Characterization of the antiplasmin activity of human thrombospondin-1 in solution

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These studies demonstrate relatively rapid association of plasmin with thrombospondin and the effects of this interaction on plasmin activity towards D-Val-L-Leu-L-Lys *p*-nitroanilide hydrochloride (S-2251) and the proteinase inhibitors α_2 -antiplasmin (α_2 AP) and α_2 -macroglobulin (α_2 M). Binding of plasmin to thrombospondin reached an apparent reversible equilibrium within 3 min at 22 °C. The amidase activity of bound plasmin was inhibited. An analysis of S-2251 hydrolysis indicated that thrombospondin is a linear mixed-type plasmin inhibitor. The dissociation constant (K_D) for the binding of plasmin to thrombospondin was 0.5 μ M, assuming one plasmin binding site per thrombospondin homotrimer. Plasmin and miniplasmin slowly cleaved thrombospondin, yielding products which were comparable with those generated by other proteinases. Tranexamic acid inhibited the digestion of thrombospondin by plasmin and miniplasmin, suggesting an important role for the kringle-5 domain in this process. When plasmin was incubated

first with thrombospondin and then with α_2 AP, plasmin that was apparently bound to thrombospondin reacted with α_2 AP at a decreased rate; however, within 20 min, all of the plasmin was recovered in complex with α_2 AP. Similar results were obtained with α_2 M. Transfer of plasmin from thrombospondin to α_2 AP or α_2 M probably required plasmin–thrombospondin-complex dissociation. A low level of reaction of α_2 AP with thrombospondin-associated plasmin could not be ruled out. These results demonstrate that the activity of plasmin, when bound to thrombospondin, is greatly diminished or eliminated. The plasmin–thrombospondin complex, which is formed within 3 min, is fully reversible and the associated plasmin is in a latent form protected from proteinase inhibitors. Therefore, thrombospondin may regulate plasmin activity in a manner which is distinct from conventional proteinase inhibitors and other extracellular-matrix proteins.

INTRODUCTION

The digestion of fibrin and fibrinogen by plasmin has been well characterized; however, recent studies have identified other physiologically significant plasmin substrates including growth factors, metalloproteinase zymogens and extracellular-matrix proteins (for review see [1]). The intact structure of [Glu¹]-plasminogen includes 791 amino-acid residues which form a C-terminal catalytic domain preceded by five triple-disulphide-bounded kringle domains [2,3]. The kringle domains mediate non-covalent binding interactions between plasmin(ogen) and numerous macromolecules including fibrin, cell-surface receptors, and matrix proteins. Plasminogen activation results from the cleavage of a single Arg⁵⁶⁰–Val⁵⁶¹ peptide bond [2]. Activation occurs more readily when plasminogen is bound to fibrin or other macromolecules [4–6]. Furthermore, plasmin bound to fibrin [7,8] or cell surfaces [1,9] is protected from inhibition by the primary plasmin inhibitors α_2 -antiplasmin (α_2 AP) and α_2 -macroglobulin (α_2 M). These complex interactions limit the proteolytic activity of plasmin to sites where this activity is necessary.

Thrombospondin-1 is a homotrimeric 420 000-*M_r* glycoprotein and apparently the product of a single locus in the human genome [10,11]. Upon activation, high levels of thrombospondin are released from platelet α -granules and incorporated into the polymerizing fibrin clot [12]. By interacting with fibrinogen and platelet receptors, thrombospondin stabilizes platelet aggregates

[13,14]. In addition, thrombospondin, which is synthesized by both normal and neoplastic cells, functions in cellular adhesion, motility and differentiation [14,15]. Since plasmin and thrombospondin co-exist in the same microenvironments (fibrin clot, basement membrane, extracellular matrix) and affect the same processes (cell motility, growth, tumour metastasis) the interaction of plasmin with thrombospondin may be of great importance.

Silverstein et al. [6,16] demonstrated that plasminogen binds thrombospondin and that binding enhances the rate of plasminogen activation by tissue-plasminogen activator (t-PA). Furthermore, these investigators demonstrated that thrombospondin-associated plasmin is protected from inhibition by α_2 AP. Based on studies with lysine analogues, an important role for the high-affinity lysine-binding site (located in the kringle-1 or K1 domain of plasminogen) in mediating the plasmin(ogen)–thrombospondin interaction was suggested. DePoli et al. [17] proposed that the plasminogen–thrombospondin interaction occurs via kringle 5 (K5) since they were able to demonstrate binding of ¹²⁵I-thrombospondin to an elastase-digestion fragment of plasminogen (miniplasminogen) which contains only K5 and the catalytic domain. The studies presented by Depoli et al. [17] were performed using plasminogen which was denatured and/or blotted on nitrocellulose, and in some experiments binding was not inhibited by lysine. Therefore, the importance of the various plasmin(ogen) kringle domains in the plasmin(ogen)–thrombospondin interaction is not clearly resolved.

