Design and Evaluation of a Thrombin-Activable Plasminogen Activator

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ABSTRACT: A new chimeric plasminogen activator with high fibrin affinity was designed to bind fibrin and to initiate clot destruction, following activation by thrombin. The chimeric activator, 59D8-scuPA-T, was made from the Fab fragment of an anti-fibrin antibody (59D8) and a C-terminal portion of a thrombin-activable low molecular weight single-chain urokinase plasminogen activator, scuPA-T, obtained by deletion of Phe-157 and Lys-158 from low molecular weight single-chain urokinase-type plasminogen activator (scuPA) by site-directed mutagenesis. The chimeric molecule had a molecular mass of 91 000, a value consistent with one 59D8 light chain (M_r = 27 000) and one 59D8 heavy-chain Fd fragment fused to low molecular weight scuPA (M_r = 64 000). According to its design, 59D8-scuPA-T was activated by thrombin but not by plasmin, whereas the control chimeric molecule, 59D8-scuPA, was activated by plasmin but not by thrombin. When activated by thrombin, 59D8-scuPA-T converted plasminogen to plasmin. *In vitro* plasma clot lysis assays showed that 59D8-scuPA-T lysed clots preformed by thrombin and that heparin and hirudin could prevent clot lysis. When incorporated as part of a thrombin-induced clot, only 59D8-scuPA-T was able to lyse the clot while 59D8-scuPA and high molecular weight scuPA were ineffective. Together these results demonstrate that 59D8-scuPA-T is a thrombin-activable plasminogen activator that offers selective thrombolysis of thrombin-rich clots over more established, aged clots, and may also act as an antithrombotic agent.

Thrombolytic therapy dramatically reduces the mortality rate in patients with acute myocardial infarction (Anderson et al., 1988; Neuhaus et al., 1988; The International Study Group, 1990), but this is accompanied by certain major side effects including haemorrhagic stroke or other systemic bleeding (Marder & Sherry, 1988; Fears, 1990; Collen, 1992). The incidence of haemorrhagic stroke was significantly higher in patients treated with fibrin-selective plasminogen activators such as t-PA than those treated with fibrin-nonspecific plasminogen activators such as streptokinase (ISIS-3 Collaborative Group, 1992), a result which was confirmed by the recent GUSTO study (The GUSTO Collaborators, 1993). To explain this finding, systemic lysis of fibrinogen or the dissolution of haemostatic plugs has been considered. The significance of systemic lysis seems less important because t-PA spares circulating fibrinogen but still causes a higher incidence of haemorrhagic stroke. It has been proposed, therefore, that dissolution of haemostatic plugs is the principal cause of haemorrhagic strokes during thrombolytic therapies (Marder & Sherry, 1988; Fears, 1990). The ideal third-generation plasminogen activator would selectively lyse a forming thrombus but spare haemostatic plugs, thereby reducing the risk of haemorrhagic strokes and other bleeding episodes (Marder & Sherry, 1988; Smitherman, 1991; Fears, 1992; Haber et al., 1992).

In the past few years, many chimeric or recombinant mutants of plasminogen activators have been designed with the goal of increasing their potency (Harris, 1987; Haber et al., 1989; Fears, 1990; Smitherman, 1991). Some of them were shown to have increased potency with higher affinity to fibrin or longer plasma half-life, but none of the mutants have been able to distinguish a thrombus from an established clot (e.g., haemostatic plug). To design a lytic agent with selectivity for thrombi, we chose thrombin as a surface marker to distinguish a thrombus from an established clot. Although there has been no direct comparison, there is evidence to support the hypothesis that a thrombus contains more thrombin than does an established clot (Castellino et al., 1983; Francis et al., 1983; Kaminski & McDonagh, 1987; Fenton et al., 1988; Hogg & Jackson, 1989): (i) thrombin is produced locally around the injury site; (ii) thrombin is trapped in a thrombus by interacting with fibrin; (iii) thrombin tends to leach out of a fibrin clot during extensive washing or further fibrin polymerization; (iv) leached-out thrombin is enzymatically active and can be rapidly inhibited by antithrombin III. Therefore, active thrombin in or near a thrombus may be a useful transient marker to distinguish a thrombus from an established clot.

In our strategy, we designed a "thrombin-activable low molecular weight single-chain urokinase" (called scuPA-T)[1] and targeted it to a thrombus by attaching a Fab fragment of a high-affinity anti-fibrin antibody (59D8; Runge et al., 1991). The chimeric plasminogen activator 59D8-scuPA-T and the wild-type chimeric 59D8-scuPA were expressed in a mouse hybridoma cell line, purified, and characterized with respect to their fibrinolytic activity.

EXPERIMENTAL PROCEDURES

Construction of a Thrombin-Activable Low Molecular Weight scuPA. A 3.0-kb human genomic DNA fragment from the expression vector pSVUKG(Ig) (Runge et al., 1991) encoding the gene from scuPA, including exons 7–11, was subcloned into the *Xho*I/*Sal*I site of a pBluescript II KS+ vector (Promega). Phagemid mutagenesis was carried out according to the manufacturer's protocol (BioRad). The

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[1] Abbreviations: scuPA-T, thrombin-activable single-chain urokinase-like plasminogen activator; scuPA, single-chain urokinase-like plasminogen activator; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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oligomer used for mutagenesis was a 28-mer (5'-ACT CTG AGG CCC CGC ATT ATT GGG GGA G-3') which corresponds to the DNA sequence encoding amino acids 152–163 of scuPA except for the deletion of six nucleotides which encoded amino acids 157 (Phe) and 158 (Lys). The nucleotide sequence of the mutant was verified by dideoxy nucleotide sequencing analysis using T7 Sequenase according to the manufacturer's protocol (United States Biochemical Corp.).

