Retention of the Glycoprotein IIb-IIIa Complex in the Isolated Platelet Cytoskeleton

Effects of Separable Assembly of Platelet Pseudopodal and Contractile Cytoskeletons

M. Elyse Wheeler, A. Chadwick Cox, and Roger C. Carroll
Department of Physiology and Biophysics, and Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center and Thrombosis/Hematology Research Laboratory, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104

Abstract. To investigate the association of the putative platelet fibrinogen receptor (glycoprotein IIb-IIIa) with the cytoskeleton, ^{125}I-surface labeled human platelets washed by gel-filtration were activated under conditions which allow selective assembly of the platelet cytoskeleton. The four conditions were activation with arachidonate or phorbol 12-myristate 13-acetate (PMA) with and without pretreatment with cytochalasin E. Activation with arachidonate generates a complete cytoskeletal core (pseudopodal and contractile elements) while PMA activation forms only an actin plus actin-binding protein pseudopodal core. Pretreatment with cytochalasin E leads to actomyosin contractile core formation if arachidonate activated, and essentially blocks cytoskeletal development if PMA activated. Cytoskeletal cores from arachidonate or PMA-activated platelets retained 26 (±3%) of the total ^{125}I-IIIa. Pretreatment with cytochalasin E followed by arachidonate or PMA activation reduced the ^{125}I-IIIa retention to near control levels (unactivated platelets: 4±2%). The role of aggregation vs. receptor occupancy in the retention of IIb-IIIa was assessed by activation of platelets with arachidonate in the presence of fibrinogen fragment D (0.6–12 mg/ml). Aggregation was blocked by increasing concentrations of fragment D reagent while cytoskeletal assembly was not altered. The IIIa retention correlated with extent of aggregation with maximal retention corresponding to full aggregation. To determine if cytoskeletal development is necessary for the expression of the fibrinogen binding site, binding studies were performed with unlabeled platelets and ^{125}I-fibrinogen. The mean number of binding sites and the mean dissociation constant were not significantly different among the four activation conditions. Although the development of a platelet cytoskeletal core is not required for the expression of the fibrinogen binding site, the retention of the glycoprotein IIb-IIIa complex is dependent on fibrinogen-supported aggregation as well as the formation of the pseudopodal cytoskeleton.

Introduction

Stimulation of platelets with ADP, arachidonic acid (AA), collagen, or thrombin exposes receptors specific for fibrinogen (1–6). The molecular interactions in the platelet surface membrane which allow expression of the fibrinogen binding site following activation are currently unknown. Several lines of evidence identify the glycoprotein complex IIb-IIIa found in the platelet surface membrane as the fibrinogen receptor (4, 7–11).

In addition to requiring calcium ions for association (12–15), the formation of the glycoprotein IIb-IIIa complex may require changes in the membrane which would create a new spatial arrangement, resulting in expression of the fibrinogen binding activity. Glycoproteins IIb and IIIa are normally exposed on the membrane surface of unstimulated platelets (16); yet, unstimulated platelets do not bind fibrinogen (1, 17). Recently, monoclonal antibodies have been isolated that rec-

Address reprint requests to Dr. Wheeler, Thrombosis/Hematology Research Laboratory, Oklahoma Medical Research Foundation, 825 Northeast Thirteenth Street, Oklahoma City, OK 73104.

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1. *Abbreviations used in this paper:* AA, arachidonic acid; CE, cytochalasin E; HBS, Hepes-buffered saline; PAGE, polyacrylamide gel electrophoresis; PMA, phorbol 12-myristate 13-acetate.

ognize the glycoprotein IIb-IIIa complex but do not bind to either individual glycoprotein (18, 19). Though unstimulated platelets do not bind fibrinogen, surface binding of these antibodies was identical in unstimulated and stimulated platelets. These findings suggest that, in the membrane of the unstimulated platelet, the glycoproteins IIb and IIIa may exist as a complex and that some additional activation-dependent alteration in the platelet membrane may be necessary for fibrinogen binding.

Glycoproteins IIb and IIIa have been suggested to be transmembranous proteins (20), which would allow for their interaction with intracellular proteins. Phillips et al. (21) have demonstrated that the glycoprotein IIb-IIIa complex is selectively retained in the detergent insoluble cytoskeletal core of thrombin-aggregated platelets. The detergent-insoluble cytoskeletal core consists primarily of actin, myosin, α-actinin, and actin-binding protein. Retention of the glycoprotein IIb-IIIa complex suggests that the complex may have a specific interaction with the contractile proteins. Similar membrane receptor-cytoskeleton interactions have been suggested in other cell types. Capping of membrane proteins is observed in round cells treated with membrane protein-specific antibodies. The antibody-induced clusters of membrane proteins appear to have a transmembranous association with actin, myosin, and α-actinin on the cytosolic side of the plasma membrane (22).

Association between the glycoprotein IIb-IIIa complex and the platelet cytoskeletal proteins is of interest, particularly in light of the suggestion that an active rearrangement of the membrane components is necessary for fibrinogen binding. Activation conditions have been developed which allow assembly of the pseudopodal cytoskeleton composed of actin, α-actinin, and actin-binding protein independent from the formation of the actomyosin microfilaments responsible for secretory granule centralization (23). In the present study, we examined the role of the cytoskeletal development and of platelet aggregation in the retention of the glycoprotein IIb-IIIa complex. The expression of the fibrinogen binding sites on the surface of the intact platelet under these conditions as well as under conditions preventing cytoskeletal assembly was also assessed.

