Interaction of Thrombospondin with Resting and Stimulated Human Platelets*

(Received for publication, January 15, 1986)

Roger Wolff, Edward F. Plow, and Mark H. Ginsberg‡
From the Department of Immunology, Research Institute of Scripps Clinic, La Jolla, California 92037

The interaction of isolated and radiiodinated thrombospondin with washed human platelets has been characterized. The ligand bound to nonstimulated and thrombin-stimulated platelets in a time-dependent manner, and apparent steady state was reached within 25 min. Binding was not due to iodination of the ligand and was inhibited by nonlabeled thrombospondin but not by unlabeled proteins, and bound ligand was identical with thrombospondin in terms of subunit structure. Nonlinear curve-fitting analyses of binding to resting platelets suggested the presence of a single class of sites which bound 3,100 ± 1,000 molecules/platelet with an apparent $K_d$ of 50 ± 20 nM. This interaction was not attributable to contaminating cells or inadver-
tant platelet activation. Binding to thrombin-stimulated platelets had a lower apparent affinity ($K_d = 250 ± 100$ nM) and higher apparent capacity (35,600 ± 9,000 molecules/platelet). Thrombin-enhanced binding was dependent upon agonist dose and platelet stimul-
tation. Fibrinogen, a monoclonal antibody to GPIIb-IIIa, temperature, and divalent ions had differential effects upon thrombospondin binding to resting and stimulated platelets, suggesting the presence of two distinct mechanisms of thrombospondin binding to platelets. While thrombospondin binding to thrombin-stimulated platelets occurs with characteristics similar to those observed for fibrinogen, fibronectin, and von Willebrand Factor, its high affinity interaction with resting platelets is unique to this adhesive glycoprotein.

During the hemostatic response, platelets adhere and spread on the subendothelial matrix and aggregate with one another. These platelet reactions can be directly mediated or influenced by specific proteins. Fibrinogen, von Willebrand Factor, and fibronectin may influence platelet adhesion and spreading on subendothelial matrices and artificial surfaces (1–3). Fibrinogen is the major regulator of platelet aggregation (4, 5), and von Willebrand Factor and fibronectin may also influence this cell-cell interaction (6, 7). These three proteins share the common properties of being large, glycosylated, and multimeric, suggesting that they may function to bridge platelets to one another or to substrata. All three glycoproteins are present within platelet $\alpha$ granules and are secreted from stimulated platelets (reviewed in Ref. 8), as well as being plasma proteins. Each of these proteins binds specifically to the surface of activated platelets (9–12), providing a mechanism for their participation in bridging functions. Similarities in the binding of these proteins to platelets, include: (a) requirements for platelet stimulation, (b) divalent ion dependence, (c) reduced binding to platelets from patients with Glanzmann’s thrombasthenia, and (d) ADP dependence (9–15). These similarities may be explained in part by the existence of shared binding sites as suggested by the capacities of the same sets of monoclonal antibodies (16) and fibrinogen $\gamma$ chain peptides (17) to inhibit their binding. Nevertheless, important differences in the number of sites and induction requirements suggest unique features for the interaction of each protein with the platelet.

Thrombospondin (TSP') has many features in common with the three adhesive glycoproteins. It is a large glycoprotein ($M_r \approx 450,000$) comprised of three subunits of similar size (18). Although present at very low levels in normal plasma (19), TSP is a major constituent of platelet $\alpha$ granules (20). When secreted from platelets, a portion of released TSP becomes associated with the cell surface in a calcium-depend-
tent interaction raising the possibility of a divalent ion receptor-mediated interaction (21). Gartner and Dockter (22), how-
ever, have recently reported that the binding of TSP to platelet membranes is divalent ion-independent, suggesting a mechanism by which George et al. (23) observed increased surface expression of TSP on thrombin-stimulated platelets in the presence of 5 mm EDTA. Several studies have implicated TSP in platelet function. It is now clear that TSP is at least in part responsible for a lectin-like activity of platelets (24–27), and antibodies to TSP can inhibit thrombin-induced platelet aggregation (28). To define a basis for the role of TSP in platelet function and to further the analogy between TSP and the other three platelet-adhesive glycoproteins, we have examined in detail the interactions of TSP with stimulated and nonstimulated human platelets.

EXPERIMENTAL PROCEDURES

Reagents—ADP, ovalbumin, BSA (once recrystallized), chloro-
mide-T, heparin from hog intestinal mucosa (156 units/mg), hirudin,
theophylline (1,3-dimethylxanthine), and prostaglandin $E_2$ were pur-
chased from Sigma. Human transferrin was obtained from Calbi-
chem. Sepharose 2B was obtained from Pharmacia. Na'T (500 mCi/
ml) and 5-hydroxy[2-14C]tryptamine creatinine sulfate ([14C]sero-
tonin at 60 mCi/mmol) were obtained from Amersham. Kodak RP
film was obtained from Eastman. All other chemicals were reagent
grade.