Construction and Expression of 59D8-scuPA and 59D8-scuPA-T. The vector p59D8-scuPA was derived from expression vector pSVUKG(Ig) (Runge et al., 1991) by deleting the CH2 domain with *Xho*I digestion of the vector. This was used as a control to express the 59D8-scuPA protein in many experiments. The 3.0-kb *Xho*I/*Sal*I fragment containing human genomic DNA encoding for the deletion mutant of scuPA (scuPA-T) was cloned into the *Xho*I/*Sal*I site of the expression vector p59D8-scuPA. Constructs having inserts in the correct orientation for p59D8-scuPA-T were screened by *Xho*I restriction mapping. The final expression vectors p59D8-scuPA and p59D8-scuPA-T (shown in Figure 1) were linearized by *Sal*I digestion, and 40 μg of each was transformed into a 59D8 light-chain-producing hybridoma cell line by electroporation as previously described (Schnee et al., 1987). The transformed cells were grown in selection media described previously (Schnee et al., 1987). Colonies growing in the selection media were screened for the production of chimeric plasminogen activator by incubating 50 μL of the culture medium in a 96-well microtiter plate coated with β7 peptide (the heptapeptide epitope for antibody 59D8) for 2 h. Bound protein was then detected by peroxidase-conjugated goat anti-mouse IgG (Runge et al., 1991) or by anti-human urokinase and then by peroxidase-conjugated rabbit anti-goat antibody (Runge et al., 1991).

Cell lines producing 59D8-scuPA and 59D8-scuPA-T were grown in a Cellquad hollow-fiber bioreactor (Cellco). Approximately 1×10^7 cells were inoculated into a polypropylene bioreactor cartridge with a capillary pore size of 0.5 μm and an extra-capillary space volume of 7.0 mL. The extra-capillary space medium and the perfusion medium consisted of AIM-V serum-free medium (Gibco BRL) supplemented with 300 IU/mL aprotinin and 10 μg/mL soybean trypsin inhibitor. Two weeks after inoculation, chimeric proteins were harvested every 1 or 2 days from the perfusion medium reservoir bottle.

Purification of 59D8-scuPA and 59D8-scuPA-T. Chimeric plasminogen activators were purified from the perfused bioreactor medium by affinity chromatography on a Sepharose matrix containing immobilized peptide ligand for 59D8 (β7) prepared as previously described (Runge et al., 1991). Chimeric proteins bound to the column were eluted with 0.2 M glycine, pH 3.5, and the eluted proteins were immediately neutralized with the addition of 0.2 volume of 1 M Tris, pH 7.8. The protein solutions were then concentrated using Centriprep-30 concentrators (Amicon). The pooled, purified samples were passed over the affinity column a second time after pretreatment with 1×10^{-4} M glutamylglycylarginine chloromethyl ketone to destroy any activated urokinase. The concentrations in solution of 59D8-scuPA and 59D8-scuPA-T were determined by the DC protein assay as described by the manufacturer (BioRad).

SDS–PAGE and Western Blotting. SDS–PAGE under reducing (using β-mercaptoethanol) and nonreducing conditions was performed as described (Laemmli, 1970). The proteins were visualized either by staining with Coomassie brilliant blue R or by transferring by electrophoresis to a Westran PVDF membrane for Western blotting. Alkaline phosphatase conjugated goat anti-mouse IgG was used to detect 59D8-Fab epitopes. Goat anti-human urokinase was used as first antibody and alkaline phosphatase conjugated rabbit anti-goat IgG as second antibody to detect scuPA epitopes.

Amino-Terminal Sequence. Intact or thrombin- or plasmin-cleaved chimeric protein was subjected to N-terminal sequence analysis using an American Biosystems 477A gas-phase sequencer.

Activation of 59D8-scuPA and 59D8-scuPA-T. 59D8-scuPA or 59D8-scuPA-T (150 nM final concentration) in TNT buffer (0.05 M Tris, pH 7.4, 0.038 M NaCl, and 0.01% Tween 80) was treated at 37 °C with either plasmin or thrombin (5 nM final concentration for 59D8-scuPA using either enzyme and 15 nM final concentration for 59D8-scuPA-T using either enzyme). At time intervals (0–60 min), the urokinase-like amidolytic activity was measured using the chromogenic substrate S-2444 (0.3 mM final concentration; Chromogenix) after stopping the reaction with either 5000 KIU/mL aprotinin for the plasmin digestions or 1 unit/mL hirudin for the thrombin digestions. Urokinase activity was expressed in International Units (IU) by comparison with the International Standard (87/594; WHO International Laboratory for Biological Standards, Hertfordshire, England).