Methods

Materials. Lyophilized human fibrinogen and plasmin were purchased from Kabi AM, Helena Laboratories, Beaumont, TX. ^{125}I Bolton-Hunter reagent and carrier-free Na^{125}I were obtained from Amersham, Radiochemica Center, Amersham, England. Sigma Chemical Co., St. Louis, MO, supplied the gelatin-agarose beads, bovine serum albumin (essentially globulin free), Triton X-100, glucose oxidase, Sepharose 2B, Coomassie Brilliant Blue R, cytochalasin E (CE), prostaglandin E_1 (PGE_1), 5'adenosine monophosphate, and protein A-sepharose CL-4B. Phorbol 12-myristate 13-acetate (PMA) was purchased from P-L Biochemicals, Inc., Milwaukee, WI. Arachidonic acid (AA) was supplied by Nu-Check-Prep, Inc., Elysian, MN. Mouse monoclonal antibody to human platelets I and ascites control were obtained from Cappel Laboratories, Westchester, PA. Bovine thrombin was a generous gift from Dr. Charles T. Esmon. Lyophilized lactoperoxidase was purchased from Calbiochem-Behring Corp., La Jolla, CA. Electrophoresis reagents were supplied by BDH Chemicals Ltd., Poole, England. High molecular weight standard protein mixture was purchased from Bio-Rad Laboratories, Richmond, CA.

Preparation of gel-filtered platelets. After obtaining informed consent, blood was drawn by standard venipuncture techniques from normal drug-free human volunteers. Whole blood was anticoagulated with 0.1 vol of 100 mM citrate acid and 130 mM glucose (pH 6.5) buffer. Platelet rich plasma was separated by centrifugation of the blood at 1000 *g* for 3 min at room temperature. Platelets for binding studies were prepared as previously described (23) with the exception that the Tangen-Hepes buffer contained 0.35% bovine serum albumin. Platelets for surface iodination were resuspended after pelleting from PRP in Tangen-Hepes buffer without bovine serum albumin but with addition of 1 μg/ml PGE_1. After gel-filtration on Sepharose 2B, the final platelet concentration was adjusted to 5×10^8 platelets/ml and warmed to 37°C prior to experimentation.

Platelet aggregometry. Aggregation studies were carried out with constant stirring in a Payton aggregometer (Payton Associates Inc., Buffalo, NY) maintained at 37°C. Aliquots of 0.5 ml were activated by the addition of 30 μM arachidonate or 10 μM PMA with and without pretreatment with 10 μg/ml CE.

Surface membrane protein labeling. Platelet surface membrane proteins were radiolabeled with ^{125}I by lactoperoxidase-catalyzed iodination. Prior to gel filtration, 0.5 mCi/ml Na^{125}I (carrier-free) and 0.02 mg/ml lactoperoxidase were added to the platelet suspension. The reaction was initiated with 0.1 U/ml glucose oxidase and the mixture was incubated at 37°C for 30 min.

Preparation of cytoskeletal cores. Platelet aliquots were activated with AA (30 μM) or PMA (10 μM) with and without pretreatment for 5–10 min with CE (5 μg/ml). Samples were stirred at 37°C to give full aggregation, at which point an equal volume of cold, twofold concentrated Triton extraction buffer (2% Triton X-100, 100 mM Tris HCl, 10 mM EGTA, 2 mM 2-mercaptoethanol, pH 7.4) was added (23). All samples were thoroughly mixed by vortexing and centrifuged at 10,000 *g* for 5 min at 4°C. The supernatant and the insoluble pellet, designated as the cytoskeletal core, were separated. Cores were resuspended by vortexing in 2 vol of one times concentrated extraction buffer and centrifuged. The supernatant was removed by aspiration.

Plasma membrane determinations. A 1-ml aliquot of gel-filtered platelets in Tangen-Hepes buffer containing 100 μM leupeptin was sonicated for 15 s at 0°C in a Branson sonifier model 350 (Branson Sonic Power Co., Danbury, CT) with a cup horn attachment. The lysed platelet membranes were isolated by ultracentrifugation at 200,000 *g* for 45 min at 0°C in a Beckman L550 ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA). Membrane preparations and cytoskeletal cores for determination of 5'nucleotidase activity were resuspended by sonication as above in 0.1 ml of 0.6 M NaCl buffered with 20 mM Hepes, pH 7.4. The dispersed samples were incubated with 0.9 ml substrate solution (5.5 mM $MgCl_2$, 55 mM Tris HCl, 11 mM AMP, pH 8.5) (24) at 37° for 1 or 2 h. The reaction was stopped and protein precipitated by the addition of cold 10% TCA. The protein-free supernatant was assayed for total inorganic phosphate. Total platelet lipid and cytoskeletal core content of lipid was determined by extraction of samples into chloroform:methanol (2:1). The chloroform fraction was transferred to acid-washed glass tubes and dried under nitrogen. The samples were washed and total phosphate determined as described by Chen et al. (25).

Immunoprecipitation of GpIIb-IIIa. Mouse monoclonal anti-human platelet I (final concentration, 12 μg/ml), which is directed against the

glycoprotein IIb-IIIa complex, was added to 0.5-ml aliquots of surface-iodinated unactivated platelets and incubated at 37°C for 5 min. An equal volume of cold Triton extraction buffer was added and the sample centrifuged at 10,000 *g* for 5 min at 4°C. Protein A-Sepharose (100 μl) was added to the supernatant and incubated at room temperature for 15 min. The protein A-Sepharose beads were pelleted by centrifugation as above and the supernatant removed. The beads were washed three times in cold Triton extraction buffer and following the final centrifugation, resuspended in 100 μl three times concentrated denaturation solution in preparation for gel electrophoresis.

