 20-fold excess of the correspondent unlabeled Fab and never exceeded 10% of total binding. The number of antibody molecules bound per platelet was estimated by measuring binding at antibody concentrations where a twofold increase in concentration gave <5% increase in binding. The association constant, K_a, was then calculated as the reciprocal of the free antibody concentration that gave half maximal binding, as derived from the binding isotherm. These binding parameters were also obtained by the method of Scatchard (38), assuming one class of non-interacting binding sites, and the results were in close agreement.

The binding of radiolabeled vWF and fibrinogen to platelets was determined after stimulation with either α-thrombin (the generous gift of Dr. J. W. Fenton II, New York State Department of Health, Albany, NY) or ADP (Sigma Chemical Co.). To measure thrombin-induced binding, platelets at a concentration of 5 × 10^8/ml were stimulated with 0.1 U/ml of thrombin for 10 min at 22–25°C. Hirudin (Sigma Chemical Co.) was then added at a 16-fold excess (U/U). After 5 min at 22–25°C, radiolabeled ligand was added at the desired concentrations and incubation continued for 30 min (platelet count at this point: 1 × 10^8/ml). Platelets were then separated from the reaction mixture as described above. The binding induced by ADP (20 μM) was measured by incubating the latter with platelets, radiolabeled ligand, and $CaCl_2$ (2 mM) all mixed at the same time. Nonspecific binding was always measured with a 20–100-fold excess of the corresponding unlabeled ligand. To test the effect of the monoclonal Fab fragments on binding, the desired concentration of antibody was added to the platelet mixture immediately before the other reagents. Nonspecific binding was substracted from total binding to calculate specific binding. Nonspecific binding was never more than 25% of total binding, and was essentially the same whether LJP5 and LJP9 were present in the mixture or not. Binding isotherms were fitted to the experimental data, each one the mean of duplicate readings which agreed within 20%, using least square regression analysis.

The binding of $^{45}Ca^{2+}$ (Amersham Corp.) to the surface of unstimulated washed platelets was measured as described by Brass and Shattil (39), using free Ca^{2+} concentrations, in EGTA buffer, between 10^{-6} and 10^{-8} M. For these studies, washed platelets devoid of calcium were prepared as described above (29), with the exception that the final suspension contained 1 mM Mg^{2+} and was incubated with 1 mM acetylsalicilic acid for 30 min at room temperature before use.

Results

The two new anti-platelet monoclonal antibodies here described were designated LJP5 and LJP9. In crossed immunoelectrophoresis, they reacted against the membrane GPIIb/IIIa complex (Fig. 1), but not against dissociated GPIIb/IIIa or GPIIIa (Fig. 2). Furthermore, immunoisolation studies using surface-labeled platelets demonstrated that both LJP5 and LJP9 reacted with two proteins exhibiting apparent molecular weight correspondent to that of GPIIb and GPIIIa (Fig. 3). The same proteins reacted with AP2, a well-characterized anti-GPIIb/IIIa monoclonal antibody (19, 37).

Monovalent Fab fragment of both LJP5 and LJP9 bound to unstimulated human platelets in a saturable manner (Fig. 4). The mean number of LJP5 Fab molecules bound per platelet, measured in 11 separate experiments with platelets from different donors, was 5.06 (range 2.41–10.9) × 10^4, with an estimated association constant, K_a, of 2.7 × 10^7 M^{-1}. The corresponding values for LJP9 Fab (10 experiments) were 5.48 (range 3.47–9.1) × 10^4 molecules/platelet, with K_a = 3.85 × 10^7 M^{-1}. Binding to thrombin-stimulated platelets (0.5 NIH U/ml) was increased up to 50% without apparent change in affinity. The binding of LJP5 to platelets was markedly reduced in the presence of a 20-fold excess of AP2, whereas the binding of LJP9 was unaffected by AP2 (Fig. 4). On the other hand, LJP9 inhibited the binding of LJP5 and vice versa, and results were compatible with a mechanism of competitive inhibition (Fig. 4). The binding of AP2, however, was blocked by LJP5 but not by LJP9 (not shown here).

