Association of Fibrin with the Platelet Cytoskeleton*

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We have previously postulated that surface membrane proteins become specifically associated with the internal platelet cytoskeleton upon platelet activation (Tuszyński, G. P., Walsh, P. N., Piperno, J., and Koshy, A. (1982) J. Biol. Chem. 257, 4557-4563). Four lines of evidence are in support of this general hypothesis since we now show that platelet surface receptors for fibrin become specifically associated with the platelet Triton-insoluble cytoskeleton. 1) Fibrin was detected immunologically in the washed Triton-insoluble cytoskeletons of thrombin-activated platelets under conditions where fibrin polymerization and resultant precipitation was blocked with Gly-Pro-Arg-Pro, a synthetic peptide that inhibits polymerization of fibrin monomer. 2) Radiolabeled fibrin bound to thrombin-activated platelets and became associated with the cytoskeleton. 3) The amount of radiolabeled fibrin bound to thrombin-activated thrombasthenic platelets and their cytoskeletons amounted to about 20% of the fibrin bound to thrombin-activated control platelets and their cytoskeletons. 4) The association of fibrin with cytoskeletons and with the platelet surface was nearly quantitatively blocked by an antibody prepared against cytoskeletons (anti-C), an antibody against isolated membranes of Pronase-treated platelets (anti-M1), and a monoclonal antibody to the platelet surface glycoprotein complex, GPIIb-GPIII (anti-GPIII). These antibodies blocked ADP and thrombin-induced platelet aggregation as well as thrombin-induced clot retraction. Analysis of the immunoprecipitates obtained with anti-C, anti-M1, and anti-GPIII from detergent extracts of 125I-surface labeled platelets revealed that these antibodies recognized GPIIb-GPIII. These data suggest that thrombin activation of platelets results in a specific association of fibrin with the platelet cytoskeleton, that this association may be mediated by the GPIIb-GPIII complex, and that these mechanisms may play an important role in platelet aggregation and clot retraction induced by thrombin.

The term "cytoskeleton" (1, 2) has been used to describe the complex network of intracellular fibrils which supports the plasma membrane and provides shape and structure to the cell and its organelles (3, 4). The resting platelet is maintained in a discoidal form by energy-requiring mechanisms and rapidly changes shape to a sphere with filopodia extending from the surface when stimulated by a variety of agonists such as thrombin (5, 6). Several cytoskeletal and contractile proteins have been identified which may be involved in these processes and in the centralization of platelet granules and secretion of their contents (5-7).

We have previously presented evidence showing a specific interaction between the platelet cytoskeleton and factor V and the hypothesis that the platelet cytoskeleton of thrombin-activated platelets may contain other specifically associated c-granule proteins (8). In support of this general hypothesis, we now show that platelet cytoskeletons contain specifically associated fibrinogen antigen present in the form of partially cross-linked fibrin. We also present evidence that the glycoprotein complex, GPIII-GPIII, is thought to function as the platelet fibrinogen receptor (9-15) may also function to bind fibrin to the platelet and its cytoskeleton.

EXPERIMENTAL PROCEDURES

Materials—Proteolytic enzyme inhibitors, buffers, and Sepharose 2B were purchased from Sigma. Reagents for sodium dodecyl sulfate gel electrophoresis were purchased from Bio-Rad. Reagents for production of antisera were purchased from Gibco. Anti-fibrinogen antibody and goat anti-rabbit IgG were purchased from N. L. Cappel Laboratories Inc. Anti-fibrinogen antibody gave one line of identity between plasma and purified fibrinogen on double immunodiffusion (data not shown). Urokinase and plasminogen were obtained from Calbiochem-Behring. Anti-fibrinogen and E were prepared by C. C. (Department of Biophysics, Medical School of Lodz, Lodz, Poland). Agarose, highest electrodosmotice grade, was obtained from Marine Colloids. Highly purified human thrombin was a gift from Dr. J. W. Fenton, II, New York State Department of Health.lodogen (1,3,4,6-tetracloro-3a,6a-diphenylglycoluril) was obtained from Pierce Chemical Co. Na125I was purchased from New England Nuclear. Purified fibrinogen was supplied by Kabl. The platelet suspension solution (Heps3-buffered Tyrode's solution) was 3.8 mM Hepes buffered with 3.8 mM NaH2PO4, pH 7.35, containing 0.137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 1 mg/ml of bovine serum albumin, and 0.1% dextrose.

1 The abbreviations used are: Heps, N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; Staph A, Staphylococcus aureus Cowan I; anti-M1, antibody to membranes of Pronase-treated platelets; anti-C, antibody to Triton-insoluble cytoskeletons; anti-GPIII, a monoclonal antibody to the platelet surface glycoprotein complex, GPIII-GPIII; this is the same as the A9A9 described by Bennett (15).

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Isolation of Platelets—Platelet-rich plasma was prepared from citrated whole blood of healthy donors or thrombathemic patients (0.38% citrate final concentration) by centrifugation at 140 × g for 15 min. The platelet-rich plasma was gel filtered through Sepharose 2B (16) equilibrated in Heps-buffered Tyrode's solution. Platelets were also washed by the method of Mustard et al. (17). Platelets were collected by Channelyzer. Thrombathemic patients (N.L. and L.M.) were the same as those described previously (18) and the GPIIb-GPIII complex present on the surface of these platelets was about 10% of controls (18, 19).

