Selective Inhibition of Platelet Macroaggregate Formation by a Recombinant Heparin-Binding Domain of Human Thrombospondin

Chantal Legrand, Veronica Morandi, Simona Mendelovitz, Hadassa Shaked, Jacob R. Hartman, Amos Panet

Abstract Thrombospondin (TSP) is a platelet α-granule adhesive protein that plays a critical role in the stabilization of thrombus by promoting the formation of platelet macroaggregates. We have recently shown that a monoclonal antibody (mAb) to the NH₂-terminal heparin-binding domain of TSP, MAII, inhibits platelet aggregation induced by thrombin in a dose-dependent manner. In this study, we have expressed in Escherichia coli two recombinant proteins comprising residues 1 to 174 (TSP18) and 1 to 242 (TSP28) of TSP. After purification, both proteins reacted equally well with mAb MAII, whereas the reactivity of TSP18 for heparin was lower than that of TSP28 or native TSP. At micromolar concentrations, TSP18 and TSP28 inhibited the second wave of platelet aggregation and the concomitant release of [³⁵S]hydroxytryptamine induced by ADP in citrated platelet-rich plasma as well as aggregation and secretion induced by a low concentration of thrombin in washed platelet suspensions. The proteins did not inhibit surface expression of endogenous TSP on activated platelets, as measured by the binding of radiolabeled mAb SG11, indicating that they did not interfere with the primary binding of TSP to the plasma membrane. In contrast, in a solid-phase binding assay, the proteins inhibited in a dose-dependent manner (IC₅₀, 0.1 and 0.06 μmol/L for TSP18 and TSP28, respectively) the binding of radiolabeled TSP to surface-adsorbed fibrinogen. Furthermore, specific and saturable binding of the proteins to immobilized fibrinogen was demonstrated by enzyme-linked immunosorbent assay. The results suggest that interaction between the heparin-binding domain of TSP and membrane-bound fibrinogen may be critical in the platelet aggregation/secretion process.

Keywords • thrombospondin • platelet aggregation • fibrinogen • thrombosis • recombinant proteins

Thrombospondin (TSP) is a large (450-kD), homotrimeric, adhesive glycoprotein that is synthesized by a variety of cells and that mediates cell-substratum and cell-cell interactions. The molecule comprises several functional domains that mediate interactions with cells, heparin, glycosaminoglycans, fibrinogen, fibronectin, plasminogen, plasminogen activators, and collagens. Platelets constitute the main reservoir of TSP in the circulation; TSP is released by platelets upon activation and plays a major role in hemostasis by promoting irreversible platelet macroaggregate formation.

In addition, there is some evidence that binding of secreted TSP to the platelet surface contributes to the amplification of the signaling process and is required for maximal platelet secretion.

The mechanism by which TSP acts on platelets remains unresolved. Several specific platelet components, including glycprotein IV (GPIV or GPIIIb), the glycoprotein IIb-IIIa complex (GPIIb-IIIa), and its ligand fibrinogen, have been shown to bind TSP in vitro and in turn have been considered as potential platelet TSP receptors. However, contradictory results have been published concerning the effects of specific antibodies to these molecules on TSP binding to platelets. Furthermore, platelets with selective deficiencies in fibrinogen (afibrinogenemic), GPIIb-IIIa (thrombasthenic), or GPIV (Nak*-negative individuals) bound normal amounts of TSP. Although these results may be explained on the basis of multiple receptors that may substitute for each other in case one or more is absent on defective platelets, it is still possible that the major receptor for TSP on activated platelets remains to be identified.

In parallel with the identification of several TSP-binding molecules on platelets, studies performed with antibodies to distinct domains of TSP have indicated that both the NH₂ and the COOH terminal parts of the molecule are involved in platelet aggregation. In addition, a small peptide, Cys-Ser-Val-Thr-Cys-Gly (CSVTCG), from the central part of the molecule, was recently shown to inhibit platelet aggregation and was proposed as a potential adhesive sequence involved in the binding of TSP to GPIV.

The results that we obtained previously with MAII, a monoclonal antibody (mAb) to the NH₂-terminal heparin-binding domain of TSP, suggested that this region could contribute to the effect of TSP in platelet aggregation. To further explore structure-function relations in this biologically important domain, we undertook the expression and functional characterization of NH₂-terminal recombinant fragments of TSP. We report here that an 18- and a 28-kD recombinant protein have the ability to inhibit platelet macroaggregate formation by...
interfering with the secretion-dependent phase of platelet aggregation.