Platelets washed in a calcium-free environment retained the ability to bind both LJP5 and LJP9 when tested promptly after isolation, but binding decreased markedly in a time-dependent manner when platelets were incubated at 37°C before adding the antibodies. The decrease in binding was largely, but usually not completely, prevented by the addition of Ca^{2+} to the platelets kept at 37°C. Platelets kept at 22–25°C in the absence of Ca^{2+} showed some decrease in the ability to bind both antibodies, but much less so than platelets kept at 37°C (Table I). Both LJP5 and LJP9 bound to unstimulated or thrombin-stimulated platelets in the presence of EDTA, at concentration as high as 6.4 mM, when platelets were kept at room temperature and the pH of the suspension was 7.4 (Table II). Under the same conditions, binding of fibrinogen and vWF to thrombin-stimulated platelets was completely blocked (Table II). Both antibodies, however, failed to bind to platelets in the presence of EDTA when the mixtures were incubated at 37°C and the pH was raised to 8. The association of $^{45}Ca^{2+}$ with the surface of unstimulated platelets was partially decreased in the presence of both LJP5 and LJP9 (Table III).

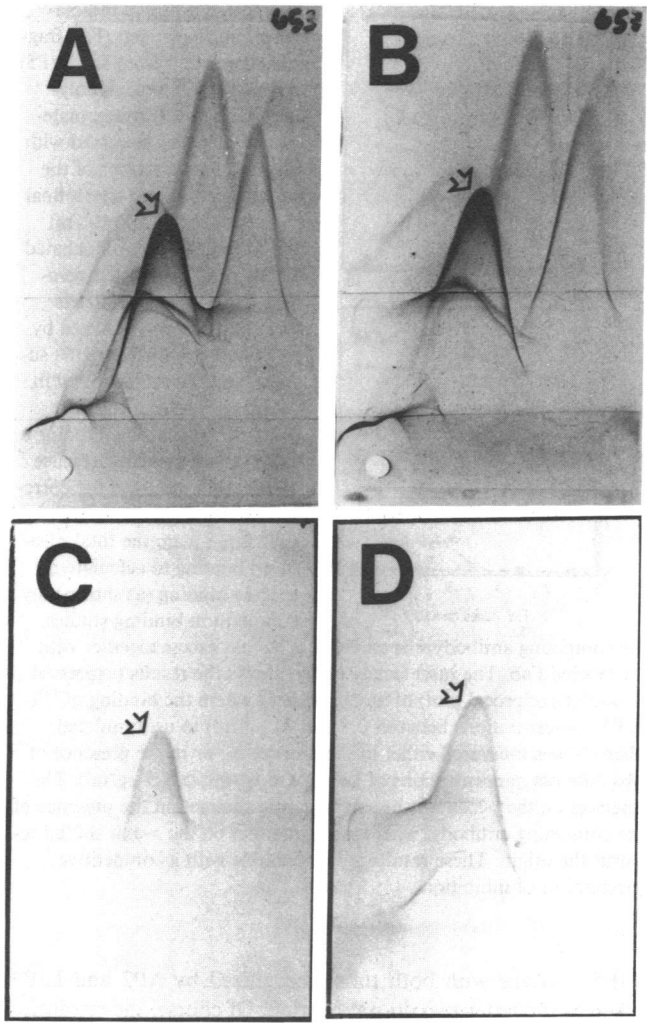

Figure 1. Specificity of LJP5 and LJP9 as determined by crossed immunoelectrophoresis. 100 μg of protein, derived from washed platelets lysed in the absence of EDTA, was electrophoresed in the first dimension and then, at 90°, into a second-dimension gel containing: in the intermediate gel, 50 μl of hybridoma culture fluid from LJP9 (gel *A*) or LJP5 (gel *B*); in the upper gel, a polyspecific rabbit anti-human platelet antibody (250 μg of purified IgG per cm^2). After completion of electrophoresis, bound monoclonal antibody was detected by incubation of gels in buffer containing ^{125}I-labeled affinity-purified rabbit anti-mouse IgG, followed by several washes to remove unbound radiolabeled antibody, and autoradiography. Coomassie blue-stained gels are depicted in *A* and *B*. The corresponding autoradiographs are shown in *C* and *D*, respectively. The precipitin arc given by the GPIIb/IIIa complex is indicated by an open arrow. Both LJP5 and LJP9 reacted with this complex.