Purified human $\alpha$-thrombin was the generous gift of Dr. John
Fenton, New York State Department of Health, Albany, NY. This
material was diluted to 100 units/ml in modified Tyrode's buffer,
stored in aliquots at -70 °C, and thawed once prior to use. Perified

1The abbreviations used are: TSP, thrombospondin; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; FG, prostaglandin; EGTA, ethylenebis(oxyethylene-nitri1o)tetraacetic acid.

* This work was supported by National Institutes of Health Grants HL 28235 and HL 16411. This is publication number 3747-1 from THE JOURNAL OF BIOLOGICAL CHEMISTRY. © 1986 by The American Society of Biological Chemists, Inc. Printed in U.S.A.
human fibrinogen (29) and fibronectin (30) were prepared as previously described. The 10E5 monoclonal was a generous gift of Dr. Barry Coller, SUNY at Stonybrook. This monoclonal antibody immunoprecipitated platelet membrane glycoprotein IIb-IIIa in the presence of calcium (31).

**TSP Purification and Radioiodination**—TSP was isolated by the procedure of Lawler et al. (18) with the modifications previously described (32). On 5% polyacrylamide gels in SDS (33), the purified protein yielded a single Coomassie Blue staining band of apparent Mr, 67,000. Homogeneity as judged by scanning densitometry (32). For selected studies, TSP was isolated from the releasate of thrombin-stimulated platelets maintaining a concentration of 2 mM calcium throughout purification.

TSP was radioiodinated by a modified chloramine-T procedure. To 500 µl of a 240 µg/ml TSP solution, 20 µl of chloramine-T at 2 mg/ml and 0.5 mCi of Na125I was added. After 5 min at 22 °C, 40 µg of sodium metabisulfite and 200 µg of KI were added, followed by 2.5 mg of BSA (pretreated with 2 mM phenylmethylsulfonyl fluoride).

The sample was then diluted with 800 µl of H2O to a final volume of 1.42 ml. Free 125I-TSP was 0.5-1.0 µCi/µg. The radioiodinated TSP exhibited a mobility identical with that of nonlabeled TSP on SDS-polyacrylamide gel electrophoresis under reducing or nonreducing conditions, and 96% of the radioactivity migrated as a single peak based on densiometric scanning of autoradiograms. In addition, ≥95% of the radioactivity was precipitated with 10% trichloroacetic acid or by monospecific polyclonal or monoclonal antibodies to TSP.

**Cell Isolation**—Platelets were isolated from acid/citrate/dextrose anticoagulated fresh human blood by differential centrifugation and gel filtration on a column (2.5 cm × 50 cm) of Sepharose 2B as previously described (11). Briefly, platelet pellets, obtained by centrifugation of platelet-rich plasma, were resuspended in 2 ml of modified Tyrode's buffer, pH 6.5, containing 2 mM MgCl2 and 0.1% BSA and gel filtered on a column (2.5 × 10 cm) of Sepharose 2B equilibrated in modified Tyrode's buffer at pH 7.4. In some experiments, platelets were prelabeled with [14C] serotonin by addition of 2 µCi/ml platelet-rich plasma as previously described (34).

Red and mononuclear cells were prepared as previously described (35). Briefly, the cell pellet, obtained by an initial low speed centrifugation (800 × g for 15 min) to obtain a platelet-rich plasma, was resuspended in platelet-poor plasma and layered onto Ficoll-Hypaque (d = 1.074 ml/g). The mononuclear cells were removed from the interface after a 15-min centrifugation at 2,500 × g and washed twice by centrifugation. Red cells pelleting through the Ficoll-Hypaque were separated from white cells by sedimentation in 3% dextran T250 and subsequently were washed by centrifugation.

**Binding Assays**—In a typical binding assay, platelets were diluted in modified Tyrode's buffer containing 0.1% BSA, pH 7.4, to 8 × 1010 platelets/ml. A 100-µl aliquot of the platelet suspension, 110 µl of buffer or unlabeled ligand, 30 µl of 1.9 µM (350 µg/ml) 125I-TSP (precentrifuged at 11,750 rpm for 5 min in a Beckman microfuge) were added, followed immediately by 10 µl of stimulus or buffer. Unless otherwise indicated, incubations were performed at 37 °C. At selected time points, triplicate 50-µl aliquots were layered onto 300 µl of 20% sucrose in modified Tyrode's buffer, pH 7.4, and centrifuged for 3 min in a Beckman microfuge. Tips were amputated and counted, and platelet-bound TSP was calculated from the specific activity of the ligand, utilizing a molecular weight of 450,000 (18). Unless otherwise indicated, the TSP recovered in the centrifuge tip in the absence of platelets was subtracted as background and was always less than 0.1% of the total added ligand.

