Time-Dependent Association Between Platelet-Bound Fibrinogen and the Triton X-100 Insoluble Cytoskeleton

By Ellinor I.B. Peerschke

Previous studies indicated a correlation between the formation of EDTA-resistant (irreversible) platelet-fibrinogen interactions and platelet cytoskeleton formation. The present study explored the direct association of membrane-bound fibrinogen with the Triton X-100 (Sigma Chemical Co, St Louis, MO) insoluble cytoskeleton of aspirin-treated, gel-filtered platelets, activated but not aggregated with 20 \( \mu \)mol/L adenosine diphosphate (ADP) or 150 mU/mL human thrombin (THR) when bound fibrinogen had become resistant to dissociation by EDTA. Conversion of exogenous \(^{125}\)I-fibrinogen to fibrin was prevented by adding Gly-Pro-Arg and neutralizing THR with hirudin before initiating binding studies. After 60 minutes at 22°C, the cytoskeleton of ADP-treated platelets contained 20% ± 12% (mean ± SD, \( n = 14 \)) of membrane-bound \(^{125}\)I-fibrinogen, representing 10% to 50% of EDTA-resistant fibrinogen binding. The THR-activated cytoskeleton contained 45% ± 15% of platelet bound fibrinogen, comprising 80% to 100% of EDTA-resistant fibrinogen binding. \(^{125}\)I-fibrinogen was not recovered with platelet cytoskeletons if binding was inhibited by the RGDS peptide, excess unlabeled fibrinogen, or disruption of the glycoprotein GP IIb-IIIa complex by EDTA-treatment. Both development of EDTA-resistant fibrinogen binding and fibrinogen association with the cytoskeleton were time dependent and reached maxima 45 to 60 minutes after fibrinogen binding to stimulated platelets. Although a larger cytoskeleton formed after platelet stimulation with thrombin as compared with ADP, no change in cytoskeleton composition was noted with development of EDTA-resistant fibrinogen binding. Examination of platelet cytoskeletons using monoclonal antibodies, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and Western blotting showed the presence of only traces of GP IIb-IIIa in the cytoskeletons of resting platelets, with no detectable increases after platelet activation or development of EDTA-resistant fibrinogen binding. These data suggest that GP IIb-IIIa-mediated fibrinogen binding to activated platelets is accompanied by time-dependent alterations in platelet-fibrinogen interactions leading to the GP IIb-IIIa independent association between bound fibrinogen and the platelet cytoskeleton.

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INTERACTIONS between fibrinogen and platelets mediate the formation of hemostatic platelet plugs and pathologic thrombi at sites of blood vessel injury. Fibrinogen binding to human platelets is divalent cation dependent, and requires platelet stimulation for receptor expression. The receptor has been identified as the glycoprotein IIb-IIIa complex (GP IIb-IIIa), a member of the cytoadhesin family involved in a variety of cell-cell and cell-substrate interactions.

Fibrinogen binding to human platelets is a multiphasic process culminating in platelet-fibrinogen interactions that are resistant to dissociation by EDTA (EDTA-resistant) or exchange with excess unlabeled fibrinogen. These platelet-fibrinogen interactions were referred to previously as “irreversible.” Stabilization of fibrinogen binding occurs via noncovalent interactions, in buffer as well as in plasma. Previous studies have shown that fibrinogen binding fails to become EDTA-resistant in the presence of inhibitors of actin polymerization. Moreover, a correlation emerged between the development of EDTA-resistant fibrinogen binding and the ability of platelets to retract fibrin clots.

The present study was undertaken to explore the hypothesis that development of EDTA-resistant fibrinogen binding involves the association of fibrinogen with the activated platelet cytoskeleton. The data support a time-dependent interaction between fibrinogen and the platelet cytoskeleton that correlates with the development of EDTA-resistant fibrinogen binding and is not directly mediated by GP IIb-IIIa.

MATERIALS AND METHODS
Platelet preparation. Blood from healthy volunteers was collected according to the guidelines of the Declaration of Helsinki. Blood collection tubes contained 0.1 vol 3.2% sodium citrate and 0.05 vol 1 mmol/L ascorbic acid per volume of whole blood. Platelet-rich plasma was obtained by centrifugation (380g, 15 minutes). Platelets were gel-filtered over Sepharose 2B (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with 0.01 mol/L HEPES (N-[2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid)-buffered modified Tyrode’s solution containing 2 mmol/L MgCl\(_2\) and 0.2 mg/mL bovine serum albumin (Sigma Chemical Co, St Louis, MO). In some studies, platelets were labeled with \(^{14}C\)-serotonin to allow quantitation of the release reaction after stimulation with adenosine diphosphate (ADP) or thrombin.

