Incorporation of Fragment X into Fibrin Clots Renders Them More Susceptible to Lysis by Plasmin†

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ABSTRACT: Bleeding, the most serious complication of thrombolytic therapy with tissue-type plasminogen activator (t-PA), is thought to result from lysis of fibrin in hemostatic plugs and from the systemic lytic state caused by unopposed plasmin. One mechanism by which systemic plasmin can impair hemostasis is by partially degrading fibrinogen to fragment X, a product that retains clottability but forms clots with reduced tensile strength that stimulate plasminogen activation by t-PA more than fibrin clots. The purpose of this study was to elucidate potential mechanisms by which fragment X accelerates t-PA-mediated fibrinolysis. In the presence of t-PA, clots containing fragment X were degraded faster than fibrin clots and exhibited higher rates of plasminogen activation. Although treatment with carboxypeptidase B, an enzyme that reduces plasminogen binding to fibrin, prolonged the lysis times of fragment X and fibrin clots, clots containing fragment X still were degraded more rapidly. Furthermore, plasmin or trypsin also degraded clots containing fragment X more rapidly than fibrin clots, suggesting that this effect is largely independent of plasminogen activation. Fragment X-derived degradation products were not preferentially released by plasmin from clots composed of equal concentrations of fibrinogen and fragment X, indicating that fragment X does not constitute a preferential site for proteolysis. These data suggest that structural changes resulting from incorporation of fragment X into clots promote their lysis. Thus, attenuation of thrombolytic therapy-induced fragment X formation may reduce the risk of bleeding.

Hemostasis, the process by which blood is maintained in a fluid state, is achieved through the actions of the coagulation and fibrinolytic pathways. Coagulation generates thrombin which cleaves fibrinogen, enabling it to polymerize and form a clot. Clot formation is balanced by the fibrinolytic system whose role is to degrade the fibrin.

Fibrinogen is composed of three pairs of chains, denoted α, *β*, and *γ*, which form an extended globular structure of 340000 Da. Thrombin-mediated conversion of fibrinogen to fibrin requires removal of the NH_2 termini of the α- and *β*-chains to expose polymerization knobs that bind to preexisting pockets near the COOH termini. Individual fibrin monomers then polymerize to form two-stranded protofibrils that then assemble into thicker fibers. Plasmin, generated by the fibrinolytic system, cleaves all three fibrin chains at numerous sites, thereby solubilizing the clot. Clotting and fibrinolysis are tightly regulated and only function locally in response to tissue damage. Under certain pathological conditions, however, unregulated activation of coagulation or fibrinolysis can lead to thrombosis or bleeding, respectively.

Tissue-type plasminogen activator (t-PA)[1] initiates fibrinolysis by converting plasminogen to plasmin. This reaction is stimulated by fibrin, which serves as a template that binds both t-PA and plasminogen, thereby promoting the rate of plasminogen activation by at least 2 orders of magnitude (*1*, *2*). The resultant plasmin degrades fibrin to generate soluble fibrin degradation products. Therefore, in addition to its structural role in clot stabilization, fibrin also has a regulatory function, serving as an essential cofactor in plasminogen activation. This property contributes to the observed fibrin specificity of t-PA-mediated plasminogen activation (*3*).

Despite enhanced plasminogen activation in the presence of fibrin relative to fibrinogen, t-PA produces fibrinogen degradation when given in pharmacological doses. Thus, when patients are given t-PA for coronary thrombolysis, over 80% of the fibrinogen molecules demonstrate proteolysis after 1 h (*4*). Large fibrinogen degradation products, particularly fragment X, an early degradation product that is missing the COOH-terminal two-thirds of its α-chains,

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[1] Abbreviations: *A*, absorbance; cIII, protease III from *Crotalus atrox* venom; COOH, carboxyl-terminal; CPB, carboxypeptidase B; Glu-plasminogen, NH_2-terminal Glu^1-plasminogen; IIa, thrombin; Lys-plasminogen, NH_2-terminal Lys^{77}-plasminogen; PAGE, polyacrylamide gel electrophoresis; TAFI, thrombin-activatable fibrinolysis inhibitor; t_m, time to lysis midpoint; t-PA, tissue-type plasminogen activator; VFK-CMK, valylphenylalanyllysyl chloromethyl ketone.

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predominate during t-PA therapy. Fragment X appears within 30 min of starting t-PA therapy and persists in the circulation for at least 24 h. More than 30% of fibrinogen is converted to fragment X after t-PA treatment (*5*). Because fragment X retains its polymerization sites and is clottable, the extent of its formation may be underestimated when fibrinogen levels are measured using clotting assays.

Bleeding, particularly intracranial hemorrhage, is the most serious side effect of thrombolytic therapy for acute myocardial infarction (*6*). Two mechanisms have been proposed to explain this phenomenon. The most widely accepted explanation is that bleeding results from the inability of t-PA to distinguish between fibrin in occlusive thrombi and fibrin in hemostatic plugs (*7*). Alternatively, bleeding may reflect the systemic lytic state wherein degradation of fibrinogen and other clotting factors by unopposed plasmin reduces the hemostatic potential of the blood (*8*, *9*). The latter explanation has received less attention because t-PA produces more intracranial bleeding than streptokinase, yet streptokinase causes more fibrinogenolysis. However, the pattern of fibrinogenolysis is different with t-PA than it is with streptokinase. Whereas t-PA induces the accumulation of fragment X, lower molecular weight nonclottable degradation products, notably fragments Y, D, and E, are generated after streptokinase administration (*5*). Because it is clottable, fragment X can be incorporated into hemostatic plugs. In contrast, lower molecular weight fibrinogen degradation products cannot be incorporated into hemostatic plugs.

In vitro, fragment X incorporation into fibrin clots renders them more susceptible to t-PA-mediated lysis (*10*). These findings prompted the hypothesis that t-PA-induced bleeding reflects, at least in part, compromised stability of hemostatic plugs because of fragment X incorporation. This study was undertaken to explore the mechanisms by which fragment X incorporation into fibrin clots increases their susceptibility to lysis.