Labeling of Proteins and Platelets—Fibrinogen was labeled with [125I]iodine as previously described (20). Stock [125I]iodine solutions contained 1 to 2 mg/ml of protein and had a specific activity of 77 μCi/mg of protein. Labeled fibrinogen solutions retained their thrombin clottability and their ability to bind ADP-stimulated platelets (21).

Platelets were surface-labeled with [125I]iodine by the iodogen method as previously described (22). Briefly, 3 × 10^8 platelets/ml, washed by gel filtration, were added to septum-capped glass vials coated on the inside with 100 μg of iodogen. Approximately, 200 μCi of carrier-free [125I]iodide was added to each vial and the platelets incubated at room temperature for 30 min with occasional agitation. The platelets were then removed from the vials, washed by centrifugation to remove unreacted [125I]iodide, and solubilized in 100 μl of Heps-buffered Tyrode's albunin-free solution containing 1% Triton, 0.2% SDS, and 1 mM diisopropyl fluorophosphate. Solutions typically contained 10–20 μCi of labeled protein and were frozen at −70 °C for further use.

Preparation of Cytoskeletons—Triton-insoluble cytoskeletons were prepared from gel-filtered platelets as previously described (8). Briefly, platelets were activated with thrombin (1 unit/ml), treated with hirudin and diisopropyl fluorophosphate, lysed with Triton, and washed by centrifugation in albumin-free Heps-buffered Tyrode's solution containing Tris-Cl (21).

Assays—Protein assays were performed according to the procedure of Lowry et al. (23) or Schaffner and Weissman (24). The amount of fibrin and/or fibrinogen present in platelets and their corresponding cytoskeletons was measured by a double-antibody equilibrium-competitive radioimmunoassay based on the amount of degradation products, Fragments D and E, generated from these preparations by the action of plasmin, as previously described (25). Briefly, a platelet pellet (2–3 × 10^9 platelets) and the cytoskeletal pellet (2–3 × 10^8 platelets) prepared as described previously (8) but in the presence of 0.1% or 1% Gly-Pro-Arg-Pro to prevent nonspecific association of fibrinogen on double immunodiffusion and did not immunoprecipitate labeled fibrinogen (data not shown). The antiserum against cytoskeletons would be referred to as anti-G FI antibody.

Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli (27). Gels were stained, dried onto paper, and autoradiograms were prepared from the dried gels using intensifying screens (DuPont Cromex Lightning-Plus screens mounted in Spectrolite Cassette, Reliance X-Ray Inc., Oreland, PA). Kodak X-Omat-AR film was used and developed according to the instructions provided with the film. Films were exposed for 1 week at −70 °C.

Antisera—Antisera to membranes prepared from Pronase-treated platelets (anti-M1 antibody) was prepared in rabbits as previously described (21). Anti-M1 antibody was employed in this study since it predominantly recognizes GPIIb and GPIII (21). The monoclonal antibody to GPIIb-GPIII (anti-GPIII) was prepared as previously described (15) and was kindly supplied by Dr. T. H. Bennett, Hematology-Oncology Section, University of Pennsylvania Hospital, Philadelphia, PA. Anti-GPIII was a purified IgG solution (stock 3 mg/ml). Antiserum to Triton-insoluble cytoskeletons was prepared as follows. Cytoskeletons from 5.2 × 10^10 platelets were first washed three times in Heps-buffered Tyrode's albumin-free solution containing 0.5% Triton and subsequently by three washes in Heps-buffered Tyrode's albumin-free solution containing no Triton. The cytoskeletons were finally suspended in 0.5 ml of Heps-buffered Tyrode's albumin-free solution containing 1% Triton, 0.2% SDS, and 1 mM diisopropyl fluorophosphate. Solutions typically contained 1–2 mg/ml of protein and had a specific activity of 20 to 40 μCi/ml.

Immunoprecipitation—Platelet suspensions containing 10^10 platelets/ml containing 10 μl of antiserum per 1 ml of a 10% suspension of Staph A in order to assure binding of the mouse IgG to the Staph A immunoadsorbent. The platelet suspensions were then washed as described by Kessler (28). Briefly, between 2–4 × 10^9 platelets/ml containing 10 μl of immunoprecipitation buffer which was Heps-buffered Tyrode's solution (modified to contain no MgCl_2 or albumin) containing 1% Triton, 0.2% SDS, 1 mM EDTA, and 1 mM diisopropyl fluorophosphate. Samples were then immunoprecipitated essentially according to the procedure of Kessler (28). After incubation, 100 μl of a washed suspension of fixed Staph A grown from a culture kindly supplied by Dr. T. H. Bennett, Hematology-Oncology Section, University of Pennsylvania Hospital, Philadelphia, PA, was added to the samples and the samples placed on ice for 10 min. In the case of anti-GPIII antibody, Staph A suspensions were preincubated with goat anti-rabbit IgG (10 μl of serum per 1 ml of 10% suspension of Staph A) in order to assure binding of the mouse IgG to the Staph A immunoadsorbent. The Staph A suspensions were then washed as described by Kessler (28), extracted with SDS sample buffer (27) containing 2% SDS either in the presence or absence of reducing agent and the SDS extracts analyzed by SDS-gel electrophoresis, followed by autoradiography as described above.