Fibrinogen binding. Binding studies were performed at 22°C using purified, \(^{125}\)I-labeled fibrinogen (25 to 250 \( \mu \)g/mL; specific activity 3248 cpm/\( \mu \)g). Platelets were stimulated with 20 \( \mu \)mol/L ADP or 150 mU/mL human thrombin (gift from Dr John Fenton, New York State Department of Health, Albany) in the presence or absence of 0.5 mmol/L Gly-Pro-Arg (Peninsula Laboratories, Inc, Belmont, CA). Thrombin was neutralized after 5 minutes with 1.5 U/mL hirudin (Sigma). Thrombin neutralization was verified by incubating unlabeled fibrinogen (1 mg/mL) with the combination of thrombin and hirudin for 60 minutes at 22°C. The reaction was stopped by brief (45 to 60 seconds) immersion in boiling water. The sample was passed through a 0.45-\( \mu \)m filter and fibrinopeptide A and B releases were measured subsequently by high performance liquid chromatography (courtesy of Dr D.K. Galanakis, State University of New York [SUNY] at Stony Brook).

Binding studies were initiated by adding \(^{125}\)I-fibrinogen. Nonspecific binding was
assessed in the presence of 10 mmol/L EDTA or excess unlabeled fibrinogen. Fibrinogen binding was quantified at various times, 5 to 60 minutes after platelet stimulation. The extent of EDTA-resistant fibrinogen binding was established 5 and 60 minutes after platelet stimulation by adding 10 mmol/L EDTA (final concentration) to separate aliquots of the platelet suspension and measuring fibrinogen dissociation 60 minutes later.6,7

Cytoskeleton preparation. Platelet cytoskeletons were precipitated 5 to 60 minutes after platelet stimulation and 125I-fibrinogen binding by adding 0.1 vol of buffer containing 10% Triton X-100, 0.15 mol/L NaCl, 0.01 mol/L Tris, pH 7.5, and 5 mg/mL leupeptin (Sigma) in the presence or absence of 100 mmol/L EDTA or 100 mmol/L EGTA (lysis buffer) per volume of platelet suspension. Cytoskeletons were allowed to form for 60 minutes at 4°C. Lysates were centrifuged (12,000g, 22°C), and the insoluble pellet washed twice with lysis buffer diluted 1:10 with 0.15 mol/L NaCl. Fibrinogen recovery with the cytoskeletal pellet was detected by measuring radioactivity associated with cytoskeletons in a gamma counter. Nonspecific precipitation of fibrinogen was evaluated by preparing cytoskeletons from platelet suspensions in which specific fibrinogen binding was inhibited either by the presence of 10 mmol/L EDTA or excess unlabeled fibrinogen. In addition, fibrinogen association with platelet cytoskeletons was evaluated when fibrinogen binding was inhibited by 100 μmol/L RGDS (Peninsula Laboratories) or disruption of the GP IIb-IIIa complex by EDTA treatment.15 The association between platelet-bound fibrinogen and the cytoskeleton was further evaluated by solubilizing stimulated platelets in the presence of 2 mg/mL DNase I (Sigma), which reverses actin polymerization.14 Fibrin polymerization in samples of thrombin-stimulated platelets was prevented by 500 μmol/L Gly-Pro-Arg (Peninsula Laboratories).16 To quantify the recovery of EDTA-resistant, platelet-associated fibrinogen with activated platelet cytoskeletons, aliquots of stimulated platelets were lysed and cytoskeletons prepared after reversibly bound fibrinogen had been dissociated by incubation with 10 mmol/L EDTA as described above.