There are several mechanisms by which fragment X incorporation may compromise the integrity of fibrin clots. Previous studies demonstrated that clots formed from fragment X have reduced tensile strength compared with those generated from intact fibrinogen (*11*). Furthermore, fragment X clots are more susceptible to mechanical disruption and exhibit a looser distribution of fibrin fibers than clots generated from fibrinogen, differences that may reflect reduced branching of fibers and fewer interconnections (*12*). Despite the fact that the clottability of fragment X is similar to that of fibrinogen, the rigidity of fragment X clots is only 1% of that of clots formed from intact fibrinogen (*13*). These observations highlight the contribution of the COOH termini of the α-chains to the lateral aggregation of fibrin protofibrils by forming intermolecular contacts with adjacent fibrin monomers during polymerization (*14*).

Another potential mechanism by which fragment X incorporation into fibrin clots may compromise their integrity is through enhanced plasminogen activation by t-PA. Thus, Lys residues at the COOH termini of the α-chains of fragment X may serve as additional binding sites for t-PA and plasminogen, thereby augmenting their interaction (*15*, *16*). To explore the relative importance of these various mechanisms, we examined the influence of fragment X on clot structure, rate of plasminogen activation, and susceptibility to plasmin-mediated lysis.

EXPERIMENTAL PROCEDURES

Materials

Human fibrinogen (plasminogen free), factor XIII, and α-thrombin were obtained from Enzyme Research Laboratories (South Bend, IN). Trypsin was obtained from Sigma-Aldrich (Oakville, Ontario, Canada). Porcine pancreatic carboxypeptidase B (CPB) was from Sigma or Calbiochem (San Diego, CA). Recombinant single-chain t-PA (lot L9504AX) was a generous gift from Dr. Bruce Keyt (Genentech Inc., South San Francisco, CA), whereas low molecular weight recombinant urokinase was kindly provided Dr. Jack Henkin (Abbott Laboratories, North Chicago, IL). $Na^{125}I$ was obtained from Perkin-Elmer Life Sciences. D-Val-Phe-Lys chloromethyl ketone (VFK-CMK) was purchased from Calbiochem. Sepharose CL-4B and benzamidine−Sepharose were from GE Health Bio-sciences (Piscataway, NJ). Chromogenic plasmin substrate S-2251 (H-D-Val-Leu-Lys-*p*-nitroaniline dihydrochloride) was purchased from DiaPharma (West Chester, OH). *Crotalus atrox* venom was obtained from Sigma, and the protease III fraction (cIII) was isolated as described (*17*). Proteins were radiolabeled with $Na^{125}I$ as described (*18*).

Human Glu^{1}-plasminogen and Lys^{77}-plasminogen were isolated from human plasma as described previously (*19*). Plasminogen was activated on Lys-Sepharose to obtain plasmin. Lys-Sepharose (7 mL) was combined with 10 mg of plasminogen in 20 mM Tris-HCl and 150 mM NaCl, pH 7.4 (TS buffer), in a total volume of 20 mL and mixed for 15 min. Urokinase (∼10000 units) was added, and the suspension was continuously mixed by inversion. At 4 min intervals, a 100 μL aliquot of the suspension was removed to monitor plasmin activity. Each aliquot was made 20 mM in 6-aminohexanoic acid, and the Lys-Sepharose was pelleted by centrifugation for 2 min at 14000*g*. Ten microliters of supernatant was removed for SDS−polyacrylamide gel electrophoresis (SDS−PAGE), while another 10 μL aliquot was removed to a cuvette containing 0.5 mL of 0.25 mM S-2251. Plasmin activity was determined by monitoring hydrolysis of S-2251 at 405 nm in a DU7400 spectrophotometer (Beckman Instruments, Palo Alto, CA). When plasmin activity reached a plateau, the suspension was loaded into a 1 × 10 cm column and washed with TS (>50 mL) to remove urokinase. Plasmin was eluted with 20 mM 6-aminohexanoic acid in TS. The bound fraction was applied to a benzamidine−Sepharose column (1.5 cm × 3 cm) that was washed with 20 mL of TS. After additional washing, bound plasmin was eluted with 10 mM benzamidine in TS. The eluate was reapplied to the Lys-Sepharose column to remove benzamidine and washed with TS, and bound plasmin was eluted with 6-aminohexanoic acid. The resultant plasmin was dialyzed against TS overnight at 4 °C, and its concentration was determined using a molecular weight of 83000 and a $\epsilon^{280}_{0.1\%}$ of 1.7 (*20*). The integrity of the plasmin was assessed by subjecting it to SDS−PAGE analysis using precast 4−15% polyacrylamide gels (Ready-Gel; Bio-Rad) (*21*). Gels were stained with Fast Stain (Zoion, Newton, MA).

Fibrinogen was processed to remove factor XIII using a 50 mL anti-fXIII antibody column (SAFXIII−IgG−Sepharose; Affinity Biologicals, Ancaster, Ontario, Canada) as previously described (*22*). Fibrinogen concentration was deter-

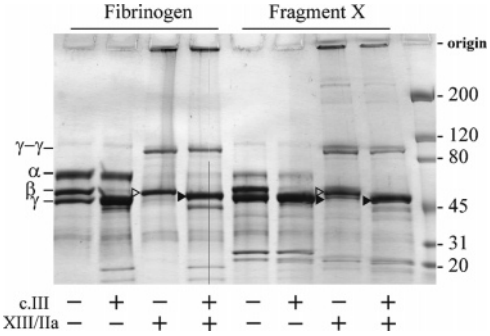

FIGURE 1: Characterization of fragment X. To assess the integrity of the NH_2 termini of the β-chains, fibrinogen and fragment X were incubated with 1 μM *C. atrox* protease III (cIII) for 60 min. Subsequently, untreated and cIII-treated fibrinogen and fragment X were cross-linked by the addition of 10 nM thrombin (IIa) and 100 nM factor XIII. The samples were subjected to electrophoresis on 4−15% polyacrylamide gradient gels under denaturing and reducing conditions. Gels were stained with Fast Stain. The positions of molecular weight markers are indicated on the right side and the α-, β-, and γ-chains and $\gamma-\gamma$ dimers on the left. In the cross-linked samples, the β-chains are highlighted by arrows for intact β- (open arrowheads) and des 1−42 β-chains (closed arrowheads).

