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Thrombospondin Is a Slow Tight-Binding Inhibitor of Plasmin†

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ABSTRACT: Thrombospondin is a multifunctional glycoprotein of platelet α-granules and a variety of growing cells. We demonstrate that thrombospondin is a slow tight-binding inhibitor of plasmin as determined by loss of amidolytic activity, loss of ability to cleave fibrinogen, and decreased lysis zones in fibrin plate assays. Stoichiometric titrations indicate that approximately 1 mol of plasmin interacts with 1 mol of thrombospondin, an unexpected result considering the trimeric nature of thrombospondin. Plasmin in a complex with streptokinase or bound to ε-aminocaproic acid is protected from inhibition by thrombospondin, thereby implicating the lysine-binding kringle domains of plasmin in the inhibition process. Thrombospondin also inhibits urokinase plasminogen activator, but more slowly than plasmin, stimulates the amidolytic activity of tissue plasminogen activator, and has no effect on the amidolytic activity of α-thrombin or factor Xa. These results, therefore, identify thrombospondin as a new type of serine proteinase inhibitor and potentially important regulator of fibrinolysis.

Thrombospondin (TSP),[1] a 450 000-dalton trimer of 150 000-dalton disulfide-bonded subunits, is a major constituent of platelet α-granules and is released upon platelet activation (Frazier, 1987). TSP is also a product of normal and transformed cells in culture, including endothelial cells (Mosher et al., 1982), smooth muscle cells (Raugi et al., 1982), fibroblasts (Jaffe et al., 1983), monocytes (Jaffe et al., 1985), and glial cells (Asch et al., 1986), and a constituent of their extracellular matrix (Jaffe et al., 1983). Embryonic tissues immunostain intensely for TSP (O'Shea & Dixit, 1988). In addition, atherosclerotic lesions exhibit marked immunostaining for TSP (Wight et al., 1985). Among known functions of TSP are binding specific to Ca^{2+} (Lawler & Simons, 1983), heparin (Dixit et al., 1984), collagen (Galvin et al., 1987), fibronectin (Lahav et al., 1984), fibrinogen (Leung & Nachman, 1982), fibrin (Bale et al., 1985), laminin (Lawler et al., 1986), plasminogen (Silverstein et al., 1984), and urokinase plasminogen activator (u-PA) (Harpel et al., 1990). Thus, TSP has the potential to play a role in tissue remodeling, development, and hemostasis.

Materials and Methods

Chemicals. H-D-Val-Leu-Lys-*p*-nitroanilide (S2251) and H-D-Ile-Pro-Arg-*p*-nitroanilide (S2288) were purchased from Kabi, Mölndal, Sweden, and *p*-nitrophenyl p-guanidinobenzoate and ε-aminocaproic acid (ε-ACA) were from Sigma Chemical Co., St. Louis, MO.

Proteins. Plasminogen was purified from fresh frozen human plasma and separated into its two carbohydrate variants according to published procedures (Castellino & Powell, 1981). Plasminogen was activated by u-PA immobilized on Sepharose 4B as described previously (Wiman & Wallén, 1973). Ac-

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[1] Abbreviations: TSP, thrombospondin; S2251, H-D-Val-Leu-Lys-*p*-nitroanilide; S2288, H-D-Ile-Pro-Arg-*p*-nitroanilide; u-PA, urokinase plasminogen activator; t-PA, tissue plasminogen activator; HEPES *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HBD, amino-terminal heparin binding domain of TSP; TSP(-HBD), TSP lacking the amino-terminal HBD; ε-ACA, ε-aminocaproic acid.

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tive-site titration of the plasmin with *p*-nitrophenyl *p*-guanidinobenzoate (Chase & Shaw, 1969) indicated that the enzyme was 78% active based on the concentration determined by the 280-nm absorbance assuming an $A_{1cm}^{1\%}$ of 1.61 and a molecular weight of 76 500 (Wallén & Wiman, 1970). TSP was purified from human platelet concentrates (Murphy-Ullrich & Mosher, 1985) and its concentration determined using an $A_{1cm}^{1\%}$ of 1.09 and a molecular weight of 450 000 (Lawler, 1986). Human fibrinogen and α-thrombin were prepared as described previously (Jakobsen & Kierulf, 1973; Owen & Jackson, 1973). Recombinant single-chain tissue plasminogen activator (t-PA) was purchased from Biopool AB, Umeå, Sweden, u-PA from Sigma Chemical Co., St. Louis, MO, and streptokinase from Kabi, Mölndal, Sweden. Rabbit anti-TSP antibodies were prepared as described previously (Jaffe et al., 1983) and tested at approximately one-fourth their concentrations in the antiserum as a globulin fraction prepared by precipitation with 40% saturated ammonium sulfate.