The composition of platelet cytoskeletons was studied by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)3 and Western blotting. Cytoskeletal pellets were solubilized in SDS-containing sample buffer in the presence or absence of 2% β-mercaptoethanol and electrophoresed into 7.5% gels. The gels were stained with Coomassie Brilliant Blue and destained to visualize cytoskeletal proteins. The extent of actin precipitation in cytoskeletons from platelet suspensions in which specific fibrinogen binding was inhibited either by the presence of 10 mmol/L EDTA or excess unlabeled fibrinogen, in addition, fibrinogen association with platelet cytoskeletons was evaluated when fibrinogen binding was inhibited by 100 μmol/L RGDS (Peninsula Laboratories) or disruption of the GP IIb-IIIa complex by EDTA treatment.15 The association between platelet-bound fibrinogen and the cytoskeleton was further evaluated by solubilizing stimulated platelets in the presence of 2 mg/mL DNase I (Sigma), which reverses actin polymerization.14 Fibrin polymerization in samples of thrombin-stimulated platelets was prevented by 500 μmol/L Gly-Pro-Arg (Peninsula Laboratories).16 To quantify the recovery of EDTA-resistant, platelet-associated fibrinogen with activated platelet cytoskeletons, aliquots of stimulated platelets were lysed and cytoskeletons prepared after reversibly bound fibrinogen had been dissociated by incubation with 10 mmol/L EDTA as described above.

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The association of GP IIb and GP IIIa with the activated platelet cytoskeleton was examined more directly by Western blotting. Western blots were probed with monoclonal anti-GP IIb (SSZ2; AMAC, Inc, Westbrook, ME) or anti-GP IIIa (SSZ2; AMAC) antibodies and developed with nitroblue tetrazolium (Kirkegaard and Perry, Gaithersberg, MD). Because GP IIIa-IIIa is known to interact with the cytoskeleton after platelet aggregation,7 control platelet suspensions were aggregated for 5 minutes at 22°C and cytoskeletons analyzed as described above.

RESULTS

Both the development of EDTA-resistant fibrinogen binding and fibrinogen incorporation into the Triton X-100 insoluble cytoskeletal core increased with time after platelet stimulation (Fig 1). As reported previously,6,7 the majority of fibrinogen bound to ADP- or thrombin-stimulated platelets was reversibly associated immediately after equilibrium binding had been achieved (5 minutes after platelet stimulation). EDTA-resistant fibrinogen binding increased markedly 60 minutes after platelet stimulation with ADP (Fig 1A) or thrombin (Fig 1B), representing 40% ± 12% and 50% ± 11% of platelet-bound fibrinogen, respectively. These percentages are based on total, specific fibrinogen binding for ADP- and thrombin-stimulated platelets. At fibrinogen concentrations of 25 μg/mL and 250 μg/mL, 2,490 ± 747 and 20,000 ± 3,200 molecules of fibrinogen bound specifically to ADP-treated platelets, compared with 6,270 ± 1,160 and 38,200 ± 7,300 molecules of fibrinogen bound to thrombin-stimulated platelets. Approximately 50% of fibrinogen bound to ADP-treated platelets in an EDTA-resistant manner coprecipitated with the cytoskeleton (coefficient of variation [CV] = 55%) compared with 80% to 100% of fibrinogen that bound to thrombin-treated platelets and became EDTA resistant (CV = 24%).

Fig 1. Development of EDTA-resistant fibrinogen binding and fibrinogen incorporation into the cytoskeleton of platelets 5 and 60 minutes after platelet stimulation with 20 μmol/L ADP (A) or 150 μmol/L thrombin (B). Fibrinogen binding occurred in the presence of 250 μg/mL 125I-fibrinogen. EDTA-resistant fibrinogen binding and fibrinogen incorporation into the Triton X-100 insoluble cytoskeleton are expressed relative to total, specific fibrinogen binding observed after platelet stimulation with ADP or thrombin. Error bars indicate mean ± SD, n = 14.
The coprecipitation of fibrinogen with platelet cytoskeletons was dependent on specific platelet-fibrinogen interactions as summarized in Table 1. When fibrinogen binding was inhibited by RGDS, excess unlabeled fibrinogen, or disruption of the GP IIb-IIIa complex, only nonspecific fibrinogen association with platelet cytoskeletons was observed. In addition, fibrinogen failed to precipitate with platelet cytoskeletons prepared in the presence of DNAse I (Table 2). These data (1) argue against the association of fibrinogen with cytoskeletal components after platelet lysis, and (2) suggest a requirement for recovery of polymerized actin.

Because the composition of platelet cytoskeletons, most notably the extent of myosin precipitation, is affected by the presence or absence of magnesium,28 similar studies were performed in which cytoskeletons were prepared in the presence of EGTA or in the complete absence of chelating agents. Table 2 illustrates that the divalent cation composition of the cytoskeleton lysis buffer had no effect on the recovery of fibrinogen with platelet cytoskeletons.