**SDS-Polyacrylamide Gel Electrophoresis**—The polyacrylamide gel system of Laemmli (33) was utilized in a vertical gel apparatus. To analyze 125I-TSP bound to platelets, all pellets were extracted with 10% SDS, 20% β-mercaptoethanol, and 6 M urea (32) for 5 min at 22 °C. Platelet extracts were then heated to 100 °C for 5 min prior to loading onto a 3% stacking gel. Electrophoresis was performed at 30 mA for 1 h, and the gels were fixed with 10% acetic acid in methanol and dried. Autoradiograms were developed using Kodak RP film and a Cronex enhancing screen at −70 °C.

**Statistical Analysis**—Binding data were fitted to models for N ligands binding to M sites utilizing the Ligand program (36) modified for the Apple II by T. Jackson, Department of Nuclear Medicine, Middlesex Hospital Medical School, London, England. This program performs linear regression of the non saturable binding, N₀, as a fitted parameter with the assumption that non-saturable binding is a constant fraction of the free ligand. Correlation coefficients were calculated on a Texas Instrument TI55 calculator.

**RESULTS**

**Binding of TSP to Unstimulated and Thrombin-stimulated Platelets**—When 125I-TSP (6 nM) was incubated with unstimulated platelets at 37 °C, time-dependent binding was observed (Fig. 1). An apparent steady state was attained at 25 min as the extent of binding did not change with an additional 20 min of incubation. Platelets, stimulated with 1.0 unit/ml thrombin, bound TSP with a similar time course. At the TSP input concentration utilized (6 nM), binding was augmented 4-fold by thrombin stimulation. Subsequent studies of 125I-TSP interaction with either thrombin-stimulated or resting platelets were evaluated at 30 min to ensure that steady state binding has been attained. Approximately 70% of the TSP bound to the resting or thrombin-activated platelets was initially reversibly bound. As has been observed with fibrinogen binding (5), bound TSP was subsequently stabilized, becoming irreversibly bound by 30 min of incubation (Table I).

To show that the binding of 125I-TSP to platelets was not due to its modification by radioiodination, varying proportions of 125I-TSP and unlabeled TSP were added to platelets, maintaining a constant total TSP concentration. As shown in Fig. 2A, a plot of the 125I-TSP bound to the resting platelets versus the per cent of 125I-TSP added was linear (correlation coefficient (r) = 0.99). A similar plot was obtained for 125I-TSP binding to thrombin-stimulated platelets (Fig. 2B). The correlation coefficient (r) was 0.98. These observations suggested that labeling of TSP did not alter its affinity for either

![Figure 1](image_url)

**Figure 1.** Time-dependent binding of TSP to resting and thrombin-stimulated platelets. The incubation mixtures consisted of the following components: platelets, 2.5 × 10⁷ cells/ml; 125I-TSP, 6 nM. The buffer was modified Tyrode's, pH 7.4, containing 2 mM CaCl₂. Incubations were performed at 37 °C, and the means ± S.E. are of triplicates.

| Time (min) | 500 | 1000 | 1500 |
|-----------|-----|------|------|
| 0         |     |      |      |
| 10        |     |      |      |
| 20        |     |      |      |
| 30        |     |      |      |

**Table I**

Reversibility of TSP binding

| Stimulus | Sample time | No inhibitor | Inhibitor at 8 min | Inhibitor at 30 min |
|----------|-------------|--------------|--------------------|-------------------|
|          |             | 5            | 30                 | 60                |
| Thrombin | 5           | 8,370        | 37,200             | 4,040             |
|          | 30          |              |                    |                   |
| None     | 5           | 4,500        | 260                | 630               |
|          | 30          |              |                    |                   |
|          | 60          | 670          | 160                | 630               |
|          |             |              |                    |                   |

The incubation mixtures consisted of the following components: platelets, 2.5 × 10⁷ cells/ml; 125I-TSP, 6 nM. The buffer was modified Tyrode's, pH 7.4, containing 2 mM CaCl₂. Incubations were performed at 37 °C, and the means ± S.E. are of triplicates.
TSP Binding to Platelets

**FIG. 2.** Effect of radiolabelling on the binding of TSP to platelets. $^{125}$I-TSP and nonlabelling TSP were added in varying proportions, maintaining a constant final TSP concentration of $2 \times 10^7$/ml in modified Tyrode's buffer, pH 7.4, containing 2 mM CaCl$_2$. Incubations were at 37°C for 30 min. With resting platelets, upper panel, the final concentration to TSP was 33 nM. With thrombin-stimulated platelets, lower panel, the final TSP concentration was 110 nM.

**Fig. 3.** Polyacrylamide gel analysis of the radiolabeled ligand bound to resting and thrombin-stimulated platelets. $^{125}$I-TSP was bound to resting or thrombin-stimulated platelets for 30 min at 37°C. The radioactivity associated with the cell pellets was extracted as indicated under "Experimental Procedures" and analyzed on 7.5% polyacrylamide gels under reducing conditions in the buffer system of Laemmli (33). Autoradiograms of the dried gel is shown. Left lane, thrombin-stimulated platelets; center lane, resting platelets; and right lane, the starting $^{125}$I-TSP. Scanning densitometry revealed >95% of radioactivity was in the major bands in each case. Note the smearing in the lower portions of the gels of the platelet-bound material due to the presence of 1 mg/ml BSA.

resting or thrombin-stimulated platelets.