Platelet Aggregation and Clot Retraction—Platelets were aggregated by thrombin in a Payton aggregometer. Platelet suspensions (100 μl) in the range of 2–4 × 10^8 platelets/ml were first incubated at 37 °C with stirring with 100 μl of controlled serum or immune serum. Aggregation was initiated with 10 μl of thrombin (final concentration 0.2 unit/ml). Extent of aggregation was determined by measuring the initial slope of the aggregation curve and comparing it to control values.

Clot retraction was measured essentially according to the procedure of Niewiarowski et al. (26). Briefly, 0.4 ml of a washed suspension of platelets (10^10 platelets/ml) containing 18 μl of a 2 mg/ml fibrinogen solution was mixed with 50 μl of Heps-buffered Tyrode's solution or 50 μl of antiserum in a glass test tube. The resulting suspension was incubated at 37 °C for 10 min, and 50 μl of a 425 unit/ml solution of thrombin were added to start the retraction and incubation continued for 90 min. After 90 min the samples were placed on ice and quickly photographed.

Production of Antiserum—Antiserum to membranes prepared from Pronase-treated platelets (anti-M1 antibody) was prepared in rabbits as previously described (21). Anti-M1 antibody was employed in this study since it predominantly recognizes GPIIb and GPIII (21). The monoclonal antibody to GPIIb-GPIII (anti-GPIII) was prepared as previously described (15) and was kindly supplied by Dr. J. J. Bennett, Hematology-Oncology Section, University of Pennsylvania Hospital, Philadelphia, PA. Anti-GPIII was a purified IgG solution (stock 3 mg/ml). Antiserum to Triton-insoluble cytoskeletons was prepared as follows. Cytoskeletons from 5.2 × 10^10 platelets were first washed three times in Heps-buffered Tyrode's albumin-free solution containing 0.5% Triton and subsequently by three washes in Heps-buffered Tyrode's albumin-free solution containing no Triton. The cytoskeletons were finally suspended in 0.5 ml of Heps-buffered Tyrode's albumin-free solution containing 1% Triton, 0.2% SDS, and 1 mM diisopropyl fluorophosphate. Solutions typically contained 1–2 mg/ml of protein and had a specific activity of 20 to 40 μCi/ml.
Pro-Arg-Pro, or 0.1 mM Gly-Pro-Arg-Pro and 100 µl of antiserum were activated with 1 unit/ml of thrombin. The samples were divided in half. One-half was washed three times in Hepes-buffered saline by centrifugation at 10,000 × g for 2 min, and cytoskeletons were prepared from the other half as previously described (8). The platelet pellets and cytoskeletons were then either counted or analyzed on SDS gels as described above.

Direct fibrin-binding studies were performed by first activating platelets with thrombin (1 unit/ml for 3 min) followed by the addition of 2.5 units/ml of hirudin. 125I-Fibrinogen (1 mg/ml) was activated with 1 unit/ml of thrombin for 3 min in the presence of 0.1 mM Gly-Pro-Arg-Pro followed by the addition of 2.5 units/ml of hirudin. Various amounts of 125I-fibrin were added to platelet suspensions containing 0.1 mM Gly-Pro-Arg-Pro, incubated for 5 min, and either the platelet pellet counted after three washes with Hepes-buffered Tyrode’s solution containing albumin or cytoskeletons prepared as previously described (8). Antibody-blocking experiments performed with anti-GPIII were performed as described above except that the antibody was added to the platelet suspension before addition of fibrin or fibrinogen.

RESULTS

Characterization of the Cytoskeletal-associated Fibrin—In order to characterize the cytoskeletal-associated fibrin, cytoskeletons prepared in the presence of 125I-fibrinogen were analyzed on SDS gels and the Coomassie blue-staining profiles compared with the corresponding autoradiograms (Fig. 1). Major radioactive bands (Fig. 1, lanes 3 and 4) corresponding to Coomassie blue bands (Fig. 1, lanes 1 and 2 and designated fibrin) migrating at 100,000, 50,000–55,000 daltons, and a faint band at 68,000 daltons were observed. The heavily stained band migrating below the β band of fibrin in Fig. 1, lane 2, designated IgG, has been identified as the heavy chain of IgG, which should be present if the anti-C immune complex is blocking the association of fibrin with the cytoskeleton (see below). In this system the β and α bands of fibrin do not resolve well and run very close together, a situation similar to that previously observed when fibrinogen bound to platelets was analyzed by SDS gels (29). This explains why there is so much radioactivity in the 50,000–55,000-dalton region of our cytoskeletons. At lower exposure of Fig. 1, lane 3, or in the anti-C-treated sample (Fig. 1, lane 4) a faint γ band migrating close to the β band is observed. These results are consistent with the presence of the γ-γ, degraded α, and the β and γ bands of fibrin. The reason for the relative increase in the amount of minor bands migrating in the region of the gel above 50,000 daltons in the control serum-treated sample as compared to the immune serum-treated sample is unknown (Fig. 1, lanes 1 and 2). Since a high background in the autoradiogram of lane 1 is also observed, it is possible that some of the differences between the polypeptide patterns of the samples in lanes 1 and 2 may be due to fibrin incompletely reduced or partially degraded. Differences in the myosin content of cytoskeletons from preparation to preparation have been observed and the reason for this is unknown.