Experiments. All experiments described were performed using 50 mM HEPES, 0.119 M NaCl, 2 mM $CaCl_2$, and 1 mg/mL PEG 6000, pH 7.4. Plasminogen activation by u-PA or t-PA was measured according to Rånby (1982), where plasmin formation is monitored continuously through production of *p*-nitroaniline from amidolysis of the chromogenic substrate S2251. Reaction volumes were 200 μL, and the increase in absorbance at 405 nm was measured using a Thermomax Molecular Devices Kinetic Microplate reader. The initial rates of plasmin generation, calculated from nonlinear least-squares fits of the progress curves, were measured for ≤5 min so as to minimize inactivation of the generated plasmin by TSP. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was developed and run according to Blobel and Dobberstein (1975), using the buffer system of Maizel (1971), and stained with Coomassie Brilliant Blue R-250. For consistency, the plasmin used for all experiments is Lys(2)-plasmin, except in Figure 3 where unfractionated plasmin was used. The same results were observed with Lys(1)-plasmin and Glu-plasmin, with one exception. The rate of inhibition of Lys(1)-plasmin by TSP was approximately 2-fold slower than the rate of inhibition of Lys(2)-plasmin. Other experimental details are found in the legends to the figures.

Results

Lysis of fibrin/plasminogen plates by t-PA or, more strikingly, by u-PA was inhibited when TSP was a component of the fibrin matrix (Figure 1A). This result has been observed previously with t-PA (Silverstein et al., 1984) and was attributed to effects of TSP on the activation of plasminogen by t-PA. However, we did not find any statistically significant effects of TSP on plasminogen activation by t-PA or u-PA in a soluble system in the absence or presence of fibrinogen or fibrin (Table I). Therefore, we tested whether the observed antifibrinolytic effect of TSP might be a result of modulation of plasmin activity and not plasminogen activation. Figure 1B,C shows the effect of TSP on the lysis of fibrin plates by purified plasmin. Fibrinolysis was inhibited when TSP was incorporated into the fibrin matrix or when TSP was preincubated with plasmin. These findings indicate, therefore, that the antifibrinolytic activity of TSP results principally from inhibition of plasmin activity and not plasmin generation.

To test this proposal further, the activities of plasmin samples incubated overnight with varying molar ratios of TSP were determined using amidolytic, fibrinogenolytic, and fibrinolytic assays (Figure 2). Complete loss of amidolytic and fibrinogenolytic activities (panels A and B, respectively, of Figure 2) and ~90% loss of fibrinolytic activity (Figure 2C, also see Figure 1C) were observed. The titration in Figure 2A is typical of the type observed for the interaction of an enzyme with a tight-binding inhibitor (Morrison & Stone, 1985). The residual fibrinolytic activity (~10%) is probably due to a nonlinear relationship between plasmin concentration and lysis area (Figure 1C) and some slow dissociation of TSP–plasmin complexes due to the 100-fold dilution of the aliquots and long duration, 20 h, of this assay. Alternatively, the incomplete inhibition is possibly caused by competition of fibrin with TSP for the binding to plasmin.

Table I: Effect of TSP on the Initial Velocity of Activation of Plasminogen by t-PA or u-PA in the Absence or Presence of Fibrinogen or Fibrin[a]

activator	effector	initial velocity (nM plasmin/min) −TSP	+TSP	% change
t-PA		0.07	0.06	−14
t-PA	fibrinogen	0.78	0.84	+8
t-PA	fibrin	1.22	1.34	+10
u-PA		2.82	2.58	−9
u-PA	fibrinogen	1.52	1.58	+4
u-PA	fibrin	1.46	1.29	−12

[a] 321 nM Glu(2)-plasminogen was incubated with 220 μM S2251 in the absence or presence of 5.0 μM fibrinogen or 4.8 nM α-thrombin/62 nM fibrinogen and 0 or 235 nM TSP. After incubation for 10 min at 25 °C, t-PA or u-PA at final concentrations of 1.3 or 1.2 nM, respectively, was added, and the increase in absorbance at 405 nm measured and analyzed as described under Materials and Methods.