mined using a molecular weight of 340000 and $\epsilon^{280}_{0.1\%}$ of 1.51 (*23*). Fragment X was prepared by limited plasmin digestion of fibrinogen. Plasmin (10 nM) was added to 165 mg of 4.7 mg/mL factor XIII-depleted fibrinogen, and the solution was incubated at 37 °C. At intervals, aliquots were removed and incubated with thrombin and $CaCl_2$ (final concentrations 10 nM and 2 mM, respectively) for 2 min. Clotted material was pelleted by centrifugation at 14000*g* for 1 min, and the A_{280} of the supernatant was compared with that of the starting fibrinogen to assess clottability. After approximately 30 min incubation, when clottability was 80−85%, plasmin was inhibited by the addition of 100 nM VFK-CMK. The preparation was concentrated to a volume of approximately 8 mL by ultrafiltration (Amicon Centriprep10; Millipore Corp.) and subjected to gel filtration on a 600 × 21.2 mm BioSep Sec3000 column (Phenomenex, Torrance, CA) attached to a high-performance liquid chromatograph (System Gold; Beckman Instruments Inc.) using 100 mM NaCl and 50 mM Tris-HCl, pH 7.0, at a flow rate of 5 mL/min. Resultant fractions were measured for optical density at 280 nm and subjected to SDS−PAGE. Fractions containing only fragment X were pooled, and the concentration was determined spectrophotometrically using a molecular weight of 240000 and $\epsilon^{280}_{0.1\%}$ of 1.4. Control experiments confirmed the absence of residual plasmin or VFK-CMK in the fragment X preparations.

The integrity of the β- and γ-chains of fragment X was assessed by SDS−PAGE analysis under reducing conditions (Figure 1). Compared with fibrinogen, fragment X exhibited reduced intensity of the α-chain band. Because the integrity of the NH_2 terminus of the β-chains affects fibrin clot structure and the β 42−43 bond is an early target for plasmin (*24*), further characterization of fragment X was undertaken. Both fibrinogen and fragment X were incubated in TS buffer containing 10 mM $CaCl_2$ for 1 h with 1 μM cIII, which only cleaves the β 42−43 bond (*25*). Subsequent incubation with 10 nM thrombin and 100 nM factor XIII cross-links the α- and γ-chains, allowing visualization of the des 1−42 β-chain, which comigrates with the γ-chain. When fragment X is treated with cIII, mobility of the β-chain increases, suggesting that the β 42−43 bond in the fragment X preparation is largely intact. When the mobility of the γ-chain is altered by cross-linking, the β-chain of fragment X reveals only a minor amount of des 1−42 β-chain. Densitometric analysis of the gel reveals 80% intact β-chain in the fragment X preparation. Thus, the β-chain of the fragment X preparation used in our studies is largely intact; only the α-chain is degraded.

Fibrinogen preparations can be contaminated with α_2-antiplasmin (*26*). A polyclonal antibody against α_2-antiplasmin (American Diagnostica Canada, Montreal, Quebec, Canada) was used to exclude this possibility. In a chromogenic assay, added α_2-antiplasmin was observed to inhibit plasmin activity in the absence or presence of fibrinogen. This inhibitory activity was neutralized by addition of the antibody against α_2-antiplasmin (data not shown). In the absence of added α_2-antiplasmin, the antibody had no effect on plasmin activity when fibrinogen was present, indicating that the fibrinogen preparation was devoid of α_2-antiplasmin.

Methods

Clot Lysis and Plasminogen Activation. The effects of fragment X on rates of plasmin formation and clot lysis times were examined in a purified system. Five microliter aliquots of thrombin and t-PA (final concentrations of 5 nM and 0.1 nM, respectively) were placed on opposite sides of wells in a flat-bottomed 96-well plate. Fibrinogen and fragment X were mixed in various proportions to yield a total final concentration of 3 μM. Aliquots (90 μL) of fibrinogen/fragment X containing 0.5 μM Glu-plasminogen or Lys-plasminogen, 0.4 mM plasmin-directed chromogenic substrate S-2251, and 2 mM $CaCl_2$ in TS containing 0.01% Tween 20 buffer (TS-Tw) were added to the wells to initiate the reaction. The plate was incubated at 23 °C, and absorbance values at 405 and 490 nm were read in a SpectraMax 340 microplate reader (Molecular Devices) at 11−30 s intervals for 1−5 h. Absorbance values at 490 nm were subtracted from those at 405 nm to correct S-2251 hydrolysis for turbidity. Corrected A_{405} values were plotted against time squared to yield the slope (A/min^2). Rates of plasminogen activation were calculated as previously described (*27*). The time to half-maximal lysis (t_m) was calculated by determining the time to reach the transition midpoint between maximum (clotted) and minimum (lysed) absorbance readings at 490 nm. In some experiments, lysis was initiated with 5 nM plasmin or 100 nM trypsin in place of t-PA and plasminogen; S-2251 was omitted in these studies. To examine the contribution of COOH-terminal Lys residues, CPB was preincubated for 10 min with the fibrinogen/fragment X, plasminogen, $CaCl_2$, and S-2251 stock solutions at a final concentration of 100 nM. No steps were taken to inactivate CPB during clot formation or lysis. Experiments with CPB were also performed in the presence of plasmin instead of t-PA, plasminogen, and S-2251.