SDS-polyacrylamide gel electrophoresis (PAGE). Cytoskeletal core pellets from 0.5 ml of platelets (5×10^8/ml) were denatured with 0.5 ml of threefold concentrated denaturing solution consisting of 6% SDS, 6% 2-mercaptoethanol, 30% glycerol, 2 mM EDTA, 12 mM EGTA, 0.03% bromophenol blue, and 450 mM Tris-HCl (pH 6.8). An 0.4-ml aliquot of each supernatant sample was treated with 0.1 ml of a fivefold concentrated denaturing buffer. A total platelet sample was prepared by mixing 0.2-ml aliquot of the platelet suspension with 0.1 ml of the threefold conentrated denaturing solution. All samples were incubated at 100°C for 3 min and electrophoresed on 6–17.5% polyacrylamide gradient SDS slab gels prepared as described by Studier (26). The gels were stained and fixed overnight at 37°C with 0.05% Coomassie blue in 25% isopropanol, and 10% acetic acid. Destaining procedure consisted of four changes of a 10% methanol, 10% acetic acid solution at 37°C. Gels were photographed, vacuum dried, and subjected to autoradiography for 14–21 d at −70°C using Kodak BB-5 Blue Brand X-ray film (Eastman Kodak Co., Rochester, NY). A Bio-Rad high molecular weight standard (Bio-Rad Laboratories) consisting of myosin, β-galactosidase, phosphorylase A, bovine serum albumin, and ovalbumin was included on each gel. Laser densitometric scanning of undried gels and autoradiograms was done with an LKB 2202 Ultroscan densitometer interfaced to a LKB 2220 recording integrator (LKB Instruments, Inc., Gaithersburg, MD).

Preparation of fibrinogen. Lyophilized human fibrinogen (Kabi grade L) was allowed to hydrate in Hepes-buffered saline (HBS) (20 mM Hepes, 0.9% sodium chloride, pH 7.4) and 10 mM benzamidine at 4°C. Then, the solution was extensively dialyzed against HBS. Commercially prepared fibrinogen frequently contains fibronectin which was removed from the rehydrated Kabi human fibrinogen by adsorption onto gelatin-agarose. The fibrinogen-fibronectin solution was batch-adsorbed for 1 h at 22°C with an equal volume of washed packed gelatin-agarose beads. The slurry was transferred to a 1 × 30-cm column and the fractions containing fibrinogen were collected. Peak fibrinogen fractions were pooled at a concentration of 19.9 to 23.0 mg/ml. When incubated with thrombin, the purified fibrinogen was >93% clottable and had a clotting time of 12±1 s with 0.5 units/ml thrombin in 1 mM $CaCl_2$.

An aliquot of the purified fibrinogen was radiolabeled with ^{125}I using the Bolton-Hunter reagent. Free and protein bound ^{125}I were separated by extensive dialysis against HBS. The radioactivity of the labeled fibrinogen was assessed on a Beckman biogamma counter (Beckman Instruments, Inc.). Protein concentration was initially estimated based on $A_{1cm}^{1\%}$ 280 nm = 15.1 (27) and confirmed by Bradford protein determination with a standard curve of 1 mg/ml unlabeled human fibrinogen. Specific radioactivity was calculated based on the Bradford protein determination.

To prepare fibrinogen fragment D, an aliquot of the purified fibrinogen (20 mg/ml) was incubated overnight at 37°C with 12 μg plasmin (Kabi) in 1 mM $CaCl_2$. SDS-PAGE under nonreduced conditions demonstrated that all the fibrinogen had been degraded to fragment D and fragment E. These fragments were isolated by gel-filtration on Sephadex G-150 in 10% acetic acid. The fragment D concentrations used in these experiments (0.6–12.0 mg/ml) were shown to displace ^{125}I-fibrinogen from the surface of AA-activated platelets. The highest concentration of fragment D reduced the ^{125}I-fibrinogen binding (at 1 K_d or 0.2 mg/ml) to the level of nonspecific binding.

The purity of the unlabeled and ^{125}I-labeled fibrinogen was routinely assessed by SDS-polyacrylamide slab gel electrophoresis. PAGE following reduction showed only the three polypeptide chains of fibrinogen (Aα, Bβ, and γ; $M_r \cong$ 66,000, 54,000, and 51,000, respectively). Laser densitometric scanning of the autoradiogram indicated that >96% of the ^{125}I-label is incorporated into the three fibrinogen chains. Radiolabeling did not alter the clotting characteristics of the fibrinogen.

Fibrinogen binding to gel-filtered platelets. All fibrinogen binding experiments were performed at 37°C. The platelet suspension was adjusted to 1 mM $CaCl_2$ and an aliquot was placed into a siliconized cuvette. ^{125}I-labeled fibrinogen from 0.02 to 0.6 mg/ml final concentration was added to the platelet suspension for assessment of total fibrinogen binding. Nonspecific fibrinogen binding was determined by the addition of a 20-fold excess of unlabeled purified fibrinogen. Platelets were activated with AA (30 μM), or PMA (10 μM), with and without pretreatment with CE (5 μg/ml). Stirring was continued until shape change and initial aggregation were observed. Platelet shape change and aggregation were monitored on a Payton aggregometer at 37°C. Samples were then incubated unstirred for 5 min at 37°C. Full aggregation was avoided in this case to allow accurate sampling and separation of bound from free label. Triplicate aliquots were layered onto Apiezon A:*n*-butyl phthalate (1:9) mixture in microfuge tubes and centrifuged at 10,000 *g* for 3 min in a Beckman microfuge 11 (Beckman Instruments, Inc.). Samples of the aqueous supernatant and the platelet pellet were separated and radioactivity was assessed on a Beckman biogamma counter (Beckman Instruments, Inc.). A sample of the platelet pellet with bound ^{125}I-labeled fibrinogen was denatured, electrophoresed as above, and autoradiographed to demonstrate that the bound material was identical to the added labeled fibrinogen.