Samples of incubations were examined using SDS–PAGE (Figure 3). There was limited degradation of TSP, as reported (Lawler & Slayter, 1981), but no evidence for formation of SDS-stable TSP–plasmin complexes.

Figure 4A shows the time course for inhibition of TSP by plasmin under the pseudo-first-order conditions, [TSP]/[plasmin] > 10. The mode of inhibition of plasmin by TSP is indistinguishable from that observed for irreversible inactivators and, therefore, has been analyzed as such. The data of Figure 4A were fit to eq 1, which describes the irreversible

$$[E]_{t=t}/[E]_{t=0} = \exp(-k_{obs}t) \quad (1)$$

inactivation of an enzyme under pseudo-first-order conditions. k_{obs} is the pseudo-first-order rate of inhibition of plasmin (E) by TSP, units of s^{-1}, which is equal to k_2[TSP], where k_2 is the second-order rate constant for inhibition of plasmin by TSP, units of M^{-1} s^{-1}. From Figure 4A, the rate of inhibition of plasmin by TSP, k_{obs}, was 1.6×10^{-3} s^{-1}, which corresponds to a k_2 of 6300 M^{-1} s^{-1}. Equation 1 predicts that k_{obs} should be a linear function of TSP concentration. Indeed, other experiments show that k_{obs} increases linearly with TSP concentration up to 0.54 μM.

When plasmin (or plasminogen) was in a complex with streptokinase, it was protected from inhibition by TSP (Figure 4A, open squares). In addition, when plasmin was bound to the lysine analogue ε-ACA, the rate of inhibition by TSP was dramatically slowed; k_{obs} decreased 27-fold to 5.9×10^{-5} s^{-1} (Figure 4A, open circles). The rate of inhibition of plasmin by TSP was also slowed by an anti-TSP polyclonal antibody (Figure 4A, closed squares) and by diisopropyl phosphate–plasmin (data not shown), but not by Ca^{2+}, heparin, or fibronectin (data not shown), other molecules that interact with TSP. The one-to-one stoichiometry, slow kinetics, and neutralizing effects of anti-TSP antibodies are evidence against the possibility that the inhibition we observed is due to a contaminant of TSP such as α_2-antiplasmin (Wiman & Collen, 1978). In addition, we could detect no bands except for the

