Molecular Requirements for the Interaction of Thrombospondin With Thrombin-Activated Human Platelets: Modulation of Platelet Aggregation

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We have investigated the molecular requirements for thrombospondin (TSP) to bind to the platelet surface and to support the subsequent secretion-dependent platelet aggregation. For this, we used two distinct murine monoclonal antibodies (MoAbs), designated MAI and MAII, raised against human platelet TSP, and three polyclonal antibodies, designated R3, R6, and R5, directed against fusion proteins containing the type 1 (Gly 385-Ile 522), type 2 (Pro 559-Ile 669), and type 3 (Asp 784-Val 932) repeating sequences, respectively. Among them, R5 and R6, but not R3, inhibited thrombin-induced aggregation of washed platelets and the concomitant secretion of serotonin. These antibodies, however, did not inhibit the expression of TSP on thrombin-activated platelets, as measured by the binding of a radiolabeled MoAb to TSP, suggesting that they may inhibit platelet aggregation by interfering with a physiologic event subsequent to TSP binding. In contrast, MoAb MAII, which reacts with an epitope located within the heparin-binding domain of TSP, inhibited both TSP surface expression and platelet aggregation/secretion induced by thrombin. In addition, this MoAb inhibited in a dose-dependent manner (IC50 = 0.5 μmol/L) the interaction of [125I]-TSP with immobilized fibrinogen and platelet glycoprotein IV, both potential physiologic receptors for TSP on thrombin-activated platelets. These results indicate that the interaction of TSP with the surface of activated platelets can be modulated at the level of a specific epitope located within the amino terminal heparin-binding domain of the molecule. Thus, selective inhibition of the platelet/TSP interaction may represent an alternative approach to the inhibition of platelet aggregation.

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THROMBOSPONDIN (TSP) is a 420-Kd multifunctional glycoprotein (GP) with the ability to bind to several macromolecules such as heparin, fibronectin, fibrinogen, plasminogen, histidine-rich GP, sulfated glycolipids, laminin, and collagen. TSP also interacts with cell surfaces and appears to be a major component of extracellular matrices. Once bound to the cells, TSP is thought to participate in cell-substratum and cell-cell interactions and to regulate the cellular proliferation.

In platelets, TSP has been earlier recognized as a major α-granule constituent that was secreted upon cell activation and became associated with the platelet membrane. The association of TSP with platelets was closely paralleled by the appearance of fibrinogen (or fibrin) on the platelet surface, thus suggesting that fibrinogen could constitute its receptor on activated platelets. Indeed, subsequent experiments performed with an anti-TSP antibody have indicated that TSP could stabilize the interaction of fibrinogen with the GPIIb-IIIa complex leading to irreversible platelet aggregate formation. However, this proposal was challenged when it was reported that platelets from thrombasthenic patients, deficient in both GPIIb-IIIa and fibrinogen, and platelets from a severe afibrinogenemic patient expressed normal amounts of TSP upon platelet activation.

More recent studies have identified GPIV (also known as GPIIIb or CD36), an integral plasma membrane GP, as a platelet receptor for TSP, therefore providing a suitable explanation to the apparent normal binding of TSP to thrombasthenic and afibrinogenemic platelets. However, there is some evidence that TSP may be interacting with both GPIV and fibrinogen on thrombin-activated normal platelets coexpressing those two molecules. This possibility is supported by the observations that antibodies to either GPIV or fibrinogen inhibit the expression of TSP on activated platelets.

Following this line, if the same molecule of TSP may actually be linked to one molecule of GPIV and one molecule of fibrinogen simultaneously, as proposed by Nachman et al, these interactions should most likely involve different domains within the TSP molecule. Indeed, previous studies have shown that platelet aggregation could be inhibited by antibodies directed against two opposite regions within the TSP molecule. However, the effect of these antibodies on the specific interaction of TSP with fibrinogen and/or GPIV was not investigated.