The $^{125}$I-TSP bound to both the resting and thrombin-stimulated platelets was characterized by polyacrylamide gel electrophoresis. Autoradiograms of the gels are shown in Fig. 3. Under reducing conditions, the starting $^{125}$I-TSP yielded a single major band. The estimated molecular weight of 170,000 is consistent with that reported for the subunits of TSP (18). The ligands extracted from both resting platelet and thrombin-stimulated platelets had mobilities identical with that of the starting $^{125}$I-TSP.

To evaluate the specificity of TSP binding to resting and thrombin-stimulated platelets, the ability of four proteins (fibronectin, ovalbumin, transferrin, and BSA) to inhibit $^{125}$I-TSP binding was analyzed (Table II). For both resting and the thrombin-activated cells, the four proteins had a minimal effect on $^{125}$I-TSP binding; inhibition of binding did not exceed 7%. In contrast, unlabeled TSP (500 μg/ml) produced 64% inhibition of $^{125}$I-TSP binding to thrombin-activated platelets and 54% inhibition of binding to resting cells.

The capacity of nonlabelling TSP to inhibit $^{125}$I-TSP binding indicates that its interaction with both resting and thrombin-stimulated platelets is mediated by a limited number of binding sites. To estimate the number of these sites and their affinity, varying concentrations of TSP, containing $^{125}$I-TSP as a tracer, were added to unstimulated platelets. The TSP molecules bound per platelet were calculated from the specific activity of the $^{125}$I-TSP and was plotted as a function of the total TSP added. The binding of TSP to resting platelets appeared to be saturable (Fig. 4A). These data could be fit to a single class of TSP binding sites by nonlinear curve-fitting analysis in the "Ligand" computer program. In the sample shown, a good fit was obtained (mean square error = 28.7) when the nonspecific binding ($N_1$) was estimated to be 7.8 x 10$^{-15}$ M. This fit gave an apparent dissociation constant ($K_d$) of 5.0 x 10$^{-4}$ M, and 4200 TSP molecules were maximally bound per platelet. With three different platelet donors, unstimulated platelets bound 3100 ± 1000 molecules/platelet with a $K_d$ of 5.0 ± 0.2 x 10$^{-4}$ M ($N_1$ = 1.44 ± 0.05 x 10$^{-3}$ M).

With thrombin-stimulated cells, binding again appeared to have a major saturable component (Fig. 4B). A fit consistent with a single class of binding sites with apparent $K_d$ of 2.0 x 10$^{-15}$ M and 28,000 TSP molecules maximally bound per platelet was obtained by computer analysis. This fit was derived with $N_1$ = 1.6 x 10$^{-3}$ and had a mean square error of 7.18. The mean values for five donors were $K_d$ = 2.5 ± 1.0 x 10$^{-7}$ M, 35,600 ± 9,000 molecules/platelet, and $N_1$ = 1.7 ± 0.5 x 10$^{-3}$ M. Subsequent analyses of TSP binding were performed utilizing radiolabeled ligand at its apparent $K_d$ of 200 nM for thrombin-stimulated platelets and 50 nM for resting platelets.

At the input concentration of 200 nM, if the nonsaturating TSP molecules bound per platelet, it would contribute ≈3000 TSP molecules bound per platelet.

To derive the binding parameters indicated above, TSP had been isolated in the presence of EDTA, removal of calcium may alter the conformation of TSP (37), thereby changing its platelet binding functions. Therefore, TSP was isolated maintaining 2 mM calcium throughout its purification. In analyses such as those shown in Fig. 4, this material bound to both resting and thrombin-stimulated platelets with similar affinities and numbers of sites as TSP isolated in the presence of EDTA.

**TABLE II**

| Proteins           | $^{125}$I-TSP binding to platelets |
|--------------------|-----------------------------------|
|                    | Thrombin-activated | Resting |
| Ovalbumin (10 mg/ml) | 1 ± 2                 | 0 ± 3    |
| Transferrin (2 mg/ml) | 7 ± 4                 | 7 ± 4    |
| Bovine serum albumin (10 mg/ml) | 0 ± 1              | 0 ± 1    |
| Fibronectin (0.45 mg/ml) | 64 ± 8               | 54 ± 7   |

**Specificity of TSP binding to platelets**

Platelets, at a final concentration of $2 \times 10^7$/ml, were incubated with the indicated final concentrations of potential inhibitors, $^{125}$I-TSP (200 nM), and 1 unit/ml thrombin for 30 min at 37°C. Identical protocols were employed for binding to unstimulated cells except for omission of thrombin and reduction of the TSP concentration to 50 nM. In the absence of the added proteins, the stimulated cells bound 22,000 TSP molecules/platelet and the resting cells bound 846 molecules/platelet. Means ± S.E. of triplicates.
the inhibitors, their effect on TSP binding to thrombin-platelets was inhibited by 73%. EDTA (5 mM) inhibited binding to stimulated cells to a similar degree and the combination of the two inhibitors was no more effective than either alone. Thus, TSP binding to unstimulated platelets, is unlikely to arise from low level platelet activation during cell preparation.