The amount of fibrinogen antigen in platelet cytoskeletons prepared from thrombin-activated platelets treated with Gly-Pro-Arg-Pro, a synthetic polypeptide that prevented the non-specific copurification of fibrin polymer with cytoskeletons (see below), was determined by a radioimmunoassay and densitometric scans of SDS gels. The first assay measured the level of fibrin or fibrinogen degradation products, Fragments D or E, produced by the action of plasmin on either total platelet lysates or washed cytoskeletons (see under “Experimental Procedures”). As a control, cytoskeletons solubilized in Triton-SDS buffer were also analyzed by the radioimmunoassay. In the second assay the amount of fibrin associated with washed cytoskeletons was quantitated from densitometric scans of cytoskeletons analyzed on SDS gels (see under “Experimental Procedures”). Both assay procedures indicated that platelet cytoskeletons contain 24% of the total platelet fibrinogen-related antigen (Table I). Thrombasthenic platelets contained approximately 4 times less fibrinogen antigen as compared to normal platelets (Table I). The level of radioactive fibrin bound to thrombasthenic cytoskeletons was also proportionally reduced (see below).

Specificity of Association of Fibrin with the Cytoskeleton—The purpose of the experiments to be described was to establish the specificity of the association of fibrin with the cytoskeleton. A major concern of ours was that secreted platelet fibrinogen was being activated to fibrin, polymerizing and sedimenting nonspecifically during the isolation of the cytoskeletons. In order to prevent this sedimentation of fibrin polymer, we employed a specific reagent, the synthetic polypeptide Gly-Pro-Arg-Pro, first shown by Laudano and Doolittle (30) to completely inhibit fibrin polymerization. When 0.1 mM Gly-Pro-Arg-Pro was added to a platelet lysate containing 14 µg/ml of 125I-fibrinogen and thrombin-activated,

TABLE I

| Sample* | Normal | Thrombasthenic |
|---------|--------|----------------|
| µg/10⁶ platelets |       |               |
| Total lysate  | 21.00 ± 1.05 5 | 5.75 ± 0.29 |
| Cytoskeletons | 4.97 ± 0.80³ | 1.58 ± 0.33 |
|           | 4.83 ± 1.56² | Not determined |
|           | 4.71 ± 0.87⁴ | Not determined |
|           | 4.55 ± 0.1⁰ |               |
|           | 4.92 ± 0.81¹ |               |

* Samples were prepared as described under “Experimental Procedures.”
Fibrin was measured by a radioimmunoassay described in “Experimental Procedures.”
* Determined from densitometric scans of stained SDS gels.
* Samples were solubilized in 2% SDS and analyzed by the radioimmunoassay.
* Determined in the presence of 1 mM Gly-Pro-Arg-Pro.
* Determined in the presence of 0.5 mM NaCl and analyzed by the radioimmunoassay.

![Fig. 1. SDS gels (8% polyacrylamide) of cytoskeletons prepared from platelets treated with 125I-fibrin in the presence of anti-C antibody. Platelets were treated as described in the legend of Table III and cytoskeletons prepared as described under “Experimental Procedures.” Lanes 1, 2, and 5 are Coomassie blue stained and lanes 3 and 4 are autoradiograms of lanes 1 and 2, respectively. Lanes 1 and 3, cytoskeletons prepared from control serum-treated platelets; lanes 2 and 4, cytoskeletons from anti-C treated platelets; lane 5, molecular weight standards.]
over 99% of the sedimentable fibrin polymer which might be expected to centrifuge down under the conditions of our cytoskeleton preparative procedure (10,000 x g for 2 min) was blocked (Fig. 2, solid triangles). Similar results were obtained when platelets were first activated by thrombin and then lysed, indicating that the presence of cytoskeletons did not affect the equilibrium between fibrin oligomers and polymers. A shift in the equilibrium would be unlikely in any event since the molar concentration of Gly-Pro-Arg-Pro in the system was 600-fold greater than the molar concentration of fibrin, and it has been shown (30) that a 100-fold excess completely inhibits fibrin polymerization. To ensure that no fibrin was nonspecifically precipitating with our cytoskeletal preparation we prepared cytoskeletons in the presence of 1 mM Gly-Pro-Arg-Pro or a 6000-fold excess and found that they contained the same amount of fibrin as those prepared in the presence of 0.1 mM Gly-Pro-Arg-Pro (Table I). Additionally the associated fibrin was solubilized in 0.5 M NaCl, conditions that dissociate cytoskeletal structures, further indicating that the bound fibrin is not precipitated as fibrin polymer (Table I). In the absence of Gly-Pro-Arg-Pro over 60% of the labeled fibrin was recovered in the sediment (Fig. 2, open symbols). In contrast, cytoskeletons prepared in the presence of the same amount of labeled fibrinogen and Gly-Pro-Arg-Pro bound over 20% of the fibrin (Fig. 2, solid circles). Cytoskeletons prepared in the absence of Gly-Pro-Arg-Pro (Fig. 2, open circles) contained levels of radiolabeled fibrin in excess of 60%. Similar amounts of fibrin were recovered in the platelet lysate under the same conditions. Therefore, the experiment presented in Fig. 2 shows that Gly-Pro-Arg-Pro completely blocked the formation of partially polymerized sedimentable fibrin present in the platelet lysate. On the other hand, this tetrapeptide reduced by only two-thirds the amount of fibrin pelleted with the Triton-insoluble platelet cytoskeleton. This experiment suggested that one-third of the fibrin was binding specifically. In addition, the cross-linking of fibrin monomers by platelet factor XIII also occurred in the presence of Gly-Pro-Arg-Pro-treated platelet lysate (data not shown). Therefore, it appears that partially cross-linked fibrin is still soluble. In order to prevent fibrin polymerization and nonspecific sedimentation all subsequent experiments were performed in the presence of at least 0.1 mM Gly-Pro-Arg-Pro.