In the present study, we have used monospecific antibodies to different domains of the TSP molecule to delineate the molecular requirements for the binding of TSP to its specific receptors on activated platelets. In so doing, we have obtained evidence that blocking a specific epitope within the heparin-binding domain of TSP inhibits its interaction with purified fibrinogen and GPIV and alters its expression on the surface of thrombin-activated platelets. This regulation appears to be functionally significant because it correlates with a decrease in the platelet aggregation/secretion response.

MATERIALS AND METHODS

TSP and Fibrinogen

TSP was purified from the supernatant of thrombin-activated human platelets by heparin-Sepharose affinity chromatography followed by Sepharose-4B gel filtration, essentially as described by Lawler et al with minor modifications. Calcium (2 mmol/L) was added to prevent fibrinogen from fibrin polymerization.

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maintained throughout the procedure. The TSP preparation was greater than 95% pure when analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Absence of thrombin, plasminogen, fibrinogen, and fibronectin contamination was further established by immunoblotting with monospecific antibodies. Purified TSP was stored in aliquots at -80°C in 10 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 2 mmol/L CaCl₂ (TBS) in the presence of 20% (wt/vol) sucrose.

Human fibrinogen was from IMCO (Stockholm, Sweden). The clotability of this fibrinogen was greater than 95%. The protein migrated as a single band on 5% SDS-PAGE with an estimated molecular weight (Mr) of 330 Kd. The reduced protein showed a typical distribution of αα-, ββ-, and γ-chains with absence of degradation products.

**Antibody Preparation and Characterization**

**Monoclonal antibodies (MoAbs).** The preparation and characterization of mouse MoAbs MAI and MAII, which were raised against human platelet TSP, has been reported. These antibodies belong to the IgG1 subclass and they recognize an epitope located in the type 3 repeating sequences and in the NH₂-terminal heparin-binding domain of TSP, respectively. The mouse MoAb FA6-152 (IgG1) was a gift from Dr. L. Edelman (Institut Pasteur, Paris, France). Its specificity towards platelet membrane GPIV and binding characteristics to platelets have been reported in earlier studies. AliG were purified from ascitic fluids by chromatography and used as an irrelevant IgG. Purified TSP was stored in aliquots at -80°C in the presence of 20% (wt/vol) sucrose.

**Polyclonal antibodies to fusion proteins.** Fusion proteins were produced using endothelial cell cDNA clones and appropriately positioned sites for restriction enzymes. The portion of cDNA that encodes for the region of the type 1 repeats between glycine (385) to isoleucine (559) was produced by digesting clone M4 with Not I and Bgl II digest of clone M10. The portion of cDNA that encodes for the region of the type 2 repeats between proline (559) to isoleucine (669) was produced by digesting clone M4 with Mnl I. The portion of cDNA that encodes for the region of the type 3 repeats between aspartic acid (784) to valine (932) was produced by digesting clone M5 with HinclI. Various size EcoRI linkers were added to provide the correct reading frame relative to β-galactosidase and the DNA was cloned into the λgt11 phage. The DNA was packaged using the Packagene system (Promega, Madison, WI) following the manufacturer’s protocol. The packaged phages were used to infect Escherichia coli strain Y1089 and lysogens were selected. Cultures were grown overnight at 30°C in L2 media containing 25 µg/mL of ampicillin. The cultures were then diluted 1:8 with the same media and incubated at 30°C for 1 hour. The cultures were then incubated at 45°C for 15 minutes, isopropyl B-D-thiogalactopyranoside (IPTG) was added to a final concentration of 10 mmol/L, and the cultures were allowed to grow at 37°C for 2 hours. The cells were pelleted by centrifugation, resuspended in 0.25 mol/L NaCl, 50 mmol/L Tris-HCl, pH 8.0, and 0.2 mg/mL lysozyme, and incubated at 0°C for 30 minutes. The cells were frozen and thawed once; diisopropyl fluorophosphate was added to a final concentration of 1 mmol/L and the cells frozen and thawed again. The sample was sonicated and centrifuged to remove cellular debris. The supernatant was applied to a Protosorb lacZ column (Promega) and eluted at high pH following the manufacturer’s protocols.