To determine the kinetic constants, activation of 59D8-scuPA (1–10 μM) by plasmin (5 nM) and of 59D8-scuPA-T (1–8 μM) by thrombin (15 nM) was measured at 37 °C in TNT buffer. Generated urokinase activity at different time intervals (0–5 min) was measured using the chromogenic substrate S-2444 (1.0 mM) after stopping the reactions by diluting the samples 10-fold and adding 5000 KIU/mL aprotinin for the plasmin reactions or 3 units/mL hirudin for the thrombin reactions.

Two-Chain Activators. Stock solutions of 59D8-tcuPA and 59D8-tcuPA-T were obtained by treating 59D8-scuPA (10 μM) with plasmin (2 mol/100 mol) and 59D8-scuPA-T (10 μM) with thrombin (1 NIH unit/2 nmol) for 30 min at 37 °C. Plasmin and thrombin were separated by passing the samples over a β7-peptide–Sepharose column. The conversion of urokinase from the one-chain to the two-chain form was monitored by SDS–PAGE on 12% gels after reduction with β-mercaptoethanol. Carbohydrate was removed from the chimeric proteins for SDS–PAGE analysis using *N*-glycanase (Genzyme).

Activation of Plasminogen. Activation of plasminogen (10–50 μM) was measured at 37 °C in TNT buffer with 59D8-tcuPA or 59D8-tcuPA-T (5 nM). Generated plasmin at different time intervals (0–5 min) was measured using the chromogenic substrate S-2251 (1.0 mM; Chromogenix) after 30-fold dilution of the samples. High molecular weight urokinase, UKIDAN (Serono), at 5 nM was used for comparison. These experiments were repeated at least 4 times.

Plasma Clot Formation. Citrated human plasma (pooled from at least 10 healthy donors) was mixed with ^{125}I-labeled fibrinogen [42.6×10^6 cpm/mg; prepared using Biorad's peroxidase–glucose oxidase enzymobeads (Procyk et al., 1991)] at a ratio of 1.25×10^6 cpm/mL of plasma. Clotting was initiated by adding $CaCl_2$ to 25 mM (final concentration) and human thrombin to 0.25 NIH unit/mL (final concentration). Clots were formed in a 0.2-mL section of a graduated plastic 1-mL pipet sealed with parafilm on one end, and allowed to clot for at least 1 h. The clots were then removed from the pipet sections, placed into polystyrene tubes, and washed with TNEA buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mg/mL BSA, and 0.01% NaN_3). For experiments in which plasminogen activators were present in the clot matrix, 0.3-mL clots were formed at the bottom of a polystyrene tube

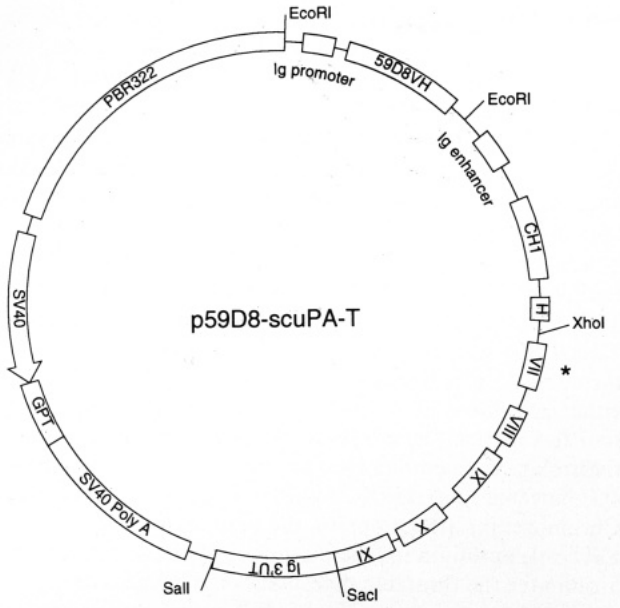

FIGURE 1: Expression plasmid for 59D8-scuPA-T. p59D8-scuPA-T contains a genomic heavy-chain variable region from fibrin-specific monoclonal antibody 59D8, the cloned genomic constant region of mouse γ2b[CH1 and H (hinge)], and the coding region from a genomic clone on scuPA (containing exons VII through XI, amino acid positions 144–411). The asterisk indicates the location of the deletion mutation for scuPA-T, which differentiates the plasmid from p59D8-scuPA.

with an indicated amount of plasminogen activator added to the citrated plasma prior to clot formation. Clots were allowed to stand at 25 °C for 10–20 min prior to use in lysis experiments.

In Vitro Clot Lysis. Clot lysis was done in 2 mL of autologous human plasma to which plasminogen activator was added. Heparin or hirudin was also added as indicated. Clots that contained plasminogen activators within their clot matrix (i.e., formed in the presence of added amounts of plasminogen activator) were lysed in 2 mL of TNEA. Tubes containing either clot type were rotated at 37 °C, and 0.1-mL aliquots were removed at various times to determine the released radioactivity. Total clot radioactivity was determined by measuring the radioactivity of each clot after formation. High molecular weight scuPA was used as a control in some experiments.