**Methods**

**Isolation of cDNA Clones and Construction of Expression Vectors**

TSP1 cDNA clones were isolated by screening a bacteriophage λgt11 cDNA library prepared from cultured human endothelial cells derived from umbilical cord veins (Clontech). 32P-labeled synthetic oligonucleotide probes made according to the published nucleotide sequence were used for hybridization. Positive clones were plaque purified, and the inserts were subcloned into the unique EcoRI restriction endonuclease site of pBR322. Clones relating to the NH2-terminal region of TSP were identified by hybridization to specific oligonucleotide probes and by DNA sequence analysis. One clone (TSPA, see Fig 1A) contains a 1300-bp-long insert spanning the 5'-untranslated region and an open reading frame encoding 379 amino acids. This clone was further manipulated for construction of expression plasmids as follows. First, by using the unique NarI site, the 5'-region containing the leader peptide sequences was replaced with a shorter, synthetic DNA fragment comprising at its 5' end an NdeI site. Thus, an ATG initiation codon was introduced that directly preceded the NH2-terminal Asn residue of mature TSP. The modified NdeI-EcoRI fragment (1100 bp long) was then inserted into the λgt11-based expression vector from pMS-E4. Furthermore, to achieve expression of specific NH2-terminal heparin-binding peptides, a termination codon was introduced. Plasmid DNA was partially cleaved with BalI and ligated to a synthetic oligonucleotide having the palindromic sequence 5'-CTAGCAGATCTGCTAG-3', which includes a unique BglII site. Following transformation of competent Escherichia coli cells and heat induction (see below), two separate clones were identified that expressed proteins with apparent molecular weights of 18 and 28 kD (TSP18 and TSP28, respectively). The COOH-terminal site of both clones was determined by restriction endonuclease mapping. TSP18 contains residues 1 to 174 with an initiating Met that precedes the first Asn residue and the sequence Arg-Ser-Ala-Ser-Gln added to the COOH-terminus, which is derived from translation of the oligonucleotide insert. TSP28 contains amino acid residues 1 to 242 with a Met residue that precedes the first amino acid.

**Bacterial Growth and Induction of Expression**

The strategy for construction of expression plasmids is shown in Fig 1B. TSP18 and TSP28 were expressed in E coli strain A4255 under control of the thermoinducible λPl promoter and CI ribosomal binding site. E coli strains harboring expression plasmids pTSP18 and pTSP28 were grown in 1 L broth supplemented with ampicillin (100 μg/mL) at 32°C. Induction of recombinant heparin-binding proteins was performed by raising the temperature to 42°C for 2 hours. Both proteins accumulated in intracellular inclusion bodies.

**Purification of Recombinant Proteins**

The protocol that we followed was very similar to that used by Yabkowitz et al, except that the presence of urea was maintained throughout the purification procedure to avoid aggregate formation. Bacterial cells were harvested by centrifugation, and the resulting pellet material was resuspended in buffer containing 50 mmol/L Tris-HCl, pH 7.4, 5 mmol/L EDTA, 50 mmol/L NaCl, and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF). The cell suspension was treated with 50 μg/mL lysozyme (Sigma Chemical Co) for 2 hours at 37°C followed by sonication at 4°C. After centrifugation in the cold, the pellet was washed twice in distilled water and frozen at −20°C. Purification of TSP18 and TSP28 followed slightly different protocols. Inclusion bodies containing TSP28 were dissolved in 8 mol/L urea, 5 mmol/L EDTA, and 1 mmol/L PMSF solution buffered with 20 mmol/L ethanolamine, pH 9.5. Insoluble material was removed by centrifugation at 12 000g for 15 minutes. Inclusion bodies containing TSP18 were solubilized in 8 mol/L urea, 5 mmol/L EDTA, and 1 mmol/L PMSF solution brought to pH 12 with NaOH and incubated, with stirring, for 15 minutes at room temperature. The pH was then adjusted to 10.5 and incubation continued for 1 hour. Ethanolamine was supplemented to achieve a final concentration of 20 mmol/L, and pH was adjusted finally to 9.5. The solution was cleared by centrifugation as described above. Protein solutions prepared by either technique were applied to DEAE-Sepharose fast-flow columns (Pharmacia LKB Biotechnology Inc) equilibrated in 8 mol/L urea (TSP28) or 6 mol/L urea (TSP18), 20 mmol/L ethanolamine, 5 mmol/L EDTA, and 1 mmol/L PMSF, pH 9.5. For both protein solutions, unbound material was collected and passed over a CM-Sepharose column equilibrated in 6 mol/L urea, 5 mmol/L EDTA, and 20 mmol/L MES, pH 6.5. Elution was performed with a 0 to 0.15 mol/L NaCl gradient, and fractions were