Antisera were produced in New Zealand white rabbits by subcutaneous injections at multiple sites of purified fusion proteins emulsified with an equal volume of Freund’s complete adjuvant. The rabbits received a booster injection after 4 to 6 weeks of antigen emulsified in Freund’s incomplete adjuvant. The sera are designated R3, R5, and R6 for the type 1, type 3, and type 2 fusion proteins, respectively (Fig 1). IgG were purified on protein A-Sepharose as described above.

**Radioiodination of Proteins**

Purified proteins were radiolabeled with carrier-free Na⁺¹¹ (CIS International, Gif-sur-Yvette, France) using the chloramine T method to a specific activity of about 0.2 µCi/µg protein. Residual free Na⁺¹¹ was removed from the protein by gel filtration through a Sephadex G25 column (Pharmacia-LKB). The radiolabeled proteins showed no structural alterations as compared with the unlabeled counterparts when analyzed by SDS-PAGE. The precipitability in 20% (wt/vol) trichloroacetic acid was more than 95%. Protein concentrations were determined by light absorption at 280 nm using published extinction coefficients. The radiolabeled proteins were stored in aliquots at -80°C in the presence of 2 mg/mL bovine serum albumin (BSA; grade V, Sigma), except TSP that was stored in the presence of 20% (wt/vol) sucrose.

Platelet surface proteins were radiolabeled using the lactoperoxidase-catalyzed ¹¹¹I-labelling procedure.

**Platelet Preparation and Aggregation Studies**

Blood was collected from healthy adult volunteers into acid/citrate/dextrose anticoagulant in accordance with the guidelines of the Declaration of Helsinki. Platelet-rich plasma was obtained by centrifugation for 15 minutes at 120g and 22°C and incubated with 0.5 µmol/L 1,5-dihydroxy-2,3-5,6-tetramethyl-4H-pyran-4-one (40 mCi/mmol; CIS International) for 30 minutes at 22°C. Platelets were then washed by repeated centrifugations in a modified Tyrode’s buffer, pH 6.5, containing 25 µg/mL ascorbate (grade I, Sigma), 0.1 µmol/L prostaglandin E₁ (Sigma), and 3.5 mg/mL BSA as previously described. The platelets were resuspended at 250,000/µL in Tyrode buffer (137 mmol/L NaCl, 3 mmol/L KCl, 12 mmol/L NaHCO₃, 0.3 mmol/L NaHPO₄, 2 mmol/L CaCl₂, 1 mmol/L MgCl₂, 5.5 mmol/L glucose, 5 mmol/L HEPES), pH 7.4, containing 3.5 mg/mL BSA.

**Fig 1. Schematic representation of the TSP subunit, adapted from Lawler and Hynes.** The NH₂-terminal part of the molecule is at left. The position of the type 1 (a), type 2 (c), and type 3 (d) repeats are indicated. The fragments corresponding to the fusion proteins are identified by their NH₂-terminal and COOH-terminal amino acid. The antibodies produced against these sequences are indicated underneath each fragment. MAI and MAII react with an epitope located in the type 3 repeating sequences and in the NH₂-terminal heparin-binding domain, respectively.
Platelet aggregation and ³C-serotonin secretion were performed as described using a dual-channel aggregometer (Chronolog Corp, Havertown, PA) in which stirred platelets were activated by the addition of 0.05 U/mL human α-thrombin (3,000 U/mg; Sigma). Aggregation was recorded as decrease in optical density. ³C-serotonin secretion was measured in the presence of 3 μmol/L chlorimipramine (Ciba Geigy, Basel, Switzerland) to avoid its reuptake.