The observed increase in fibrinogen recovery with cytoskeletons of thrombin-stimulated platelets was also not the result of fibrin formation and the previously described insolubility of fibrin in nonionic detergent as (1) thrombin was neutralized with hirudin before initiating fibrinogen binding; (2) concentrations of hirudin used to inactivate thrombin-inhibited detectable fibrinopeptide A and B release as determined by high performance liquid chromatography in separate studies; and (3) similar proportions of bound fibrinogen were recovered with cytoskeletons from platelets activated with thrombin in the presence and absence of Gly-Pro-Arg (Table 1). However, in the presence of peptide, some inhibition in total fibrinogen binding was shown, as described by others.30

Cytoskeletons of platelets activated but not aggregated with either ADP or thrombin were composed of actin, myosin heavy and light chains, α-actinin, and actin binding protein (Fig 2). The extent of actin precipitation with the cytoskeleton is shown in Table 3, and agrees with values reported in the literature.21 125I-fibrinogen associated with these cytoskeletons was indistinguishable from native fibrinogen (Fig 3). Fibrinogen α-chains were weakly iodinated compared with β- and γ-chains, which comigrated during electrophoresis using the Laemmli system.30

Comparison of cytoskeletons derived from thrombin- and ADP-treated platelets showed increases in all cytoskeletal components after platelet stimulation with thrombin (Fig 2). In the absence of Gly-Pro-Arg, fibrin derivatives, present at the top of the gel coprecipitated with cytoskeletons from thrombin-treated platelets, reflecting the conversion of released platelet fibrinogen to fibrin before binding studies were initiated. Indeed, studies with 14C-serotonin-labeled platelets indicated the release of 65% ± 10% of platelet dense granule contents following stimulation with 150 μU/mL thrombin at 22°C, compared with 7% ± 3% release after stimulation with 20 μmol/L ADP (mean ± SD, n = 3). However, it is interesting to note that significant fibrin association with cytoskeletons from thrombin-stimulated platelets failed to occur when platelets were stimulated in the presence of EDTA when fibrinogen binding was inhibited (Fig 2).

Consistent with reports demonstrating maximum cytoskeleton formation 5 to 15 minutes after platelet stimulation,39 no changes in individual cytoskeletal proteins were noted 5 and 60 minutes after platelet stimulation, ie, during the time required for development of EDTA-resistant fibrinogen binding. Maximum fibrinogen incorporation into Triton insoluble cytoskeletons occurred 45 to 60 minutes after fibrinogen binding to thrombin-stimulated platelets. This correlated with the time course for developing EDTA-resistant fibrinogen binding (Fig 4).

The coprecipitation of GP IIb-IIIa with platelet cytoskeleton was investigated by two independent approaches. First, recovery of GP IIb-IIIa was explored using the 10E5 MoAb. Because GP Ib has been described in Triton X-100 insoluble platelet residues,22,23 control studies were performed using the 6D1 MoAb. The data (Table 2) suggest that relative to the amount of platelet-bound fibrinogen, only traces of surface-bound 10E5 were recovered with platelet cytoskeletons prepared either 5 or 60 minutes after fibrinogen binding to thrombin-stimulated platelets. At the same times, 49% ± 12% and 44% ± 9% of surface-bound 6D1 was recovered.

Similar results were obtained when platelet membrane GP incorporation into the Triton insoluble cytoskeleton was examined more directly by SDS-PAGE and Western blotting (Fig 5). Although both GP IIb and GP IIIa could

### Table 1. Binding of 125I-Fibrinogen to Platelets and Incorporation of Bound Fibrinogen Into the Triton X-100 Insoluble Cytoskeleton 60 Minutes After Platelet Stimulation With Thrombin

<table>
<thead>
<tr>
<th>Platelet Stimulation</th>
<th>125I-Fibrinogen Binding (cpm)*</th>
<th>125I-Fibrinogen Recovery With Cytoskeleton (cpm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In the presence of:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer</td>
<td>14,596 ± 1,497</td>
<td>9,022 ± 1,053</td>
</tr>
<tr>
<td>RGDS (100 μmol/L)</td>
<td>2,381 ± 206</td>
<td>514 ± 75</td>
</tr>
<tr>
<td>Fibrinogen (10 mg/mL)</td>
<td>3,846 ± 1,205</td>
<td>3,756 ± 943</td>
</tr>
<tr>
<td>GLY-PRO-ARG (50 μmol/L)</td>
<td>6,875 ± 1,566</td>
<td>6,006 ± 1,603</td>
</tr>
<tr>
<td><strong>After pretreatment with:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTAT</td>
<td>3,398 ± 600</td>
<td>3,114 ± 1,140</td>
</tr>
<tr>
<td>CA1- EDTAT</td>
<td>11,313 ± 1,387</td>
<td>7,305 ± 1,406</td>
</tr>
</tbody>
</table>

