Complex Formation of Platelet Thrombospondin with Plasminogen

Modulation of Activation by Tissue Activator

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Abstract. Thrombospondin (TSP), a multifunctional α-granule glycoprotein of platelets, binds fibrinogen, fibronectin, heparin, and histidine-rich glycoprotein and thus may play an important role in regulating thrombotic influences at vessel surfaces. In this study we have demonstrated that purified human platelet TSP formed a complex with purified human plasminogen (Plg). Complex formation was detected by rocket immunoelectrophoresis of mixtures of the purified radiolabeled proteins. Significant complex formation of fluid-phase Plg with adsorbed TSP was also demonstrated by enzyme-linked immunosorbent assay (ELISA). The complex formation was specific, saturable, and inhibited by excess fluid-phase TSP, with an apparent K_D of $\sim$35 nM. In both ELISA and rocket immunoelectrophoresis systems, complex formation was inhibited by 10 mM ϵ-amino-n-caproic acid, implying that there is a role for the lysine binding sites of Plg in mediating the interaction. TSP also formed a complex with plasmin as detected by ELISA but did not directly inhibit plasmin activity measured with a synthetic fluorometric substrate or with a ^{125}I-fibrin plate assay. TSP, when incubated with Plg before addition to ^{125}I-fibrin plates significantly inhibited the generation of plasmin activity by tissue plasminogen activator (TPA) in a manner that was calcium dependent. A kinetic study of Plg activation by TPA in the presence of TSP demonstrated that Michaelis-Menten kinetics were followed and that TSP acted as a noncompetitive inhibitor. These studies support the hypothesis that TSP,

acting as a multifunctional regulator in focal areas of active hemostasis, could serve as a prothrombotic influence, leading to increased deposition of fibrin.

Introduction

Thrombospondin (TSP),[1] a 450-kD filamentous multinodular glycoprotein (1, 2) consisting of three 140-kD subunits (3), is a major α-granule protein of human platelets (4, 5) and is expressed on the platelet surface upon thrombin stimulation (6). Although circulating plasma levels are low (20–300 ng/ml) (7, 8), human fibroblasts and endothelial cells can release large amounts into their microenvironments (9, 10). TSP has also been identified as a synthetic product of human monocytes (11).

TSP appears to be a molecule of remarkable functional diversity. It may be important in mediating cell-to-cell communication. It is the endogenous lectin of stimulated platelets (12, 13). TSP binds fibrinogen (14), and monospecific anti-TSP Fab' partially inhibits both platelet aggregation and platelet fibrinogen binding (15). TSP also binds collagen and fibronectin (14, 16) and is incorporated into the extracellular matrix of fibroblasts in culture (10). TSP may play an important role in the coagulation and fibrinolytic systems, as evidenced by its ability to form complexes with histidine-rich glycoprotein (HRGP) (17), an 80-kD plasma protein with high affinity for heparin (18), and the lysine binding sites (LBS) of plasminogen (19).

The TSP-HRGP complex in a focal area of active hemostasis and thrombosis such as the vicinity of the platelet membrane could augment thrombotic influences, leading to increased

1. *Abbreviations used in this paper:* AFC, 7 amino-4-trifluoromethyl coumarin; EACA, ϵ-amino-n-caproic acid; ELISA, enzyme-linked immunosorbent assay; HRGP, histidine-rich glycoprotein; I_{50}, 50% inhibitory concentration; k_{cat}, catalytic rate constant; LBS, lysine binding sites; Plg, plasminogen; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TPA, tissue plasminogen activator; TSP, thrombospondin; VIIIR:Ag, Factor VIII-related antigen.

deposition of fibrin. To define further the participation of TSP in fibrinolysis, the interaction of TSP with plasminogen (Plg) was studied. In this paper we report that TSP forms a specific complex with plasminogen and modulates its activation to plasmin by tissue plasminogen activator (TPA).

Methods

Materials. Microtitration plates for enzyme-linked immunosorbent assays (ELISA) and Linbro tissue culture plates (area, 2.0 cm^2; capacity, 3.5 ml) for fibrin plate studies were purchased from Flow Laboratories Inc. (McLean, VA). Electrophoresis grade agarose (Seakem) was purchased from FMC Corp. (Rockland, ME). A fluorometric plasmin substrate, D-val-leu-lys-7 amino-4-trifluoromethyl coumarin (AFC) was purchased from Enzyme Systems Products (Livermore, CA). ε-Amino-*n*-caproic acid (EACA) was purchased from Calbiochem-Behring Corp. (La Jolla, CA). Type VII calf mucosal alkaline phosphatase, *p*-nitrophenyl phosphate, and soybean trypsin inhibitor were purchased from Sigma Chemical Co. (St. Louis, MO). All reagents were of analytical grade.