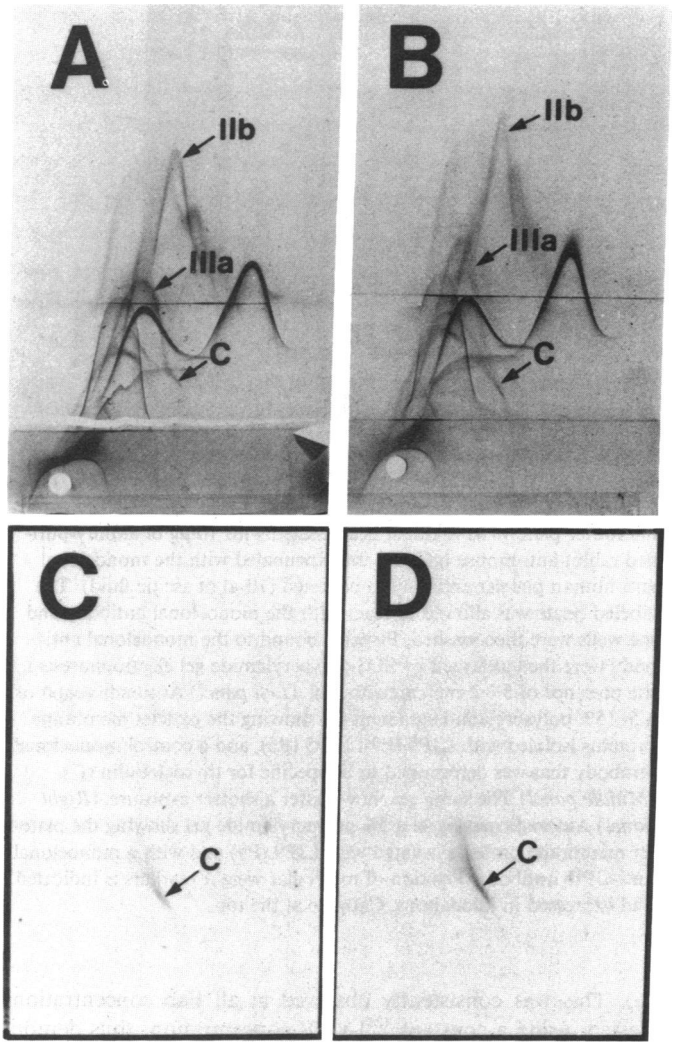

Figure 2. Specificity of LJP5 and LJP9 as determined by crossed immunoelectrophoresis. Legend as in Fig. 1, except that protein derived from platelets lysed in the presence of EDTA was analyzed. The precipitin arc containing the GPIIb/IIIa complex (*C*) is markedly diminished, although still detectable. Additional precipitin arcs containing dissociated GPIIb and GPIIIa are indicated. Note that both LJP9 (gels *A* and *C*) and LJP5 (gels *B* and *D*) react with the residual GPIIb/IIIa complex, but neither with dissociated GPIIb nor GPIIIa.

The binding of vWF to ADP-stimulated platelets was completely blocked by LJP5 as well as LJP9 (Fig. 5, *upper panel*). The latter antibody also blocked the binding of fibrinogen, whereas LJP5 had no effect in this regard (Fig. 5, *lower panel*). Similar results were obtained with thrombin-stimulated platelets. The amount of each antibody necessary to achieve maximal inhibitory effect was in agreement with the amount that gave saturation of binding to platelets (compare Fig. 4 and Fig. 5). Nevertheless, LJP5 Fab had no effect on fibrinogen binding even when added in a 10-fold excess over the saturating amounts. The concentration of vWF or fibrinogen added to the platelets had no effect on the extent of inhibition obtained with the antibodies, and results were compatible with a noncompetitive inhibitory mechanism (Fig. 5). The binding of vWF added at concentrations between 0.5 and 30 μg/ml could be completely inhibited by LJP5 and LJP9. LJP9 also markedly inhibited the binding of fibrinogen when the latter was added at concentrations between 5 and 600 μg/ml, whereas LJP5 was consistently without effect at all concentrations of fibrinogen tested. Experiments on the inhibition of vWF and fibrinogen binding were performed several times (only three are reported in Fig. 5) using platelets from different donors and different Fab preparations. In all cases, the results were in close agreement. Neither LJP5 nor LJP9 affected vWF binding to platelets in the presence of ristocetin.

The inhibitory effect of LJP5 Fab on vWF binding to platelets was inversely related to the platelet count in the mixture (Fig.