Results

Retention of glycoproteins IIb and IIIa in cytoskeletal cores. The platelets were activated in the presence of 0.2 mg/ml fibrinogen and incubated for 5 min at 37°C with stirring to give maximal aggregation (Fig. 1) prior to extraction. Detergent-insoluble cytoskeletal cores from ^{125}I-labeled platelets were recovered by centrifugation following platelet activation with AA (30 μM) or PMA (10 μM) with and without pretreatment with CE (5 μg/ml). SDS-PAGE slab gels stained with Coomassie blue are shown in Fig. 2 *A*. The AA-activated platelets formed a complete cytoskeletal structure as evidenced by the inclusion of actin-binding protein, myosin heavy and light chains, actin, and α-actinin in the detergent-insoluble cytoskeletal cores (lane 1). Pretreatment of AA-stimulated platelets with CE blocked the incorporation of the actin-binding protein and α-actinin (lane 2). PMA-activated platelets formed pseudopodal cytoskeletons composed of actin-binding protein, α-actinin, and actin with a greatly reduced amount of myosin (lane 3).

The monomeric actin content of the unactivated platelets has been previously shown (23) and reconfirmed in this study

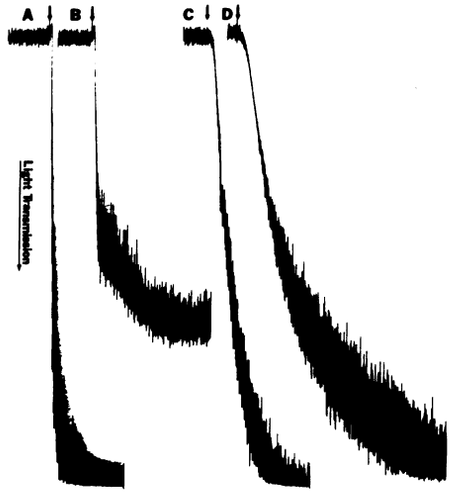

Figure 1. Aggregometer tracings of platelet aliquots. Tracings of platelet aggregation (5×10^8/ml) were obtained on a Payton aggregometer at 37°C. Platelets were activated with 30 μM arachidonate (*A*); pretreated for 15 min at 37°C with 10 μg/ml CE and then activated with 30 μM arachidonate (*B*); with 10 μM PMA (*C*); or pretreated for 15 min at 37°C with 10 μg/ml CE and then activated with 10 μM PMA (*D*). Arrows indicate addition of activator.

(data not shown) to be ~69% of the total actin content. Upon thrombin or PMA activation, the monomer actin value is reduced to 20±4% with almost all of the filamentous actin recoverable in the cytoskeletal core (23). Similarly, activation with AA reduces the G-actin content to ~25% (28). Pretreatment with cytochalasin B reduces the core content of thrombin activated platelets to 47±9% and completely inhibits the actin polymerization response to PMA (23). Identical results have been obtained with CE in this study (data not shown) such that there is almost a 40% reduction in the AA plus CE core (lane 2), and little additional cytoskeletal core material is recovered after activation with PMA plus cytochalasin E (lane 4) as compared with control, unactivated platelet cores (lane 5).

Autoradiography of the SDS-PAGE slab gel (Fig. 2 *B*) demonstrated that the complete cytoskeletal cores (AA-activated) (lane 1) and the modified core consisting primarily of actin-binding protein, α-actinin, and actin (PMA-activated) (lane 3) retained ^{125}I-labeled glycoproteins IIb and IIIa. The identity of the ^{125}I-labeled bands as glycoprotein IIb and IIIa was confirmed by immunoprecipitation with a monoclonal antibody against human glycoprotein IIb-IIIa complex (Fig. 2 *C*). Despite extensive platelet aggregation (Fig. 1), pretreatment with CE significantly reduced the retention of glycoproteins IIb and IIIa in platelets activated with AA (lane 2) or PMA (lane 4). In addition, platelets were activated with thrombin (1 U/ml) with and without pretreatment with CE. Our results with thrombin activation are comparable to those reported by Phillips et al. (21). Similar to the results with AA and despite maximal platelet aggregation, pretreatment with CE significantly reduced pseudopodal core development, as previously reported (23), and the retention of the glycoprotein IIb-IIIa in the thrombin-generated core.

Laser densitometric scanning of autoradiograms like that shown in Fig. 2 *B* and integration of the areas under the curves indicated that the complete cytoskeletal core (AA-activated) retained 30±6% glycoprotein IIb and 26±3% glycoprotein IIIa (Table I). Similarly, cytoskeletal cores from PMA-activated platelets retained 24±4% of glycoprotein IIIa. The remainder was quantitatively recovered in the detergent supernatant. Pretreatment with CE followed by AA or PMA activation reduced the glycoprotein IIIa retention to 6±2%. Control cores retained 4±2% of the labeled glycoprotein IIIa. In control and CE pretreated cores, the retained ^{125}I-label associated with glycoprotein IIb was too low to be accurately detected.

To evaluate the specificity of the glycoprotein IIb-IIIa retention and to assess the extraction efficiency under the activation conditions, the phospholipid content and 5′-nucleotidase activity of the cytoskeletal cores are also given in Table I. The 5′-nucleotidase activity was ~15% of the total membrane activity for cytoskeletons harvested from AA, and PMA-activated and CE pretreated AA-activated platelets. The reduced content in the CE pretreated PMA-activated platelets probably reflects little cytoskeletal core formation beyond that already in control platelets. Similarly, the core content of inorganic phosphate as a reflection of phospholipid remains ~10% for all cores except the CE pretreated PMA-generated core in which the content is similarly reduced to almost control unactivated platelet levels.