To assess the contribution of contaminating cells to TSP binding, the interaction of the ligand with red and mononuclear cell preparations was examined. \(^{125}\)I-TSP binding to these cells was minimal, and thrombin had no effect on the limited binding observed. A platelet preparation from the same donor contained red cells and mononuclear cells at levels of 0.3 and 0.03%, respectively, relative to platelets. At these levels of contamination, the red and mononuclear cells contributed less than 5 molecules to the total of 767 molecules bound per cell to the nonstimulated platelet preparation.

**Stimulus Requirements for Enhanced TSP Binding—** Experiments were performed to establish that thrombin augmented binding of TSP to platelets was due to thrombin stimulation of the platelets rather than to proteolysis of the ligand (Table IV). Thrombin was preincubated with platelets prior to the addition of hirudin and \(^{125}\)I-TSP. Under this condition, binding was virtually identical with that observed in the absence of the thrombin inhibitor. In contrast, incubation of the TSP with thrombin, followed by the addition of hirudin and platelets, resulted in TSP binding similar to that seen in the absence of stimulus (Table IV). This result, coupled with the near complete inhibition of serotonin release, indicates that the hirudin was an effective thrombin antagonist and suggests that the critical interaction of thrombin is with the platelet rather than with the ligand.

The ability of ADP, a stimulus for fibrinogen binding, to induce TSP binding was also assessed. Under conditions where thrombin-stimulated platelets bound 10,150 ± 60 molecules/platelet, ADP (5 µM)-stimulated cells bound 1,070 ± 140 molecules/platelet and nonstimulated cells bound 850 ± 90 molecules/platelet. In contrast, the same ADP-stimulated cells bound 12 times more fibrinogen than resting platelets (9,300 ± 500 molecules/platelet versus 720 ± 60 molecules/platelet), confirming the activity of the ADP. ADP doses as high as 40 µM produced no increment in TSP binding. The thrombin requirement for augmented TSP binding was evaluated.

![Graph](image)

**Fig. 4.** A, saturable binding of TSP to resting platelets. Varying concentrations of TSP were added to platelets at a final concentration of \(2 \times 10^7 \)/ml in modified Tyrode’s buffer containing 2 mM CaCl\(_2\). Incubations were at 37°C for 30 min. The open circles are the experimentally determined data points, and the total binding curve is the best fit. The solid line is the nonsaturable binding estimated in the Ligand program. The solid circles represent saturable binding which was calculated by subtracting the nonsaturable component from the total binding. B, saturable binding of TSP to thrombin-stimulated platelets. The platelets were stimulated with a final concentration of 1 unit/ml α-thrombin. The incubation conditions and the symbols in the figure are the same as in 4A. Note the difference in ordinates.

**TABLE III**

*Effect of inhibitors on TSP binding*

| Stimulus          | Inhibitor       | TSP bound (molecules/platelet) |
|------------------|-----------------|-------------------------------|
| Thrombin         | None            | 11,550 ± 350                  |
|                  | EDTA            | 2,950 ± 220                   |
|                  | PGE\(_1\) + theophylline | 3,100 ± 110               |
|                  | PGE\(_1\) + theophylline | 2,870 ± 289               |
| None             | None            | 1,160 ± 80                    |
|                  | PGE\(_1\) + theophylline | 1,260 ± 190               |
|                  | EDTA            | 1,050 ± 80                    |
|                  | EDTA + PGE\(_1\) + theophylline | 1,360 ± 60               |

**Specificity of TSP Binding to Unstimulated Platelets**—The role of activated platelets and other cell types in the binding of TSP to nonstimulated platelet preparations was assessed. PGE\(_1\)-theophylline mixtures were used as inhibitors of platelet activation. As shown in Table III, this combination of antagonists did not reduce TSP binding to resting platelets (990 versus 1020 molecules/platelet). To verify the activity of the inhibitors, their effect on TSP binding to thrombin-activated cells was assessed in parallel. At the doses of PGE\(_1\) and theophylline used, TSP binding to thrombin-activated platelets was inhibited by 73%. EDTA (5 mM) inhibited binding to stimulated cells to a similar degree and the combination of the two inhibitors was no more effective than either alone. Thus, TSP binding to unstimulated platelets, is unlikely to arise from low level platelet activation during cell preparation.