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**Fig 1.** Schematic description of the various thrombospondin (TSP) clones (A) and expression plasmids (B) showing inserts for two TSP fragments comprising amino acids 1 to 174 for TSP18 and 1 to 242 for TSP28. pTSP expresses TSP18 or TSP28 under control of the thermoinducible λPl promoter.
pooled according to sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) analysis. Material containing TSP was adjusted to 20 mmol/L NaCl and applied to a heparin-Sepharose column equilibrated in 4 mol/L urea, 20 mmol/L sodium phosphate, 1 mmol/L EDTA, 1 mmol/L PMSF, and 0.1 mmol/L diithiothreitol (DTT), pH 7.6. The column was extensively washed and subjected to stepwise elution with NaCl. TSP eluted at 0.1 mol/L NaCl and was refolded during sequential dialysis steps against 2 and 1 mol/L urea solutions buffered with 20 mmol/L NaHCO₃, pH 8.8, in the presence of 0.1 mmol/L DTT. TSP28 was finally dialyzed against 1 mmol/L NaHCO₃, pH 8.8, in the presence of 1 mmol/L DTT and lyophilized.

Oxidation of TSP28 was carried out by adjusting the protein concentration of the CM-Sepharose pool to 200 µg/mL and dialyzing against 4 mol/L urea and 20 mmol/L NaHCO₃, pH 10.5, in the presence of 100 µmol/L oxidized glutathione. Reduction in urea concentration and folding was achieved by sequential dialysis against 2 and 1 mol/L urea solutions buffered with 20 mmol/L NaHCO₃, pH 10.5. Urea was finally removed by extensive dialysis against 2 mol/L NaHCO₃, followed by 2 mmol/L NaHCO₃, pH 10.5, and the protein was lyophilized.

Progress in folding was monitored throughout dialysis by gel chromatography on Superose-6 columns (Pharmacia). The authenticity of TSP18 and TSP28 was confirmed by sequencing the N-terminal residues of the peptides obtained by cyanogen bromide cleavage and analysis of amino acid composition.

The recombinant proteins were reconstituted by dissolution in distilled water and were desalted before use by chromatography on a Sephadex G-25 column preequilibrated with 10 mmol/L NaHCO₃, pH 9.5. Recombinant proteins were analyzed by SDS-PAGE on an 18% polyacrylamide gel followed by Coomassie brilliant blue R-250 staining, and their reactivity with mAb MAII was assessed by immunoblotting.

A recombinant fragment of human fibroectin (FN33) containing the cell-binding domain, amino acids 1329 to 1722, has also been purified from inclusion bodies by fractionation on a DEAE-Sepharose column in the presence of urea and has been characterized for its effect on platelet function.

### Anti-TSP Antibodies

The anti-TSP antibodies used in this study were as follows. MAI and MAII are mouse mAbs that recognize a sequence within the type 3 repeats and the heparin-binding domain, respectively. SG11 is a rat mAb that reacts with a 15-kD proteolytic fragment of TSP that is distinct from the heparin-binding domain. These antibodies were purified as previously described. A polyclonal antibody to TSP28 was raised in rabbits, and the serum was used in an enzyme-linked immunosorbent assay (ELISA).

### Heparin-Binding Affinity

Native platelet TSP, TSP18, or TSP28 was applied to a heparin-Sepharose column in buffer containing 10 mmol/L Tris-HCl, pH 7.9, 40 mmol/L NaCl, and 1 mol/L urea. The bound proteins were eluted by a linear gradient of 40 to 700 mmol/L NaCl in 10 mmol/L Tris-HCl, pH 7.9, and 1 mol/L urea. The eluted fractions were analyzed by SDS-PAGE for the presence of TSP or recombinant proteins.