Abbreviations used: α_2 AP, α_2 -antiplasmin; α_2 M, α_2 -macroglobulin; t-PA, tissue-plasminogen activator; PNPGB, *p*-nitrophenyl *p*'-guanidinobenzoate hydrochloride; BAPNA, *N*^ε-benzoyl-DL-arginine *p*-nitroanilide hydrochloride; TEA, tranexamic acid; εAHA, ε-amino-*N*-hexanoic acid; PPACK, D-Phe-L-Pro-L-Arg-chloromethyl ketone; S-2251, D-Val-L-Leu-L-Lys *p*-nitroanilide hydrochloride; K5, kringle-5 domain; TBS-C-T, buffer containing 50 mM Tris/HCl, 150 mM NaCl, 1 mM CaCl₂, 0.05 % Tween 80, pH 7.6; *t*_{0.5}, time required for 50 % inhibition of initial plasmin activity; *t*_{0.95}, time required for 95 % inhibition of initial plasmin activity.

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The studies by Silverstein et al. [16] demonstrated that binding of plasmin to thrombospondin does not directly affect plasmin activity. Recently, however, this conclusion has been brought into question. Based on fibrin plate-lysis studies and chromogenic-substrate-hydrolysis experiments, Hogg et al. [18] proposed that plasmin binds slowly but irreversibly to thrombospondin. As a result of binding, plasmin is completely inactivated so that, in effect, thrombospondin may be considered a slow tight-binding plasmin inhibitor ($K_D < 1.0$ nM).

In this investigation, we demonstrate relatively rapid association of plasmin with thrombospondin. As a result of this interaction, which reaches an apparent equilibrium in approx. 3 min, the plasmin is completely or almost completely inhibited. Our conclusion, that thrombospondin inhibits plasmin, supports the study by Hogg et al. [18]; however, the interaction reported here was reversible and of significantly lower affinity (0.5 μ M) than previously reported. As demonstrated in thrombospondin-digestion experiments, the kringle domains and particularly K5 play an important role in promoting the rapid association of plasmin with thrombospondin.

MATERIALS AND METHODS

Materials

p-Nitrophenyl *p*'-guanidinobenzoate hydrochloride (PNPGB), *N* α -benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BAPNA), tranexamic acid (TEA) and ϵ -aminohexanoic acid (ϵ AHA) were purchased from Sigma. D-Phe-L-Pro-L-Arg-chloromethyl ketone (PPACK) was from Calbiochem. D-Val-L-Leu-L-Lys *p*-nitroanilide hydrochloride (S-2251) was from Kabi Vitrum, Stockholm, Sweden. Na¹²⁵I was from Amersham International, and Iodobeads were from Pierce.

Proteins

[Glu¹]Plasminogen was purified from human plasma by the method of Deutsch and Mertz [19]. The final preparations included carbohydrate variants I and II, with variant II present in slight excess. Miniplasminogen was prepared by digesting plasminogen with pig pancreatic elastase and purified by chromatography on lysine-Sepharose [20].

Miniplasminogen and plasminogen were activated with low-molecular-mass urokinase (Calbiochem). The concentration of active proteinase was determined by active-site titration [21] or by titration with α_2 AP. α_2 M and α_2 AP were purified from human plasma by the methods of Imber and Pizzo [22] and Wiman [23] respectively.

Human thrombospondin was purified from outdated pooled human platelet concentrates kindly provided by the Virginia Blood Service (Richmond, VA, U.S.A.). The method of Santoro and Frazier [24] was followed except that the thrombin (5.0 nM) used to release platelet α -granules was inactivated with 5.0 μ M PPACK. The thrombospondin was homogeneous as determined by SDS/PAGE. A single band with an apparent $M_r < 400\,000$ was present before reduction. The mobility of thrombospondin purified in this laboratory (with and without reduction) was identical to a preparation kindly provided by Dr. Deane Mosher (University of Wisconsin). The concentration of purified thrombospondin was determined by measuring the absorbance at 280 nm using a coefficient ($A_{1\text{cm}}^{1\%}$) of 9.1 [25].

