Complex Formation of Platelet Membrane Glycoproteins IIb and IIIa with Fibrinogen

RALPH L. NACHMAN and LAWRENCE L. K. LEUNG, *Department of Medicine, Division of Hematology-Oncology, New York Hospital-Cornell Medical Center, New York 10021*

ABSTRACT We have recently reported the isolation of purified platelet membrane glycoproteins IIb and IIIa and the generation of monospecific antisera to these membrane proteins. Using these monospecific antisera in an enzyme-linked immunosorbent assay system, it is now demonstrated that glycoprotein IIb (GPIIb) and glycoprotein IIIa (GPIIIa) form a complex with purified human fibrinogen. The formation of this GPIIb-GPIIIa fibrinogen complex is calcium dependent, fibrinogen specific, saturable, and inhibited by specific amino sugars and amino acids. These observations suggest that the GPIIb-GPIIIa macromolecular complex on the platelet surface acts under the proper physiologic circumstances as the fibrinogen binding site required for normal platelet aggregation.

INTRODUCTION

Platelet membrane glycoprotein IIb (GPIIb)[1] and glycoprotein IIIa (GPIIIa) are major components of the platelet plasma membrane that may mediate platelet-platelet interactions (1, 2). GPIIb and GPIIIa are markedly diminished to absent in platelets in Glanzmann's thrombasthenia, a hereditary bleeding disorder characterized by defective platelet aggregation. An IgG alloantibody isolated from a polytransfused thrombasthenic patient interacts with GPIIb and GPIIIa (3) and induces a thrombasthenia-like state in normal platelets (4, 5). ADP, epinephrine, and thrombin induce fibrinogen binding to normal platelets, which correlates with platelet aggregability (6–9). Thrombasthenic platelets do not bind fibrinogen (7, 9). These observations suggest that GPIIb and/or GPIIIa are involved in platelet fibrinogen receptor function. We have recently reported the isolation of purified GPIIb and GPIIIa and the generation of monospecific antisera to these membrane proteins (10). In this report, we present evidence demonstrating complex formation of GPIIb and GPIIIa with purified human fibrinogen.

Received for publication 7 August 1981 and in revised form 19 October 1981.

[1] *Abbreviations used in this paper*: ELISA, enzyme-linked immunosorbent assay; GP, glycoproteins; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

METHODS

Materials. Lentil lectin (lens culinaris hemagglutinin), glucosamine, mannosamine, *N*-acetyl glucosamine, *N*-acetyl mannosamine, arginine, glycine, *p*-nitrophenyl phosphate, and type VII calf mucosa alkaline phosphatase were obtained from Sigma Chemical Co., St. Louis, Mo. Protein A was obtained from Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J. Microtitration plates and a Titertek Multiscan photometer were purchased from Flow Laboratories, Inc., Rockville, Md. All reagents were of analytical grade.

Protein purification. Chromatographically pure peak 1 human fibrinogen prepared by the method of Finlayson and Mosesson (11) (kindly supplied by Dr. M. Mosesson, Downstate Medical Center, New York) was free of factor XIII, plasminogen, factor VIII antigen (VIII:AGN), and fibronectin as determined by lack of reactivity using monospecific antisera to these potential contaminants in an enzyme-linked immunosorbent assay (ELISA). The clottability of this fibrinogen was ~96%. The protein migrated as a single band on 5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with an estimated M_r of 330,000. The reduced protein in SDS-PAGE revealed a typical distribution of Aα, Bβ, and γ chains with absence of degradation products. When chromatographed on a Sepharose CL-6B column (1.5 × 45 cm) in 0.25 M NaCl, 0.05 M Tris-HCl, pH 8.6, fibrinogen eluted as a single sharp peak indicating freedom from detectable quantities of fibrin monomers (6). Plasminogen isolated from plasma by lysine affinity chromatography (12) and gel filtration chromatography was kindly provided by Dr. Peter Harpel, Cornell University Medical College, New York. Factor VIII:AGN and fibronectin were isolated from human plasma as described (13, 14). Purified human albumin was obtained from Calbiochem-Behring Corp., American Hoechst, San Diego, Calif.

Platelet membrane GPIIb and GPIIIa were isolated and purified using lentil lectin affinity chromatography and electrophoretic elution from SDS-PAGE gels as described (10). Platelet GPIb was prepared as described (15). A crude preparation of platelet glycoprotein G or thrombospondin was prepared according to the method of Phillips et al. (16).

Washed platelets were incubated with thrombin in the presence of 1 mM EDTA without stirring. The platelets were removed by centrifugation at 8370 *g* for 3 min using a Beckman microfuge (Beckman Instruments, Inc., Fullerton, Calif.). The supernate was incubated with 10% by volume of insoluble antifibrinogen beads. After reduction on SDS-PAGE, this protein mixture contained major bands at M_r 185,000 (thrombospondin) and at M_r 68,000, probably representing GPV fragment (17). Platelet factor 4 and β-thromboglobulin were also detected at the lower M_r range.