**Binding Assay for Surface Expression of Platelet TSP**

Surface-bound platelet TSP was determined by measuring the binding of a ¹²⁵I-labeled monoclonal anti-TSP antibody (5G11) to washed platelets preloaded with ³C-serotonin, as we previously described. Briefly, 500 μL aliquots of platelets at 250,000/μL, preloaded with ³C-serotonin, were activated for 3 minutes at 37°C with 0.05 U/mL α-thrombin. Hirudin (2 U/mL) was added at the end of the incubation and aliquots (200 μL) were taken for determination of ³C-serotonin release. The platelets were then diluted to 125,000/μL with Tyrode buffer and 20 μg/mL ¹²⁵I-5G11 was added. After 30 minutes at 22°C, triplicate volumes (100 μL) were layered over 0.5 mL of 20% (wt/wt) sucrose and centrifuged for 5 minutes at 12,000g. The radioactivity associated with the pellets was counted in a gamma counter (Beckman Instruments Inc, Fullerton, CA) and corrected for background radioactivity.

**Competitive Radioimmunoassay for Anti-TSP Antibodies**

Polystyrene microtiter wells (Costar Corporation, Cambridge, MA) were coated in duplicate with MoAb 5G11 by incubating each well with 100 μg 5G11 at 10 μg/mL in TBS. After 18 hours at 22°C, the wells were rinsed twice with TBS, pH 7.4, containing 2 mmol/L CaCl₂, 1 mmol/L MgCl₂, and 0.05% (vol/vol) Tween 20 (TBS-Tween), and then incubated for 1 hour in the same buffer with 1.5% (wt/vol) BSA to block unreacted sites. Wells were rinsed twice and incubated with ¹²⁵I-TSP (0.1 μg) in TBS-Tween, alone or in the presence of increasing concentrations of anti-TSP antibodies. After 3 hours at 22°C, the wells were washed four times with TBS-Tween, then cut out, and the radioactivity associated with each well was counted. Specific binding was calculated by subtracting nonspecific binding (less than 5%) corresponding to ¹²⁵I-TSP binding to wells coated with an irrelevant IgG (MOPC 21) or microtiter wells coated with FA6-152 were incubated with buffer lacking platelet lysate. Specific binding was obtained by subtracting background radioactivity (less than 15%) from total radioactivity.

**Western Blot Procedure**

Washed platelets were solubilized in 10 mmol/L Tris, pH 7.0, 150 mmol/L NaCl, 3 mmol/L EDTA, 5 mmol/L N-ethylmaleimide by heating at 100°C for 5 minutes in the presence of 2% (wt/vol) SDS, with or without 5% (vol/vol) 2-mercaptoethanol. Samples (50 μg) were electrophoresed on a 5 to 12% gradient acrylamide gel according to Laemmli. The proteins were electrophoretically transferred to nitrocellulose papers and probed with anti-TSP antibodies, essentially as described by Kieffer et al. Bound IgG were detected by incubation with radiolabeled protein A (280,000 cpn/mg; New England Nuclear, Boston, MA) followed by autoradiography on Kodak X-Omat MA films (Kodak-Pâthe, Paris, France).

**RESULTS**

**Characterization of the Anti-TSP Antibodies**

Polyclonal rabbit antibodies raised against fusion proteins encompassing different domains of the TSP molecule (Fig 1) reacted with the intact TSP molecule contained in solubilized platelet samples, both in its unreduced and in its reduced form (Fig 2). Under reduced conditions, the principal band reacting with the antibodies corresponded to the position of intact TSP chains (Mr, 190 Kd). A degradation product (Mr, 160 Kd) formed by proteolytic cleavage of the NH-terminal heparin-binding domain was also detected. In addition, faint bands at Mr 120 to 130 Kd and Mr 110 Kd were observed with R6 and, to a lesser degree, with R5. These bands were not observed under unreduced conditions and may therefore correspond to fragments of TSP that remain attached to the whole molecule by disulphide bridges. Two previously characterized MoAbs to TSP, MAI and MAII, also reacted with intact TSP and with its reduced subunits.