Aggregation studies. Increasing concentrations of fragment D (0.6–12 mg/ml) successively blocked arachidonate-stimulated platelet aggregation (Fig. 3). Conversely, the extent of aggregation of platelets pretreated with aspirin and then activated with ADP and epinephrine correlated with increasing concentration of added fibrinogen. Under both of these conditions, the extent of cytoskeletal core development as assessed by densitometry of Coomassie Blue protein staining was equivalent in all samples. Autoradiography of SDS-PAGE slab gel and laser densitometric scanning of the autoradiogram demonstrated that the retention of GpIIIa in the core correlated with the extent of aggregation and data from both experiments are shown in Fig. 4. Maximal aggregation retained 26±3% of the labeled GpIIIa.

Fibrinogen binding to platelets. The ^{125}I-fibrinogen binding to platelets activated with 30 μM arachidonate and incubated for 5 min at 37°C is illustrated in Fig. 5 *A*. The binding was saturable and reached a maximum at ~6 pmol ^{125}I-fibrinogen bound/10^8 platelets. Scatchard analysis of the fibrinogen binding data is shown in the inset. Pretreatment of the platelets with CE, which blocks actin-binding protein, α-actinin, and actin incorporation into the cytoskeletal core did not inhibit the extent of fibrinogen binding nor alter the apparent equilibrium