Human erythrocyte membranes were prepared according to the method of Dodge et al. (18). The membrane proteins were solubilized with 1% sodium deoxycholate, and lentil lectin affinity chromatography was performed as described for platelet membrane proteins (10). Densitometric scans of Coomassie Blue-stained SDS-PAGE gels were carried out in a Gilford model 240 spectrophotometer equipped with a gel scanning attachment (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) and a Densicord recorder equipped with an integrator (Photovolt Corp., New York).

Antisera. Individual monospecific antisera have been raised in rabbits to the separate isolated GPIIb and GPIIIa (10). The antisera were absorbed with washed platelets from a patient with classical Glanzmann's thrombasthenia. By two-dimensional immunoelectrophoresis (19) anti-GPIIb reacted with a single antigen of apparent M_r 140,000 in a mixture of solubilized platelet membrane proteins. Anti-GPIIIa similarly reacted with a single antigen of apparent M_r 95,000 in a mixture of solubilized platelet membrane proteins. The antisera either singly or in combination failed to react with fibrinogen, albumin, fibronectin, plasminogen, Factor VIII-AGN, platelet GPIb or a crude thrombospondin mixture. For these studies an ELISA system was used in which the antigen tested was coated at concentrations of 1–6 μg/ml and probed with anti-GPIIb and/or anti-GPIIIa γ globulin solutions (1 mg/ml) in varying dilutions from 1:50 to 1:1,000. Antiplasminogen, antifibrinogen, and antialbumin sera were obtained from Calbiochem-Behring Corp. Anti-GPIb was prepared as described (20). Antierythrocyte membrane sera were obtained from Accurate Chemical and Scientific Corp., Hicksville, N. Y. γ-globulin fractions and Fab'_2 fragments of antisera were prepared as described (20). Antifibrinogen γ-globulin was coupled to CNBR-activated sepharose 4B beads as described (21).

ELISA. The performance of the assay was essentially as described by Voller et al. (22). Microtitration plates were coated with purified human fibrinogen. 0.2-ml portions of the fibrinogen in the bicarbonate coating buffer (0.1 M sodium carbonate, pH 9.6, 0.02% NaN_3) were incubated in a humid chamber overnight at 4°C. Optimum binding occurred at fibrinogen concentrations of 4–6 μg/ml. Contents of the microtitration plates were removed and the wells washed three times for 3 min each in Tris-Tween buffer (0.01 M Tris, 0.15 M NaCl, pH 7.4 with 0.5 mM $CaCl_2$, containing 0.05% Tween 20). The solutions containing the partially purified platelet GPIIb and GPIIIa (lentil lectin affinity column eluate) (10) were diluted in Tris-Tween buffer and added in duplicate to coated wells and the plates incubated overnight at 4°C in a humid chamber. The washing procedure was repeated and a mixture of anti-GPIIb and anti-GPIIIa γ-globulin (1 mg/ml), each diluted to final concentration of 1:100 in Tris-Tween buffer, was added for 24 h, 4°C. The washing procedure was repeated and the alkaline phosphatase-labeled protein A prepared as described below was added for 3-h incubation in a humid chamber at 4°C. The wells were emptied, the wash step repeated, and 0.2 ml of the substrate *p*-nitrophenyl phosphate (1 mg/ml in 10% diethanolamine buffer, pH 9.8) was added. The color development was followed at 5–15-min intervals by repeated readings at 405 nm in a Titertek Multiscan photometer. Color development with time was plotted and the best fit curve was calculated by linear regression analysis. The formation of the GPIIb-GPIIIa-fibrinogen complex was expressed as the enzymatic activity of the bound alkaline phosphatase ($\Delta A405\ min^{-1}$).

Alkaline phosphatase-labeled protein A. Protein A was labeled with alkaline phosphatase as described by Engvall (23). The labeled protein was stored at 4°C with 1% bovine serum albumin and 0.02% NaN_3 in Tris buffered saline, pH 7.4. The activity of the labeled protein A was assessed by coating the microtitration plates with fibrinogen (1–6 μg/ml) in coating buffer for 18 h in a humid chamber at 4°C. After washing three times for 3 min each in Tris-Tween, dilutions of fibrinogen antisera in Tris-Tween were added for 18 h. The washing step was repeated and Protein A-alkaline phosphatase (0.125 mg/ml) in various dilutions of Tris-Tween were added for 3 h. The wash step was again repeated and the substrate added. Maximum color developed using protein A-alkaline phosphatase at 1:1,000 dilution.