Radio-iodination

Plasminogen and miniplasminogen were radio-iodinated with Iodobeads as described by the manufacturer (Pierce). Desalting

was performed on a Sephadex G-25 column (Pharmacia). The specific activities of ¹²⁵I-plasminogen and ¹²⁵I-miniplasminogen were 0.5–1.4 μ Ci/ μ g of protein and 0.8–1.1 μ Ci/ μ g of protein respectively. After activation with urokinase, the activities of ¹²⁵I-plasmin and ¹²⁵I-miniplasmin were decreased by less than 5% compared with non-radio-iodinated proteinase, as determined by the rate of S-2251 hydrolysis.

Plasmin amidase activity in the presence of thrombospondin

Plasmin (18 nM) and different concentrations of thrombospondin (0.2–1.2 μ M) were incubated in the sample cuvette of a Hewlett-Packard 8450 diode-array spectrophotometer for 5 min at 22 °C. S-2251 (0.09–0.72 mM) was then added and the absorbance at 406 nm was measured every 2 s for 200 s. The velocity of substrate hydrolysis was determined by transforming absorbance measurements using the first-derivative function (dA_{406}/dt). Results were analysed by Lineweaver–Burk plots, which were uniformly linear with correlation coefficients ranging from 0.99 to 1.00.

The buffer for these amidase activity experiments and all other presented studies (except when specified) was 50 mM Tris/HCl/150 mM NaCl/1 mM CaCl₂/0.05% Tween 80, pH 7.6 (TBS-C-T). The hydrolysis of S-2251 by plasmin and the effects of thrombospondin on plasmin amidase activity were unchanged when Tween 80 was omitted from the buffer; however, high concentrations of thrombospondin (> 0.5 μ M) could not be consistently maintained in solution without Tween 80.

In some amidase activity experiments, plasmin (18 nM) and thrombospondin (0.2–1.2 μ M) were incubated for 30 min (instead of 5 min) before addition of S-2251. In other studies, thrombospondin (0.2–1.2 μ M) and S-2251 (0.54 mM) were added simultaneously to a cuvette containing plasmin (18 nM). Experiments which were used to generate Lineweaver–Burk plots were performed in quadruplicate. All other experiments were performed at least in duplicate.

Irreversible inhibition of plasmin by thrombospondin

The inhibition of plasmin by thrombospondin detected by Hogg et al. [18] was irreversible in the presence of ϵ AHA [18]. To detect this form of inhibition, plasmin (24 nM) was incubated with thrombospondin (260 and 400 nM) at 37 °C for 2 h in the presence and absence of 10 mM ϵ AHA. Samples of each solution were removed at various times. S-2251 and ϵ AHA were then added simultaneously at final concentrations of 0.54 mM and 25 mM respectively. Absorbance values were determined at 406 nm.

Thrombospondin digestion by plasmin and miniplasmin

Plasmin or miniplasmin (18 nM) were incubated with thrombospondin (0.74 μ M) in the presence or absence of 10 mM TEA at 37 °C. Aliquots of the reaction mixtures were removed at various times. The proteinases were inactivated with 0.4 mM PNPGB and the samples were denatured in the presence of 22 mM dithiothreitol. SDS/PAGE was performed on 8% (w/v) slabs using a HEPES–imidazole, pH 7.3, buffer system. Fragments were identified by comparison with Bio-Rad molecular-mass standards. The digestion of thrombospondin was also studied in the presence of 2 mM EDTA (no CaCl₂).

Reaction of plasmin with α_2 AP in the presence of thrombospondin

Plasmin inhibition by α_2 AP in the presence of thrombospondin was studied by two different techniques. In the first set of

experiments, plasmin (18 nM) and thrombospondin (0.1–0.4 μM) were incubated for 5 min at 22 °C. S-2251 (0.54 mM) was then added and the absorbance at 406 nm was determined every 2 s for 100 s to establish a baseline rate of substrate hydrolysis. After adding $\alpha_2\text{AP}$ (18 nM), monitoring was continued for an additional 300 s. The presented values ($t_{0.5}$ and $t_{0.95}$) are the times required for 50% and 95% inhibition of the initial plasmin activity. The $t_{0.5}$ and $t_{0.95}$ were not corrected for the effect of S-2251 on the reaction of plasmin with $\alpha_2\text{AP}$.