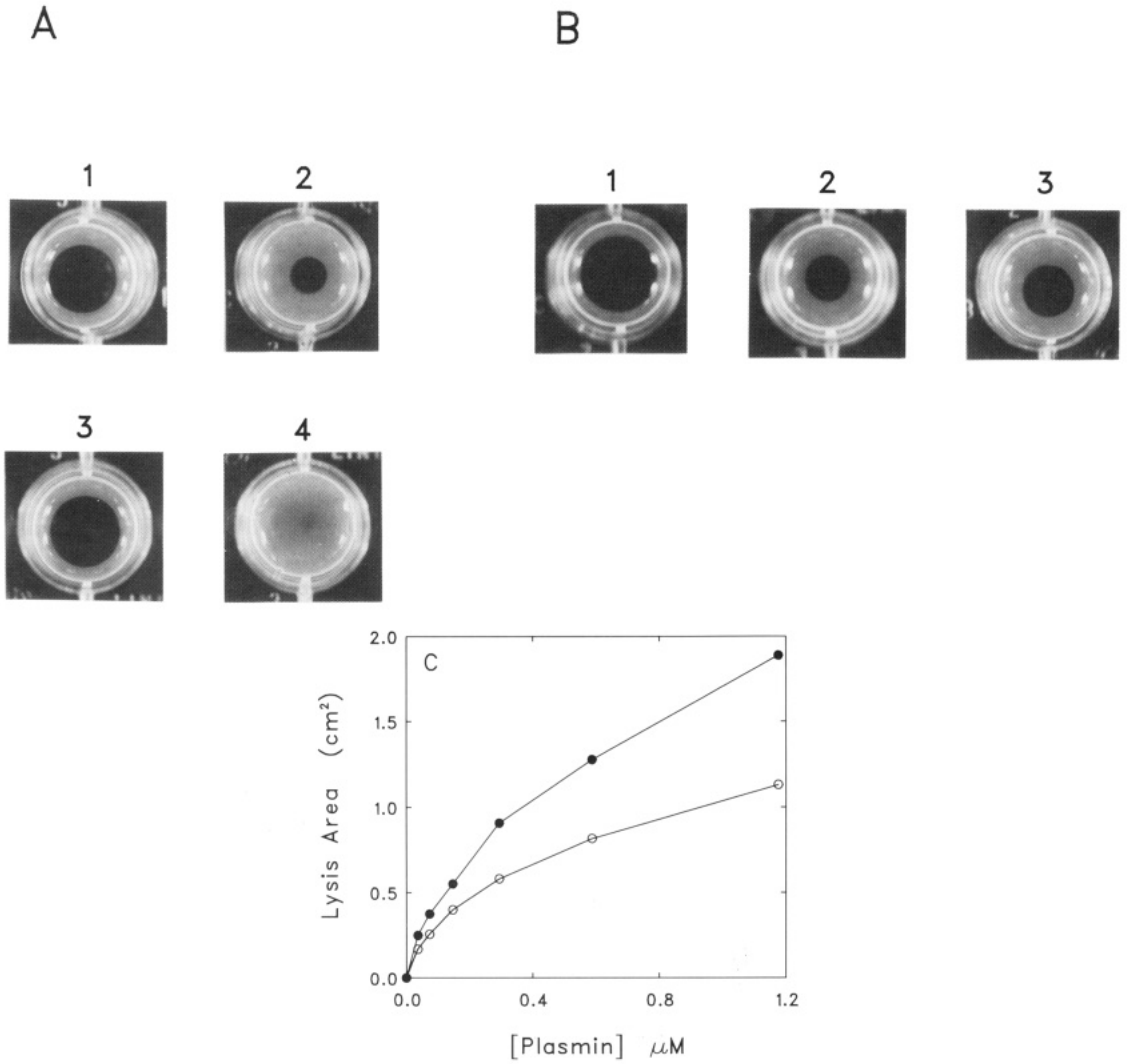

FIGURE 1: Effect of TSP on the lysis of fibrin plates by t-PA/plasminogen, u-PA/plasminogen, and plasmin. (A) Fibrin plates (0.5 mL) were prepared using 5.4 μM fibrinogen, 0.21 μM Glu(2)-plasminogen, and 1.5 nM α-thrombin. TSP, 0.60 μM, was incorporated into plates 2 and 4. t-PA, 5 μL of 0.13 nM, was added to the center of plates 1 and 2, and u-PA, 5 μL of 0.12 nM, was added to plates 3 and 4 which were then incubated for 20 h at 37 °C in a humid environment. (B) Fibrin plates were prepared using 5.4 μM fibrinogen and 1.5 nM α-thrombin. TSP, 0.62 μM, was incorporated into plate 3. A 5-μL aliquot of 0.59 μM plasmin was added to the center of plates 1 and 3. In plate 2, a 5-μL aliquot of a mixture of 0.59 μM plasmin and 0.79 μM TSP preincubated for 5 h at 37 °C was added. The plates were treated as described in (A). (C) Aliquots (5 μL) of varying plasmin concentrations were added to the center of fibrin plates containing no (●) or 0.43 μM TSP (○), and the area of lysis zones was determined. The fibrin plates were prepared and treated as described in (B). Lysis areas were determined from the average of two diameters measured at right angles. The data points represent the average of duplicate experiments.

150-kDa subunit of TSP by staining with Coomassie brilliant blue when 50 μg of reduced purified protein was separated in SDS on a gradient polyacrylamide gel that separated proteins 5–200 kDa in size.