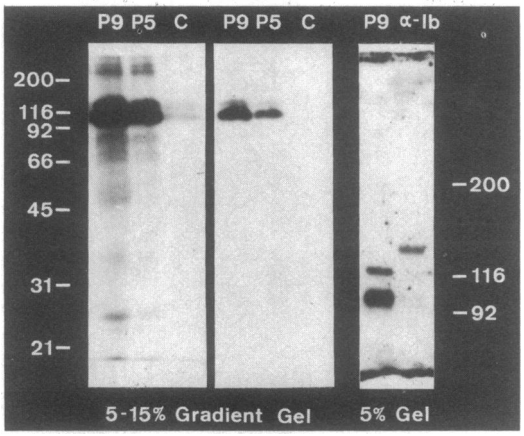

Figure 3. Specificity of LJP5 and LJP9 as determined by immunoisolation. Washed platelets (4×10^9/ml) were surface labeled with ^{125}I and lysed with Triton X-100. 100 μl of the labeled lysate was added to microtiter plate wells that had been coated with 10 μg of affinity-purified rabbit anti-mouse IgG and then incubated with the monoclonal anti-human platelet antibody to be tested (10 μl of ascitic fluid). The labeled lysate was allowed to react with the monoclonal antibody and the wells were then washed. Proteins bound to the monoclonal antibody were then analyzed by SDS-polyacrylamide gel electrophoresis in the presence of 5% 2-mercaptoethanol. (*Left panel*) Autoradiograph of a 5–15% polyacrylamide gradient gel showing the platelet membrane proteins isolated with LJP9 (P9), LJP5 (P5), and a control monoclonal antibody that was determined to be specific for thyroglobulin (*C*). (*Middle panel*) The same gel shown after a shorter exposure. (*Right panel*) Autoradiography of a 5% polyacrylamide gel showing the platelet membrane proteins isolated with LJP9 (P9) and with a monoclonal anti-GPIb antibody. Position of molecular weight markers is indicated and expressed in kilodaltons. Cathode at the top.

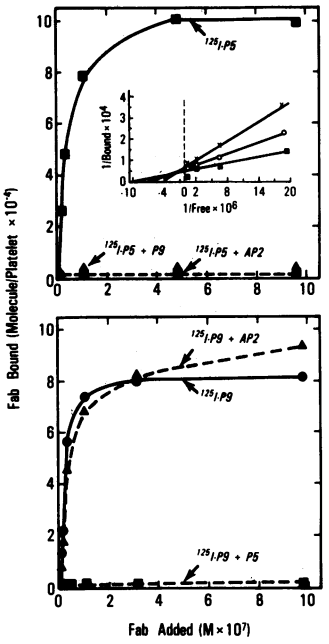

Figure 4. Binding of radiolabeled Fab to platelets. Fab fragments prepared from LJP5 (P5) and LJP9 (P9) were radiolabeled with ^{125}I. Citrated platelet-rich plasma was mixed with varying concentrations of the radiolabeled Fab to give a final platelet count of 3×10^7/ml and the mixture was incubated for 30 min at room temperature. Separation of platelets from free Fab was attained by centrifugation through 20% sucrose, and the radioactivity in the platelet pellets was then counted. Nonspecific binding was measured in the presence of a 20-fold excess of the correspondent unlabeled Fab, and subtracted from the total measured binding to calculate the specific binding (as shown). In competition binding studies, the competing antibody was added at a 20-fold excess together with the labeled Fab. The inset (*upper panel*) shows the results (expressed as double reciprocal plot) of an experiment where the binding of ^{125}I-LJP5 (concentrations between 0.5 and 31 μg/ml) to unstimulated platelets was measured either in the absence (■) or in the presence of two different concentrations of LJP9 (×, 6 μg/ml; ○, 1.5 μg/ml). The intersect on the *y*-axis was not significantly changed in the presence of the competing antibody, whereas the intersect on the *x*-axis shifted towards the origin. These results are compatible with a competitive mechanism of inhibition.

6). This was consistently observed at all Fab concentrations tested, using a constant ^{125}I-vWF concentration, thus demonstrating that the effect of LJP5 Fab on vWF binding was not related to alterations induced in the vWF molecule, but rather to occupancy of the corresponding epitope on GPIIb/IIIa.