Determination of protein coating efficiency on microtitration plate. Purified human fibrinogen, fibronectin, plasminogen, VIII-AGN, and GPIIb-GPIIIa mixtures were labeled with I^{125} using the modified chloramine-T method (6). Microtitration plates were coated in duplicate with 0.2-ml portions of the labeled proteins at increasing concentrations in the bicarbonate coating buffer at 4°C overnight as described above. After washing three times with Tris-Tween buffer, each well was cut out, radioactivity of the coated protein counted, and the coating efficiency for each protein was determined. The saturating amount of proteins that could be coated per well were 0.7 pm (fibrinogen), 1.2 pm (GPIIb-GPIIIa, assuming M_r of 235,000), 3.4 pm (plasminogen), 1.2 pm (fibronectin), and 0.3 pm (VIII:AGN). Optimal coating concentration was 4–6 μg/ml for the various proteins. Thus the coupling efficiency for the proteins tested, i.e., fibrinogen, GPIIb-GPIIIa, plasminogen, fibronectin, and Factor VIII:AGN were comparable.

Binding of ^{125}I fibrinogen to platelets. The method of Peerschke et al. (9) was followed with minor modifications. Purified fibrinogen (see above) was labeled with ^{125}I using the modified chloramine-T method (6). Isolated human platelets were gel filtered on a Sepharose 2B column in divalent ion-free Tyrode's buffer at pH 7.35 containing 0.35% bovine serum albumin. Samples of gel-filtered platelets were mixed with labeled fibrinogen, $CaCl_2$ (1 mM final concentration), and buffer. Anti-GPIIb and/or anti-GPIIIa Fab'_2 were added at a final concentration of 0.5 mg/ml. The mixture was incubated at 37°C for 10 min. ADP (10 μM, final concentration) was then added. After an initial agitation to insure mixing, incubation was continued at 37°C for 10 min without stirring. The platelet suspensions were then layered on 0.5 ml of silicone oil in a 1.5-ml conical centrifuge tube. Free and platelet bound labeled fibrinogen was separated by centrifugation through the silicone oil mixture at 15,600 *g* for 5 min in an Eppendorf centrifuge. After centrifugation, the tip of the centrifuge tube containing the platelet pellet was sliced off and counted for ^{125}I. Each assay was done in duplicate. Nonspecific fibrinogen binding was measured by performing the binding assays in the absence of ADP stimulation. Specific binding was expressed as nanograms of fibrinogen bound per 10^8 platelets.

RESULTS

GPIIb-GPIIIa mixture. The platelet glycoproteins were isolated from solubilized platelet membranes

using lentil lectin affinity chromatography. The mixture contained primarily two major polypeptides of apparent M_r 140,000 and M_r 95,000 (unreduced). Gels scan analysis of Coomassie Blue-stained gels of the lentil lectin eluate showed that these major polypeptides constituted 87% of the protein mixture. These bands have been characterized as GPIIb and GPIIIa (10).

ELISA of the GPIIb-GPIIIa mixture. Monospecific anti-GPIIb and anti-GPIIIa reacted with the GPIIb-GPIIIa mixture in an ELISA assay. The glycoprotein mixture in increasing concentrations was passively adsorbed to the wells of the microtitration plate. Anti-GPIIb and anti-GPIIIa γ-globulin were added. The amount of antibody bound to the adsorbed glycoproteins was monitored by incubation with alkaline phosphatase-labeled protein A. The resulting hydrolysis of the substrate *p*-nitrophenyl phosphate was linear with the concentration of platelet glycoproteins added to the well up to 1 μg/ml.

Using ^{125}I-labeled platelet glycoproteins, the actual amount of GPIIb and GPIIIa bound to the plastic well was determined at each concentration of protein added, and this was correlated with color generated in the ELISA (Fig. 1). It should be noted that no differences in reactivity with specific antibody were noted when nonlabeled glycoproteins were compared to ^{125}I-labeled glycoproteins.

Complex formation of GPIIb-GPIIIa with fibrinogen. GPIIb and GPIIIa attached and formed a complex with the purified fibrinogen passively adsorbed to the wells of the plastic microtitration plate. The formation of the GPIIb-GPIIIa complex with adsorbed fibrinogen was dependent on the presence of Ca^{2+}. In the absence of added Ca^{2+}, no significant complex formation was demonstrated using a mixture of anti-GPIIb and anti-GPIIIa γ-globulin (Table I). Maximum complex formation was demonstrated in the presence of 0.5 mM Ca^{2+}. A mixture of anti-GPIIb and anti-GPIIIa γ-globulin appeared to detect a greater degree of membrane glycoprotein complex formation with fibrinogen than the sum detectable by using each antiserum alone at an identical concentration.