Glu-plasminogen to Lys-plasminogen Conversion. Conversion of Glu-plasminogen to Lys-plasminogen was monitored as described (*28*). Ninety microliter aliquots of fibrinogen or fragment X (3 μM), Glu-plasminogen (0.5 μM), ^{125}I-Glu-plasminogen (8 nM), $CaCl_2$ (2 mM), and S-2251 (0.4 mM) were added to thrombin and t-PA, as described

above. Clot formation and lysis were monitored at 490 nm in a plate reader. Parallel reactions in a separate plate were terminated at 10 min intervals with the addition of an equal volume of 0.9 M acetic acid. The extent of Glu-plasminogen to Lys-plasminogen conversion was examined by subjecting aliquots of these samples to acid/urea PAGE analysis. The gels were dried and exposed to Xray film and the radioactive bands excised and counted for radioactivity.

Release of ^{125}I-Labeled Fibrin and Fragment X Degradation Products. A series of 100 μL clots of fibrinogen (1.5 μM), fragment X (1.5 μM), $CaCl_2$ (2 mM), and ^{125}I-fibrinogen or ^{125}I-fragment X (40 nM) were formed in plastic tubes in the presence of thrombin (5 nM) and plasmin (10 nM). Clot formation and lysis were monitored in a parallel reaction at 490 nm in a plate reader. Reactions in individual tubes were terminated at 10 min intervals by the addition of VFK-CMK to 20 μM. Clots were compacted by centrifugation for 2 min at 14000*g*, and 10 μL of supernatant was removed and counted for radioactivity. Additional 10 μL aliquots were removed and subjected to SDS−PAGE analysis.

Determination of the K_i of Fibrinogen and Fragment X for Plasmin. Because fragment X has already undergone limited plasmin proteolysis, fewer cleavages may be required to lyse clots containing fragment X than those containing fibrinogen. This predicts that fragment X should be a weaker competitor of plasmin activity. To explore this possibility, the inhibitory effects of fibrinogen and fragment X on plasmin were determined as described (*29*). Briefly, plasmin (20 nM) hydrolysis of S-2251 at concentrations of 0.25 and 0.75 mM was monitored in the presence of 0−10 μM fibrinogen or fragment X in TS buffer. A Dixon plot of inverse rate versus competitor concentration was used to determine K_i.

Statistical Methods. The data presented represent the mean of at least three experiments, and the error bars denote the standard deviation.

RESULTS

Effect of Fragment X on Clot Lysis Times. Previous studies in vitro have demonstrated that fragment X promotes fibrinolysis. This result was confirmed in a t-PA-stimulated clot lysis experiment where the proportion of fragment X varied from 0 to 100%. Samples containing 3 μM fibrinogen species, 2 mM $CaCl_2$, 0.5 μM plasminogen, and 0.1 nM t-PA were clotted with 10 nM thrombin, and turbidity was monitored at 490 nm (Figure 2). The maximum turbidity increased in a dose-dependent manner as the proportion of fragment X increased. Over the range of fragment X concentrations tested, maximum turbidity was up to 100% higher with 3 μM fragment X clots than it was with 3 μM fibrin clots. Because turbidity is a function of fiber thickness, these results suggest that thicker fibers are formed in the presence of fragment X, an observation supported by previous studies (*24*, *30*). Fragment X addition produced similar increases in turbidity in the presence of either Glu- or Lys-plasminogen (data not shown). Turbidity profiles were also used to monitor clot lysis. Lysis time was defined as the time required to reduce the absorbance by 50%. During the final phase of lysis, transient increases in turbidity may reflect reorganization of fibrin fibers as the clot lyses, a phenomenon reported by other investigators (*31*, *32*). As previously reported (*10*), fragment X shortened lysis times in a concentration-dependent fashion (Figure 2, inset). Although, lysis times were shorter with Lys-plasminogen than with Glu-plasminogen, fragment X shortened the lysis times to a similar extent with both plasminogen species. Thus, with Glu-plasminogen, lysis times of clots composed entirely of fragment X were 48 ± 8% shorter than those of fibrin clots, whereas with Lys-plasminogen lysis times were 37 ± 8% shorter.

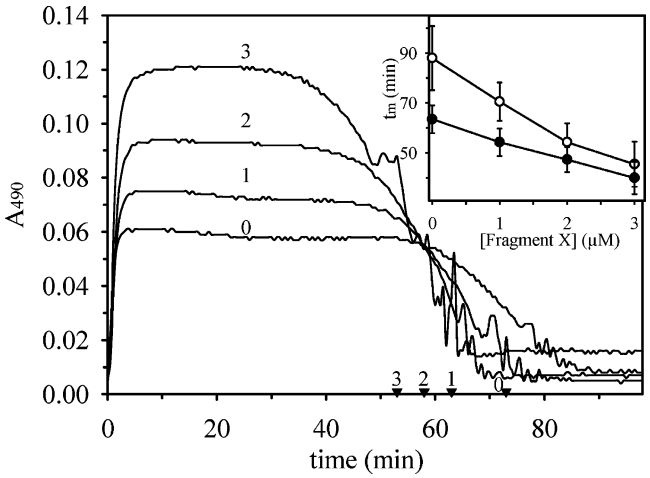

FIGURE 2: Effect of fragment X incorporation on t-PA-mediated lysis of fibrin clots. Samples containing 0.5 μM Glu-plasminogen, 0.1 nM t-PA, and 2 mM $CaCl_2$ in TS buffer were placed in individual wells of a microplate. Fibrinogen and fragment X were mixed in varying proportions to give a total final concentration of 3 μM. The concentration (0−3 μM) of fragment X in each well is indicated in the figure. Thrombin was added to 5 nM to initiate clotting, and the plate was monitored for turbidity at 490 nm in a plate reader. Lysis midpoint times were determined by calculating the time at which the turbidity had decreased by half (arrowheads on the *x*-axis). In the inset, lysis midpoints (t_m) are plotted versus the fragment X concentration. The experiment was performed using Glu-plasminogen (open circles) or Lys-plasminogen (closed circles).