*Fibrinogen binding is expressed in raw cpm per platelet pellet derived from 1 mL of platelets (250,000/μL). No adjustment is made for nonspecific binding. Non-specific binding, assessed in the presence of 10 mmol/L EDTA, comprised 2,986 ± 165 cpm/platelet pellet. Fibrinogen recovered with the cytoskeletons is expressed in raw cpm per cytoskeleton preparation obtained from 1 mL of platelet suspensions. Nonspecific precipitation of fibrinogen with the cytoskeleton comprised 3,061 ± 560 cpm. Data represent the mean ± SD of six separate experiments. Platelets were stimulated with 150 μU/mL thrombin in the presence or absence of RGDS, unlabeled fibrinogen, or GLY-PRO-ARG. Thrombin was neutralized with hirudin and 125I-fibrinogen (250 μg/mL) added as described in Materials and Methods. After 60 minutes, aliquots of the platelet suspension were removed to assess fibrinogen binding. Separate aliquots were solubilized in Triton X-100 lysis buffer for cytoskeleton preparation.

†EDTA treatment consisted of preincubating unstimulated platelets with 10 mmol/L EDTA (37°C, pH 8.1, 60 minutes) to dissociate the GP IIb-IIIa complex.13 Control platelets were preincubated with Ca2+ -EDTA.
Table 2. Coprecipitation of Fibrinogen, Anti-GP Ib (6D1), and Anti-GP IIb-IIIa (10E5) MoAbs With the Cytoskeleton of Thrombin-Treated Platelets

<table>
<thead>
<tr>
<th>Ligand: Time After Fibrinogen Binding</th>
<th>EDTA</th>
<th>EGTA</th>
<th>No Chelator</th>
<th>DNAse I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min</td>
<td>10 ± 5</td>
<td>8 ± 3</td>
<td>11 ± 5</td>
<td>4 ± 3</td>
</tr>
<tr>
<td>60 min</td>
<td>44 ± 15</td>
<td>49 ± 12</td>
<td>48 ± 9</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>10E5:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min</td>
<td>0.4 ± 0.3</td>
<td>0.3 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>ND</td>
</tr>
<tr>
<td>60 min</td>
<td>0.5 ± 0.3</td>
<td>0.3 ± 0.1</td>
<td>0.3 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td>6D1:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min</td>
<td>49 ± 12</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>60 min</td>
<td>44 ± 9</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Platelets were stimulated with 150 mU/mL thrombin as described in Materials and Methods. Labeled or unlabeled fibrinogen (250 μg/mL) was added. Five and 60 minutes after fibrinogen binding at 22°C, samples incubated with labeled fibrinogen were processed to assess fibrinogen binding and precipitation with cytoskeletons prepared in the presence or absence of EDTA, EGTA, or DNAse I. Saturating concentrations of MoAbs were added to separate aliquots of platelet suspensions incubated with unlabeled fibrinogen. After 30 minutes, antibody binding to intact platelets and recovery with cytoskeletons was quantified as described in Materials and Methods. Data represent mean ± SD, n = 3.

**DISCUSSION**

Fibrinogen is a cofactor for normal platelet aggregation,1 It binds specifically to platelet membrane GP IIb-IIIa complexes, which are capable of interacting with a variety of adhesive proteins including fibronectin and von Willebrand factor. Platelet stimulation by a variety of agonists results in the exposure of binding sites for adhesive proteins at the platelet surface. In plasma, it is likely that fibrinogen, which is considerably more abundant than fibronectin or von Willebrand factor, binds preferentially to stimulated platelets. Although considerable attention has focused on events leading to and supporting the interaction of platelets with adhesive glycoproteins, little is known about postligand binding events.