The ability of TSP to inhibit other serine proteinases was compared to inhibition of plasmin (Figure 4B). TSP inhibited the amidolytic activity of u-PA, but at a very slow rate (k_{obs} = 1.7×10^{-5} s^{-1}), stimulated the amidolytic activity of t-PA, and had no significant effect on the amidolytic activity of human α-thrombin or bovine factor Xa (data not shown). The greater inhibitory effect of TSP on the lysis of fibrin/plasminogen plates by u-PA as compared to t-PA (Figure 1A) is, therefore, probably a consequence of the inhibition of u-PA versus activation of t-PA by TSP. Although the effects of TSP on u-PA and t-PA activity develop slowly, the rates of these processes are not incompatible with the rate of clot lysis in vivo.

Discussion

TSP inhibits plasmin at equimolar concentrations of enzyme and inhibitor (Figure 2) in a fashion which is indistinguishable from that observed for irreversible inactivators (Figure 4A). Because inhibition is not immediate and SDS-stable complexes are not formed (Figure 3), TSP may be classified as a "slow tight-binding" inhibitor of plasmin (Morrison & Stone, 1985). Subsequent studies have shown that the K_d for plasmin–TSP is <1 nM as determined in assays that continuously monitor plasmin inhibition through competitive chromogenic substrate hydrolysis (P. J. Hogg, unpublished results), considerably tighter than the estimate of binding of plasminogen to substrate-adsorbed TSP (Silverstein et al., 1984). The second-order rate constant for inhibition of plasmin by TSP, 6300 M^{-1} s^{-1}, is comparable to the rate constant for inactivation of thrombin by antithrombin III, 7400 M^{-1} s^{-1} (Olson & Shore, 1982), a process of known physiological importance.

Our results are different from previously reported descriptions of enzymatic activity of plasmin–TSP (Silverstein et al., 1984) and u-PA–TSP (Harpel et al., 1990) complexes. We cannot explain why previous investigators failed to note inhibition. Many of the previously reported experiments were carried out with one or more of the proteins adsorbed to microtitration plates.