**TABLE IV**

*Enhanced TSP Binding—*The platelets (PLTS) were at a concentration of 8 x 10^7 platelets/ml and were added in a 50-µl aliquot. The thrombin (Thr), 20 units/ml, was added in 10 µl as was the hirudin (hir) at 200 units/ml. The \(^{125}\)I-TSP was added at a final concentration of 200 nM. [\(^{14}\)C]Serotonin release was measured as previously described under “Experimental Procedures.” All incubations were at 37°C. Tyr is the abbreviation for Tyrode’s buffer.

The reaction mixtures were:

1. **PLTS + Thr** \(-^{125}\)I-TSP 30’
2. **PLTS + Thr** \(-^{125}\)I-TSP 30’
3. **PGE\(_1\) + Thr** \(-^{125}\)I-TSP 30’
4. **PLTS** \(-^{125}\)I-TSP 30’
5. **PLTS** \(-^{125}\)I-TSP 30’

| Reaction number | TSP-bound | [\(^{14}\)C]Serotonin release |
|-----------------|-----------|------------------------------|
|                 | molecules/platelet | %              |
| 1               | 14,670 ± 940      | 87 ± 1.2         |
| 2               | 16,500 ± 620      | 89 ± 1.7         |
| 3               | 2,470 ± 430       | 16 ± 3.4         |
| 4               | 2,690 ± 110       | 0 ± 3.4          |
| 5               | 2,670 ± 150       | 0 ± 1.0          |
uated. The thrombin effect was maximal at 0.25 units of thrombin/ml (Fig. 5).

Comparison of Requirements for TSP Binding to Unstimulated and Thrombin-stimulated Platelets—The divalent ion requirements for the binding of 125I-TSP to resting and thrombin-activated platelets has been further evaluated in Table V. Platelets were prepared in divalent ion-free Tyrode's buffer (using Chelex 100) to reduce calcium and magnesium to <0.1 μM. TSP binding to thrombin-stimulated platelets was similar in the presence of added calcium or magnesium and were enhanced relative to the absence of added divalent cation. When EGTA was added to reduce extracellular calcium, TSP binding was diminished by 5-fold. Addition of EDTA to the medium.

In contrast, TSP binding to resting platelets was temperature-independent in the 4–27°C range. In contrast, binding of the ligand to thrombin-activated platelets was temperature-sensitive. Although binding to the thrombin-stimulated cells was similar at 22 and 37°C, the interaction was reduced approximately 75% at 4°C. The level of residual binding at 4°C is consistent with the extent of TSP binding to nonstimulated platelets at the 125I-TSP input concentration.

As shown in Fig. 6B, fibrinogen had no effect on the binding of TSP to resting platelets. In contrast, fibrinogen inhibited TSP binding to thrombin-stimulated cells (Fig. 6A). This inhibition was dose-dependent, and 50% inhibition occurred at 40 nM fibrinogen. The maximal inhibition of TSP binding by fibrinogen was 82% which corresponded to 3600 TSP molecules bound per platelet. This level was consistent with the binding of TSP to unstimulated platelets at the TSP input concentration utilized.

Monoclonal antibody 10E5 immunopurifies glycoprotein GPIIb-IIIa in the presence of calcium (31). Dilutions of 10E5 inhibited TSP binding to thrombin-stimulated platelets in a dose-dependent fashion, and, at high concentrations of the antibody, 94% inhibition was achieved (Fig. 7). These same doses of 10E5 lowered 125I-TSP binding to the resting cells by less than 11%.

**DISCUSSION**

Both unstimulated and thrombin-activated platelets bound 125I-TSP. These interactions were time-dependent, not due to...
Thrombin-stimulated platelets. Conditions are the same as in Fig. 6A. B, the effect of monoclonal antibody 10E5 on TSP binding to resting platelets. Conditions are the same as in Fig. 6B.

The interaction of TSP with resting platelets had an apparent dissociation constant, $K_d$, of 50 nM, with 3100 ± 1000 TSP molecules maximally bound per platelet. This binding was not due to the presence of a small number of activated platelets or to other contaminating cells within platelet preparations. The presence of a specific binding site for TSP on unstimulated platelets distinguishes this ligand from fibrinogen, fibronecacin, and von Willebrand Factor as saturable binding of these other three adhesive proteins to unstimulated platelets distinguishes this ligand from fibrinogen and fibronecacin during spreading. The presence of less than 25 ng/ml (50 pm) TSP (19), and this level would not result in significant occupancy of the TSP binding site on resting platelets.