As shown in Fig. 4, excess LJP5 could block LJP9 binding to platelets. Accordingly, excess LJP5 Fab could effectively prevent the inhibitory effect of LJP9 Fab on fibrinogen binding (Fig. 7). Moreover, LJP5 Fab had no effect on ADP-induced platelet aggregation, whereas LJP9 Fab completely inhibited it (Fig. 8). Identical results were observed when α-thrombin or collagen were used as agonists instead of ADP. In accordance with the results of fibrinogen binding studies, excess LJP5 Fab could prevent the inhibitory effect of LJP9 on aggregation (Fig. 8). The results of aggregation studies were identical for either citrated platelet-rich plasma or washed platelet suspensions containing purified fibrinogen and 2 mM $CaCl_2$.

Discussion

Our results demonstrate that the two new monoclonal antiplatelet antibodies described in this report, LJP5 and LJP9, recognize distinct epitopes on the GPIIb/IIIa complex that are also different from the one recognized by AP2. In fact, LJP9 and AP2 bound to platelets independently of each other, whereas LJP5 and AP2 competed for binding. Competition for binding was also observed between LJP5 and LJP9. The most likely explanation for our findings is that the epitope recognized by LJP5 overlaps with both those recognized by AP2 and LJP9, whereas the latter two do not overlap. Of course, the resolution of these inhibition binding studies is limited by the size of the

Table I. Effect of Temperature on LJP5 and LJP9 Binding to Platelets in the Absence of Added Extracellular Calcium

		Antibody bound	
Temperature	Incubation time	LJP5	LJP9
°C	*min*	%	
37	0	100	100
	15	38	34
	30	22	22
	45	13	12
	60	4	7
	60 (Ca^{2+})	68	73
22–25	60	85	69

Platelets were incubated in the absence of added extracellular calcium (unless indicated) for variable periods of time before addition of the labeled antibodies. After this, incubation was continued at room temperature (22–25°C) for additional 30 min. When indicated, calcium was added at 2 mM from the beginning of the incubation. Both antibodies were added at a concentration of 6 μg/ml (1.2×10^{-7} M) and the binding measured corresponded to that shown in Fig. 4. The platelet count was 1×10^8/ml in the incubation mixtures, and the pH was 7.4.

Table II. Effect of EDTA on the Binding of LJP5, LJP9, vWF and Fibrinogen to Thrombin-stimulated Platelets Kept at Room Temperature and pH 7.4

EDTA	Thrombin stimulation	Binding			
		LJP5	LJP9	vWF	Fibrinogen
mM	*0.15 NIH U/ml*	%	%	%	%
0	no	100	100	0	0
0	yes	148	115	100	100
0.1	yes	129	107	91	89
0.4	yes	147	99	0	0
1.6	yes	121	125	0	0
6.4	yes	107	122	0	0

Platelets were stimulated with thrombin in the presence of EDTA for 10 min, then hirudin was added followed by the radiolabeled ligands (see Methods). All incubations were at room temperature (22–25°C) and the pH of the mixtures was 7.4. LJP5 and LJP9 were added at a concentration of 6 μg/ml (1.2×10^{-7} M), fibrinogen was at 60 μg/ml, and vWF at 5 μg/ml. Binding defined as 100% corresponded to that shown in Fig. 4 for the two antibodies, to 64,000 molecules/platelet for fibrinogen, and to 1.2 μg/10^8 platelets for vWF.

Fab fragments used. Theoretically, conformational changes of GPIIb/IIIa following the binding of monoclonal antibody might also be responsible for the competition observed between LJP5 and the other two antibodies, even if the corresponding epitopes were not close to one another. The observed mechanism of competitive inhibition between LJP5 and LJP9, however, is not in favor of the latter hypothesis.

The average number of LJP5 and LJP9 molecules bound per platelet was ~50,000, a number not dissimilar from the ones previously reported for other anti-GPIIb/IIIa monoclonal antibodies (40–44). The range previously observed varied from 21,380 (43) to 57,400 (40) molecules/platelet, with one investigator reporting extreme values of 12,000–83,000 (42). We also observed a wide range of values among different individual

Table III. Effect of LJP5 and LJP9 on the Association of $^{45}Ca^{2+}$ with the Surface of Unstimulated Platelets

Antibody present	$^{45}Ca^{2+}$ bound
	sites/platelet
None	24,900
LJP5	12,200
LJP9	13,050

Platelets were washed free of Ca^{2+} and with 1 mM Mg^{2+} present (see Methods). Free $^{45}Ca^{2+}$ was then added at a concentration of 7.8×10^{-7} M, in 0.5 mM EGTA buffer, and incubated with platelets (2×10^8/ml) for 10 min at 22–25°C, either in the presence or absence of LJP5 and LJP9 (30 μg/ml). Separation of bound from free $^{45}Ca^{2+}$ was achieved by centrifugation through 20% sucrose. The amount of $^{45}Ca^{2+}$ bound to the platelet membrane was obtained by subtracting from the total value associated with the platelet pellet the amount that could not be displaced by the addition of 3 mM EGTA 2 min before separation of bound from free ligand. The latter was considered to represent intracellular $^{45}Ca^{2+}$ that could not readily exchange with the extracellular medium (see Reference 39).