In the second set of experiments, ^{125}I -plasmin (18 nM) was incubated with 1.2 μM thrombospondin for 5 min at 22 °C. $\alpha_2\text{AP}$ (0.9 μM) was then added. At various times, samples were removed and the plasmin inactivated with PNPGB. SDS/PAGE was performed on 5% (w/v) slab gels. $\alpha_2\text{AP}$ –plasmin complex was clearly resolved from unreacted ^{125}I -plasmin. Gels were autoradiographed and each lane was sliced into 3-mm-long sections which were counted in an LKB model 1275 Minigamma γ counter. This procedure quantifies free ^{125}I -plasmin and ^{125}I -plasmin bound to $\alpha_2\text{AP}$ [8,9].

Reaction of plasmin with $\alpha_2\text{M}$ in the presence of thrombospondin

^{125}I -Plasmin (18 nM) was incubated with thrombospondin (0.2–1.2 μM) for 5 min at 22 °C. $\alpha_2\text{M}$ (180 nM) was then added and incubation was continued. At various times, samples were removed and the plasmin inactivated with PNPGB. Samples were denatured under non-reducing conditions and subjected to SDS/PAGE on 5% (w/v) slab gels. $\alpha_2\text{M}$ –plasmin complex was clearly resolved from unreacted ^{125}I -plasmin. Gels were autoradiographed and analysed as described above.

Plasmin inhibition by mixtures of $\alpha_2\text{M}$ and $\alpha_2\text{AP}$

^{125}I -Plasmin was incubated with thrombospondin (0.2–1.2 μM) for 5 min at 22 °C. A premixed solution of $\alpha_2\text{AP}$ and $\alpha_2\text{M}$ was then added. The final concentrations of plasmin, $\alpha_2\text{AP}$, and $\alpha_2\text{M}$ were 3.3 nM, 33 nM, and 2.04 μM respectively. These concentrations were selected so that the plasmin would distribute almost equally between the two inhibitors in the absence of thrombospondin. After incubation for 30 min at 22 °C, reactions were terminated with 0.4 mM PNPGB and analysed by SDS/PAGE, autoradiography, and gel slicing.

In a second set of experiments, ^{125}I -plasmin (18 nM) was incubated with increasing concentrations of thrombospondin (0.2–1.2 μM) for 5 min at 22 °C in TBS-C-T. $\alpha_2\text{M}$ (0.18 μM) was added and allowed to react for 5 min, at which point 0.9 μM $\alpha_2\text{AP}$ was added to the solution. The reaction was then continued for an additional 30 min and the products were analysed by SDS/PAGE.

RESULTS

Effect of thrombospondin on plasmin amidase activity

The binding of plasmin to thrombospondin may involve the kringle domains and/or the plasmin active site. Either of these interactions may affect plasmin activity. In initial studies, thrombospondin (0.2–1.2 μM) and S-2251 (0.54 mM) were added simultaneously to solutions of plasmin (18 nM). The velocity of S-2251 hydrolysis decreased progressively for up to 3 min. Amidase activity was then stable for up to 10 min, at which time monitoring was discontinued. Based on these studies, we assumed that a state of apparent equilibrium was achieved within 5 min.

Plasmin was incubated with thrombospondin for 5 min and then with different concentrations of S-2251. Thrombospondin

decreased the initial velocity of substrate hydrolysis (average dA_{406}/dt in first 200 s) and the extent of the decrease depended on the thrombospondin concentration. Initial velocities of substrate hydrolysis were used to generate the Lineweaver–Burk plots shown in Figure 1. When plasmin was incubated with thrombospondin for 30 min before adding S-2251 instead of 5 min, equivalent results were obtained.

In order to determine whether addition of S-2251 might shift the equilibrium between plasmin and thrombospondin (promoting dissociation), substrate hydrolysis was monitored for 15 min after pre-incubating plasmin and thrombospondin for 5 min. The average dA_{406}/dt was determined for each 60 s interval for 15 min. A significant change in the rate of substrate hydrolysis (compared with the initial velocity of substrate hydrolysis as defined above) was not observed. These results suggest that the apparent equilibrium established before substrate addition is stable within the detection limits of the S-2251 hydrolysis method.