Purified proteins. Purified human TSP was prepared as described previously (17) by a modification of the method of Lawler et al. (1). 4 to 8 U of fresh human platelets were washed and the TSP was isolated from the thrombin-stimulated releasate by Sepharose 4B gel exclusion chromatography followed by heparin Sepharose affinity chromatography. The TSP was 95% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (17). Purified human glu-Plg was isolated by lysine affinity chromatography (20) followed by gel filtration and was determined to be free of HRGP and fibrinogen by an ELISA specific for these proteins. For the fibrin plate studies, purified human plasminogen (IMCO, Stockholm) was purchased from Mico, USA, New York and was free of TPA activity as determined by the ^{125}I-fibrin plate assay. This preparation was >95% pure by SDS-PAGE and consisted of only the glu form of Plg. Authentic glu and lys Plg were kindly supplied by Dr. F. J. Castellino (University of Notre Dame, IN) and were used as standards for the SDS-PAGE. Purified human plasmin was obtained by activation of purified Plg with insolubilized urokinase, as described previously (21), and stored at −70°C in 50% glycerol. Antithrombin III, albumin, gamma-globulin, Factor VIII-related antigen (VIIIR:Ag), α$_2$-plasmin inhibitor, platelet glycoproteins IIb and IIIa, complement components (C$_{1q}$, C$_3$, C$_4$, C$_5$), high molecular weight kininogen, coagulation Factors X and XII, and fibronectin, which were used as control proteins for the ELISA and fibrin plate studies, were obtained and purified as described in detail previously (14, 17). Plg-free human fibrinogen was obtained from IMCO, purified bovine fibrinogen from Calbiochem-Behring Corp. Human melanoma cell line TPA (70,000 IU/mg) was a generous gift of Dr. Desiré Collen, University of Leuven, Belgium.

Antisera. Antisera to purified Plg and TSP were raised in rabbits by standard techniques (22). Anti-Plg did not react as determined by ELISA with purified human fibrinogen, IgG, albumin, fibronectin, TSP, or Factor VIIIR:Ag. Preparation and monospecificity of other antisera has been documented in detail previously (14, 17). Anti-TSP did not react with Plg as determined by ELISA.

Radioisotope labeling. Purified proteins were labeled with ^{125}I by the modified chloramine-T method (23). For the rocket immunoelectrophoresis studies, specific activity of the Plg was 3.7 × 10^5 cpm/µg, and specific activity of the TSP was 8.2 × 10^4 cpm/µg. For the fibrin plate studies the specific activity of the fibrinogen was 2 × 10^5 cpm/pmol.

Reductive alkylation of thrombospondin. A sample of purified TSP was reduced in 3% 2-mercaptoethanol for 3 h at room temperature and followed by alkylation in 0.25 M iodoacetamide, pH 8.0. Complete reduction was confirmed by SDS-PAGE of this material, which revealed a single band of M$_r$ 180,000, identical to that of TSP monomer.

Rocket immunoelectrophoresis. Agarose stocks were prepared by melting electrophoresis grade agarose in 0.05 M phosphate buffer, pH 7.4, at a concentration of 1%. The melted agarose was cooled to 45°C and mixed with the indicated volume of prewarmed antiserum. 3 to 5 ml of antibody-agarose mixture was layered onto glass microscope slides. Antigen samples were applied into 4-mm diam wells and the individual slides were subjected to 50 V and 3 mA for 20 h at 22°C. After electrophoresis the slides were washed exhaustively with saline containing 0.1% azide and followed by a 30-min distilled-water wash, dried, and stained with 1% amido black in 40% ethanol-10% acetic acid and destained in 90% methanol-5% acetic acid.

Fibrin-agar indicator gels. The fibrin-agar indicator gels were prepared by the method of Reich (24) with the following solutions: (*a*) 2.5% agar in water boiled for 10 min before use and kept at 45°C; (*b*) phosphate-buffered saline (PBS) supplemented with thrombin (0.2 U/ml) and TPA (0.2 U/ml); and (*c*) PBS with Plg-free fibrinogen (10 mg/ml). Solution *a* (3.2 ml) was added to solutions *b* (1.9 ml) and *c* (1.3 ml), and the mixture was transferred to prewarmed templates to form a gel free of bubbles. Gels for rocket immunoelectrophoreses were prepared as described above, placed carefully on top of the indicator gel, and sealed in plastic wrap for 18 h in a humid chamber at 37°C. Lysis zones were identified as clear areas in the cloudy background of the unlysed fibrin. The upper immunoelectrophoresis gel was removed and the underlying fibrin layer was stained for 10 min with 0.5% amido black in 40% ethanol-10% acetic acid and destained in 90% methanol-5% acetic acid. The upper gel layer was also stained to determine the completeness of the transfer of the Plg-containing immunoprecipitate.

ELISA. The assay was performed according to the method of Voller et al. (25) as previously described (14, 17). Microtitration plates were coated with 0.2 ml purified TSP, 4 µg/ml, in bicarbonate coating buffer (15 mM Na$_2$CO$_3$, 35 mM NaHCO$_3$, pH 9.6, 0.02% NaN$_3$) for 3 h at 37°C. The wells were washed three times with Tris-Tween buffer (10 mM Tris-HCl, 0.15 M NaCl, 0.05% Tween 20, pH 7.4), and then purified Plg was added in duplicate (diluted in the same buffer) for 18 h at 4°C. After washing to remove unbound Plg, alkaline phosphatase-conjugated anti-Plg F(ab′)$_2$ prepared as previously described (21) at 1:1,600 dilution was added for 3 h at 22°C. After washing, the substrate *p*-nitrophenyl phosphate was added and color development was followed by repeated readings in a photometer (Titertek Multiskan; Flow Laboratories Inc.). The formation of complex was expressed as the enzymatic activity of the bound alkaline phosphatase (ΔA405 min^{-1}).