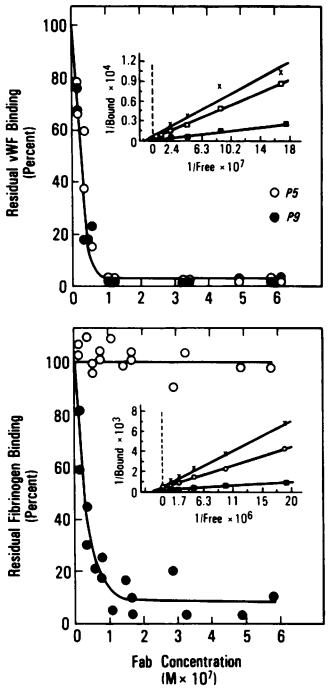

Figure 5. Effect of LJP5 and LJP9 on fibrinogen and vWF binding to platelets. Washed platelet suspensions (1×10^8/ml) were mixed with 100 μg/ml of ^{125}I-fibrinogen (2.9×10^{-7} M) (*lower panel*), or 5 μg/ml of ^{125}I-vWF (*upper panel*). This was followed by 0.002 M $CaCl_2$, the indicated concentration of Fab from LJP5 (P5) or LJP9 (P9), or an equal volume of Tyrode buffer, and 20 μM ADP. All indicated concentrations were final. The mixture, containing also 2% bovine serum albumin, was incubated for 30 min at room temperature, after which the platelets were separated from free ligand by centrifugation through 20% sucrose. The radioactivity in the platelet pellets was then counted. Nonspecific binding was measured in the presence of a 100-fold excess of unlabeled ligand. Specific binding was calculated by subtracting nonspecific from total binding, and expressed as percentage of the binding measured in the absence of Fab (as shown). The latter values corresponded to 38,600 molecules/platelet for fibrinogen and 0.6 μg/10^8 platelets for vWF. These results are the aggregate of three separate experiments performed with different platelet preparations. The *inset* in the upper panel shows the results of a competition binding study where increasing amounts of ^{125}I-vWF (between 1.3 and 37 μg/ml) were added to platelets either in the absence (■) or in the presence of LJP5 (×, 1.5 μg/ml; □, 0.8 μg/ml). The reciprocal of specific binding was plotted against the reciprocal of the free vWF concentration added. Even at the highest concentrations of vWF tested, the inhibitory effect of LJP5 could not be overcome. A change in position of the intersect on the *y*-axis, but not on the *x*-axis, was observed in the presence of the antibody, a finding compatible with a noncompetitive mechanism of inhibition. The *inset* in the lower panel shows the results of a similar experiment performed with ^{125}I-fibrinogen (concentrations between 15 and 366 μg/ml) either in the absence (■) or in the presence of LJP9 (×, 15 μg/ml; ○, 3 μg/ml). Results could be interpreted in a similar way as those obtained in the experiment with vWF. In both instances, platelets were stimulated with 0.1 NIH U/ml of α-thrombin (see *Methods*).

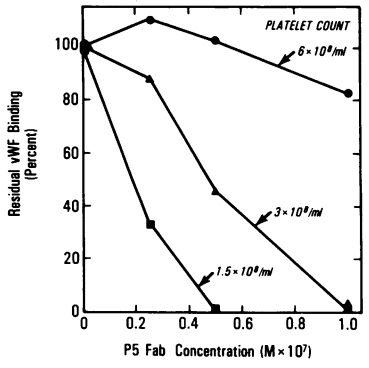

Figure 6. Effect of platelet count on inhibition of vWF binding by LJP5. The binding of ^{125}I-vWF to platelets was measured as indicated in the legend to Figure 5, with the following modifications. The platelet count in the mixture varied between 1.5 and 6×10^8/ml (as indicated); and the platelets were stimulated with 0.1 NIH U/ml of α-thrombin (see Methods). Binding of ^{125}I-vWF in the presence of increasing concentrations of LJP5 Fab was expressed as percentage of the binding measured in the absence of LJP5 Fab. The latter value corresponded to 0.52 μg/10^8 platelets.