RESULTS

Construction and Expression of 59D8-scuPA and 59D8-scuPA-T. The 59D8-scuPA and 59D8-scuPA-T proteins were expressed separately using a 59D8 light-chain-producing cell line transfected with the expression plasmids p59D8-scuPA or p59D8-scuPA-T, respectively. The cDNA construct of p59D8-scuPA-T shown in Figure 1 is similar to that of p59D8-scuPA except that two amino acids (Phe-157 and Lys-158) between the plasmin and thrombin cleavage sites in scuPA were removed by site-directed mutagenesis. These two molecules are shown schematically in Figure 2.

Characterization of 59D8-scuPA and 59D8-scuPA-T. The chimeric proteins were purified by affinity chromatography and characterized by SDS–PAGE. The Coomassie-stained gel shown in Figure 3a showed one band for each chimera at 91 kDa under nonreducing conditions and two bands at 27 and 64 kDa under reducing conditions. The identity of each band was deduced by comparing the staining pattern of these bands when antisera against urokinase (Figure 3B) and mouse IgG (Figure 3C) were used as immunoblotting reagents. Epitopes for urokinase were detected in the 91-kDa bands (Figure 3B, lanes 2 and 3) and the 64-kDa bands (lanes 4 and 5). Epitopes for the mouse immunoglobulin were present in the 91-kDa bands (Figure 3C, lanes 2 and 3) and the 27-kDa bands (lanes 4 and 5). Subsequently, we learned that these reagents did not stain the mouse Fd fragment that had been treated with β-mercaptoethanol (not shown). Together, we inferred from these results that 59D8-scuPA-T and 59D8-scuPA, like their predecessor r-scuPA(32)-59D8 (Runge et al., 1991), are heterodimers composed of one 59D8 light chain (27 kDa) and one fused protein (64 kDa) that contains 59D8 Fd and low molecular weight single-chain urokinase. The 59D8 light chain and the fused protein were linked together by a disulfide bond.

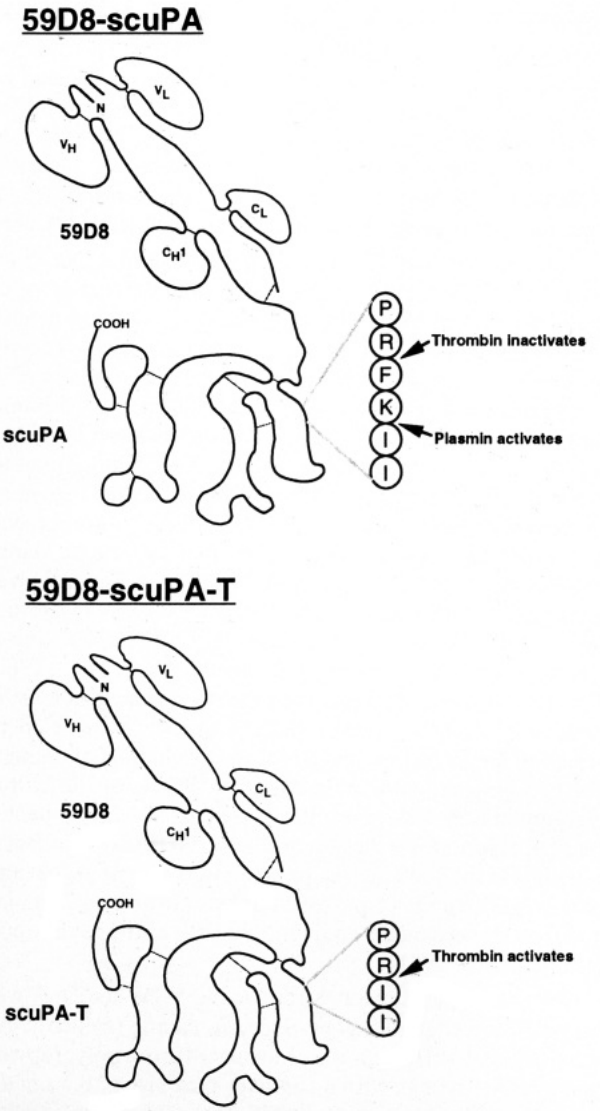

FIGURE 2: Schematic drawing of 59D8-scuPA and 59D8-scuPA-T. Thrombin and plasmin cleavage sites are indicated by arrows.

Plasmin and Thrombin Treatment of 59D8-scuPA and 59D8-scuPA-T. The susceptibility of 59D8-scuPA and 59D8-scuPA-T toward both plasmin and thrombin cleavage was assessed by SDS–PAGE under reducing (Figure 4) and nonreducing conditions (not shown). As seen in Figure 4, the 64-kDa band of 59D8-scuPA was cleaved by both thrombin and plasmin and gave lower molecular mass bands of 33 and 31 kDa, presumably urokinase and the 59D8 Fd. This pattern was expected based upon the susceptibility of scuPA toward plasmin and thrombin. By design, we anticipated that scuPA-T would retain its thrombin susceptibility and not be affected by plasmin. The 64-kDa band of 59D8-scuPA-T (Figure 4, lane 5) was unaffected by plasmin digestion (lane 7) but became converted to two bands consisting of 33 and