**Effect of the Antibodies on Platelet Aggregation**

The antibodies were tested for their effect on thrombin-induced aggregation of washed platelets. Experiments were performed on six different platelet preparations, the
MoAbs and the polyclonal antibodies being used at 0.2 mg/mL and 0.4 mg/mL, respectively. As indicated in Fig 3, inhibition of platelet aggregation and 14C-serotonin secretion was observed with R5 and R6, but not with R3 or a control rabbit nonimmune IgG. A low but reproducible inhibition was also observed with MoAb MAI as compared with MOPC21. However, the strongest inhibition was observed with MoAb MAII, which, in some occasions, could almost completely inhibit platelet aggregation when used at 0.2 mg/mL. The concentration-dependent inhibition of platelet aggregation by MoAb MAII is depicted in Fig 4.

Effect of the Antibodies on Endogenous TSP Binding to the Platelet Surface

The antibodies were tested for their effect on thrombin-induced surface expression of endogenous TSP. Radiolabeled 5G11, a rat MoAb to TSP, was used to quantify membrane-bound TSP, as previously reported.10 Because in these experiments the various antibodies were added to the platelets during the activation process and could thus

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Fig 2. Western blot of anti-TSP antibodies. Platelet proteins (50 μg), solubilized with SDS in the absence (A) or in the presence (B) of 5% β-mercaptoethanol, were separated on 5% to 12% gradient acrylamide gels. On lane 1, the proteins were visualized by Coomassie blue R-250 staining. On lanes 2 through 6, the proteins were electrotransferred to nitrocellulose membranes and tested for reactivity with various antibodies (10 μg/mL). Bound IgG was located using 125I-protein A and the labeled bands were visualized by autoradiography. Lane 2, MAI; lane 3, MAII; lane 4, R3; lane 5, R5; lane 6, R6. The position of TSP on the platelet profile is indicated.

Fig 3. Effect of anti-TSP antibodies on platelet (□) aggregation and (□) secretion. Washed platelets (250,000/μL), preloaded with 14C-serotonin, were stimulated with 0.05 U/mL α-thrombin in the presence of buffer or in the presence of the various antibodies. The extent of platelet aggregation and 14C-serotonin release was measured 3 minutes after the onset of aggregation, as described in Materials and Methods, and expressed in percentage of inhibition of the control. MOPC21, MAI, and MAII were used at 0.2 mg/mL. Nonimmune rabbit IgG, R3, R5, and R6 were used at 0.4 mg/mL. Values are the average ± SD of five experiments. *P < .01, Student’s t-test on paired samples comparing values with and without antibody.

Fig 4. Concentration-dependent inhibition of platelet aggregation by MoAb MAII. Washed platelets (250,000/μL) were activated with 0.05 U/mL α-thrombin in the presence of increasing concentrations of the antibody. In the control (lower tracing), no MoAb was added. Aggregation of the platelets is indicated by the decrease in optical density. 14C-serotonin secretion is indicated by the values in parentheses. The first arrow indicates the addition of the antibody.
become fixed to TSP on the platelet surface, it was first necessary to establish that they would not inhibit the subsequent binding of $^{125}$I-5G11 because of the close proximity of their respective epitopes on the TSP molecule. For this, we developed a competitive solid-phase assay in which the binding of $^{125}$I-TSP (1 $\mu$g/mL) to immobilized 5G11 was measured in the presence of the various antibodies. As shown in Fig 5, preincubation of $^{125}$I-TSP with MoAbs MOPC 21, MAI, and MAII (Fig 5A) or with polyclonal antibodies R3, R5, and R6 (Fig 5B) did not inhibit the interaction of TSP with immobilized 5G11. As expected, inhibition of TSP binding to immobilized 5G11 was observed when $^{125}$I-TSP was preincubated with the homologous antibody (5G11) or with a polyclonal antibody prepared against the native TSP (R1). Similar results were obtained when TSP was adsorbed to plastic wells and the binding of $^{125}$I-5G11 measured after the preincubation of TSP with the various antibodies (data not shown). These results showed that $^{125}$I-5G11 reacted identically with free TSP and TSP complexed by any of the antibodies. Thus, any decrease in $^{125}$I-5G11 binding to thrombin-activated platelets would result from a decreased expression of TSP on the platelet surface and not from a decreased accessibility of 5G11 to its epitope on the TSP molecule.