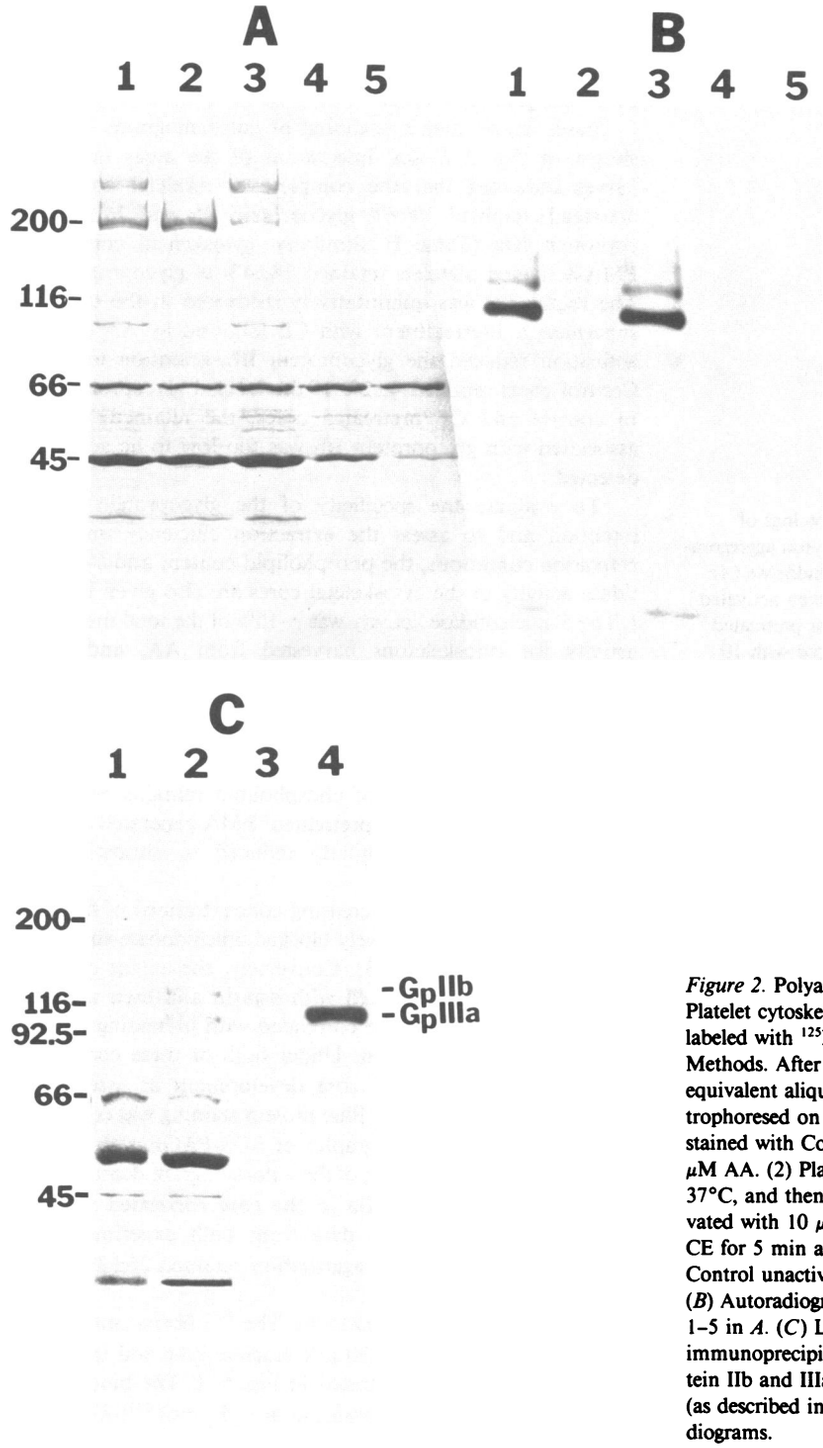

Figure 2. Polyacrylamide gradient-SDS gels of platelet cytoskeletons. Platelet cytoskeletons were prepared from whole platelets surface-labeled with ^{125}I by extraction with 1% Triton X-100 as described in Methods. After denaturation with SDS and 2-mercaptoethanol, equivalent aliquots with respect to platelet concentration were electrophoresed on 6–17.5% polyacrylamide gradient-SDS slab gels and stained with Coomassie blue (*A*). (1) Platelets were activated with 30 μM AA. (2) Platelets were pretreated with 5 μg/ml CE for 5 min at 37°C, and then, activated with 30 μM AA. (3) Platelets were activated with 10 μM PMA. (4) Platelets were pretreated with 5 μg/ml CE for 5 min at 37°C, and then, activated with 10 μM PMA. (5) Control unactivated platelets were extracted as described in Methods. (*B*) Autoradiogram of gel in Part A. Lanes 1–5 correspond to lanes 1–5 in *A*. (*C*) Lanes 1 and 2, respectively, are Coomassie blue-stained immunoprecipitates of control and monoclonal antibody to glycoprotein IIb and IIIa from unactivated ^{125}I-labeled whole platelet extract (as described in Methods). Lanes 3–4 are the corresponding autoradiograms.

dissociation constant (Fig. 5 *B*). Similarly, platelets activated with PMA, which allowed assembly of the actin-binding protein:α-actinin:actin cytoskeleton with little incorporation of myosin, bound ^{125}I-fibrinogen to the same extent as the AA-activated platelets (Fig. 6 *A*). The combination of the CE pretreatment followed by activation with PMA inhibits for-

Table I. Retention in Cytoskeletal Cores

Activation condition	Percent GpIIIa (±SEM)	Percent 5'nucleotidase* (±SEM)	Percent phospholipid‡ (±SEM)
Control	4±2	<1%	<1%
AA	26±3	15±1	11±3
CE + AA	6±2	17±1	11±2
PMA	24±4	14±2	9±1
CE + PMA	6±2	<10%	3±1

* Membrane activity = 18±3 nM/10^9 platelets/h.
‡ Whole platelet phospholipid = 315±21 nM P_i/10^9 platelets.

mation of a detergent-insoluble cytoskeletal structure, and indeed under these conditions, little actin polymerization is observed (23). However, platelets activated under these conditions do express fibrinogen binding activity approaching that of the AA-stimulated platelets (Fig. 6 *B*). One way analysis of variance of the mean number of fibrinogen-binding sites and the mean apparent dissociation constant (K_d) for each activation condition indicates that the mean number of binding sites expressed and the K_d for each activation condition is not significantly different ($P > 0.05$) (Table II).

Discussion

The complete cytoskeletal core (AA-activated, fully aggregated platelets) retained significant quantities of glycoproteins IIb and IIIa. This finding is in agreement with the work of Phillips et al. (21) using thrombin-activated aggregated platelets. Activation with PMA which allows formation of the pseudopodal structure but inhibits the actin-myosin interaction to form the contractile gel leads to retention of the glycoproteins IIb and IIIa to the same extent as in the complete cytoskeletal cores. However, pretreatment of the platelets with CE, which partially inhibits the incorporation of actin filaments and completely blocks the incorporation of actin-binding protein and α-actinin into the cytoskeletal core, reduced retention of the surface labeled glycoproteins. These findings suggest that there is an interaction between the glycoprotein IIb-IIIa complex and one or more of the pseudopodal cytoskeletal proteins (α-actinin, actin-binding protein, actin, or as yet unidentified component such as vinculin [29]) which results in selective retention of these glycoproteins in the insoluble cytoskeletal cores of aggregated platelets.

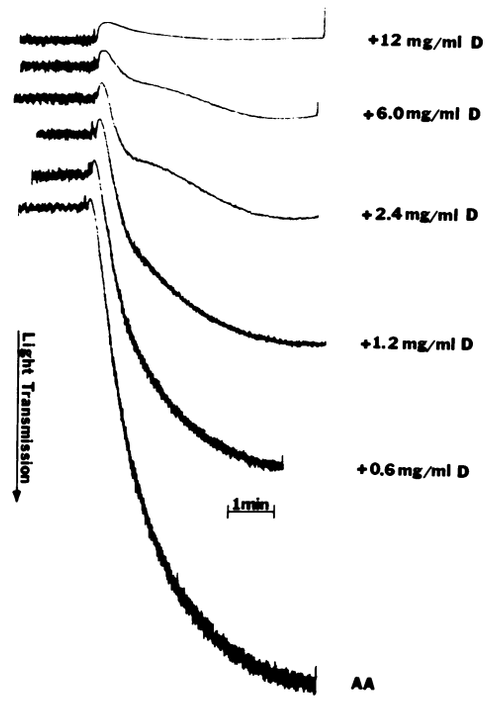

Figure 3. Inhibition of AA-induced aggregation by fibrinogen fragment D. Gel-filtered platelets were activated with 30 μM AA. Maximal aggregation is illustrated in the bottom tracing. From the bottom, increasing concentrations of fibrinogen fragment D (D) from 0.6 to 12 mg/ml were added prior to activation. A 1-min interval is indicated by the bar.

Table II. ^{125}I-Fibrinogen Binding to Activated Platelets

Activation condition	N	Fibrinogen binding sites (±SEM)*	K_d (μM±SEM)*
AA	5	51,060±3,750	0.71±0.09
AA + CE	5	49,060±10,180	0.75±0.19
PMA	5	50,920±1,695	0.76±0.07
PMA + CE	3	40,025±6,330	0.91±0.15

* determined by Scatchard analysis.
AA: 30 μM arachidonic acid, CE: 5 μg/ml cytochalasin E, PMA: 10 μM phorbol 12-myristate 13-acetate.