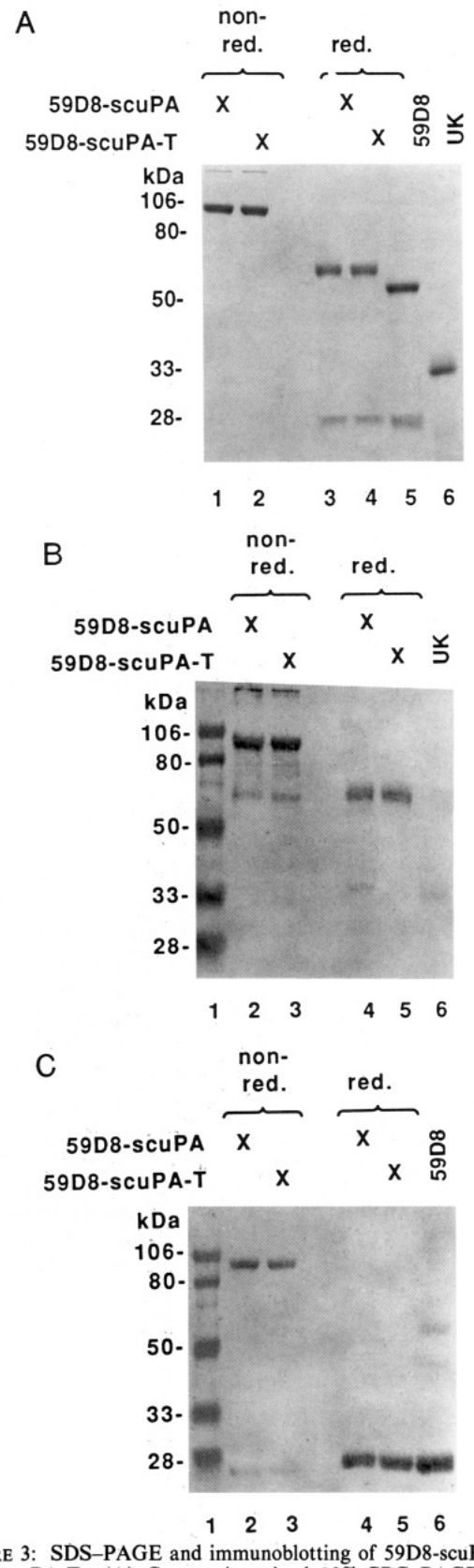

FIGURE 3: SDS–PAGE and immunoblotting of 59D8-scuPA and 59D8-scuPA-T. (A) Coomassie-stained 12% SDS–PAGE. The samples were run under nonreducing conditions in lanes 1 and 2, and under reducing conditions using β-mercaptoethanol in lanes 3–6. 59D8 antibody was run in lane 5 and low molecular weight urokinase in lane 6. (B) The gel was immunoblotted with goat anti-human urokinase. Prestained standards are shown in lane 1. The samples were run under nonreducing conditions in lanes 2 and 3, and under reducing conditions using β-mercaptoethanol in lanes 4–6. Urokinase was run as a control in lane 6. (C) The gel was immunoblotted with goat anti-mouse IgG. Lane assignments are the same as for (B), except that 59D8 was run as a control in lane 6. This second antibody could not detect the reduced 59D8 heavy-chain fragment.

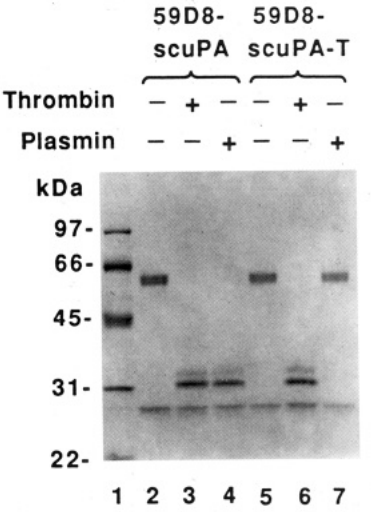

FIGURE 4: SDS–PAGE of 59D8-scuPA and 59D8-scuPA-T treated with thrombin or plasmin. Samples were also treated with *N*-glycanase and reduced prior to analysis on a 12% SDS–PAGE gel and Coomassie staining.

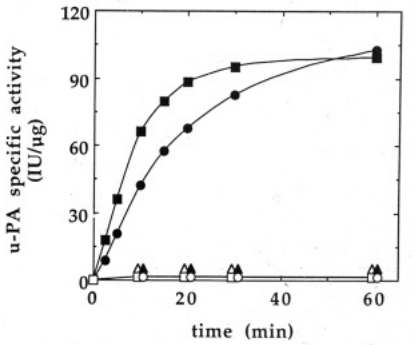

FIGURE 5: Time course of chimeric plasminogen activator activation. 59D8-scuPA (150 nM) was untreated (△), treated with 5 nM plasmin (■), or treated with 5 nM thrombin (□). 59D8-scuPA-T (150 nM) was untreated (▲), treated with 15 nM plasmin (○), or treated with 15 nM thrombin (●). u-PA activity was assayed with S-2444 as described under Experimental Procedures. Data points are representative of a typical experiment.

31 kDa when treated by thrombin (lane 6). The samples in Figure 4 were treated with *N*-glycanase to remove carbohydrate. This was necessary in order to prevent multiple bands in the SDS–PAGE gel for the cleavage products of urokinase due to a glycosylation site at Asn-302 (Bachmann, 1987).