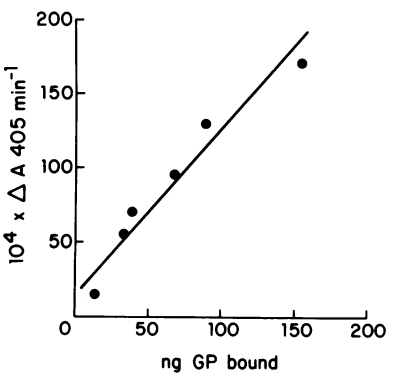

FIGURE 1 Correlation of the amount of GPIIb-GPIIIa bound and the color generated in the ELISA. The radiolabeled glycoprotein mixture varying from 0.5 to 12 μg/ml in coating buffer was applied to the plastic wells for 18 h at 4°C. After washing, a mixture of anti-GPIIb and anti-GPIIIa γ-globulin (10 μg/ml each) was added for 24 h at 4°C. After washing, alkaline phosphatase-labeled protein A (0.125 μg/ml) was added for 3 h at room temperature. After washing the substrate *p*-nitrophenyl phosphate was added and color development followed in a Titertek Multiscan photometer. The reaction was expressed as the enzymatic activity of the bound alkaline phosphatase (10^4 × ΔA405 min^{-1}). The wells were then washed, cut out, and counted and the amount of protein in each well determined. Y=1.116x + 13.73, R=0.97.

Specificity and stoichiometry of GPIIb-GPIIIa complex formation with fibrinogen. Complex formation of GPIIb-GPIIIa with adsorbed fibrinogen was determined in the presence of excess fluid phase fibrinogen. Following the coating of fibrinogen (3 μg/ml) on the microtitration plate, the platelet glycoprotein mixture (2.4 μg/ml) was incubated with Tris-Tween buffer with 0.5 mM Ca^{2+} in the presence of 6 μg/ml fibrinogen. The amount of GPIIb and GPIIIa that was detectable as a complex with the adsorbed fibrinogen was decreased practically to the control level (Table II). The small amount of GPIIb and GPIIIa that complexed to adsorbed fibrinogen in the presence of soluble fibrinogen was considered "nonspecific" binding. No inhibition of GPIIb-GPIIIa complex formation with fibrinogen was detected when the experiments were repeated in the presence of comparable amounts of fluid phase purified human albumin.

Saturation of the binding or complexing of the glycoproteins to the fibrinogen was determined by plotting the specific binding (total minus nonspecific) of incremental amounts of GPIIb and GPIIIa to a fixed amount of adsorbed fibrinogen (Fig. 2). Saturation was achieved at a GPIIb-GPIIIa concentration of 2.4 μg/ml. The color generated in the ELISA by this amount of bound GPIIb-GPIIIa (35 × 10^{-4} ΔA405 min^{-1}) cor-

TABLE I
ELISA Detection of GPIIb-GPIIIa-Fibrinogen Complexes

Antiserum	10^4 × ΔA405 min^{-1} Calcium	
	0	*0.5 mM*
Anti-IIb-anti-IIIa	11.2±1.5	106.5±3.0
Anti-IIb	12.8±1.8	36.3±2.5
Anti-IIIa	12.9±0.5	42.6±2.1
Antialbumin	7.7±0.65	10.1±1.2

Fibrinogen (6 μg/ml) in coating buffer was applied to the plastic wells for 18 h, 4°C. After washing, the glycoprotein mixture (2.88 μg/ml) in Tris-Tween with or without added Ca^{2+} was added for 24 h at 4°C. The remaining steps were carried out as described in Fig. 1. Control studies performed with antialbumin substituing for anti-GPIIb and anti-GPIIIa.

TABLE II
Inhibition of Complex Formation by Fluid Phase Fibrinogen

Mixture*	$10^4 \times \Delta A405\ min^{-1}$
GPIIb-GPIIIa	52.1±1.1
GPIIb-GPIIIa plus fluid phase fibrinogen	10.8±1.4
GPIIb-GPIIIa plus fluid phase albumin	51.8±1.6

* Fibrinogen (3 μg/ml) in coating buffer was applied to the plastic wells for 18 h, 4°C. After washing, the GPIIb-GPIIIa mixture (2.4 μg/ml) in Tris-Tween Ca^{2+} buffer was added alone or in the presence of fibrinogen (6 μg/ml) or albumin (12 μg/ml). The remaining steps were carried out as described in Fig. 1.