To test whether platelet activation was necessary for association of radiolabeled fibrin with platelets and their cytoskeletons radiolabeled fibrin was added in a dose-dependent manner to activated and unactivated platelets. As can be seen from the data of a typical experiment, unactivated platelets as well as their cytoskeletons bound negligible amounts of fibrin (less than 5% of the fibrin bound to activated platelets) whereas activated platelets and their cytoskeletons bound fibrin in a saturable manner (Fig. 3). The data are presented as a double reciprocal plot although true equilibrium binding has not been established. The average of four experiments performed on different donor platelets shows that despite considerable variability from donor to donor, cytoskeletons and platelets bound nearly the same amount of fibrin and show half-saturation at roughly the same fibrin concentrations (Table II). The binding to cytoskeletons was inhibited in the presence of a 100-fold excess of cold fibrinogen (Table II). These results indicate that platelet activation is necessary for fibrin association to platelets and cytoskeletons and that this association depends on the presence of specific receptors.

To better establish that the association of fibrin with the platelet cytoskeleton was specific and occurring through a platelet surface receptor, we compared the amount of fibrin associated with platelets and cytoskeletons of normal and thrombathenic platelets obtained from patients who have been shown not to expose fibrinogen receptors upon activation.
by ADP (31). We hypothesized that thrombasthenic platelets that fail to aggregate in response to most platelet activators including thrombin (32) might also be defective in fibrin-binding receptors. We, therefore, postulated that these thrombasthenic platelets and their cytoskeletons should bind less fibrin than controls, provided that the formation of fibrin polymers is prevented by Gly-Pro-Arg-Pro. As can be seen from the data in Fig. 4, thrombasthenic platelets and their cytoskeletons bound approximately 80% less fibrin than controls. In addition, these experiments indicated that over 80% of the fibrin formed by the addition of thrombin on the platelet surface was recovered with the platelet cytoskeleton.

The polypeptide composition of cytoskeletons prepared from normal platelets (see below) or cytoskeletons (data not shown) can only be demonstrated by surface labeling. Therefore, it would appear that receptors for fibrin are present on the platelet surface and become bound to the platelet cytoskeletons and that these receptors are either deficient or present in a non-functional form on thrombasthenic platelets.

Finally we employed a third completely independent experimental approach to confirm the specific receptor-mediated association of fibrin with the platelet cytoskeleton. For this approach we utilized three different antibodies. The first antibody raised against membranes prepared from Pronase-treated platelets (anti-M1 antibody) has been extensively characterized and has been shown to block ADP-induced platelet aggregation and fibrinogen binding (21). The second antibody raised against platelet Triton-insoluble cytoskeletons (anti-C antibody) also blocks fibrinogen binding to ADP-stimulated platelets (data not shown). The third antibody is a monoclonal antibody to the GPIIh-GPIII complex and has also been extensively characterized (15). These antibodies blocked clot retraction (Fig. 6) and thrombin-induced platelet aggregation (Fig. 7). We reasoned that since these antibodies were directed against platelet surface receptors important in platelet aggregation and clot retraction they might also block the association of fibrin with platelets and their cytoskeletons. Indeed this was the case. When cytoskeletons were prepared from platelets treated with Gly-Pro-Arg-Pro, 125I-fibrinogen and either control serum, anti-M1 antibody, anti-C antibody, or anti-GPIII, all three antibodies blocked association of fibrin to both platelets and their cytoskeletons (Table III). SDS gels of cytoskeletons from the antibody-treated cells show that the major proteins of the cytoskeleton (actin and myosin) are not altered by antibody treatment of the platelets. For example, when platelets were treated with anti-C antibody only the bands corresponding to fibrin were blocked from appearing in the cytoskeleton (Fig. 1, compare lanes 1 and 2 with lanes 3 and 4). These results, therefore, strongly suggest that the association of fibrin both to whole platelets and their cytoskeletons is specific and that anti-C antibody, anti-M1 antibody, and anti-GPIII antibody