From the Lineweaver–Burk plots shown in Figure 1, kinetic parameters for the hydrolysis of S-2251 by plasmin were determined. The K_M and k_{cat} values in the absence of thrombospondin were $150 \pm 12 \mu\text{M}$ and $11.3 \pm 0.3 \text{ s}^{-1}$ respectively. In the presence of thrombospondin, the apparent k_{cat} decreased and the apparent K_M increased. The slopes from the Lineweaver–Burk graphs were plotted against the initial thrombospondin concentration, yielding a straight line (correlation coefficient, 0.99). This analysis indicated that thrombospondin is a linear mixed-

Figure 1 Steady-state plasmin amidase activity in the presence of thrombospondin

(a) Plasmin was allowed to react with S-2251 at 22 °C after incubation with the following concentrations of thrombospondin: 0 ($\blacklozenge$), 0.2 μM ($\blacktriangle$), 0.4 μM ($\bullet$), 0.55 μM ($\square$), 0.8 μM ($\diamond$), 0.9 μM ($\triangle$) and 1.2 μM ($\circ$). The slopes from (a) were plotted against thrombospondin concentration in (b).

type inhibitor of S-2251 hydrolysis. The dissociation constant (K_i) for the interaction of plasmin with thrombospondin, assuming a single plasmin-binding site per thrombospondin homotrimer (intact or partially digested), was $0.5 \mu\text{M}$.

The characterization of thrombospondin as a linear mixed-type plasmin inhibitor may be explained if plasmin, which is bound to thrombospondin, has decreased affinity for S-2251 and if S-2251 binding to plasmin-thrombospondin complex does not result in *p*-nitroanilide generation. Owing to thrombospondin solubility, the number of experiments performed with thrombospondin concentrations exceeding the K_i ($0.5 \mu\text{M}$) was limited. Therefore, our analysis may not have been sufficient to rule out a low level of residual plasmin activity for the complex of plasmin with thrombospondin.

Irreversible plasmin inhibition by thrombospondin

Plasmin (24 nM) was incubated with four separate preparations of purified thrombospondin (260 and 400 nM) for up to 2 h at 37°C . At various times, plasmin amidase activity was determined by adding S-2251 and ϵAHA simultaneously. The maximum loss of plasmin activity was less than 10% , suggesting that the binding of plasmin to thrombospondin was reversible by ϵAHA . In control experiments, plasmin amidase activity was determined after incubating plasmin for 2 h in the absence of thrombospondin or for 2 h in the presence of thrombospondin and 10 mM ϵAHA . In both cases, plasmin amidase activity was decreased by less than 10% .

Thrombospondin digestion by plasmin and miniplasmin

Thrombospondin monomer (M_r $150\,000$) was relatively free of contaminants as demonstrated by SDS/PAGE (Figure 2). When incubated with plasmin (18 nM) for up to 2 h , the intact monomer was slowly digested, yielding two major fragments with apparent M_r values of $125\,000$ and $35\,000$. Similar thrombospondin fragmentation patterns have been generated with other proteinases [26]; the heparin-binding domain (M_r $35\,000$) is readily cleaved from the remainder of the protein.

Thrombospondin was incubated with plasmin in 10 mM TEA. This concentration of TEA almost completely inhibits the digestion of fibrin monomer by plasmin without affecting plasmin amidase activity [27]. As shown in Figure 2, thrombospondin digestion was partially inhibited by 10 mM TEA; however, the inhibition was less complete than with fibrin.

Figure 2 Thrombospondin digestion by plasmin and miniplasmin

Plasmin (18 nM) and miniplasmin (18 nM) were incubated with thrombospondin ($0.72 \mu\text{M}$) in the presence or absence of 10 mM TEA at 37°C . The time of reaction is indicated at the top of the figure.

Miniplasmin digested thrombospondin into products that were comparable with the plasmin products, as determined by SDS/PAGE. The rate of digestion by miniplasmin was slightly slower. TEA (10 mM) significantly inhibited thrombospondin digestion by miniplasmin; however, inhibition was not complete. Since miniplasmin lacks K1–K4, the inhibitory activity of TEA suggests that binding of the plasmin K5 domain to thrombospondin is critical for thrombospondin digestion.

Thrombospondin digestion in the presence of EDTA

Thrombospondin was incubated with plasmin and miniplasmin in the presence of 2.0 mM EDTA (Figure 3). EDTA chelates calcium which is bound to thrombospondin; the loss of calcium forces the structure of thrombospondin to unwind and open [28,29]. The resulting conformation is more susceptible to digestion by most proteinases and as shown in Figure 3, cleavage of thrombospondin by plasmin and miniplasmin was more rapid as well. The major products were not substantially different in the presence of EDTA; however, the inhibitory activity of TEA was more apparent. These results strongly suggest that the lysine-binding sites, and in particular K5, assist in the anchoring of plasmin and miniplasmin to thrombospondin and the subsequent digestion of the substrate.