Effect of Fragment X on Rates of Plasmin Generation. Having confirmed that fragment X promotes clot lysis, it was of interest to determine whether the effect was the result of accelerated plasminogen activation. Rates of t-PA-stimulated plasminogen activation in clots prepared from varying proportions of intact fibrinogen and fragment X were determined by quantifying plasmin concentrations using a plasmin-directed substrate. With both Glu- and Lys-plasminogen, rates of plasmin formation increased with increasing fragment X concentrations (Figure 3). Thus, rates of plasmin formation with Glu-plasminogen and Lys-plasminogen were 9.8 ± 2.8- and 3.5 ± 0.9-fold higher, respectively, in 3 μM fragment X clots than they were in 3 μM fibrin clots. The higher rates of activation observed with Lys-plasminogen are consistent with this form being a better substrate for t-PA than Glu-plasminogen. Retention of the profibrinolytic effect with Lys-plasminogen suggests that fragment X does not promote activation exclusively by enhancing Glu-plasminogen binding because Lys-plasminogen possesses higher affinity for fibrin and fibrin degradation products than Glu-plasminogen (*27*). This finding suggests involvement of other mechanisms.

Effect of Fragment X on Conversion of Glu-plasminogen to Lys-plasminogen. Because the conversion of Glu-plasmi-

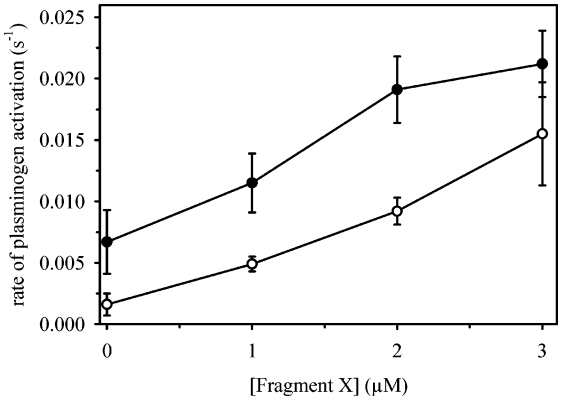

FIGURE 3: Effect of fragment X on the rate of t-PA-mediated plasminogen activation. Samples containing 0.5 μM Glu- (open circles) or Lys-plasminogen (closed circles), 0.1 nM t-PA, 0.4 mM S-2251, and 2 mM $CaCl_2$ in TS were prepared in the presence of varying amounts of fibrinogen and fragment X totaling 3 μM. Samples were clotted with 5 nM thrombin in microplate wells. S-2251 hydrolysis was monitored at 405 nm, and these data were used to calculate rates of plasminogen activation.

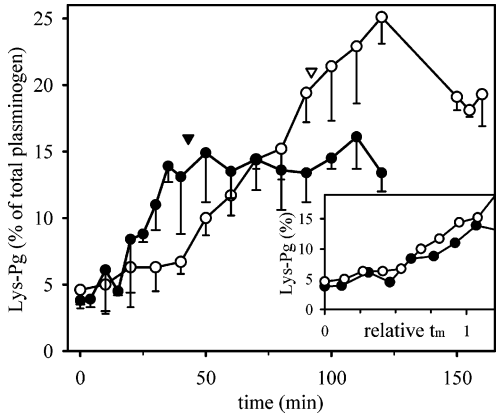

FIGURE 4: Effect of fragment X on conversion of Glu-plasminogen to Lys-plasminogen. Two stock solutions containing 0.5 μM Glu-plasminogen, 8 nM ^{125}I-Glu-plasminogen, 0.1 nM t-PA, 2 mM $CaCl_2$, and 3 μM fibrinogen (open symbols) or fragment X (closed symbols) were prepared. Aliquots were dispensed to tubes and clotted by the addition of thrombin to 5 nM. A representative sample from each series was monitored at 490 nm for turbidity. At various times, reactions in individual tubes were quenched by the addition of an equal volume of 0.9 M acetic acid. Following lysis, samples were lyophilized, resuspended in sample buffer, and subjected to acid/urea PAGE using 7.5% polyacrylamide gels. The gels were stained, dried, and exposed to Xray film. Areas of the gel corresponding to the position of radioactive species were excised and counted for radioactivity. The percent Lys-plasminogen of the total radioactivity is plotted versus time. The open and closed arrowheads denote the completion of lysis in the fibrinogen and fragment X clots, respectively. The inset shows the same data plotted on a relative time scale, normalized to the lysis midpoint for the two forms of fibrinogen.

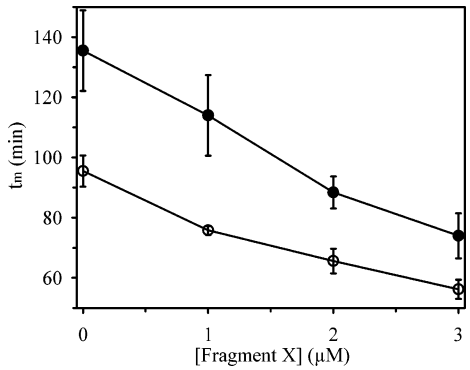

FIGURE 5: Effect of fragment X on t-PA-mediated lysis in the presence of CPB. Samples containing 0.5 μM Glu-plasminogen, 0.1 nM t-PA, 2 mM $CaCl_2$, and varying concentrations of fibrinogen and fragment X were clotted with 5 nM thrombin, as described in Figure 2. Lysis was monitored by turbidity, and lysis midpoint times are plotted versus the fragment X concentration. The experiment was performed in the absence (open circles) or presence (closed circles) of 100 nM CPB.

nogen to Lys-plasminogen within clots promotes fibrinolysis (*28*), formation of this intermediate was quantified to determine whether fragment X enhances this positive feedback reaction. In these lysis experiments, a series of clots containing varying amounts of fragment X were clotted in the presence of a trace amount of ^{125}I-Glu-plasminogen. The progress of clot lysis was monitored by turbidity. Individual samples were quenched at various time points, and aliquots were subjected to acid/urea PAGE analysis to resolve Glu- and Lys-plasminogen forms. Quantification of radioactivity of the bands excised from the gels (Figure 4) demonstrated that Lys-plasminogen formation coincided with the decrease in turbidity that signaled clot lysis. From a background amount of 5% Lys-plasminogen at time 0, about 20−25% of plasminogen was converted to the Lys form in fibrin clots. After lysis (arrowhead), the amount of Lys-plasminogen did not change significantly. Similar results were obtained with clots composed of fragment X, where the amount of Lys-plasminogen increased until the time of lysis. With fragment X clots, however, the amount of Lys-plasminogen formed was half that formed with fibrin clots. Because the processes of lysis and Lys-plasminogen formation are intimately linked (*28*), normalization of lysis times of fibrinogen and fragment X clots removes differences in the time courses and amounts of Lys-plasminogen formed (Figure 4, inset). Thus, there is no evidence of increased conversion of Glu-plasminogen to Lys-plasminogen in fragment X clots.