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**TABLE II**

| Sample            | Maximal fibrin bound* | Concentration of fibrin added that gives half-maximal bound* |
|-------------------|-----------------------|-----------------------------------------------------------|
| Activated platelets| 3.7 ± 4.2             | 4.3 ± 2.6                                                 |
| Cytoskeletons of activated platelets | 2.6 ± 1.9             | 3.4 ± 3.0                                                 |
| Unactivated platelets| 0.013 ± 0.004*        |                                                           |
| Cytoskeletons of unactivated platelets | 0.010 ± 0.004*        |                                                           |

*Fibrin binding was performed as described under "Experimental Procedures." Maximal and half-maximal fibrin bound were determined from double reciprocal plots as shown in Fig. 3. The values given are the mean of four determinations performed on different donor platelets followed by the standard deviation.

*Unactivated platelets were treated with 14 μg/ml of radiolabeled fibrin and cytoskeletons prepared as described for activated platelets under "Experimental Procedures." The values are the mean of two determinations. Amount of fibrin bound to cytoskeletons when platelets were treated with a 100-fold excess of unlabeled fibrinogen was identical to the values obtained for unactivated platelets or cytoskeletons of unactivated platelets.
Fig. 6. The effect of anti-C, anti-M1, and anti-GPIII antibodies on clot retraction. Clot retraction was measured as described under “Experimental Procedures.” Sample 1, control serum; sample 2, anti-C antibody; sample 3, anti-M1 antibody, sample 4, control serum sample for sample 5; and sample 5, anti-GPIII antibody. Samples 4 and 5 were performed under different lighting conditions and the white areas close to the top of the solutions are artifacts of lighting (reflections). The retracted clot in sample 4 is at the bottom of the tube rather than at the top of the tube as is the case for sample 1.

Fig. 7. The effect of anti-C, anti-M1, and anti-GPIII antibodies on thrombin-induced platelet aggregation. 400-μl platelet suspensions (3.0 × 10^5 platelets/ml) containing either 100 μl of control serum, 100 μl of anti-C antibody, 100 μl of anti-M1 antibody, or 50 μl/ml of anti-GPIII IgG were aggregated with 0.2 unit/ml thrombin suspensions (3.0 μl). The effect of anti-C, anti-M1, and anti-GPIII antibodies on the amount of 125I-fibrin associated with platelets and cytoskeletons

Cytoskeletons or washed platelet pellets were prepared as described under “Experimental Procedures” from 0.5-ml platelet suspensions (3 × 10^5 platelets/ml) containing the following additions: 14 μg/ml of radiolabeled fibrinogen and 0.1 mg of Gly-Pro-Arg-Pro, and either 50 μl of antiserum or 50 μg/ml of anti-GPIII. Values are the mean of two determinations (separate donors) followed by the standard deviation.

| Antisera   | Pellets | Cytoskeletons | Inhibition of pellet | Inhibition of cytoskeletons |
|------------|---------|---------------|----------------------|-----------------------------|
|            | cpm     | %             |                      |                             |
| Control serum | 43,350 ± 1,532 | 38,350 ± 8,086 | 0                    | 0                           |
| Anti-C     | 7,666 ± 1,178 | 6,995 ± 1,870 | 82                   | 82                          |
| Anti-M1    | 14,440 ± 2,210 | 15,287 ± 1,986 | 67                   | 60                          |
| Anti-GPIII | 6,862 ± 3,255 | 3,606 ± 1,073 | 84                   | 91                          |

may be directed against platelet fibrin receptors, important in aggregation and clot retraction.

Identification of Platelet Surface Components Associated with Fibrin Receptors—Since both anti-M1 antibody and anti-C antibody block platelet aggregation and platelet fibrin association, it seemed reasonable to postulate that these heterogeneous antisera might recognize potential platelet fibrin receptors. In particular, a comparison of the proteins immunoprecipitated by these antibodies from 125I-surface-labeled detergent extracts of normal and thrombasthenic platelets would be expected to give information as to the molecular nature of the platelet fibrin receptor. In Fig. 8, the results of such an immunoprecipitation experiment are shown. The autoradiograms of the SDS gels show that total surface-labeled detergent extracts of normal and thrombasthenic platelets differ predominately in the diminished labeling of the thrombasthenic platelets in a 96,000-dalton species and a heterogeneous band of 130,000 daltons (Fig. 8, compare lane 1 with lane 2). The 96,000-dalton band and the 130,000-dalton band migrated 120,000 and 116,000 daltons, respectively, under reducing conditions (data not shown). These results are consistent with the reduced level of the glycoprotein complex GPIIb–GPIII in the platelets of these thrombasthenic patients, as has previously been reported (18, 19, 32). Anti-C antibody recognized the putative GPIIb–GPIII complex from both normal and thrombasthenic platelet extracts (Fig. 8, compare lane 5 with lane 6). Anti-M1 antibody recognized predominantly GPIIb–GPIII (Fig. 8, lanes 7 and 8). And, as expected, Anti-GPIII, recognized the GPIIb–GPIII complex (Fig. 8, lanes 9 and 10). In addition, anticytoskeleton antibody immunoprecipitated a 58,000-dalton protein (which analyzes as a 70,000-dalton protein on reduced gels, data not shown) and a large complex at about 220,000 daltons from both normal and thrombasthenic platelet extracts (Fig. 8, compare lane 5 with lane 6). Despite the fact that equal numbers of counts were immunoprecipitated (Fig. 8, compare lane 1 with lane 2), all three antibodies immunoprecipitated significantly less GPIIb–GPIII from thrombasthenic platelet extracts than from controls. This is in agreement with previous observations (18, 19). That platelets of our thrombasthenic patients contain less