TABLE III
Interaction of GPIIb-GPIIIa Complexes With Other Proteins

Protein coat	$10^4 \times \Delta A405\ min^{-1}$
None	5.1±.88
Fibronectin	6.6±1.1
Plasminogen	8.1±2.0
VIII:AGN	7.9±1.2
Fibrinogen	116.0±1.4

The proteins (6 μg/ml) in coating buffer were applied to the plastic wells for 18 h, 4°C. After washing, the GPIIb-GPIIIa mixture (2.88 μg/ml) in Tris-Tween calcium buffer was added for 24 h, 4°C. The remaining steps were carried out as described in Fig. 1.

responded to 19 ng GPIIb-GPIIIa bound (Fig. 1). Using radiolabeled fibrinogen, the coating efficiency of fibrinogen at 3 μg/ml was 35.8%, corresponding to 215 ng bound to the well. Assuming a M_r of 235,000 for a 1:1 GPIIb-GPIIIa complex and M_r of 330,000 for fibrinogen, the data suggest a stoichiometric relationship of fibrinogen to GPIIb-GPIIIa of 8:1.

The specificity of the GPIIb-GPIIIa interaction with fibrinogen was further studied by incubating GPIIb-GPIIIa mixtures with adsorbed fibronectin, plasminogen, or factor VIII:AGN. The coating efficiency for these proteins to the microtitration plate was determined using ^{125}I-labeled proteins and was comparable to that of fibrinogen. No evidence of complex formation was detected (Table III). Platelet membrane GPIb, purified human albumin, and a mixture of human erythrocyte membrane glycoproteins (lentil lectin affinity column eluate) also did not show any complex formation with adsorbed fibrinogen, using monospecific antisera to these proteins in an ELISA assay (Table IV).

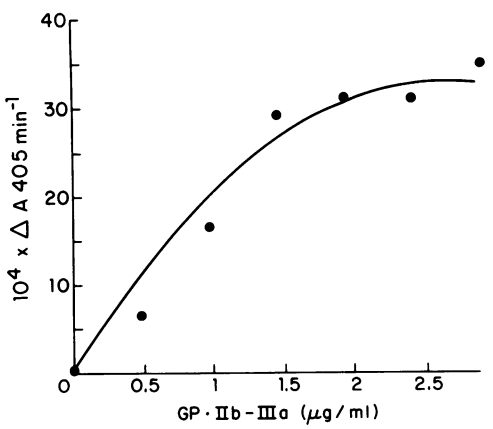

FIGURE 2 Saturation of GPIIb-GPIIIa complex formation with adsorbed fibrinogen as measured by the ELISA. Fibrinogen (3 μg/ml) in coating buffer was applied to the plastic wells for 18 h, 4°C. After washing varying amounts of the GPIIb-GPIIIa mixture in Tris-Tween Ca^{2+} buffer with or without fluid phase fibrinogen (6 μg/ml) was added for 24 h at 4°C. The remaining steps were carried out as described in Fig. 1. The extent of specific GPIIb-GPIIIa complex formation with adsorbed fibrinogen (total minus nonspecific) was plotted as a function of increasing GPIIb-GPIIIa concentration.

Effect of amino sugars and amino acids on GPIIb-GPIIIa complex formation with fibrinogen. Previous studies have suggested that specific amino sugars such as glucosamine, mannosamine, and amino acids such as arginine block platelet lectin activity and may interfere with platelet fibrinogen binding (24, 25). Glucosamine, mannosamine, and arginine significantly inhibited GPIIb-GPIIIa complex formation with fibrinogen (Table V). In contrast, *N*-acetyl glucosamine, *N*-acetyl mannosamine, and glycine at similar concentrations had no effect.

Lack of complex formation using purified GPIIb-GPIIIa. It has previously been demonstrated that the eluate from the lentil lectin affinity chromatgraphy of solubilized platelet membrane proteins contains other minor proteins in addition to GPIIb and GPIIIa (26). These include GPIb and thrombospondin. Antisera raised to the purified GPIIb and GPIIIa isolated from SDS-PAGE gels appeared to be monospecific and did not react with thrombospondin or GPIb. The SDS-PAGE gel isolated platelet GPIIb and GPIIIa were

TABLE IV
Interaction of Other Proteins With Adsorbed Fibrinogen

Fluid phase protein	$10^4 \times \Delta A405\ min^{-1}$
GPIb	5.1±.74
Albumin	2.0±0.4
RBC-glycoprotein	4.0±0.7

Fibrinogen (6 μg/ml) in coating buffer was applied to the plastic wells for 18 h, 4°C. After washing the proteins (12 μg/ml) in Tris-Tween calcium buffer were added for 24 h, 4°C. The remaining steps were carried out as described in Fig. 1. The antisera to the individual proteins were used at 1:100 dilution.