### Preparation of Blood Samples

Blood was taken from healthy adult volunteers who denied having taken any drug for at least 1 week. Platelet-rich plasma was prepared from blood freshly drawn into a 1/10 volume of 0.11 mol/L trisodium citrate and centrifuged at 1200g for 15 minutes. Washed platelets were prepared from acid-citrate-dextrose–anticoagulated blood by repeated centrifugations as described. Platelets were resuspended at a final concentration of 2.5 × 10⁸/mL in Tyrode's buffer, pH 7.4, containing 5 mmol/L HEPES, 1 mmol/L MgCl₂, 2 mmol/L CaCl₂, and 3.5 mg/mL bovine serum albumin (BSA).

### Platelet Aggregation and Secretion

Aggregation studies were done with citrated platelet-rich plasma or washed platelets in an aggregometer (Chronolog Corp) at 37°C with constant stirring (1200 rpm) as described. The release reaction was measured in the presence of 3 µmol/L chlorimipramine (Ciba Geigy) on platelet samples prelabeled with [³¹]S-hydroxytryptamine.

### Surface Expression of Endogenous TSP

Surface-bound platelet TSP was determined by measuring the binding of radiolabeled mAb SG11 as described. Briefly, 20 µg/mL SG11 was added to nonstimulated or thrombin-stimulated (0.05 U thrombin per milliliter) platelets at 1.25 × 10⁶/mL. Binding was measured after a 30-minute incubation at 22°C by centrifugation of triplicate 100-µL aliquots of the reaction mixtures through 20% (wt/wt) sucrose.

### TSP Purification and Radiiodination

TSP was purified from fresh, thrombin-stimulated human platelets as described previously. The protein was radiiodinated to a specific activity of 0.1 to 0.3 µCi/µg by the chloramine T method as described.

### TSP Binding to Fibrinogen

Binding of radiolabeled TSP to fibrinogen (Imco) was determined with a solid-phase assay as described. Briefly, [³¹]I-labeled TSP (5 nmol/L) was incubated in fibrinogen-coated wells in Tris-Tween buffer (10 mmol/L Tris, 150 mmol/L NaCl, 2 mmol/L CaCl₂, and 0.05% Tween 20, pH 7.4) for 2 hours at 22°C, and the radioactivity associated with each well was counted. Specific binding was calculated by subtracting nonspecific from total binding (nonspecific binding was <5%, corresponding to [³¹]I-labeled TSP binding to BSA-coated wells).

### Interaction of TSP18 and TSP28 With Fibrinogen

Binding of recombinant proteins to fibrinogen-coated wells was performed under the same conditions as binding of [³¹]I-TSP, except that binding was monitored by sequential addition of a rabbit antiserum to TSP28 (1:500) followed by addition of peroxidase-conjugated, affinity-isolated goat immunoglobulins to rabbit IgG (1:500) and ABTS enzyme substrate (Boehringer Mannheim). Absorbance at 405 nm was read in a Titertek plate Twinreader.

### Results

**Characterization of Recombinant Fragments**

Purified, bacterially produced TSP18 and TSP28 migrated as a single band on SDS-PAGE with the expected sizes of 18 and 28 kD under nonreducing conditions (Fig 2A). Consistent with the presence of an intrachain disulfide bond within TSP28, the mobility of this protein was slightly reduced after disulfide bond reduction (Fig 2A; cf lane 4 and lane 2). Both proteins reacted equally well with mAb MAII by immunoblotting (Fig 2B), indicating that the epitope that we previously showed to be functionally important in platelet aggregation was normally expressed. The identical reactivity of TSP18 and TSP28 with mAb MAII was confirmed in solid-phase binding assays (not shown).