Fig. 8. Immunoprecipitation analysis of detergent extracts of 125I-surface-labeled platelets. Platelets were labeled and immunoprecipitated with anti-C, anti-M1, and anti-GPIII, and the immunoprecipitates analyzed on 8% polyacrylamide SDS slab gels as described under “Experimental Procedures.” Gels were stained, dried, and autoradiograms prepared as described under “Experimental Procedures.” Lane 1, total platelet extract of normal platelets (100,000 cpm applied); lane 2, total platelet extract of thrombasthenic platelets (100,000 cpm applied); lane 3, control serum immunoprecipitate of normal platelets; lane 4, control serum immunoprecipitate of thrombasthenic platelets; lane 5, anti-C immunoprecipitate of normal platelets; lane 6, anti-C immunoprecipitate of thrombasthenic platelets; lane 7, anti-M1 immunoprecipitate of normal platelets; lane 8, anti-M1 immunoprecipitate of thrombasthenic platelets; lane 9, anti-GPIII immunoprecipitate of normal platelets; lane 10, anti-GPIII immunoprecipitate of thrombasthenic platelets.
than 10% of control levels of GPIIb-GPIII. Preimmune serum precipitates from control platelets revealed low levels of the 96,000-dalton species while the immunoprecipitates from thrombasthenic platelets revealed negligible radioactivity (Fig. 8, compare lane 3 with lane 4). These proteins represented the background levels of material nonspecifically adhering to the fixed bacterial immunoadsorbent in the presence of control serum (see under “Experimental Procedures”).

In summary, these results suggest that the GPIIb-GPIII complex may function in binding of fibrinogen or fibrin to the platelet and cytoskeleton, although these observations do not exclude the possibility that other proteins may play a role in the receptor complex.

**DISCUSSION**

The exact protein composition of the platelet cytoskeleton depends on its method of isolation but most agree that the Triton-insoluble residue of thrombin-stimulated platelets is composed primarily of actin filament bundles cross-linked with short myosin filaments (25). Recent evidence suggests a specific interaction between cytoskeletal proteins and plasma membrane components that bind various plasma ligands. Cytoskeletons prepared from platelets aggregated by thrombin have been shown to contain the surface membrane glycoproteins GPIIb and GPIII (33, 34). These glycoproteins are deficient or absent from thrombasthenic platelets (26, 37) which when stimulated with ADP fail to aggregate and to bind fibrinogen (13, 31). Exposure of fibrinogen receptors by ADP is a requirement for platelet aggregation (13, 14, 31). We have pursued these observations further by investigating the association with cytoskeletons of ligands which bind to the platelet surface, i.e. factor Va and factor Xa (8). We have presented evidence that factor Va becomes associated with cytoskeletal proteins from a site on the surface of the platelets only if it is first released from &alpha;-granules. Factor Xa was bound to thrombin-treated platelets and to cytoskeletons with identical binding constants. From these observations it was suggested that platelets are stimulated by thrombin to assemble cytoskeletons and release &alpha;-granule constituents including factor Va, which becomes bound to the platelet surface through receptors that irreversibly associate with cytoskeletal elements.

The present studies are consistent with the proposal that platelet fibrinogen is released from platelets, converted to fibrin by the action of thrombin either in solution or on the platelet surface, and becomes associated with cytoskeletons by a mechanism similar to that proposed for factor Va (8). The evidence for a specific association of fibrin with the platelet cytoskeleton is detailed in the following paragraphs.

(a) In the presence of Gly-Pro-Arg-Pro, a synthetic peptide that inhibits fibrin polymerization, cytoskeletons prepared from platelets treated with radiolabeled fibrinogen still bound over 20% of the 125I-radioactivity under conditions in which formation of insoluble fibrin polymers was blocked by more than 99% (Fig. 2). These results suggested that fibrin polymer was not simply copurifying nonspecifically by virtue of its insolubility during preparation of the cytoskeletons. Control experiments performed to show the effect of Gly-Pro-Arg-Pro on fibrinogen binding to ADP-stimulated platelets revealed that the levels of Gly-Pro-Arg-Pro used for this study (>1 mM) did in fact inhibit fibrinogen binding by as much as 50% as has been previously shown (38). However, thrombin-induced platelet aggregation was not affected. Therefore, Gly-Pro-Arg-Pro may not inhibit fibrin binding to the same extent as fibrinogen binding. All subsequent experiments were performed in the presence of Gly-Pro-Arg-Pro.