TABLE V

Effects of Aminosugars and Amino Acids on GPIIb-GPIIIa-Fibrinogen Complex Formation

GPIIb-GPIIIa mixture	Complex formation
plus	%
Buffer	100
Glucosamine	37.1
Mannosamine	40
Arginine	30.2
N-acetyl glucosamine	112
N-acetyl mannosamine	82
glucose	129
glycine	86

Fibrinogen (6 μg/ml) in coating buffer was applied to the plastic wells for 18 h, 4°C. After washing, the GPIIb-GPIIIa mixture (2.88 μg/ml) in Tris-Tween Ca^{2+} buffer containing the above was added for 24 h, 4°C. The aminosugars and amino acids were used at final concentration of 120 mM except for mannosamine and *N*-acetyl mannosamine, which were used at 30 mM. The pH of the glycoprotein mixture in all cases was adjusted to 7.4 with HCl. The remaining steps were carried out as described in Fig. 1. The extent of complex formation was expressed as the enzymatic activity of bound alkaline phosphatase ($\Delta A405\ min^{-1}$). Control was considered 100% complex formation.

studied for complex formation with adsorbed fibrinogen (Table VI). The isolated partially denatured purified GPIIb and GPIIIa did not form complexes in the presence or absence of calcium.

The effect of anti-GPIIb and anti-GPIIIa Fab'$_2$ fragments on platelet fibrinogen binding. Incubation of washed human platelets with anti-GPIIb Fab'$_2$ fragments alone, anti-GPIIIa Fab'$_2$ fragments alone, or a mixture of both failed to block ^{125}I-fibrinogen binding (Table VII). Previous studies have shown that both anti-GPIIb and anti-GPIIIa Fab'$_2$ fragments caused platelet agglutination, indicating that they were directed against platelet surface components

TABLE VI

Complex Formation of Isolated Purified GPIIb and GPIIIa with Fibrinogen

Glycoprotein	$10^4 \times \Delta A405\ min^{-1}$
GPIIb-GPIIIa, purified	7.1±0.65
GPIIb-GPIIIa mixture, eluate of affinity column	112.3±2.6
Control	8.1±1.4

Fibrinogen (6 μg/ml) in coating buffer was applied to the plastic wells for 18 h, 4°C. After washing, the isolated purified GPIIb and GPIIIa (1.44 μg/ml each) or the GPIIb-GPIIIa mixture (2.88 μg/ml) was added for 24 h at 4°C. The remaining steps were carried out as described in Fig. 1. Control included purified human albumin (12 μg/ml).

TABLE VII

Effects of Anti-GPIIb and Anti-GPIIIa Fab'$_2$ on the Binding of [I^{125}]Fibrinogen to Platelets

Stimulant	Fibrinogen specifically bound (ng/10^8 platelets)
mg/ml	
ADP	405
ADP + preimmune Fab'$_2$, 0.5	369
ADP + anti-GPIIb Fab'$_2$, 0.5	668
ADP + anti-GPIIIa Fab'$_2$, 0.5	400
ADP + anti-GPIIb Fab'$_2$, 0.25 anti-GPIIIa Fab'$_2$, 0.25	645

Binding assays were performed at fibrinogen concentrations of 300 μg/ml. ADP was used in final concentration of 10 μM. Representative experiment of two similar studies. Each value represents the average of duplicate assays.

(10). The reason for the increased binding detected in the presence of anti-GPIIb Fab'$_2$ was not determined.

DISCUSSION

In this study we have demonstrated that a mixture of undenatured platelet membrane glycoproteins eluted from a lentil lectin affinity column forms a complex with human fibrinogen. Using monospecific antisera to isolated purified GPIIb and GPIIIa (10), we have demonstrated that platelet GPIIb and GPIIIa in the membrane glycoprotein mixture are the specific molecular components that complex with fibrinogen. The formation of the GPIIb-GPIIIa fibrinogen complex is calcium dependent, fibrinogen specific, saturable, and inhibited by specific amino sugars and amino acids. These observations suggest that GPIIb and GPIIIa, which probably exist in the platelet membrane as a macromolecular complex (3, 28, 29), act under the proper physiologic circumstances as the fibrinogen binding site required for normal platelet aggregation. It is not clear from our studies whether GPIIb alone, GPIIIa alone, or the GPIIb-GPIIIa complex bears the actual binding site. The ELISA studies demonstrating complex formation were possible only using undenatured mixtures of the membrane glycoproteins. When GPIIb and GPIIIa were individually isolated by electrophoretic elution from SDS-PAGE gels, no complex formation was demonstrable (Table VI). It is thus highly probable that the binding site(s) of the membrane glycoproteins was denatured in the process of purification. The premise is further supported by the fact that the monospecific antisera to GPIIb and GPIIIa neither inhibited platelet aggregation to physiologic stimuli (10) nor blocked fibrinogen binding by ADP stimulated platelets (Table VII). The possibility should be considered that the binding of GPIIb-GPIIIa

to fibrinogen is facilitated by an additional component or cofactor that is present in the lentil lectin eluate but is absent in the mixture of purified proteins. Kornecki et al. (27) have described a high affinity fibrinogen receptor present on normal as well as thrombasthenic platelets that is exposed by chymotrypsin. It is not known how this latter activity is related to the GPIIb-GPIIIa system.