Fluorometric plasmin assay. A fluorometric plasmin substrate, D-val-leu-lys-AFC was added to PBS-Tween buffer containing a mixture of purified plasmin (64 ng/ml) plus TSP. The final volume was 300 µl and contained 33 ng substrate and the following molar ratios of TSP/plasmin: 0:1; 1:1; 49:1; and 98:1. Substrate hydrolysis was monitored in a spectrofluorometer (Perkin-Elmer Corp., Instrument Div., Norwalk, CT) at slit width 10 µm, excitation 400 nm, and emission 505 nm.

Preparation of ^{125}I-fibrin plates. ^{125}I-fibrin plates were prepared by the method of Unkeless et al. (26). To each well of a Linbro tissue culture plate was added 200 µl of a solution containing 20 pmol ^{125}I-

labeled bovine Plg-free fibrinogen with specific radioactivity of $\sim 10^5$ cpm/pmol. The wells were then air dried at 37°C for 15 h, clotted with thrombin (200 μl/well of a solution containing 0.04 μg/ml), and extensively washed. Plates were used within 10 d of preparation and washed three times with buffer before use.

Digestion of ^{125}I-fibrin films by plasmin or by Plg activated with TPA. Purified human plasmin (0.1 to 1.0 nM) in 200 μl Tris-saline buffer (20 mM Tris-HCl, 0.15 M NaCl, pH 7.4) containing 0.25% gelatin and 0.006% Tween 80 was added in duplicate to wells of ^{125}I-fibrin coated plates. These were gently rocked at 37°C and aliquots were removed at specific time intervals and counted to quantify cumulative release of ^{125}I-fibrin degradation products.

Purified human Plg (100 to 800 nM) in 190 μl Tris-saline buffer containing 0.25% gelatin, 0.006% Tween 80, and 2.0 mM $CaCl_2$ was added in duplicate to wells of ^{125}I-fibrin coated plates, which were gently rocked at 37°C. 10 μl of TPA (final concentration, 0.24 U/ml) was then added to the wells and 10-μl aliquots were taken at intervals and counted.

The influence of TSP on the above reaction(s) was studied by incubating Plg or plasmin in buffer as above with TSP (0–200 nM) for 1 h at room temperature before addition to the ^{125}I-fibrin coated wells. Control studies substituted Factor VIIIR:Ag or fibronectin at 200 nM for TSP.

Kinetics of Plg activation by TPA in the presence of TSP. Plg (125 to 800 nM) was incubated with TSP (0 to 150 nM) for 1 h at room temperature in Tris-saline buffer containing 0.25% gelatin, 0.006% Tween 80, and 1.5 mM $CaCl_2$ before addition to the wells of ^{125}I-fibrin plates. The Plg was activated with TPA (0.24 U/ml) for 30 min at 37°C and followed by the addition of 10 μl EACA (final concentration, 80 μM) to terminate the Plg activation. This interval was selected because Hoylaerts et al. (27), using a similar assay, have shown that activation is complete by 20 to 30 min. Our preliminary studies (data not shown) demonstrated >80% activation at 30 min, with no inhibition of activity of formed plasmin by this concentration of EACA. The cumulative solubilization of fibrin by the generated plasmin was measured as described above and the initial reaction rate was calculated as the amount of plasmin generated per second. All studies were done in duplicate.

Results

Demonstration of TSP-Plg complex formation by rocket immunoelectrophoresis

Radiolabeled Plg mixed with TSP (molar ratio 3:1) and electrophoresed into anti–Plg-containing agar migrated more rapidly than Plg alone and formed an anodally moving sharp peak, which contained Plg as detected by immunoprecipitation with monospecific antibody and by autoradiography (Fig. 1, *A* and *B*). To demonstrate that the anodal moving peak contained TSP complexed to the Plg, studies were performed using ^{125}I-TSP and unlabeled Plg. The fast moving Plg-containing peak demonstrable in antibody agar was radioactive (Fig. 2, *A* and *B*). Plg mixed with albumin, gamma-globulin, or Factor VIIIR:Ag at similar molar ratios to that employed with TSP (3:1) showed no alteration in mobility by rocket immunoelectrophoresis.
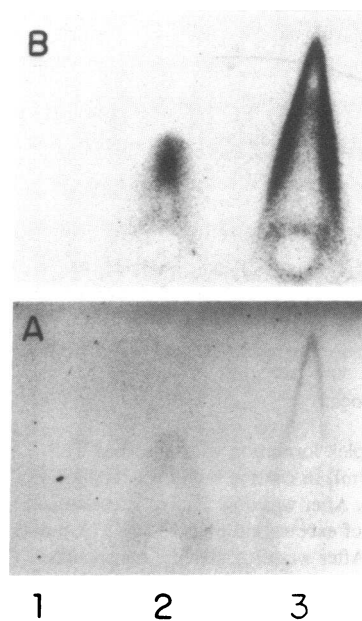
Figure 1. Radioactive Plg-TSP rocket immunoelectrophoresis. Well 1, TSP (4.9 μg); well 2, ^{125}I-Plg (3.2 μg, specific activity 9,400 cpm/μg); well 3, TSP (4.9 μg) plus ^{125}I-Plg (3.2 μg). Proteins were electrophoresed into agarose containing 0.08% monospecific anti-Plg serum. (*A*) Amido black stain of washed agarose plate. (*B*) Autoradiograph of *A*.