With thrombin-stimulated platelets, TSP bound with an apparent dissociation constant of 250 nM and 35,600 ± 9,000 molecules were maximally bound per platelet. Although the fit of the experimental data to a single site model was quite good, the release of endogenous platelet TSP (4 nM released under the conditions of the binding anlyses (42)), as well as fibrinogen, which inhibits TSP binding, and the probable presence of the nonstimulated TSP site on thrombin-stimulated platelets (see below) indicate that these parameters must be forwarded with considerable caution. The capacity of thrombin to enhance TSP binding was due to the effect of this agonist on platelets rather than to its proteolytic modification of the ligand. This conclusion is based upon the experiments using a thrombin inhibitor hirudin, as well as on the analysis of the ligand bound to thrombin-stimulated platelets. The doses of thrombin required for optimal augmentation of TSP binding are compatible with those required for induction of platelet secretion and fibronecacin receptor expression (11). ADP failed to augment TSP binding, although it did induce fibrinogen receptors on the same platelet preparation. The capacity of thrombin but not ADP to support specific binding also occurs with fibronecacin (11) and distinguishes TSP binding from that of fibrinogen (9) and von Willebrand Factor (43) which bind with high affinity to ADP-stimulated cells.

In addition to the differences in estimated binding parameters, several lines of evidence indicate that the TSP binding sites on unstimulated and thrombin-activated platelets are distinct. The differential effects of inhibitors of platelet activation (PGE, + theophylline), temperature, fibrinogen, monoclonal antibody 10E5, and divalent ions all point to the nonidentity between the TSP binding sites. Phillips et al. (21) reported that the association of endogenous TSP with the surface of thrombin-stimulated platelets was calcium dependent. In contrast, Gartner and Dockter (22) measured TSP binding to platelet surfaces and found it to be divalent ion-independent. This apparent dichotomy can now be explained by the identification of two independent TSP binding sites, one divalent ion-dependent and one divalent ion-independent. It seems most likely that both the binding sites co-exist on the surface of thrombin-stimulated platelets. Inhibitors of platelet activation, 4 °C and fibrinogen reduced $^{125}$I-TSP binding to thrombin-stimulated platelets by 75–85%. The level of residual TSP binding was compatible with the occupancy of the unstimulated binding sites at the TSP input concentration utilized. Monoclonal antibody 10E5 had a somewhat greater effect on TSP binding to thrombin-stimulated platelets, inhibiting the interaction by 94% rather than by the predicted 75–85%. The antibody, however, also had a slight effect on TSP binding to nonstimulated platelets (11% inhibition).

The effects of fibrinogen and fibronecacin on TSP binding to thrombin-stimulated platelets merit comment. TSP has been reported to interact with fibrinogen in the solid phase with dissociation constants of 3.4 nM (44). While the affinity of TSP for thrombin-stimulated platelets appears to be 2 orders of magnitude lower, the possibility that platelet fibrinogen or fibrin, expressed on the cell surface as a result of thrombin stimulation (45), serves as a TSP binding site must be considered. This possibility provides one explanation for the inhibition of TSP binding by fibrinogen. Alternative explanations for the inhibitory effect of fibrinogen may be competition for a shared or sterically related binding site, or interaction of the two molecules in solution preventing TSP binding to the platelets. Lahav et al. (46) have shown that platelet TSP comes in close proximity to surface-bound fibronecacin during spreading. The presence of less than 4000 molecules of fibronecacin per platelet (30) makes it unlikely that fibronecacin serves as a univalent TSP receptor for thrombin-activated platelets.

In sum, evidence has been provided for two classes of TSP binding sites on platelets. One class of sites is thrombin-inducible, and this interaction is calcium ion-dependent. The identification of this inducible site extends the analogy between thrombomodulin and the other three adhesive proteins, fibrinogen, fibronecacin, and von Willebrand Factor. The second class of sites is expressed by unstimulated platelets, and TSP binding to the site is divalent ion-independent. The existence of this site distinguishes the interaction of TSP with platelets from those of fibrinogen, fibronecacin, and von

![Graph](https://example.com/graph.png)
Willebrand Factor, and establishes a potentially unique mechanism for thrombospondin to influence platelet function.