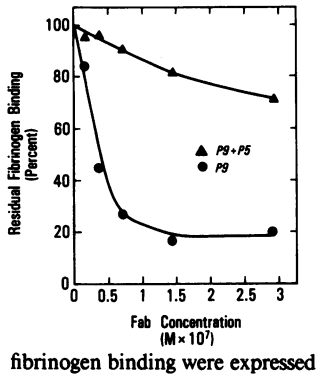

Figure 7. Effect of LJP5 on inhibition of fibrinogen binding by LJP9. The binding of ^{125}I-fibrinogen to platelets was measured as indicated in the legend to Fig. 5, with the following modifications. In one series of mixtures, LJP9 Fab (P9) was present at the concentrations indicated on the abscissa. In a parallel series, LJP9 Fab was present at the same concentrations, but LJP5 Fab (P5) was also added at a 20-fold excess. The results of ^{125}I-fibrinogen binding were expressed as percentage of the binding measured in the absence of Fab.

platelets. These results may reflect technical variability of the assays, but are also likely to reflect individual variations in the number of GPIIb/IIIa molecules expressed on the platelet membrane. In our studies, we used monovalent Fab rather than divalent IgG (40, 41, 43, 44) or F(ab')$_2$ (42). This may be responsible, at least in part, for some of the differences observed, since antibodies may bind to cell surface antigens divalently, and the proportion of divalent to monovalent binding may vary (45, 46). The reason why thrombin-stimulated platelets bound up to 50% more LJP5 and LJP9 Fab than unstimulated platelets may reflect the fact that a pool of GPIIb/IIIa molecules contained in the α-granule membrane becomes exposed on the platelet surface following secretion (47), thereby increasing the number of epitopes interacting with the monoclonal antibodies.

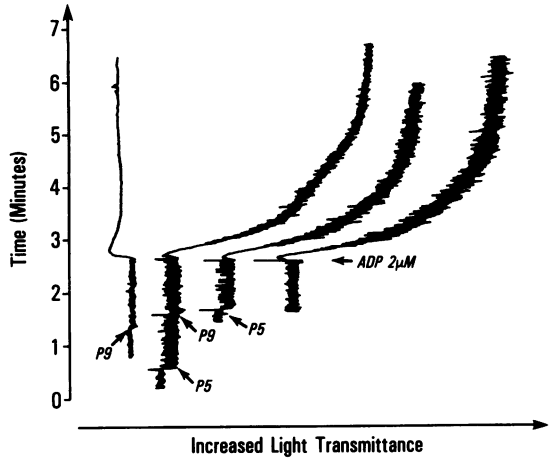

Figure 8. Effect of LJP5 and LJP9 on platelet aggregation. Washed platelet suspensions (2×10^8/ml) were added to an aggregometer cuvette, under stirring conditions, and mixed with 0.002 M $CaCl_2$ and 300 μg/ml of fibrinogen (8.8×10^{-7} M). To the mixture was then added either LJP5 (P5) or LJP9 (P9) Fab separately (20 μg/ml each, $\sim 4.2 \times 10^{-7}$ M), or LJP5 (200 μg/ml) followed by LJP9 (20 μg/ml). A control mixture contained Tyrode's buffer instead of Fab (curve on the right). All indicated concentrations were final. After equilibration and establishment of a straight baseline, ADP (2 μM, final concentration) was added to the mixtures (at arrow) and platelet aggregation recorded as increased light transmittance through the cuvette. The decrease in light transmittance immediately after addition of ADP represents the platelet shape change. Note the inhibitory effect of LJP9 on platelet aggregation, but its lack of inhibition of the platelet shape change. Note also the lack of inhibition in the presence of LJP5 and the blocking effect of the latter on the inhibition exerted by LJP9.