Demonstration of TSP-Plg complex formation by ELISA

The ELISA binding assay demonstrated that fluid-phase Plg bound significantly to purified TSP that was passively adsorbed to wells of a plastic microtitration plate. The extent of binding was quantified by the enzymatic activity of the bound alkaline phosphatase-conjugated anti-Plg $F(ab')_2$ ($10^4 \times \Delta A405 \ min^{-1}$) (Fig. 3). Complex formation was specific and saturable as demonstrated by complete inhibition of binding in the presence of excess fluid-phase TSP (Fig. 3). Excess fluid-phase albumin did not inhibit complex formation. The apparent K_D for TSP-Plg complex formation, approximated from the 50% binding point on the saturation curve (Fig. 3), was ~ 35 nM. Similarly, the I_{50} (50% inhibitory concentration) of fluid phase TSP as a competitive inhibitor of complex formation, determined from a titration experiment over a range of TSP concentrations from 0 to 50 nM, was ~ 18 nM, suggesting affinity in fluid phase similar to that in solid phase. No difference in the extent of complex formation was observed when either 1.5 mM
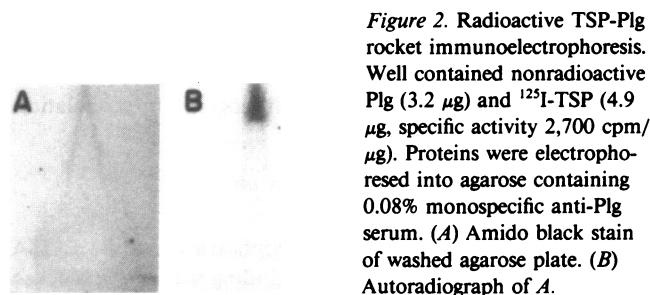
Figure 2. Radioactive TSP-Plg rocket immunoelectrophoresis. Well contained nonradioactive Plg (3.2 μg) and ^{125}I-TSP (4.9 μg, specific activity 2,700 cpm/μg). Proteins were electrophoresed into agarose containing 0.08% monospecific anti-Plg serum. (*A*) Amido black stain of washed agarose plate. (*B*) Autoradiograph of *A*.
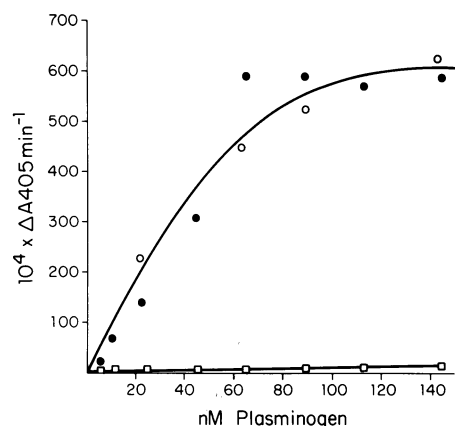
Figure 3. Saturation of Plg complex formation with adsorbed TSP measured by ELISA. TSP (4 µg/ml) in coating buffer was applied to the plastic wells for 3 h at 37°C. After washing, Plg (●), plasmin (○), or plasminogen in the presence of excess fluid phase TSP (23.8 pmol) (□) was added for 18 h at 4°C. After washing, alkaline phosphatase-conjugated anti-Plg F(ab')$_2$ was added. Curves show complex formation expressed as the enzyme activity of the bound alkaline phosphatase ($10^4 \times \Delta A405$ min^{-1}).

CaCl$_2$ or 1.0 mM EDTA was included in the buffer with Plg. Reduced alkylated TSP (TSP monomer) adsorbed to microtitration plates also bound Plg in these studies. The extent of binding was equivalent to that of whole TSP, suggesting that one TSP molecule can potentially bind three Plg molecules.

TSP complex formation with plasmin was also detected by ELISA. Plasmin incubated with a fivefold molar excess of soybean trypsin inhibitor to block its active site was added to TSP-coated microtiter plates, and saturable binding was observed (Fig. 3). The protease inhibitor was necessary to prevent proteolytic removal of TSP from the plastic plates by plasmin, since it has been shown that TSP is a substrate of plasmin (28).

Further specificity of complex formation was demonstrated by the inability of fluid-phase Plg to bind other adsorbed proteins. No complex formation was detected with fibronectin, antithrombin III, or Factor VIIIR:Ag (Table I). These proteins were adequately adsorbed to the plastic wells, as shown by binding of monospecific alkaline phosphatase-conjugated IgG and by ^{125}I-protein A binding studies (14). We have previously shown that adsorbed TSP did not bind fluid-phase albumin, gamma globulin, α_2-plasmin inhibitor, antithrombin III, platelet glycoproteins IIb and IIIa, complement components C1$_q$, C3, C4, C5, high molecular weight kininogen, or coagulation Factors X and XII (14, 17).

Influence of Plg lysine binding sites on TSP-Plg complex formation

With both the rocket immunoelectrophoresis and the ELISA studies, interference with the lysine binding sites of Plg blocked

Table I. Interaction of Plg with Adsorbed Proteins

Proteins*	$10^4 \times \Delta A405$ min^{-1}
TSP	307.5±3.6
AT-III	17.0±2.4
Fibronectin	21.1±1.4
VIIIR:Ag	19.1±2.1

AT-III, antithrombin III.
* Proteins (4 µg/ml) in coating buffer were applied to plastic wells for 18 h at 4°C. After washing, Plg (4 µg/ml) was added and binding was determined as described in Fig. 3.