The heparin-binding capacity of recombinant proteins was evaluated by affinity chromatography on a heparin-Sepharose column, followed by elution with a linear salt gradient. The assay was conducted in the presence of 1 mol/L urea to avoid precipitation of recombinant proteins at higher salt concentrations. Under these experimental conditions, the native trimeric molecule eluted at a peak salt concentration ranging...
from 0.29 to 0.36 mol/L NaCl. A major fraction of monomeric TSP28 eluted as trimeric TSP at a peak salt concentration ranging from 0.28 to 0.36 mol/L NaCl. Minor peaks were eluted at 0.19 to 0.25 mol/L NaCl, probably relating to a few aggregated forms. As reported by others,37 TSP18 displayed a lower affinity for heparin compared with monomeric TSP28 or trimeric TSP, since TSP18 was eluted at a peak salt concentration ranging from 0.18 to 0.31 mol/L NaCl. However, there was no evidence of aggregated forms in this preparation when it was analyzed by gel filtration or electrophoresis under native conditions.

**Inhibition of Platelet Aggregation**

The effects of recombinant proteins on platelet aggregation reactions induced by two different stimuli, ADP and thrombin, were investigated for several healthy donors. Results of a typical experiment with TSP28 are shown in Fig 3. At micromolar concentrations, TSP28 did not interfere with the primary phase of aggregation induced by 3 μmol/L ADP in citrated platelet-rich plasma but inhibited the secondary phase of aggregation and the concomitant release of [14C]5-hydroxytryptamine (Fig 3A). TSP28 also inhibited the rate and extent of platelet aggregation as well as [14C]5-hydroxytryptamine release induced by low-dose thrombin in washed platelet suspensions (Fig 3B). This decreased the size of the aggregates formed, as shown by the reduction in amplitude of the oscillations in the aggregometer tracings. Examination of the aggregates by light microscopy confirmed the presence of much smaller aggregates in the presence of the recombinant protein. Identical results were obtained with TSP18 used at similar concentrations. The mean percent aggregation and secretion responses obtained from experiments performed on blood from four individual donors are given in the Table. It should be noted that the effects of recombinant proteins were almost totally overcome by increasing the concentration of the agonist. In these experiments, the recombinant cell-binding domain of fibronectin (FN33), used at micromolar concentrations, inhibited the primary phase of aggregation induced by ADP in citrated platelet-rich plasma, as reported previously.34

![Fig 3](http://atvb.ahajournals.org/)

**Fig 3.** Tracings showing inhibition of platelet aggregation by recombinant protein TSP28. Platelets were activated in a Chronolog aggregometer in the absence or presence of TSP28 at the indicated concentrations. Platelet aggregation was monitored by the increase in light transmission, and the percentage of [14C]5-hydroxytryptamine released at 3 minutes is indicated in parentheses. A. Platelets in citrated platelet-rich plasma were activated with 3 μmol/L ADP. B. Washed platelets were activated with 0.05 U/mL (0.5 nmol/L) α-thrombin (3000 National Institutes of Health U/mg; Sigma). thr indicates thrombin.
Inhibition of Platelet Aggregation and Secretion by Recombinant TSP18

<table>
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<th>Stimulus</th>
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<th>Percent Inhibition Aggregation</th>
<th>Percent Inhibition Secretion</th>
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<td>42±14</td>
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<tr>
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<tr>
<td></td>
<td>8</td>
<td>29±5</td>
<td>3±2</td>
</tr>
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Platelet aggregation was monitored as described in Fig 3, and the changes in light transmittance and [14C]5-hydroxytryptamine secretion were measured 3 minutes after addition of the stimulus. Results are mean±SD of aggregations performed on blood from four individual donors. TSP indicates thrombospondin.

Expression of TSP on Activated Platelets

The effects of recombinant proteins on thrombin-induced surface expression of endogenous TSP were measured as the binding of radiolabeled mAb 5G11, as previously reported. We anticipated that TSP18 and TSP28 would inhibit expression of the endogenous protein by blocking its interaction with membrane receptors. On the contrary, we found either no decrease or only a slight increase in the binding of mAb 5G11 when we activated the platelets in the presence of TSP18 or TSP28 at concentrations that inhibited platelet aggregation (Fig 4). An increase in 5G11 binding to thrombin-activated platelets was also measured in the presence of 1 µmol/L FN33.