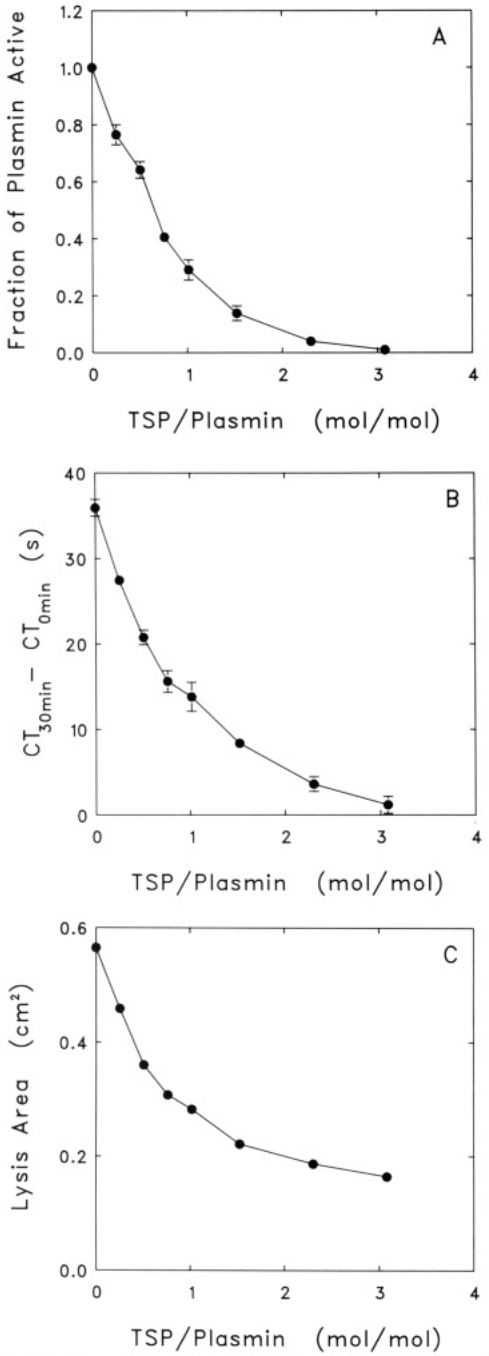

FIGURE 2: Titration of plasmin with TSP as determined by amidolytic, fibrinogenolytic, and fibrinolytic assays. (A) Plasmin (0.24 μM) was incubated with varying molar ratios of TSP for 16 h at room temperature. Remaining active plasmin concentration was measured amidolytically using the chromogenic substrate S2251. (B) Samples of the TSP/plasmin complexes from the experiment described in part A were diluted 50-fold into 5.4 μM fibrinogen and incubated at 37 °C. The clottability of the fibrinogen was determined at 0 and 30 min by sampling the reactions into 7.2 nM α-thrombin and measuring the clotting time (CT) using a Amelung coagulometer KC4A. (C) Samples (5 μL) of the TSP/plasmin complexes from the experiment described in part A were added to the center of fibrin plates which were prepared and incubated as described in the legend to Figure 1C. Data points represent the average of duplicate experiments; error bars indicate two standard errors of the mean.

The titrations in Figure 2 indicate that approximately 1 mol of TSP interacts with 1 mol of plasmin. This was an unexpected result in view of the trimeric structure of TSP. The result suggests that plasmin interacts with TSP through a unique region of the trimeric TSP molecule.

When plasmin is in a complex with streptokinase or bound

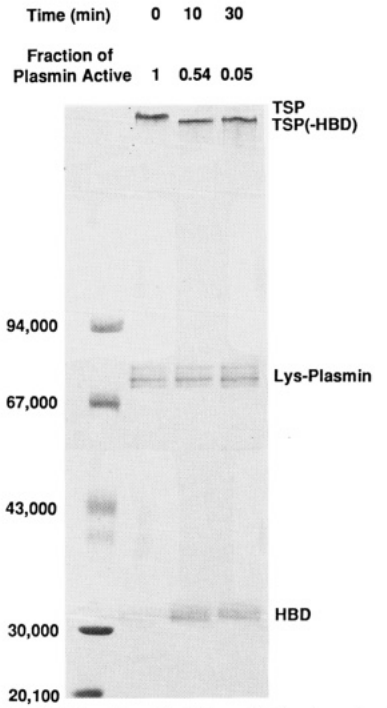

FIGURE 3: SDS–PAGE and amidolytic activity of equimolar mixtures of TSP and plasmin incubated for different times. TSP (1.5 μM final concentration) and plasmin (1.4 μM final concentration) were mixed and sampled at 0, 10, and 30 min. Five microliters of the samples was immediately assayed for plasmin amidolytic activity using S2251, while 70 μL was quenched with SDS treatment buffer (lacking reducing agent) and subjected to SDS–PAGE on a 7–12% gradient gel as described under Materials and Methods. Molecular weight marker proteins are in the left lane. The amino-terminal heparin-binding domain (HBD) of TSP, which is cleaved from TSP by plasmin, is indicated near the bottom of the gel, and the disulfide-linked trimer lacking the heparin-binding domain [TSP(–HBD)] is shown just below intact TSP.

to the lysine analogue ϵ-ACA, it is protected from inhibition by TSP (Figure 4A). These features are in common with inactivation of plasmin by α_2-antiplasmin (Wiman & Collen, 1978; Cederholm-Williams et al., 1979) and implicate the lysine-binding kringle domains of plasmin in the inhibition process (Castellino & Powell, 1981).

The finding that TSP inhibits u-PA-mediated fibrin dissolution more than it does t-PA-mediated lysis (Figures 1 and 4B) suggests that processes involving u-PA action may be preferentially regulated by TSP. Also, the observation that plasmin/streptokinase is not inhibited by TSP (Figure 4A) suggests that this complex should be more effective in dissolving thrombospondin-rich clots than either of the plasminogen activators.

These results identify TSP as a new type of serine proteinase inhibitor. The findings have important implications for the role of TSP in tissue remodeling, development, and haemostasis. Plasmin activity is important in all these processes, and the identification of TSP as a tight-binding plasmin inhibitor suggests a regulatory role for this multifunctional protein. For instance, TSP release from platelets is incorporated in large amounts into fibrin clots (Bale et al., 1985). This TSP, like fibrin-bound α_2-antiplasmin derived from plasma (Sakata & Aoki, 1982), may control orderly lysis of the fibrin network by infiltrating leukocytes and stromal cells. Also, the antifibrinolytic activity of TSP may account for the finding that platelet-rich clots are more resistant to thrombolysis than platelet-poor clots (Jang et al., 1989). The observation that the kringle modules of plasmin are involved in the inhibition by TSP hints as additional regulation by molecules that in-