TSP-Plg complex formation. Incorporation of 10 mM EACA into the anti-Plg antibody agar blocked formation of the rapidly moving anodal peak shown to contain the TSP-Plg complex (Fig. 4). Similarly, EACA added to fluid-phase Plg inhibited TSP-Plg complex formation as detected by ELISA. An inhibition titration over a range of EACA concentration from 10 µM to 10 mM demonstrated an I$_{50}$ of ~200 µM (Table II). This is consistent with involvement of the high affinity lysine binding site in the TSP-Plg interaction.

Effect of TSP on digestion of fibrin by Plg activated by TPA

Fibrin agar indicator gels. When rocket immunoelectrophoresis gels prepared as above in anti-Plg-containing agar were overlaid on agar gels containing fibrin and TPA, a clear zone of lysis was easily seen under the immunoprecipitate containing Plg (Fig. 5 B, well 2). A zone of lysis was also seen under the immunoprecipitate containing the TSP-Plg complex, but the degree of clearing was decreased (Fig. 5 B, well 3), suggesting that TSP interfered with fibrinolysis. To study further the functional implications of the TSP-Plg interaction, a ^{125}I-fibrin plate system was used.

^{125}I-fibrin plates. Purified human plasmin at different concentrations added to wells of ^{125}I-fibrin-coated plates digested the fibrin, yielding a family of sigmoidal solubilization curves (cumulative release of ^{125}I-fibrin degradation products). Each of these curves began at the origin and ended at the same plateau of maximum lysis. The time to reach 50% of total lysis (S$_{50}$) was an accurate reflection of the amount of plasmin
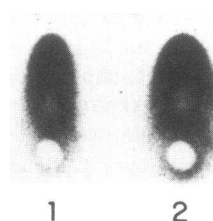

Figure 4. Radioactive TSP-Plg rocket immunoelectrophoresis in presence of EACA. Well 1 contained ^{125}I-Plg (3.2 µg) and well 2 contained ^{125}I-Plg (3.2 µg) plus TSP (4.9 µg). Proteins were electrophoresed in 0.08% anti-Plg and 10 mM EACA before autoradiography.

Table II. Influence of Lysine Binding Sites on Complex Formation Between TSP and Plg

Plg plus	Complex formation
Buffer	100%
EACA (20 μM)	100%
EACA (100 μM)	76.5%
EACA (500 μM)	30.5%
EACA (1 mM)	23.5%
EACA (10 mM)	0.6%

TSP (4 μg/ml) in coating buffer was applied to plastic wells for 18 h at 4°C. After washing, Plg (4 μg/ml) in buffer or in buffer containing glycine, EACA, or lysine was added. Plg binding was determined as described in Fig. 3.

added to the wells and was linearly related to the log plasmin concentration, as described previously (27). Such a plasmin standard curve was obtained for each set of ^{125}I-fibrin plates used.

Similarly, the addition of Plg plus TPA to ^{125}I-fibrin-coated plates digested the fibrin and yielded highly reproducible sigmoidal solubilization curves. The S_{50} in these studies reflected the amount of plasmin generated from the Plg by TPA. When Plg was first incubated with TSP before addition to the wells, these curves (and hence S_{50} values) were shifted to the right, reflecting a lower amount of plasmin generated from the same amount of Plg. A representative family of curves for a single Plg concentration (200 nM) is shown in Fig. 6 *A*. Fig. 6 *B* depicts results from a series of similar experiments done at four different Plg concentrations, each at five different TSP
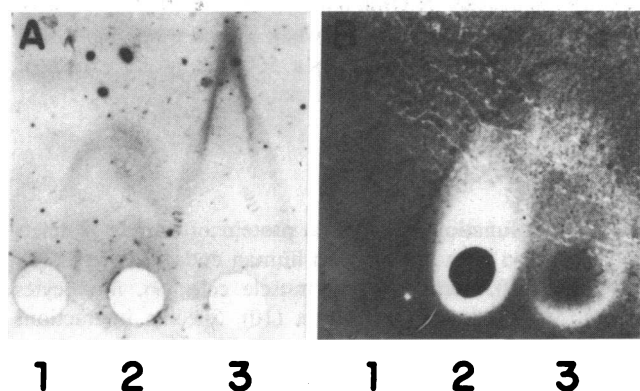