Inhibition of TSP Interaction With Fibrinogen

TSP has been shown to have a strong affinity for fibrinogen and to promote platelet aggregation by stabilizing the interaction of fibrinogen with its receptor on activated platelets, the GPIIb-IIIa complex. We therefore analyzed the effects of recombinant proteins on the interaction of TSP with fibrinogen, as measured in a solid-phase binding assay. We have previously shown with this system that [125I]-labeled TSP binds to immobilized fibrinogen in a specific and saturable manner and that this binding is inhibited by mAb MAI,11,39 TSP18 and TSP28 inhibited binding of [125I]-TSP (2 µg/mL) to fibrinogen in a dose-dependent manner, with IC50 values of 0.1 and 0.06 µmol/L, respectively (Fig 5), whereas the 33-kD fragment of fibronectin had no inhibitory effect at 1 µmol/L (not shown). It should be noted that similar concentrations of recombinant proteins...
teins were required to inhibit platelet aggregation (Fig 3) and binding of TSP to fibrinogen (Fig 5), considering that approximately 20 μg/mL of TSP is being released from platelets upon activation by thrombin.40

Interaction of Recombinant Proteins With Fibrinogen

Direct interaction of recombinant proteins with immobilized fibrinogen was assessed in an ELISA system with a polyclonal antibody prepared against TSP28 for detection of the bound proteins. TSP18 and TSP28 bound to fibrinogen-coated wells in a dose-dependent and saturable manner at concentrations ranging from 0.02 to 0.2 μmol/L, whereas they did not bind significantly to BSA-coated wells (Fig 6A). As a control for specificity, we did not measure the interaction of the 33-kD fragment of fibrinectin (ie, FN33) to immobilized fibrinogen with an anti-fibrinectin antibody for detection. Binding of TSP18 and TSP28 was inhibited by 90% after preincubating the recombinant proteins with a 10-fold molar excess of mAb MAI, whereas mAb MAII, which recognizes an epitope in the type 3 repeat of TSP, had no effect (Fig 6B). Interestingly, excess soluble fibrinogen could not compete for the binding of recombinant proteins to immobilized fibrinogen, whereas fibrinogen did compete for the binding of the whole TSP molecule under similar conditions.41 Moreover, using the same technical approach, we failed to demonstrate an interaction between soluble fibrinogen and the immobilized recombinant proteins (data not shown).

Discussion

Platelet aggregation that is initiated by the binding of plasma fibrinogen to its receptor on activated platelets, the GPIb-IIIa complex, becomes almost irreversible when aggregation is induced by agents that cause the release of granule contents.41 Among other α-granule-secreted glycoproteins, TSP has been shown to contribute to the stabilization of platelet aggregation by promoting formation of large aggregates of thrombin-stimulated platelets.7,8

In this study, we used recombinant proteins encompassing amino acid residues 1 to 174 (TSP18) and 1 to 242 (TSP28) of human TSP. Both proteins exhibited functional characteristics expected of a structurally intact, heparin-binding domain, as shown by their capacity to interact with heparin (this study) and to modulate endothelial cell adhesion, proliferation, and motility.42 In agreement with previous work,33,37 we found that the affinity of monomeric TSP28 for heparin was similar to that of the whole trimeric TSP. On the other hand, monomeric TSP18 displayed a decreased affinity for heparin, indicating that the intrachain disulfide bond that links Cys153 and Cys214 (which is present in TSP28 but absent in TSP18) is required for optimal binding to heparin.37 Nevertheless, the reactivity of TSP18 with mAb MAI was identical to that of TSP28, indicating that the epitope that is functionally important for platelet aggregation11 was correctly expressed in the shorter peptide despite the absence of the disulfide loop.