Plasmin- and thrombin-cleaved molecules were subjected to amino-terminal sequence analysis to determine the sites of cleavage. As expected, 59D8-scuPA was cleaved by plasmin between Lys-158 and Ile-159 and by thrombin between Arg-156 and Phe-157. The new amino-terminal sequence for 59D8-scuPA-T after thrombin treatment was Ile-Ile-Gly-Gly-Glu-Phe-Thr-Thr with a yield of 98%, indicating that cleavage occurred between Arg-156 and Ile-157 (formally Ile-159) of the engineered molecule.

In terms of activity, thrombin catalyzed a time-dependent conversion of 59D8-scuPA-T to its active two-chain derivative, similar to plasmin acting on 59D8-scuPA (Figure 5). A thrombin to 59D8-scuPA-T ratio 3-fold higher than the plasmin to 59D8-scuPA ratio was selected to obtain similar activation rates. Kinetic analysis using linear double-reciprocal plots (not shown) indicated that thrombin cleaved 59D8-scuPA-T with a $K_m = 1.5\ \mu M$ and $k_{cat} = 0.11\ s^{-1}$ and plasmin cleaved 59D8-scuPA with a $K_m = 3.4\ \mu M$ and $k_{cat} = 0.41\ s^{-1}$. The catalytic efficiencies (k_{cat}/K_m) obtained, 0.074 and 0.120 $\mu M^{-1}\ s^{-1}$, respectively, indicate similar sensitivities of the chimeric molecules to activation. The previously reported

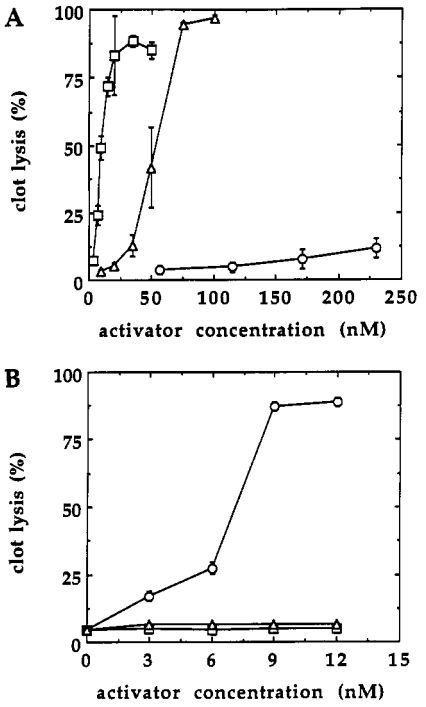

FIGURE 6: Exogenous and endogenous lysis of ^{125}I-fibrin-labeled human plasma clots by plasminogen activators. Percent clot lysis was determined after 2-h incubation at 37 °C. Data represent the mean of four experiments with the standard deviation of the mean shown. (A) Exogenous lysis done in human plasma containing increasing concentrations of 59D8-scuPA (□), 59D8-scuPA-T (○), or high molecular weight scuPA (△). (B) Endogenous lysis. Clots formed in the presence of different activators [59D8-scuPA (□), 59D8-scuPA-T (○), or high molecular weight scuPA (△)] and permitted to lyse in TNEA without any further addition of exogenous activator.

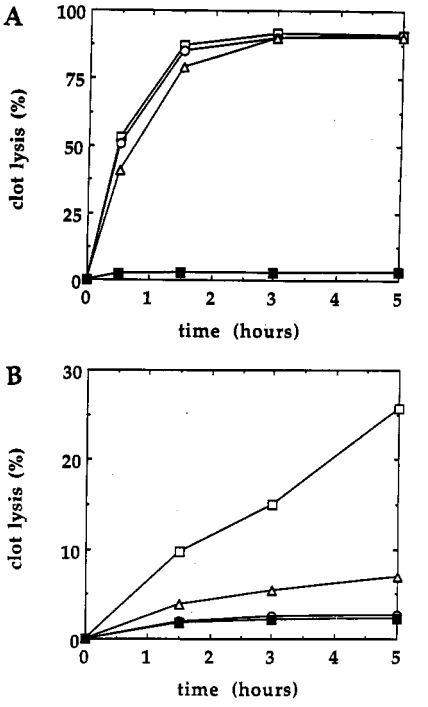

FIGURE 7: Effect of heparin and hirudin on ^{125}I-fibrin-labeled human plasma clot lysis. (A) Lysis done in human plasma (■) or in plasma containing 34 nM 59D8-scuPA (□), activator plus 20 units/mL heparin (○), or activator plus 2.5 units/mL hirudin (△). (B) Lysis done in human plasma (■) or in plasma containing 230 nM 59D8-scuPA-T (□), activator plus 20 units/mL heparin (○), or activator plus 2.5 units/mL hirudin (△). Data represent the average of four experiments.

(de Munk et al., 1991) catalytic efficiency for thrombin inactivation of scuPA (0.39 μM^{-1} s^{-1}), in comparison to thrombin activation of 59D8-scuPA-T, also indicates that the altered chimeric molecule is similar to wild-type scuPA in sensitivity to thrombin cleavage. As shown in Figure 5, urokinase activity for both two-chain derivatives reached approximately 100 IU/μg. 59D8-scuPA treated with thrombin did not result in any urokinase activity while 59D8-scuPA-T was unaffected by plasmin treatment, further indicating that the engineered plasminogen activator is activated by a novel mechanism. Incubation of the chimeric plasminogen activators at 37 °C in the absence of thrombin or plasmin did not lead to activation or expression of u-PA activity (Figure 5).