1   2   3   1   2   3

Figure 5. Effect of TSP on digestion of fibrin by Plg activated by TPA demonstrated by a fibrin agar indicator gel assay. (*A*) Amido black stain of TSP-Plg rocket immunoelectrophoresis in agar containing 0.08% monospecific anti-Plg serum. Well 1, TSP (4.9 μg); well 2, Plg (3.2 μg); and well 3, TSP (4.9 μg) plus Plg (3.2 μg). (*B*) Lysis zones (seen as clear area in cloudy background) in fibrin-agar indicator gel containing TPA (0.2 U/ml) overlaid for 18 h at 37°C.
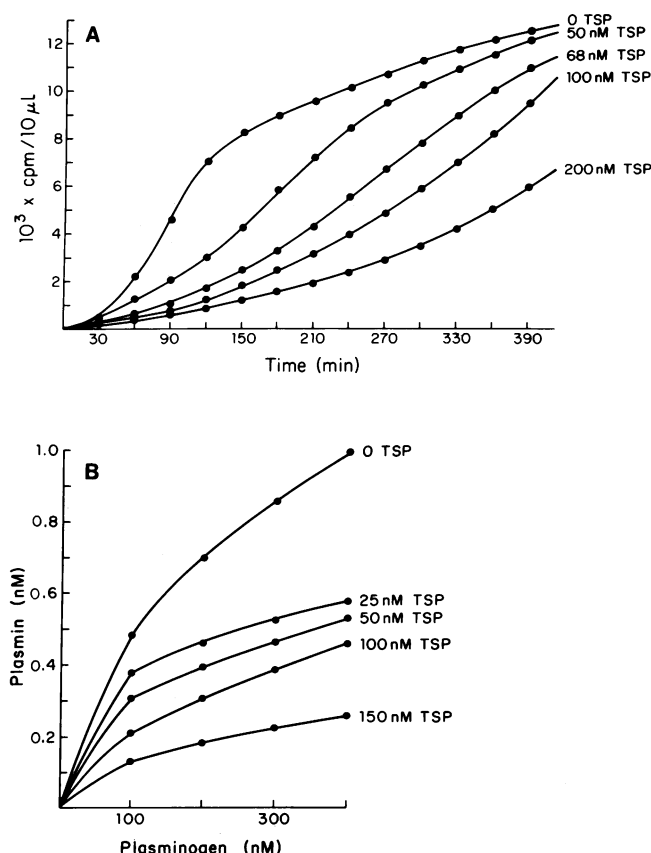

Figure 6. Effect of TSP on solubilization of ^{125}I-fibrin by Plg activated by TPA. (*A*) A solution containing Plg (200 nM), CaCl$_2$ (2 mM), and different amounts of TSP (0–200 nM) was incubated for 1 h at room temperature and added to ^{125}I-fibrin coated wells. TPA (final concentration, 0.24 U/ml) was added to the wells at time 0 and aliquots were taken at timed intervals to quantify release of ^{125}I-fibrin degradation products. Curves depict cumulative release of radioactivity. (*B*) Studies similar to that shown in *A* were done at different Plg concentrations (100–400 nM) and 1.5 mM CaCl$_2$, and from S_{50} values the amount of plasmin generated was calculated. TSP concentration was varied from 0 to 150 nM.

concentrations (0–150 nM). The data are expressed as the amount of plasmin generated (determined from S_{50} values) under each set of conditions. The family of curves generated demonstrated that at ~60 nM TSP the generation of plasmin is 50% inhibited (I_{50}, ~60 nM). This inhibition was calcium dependent with maximal inhibition at physiological calcium concentrations of 1.5 to 2.0 mM. Specificity of the inhibition was shown by the failure of other platelet α-granule glycoproteins (fibronectin or Factor VIIIR:Ag) at similar concentrations (up to 200 nM) to influence solubilization (Fig. 7). A molecular weight of one million was used to calculate Factor VIIIR:Ag concentrations.
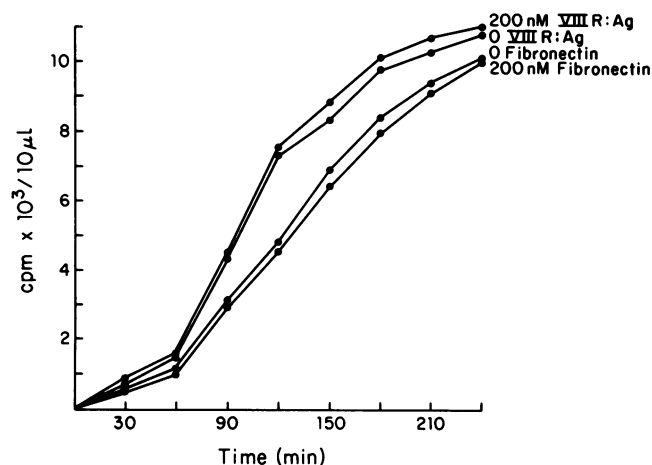
Figure 7. Influence of fibronectin or Factor VIIIR:Ag on solubilization of ^{125}I-fibrin by Plg plus TPA. Conditions were similar to those of Fig. 6 A except that fibronectin or Factor VIIIR:Ag (200 nM) was substituted for TSP.
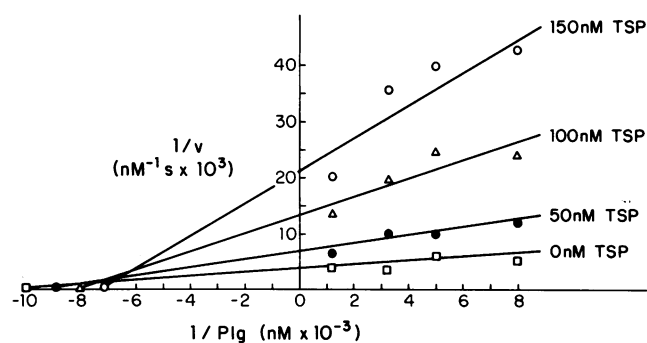

Figure 8. Lineweaver-Burke plot of activation of Plg on ^{125}I-fibrin by TPA in the presence of TSP. A solution containing Plg (125 to 800 nM), CaCl$_2$ (1.5 mM) and TSP (0–150 nM) was added to ^{125}I-fibrin plates and activated by addition of TPA (final concentration, 0.24 U/ ml). Activation was terminated by the addition of EACA (final concentration, 80 μM) at 30 min. The amount of plasmin generated by the 30-min activation was calculated from S_{50} values.