Interestingly, a recently described monoclonal antibody to the platelet membrane GPIIb-GPIIIa complex also did not interfere with ADP or thrombin-induced platelet aggregation (28). It is probable that these antibodies do not provide enough steric hindrance to indirectly block fibrinogen binding.

The stoichiometry of the fibrinogen GPIIb-GPIIIa interaction on the plastic microtitration plates suggests that one GPIIb-GPIIIa complex was bound per eight fibrinogen molecules—assuming a monomeric 1:1 relationship between GPIIb and GPIIIa. This stoichiometric relationship must be interpreted with some caution before extrapolating to the real biologic event. Thus these studies use a plastic surface with coated fibrinogen molecules. It is most probable that the adsorbed fibrinogen molecules are oriented in a highly random manner with significant steric restriction. Thus only a fraction of the bound fibrinogen may be functionally available for binding of the soluble GPIIb-GPIIIa complex. These considerations make it probable that the 8:1 stoichiometry is an overestimation. A more biologically relevant experimental surface such as a liposome might be more appropriate for defining the true stoichiometry of this complex formation.

Presently available information from several different groups suggest that there is a single class of fibrinogen binding sites (6–8) estimated to be ~40,000 per platelet. This estimation nicely fits the monoclonal antibody studies that identified ~40,000 GPIIb-GPIIIa complexes per platelets (28). Other groups have demonstrated fewer receptors with evidence of two classes of binding sites with different affinities (9, 30). Further studies are required to determine whether additional platelet membrane constituents other than GPIIb and GPIIIa bind to fibrinogen after physiologic stimulation.

ACKNOWLEDGMENTS

We thank Dr. M. Mosesson for the supply of purified Peak I human fibrinogen and Drs. Marjorie Zucker, Margaret Johnson, and Harvey Weiss for the supply of thrombasthenic platelets. The invaluable technical assistance of Barbara Ferris is gratefully acknowledged.

This work was supported by grant HL 18828 (Specialized Center of Research in Thrombosis) from the National Institutes of Health. Dr. Leung is a recipient of a Clinical Investigator award (KO8 HL00877-01) from the National Heart, Lung, and Blood Institute. Additional support was provided by the Arnold Krakower Foundation.

REFERENCES

1. Phillips, D. R., L. K. Jennings, and H. H. Edwards. 1980. Identification of membrane proteins mediating the interaction of human platelets. *J. Cell Biol.* **86:** 77–86.
2. Nurden, A. T., and J. P. Caen. 1979. The different glycoprotein abnormalities in thrombasthenic and Bernard-Soulier platelets. *Semin. Hematol.* **16:** 234–250.
3. Hagen, I., A. Nurden, O. J. Bjerrum, N. O. Solum, and J. Caen. 1980. Immunochemical evidence for protein abnormalities in platelets from patients with Glanzmann's thrombasthenia and Bernard-Soulier syndrome. *J. Clin. Invest.* **65:** 722–731.
4. Degos, L., A. Dautigny, J. C. Brouet, M. Colombani, N. Ardaillou, J. Caen, and J. Colombani. 1975. A molecular deficit in thrombasthenic platelets. *J. Clin. Invest.* **56:** 235–240.
5. Levy-Toledano, S., G. Tobelem, C. Legrand, R. Bredoux, L. Degos, A. Nurden, and J. P. Caen. 1978. Acquired IgG antibody occurring in a polytransfused thrombasthenic patient: its effect on human platelet function. *Blood.* **51:** 1065–1071.
6. Marguerie, G. A., E. F. Plow, and T. S. Edgington. 1979. Human platelets possess an inducible and saturable receptor specific for fibrinogen. *J. Biol. Chem.* **254:** 5357–5363.
7. Bennett, J. S., and G. Vilaire. 1979. Exposure of platelet fibrinogen receptors by ADP and epinephrine. *J. Clin. Invest.* **64:** 1393–1401.
8. Plow, E. F., and G. A. Marguerie. 1980. Participation of ADP in the binding of fibrinogen to thrombin-stimulated platelets. *Blood.* **56:** 553–555.
9. Peerschke, E. I., M. B. Zucker, R. A. Grant, J. J. Egan, and M. M. Johnson. 1980. Correlation between fibrinogen binding to human platelets and platelet aggregability. *Blood.* **55:** 841–847.
10. Leung, L. L. K., T. Kinoshita, and R. L. Nachman. 1981. Isolation, purification and partial characterization of platelet membrane glycoproteins IIb and IIIa. *J. Biol. Chem.* **256:** 1994–1997.
11. Finlayson, J. S., and M. W. Mosesson. 1963. Heterogeneity of human fibrinogen. *Biochemistry.* **2:** 42–46.
12. Deutsch, D. G., and E. T. Mertz. 1970. Plasminogen: purification from human plasma by affinity chromatography. *Science (Wash., D. C.).* **170:** 1095–1096.
13. Nachman, R. L., and E. A. Jaffe. 1975. Subcellular platelet factor VIII antigen and von Willebrand's factor. *J. Exp. Med.* **141:** 1101–1113.
14. Mosesson, M. W., and R. A. Umfleet. 1970. The cold-insoluble globulin of human plasma. *J. Biol. Chem.* **245:** 5728–5736.
15. Nachman, R. L., T. Kinoshita, and B. Ferris. 1979. Structural analysis of human platelet membrane glycoprotein I complex. *Proc. Natl. Acad. Sci. U.S.A.* **76:** 2952–2954.
16. Phillips, D. R., L. K. Jennings, and H. R. Prasanna. 1980. Ca^{2+}-mediated association of glycoprotein G (Thrombin-sensitive protein, Thrombospondin) with human platelets. *J. Biol. Chem.* **255:** 11629–11632.
17. Berndt, M. C., and D. R. Phillips. 1981. Purification and preliminary physicochemical characterization of human platelet membrane glycoprotein V. *J. Biol. Chem.* **256:** 59–65.
18. Dodge, J. T., C. Mitchell, and D. J. Hanahan. 1963. The preparation and chemical characterization of hemoglo-