In experiments designed to test the effects of the antibodies on TSP binding to activated platelets, we used the same ratios of antibodies to platelets as those used in the aggregation assay. Only MoAb MAII was found to inhibit the binding of $^{125}$I-5G11 to thrombin-stimulated platelets (Table 1). Inhibition of TSP expression was not due to inhibition of the release reaction because under nonstirring conditions, ie, in the absence of platelet aggregate formation, MAII did not significantly inhibit the release of $^{14}$C-serotonin. Increasing the concentration of the antibody in the assay up to 0.4 mg/mL did not result in a significant increase of its inhibitory effect as compared with the results obtained at 0.2 mg/mL (Table 1).

**Table 1. Effect of Anti-TSP Antibodies on Endogenous TSP Surface Expression**

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Concentration (µg/mL)</th>
<th>5G11 Binding (molecules/platelet)</th>
<th>$^{14}$C-Serotonin Release (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>12,450 ± 3,700</td>
<td>45.2 ± 5.8</td>
</tr>
<tr>
<td>MOPC21</td>
<td>200</td>
<td>12,320 ± 5,900</td>
<td>43.9 ± 6.0</td>
</tr>
<tr>
<td>MAI</td>
<td>400</td>
<td>12,820 ± 4,600</td>
<td>43.6 ± 6.6</td>
</tr>
<tr>
<td>MAII</td>
<td>400</td>
<td>12,840 ± 7,680</td>
<td>48.9 ± 9.4</td>
</tr>
<tr>
<td>R3</td>
<td>400</td>
<td>11,570 ± 9,000</td>
<td>46.4 ± 9.4</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>200</td>
<td>9,412 ± 4,300</td>
<td>42.0 ± 10.1</td>
</tr>
<tr>
<td>R5</td>
<td>400</td>
<td>7,180 ± 2,400</td>
<td>36.3 ± 2.1</td>
</tr>
<tr>
<td>R6</td>
<td>400</td>
<td>14,770 ± 7,220</td>
<td>55.4 ± 6.8</td>
</tr>
</tbody>
</table>

Washed platelets (250,000/µL) were activated for 3 minutes at 37°C with 0.05 U/mL $\alpha$-thrombin. Hirudin (2 U/µL) was added and samples were taken for $^{14}$C-serotonin release or diluted to 125,000 platelets/µL and incubated for 30 minutes at 22°C with 20 µg/mL $^{125}$I-5G11 as detailed in Materials and Methods. Values are mean ± SD of five experiments. P values reflect Student's t-test on paired samples comparing values without and with antibody.

$^{*}P < .01$.

$^{+}P < .005$.

Effect of the Antibodies on TSP Interaction With Fibrinogen

A solid-phase assay was used to examine the effects of the antibodies on the binding of TSP to immobilized fibrinogen. Fibrinogen in increasing concentrations was passively adsorbed to microtiter wells. In preliminary experiments, $^{125}$I-fibrinogen was used to evaluate the amount of fibrinogen bound to the plastic well. The coating efficiency at 10 µg/mL (ie, 1 µg added to the wells) was about 50% corresponding to 0.5 µg of fibrinogen bound per well. Unlabeled immobilized fibrinogen was found to react with $^{125}$I-TSP (1 to 20 µg/mL) in a saturable manner. The maximum amount of TSP bound per well was determined to be 0.165 µg. Assuming an Mr of 420 Kd for TSP and an Mr of 340 Kd for fibrinogen, the data suggest a stoichiometric relationship of TSP to fibrinogen of 1:4 similar to that previously published by other investigators. The specificity of the reaction was attested by the fact that the interaction of $^{125}$I-TSP with immobilized fibrinogen was inhibited up to 98% in the presence of increasing concentrations of soluble fibrinogen (Fig 6). Moreover, binding of $^{125}$I-TSP to albumin-coated wells represented less than 5% of its binding to fibrinogen-coated wells.