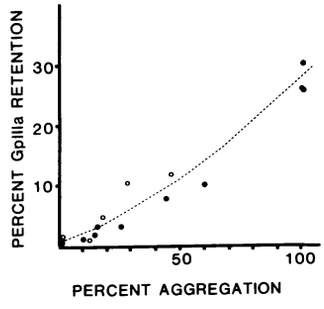

Figure 4. Correlation between aggregation and glycoprotein IIIa (GpIIIa) retention. The extent of arachidonate-activated platelet aggregation in the presence of fibrinogen fragment D (●) or platelets pretreated with aspirin and activated by ADP and epinephrine in the presence of increasing concentrations of exogenous fibrinogen (○) is plotted vs. the percent retention of ^{125}I-labeled GpIIIa.

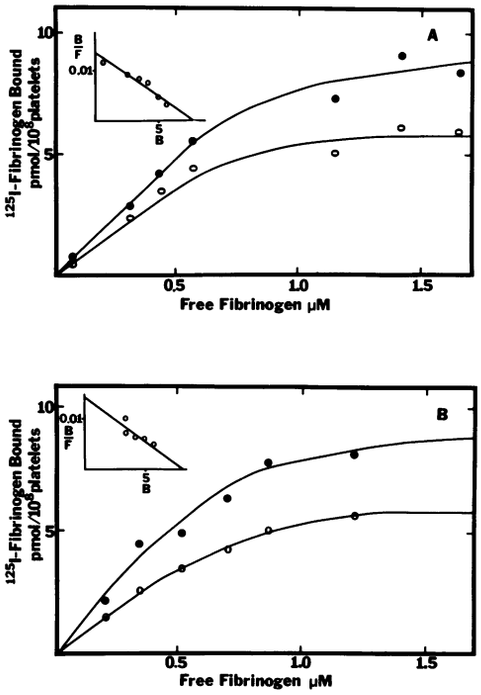

Figure 5. Binding of ^{125}I-labeled fibrinogen to platelets activated with AA with and without CE pretreatment. Increasing concentrations of ^{125}I-fibrinogen were added to gel-filtered platelets. The platelet suspensions (5×10^8 platelets/ml) were activated with 30 μM arachidonate (*A*) or pretreated with 5 μg/ml CE followed by activation with 30 μM arachidonate (*B*). All the mixtures were incubated for 5 min at 37°C before separation of bound from free labeled fibrinogen as described in Methods. Each point is the mean of triplicate determinations in one representative experiment. Closed circles represent the total ^{125}I-fibrinogen binding to activated platelets. Open circles represent the specific binding as determined by total binding minus nonspecific binding. The insets are the Scatchard analysis of these experiments (B/F = picomoles per 10^8 platelets/μM; B = picomoles per 10^8 platelets). Scatchard analysis indicated the number of fibrinogen sites and the K_d's to be 47,800, 0.63 μM; and 45,200, 0.50 μM; respectively.

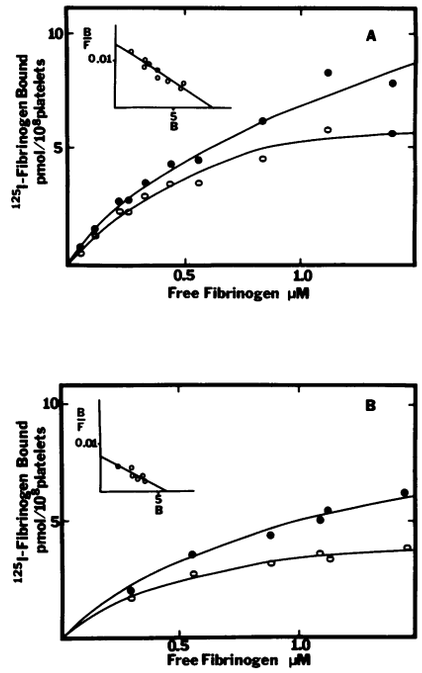

Figure 6. Binding of ^{125}I-labeled fibrinogen to platelets activated with PMA with and without CE pretreatment. The conditions and explanations of the graphs are the same as given in Fig. 5 except for the activations. The platelet suspension (5×10^8 platelets/ml) were activated with 10 μM PMA (*A*) or pretreated with 5 μg/ml CE followed by activation with 10 μM PMA (*B*). Binding was assessed as described in Fig. 3 and Methods. The insets are the Scatchard analyses (B/F = picomoles per 10^8 platelets/μM; B = picomoles per 10^8 platelets) of these experiments which indicate the number of fibrinogen binding sites and the K_d to be 53,000, 0.71 μM; and 41,000, 0.54 μM; respectively.

The cytochalasins have been suggested to interfere with actin polymerization (30). However, as previously reported (23), cytochalasin B does not prevent actin polymerization in platelets activated by thrombin. It was proposed, therefore, that cytochalasin B blocks incorporation of actin-binding protein into the platelet cytoskeletal core by preventing the development of the long filaments of actin necessary for pseudopodal formation (23). Actin-binding protein and α-actinin may still interact with the short actin filaments but may not be able to sufficiently crosslink the filaments into a precipitable gel (30). Additionally, Schollmeyer et al. (31) have suggested that cytochalasin B directly influences the interaction of α-actinin and actin-binding protein with purified actin containing troponin-tropomyosin. Further studies would be needed to determine if the cytochalasins can directly inhibit the interaction between glycoprotein IIb-IIIa complex and the pseudopodal cytoskeleton or if the glycoprotein IIb-IIIa complex is still linked to the nonprecipitable cytoskeletal fragments.