A variety of proteins have been reported previously to associate with the Triton X-100 insoluble skeleton of activated platelets.24,25 The association of fibrin or fibronectin with the activated platelet cytoskeleton requires the initial binding of these ligands to the GP IIb-IIIa complex. Although the role of GP IIb-IIIa in either fibronectin or fibrin interactions with the platelet cytoskeleton has not been reported, the present study provides direct evidence that events leading to the specific association between fibrinogen and the activated platelet cytoskeleton are not mediated by GP IIb and/or GP IIIa.

The data suggest a close temporal relationship between activated platelet cytoskeletons. Cytoskeletons were prepared in EDTA and leupeptin containing lysis buffer as described in Materials and Methods, solubilized in sample buffer containing 1% SDS, and electrophoresed into 7.5% gels following reduction with 2% β-mercaptoethanol. Cytoskeletons were derived from platelets stimulated with 20 μmol/L ADP (lanes 1 through 4) or 150 mU/mL thrombin (lanes 5 through 8) in the presence (lanes 1, 2, 5, and 6) or absence (lanes 3, 4, 7, and 8) of EDTA. Cytoskeletons were prepared 5 minutes (lanes 1, 3, 5, and 7) or 60 minutes (lanes 2, 4, 6, and 8) after platelet stimulation and initiation of fibrinogen binding in the presence of 25 μg/mL [3H]-fibrinogen. Platelet stimulation with thrombin was performed in the absence of Gly-Pro-Arg. The migration of molecular weight standards is shown at left. The gel was stained with Coomassie Brilliant Blue.
Table 3. Actin Content of Platelet Cytoskeletons

<table>
<thead>
<tr>
<th>Platelet Activation</th>
<th>Actin Content (% of total)*</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>17 ± 8</td>
</tr>
<tr>
<td>ADP (5 min)</td>
<td>65 ± 25</td>
</tr>
<tr>
<td>(60 min)</td>
<td>67 ± 28</td>
</tr>
<tr>
<td>Thrombin (5 min)</td>
<td>88 ± 30</td>
</tr>
<tr>
<td>(60 min)</td>
<td>85 ± 35</td>
</tr>
<tr>
<td>Thrombin (60 min) + DNAse I</td>
<td>20 ± 12</td>
</tr>
</tbody>
</table>

*Determined by densitometric scanning of bands resolved on SDS-polyacrylamide gels (mean ± SD, n = 4). Platelets were stimulated as described in Materials and Methods and lysed at the indicated times in the presence of EDTA-containing buffer with and without DNAse I. Cytoskeletons were analyzed by SDS-PAGE using 7.5% gels.

the development of EDTA-resistant fibrinogen binding and the recovery of fibrinogen in the Triton X-100 insoluble cytoskeleton, particularly after platelet stimulation with thrombin. To ensure that fibrinogen association with the activated platelet cytoskeleton did not occur in the lysis buffer, fibrinogen-cytoskeleton interactions were evaluated using platelets stimulated in the presence of RGDS, or excess unlabeled fibrinogen or platelets whose GP IIb-IIIa receptor complex was disrupted by EDTA-treatment when specific fibrinogen binding was inhibited. Under these conditions, fibrinogen recovery with activated platelet cytoskeletons was neither specific nor saturable (data not

Fig 3. Autoradiograph of fibrinogen recovered in the Triton X-100 insoluble cytoskeleton of platelets 60 minutes after stimulation with ADP (20 μmol/L) or thrombin (150 μU/mL) in the presence of 25 μg/mL 125I-fibrinogen. Cytoskeletons were solubilized in sample buffer containing 1% SDS and electrophoresed into 7.5% polyacrylamide gels after reduction with 2% β-mercaptoethanol. (1) Native 125I-fibrinogen; (2) 125I-fibrinogen recovered with cytoskeletons derived from thrombin-treated platelets; and (3) 125I-fibrinogen recovered with the cytoskeleton of ADP-treated platelets, as described in Materials and Methods.

Fig 4. Time course for development of EDTA-resistant fibrinogen binding (A) and fibrinogen incorporation into platelet cytoskeletons (B). Platelets were activated with 150 μU/mL thrombin. Thrombin was neutralized with hirudin, and fibrinogen binding and association with the cytoskeleton quantified as described in Materials and Methods in the presence of 250 μg/mL 125I-fibrinogen. EDTA-resistant fibrinogen binding and fibrinogen incorporated into the cytoskeleton are expressed relative to specific fibrinogen binding to intact platelets. Error bars reflect mean ± SD, n = 3.