When used at micromolar concentrations, the recombinant proteins specifically inhibited the secondary phase of aggregation induced by ADP in citrated, platelet-rich plasma as well as platelet aggregation induced by low-dose thrombin (0.05 U/mL) in platelet suspensions. This resulted in the formation of smaller aggregates, as we previously observed with mAb MAII.11 In agreement with previous work that demonstrated the role of endogenous or exogenously added TSP in platelet aggregation,10,43 we found that higher concentrations of ADP (10 μmol/L) or thrombin (0.2 U/mL) could overcome the inhibitory effect of the recombinant proteins. These results underline the important role played by TSP in promoting platelet aggregation at low concentrations of stimulating agents.3,7,10,43 Obviously, other mechanisms are involved in platelet aggregation induced by high concentrations of agonists, as Gray platelets, which lack α-granule constituents, aggregate nearly normally in response to ≥0.15 U/mL thrombin.44 Interestingly in our study, the inhibitory effect of recombinant proteins on platelet aggregation induced by a low concentration of ADP or thrombin was accompanied by an inhibition of platelet secretion, an effect that we11 and others9 have also observed with antibodies directed against the heparin-binding domain of TSP. These results strongly suggest that the binding of secreted TSP to the platelet surface during the activation process in some way contributes to amplification of the release reaction. In this regard, we were surprised to find that the recombinant proteins did not inhibit surface expression of TSP on thrombin-activated
platelets, as measured by the binding of a radiolabeled antibody (mAb 5G11) to TSP. On the contrary, we measured a slight increase in binding of this mAb at the highest concentration of recombinant proteins. This finding does not necessarily indicate an increase in the level of TSP associated with the membrane, as an increase in mAb 5G11 binding may also be related to an increase in binding capacity of the antibody. For instance, we assume that the antibody may have a better chance to gain access to all three chains of membrane-bound TSP when the latter is prevented from a secondary interaction with membrane-bound fibrinogen, as discussed below. Consistent with this assumption, we also observed an increase in binding of mAb 5G11 to thrombin-activated platelets in the presence of the recombinant cell-binding domain of fibronectin that inhibits the binding of fibrinogen to the GPIIb-IIIa complex, thus preventing a possible interaction of membrane-bound TSP with fibrinogen on the platelet surface. Confirmation of this hypothesis, however, will require further experimental evidence on the basis of the use of a series of anti-TSP mAbs.

A possible explanation for the failure of recombinant proteins to inhibit surface expression of TSP on thrombin-activated platelets is that the proteins affected a postbinding event, such as the interaction of TSP with membrane-bound fibrinogen. We indeed demonstrated that the recombinant proteins competed for the binding of radiolabeled TSP to immobilized fibrinogen, thus confirming previous results obtained with mAb MAII. Moreover, using an ELISA, we demonstrated a direct interaction between the heparin-binding domain of TSP and surface-immobilized fibrinogen. Using radiolabeled fragments of TSP, Lawler et al demonstrated an interaction between immobilized fibrinogen and a 70-kD chymotryptic fragment of TSP located within the central part of the molecule, whereas they failed to demonstrate an interaction with the 25-kD NH2-terminal fragment. A possible explanation for this apparent discrepancy may relate to the procedure used by these authors to radiolabel the fragment (labeling was performed at 0°C overnight, followed by extensive dialysis), as we did measure specific and saturable binding of TSP by radiolabeling the fragment for a shorter time (15 minutes at 0°C, followed by gel filtration). Evidence for the existence of two different binding sites for fibrinogen within the TSP molecule was first provided by the observation that TSP may interact with two distinct sequences within the Aα- and Bβ-chains of fibrinogen. Consistent with this hypothesis, mAb MAII almost completely blocked interaction of the heparin-binding domain of TSP with immobilized fibrinogen (this study), whereas MAII only partially inhibited binding of the whole TSP molecule. Interestingly in this study, soluble fibrinogen could not compete for binding of recombinant proteins to immobilized fibrinogen, as it did for the binding of the whole TSP molecule. These results suggest the existence of two different mechanisms for the binding of TSP to fibrinogen: one that occurs to both soluble and immobilized fibrinogen, possibly involving the central part of the molecule, and the other that would specifically relate to interaction of the heparin-binding domain of TSP with surface-adsorbed fibrinogen. This may be the reason why (in this study) micromolar concentrations of recombinant proteins inhibited platelet aggregation in citrated plasma despite a high concentration of soluble fibrinogen. Thus, we actually hypothesize that TSP may bind to specific membrane receptors, possibly through its CSVTCG sequence, and then interacts with membrane-bound fibrinogen through its heparin-binding domain.

In conclusion, our results demonstrate that recombinant fragments from the heparin-binding domain of TSP are potent inhibitors of the platelet aggregation secretion process in vitro. Selective inhibition of platelet macroaggregate formation may provide an alternative approach to the blockade of the GPIIb-IIIa complex. If these recombinant fragments are efficient inhibitors of thrombus formation in vivo, their production in large-scale quantities should prove useful for the development of new antithrombotic molecules.

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