Zucker and Masiello (32) reported that the binding of the bilaterally symmetrical fibrinogen to the platelet surface did not cause an association between the surface proteins and the "Triton-insoluble residue." The requirement for platelet aggregation led them to conclude that the residual phospholipid in the cytoskeletal core of aggregated platelets reflected inadequate membrane lysis and that the glycoprotein IIb-IIIa retention in the cytoskeletal core is an artifact. In contrast, the cytoskeletal cores in our preparations consistently retain 9–12% of the total platelet phospholipid and demonstrate <15% activity of a membrane-associated enzyme marker (33) irrespective of cytoskeletal assembly (complete, pseudopodal, or contractile). The receptor retention, however, is significantly reduced when

the pseudopodal cytoskeletal core formation is inhibited even though extensive platelet aggregation has occurred. These findings argue for the specificity of the glycoprotein IIb-IIIa association with the pseudopodal cytoskeleton. A similar efficiency of detergent (Triton X-100) extraction was seen with nonaggregated neutrophils containing a formed cytoskeletal structure which yields centrifuge-recoverable "ghosts." These ghosts were devoid of membrane-bound organelles and soluble proteins; yet, they retained 10% of the total phospholipid (34). The findings that detergent-extracted cytoskeletal cores from cells which exhibit transmembrane linkage between surface receptors and the cytoskeleton retain a measure of the surface membrane phospholipid may simply reflect a phospholipid component which is tightly associated with these surface receptors.

The demonstration that the retention of the glycoprotein complex in the cytoskeletal core is dependent on the extent of aggregation supports the observation of Phillips et al. (21) that activated platelets that were not allowed to aggregate did not show significant retention in the core. Aggregation was prevented either by not stirring the preparation or by the addition of EGTA to chelate the external calcium. Since fibrinogen binding to the receptor is calcium-dependent, chelation of the external calcium would prevent the receptor from binding fibrinogen. If the receptor is unoccupied, the receptor-cytoskeleton interaction may not be stable enough to survive the detergent extraction. As previously shown (2) and confirmed in this study, fibrinogen fragment D serves as a monovalent ligand which will compete with the platelet fibrinogen for the glycoprotein receptor site. In this way, aggregation is inhibited; yet the receptor is occupied. However, the correlation between the extent of aggregation and glycoprotein retention remains the same as the correlation produced when the extent of aggregation is increased by titration of exogenous fibrinogen to platelets pretreated with aspirin and activated with ADP and epinephrine. The apparent requirement for aggregation to obtain retention in the core suggests that even when occupied by dimeric fibrinogen molecule, the receptor cytoskeleton interaction is relatively labile. Stabilization of the receptor cytoskeleton complex may require multivalent binding reactions which have the potential to greatly increase transmembrane interactions (35). Enhancement of the transmembrane interaction may be important with respect to ligands with low affinities such as fibrinogen.

The apparent K_d (7×10^{-7} M) derived from our studies suggest that we were examining the low affinity fibrinogen binding site previously reported (4, 6). However, no attempt was made to correct the binding for fibrinogen that was secreted from the platelets. The granular fibrinogen content has been reported to be 70–100 $\mu g/10^9$ platelets (36, 37). If this fibrinogen was totally secreted and completely indistinguishable from the plasma fibrinogen by its binding properties to platelets, then the K_d value would be 6×10^{-7} M, but the number of sites would be the same. Although complete alpha granule secretion was unlikely, even a modest secretion precluded our investigations of high-affinity binding regardless of whether this fibrinogen binding results from separate sites (4) or negative cooperativity (6). However, it should be pointed out that analysis of fibrinogen binding to surface platelet proteins by Gogstad et al. (8) suggests that the glycoprotein IIb-IIIa complex is the only surface entity that binds fibrinogen. They proposed, therefore, that the glycoprotein IIb-IIIa complex is a homogeneous class of receptors forming the low affinity binding site expressed upon platelet activation and that this site mediates aggregation.

Expression of the fibrinogen binding site on the surface of activated platelets, however, is not dependent upon the formation of a cytoskeletal core. Pretreatment with CE followed by activation with either AA or PMA did not alter the expression of the fibrinogen binding sites. Additionally, in the case of activation with PMA, pretreatment with CE almost completely blocks actin polymerization and cytoskeletal core formation. Activation under any of these conditions did not significantly change the number of binding sites expressed nor the apparent dissociation constant of those sites. The finding that pretreatment with the cytochalasins does not block fibrinogen binding is in agreement with the work of Peerschke and Zucker (38), who demonstrated that cytochalasin B and colchicine did not inhibit the ability of ADP-stimulated platelets to bind fibrinogen. They concluded that fibrinogen binding is associated with platelet aggregability, but not with platelet shape changes during activation. The effects of the phorbol esters (±cytochalasin B or E) on fibrinogen binding have not been previously assessed.

The mechanism by which the transmembrane glycoproteins IIb and IIIa associate to form the platelet fibrinogen binding site is not currently known. The retention of the glycoprotein IIb-IIIa complex in the detergent-insoluble cytoskeletal core suggests a specific interaction of the complex with the cytoskeleton. Previous studies have suggested that the glycoprotein IIb-IIIa complex may be the site of actin attachment to the platelet membrane (39, 40). Recently, vinculin has been suggested as the linking component between the membrane and the cytoskeleton (29). This study demonstrated that it is the pseudopodal elements (filamentous actin crosslinked by actin-binding protein and α-actinin) that are necessary for the retention of the glycoprotein complex in the cytoskeletal core. However, the expression of the fibrinogen binding site is not affected by blocking the development of the interaction between α-actinin, actin-binding protein, and actin or by substantially blocking actin polymerization. These findings do not establish if the effect of the cytochalasins is to block the association of the glycoprotein complex with the cytoskeleton or to render the glycoprotein-cytoskeleton units nonprecipitable. Therefore, one or more of the pseudopodal elements may be involved in a local membrane event resulting in the formation of the fibrinogen binding site and its subsequent linkage into the cytoskeletal structure.

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