The thrombospondin-digestion experiments suggested that plasmin may interact with both conformations of thrombospondin (in calcium or in EDTA) in a manner which is at least partially dependent on K5. To study the binding of plasmin to thrombospondin in the presence of EDTA further, the two proteins were pre-incubated for 5 min and then reacted with S-2251. In three separate experiments, thrombospondin at concentrations up to $0.4 \mu\text{M}$ decreased the amidase activity of plasmin in EDTA. The extent of the decrease was within 15% of that demonstrated in TBS-C-T buffer (results not shown).

Reaction of plasmin with $\alpha_2\text{AP}$

$\alpha_2\text{AP}$ is the primary inhibitor of plasmin in human plasma; the inhibition rate constant is approx. $2.0 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ [30]. Rapid inhibition depends on non-covalent association of $\alpha_2\text{AP}$ with the K1–K3 domains of plasmin [7,30]. The rate of plasmin inhibition by $\alpha_2\text{AP}$ in the presence of thrombospondin was studied initially using a continuous assay measuring chromogenic-substrate hydrolysis. When plasmin (18 nM) and $\alpha_2\text{AP}$ (18 nM) were

Figure 3 Thrombospondin digestion by plasmin and miniplasmin in the presence of EDTA

Plasmin (18 nM) and miniplasmin (18 nM) were incubated with thrombospondin ($0.72 \mu\text{M}$) in 2.0 mM EDTA and in the presence or absence of 10 mM TEA at 37°C .

Table 1 Reaction of plasmin with α_2 AP in the presence of thrombospondin as determined by plasmin activity

Plasmin (18 nM) was pre-incubated with thrombospondin. A baseline level of substrate hydrolysis was determined by incubation with S-2251 for 100 s. α_2 AP (18 nM) was then added. The times required for 50% and 95% inhibition of the initial plasmin activity are presented (mean $\pm$ S.E.M., $n = 4$).

Thrombospondin (nM)	$t_{0.5}$ (s)	$t_{0.95}$ (s)
0	13.2 ± 3.0	34.3 ± 2.8
100	16.7 ± 4.0	42.8 ± 3.8
200	24.2 ± 0.5	59.9 ± 7.9
400	21.3 ± 3.0	55.2 ± 5.2

Figure 4 Reaction of plasmin with α_2 AP

125 I-Plasmin (18 nM) was incubated alone (●) or with 1.2 μ M thrombospondin (○) and then with α_2 AP (0.9 μ M) at 22 °C. α_2 AP-plasmin-complex formation was detected by SDS/PAGE.

allowed to react in the absence of thrombospondin, the plasmin activity was completely inhibited within less than 40 s; the half-life for plasmin inhibition ($t_{0.5}$) was 13.2 ± 3.0 s (Table 1).

In the presence of thrombospondin (0.1–0.4 μ M), the initial plasmin activity was decreased as expected; however, the residual plasmin activity was rapidly and completely inhibited by α_2 AP. A substantial decrease in the rate of reaction of plasmin with α_2 AP was not detected. In this regard, thrombospondin differed from fibrin or plasmin(ogen) cell-surface receptors [8,9]. Higher concentrations of thrombospondin (up to 1.2 μ M) were also studied. In these experiments, the reaction of plasmin with α_2 AP proceeded rapidly to completion; however, due to the low level of initial plasmin activity (before addition of α_2 AP), $t_{0.5}$ values were not determined.

Because of the significant decrease in plasmin amidase activity upon binding thrombospondin, the chromogenic-substrate method used to study the reaction of plasmin with α_2 AP may have detected only plasmin which was free in solution. This

Figure 5 Reaction of plasmin with α_2 M

125 I-Plasmin (18 nM) was incubated with thrombospondin at the following concentrations: 0 (●), 0.2 μ M (◇), 0.4 μ M (△), 0.8 μ M (□), and 1.2 μ M (○) and then reacted with 0.18 μ M α_2 M at 22 °C. α_2 M-plasmin complex formation was detected by SDS/PAGE.

would explain why thrombospondin altered the apparent rate of reaction of plasmin with α_2 AP minimally. To account for all of the plasmin present in the system, α_2 AP (0.9 μ M) was reacted with 125 I-plasmin (18 nM) which had been pre-incubated with 1.2 μ M thrombospondin for 5 min. α_2 AP-plasmin complex was detected by SDS/PAGE. As shown in Figure 4, about 50% of the 125 I-plasmin reacted with α_2 AP at nearly the same rate as determined in the absence of thrombospondin. The remaining 125 I-plasmin reacted more slowly, but apparently as a first-order process. Within 20 min, the reaction of plasmin with α_2 AP in the presence of thrombospondin was essentially complete. If plasmin which is bound to thrombospondin is completely incapable of reacting with α_2 AP, then the later phase of the reaction shown in Figure 4 provides a minimum estimate of the plasmin-thrombospondin complex dissociation rate constant (5.0×10^{-3} s $^{-1}$).