Effect of CPB on the Profibrinolytic Properties of Fragment X. Because it is a plasmin-derived fragment of fibrinogen, fragment X possesses COOH-terminal Lys residues that have been implicated in its capacity to accelerate clot lysis (*16*, *33*). Such residues are the target of the activated form of thrombin-activatable fibrinolysis inhibitor (TAFIa), a CPB-like enzyme that attenuates fibrinolysis. To examine the contribution of COOH-terminal Lys residues to the profibrinolytic properties of fragment X, lysis experiments were performed in the presence of CPB. At all fragment X concentrations, CPB prolonged t-PA-mediated lysis times by 30−50% (Figure 5). However, the profibrinolytic effect of fragment X persisted even in the presence of CPB. Thus, in the presence or absence of CPB, lysis times of fragment X clots were 41% and 46% shorter than those of fibrin clots, respectively. These results suggest that COOH-terminal Lys residues are not responsible for the profibrinolytic properties of fragment X clots.

Structural Differences between Fragment X and Fibrin Clots. A series of experiments were performed to examine whether structural differences between fragment X and fibrin clots account for the enhanced susceptibility of fragment X clots to lysis. Previous studies have shown that thick fibrin fibers exhibit higher rates of plasminogen activation and lysis than thin fibers (*34*, *35*). Because fragment X incorporation into clots increases maximum turbidity, a finding consistent

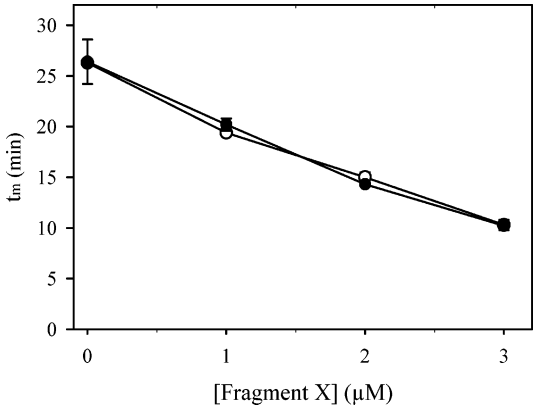

FIGURE 6: Effect of fragment X on plasmin-mediated clot lysis. Aliquots containing fragment X and fibrinogen (mixed in proportions totaling 3 μM) and 2 mM $CaCl_2$ were clotted with 10 nM thrombin in the presence of 25 nM plasmin. Parallel series were prepared either without (closed symbols) or with (open symbols) 100 nM CPB. Turbidity was monitored at 340 nm, and lysis midpoints were determined and plotted versus the concentration of fragment X.

with formation of thicker fibers (*24*), we explored the possibility that fragment X clots are more susceptible to lysis by plasmin. Lysis times with plasmin were shorter as more fragment X was incorporated in the clot (Figure 6). This sensitivity was retained in the presence of CPB, confirming that the COOH-terminal Lys residues of fragment X are not responsible for the shorter lysis times. These results indicate that clots composed of fragment X are more susceptible to plasmin-mediated lysis.

To determine whether enhanced plasmin sensitivity reflects increased fiber thickness of fragment X clots, the fiber thickness of clots prepared from intact fibrinogen was increased by lowering ionic strength. Despite increases in turbidity similar to those observed with fragment X clots, these modified fibrin clots exhibited plasmin lysis times similar to those of unmodified clots (not shown). These results reveal that fiber thickness alone does not promote plasmin-mediated lysis. Instead, the effect appears to be specific for fragment X. This concept was further investigated by substituting trypsin, an enzyme that does not bind fibrin, for plasmin. Previous studies have demonstrated that trypsin efficiently lyses fibrin clots (*36*). Trypsin effected more rapid lysis of fragment X clots than fibrin clots (Figure 7). Thus, clots formed from fragment X are more susceptible to proteolysis by both plasmin and trypsin, findings that suggest that fragment X clots are intrinsically more labile than fibrin clots. In our studies, 100 nM trypsin was required to lyse 1 μM fibrin clots at a rate comparable to that achieved with 5 nM plasmin. In contrast, other investigators have reported that trypsin is a more potent fibrinolytic agent than plasmin (*36*). These differences are not the result of reduced trypsin activity because active site titration of the trypsin used in the our studies revealed 73% activity.

Lysis of Radiolabeled Fragment X and Fibrin Clots. Additional studies were done to examine the possibility that fragment X incorporated into clots serves as a nidus for plasminogen activation and plasmin action. A series of clots formed from 1.5 μM fibrinogen and 1.5 μM fragment X and containing trace amounts of either ^{125}I-fibrinogen or ^{125}I-fragment X were lysed with plasmin. At intervals, plasmin in individual samples was quenched, and after compaction by centrifugation, aliquots of the supernatant were counted for ^{125}I-labeled degradation products originating from labeled fibrinogen or fragment X (Figure 8). There were no differences in the release of fibrinogen- or fragment X-derived degradation products, suggesting that fragment X does not serve as a focal point of fibrinolysis when incorporated into clots.