Influence of TSP on the kinetics of Plg activation by TPA

The kinetic analysis of Plg activation by TPA on ^{125}I-fibrin plates was determined by measuring the amount of plasmin generated from a 30-min activation of Plg by TPA (0.24 U/ ml) as determined from S_{50} values and a plasmin standard curve. Activation followed Michaelis-Menten kinetics with an apparent K_m of 0.10 μM and a catalytic rate constant, k_{cat}, of 7.6 s^{-1} × 10^3 (Table III). The influence of TSP on this reaction was studied and linear plots of 1/v vs. 1/Plg were obtained for each TSP concentration using a least squares linear regression analysis (Fig. 8). These lines show a progressive increase in vertical intercept (1/V_{max}) as TSP was increased, with only a corresponding small shift in the horizontal intercept (1/K_m), consistent with mixed type inhibition. Although the major component of inhibition was noncompetitive (decrease in V_{max}), there may also be a small competitive effect. The second-order rate constants k_{cat}/K_m, a measure of the overall

catalytic efficiency of Plg activation by TPA, showed that the overall reaction was markedly slowed in the presence of TSP.

TSP influence on plasmin activity

Using a synthetic tripeptide fluorometric plasmin substrate (D-val-leu-lys-AFC) it was found that TSP did not inhibit plasmin activity in vitro. No difference in substrate hydrolysis as measured in relative fluorometric units was seen when 6.4 ng/ ml plasmin was mixed with buffer alone or with 1:1, 49:1, or 98:1 molar ratios of TSP/plasmin. Incubation of TSP and plasmin for 30 min before addition of the substrate also had no effect.

Similarly, no inhibition of plasmin activity was seen on a ^{125}I-fibrin plate assay. No difference in the rate of solubilization of the fibrin was demonstrated when plasmin was incubated with buffer alone or with up to a 500-fold molar excess of TSP before addition to the fibrin plate.

Discussion

TSP, a multifunctional α-granule protein of human platelets (4, 5), has also been identified in human endothelial cells (7), fibroblasts (10), aortic smooth muscle cells (9), monocytes (11), and the extracellular matrix (10). Specific interactions have been identified with heparin (18), fibrinogen (14), fibronectin, collagen (16), and HRGP (17). These interactions suggest multiple functional roles, for example, in modulating cell-cell communication (12), cell-substratum interactions, platelet aggregation (15), and regulation of thrombotic influences at vessel surfaces.

Plg, the circulating zymogen of the serine protease plasmin, is the major enzyme of the fibrinolytic system (29) and in addition may be important in effecting other proteolytic pro-

Table III. Kinetic Parameters of Plg Activation By TPA on Fibrin Plates in the Presence of TSP

TSP	K_m*	V_{max}	k_{cat}	k_{cat}/K_m
nm	nm	nm s^{-1} × 10^3	s^{-1} × 10^3	nm s^{-1} × 10^6
0	100	0.290	7.6	76
50	120	0.160	4.2	36
100	112	0.072	1.9	17
150	142	0.047	1.2	9

* Kinetic parameters were obtained by use of the ^{125}I-fibrin plate assay as detailed in Methods, with the fibrin concentration at 20 pmol/well and TPA concentration at 0.24 U/ml.
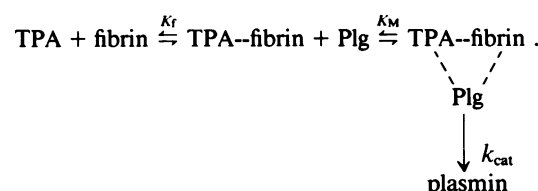
cesses such as inflammation and tumor cell invasion and migration (24, 30). In this study we have demonstrated by two independent lines of evidence that purified platelet TSP formed a complex with human Plg. By rocket immunoelectrophoresis it was demonstrated that Plg mixed with TSP when electrophoresed into antibody agar moved more rapidly than Plg alone and formed an anodally moving sharp peak which contained Plg as well as TSP (Figs. 1 and 2). In addition, an ELISA binding assay, which we have previously used to demonstrate specific TSP interaction with fibrinogen and HRGP (14, 17), showed specific and saturable binding of Plg to TSP adsorbed on microtitration plates, with an apparent K_D of 35 nM (Fig. 3).