Reaction of plasmin with α_2 M

α_2 M is the second major inhibitor of plasmin in human plasma; unlike α_2 AP, the mechanism by which α_2 M inhibits plasmin does not involve the plasmin kringle domains [27,31]. 125 I-Plasmin (18 nM) was incubated with different concentrations of thrombospondin for 5 min and then reacted with α_2 M (180 nM). The curves showing formation of α_2 M-plasmin complex were non-linear (Figure 5). In each experiment, a substantial fraction of the plasmin was relatively resistant to reaction with α_2 M. Although the SDS/PAGE method was not entirely sensitive to small differences in the size of the α_2 M-resistant fraction, the greatest

Figure 6 Reaction of plasmin with α_2 AP after pre-incubation with thrombospondin and α_2 M

¹²⁵I-Plasmin (18 nM) was incubated with thrombospondin for 5 min and then with α_2 M (0.18 μ M) for an additional 5 min at 22 °C. α_2 AP was added for an additional 30 min. Complex formation between plasmin and α_2 M (○) or α_2 AP (□) was determined by SDS/PAGE.

extent of resistance was observed with the highest concentration of thrombospondin (1.2 μ M). Reaction of plasmin with α_2 M after the first 5 min probably reflected plasmin dissociation from thrombospondin; however, a low level of reaction of α_2 M with thrombospondin-associated plasmin cannot be ruled out.

Reaction of thrombospondin-associated plasmin with mixtures of α_2 AP and α_2 M

¹²⁵I-Plasmin (3.3 nM) was pre-incubated with thrombospondin for 5 min and then reacted simultaneously with α_2 AP (33 nM) and α_2 M (2.04 μ M) for 30 min. The proteinase inhibitor concentrations were selected so that the plasmin would react equally with α_2 AP and α_2 M in the absence of thrombospondin. More than 90 % of the plasmin was recovered in complex with α_2 AP or α_2 M, even after pre-incubation with up to 1.2 μ M thrombospondin. This result was expected because of the relatively long incubation time (30 min). Thrombospondin did not significantly affect the distribution of ¹²⁵I-plasmin between α_2 AP and α_2 M. Within the entire thrombospondin concentration range approx. 50 % of the plasmin bound to α_2 AP and 50 % to α_2 M (results not shown). These results could be explained if plasmin reacted with α_2 AP and α_2 M at proportionately decreased rates when bound to thrombospondin. A more likely explanation, however, is that reaction of ¹²⁵I-plasmin with α_2 AP or α_2 M depended primarily on plasmin dissociation from thrombospondin.

In a final set of experiments, ¹²⁵I-plasmin (18 nM) was incubated with thrombospondin (0.2–1.2 μ M) for 5 min and then with α_2 M (0.18 μ M) for an additional 5 min before adding α_2 AP (0.9 μ M) for 30 min. In the absence of thrombospondin, more than 95 % of the plasmin bound to α_2 M as determined by SDS/PAGE, almost certainly reflecting reaction that occurred before α_2 AP was added (Figure 6). Thrombospondin increased the recovery of α_2 AP–plasmin complex in a concentration-

dependent manner. This shift in recovery reflects plasmin that did not react with α_2 M in the first 5 min and therefore was probably bound to thrombospondin. These results confirm that plasmin that is resistant to inhibition by proteinase inhibitors, due to association with thrombospondin, is not irreversibly inactivated.

DISCUSSION

Thrombospondin is an adhesive glycoprotein which may be found at many sites *in vivo*, including the surfaces of cells such as platelets, endothelial cells, fibroblasts, smooth muscle cells, monocytes, and macrophages, in the extracellular matrix, basement membranes and in fibrin blood clots [14,15,26]. In all of these sites, thrombospondin co-exists with proteinases such as plasmin. The proteinases remodel cellular microenvironments, support tumour-cell invasion, alter the progress of bacterial infection and promote the immune response. In order for plasmin to function, plasminogen must be activated and then protected from the rapid antiplasmins, α_2 AP and α_2 M.