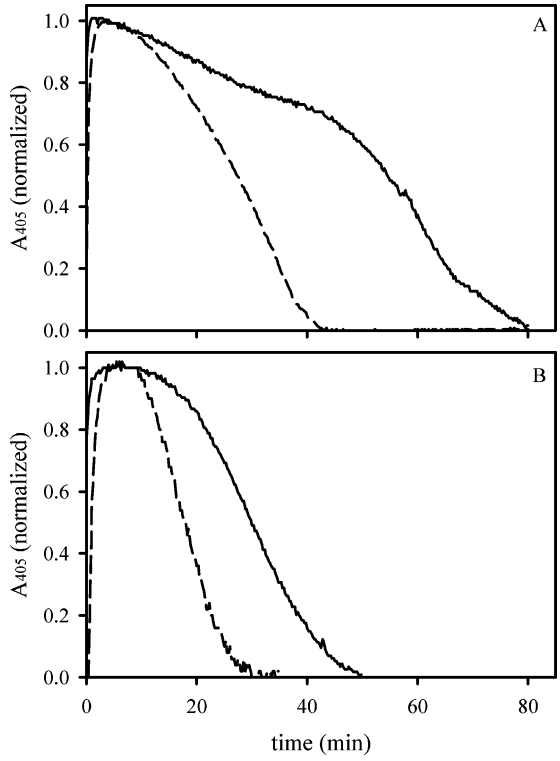

FIGURE 7: Lysis of fibrinogen and fragment X clots with plasmin or trypsin. Samples containing 1 μM fibrinogen (solid lines) or fragment X (hatched lines) and 2 mM $CaCl_2$ were clotted with 10 nM thrombin in the presence of 10 nM plasmin (panel A) or 100 nM trypsin (panel B). Turbidity at 405 nm was monitored. Turbidity values, normalized to the maximal reading of each sample, are plotted versus time.

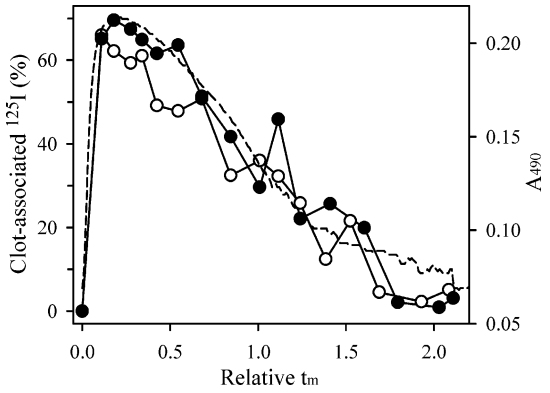

FIGURE 8: Release of radiolabeled fibrinogen- or fragment X-derived degradation products by plasmin. Stocks containing 0.5 μM Glu-plasminogen, 0.1 nM t-PA, 2 mM $CaCl_2$, 1.5 μM fibrinogen, 1.5 μM fragment X, and 40 nM ^{125}I-fibrinogen (open symbols) or ^{125}I-fragment X (closed symbols) were dispensed into tubes containing thrombin (5 nM) and plasmin (10 nM). A representative sample from each series was monitored at 490 nm for turbidity (hatched line). At various times, individual reactions were quenched by the addition of VFK-CMK to 100 nM. Clots were pelleted by centrifugation, and aliquots of the supernatant were removed and counted for radioactivity. The percent of radioactivity associated with the clot (pellet) was calculated and plotted versus the relative lysis midpoint time.

DISCUSSION

Clinical studies have documented formation and persistence of fibrinogen fragment X in the circulation of patients undergoing thrombolytic therapy with t-PA (*5*, *7*, *37*−*40*). Because fragment X is clottable (*15*, *41*), it may be incorporated into hemostatic plugs. This could impair thrombus stability because, compared with intact fibrin, clots formed from fragment X have reduced structural integrity (*13*) and exhibit enhanced plasminogen activation (*15*). Consequently, fragment X generation may contribute to t-PA-induced bleeding. In support of this concept, we previously demonstrated in rabbits that (a) attenuation of t-PA-induced fragment X formation by administration of supplemental α_2-antiplasmin reduces bleeding (*10*), (b) vampire bat plasminogen activator, which produces little fibrinogenolysis and fragment X, causes less bleeding than t-PA when the two fibrinolytic agents are given in doses that produce equivalent lysis of jugular vein thrombi (*42*), and (c) fragment X infusion enhances t-PA induced bleeding (unpublished data). The purpose of this study was to elucidate the profibrinolytic role of fragment X in fibrin clot lysis. Two of the central possibilities are that fragment X promotes plasminogen activation and that incorporation of fragment X into clots renders them more susceptible to lysis. The data presented here suggest that both mechanisms function to compromise the integrity of fibrin. Each of these will be discussed separately.

Plasminogen Activation. Fragment X has the potential to modulate two positive feedback steps that promote plasminogen activation by t-PA: conversion of Glu-plasminogen to Lys-plasminogen and generation of nascent plasminogen and t-PA binding sites on fibrin. Although both processes were documented here, their involvement in fragment X-mediated promotion of fibrinolysis is likely indirect. Lys-plasminogen has a higher affinity for fibrin and is a better substrate for activation by t-PA than Glu-plasminogen (*1*, *28*). Shortening of lysis times and elevated rates of activation occurred in the presence of fragment X even when the Glu- to Lys-plasminogen conversion was bypassed by including the Lys form at the onset of the experiment. In addition, less Lys-plasminogen formed in clots prepared from fragment X than from fibrinogen. Therefore, promotion of Lys-plasminogen formation does not appear to be a mechanism by which fragment X promotes fibrinolysis.

The second positive feedback step in plasmin-mediated fibrinolysis is the generation of binding sites for t-PA and plasminogen via newly exposed COOH-terminal Lys residues on degrading fibrin. These binding sites promote t-PA-mediated plasminogen activation because fibrin serves as a template onto which t-PA and plasminogen assemble (*1*, *2*). Because it already possesses COOH-terminal Lys residues, fragment X has the potential to bypass the need for plasmin to degrade fibrin. Consistent with this concept, clots prepared from fragment X display enhanced binding of t-PA and plasminogen (*15*, *16*). Our data also support this hypothesis. Thus, clots prepared from fragment X had 8−10-fold elevated rates of Glu-plasminogen activation compared with fibrin clots. The contribution of COOH-terminal Lys residues to this phenomenon was assessed by using CPB, a TAFIa homologue that attenuates fibrinolysis by removing these residues (*43*). Even though CPB prolonged lysis times, the profibrinolytic effect of fragment X persisted. Thus, even in the presence of CPB, fragment X shortened lysis times and increased the rate of plasminogen activation. These findings suggest that the COOH-terminal Lys residues in fragment X are not solely responsible for its capacity to promote fibrinolysis. It is worth considering that circulating fibrinogen contains some COOH-terminal Lys residues as a result of endogenous proteolysis (*44*). Thus, the presence of partially degraded fibrinogen molecules in the circulation may represent the physiological state.