The binding of Plg and TSP is mediated by the LBS of Plg. Saturation of these sites with 10 mM EACA or lysine completely inhibited complex formation in both ELISA and rocket immunoelectrophoresis systems. The I_{50} of EACA in the competitive inhibition study of $\sim$200 μM (Table II) is most consistent with involvement of the high affinity LBS. The protease plasmin that contains the LBS on its A-chain also specifically complexed with TSP (Fig. 3). The active site of the enzyme was apparently not involved in the complex formation, as incubation with soybean trypsin inhibitor did not prevent binding (Fig. 3).
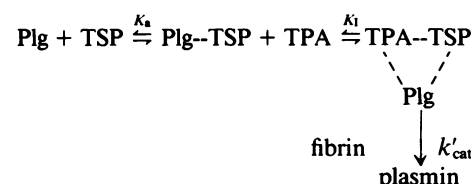
The LBS of Plg are critically important in regulating Plg activation (31, 32), and in mediating the interaction of plasmin(ogen) with fibrin(ogen) and α_2-plasmin inhibitor, as well as with HRGP and the antifibrinolytic amino acids. The kinetics of conversion of Plg to plasmin by TPA are highly unfavorable in the fluid phase. However, during blood coagulation both TPA and Plg bind to fibrin, changing the conformation of the proteins so that rapid activation ensues (27). Since the LBS of Plg appear to mediate complex formation with TSP and since TSP is likely to be present at significant concentrations in the local environment of a clot (via platelet activation and secretion), we have studied the effect of TSP on Plg activation by TPA. A fibrin-agar indicator gel system demonstrated less lysis by the TSP-Plg complex than by equal amounts of Plg alone (Fig. 5), suggesting that the TSP-Plg complex may be a less efficient substrate for TPA than is pure Plg. That this inhibition of lysis was not due to an anti-plasmin effect of TSP was shown by failure of TSP to inhibit plasmin activity both on a ^{125}I-fibrin plate assay or in fluid phase using a fluorometric plasmin substrate. In the ^{125}I-fibrin plate system, however, TSP significantly inhibited the generation of plasmin activity by TPA from Plg (Fig. 6) with an I_{50} of $\sim$60 nM. This inhibition was calcium dependent, being maximal at physiological levels of 1.5 to 2.0 mM.

Kinetic studies of Plg activation on fibrin are complicated since fibrin is both a substrate for the end product of the reaction, plasmin, and a strong positive effector of the reaction (27). Hoylaerts et al., however, have shown that the activation step (i.e., conversion of Plg to plasmin) is much faster than the actual solubilization of the fibrin film, so that by terminating

activation with EACA the two processes can be separated in time, allowing measurement of an initial activation rate. Using this method we have demonstrated, in agreement with previously published results (27), that Plg activation follows Michaelis-Menten kinetics and have demonstrated in the present study that TSP acts as a mixed inhibitor of Plg activation, with the major component being noncompetitive; i.e., the K_M was only minimally affected while V_{max} was considerably slowed (Fig. 8). Plg activation probably proceeds via the sequential formation of a TPA-fibrin-Plg ternary complex (27):

$$\text{TPA} + \text{fibrin} \overset{K_f}{\rightleftharpoons} \text{TPA--fibrin} + \text{Plg} \overset{K_M}{\rightleftharpoons} \text{TPA--fibrin}\,.$$
$$\diagdown\diagup$$
$$\text{Plg}$$
$$\Big\downarrow k_{cat}$$
$$\text{plasmin}$$

TSP is a noncompetitive inhibitor and thus may independently form another ternary complex with equal affinity for TPA, which is catalytically less active ($k'_{cat} \ll k_{cat}$):

$$\text{Plg} + \text{TSP} \overset{K_a}{\rightleftharpoons} \text{Plg--TSP} + \text{TPA} \overset{K_i}{\rightleftharpoons} \text{TPA--TSP}$$
$$\diagdown\diagup$$
$$\text{Plg}$$
$$\text{fibrin}\,\Big\downarrow k'_{cat}$$
$$\text{plasmin}$$

Recent studies using isolated purified constituents strongly suggest that TSP, Plg, and TPA form a ternary complex (Silverstein, R. L., R. Nachman, L. L. K. Leung, and P. C. Harpel, manuscript in preparation). The role of possible binary TSP-TPA complex formation in affecting the inhibition of plasmin generation remains to be determined.

These studies support the hypothesis that TSP acting as a multifunctional regulator in focal areas of active hemostasis and thrombosis could serve as a prothrombotic influence, leading to increased deposition of fibrin. We have previously shown that the interaction of TSP with fibrinogen on the activated platelet surface is an important step in the platelet aggregation process (15) and have recently shown that TSP specifically complexes with HRGP (17), a plasma and platelet α-granule protein with high affinity for heparin (18). This interaction may be important in concentrating the heparin-neutralizing action of HRGP to the vicinity of a platelet plug. The findings in the present study raise the possibility that by modulating the activation of Plg by TPA, TSP could further enhance fibrin deposition at platelet-blood vessel wall interfaces. It remains to be shown how the interaction of Plg with HRGP (19) affects these interactions.

The full biological significance of the TSP-Plg interaction remains an important subject for future studies. In addition to removal of fibrin from the vascular bed, the Plg activator-plasmin system is important in other physiological and patho-

logical processes (30), such as inflammation (33), neoplasia (34), and embryogenesis (35, 36, 37). Through a complex system of controls including the level of tissue production of TPA (38), localized degradation and inactivation of TPA (39, 40), inactivation of plasmin by circulating inhibitors (21), and microenvironmental influences on the kinetics of Plg activation, Plg activation may be a general mechanism for generating localized proteolysis in a microenvironment (30). TSP may play an important role in such non–fibrin mediated Plg activator systems. Although it circulates at very low levels, TSP may be present in significant concentrations in the microenvironment where Plg activation occurs, e.g., by platelet degranulation, as can occur at sites of inflammation or tumor cell metastasis, as part of the normal connective tissue or endothelial extracellular matrix or in association with cellular surfaces. Since TSP is functionally immobilized (like fibrin), it may modulate the kinetics of TPA-induced plasminogen activation at extravascular sites.

Acknowledgments

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