(b) The majority of fibrin associated with platelets is retained on their cytoskeletons (Figs. 3 and 4, Tables II and III) suggesting that surface receptors for fibrin present on the platelet surface are associated with the platelet cytoskeleton. The amount of radiolabeled fibrin associated with cytoskeletons is greater when radiolabeled fibrinogen is added to platelets prior to thrombin activation than when fibrin is added to thrombin-activated platelets presumably because in the latter case endogenous fibrin can compete for cytoskeletal binding sites. Endogenous cytoskeletal fibrin amounts to about 20% of the total platelet fibrinogen available for fibrin formation (Table I). Similarly, thrombasthenic platelets retain on their cytoskeletons the fibrin present on the platelet surface. However, thrombasthenic platelets bind approximately 80% less fibrin than controls both to whole platelets and their cytoskeletons (Figs. 4 and 5). These results firmly suggest that thrombasthenic platelets that fail to aggregate to most physiological platelet activators such as thrombin and ADP (32) and do not expose fibrinogen receptors upon ADP stimulation (13, 31) are deficient in platelet fibrin receptors.

(c) Three antibodies, one prepared against platelet cytoskeletons, one against membranes of Pronase-treated platelets (21), and the other a monoclonal antibody against the GPIIb-GPIII complex (15) blocked both thrombin-induced platelet aggregation (Fig. 7), ADP-induced aggregation (15, 21), clot retraction (Fig. 6), and fibrin association to both whole platelets and their cytoskeletons (Table III, Fig. 1). These experiments suggest that fibrin binding to platelets and cytoskeletons is a receptor-mediated process and that the platelet fibrin receptors recognized by these two antibodies are important in thrombin-induced platelet aggregation and in clot retraction.

In summary, the above series of experiments strongly suggest that fibrin becomes specifically associated with the platelet surface and platelet cytoskeleton presumably via integral membrane fibrin receptors that either directly interact with the platelet cytoskeleton or indirectly interact with the platelet cytoskeleton through some transmembrane protein. The identity of possible platelet fibrin receptors was probed by three antibodies that: (a) blocked platelet aggregation induced by thrombin and ADP (Fig. 7); (b) blocked clot retraction (Fig. 6); (c) blocked fibrin association to platelets and cytoskeletons (Fig. 1, Table III). These antibodies did not significantly change the basic composition of the cytoskeleton (Fig. 1).

The antibodies, one prepared against membranes from Pronase-treated membranes (21), one prepared against cytoskeletons, and the other, a monoclonal antibody to GPIIb-GPIII complex (15), recognized the following proteins from detergent extracts of 125I-surface-labeled normal and thrombasthenic platelets (Fig. 8): (a) the putative GPIIb-GPIII complex (recognized by anti-M1, anti-C, and anti-GPIII); (b) a large complex in excess of 220,000 daltons (recognized by anti-C); (c) a 58,000-dalton protein (70,000 daltons on reduced gels, recognized by anti-C). The labeling of the GPIIb-GPIII complex in thrombasthenic platelets relative to controls was always low, consistent with the deficiency of this complex in thrombasthenic platelets. The 58,000-dalton species is distinct from the 66,000-dalton cleavage product of GPIII present on chymotrypsin and Pronase-treated platelets (21).

From our immunoprecipitation data it is likely that the GPIIb-GPIII complex that is recognized by these antibodies tested plays an important role in binding fibrin to the platelet and its cytoskeleton. It is possible that the fibrin on the

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cytoskeleton first binds the platelet surface in the form of fibrinogen which then is converted to fibrin by the action of thrombin. Alternatively, fibrin and fibrinogen may bind to different sites on the platelet surface, which is most likely the case for polymerizing fibrin at its late stage (39).

In conclusion, the studies presented in this report are consistent with our previous observation (8) showing that factor Va becomes specifically associated with the platelet cytoskeleton presumably through platelet surface receptors. Both fibrinogen (40, 41) and factor V (42) are stored in the platelet α granules and released upon thrombin activation. We postulate that the released fibrinogen either binds to the platelet surface first and then it is converted to fibrin or it may be converted to fibrin in solution and subsequently become tightly bound to surface receptors that are associated with the internal cell cytoskeleton. This anchoring of cell surface receptors for fibrin to the internal cytoskeleton contains the platelet’s contractile mechanism may explain the phenomenon of clot retraction. In addition, the immobilization of cell surface receptors for fibrin and factor Va by means of the internal cytoskeleton may be an important mechanism by which the platelet stabilizes the platelet-fibrin clot and generates thrombin (43). Recent evidence from our laboratories also indicates association of another α granule protein, platelet factor 4, with the Triton-insoluble platelet cytoskeleton (44). The full characterization of the cell surface receptors and cytoskeletal elements important in the association of such important coagulation proteins as fibrin and factor Va with the platelet cytoskeleton awaits further study.

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