Clot Structure. Numerous studies have shown that fibrin structure affects the rate of clot lysis. The rate of fibrinolysis correlates with fiber thickness, reflecting increased rates of plasminogen activation in clots composed of thick fibers (*34*, *35*, *45*, *46*). On the basis of measurements of turbidity, fragment X incorporation into fibrin clots produces a concentration-dependent increase in fiber thickness. These findings are in agreement with work by other researchers showing greater fiber thickness in the presence of fragment X (*12*, *24*, *30*, *47*). Consistent with these previous studies, therefore, accelerated fibrinolysis was observed in the presence of fragment X. However, when the activation step was bypassed by the substitution of plasmin for plasminogen and t-PA, lysis times remained shorter when fragment X was present. Therefore, structural changes induced by the presence of fragment X promote greater plasmin sensitivity in addition to promoting plasminogen activation.

Enhanced sensitivity of fragment X-containing clots to plasmin-mediated lysis could be attributed to changes in fiber thickness. Microscopy studies suggest that plasmin-mediated clot lysis is increased in clots composed of thicker fibers (*48*). However, when we modulated fiber thickness of fibrin clots by altering the ionic strength, there was no change in the rate of plasmin-mediated lysis. Altered fiber thickness also influences lysis of clots composed of a recombinant fibrinogen molecule that lacks αC domains (*49*). The relative weakness of these clots was attributed to impaired mechanical stability, possibly related to the inability of factor XIIIa to induce formation of α-polymers. Differences in factor XIIIa-mediated cross-linking cannot explain the profibrinolytic effect of fragment X, however, because fragment X promoted fibrinolysis to the same extent in the absence of factor XIII as it did in its presence (data not shown).

Another determinant of clot structure relates to the integrity of the NH_2 terminus of the β-chain which promotes clot stability by supporting lateral aggregation (*50*, *51*). Because the β 42−43 bond is an early target of plasmin, the integrity of the NH_2 terminus of the β-chain of fragment X could be compromised, an event that could result in clots with reduced stability (*52*). Two lines of evidence suggest that the enhanced susceptibility of fragment X clots to lysis by plasmin is not the result of loss of Bβ 1−42. First, SDS−PAGE analysis demonstrates that the NH_2 termini of the β-chains are largely intact in the fragment X preparation used in our studies. Second, des β 1−42 fibrinogen forms clots with thinner fibers (*51*). In contrast, on the basis of turbidity measurements, fragment X produces clots with increased fiber thickness.

Another potential explanation for the enhanced susceptibility of fragment X clots to plasmin-mediated lysis is higher affinity of plasmin for fragment X clots than for fibrin. This is unlikely to be the explanation because plasmin lysis times

were unaffected by CPB. Furthermore, fragment X clots also underwent more rapid lysis with trypsin than fibrin clots. Thus, even proteolytic enzymes that have no affinity for fibrin degrade fragment X clots more readily than fibrin clots. These findings suggest that fragment X-induced changes in clot structure render clots more susceptible to proteolysis.

Therefore, it appears that it is the greater sensitivity to plasmin degradation that compromises the integrity of fragment X clots. Studies examining how the fibrin architecture is dismantled by plasmin suggest that plasmin cleaves across whole fibers, transecting them rather than dissolving them into gradually thinner fibers (*48*, *53*). This results in liberation of long fiber segments containing numerous monomers (*54*). Thus, incorporation of fragment X into a growing protofibril composed of native fibrinogen may introduce weak links. It remains unclear, however, whether a fragment X molecule would be more easily severed because, just as with an intact fibrin monomer, plasmin still must cleave all three chains at the D−E domain interface. This would argue against fragment X functioning as a weak link, a concept supported by our observation that degradation products derived from clots generated from an equimolar mixture of fibrinogen and fragment X do not originate preferentially from fragment X. Although it is possible that, in the absence of αC domains, plasmin could act directly on the coiled-coil regions of fragment X to effect more rapid lysis, the data argue against this concept. First, there is no evidence of preferential degradation of fragment X based on release of ^{125}I-fragment X from clots. Second, when fibrinogen and fragment X were compared in their ability to act as competitive inhibitors of plasmin chromogenic activity, the K_i value for fragment X was only 3-fold higher than that of fibrinogen (9.5 ± 2.9 and 3.8 ± 1.6 μM, respectively; data not shown). Finally, the early plasmin cleavage sites comprise only about 10% of the total cleavage sites in fibrinogen, and they are cleaved about 10 times faster than the remaining sites (*55*). Thus, the plasmin cleavage sites in the αC domain of intact fibrinogen are unlikely to be a major impediment to plasmin's lytic function. Taken together, these data suggest that fragment X does not promote clot lysis by simply serving as a weak link in the fibrin polymer.

In conclusion, both enhanced plasminogen activation and sensitivity to plasmin combine to contribute to the profibrinolytic property of fragment X. Because the profibrinolytic effect of fragment X also is observed with plasmin, however, the influence of fragment X on the rate of plasminogen activation must be secondary to the greater sensitivity to proteolysis. Furthermore, the profibrinolytic effect of fragment X-containing clots is specific and not the result of simple structural change because reproducing the turbidity changes that occur when fragment X is incorporated into clots by altering ionic strength does not influence the lysis of fibrin clots. Therefore, incorporation of fragment X impairs the integrity of fibrin clots such that they are rendered more susceptible to plasmin. Consequently, attenuation of fragment X formation may represent an approach to improving the safety of thrombolytic therapy (*10*).

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