The effects of thrombospondin on plasminogen activation have been studied previously [6]. Thrombospondin accelerates the activation of plasminogen by t-PA. Nevertheless, when the lysis of fibrin by mixtures of t-PA and plasminogen was examined, thrombospondin was an inhibitor [16]. The present investigation was designed to examine the effects of thrombospondin on the activity of plasmin directly.

All of the studies presented here confirm that plasmin binds to thrombospondin in solution. This association results in thrombospondin digestion, indicating the involvement of the plasmin active site. TEA inhibited the digestion of thrombospondin when present at concentrations that were insufficient to inhibit plasmin amidase activity. This result indicates that the plasmin kringle domains are also involved in the plasmin–thrombospondin interaction. From among the kringle domains, we propose that K5 plays an important role, since TEA inhibited thrombospondin digestion by plasmin and miniplasmin similarly. This conclusion supports the work of DePoli et al. [17].

How thrombospondin affects the activity of plasmin has not been resolved. The studies presented here indicate that thrombospondin is a plasmin inhibitor, as demonstrated by Hogg et al. [18]; however, the interaction described here is different from that described by Hogg et al. [18] in the following ways: (1) association occurs relatively rapidly so that an apparent equilibrium is achieved in about 3 min; (2) binding is completely reversible in the presence of ϵ AHA or other antiplasmins; and (3) the affinity of the plasmin–thrombospondin interaction described here is much lower (0.5 μ M) than previously reported (< 1.0 nM).

One model to explain our results intimately links the roles of thrombospondin as a plasmin inhibitor and a plasmin substrate. When plasmin binds thrombospondin, cleavage of susceptible peptide bonds occurs with slow turnover. While enzyme (plasmin) and substrate (thrombospondin) are engaged in what is apparently a multiple-site interaction, the plasmin expresses little or no activity against other substrates/inhibitors including S-2251, α_2 AP and α_2 M.

A concern regarding interpretation of the chromogenic-substrate-hydrolysis experiments was the possibility that our thrombospondin preparations might be contaminated by trace levels of an irreversible plasmin inhibitor. This concern was addressed in experiments with ϵ AHA. When ϵ AHA was added with S-2251, the decrease in plasmin amidase activity was reversed. The reversibility of the plasmin–thrombospondin interaction was supported by additional experiments with α_2 AP and

α_2 M. Under a variety of conditions, we demonstrated that plasmin which was apparently bound to thrombospondin could be completely transferred to α_2 AP in a relatively short period of time. Since plasmin must express proteolytic activity in order to react with α_2 AP, irreversible plasmin inhibition could not have occurred. There are two possible explanations for the complete reaction of plasmin with α_2 AP after pre-incubation with thrombospondin; either plasmin that is bound to thrombospondin reversibly dissociates and then reacts with α_2 AP, or plasmin retains limited proteolytic activity when bound to thrombospondin which was not detected in our assays (Figure 1). While the first explanation is favoured, these two possibilities are not mutually exclusive.

It is conceivable that the relatively rapid, low-affinity, reversible binding of plasmin to thrombospondin described here could be followed by a second step in which an irreversible complex, such as that described by Hogg et al. [18], is formed. In experiments with four separate thrombospondin preparations purified in this laboratory, we were not able to demonstrate irreversible plasmin inhibition; however, we could demonstrate irreversible plasmin binding to the thrombospondin provided by Dr. Mosher (results not shown). The reason why this thrombospondin, which was equivalent to our preparations by SDS/PAGE, was able to bind plasmin with higher affinity is unclear and currently under investigation.

The slow digestion of thrombospondin by plasmin coupled with the plasmin inhibitory activity of thrombospondin suggests a novel role for this protein in the regulation of fibrinolysis and other plasmin-mediated biological processes. By binding plasmin in a manner that eliminates or significantly inhibits catalytic activity, thrombospondin functions as an antiplasmin. Since the thrombospondin-plasmin complex is at least initially reversible, thrombospondin may carry plasmin in a latent form into a fibrin clot or into the extracellular matrix. While in the latent, thrombospondin-associated state, plasmin is resistant to α_2 AP. Therefore, the latent state provides a mechanism for enhanced survival of plasmin in proteinase-inhibitor-rich environments and the delivery of plasmin to sites where plasminogen activators may not be present.

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