REFERENCES

1. Tchoppy, T., Weiss, J. H., and Baumgartner, H. R. (1974) J. Lab. Clin. Med. 83, 296–305
2. Grinnell, F., Feld, M., and Snell, W. (1979) Cell Biol. Inter. Rep. 3, 586–592
3. Zucker, M. B., and Vroman, L. (1969) Proc. Soc. Exp. Biol. Med. 131, 318–320
4. Mustard, J. F., Perry, D. W., Kinlan-Rathbone, R. L., and Packham, M. A. (1975) Am. J. Pathol. 228, 1757–1765
5. Marguerie, G. A., and Plow, E. F. (1983) Annu. N. Y. Acad. Sci. 408, 556–566
6. Santoro, S. O. (1983) Biochem. Biophys. Res. Commun. 116, 135–140
7. Miller, J. L., Kupinski, J. M., Castella, A., and Ruggeri, Z. M. (1983) J. Clin. Invest. 72, 1532–1542
8. Plow, E. F., Marguerie, G. A., and Ginsberg, M. H. (1984) in The Biology of Platelets (Phillips, D. R., and Shuman, M. A., eds) Academic Press, New York
9. Marguerie, G. A., Plow, E. F., and Edgington, T. S. (1979) J. Biol. Chem. 254, 5357–5363
10. Bennett, J. S., and Vilaire, G. (1979) J. Clin. Invest. 64, 1393–1401
11. Plow, E. F., and Ginsberg, M. (1981) J. Biol. Chem. 256, 9477–9482
12. Fujimoto, T., Ohara, S., and Hawiger, J. (1982) J. Clin. Invest. 69, 1212–1222
13. Ginsburg, M. H., Forseyth, J., Lightsey, A., Chediak, J., and Plow, E. F. (1983) J. Clin. Invest. 71, 619–624
14. Ruggeri, Z. M., Badar, R., and De Marco, L. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 6036–6041
15. Plow, E. F., and Marguerie, G. A. (1980) Blood 56, 553–555
16. Ginsburg, M. H., Wolf, R., Marguerie, G., Coller, B., McEver, R., and Plow, E. F. (1985) Blood, in press
17. Plow, E. F., Srouji, A. H., Meyer, D., Marguerie, G., and Ginsberg, M. H. (1984) J. Biol. Chem. 259, 5386–5391
18. Lawler, J. W., Slattery, H. S., and Coligan, J. E. (1978) J. Biol. Chem. 253, 8609–8616
19. Saglio, S. D., and Slattery, H. S. (1982) Blood 59, 162–166
20. Gerrard, J. M., Phillips, D. R., Raos, G. H. R., Plow, E. F., Watz, D. A., Harber, L. A., and White, J. G. (1980) J. Clin. Invest. 66, 102–109
21. Phillips, D. R., Jennings, L. K., and Prasanna, H. R. (1980) J. Biol. Chem. 255, 11829–11832
22. Gartner, T. K., and Dockter, M. E. (1983) Thromb. Res. 33, 19–30
23. George, J. N., Lyons, R. M., and Morgan, R. K. (1983) J. Clin. Invest. 66, 1–9
24. Gartner, T. K., Williams, D. C., and Phillips, D. R. (1977) Thromb. Res. 7, 43–57
25. Gartner, T. K., Williams, D. C., Minion, F. C., and Phillips, D. R. (1978) Science 200, 1281–1283
26. Gartner, T. K., Gerrard, J. M., White, J. G., and Williams, D. C. (1981) Nature 289, 688–690
27. Jaffe, E. A., Leung, L. K., Nachman, R. L., Levin, R. L., and Mosher, D. F. (1982) Nature 295, 246–248
28. Norden, A. T., Haselt, M., and Rosa, J.-P. (1983) Thromb. Haemostasis 50, 401 (abstr.)
29. Doollittle, R. F., Schubert, D., and Schwartz, S. A. (1967) Arch. Biochem. Biophys. 118, 456–467
30. Plow, E. F., Birdwell, C., and Ginsberg, M. H. (1979) J. Clin. Invest. 63, 540–543
31. Coller, B. S., Peerschke, E. I., Scudder, L. E., and Sullivan, C. A. (1983) J. Clin. Invest. 72, 278–286
32. Wencel-Drake, J. D., Plow, E. F., Zimmerman, T. S., Painter, R. G., and Ginsberg, M. H. (1984) Am. J. Pathol. 115, 156–164
33. Lammli, U. K. (1970) Nature 227, 680–685
34. Ginsburg, M. H., Koizn, F., O’Malley, M., and McCarty, D. J. (1977) J. Clin. Invest. 60, 989–1007
35. Plow, E. F., and Collen, D. (1981) Blood 58, 1069–1074
36. Munson, P. J., and Rodbard, D. (1980) Anal. Biochem. 107, 220–239
37. Lawler, J., Chao, F. C., and Cohen, C. M. (1982) J. Biol. Chem. 257, 12257–12265
38. McPherson, J., Sage, H., and Bornstein, P. (1981) J. Biol. Chem. 256, 11380–11386
39. Mosher, D. F., Doyle, M. J., and Jaffe, E. A. (1982) J. Cell. Biol. 93, 343–348
40. Ratnoff, O. D., Mummy, S. M., Airhart-Brown, D., and Bornstein, P. (1982) J. Biol. Chem. 95, 351–354
41. Jaffe, E. A., Ruggiero, J. T., Leung, L. K., Doyle, M. J., McKeown-Longo, M. J., and Mosher, D. F. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 988–1002
42. Ginsburg, M. H., Wencel, J. D., White, J. G., and Plow, E. F. (1983) J. Cell. Biol. 97, 5714–5717
43. Fujimoto, T., and Hawiger, J. (1982) Nature 297, 154–166
44. Leung, L. K., and Nachman, R. L. (1982) J. Clin. Invest. 69, 542–549
45. Courtois, G., Ryckwaert, J. J., Ginsberg, M., Woods, V., Jr., Plow, E., and Marguerie, G. (1984) Circulation Suppl. 70, 358 (abstr.)
46. Lahav, J., Schwartz, M. A., and Hynes, R. O. (1982) Cell 31, 253–262