bin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* **100:** 119–130.
19. Converse, C. A., and D. S. Papermaster. 1975. Membrane protein analysis by two-dimensional immunoelectrophoresis. *Science (Wash., D. C.).* **189:** 469–472.
20. Nachman, R. L., E. A. Jaffe, and B. B. Weksler. 1977. Immunoinhibition of ristocetin induced platelet aggregation. *J. Clin. Invest.* **59:** 143–148.
21. Cuatrecasas, P. 1970. Protein purification by affinity chromatography. *J. Biol. Chem.* **245:** 3059–3065.
22. Voller, A., D. Bidwell, and A. Bartlett. 1976. Microplate enzyme immunoassays for the immunodiagnosis of virus infections. *In* Manual of Clinical Immunology. N. R. Rose and H. Friedman, editors. American Association for Microbiology, Washington, D. C. 506–512.
23. Engvall, E. 1978. Preparation of enzyme-labeled Staphylococcal protein A and its use for detection of antibodies. *Scan. J. Immunol.* 8(Suppl. 7): 25–31.
24. Gartner, T. K., D. C. Williams, F. C. Minion, and D. R. Phillips. 1978. Thrombin-induced platelet aggregation is mediated by a platelet plasma membrane-bound lectin. *Science (Wash., D. C.).* **200:** 1281–1283.
25. Kinlough-Rathbone, R. L., A. Chahil, D. W. Perry, M. A. Packham, and J. F. Mustard. 1979. Effect of amino sugars that block platelet lectin activity on fibrinogen binding to washed rabbit or human platelets. *Blood.* **54**(Suppl. 1): 249a.
26. McGregor, J. L., K. J. Clemetson, E. James, T. Greenland, E. F. Lüscher, and M. W. Dechavanne. 1980. Studies on platelet glycoproteins in Glanzmann's thrombasthenia using ^{125}I-labeled lectins. *Br. J. Haematol.* **46:** 99–107.
27. Kornecki, E., S. Niewiarowski, T. A. Morinelli, and M. Kloczewiak. 1981. Effects of chymotrypsin and adenosine diphosphate on the exposure of fibrinogen receptors on normal human and Glanzmann's thrombasthenic platelets. *J. Biol. Chem.* **256:** 5696–5701.
28. McEver, R. P., N. L. Baenziger, and P. W. Majerus. 1980. Isolation and quantitation of the platelet membrane glycoprotein deficient in thrombasthenia using a monoclonal hybridoma antibody. *J. Clin. Invest.* **66:** 1311–1318.
29. Polley, M. J., L. L. K. Leung, F. Y. Clark, and R. L. Nachman. 1981. Thrombin induced platelet membrane glycoprotein IIb and IIIa complex formation. An electron microscope study. *J. Exp. Med.* **154:** 1058–1068.
30. Niewiarowski, S., A. Z. Budzynski, T. A. Morinelli, T. M. Brudzynski, and G. J. Stewart. 1981. Exposure of fibrinogen receptor on human platelets by proteolytic enzymes. *J. Biol. Chem.* **256:** 917–925.