Fig 5. Western blot of GP IIb (A) and GP IIIa (B) associated with cytoskeletons from platelets 5 minutes (lanes 2, 4, and 6) and 60 minutes (lanes 3, 5, and 7) after platelet stimulation with 150 μU/mL thrombin and initiation of fibrinogen binding in the presence of 250 μg/mL 125I-fibrinogen. Cytoskeletons were prepared in the presence of EDTA (lanes 2 and 3), EGTA (lanes 4 and 5), or in the absence of chelator (lanes 1, 6, 7, and 8), solubilized in SDS, electrophoresed into 7.5% polyacrylamide gels without reduction, and blotted onto nitrocellulose. Whole lysed platelets (lane 1) and cytoskeletons obtained from platelets aggregated in the presence of thrombin for 5 minutes (lane 8) served as controls. GP IIb and IIIa were probed with MoAbs and visualized with nitroblue tetrazoleum.
shown). Thus, the association of fibrinogen with platelet cytoskeletons appears to be dependent on the ability of fibrinogen to bind to platelet membrane receptors. Moreover, fibrinogen association with the cytoskeleton of activated platelets was dependent on the length of time of platelet-fibrinogen interaction, as maximal fibrinogen recovery occurred 45 minutes after fibrinogen binding to stimulated platelets, and failed to occur when actin incorporation into the cytoskeleton was inhibited by DNase I.

Because fibrin has been reported to precipitate nonspecifically with platelet cytoskeletons because of its insolubility in nonionic detergents, fibrin formation of exogenously added I-labeled fibrinogen was prevented by stimulating platelets with thrombin and neutralizing thrombin with hirudin before binding studies were begun. In addition, studies were performed in the presence of the Gly-Pro-Arg peptide, which prevents fibrin assembly, and similar results were obtained. Moreover, fibrinogen association with the cytoskeleton of activated platelets was not limited to platelet stimulation with thrombin. Platelets stimulated with ADP also showed enhanced fibrinogen recovery in the cytoskeleton prepared 60 minutes after platelet stimulation, when the development of EDTA-resistant fibrinogen binding was complete. However, in comparison with fibrinogen incorporation into thrombin cytoskeletons, the extent of fibrinogen incorporation into the cytoskeleton of ADP-treated platelets was more variable, and rarely accounted for more than 50% to 60% of irreversibly bound fibrinogen. The enhanced incorporation of fibrinogen into the thrombin cytoskeleton may reflect not only the amount of cytoskeleton formed when platelets are stimulated with thrombin, but also the release of platelet granule contents, particularly thrombospondin, which has been reported to enhance the stabilization of fibrinogen binding.

The increased association of fibrinogen with platelet cytoskeletons prepared 60 minutes, compared with 5 minutes, after platelet stimulation with either ADP or thrombin may reflect specific alterations in the physical and/or biochemical properties of platelet-fibrinogen interactions. Indeed, recent studies have shown time-dependent changes in platelet-bound fibrinogen accessibility to antibody and enzyme probes.

Furthermore, present data illustrate that although fibrinogen incorporation into activated platelet cytoskeletons requires an initial interaction between fibrinogen and GP IIb-IIIa, only trace levels of GP IIb and GP IIIa were detected in cytoskeletons of ADP- or thrombin-activated platelets. The absence of significant amounts of GP IIb-IIIa from activated platelet cytoskeletons was verified using the 10E5 MoAb and was unaffected by the divalent cation content of the cytoskeleton lysis buffer.

As similar amounts of GP IIb and GP IIIa were recovered from resting as well as activated platelets, these glycoproteins may represent a previously described subtraction of GP IIb-IIIa associated with the Triton insoluble cytoskeleton of resting platelets. Platelet aggregation resulted in a significant increase in the incorporation of both GP IIb and GP IIIa with the platelet cytoskeleton. It has been estimated that 24% to 30% of platelet GP IIb-IIIa can be recovered with cytoskeletons of aggregated platelets.

Thus, the present study provides direct evidence for alterations in platelet-fibrinogen interactions leading to the development of EDTA-resistant fibrinogen binding and the GP IIb-IIIa independent association of fibrinogen with the Triton X-100 insoluble cytoskeleton. In light of recent data demonstrating the uptake of exogenous fibrinogen into platelet α-granules, these observations may represent the initial steps involved in this process.

ACKNOWLEDGMENT

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Time-dependent association between platelet-bound fibrinogen and the Triton X-100 insoluble cytoskeleton

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