Kinetics of Plasminogen Activation. Activation of plasminogen by 59D8-tcuPA and 59D8-tcuPA-T (the two-chain derivatives of 59D8-scuPA and 59D8-scuPA-T) obeyed Michaelis–Menten kinetics as evidenced by linear double-reciprocal plots of activation rate versus plasminogen concentration (not shown). Although the k_{cat} values for both proteins were similar (2.16 s^{-1} for 59D8-tcuPA and 1.75 s^{-1} for 59D8-tcuPA-T), the K_m for 59D8-tcuPA-T (66.3 μM) was approximately 3-fold higher than that for 59D8-tcuPA (22.5 μM). Higher molecular weight urokinase had a K_m = 20.7 μM and a k_{cat} = 4.11 s^{-1}, kinetic constants very similar to 59D8-tcuPA.

In Vitro Plasma Clot Lysis. ^{125}I-Fibrin-labeled clots were prepared and immersed in either citrated human plasma or buffer. When the plasminogen activator was used exogenously, i.e., present in the plasma milieu used for incubation during lysis, 59D8-scuPA (and scuPA) was more effective at lysing the clot than 59D8-scuPA-T (Figure 6A). Thrombin inhibitors present in the plasma probably inhibit scuPA-T activation and by consequence clot lysis. The inhibitory effect could be overcome by using a greater concentration of 59D8-scuPA-T; i.e., a concentration of 230 nM 59D8-scuPA-T in the plasma milieu yielded approximately 25% clot lysis after 5 h (see Figure 7B). Both heparin and hirudin, two thrombin inhibitors with differing mechanisms of action, inhibited the fibrinolytic activity of 59D8-scuPA-T (Figure 7B) but not of 59D8-scuPA (Figure 7A). By contrast, heparin did not inhibit the fibrinolytic activity of 59D8-tcuPA-T that was previously generated by thrombin (data not shown). These results demonstrate that the activating agent for 59D8-scuPA-T in the plasma clot lysis experiments is thrombin. In the experiment shown in Figure 6B, the chimeric plasminogen activator was added to the unclotted sample prior to the addition of thrombin. 59D8-scuPA-T was able to lyse this type of clot, indicating that thrombin present in the clot cleaved 59D8-scuPA-T to its active two-chain derivative. 59D8-scuPA (and scuPA) when present in the clot matrix was not able to lyse the clot, presumably due to inactivation by thrombin.

DISCUSSION

Our design of scuPA-T was based upon thrombin's ability to cleave scuPA into a two-chain molecule that is inactive. Ichinose et al. (1986) have proposed that this thrombin cleavage of the Arg-156 and Phe-157 peptide bond in scuPA serves a regulatory role by suppressing the lytic activity of urokinase during coagulation when thrombin is being generated. We viewed thrombin as a potential surface marker to distinguish a thrombus from an established clot and therefore designed a scuPA mutant that is activated instead in inactivated by thrombin. To achieve this goal, we deleted two amino acids, Phe-157 and Lys-158, from scuPA by site-directed mutagenesis to make a mutant with the sequence Arg(154)-Pro-Arg-Ile-Ile(158) around the native thrombin/plasmin sites (Figure 2). Because the thrombin recognition site, Arg(154)-

Pro-Arg (Chang, 1985), was unaltered and the plasmin recognition site, Lys-158, was removed, the mutant was expected to be cleaved only by thrombin but not by plasmin. Also, the new N-terminal amino acid from the thrombin-cleaved two-chain form of urokinase (tcuPA-T) would be Ile-157, which has been shown to be essential for the activity of the plasmin-cleaved tcuPA (Lijnen et al., 1988). These expectations have been realized.

For the purpose of the present investigation, the 59D8 part of the 59D8-scuPA-T protein shown in Figure 2 provided us with a useful handle to purify and characterize the 59D8-scuPA-T protein. Because the monoclonal antibody 59D8 (Hui et al., 1983) was elicited with a synthetic peptide corresponding to the amino terminus of fibrin β chains, we could use Gly-His-Arg-Pro-Leu-Asp-Lys–Sepharose to conveniently purify 59D8-scuPA-T or 59D8-scuPA that we used as control. The rationale and virtues of such antibody-targeted plasminogen activators have already been discussed (Haber et al., 1989).

Starting with affinity-purified 59D8-scuPA-T, we confirmed by SDS–PAGE analyses that this chimeric protein was a heterodimer, composed of one antibody 59D8 light chain (27 kDa) and one fusion protein (64 kDa) that contained a 59D8 heavy-chain Fd fragment and a low molecular weight scuPA-T. The heterodimer was covalently stabilized by a disulfide bond (Figure 3A), presumably the one that normally holds antibody heavy and light chains together. A small but detectable fraction of heterodimer, however, was not covalently stabilized because faint bands corresponding to 64 and 27 kDa (Figure 3B,C) were detected by immunoblotting even under a nonreducing SDS–PAGE conditions. The 64-kDa fusion protein in 59D8-scuPA-T, unlike its control protein in 59D8-scuPA, was cleaved only by thrombin and not by plasmin (Figure 4). In addition, as we expected, the thrombin cleavage site was at the peptide bond between Arg-156 and Ile-157.