Preincubation of $^{125}$I-TSP (2 µg/mL) with MoAb MAII resulted in a concentration-dependent inhibition of $^{125}$I-TSP binding to immobilized fibrinogen (Fig 6). No inhibition was observed with any of the other antibodies used at 100 µg/mL, but a significant increase in $^{125}$I-TSP binding ($P < .05$) was noted with R5. In contrast to its effect on the binding of $^{125}$I-TSP to immobilized fibrinogen, MAII did not inhibit the binding of $^{125}$I-TSP to immobilized plasminogen (data not shown).
Fig 6. Effect of anti-TSP antibodies on TSP binding to fibrinogen. Microtiter wells precoated with fibrinogen were incubated with \(^{125}\text{I}-\text{TSP}\) at 2 \(\mu\text{g/mL}\) (=100,000 cpm/well) after its incubation with buffer (control) or with soluble fibrinogen (Fg) or with the various antibodies at the indicated concentrations. The extent of specific binding of TSP to adsorbed fibrinogen is expressed as the percent of control binding. Results are the average \(\pm\)SD of several determinations (n), each performed in duplicate. MAI, MAII (20 and 50 \(\mu\text{g/mL}\)), R3, R5, R6 (n = 7); Fg and MAI 100 \(\mu\text{g/mL}\) (n = 5). *\(P < .05\), ** \(P < .01\), Student's t-test on paired samples comparing values with and without antibody.

Effect of the Antibodies on TSP Interaction With GPIV

An immunocapture assay, recently developed in our laboratory to quantify platelet GPIV,\(^{26}\) was used to study the effects of the various antibodies on the binding of TSP to immobilized GPIV. FA6-152, an MoAb to platelet GPIV,\(^{14,15,22}\) was adsorbed to plastic wells and then incubated with detergent-solubilized platelet proteins to specifically retain GPIV onto the microtiter wells. The specificity of this immunocapture assay was indeed demonstrated using the lysate from \(^{125}\text{I}-\text{surface-labeled platelets}\). As shown on Fig 7, GPIV and no other surface protein was identified when the captured polypeptides were visualized by SDS-PAGE. In addition, we could not detect the presence of \(\alpha\)-granular proteins in the captured material using radiolabeled specific antibodies to fibrinogen and TSP. In subsequent experiments, the captured GPIV was incubated with \(^{125}\text{I}-\text{TSP}\) (1 to 20 \(\mu\text{g/mL}\)); this resulted in saturable TSP binding to the immobilized receptor, whereas little saturable binding (less than 15\%) was measured in control samples (see the Materials and Methods). The specificity of the binding of \(^{125}\text{I}-\text{TSP}\) to immobilized GPIV was shown by the fact that no binding was measured using \(^{125}\text{I}-\text{fibrinogen}\) or \(^{125}\text{I}-\text{fibronectin}\) in place of \(^{125}\text{I}-\text{TSP}\). Furthermore, the binding of \(^{125}\text{I}-\text{TSP}\) was abolished when the platelet lysate had been preincubated with soluble FA6-152 to block the specific immunocapture of GPIV by immobilized FA6-152 (data not shown). As shown in Fig 8, the preincubation of \(^{125}\text{I}-\text{TSP}\) (2 \(\mu\text{g/mL}\)) with either soluble fibrinogen or MoAb MAII resulted in a concentration-dependent inhibition of \(^{125}\text{I}-\text{TSP}\) binding to GPIV, whereas the other antibodies tested were without significant effect.

DISCUSSION

There is now overwhelming evidence that TSP, which becomes expressed on the surface of activated platelets, plays an important role in platelet aggregation by promoting the formation of macroaggregates.\(^{7,10}\) However, the nature of the receptors involved in this process, most likely fibrinogen and/or GPIV, as well as the molecular requirements at the TSP level, are not clearly defined. In this study, we have used antibodies that bind to different regions within the TSP molecule to identify those active regions that are involved in TSP binding to the surface of thrombin-activated platelets and in subsequent secretion-dependent platelet aggregation.