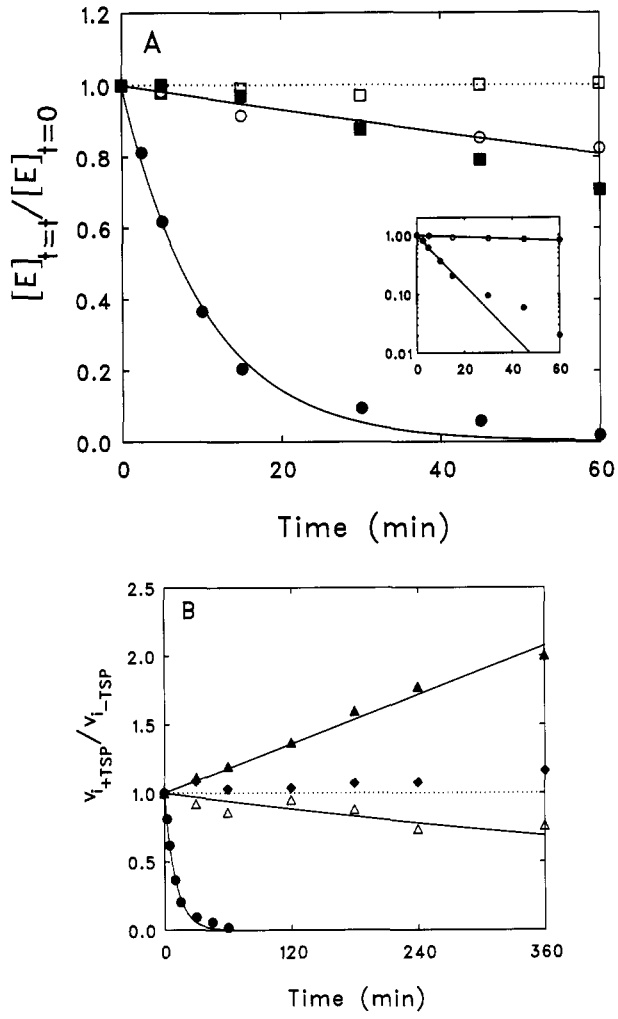

FIGURE 4: Time course of inhibition of plasmin, plasmin/streptokinase, and u-PA and activation of t-PA by TSP. (A) TSP (0.26 μM) or buffer was incubated with plasmin (24 nM) in the absence (●) or presence of 10 mM ϵ-ACA (○), 0.12 μM streptokinase (□), or anti-TSP polyclonal antibody (■) at 37 °C. At the indicated times, aliquots of the incubations were added to additional ϵ-ACA (25 mM final concentration) and assayed immediately for the concentration of active plasmin by measuring the rate of amidolysis of 147 μM S2251. Data points represent the average of duplicate experiments. The solid lines represent the best fit (Duggleby, 1984) of the data to eq 1 with $k_{obs} = 1.6 \times 10^{-3}$ s^{-1} (●) and 5.9×10^{-5} s^{-1} (○). The inset shows the logarithmic transform of the data in the absence (●) and presence (○) of 10 mM ϵ-ACA. The dotted line represents no change in plasmin activity. (B) TSP (0.26 μM) or buffer was incubated with u-PA [37 nM (△)], t-PA [30 nM (▲)], or α-thrombin [29 nM (♦)] at 37 °C. At the indicated times, aliquots of the incubations were assayed for the initial velocity (v_i) of amidolysis of 524 μM S2288. Data points represent the average of duplicate experiments. The data for u-PA (△) were fit by least-squares regression (Duggleby, 1984) to eq 1 with $k_{obs} = 1.7 \times 10^{-5}$ s^{-1}. The data for t-PA (▲) have been fit arbitrarily to a straight line with the ordinate intercept fixed at 1. The solid circles (●) and fit line are from the experiment with plasmin described in part A. The dotted line represents no change in amidolytic activity.

teract with plasmin through this region (Castellino & Powell, 1981).

Registry No. PA, 105913-11-9; plasmin, 9001-90-5; proteinase inhibitor, 37205-61-1.

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