The overall functional features of the scuPA-T chimeric protein have been retained. This is not surprising since scuPA-T lacks only two amino acids as compared to scuPA. Like the control chimeric protein (Runge et al., 1991), scuPA-T could also be purified with an immobilized peptide antigen originally used to elicit antibody 59D8. As an enzyme, 59D8-scuPA-T was inactive in the zymogen form, inactive when treated with plasmin, and active when treated with thrombin (see Figure 5). There were two significant differences as compared to 59D8-scuPA: the time dependence of activation and the K_m of scuPA-T toward plasminogen. The first difference is understandable considering that thrombin instead of plasmin was used to activate 59D8-scuPA-T. It was evident from Figure 5 that thrombin generates urokinase activity from 59D8-scuPA-T with a slower time course than plasmin does from 59D8-scuPA. On the other hand, the 3-fold increase in the K_m of activated scuPA-T is puzzling because thrombin cleavage of urokinase generates the same catalytic domain as plasmin does when it activates 59D8-scuPA. We do not understand this, but suggest without data that following thrombin cleavage of scuPA-T the thrombin·product complex might remain associated via thrombin's exosite in a manner similar to the binding of thrombin to fibrin (Stubbs & Bode, 1993). As a result, the active site of the urokinase domain in scuPA-T·thrombin might be less accessible to incoming plasminogen substrate molecules.

In consideration of the novel thrombin-mediated activation of 59D8-scuPA-T, we designed two different plasma clot lysis experiments to evaluate the *in vitro* activity of 59D8-scuPA-T. For the first assay, 59D8-scuPA-T and control plasminogen activators were added exogenously to the solution used to incubate plasma clots containing ^{125}I-fibrin; for the second, the plasminogen activators were included in the sample prior to clotting, but not in the incubation solution. When plasminogen activators were introduced exogenously, lysis was observed in the following order of potency: 59D8-scuPA > scuPA > 59D8-scuPA-T (Figure 6A). The result that the potency of 59D8-scuPA-T was lower than those of 59D8-scuPA and scuPA could be due to the combination of two possible reasons: (i) there was more active plasmin than thrombin around the clot and therefore more 59D8-tcuPA or tcuPA rather than 59D8-tcuPA-T was generated; (ii) the activity of 59D8-tcuPA was 3 times higher than that of 59D8-tcuPA-T as evidenced by kinetic studies. On the other hand, when plasminogen activators were introduced into the forming clot, lysis was clearly observed for 59D8-scuPA-T but not at all for 59D8-scuPA or scuPA (Figure 6B). This is best explained by the presence of more thrombin in a forming clot than in an established clot; the excess thrombin activated 59D8-scuPA-T but inactivated 59D8-scuPA and scuPA. The potency of 59D8-scuPA-T was different in these two experiments, suggesting that the plasma clot lysis initiated by 59D8-scuPA-T is dependent on the thrombin concentration around the clots. This unique property of 59D8-scuPA-T may permit selective lysis of thrombin-rich clots (i.e., forming or newly formed thrombi) but not thrombin-depleted clots (i.e., established clot; haemostatic plugs).

In order to further confirm that thrombin was responsible for the activation of 59D8-scuPA-T during plasma clot lysis, lysis was performed in the presence or absence of either heparin or hirudin (Figure 7). Heparin inhibits thrombin by first associating with antithrombin III, rendering this serpin extremely potent. Hirudin, on the other hand, binds tightly and directly to thrombin as another potent inhibitor. The results showed that both heparin and hirudin prevented plasma clot lysis induced by 59D8-scuPA-T but had no similar inhibitory effect on lysis induced by 59D8-scuPA. This indicated to us that 59D8-scuPA-T is activated solely by free, soluble thrombin and not by fibrin-bound thrombin.

Recent studies have shown that thrombin inactivation of scuPA is strongly promoted by the presence of thrombomodulin, an integral membrane protein that forms a one to one complex with thrombin and alters its specificity (de Munk et al., 1991, 1993; Molinari et al., 1992). Since the thrombin recognition site in 59D8-scuPA-T is similar to that in 59D8-scuPA or low molecular weight scuPA, we suspect that thrombomodulin may potentially enhance thrombin activation of the 59D8-scuPA-T molecule.

In summary, 59D8-scuPA-T is a chimeric plasminogen activator that has two unique properties: (i) affinity for fibrin and (ii) activation by thrombin. These two properties suggest that 59D8-scuPA-T will be a novel thrombolytic agent, one with the potential to discriminate a thrombus from an established clot (e.g., a haemostatic plug). Furthermore, 59D8-scuPA-T might find use as an antithrombotic agent because we find that it works most efficiently on forming clots such as those that might form after angioplasty or conventional thrombolytic therapy in up to 15–25% of patients (Topol et al., 1987). It has been suggested that fibrin-bound thrombin is reexposed during thrombolysis and may again generate fibrin (Rapold & Collen, 1992). 59D8-scuPA-T may therefore be useful as a thrombolytic agent which prevents reocclusion during ongoing treatment by continuous activation by reexposed fibrin-bound thrombin. These opportunities must be validated by future *in vivo* characterization of 59D8-scuPA-T.

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