The murine MoAbs, designated MAI and MAII, were raised against human platelet TSP.\(^{25}\) They have been used in earlier studies to probe the structure of TSP originated from human platelets\(^{21}\) or from culture supernatants of endothelial cells and fibroblasts.\(^{22}\) The epitope for MAII is located in the NH\(_{2}\)-terminal 25-Kd heparin-binding domain of the molecule, while the epitope for MAI is located in a 47-Kd tryptic fragment adjacent to the 25-Kd COOH-terminal fragment.\(^{23}\) The polyclonal antibodies R3, R6, and...
In previous studies, it has been shown that TSP (thrombospondin) plays a critical role in the regulation of platelet aggregation. 

We have previously drawn a similar conclusion that TSP is involved in the binding of TSP to both immobilized fibrinogen and GPIV. For this purpose, we developed solid-phase binding assays that allowed the simultaneous measurement of the binding of purified radiolabeled TSP to either plasma fibrinogen or to platelet GPIV immunocaptured by FA6-152. In these assays, MAI was found to inhibit the interaction of TSP with both ligands to a similar extent, with an IC50 of ~0.5 μmol/L at a TSP concentration of 2 μg/mL. The molar ratio of the antibody to TSP (40:1) was similar to that required to inhibit TSP binding to intact cells and platelet aggregation. The fact that MAI had no effect on the interaction of TSP with another immobilized protein, plasminogen, suggested that its effect was specific for fibrinogen and GPIV and was not due to a gross alteration of the configuration of the protein, as already shown by electron microscopy. Therefore, the present study suggests that the same region in the TSP molecule was involved in its interaction with both fibrinogen and GPIV. This hypothesis was further substantiated by the observation that soluble fibrinogen could compete with the binding of MAI to TSP, while fibrinogen preadsorbed to plastic wells or to platelet GPIV immunocaptured by FA6-152 did not inhibit TSP binding to intact cells and platelet aggregation. The fact that these antibodies have been shown to inhibit fibrinogen and GPIV interaction with the same site or with closely related sites in the TSP molecule suggests that TSP can indeed be simultaneously linked to fibrinogen and GPIV on the platelet surface, as proposed by Nachman et al.

The present observation that MoAb MAI inhibits the binding of TSP to fibrinogen deserves a further comment. To date, experiments performed with proteolytic fragments of the TSP molecule have localized a fibrinogen binding domain in a 70-Kd chymotryptic fragment adjacent to the NH2-terminal heparin-binding domain. This fragment, which extends from Ile 241, contains all of the type 1 and most of the type 2 repeating sequences. In this study, however, we could not demonstrate inhibition of complex formation between TSP and fibrinogen using the polyclonal antibodies R3 and R6 that recognize two different sequences, Gly 385-Ile 522 and Pro 559-Ile 669, that are included within the 70-Kd fragment.
raised antibodies against the cysteine-rich NH$_2$-terminal part of the 70-Kd fragment, which shows homologies with other adhesive proteins, such as fibronectin and von Willebrand factor, and could thus possibly be involved in a common aspect of adhesive interactions between matrix proteins.\(^1\)

On the other hand, in one of the previous studies\(^2\) it was noted that the apparent association constant for the binding of TSP to fibrinogen was different from that measured for the binding of the 70-Kd chymotryptic fragment to fibrinogen. This observation raised the possibility that a distinct binding site not included in the 70-Kd fragment was functioning in the intact molecule. The hypothesis of two different fibrinogen binding sites within the TSP molecule is indeed compatible with recent data showing that TSP interacts with two distinct sequences located within the Aα- and the Bβ-chains of fibrinogen.\(^3\)

In conclusion, the present study shows that the binding of TSP to the surface of activated platelets can be modulated at the level of a specific epitope located in the heparin-binding domain of the molecule. This observation should prove important for developing new antithrombotic molecules that would be potent inhibitors of macroaggregates formation without blocking the primary hemostatic function of platelets. Further experiments are in progress to determine whether the epitope for MAII may directly bind to the platelet surface or whether its occupancy may trigger some discrete conformational change in the TSP molecule that would modify its binding capacities.

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Molecular requirements for the interaction of thrombospondin with thrombin-activated human platelets: modulation of platelet aggregation

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