Our results demonstrate that LJP5 and LJP9 recognize distinct epitopes on the platelet membrane whose appropriate conformation for binding the antibodies is calcium dependent. Similar results have been recently obtained with another anti-GPIIb/IIIa monoclonal antibody (48). These findings do not prove that the epitopes recognized by LJP5 and LJP9 are formed by the association of GPIIb and GPIIIa, in that they might exist on either one of the two individual glycoproteins but only when the two are in complex. Nevertheless, the two epitopes can certainly be considered as complex dependent, inasmuch as they exist only under the conditions required for maintaining integrity of the GPIIb/IIIa complex (31, 49–52). Moreover, it is clear that the interaction of both LJP5 and LJP9 with the corresponding epitopes, while requiring the presence of the calcium-dependent heterodimer formed by GPIIb and GPIIIa, is independent of additional Ca^{2+} in the medium.

The two new antibodies here described, LJP5 and LJP9, showed marked differences in their effect on platelet function. The latter was a potent inhibitor of aggregation induced by ADP, collagen, or α-thrombin, whereas the former was without effect in this regard. Accordingly, LJP9 blocked fibrinogen binding to stimulated platelets, but LJP5 did not. Both antibodies, however, completely blocked vWF binding to platelets stimulated by either ADP or thrombin. Recent studies have suggested that fibrinogen and vWF share a common binding site on GPIIb/IIIa (10, 14, 15). The most compelling evidence for this is provided by experiments performed with peptides representing the carboxyl terminal of the human fibrinogen γ chain, which inhibit in a parallel fashion the binding of both fibrinogen and vWF to stimulated platelets (10, 14). Similar results have recently been obtained using monoclonal antibodies directed against GPIIb/IIIa (15). Our finding that LJP5 has contrasting effects on vWF and fibrinogen binding suggests, however, that the mechanisms involved in the interaction of these two ligands with platelets are, at least in part, distinct. This does not necessarily exclude the existence of a common receptor. Moreover, it is clear that the effects exerted by LJP5 and LJP9 are related to occupancy of the corresponding epitopes, in that LJP5 did not alter the binding properties of vWF, and displacement of LJP9 by LJP5 resulted in normal fibrinogen binding and platelet aggregation in the presence of both antibodies.

At present, it is not possible to explain in a definitive manner the mechanisms by which LJP5 and LJP9 have a differential effect on fibrinogen and vWF binding to stimulated platelets. In some aspects, the results of the present study are compatible with the concept that neither antibody directly blocks the specific binding site(s) for the two adhesive glycoproteins, because they both act as noncompetitive inhibitors of fibrinogen and/or vWF interaction with platelets. This conclusion must be taken with caution, however, as it relates to complex macromolecules whose binding to platelets is not fully reversible (2, 29, 40). Two additional observations seem to support the concept that the epitopes recognized by LJP5 and LJP9 cannot be identified with the vWF and fibrinogen binding site(s). In fact, LJP5 and LJP9, but not fibrinogen nor vWF, bind to unstimulated platelets. Moreover, additional extracellular calcium, besides that required for formation of the GPIIb/IIIa complex, is not necessary for binding of the two antibodies to their respective epitopes, but it is for binding of vWF and fibrinogen to their receptor(s). These findings might be interpreted as evidence that the epitopes recognized by LJP5 and LJP9 are expressed independently of the vWF and fibrinogen binding site(s). However, they might also

reflect the fact that calcium-dependent and stimulation-dependent changes in the microenvironment surrounding the GPIIb/IIIa complex are necessary for the binding of vWF and fibrinogen, but not of LJP5 and LJP9, possibly because of the larger size of the two adhesive glycoproteins as compared to the antibody molecules (53). The observations that smaller peptides derived from the fibrinogen γ chain bind to unstimulated platelets (54), and that fibrinogen itself forms a complex with isolated GPIIb/IIIa (8), are both in agreement with the latter hypothesis. Therefore, because vWF is obviously a much larger molecule than fibrinogen, it is possible that the steric constraints imposed by LJP5 on the microenvironment surrounding GPIIb/IIIa are less tight than those imposed by LJP9, so that only binding of the large vWF, but not fibrinogen, is affected by the former antibody. Both LJP5 and LJP9 interfered with the association of calcium with the membrane of unstimulated platelets, but only partially and to about the same extent. Therefore, changes in the Ca^{2+} associated with the GPIIb/IIIa complex are not likely to explain the different effect of the two antibodies on vWF and fibrinogen binding.

In conclusion, the present study demonstrates that the binding of at least two different adhesive glycoproteins to stimulated platelets can be independently modulated. This concept should prove important for unraveling the molecular bases of platelet interaction with specific ligands and for better understanding the mechanisms of platelet function.

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