Actin Accelerates Plasmin Generation by Tissue Plasminogen Activator*

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Actin has been found to bind to plasmin's kringle regions, thereby inhibiting its enzymatic activity in a noncompetitive manner. We, therefore, examined its effect upon the conversion of plasminogen to plasmin by tissue plasminogen activator. Actin stimulated plasmin generation from both Glu- and Lys-plasminogen, lowering the $K_m$ for activation of Glu-plasminogen into the low micromolar range. Accelerated plasmin generation did not occur in the presence of $\epsilon$-amino caproic acid or if actin was exposed to acetic anhydride, an agent known to acetylate lysine residues. Actin binds to tissue plasminogen activator (t-PA) ($K_d = 0.55 \mu M$), at least partially via lysine-binding sites. Actin's stimulation of plasmin generation from Glu-plasminogen was inhibited by the addition of aprotinin and was restored by the substitution of plasmin-treated actin, indicating the operation of a plasmin-dependent positive feedback mechanism. Native actin binds to Lys-plasminogen, and promotes its conversion to plasmin even in the presence of aprotinin, indicating that plasmin's cleavage of either actin or plasminogen leads to further plasmin generation. Plasmin-treated actin binds Glu-plasminogen and t-PA simultaneously, thereby raising the local concentration of t-PA and plasminogen. Together, but not separately, actin and t-PA prolong the thrombin time of plasma through the generation of plasmin and fibrinogen degradation products. Actin-stimulated plasmin generation may be responsible for some of the changes found in peripheral blood following tissue injury and sepsis.

Plasminogen is a plasma glycoprotein of 791 amino acids that is converted to the active enzyme plasmin by the cleavage of the Arg $^{176}$-Val $^{176}$ peptide bond (1). The first 76 amino acids comprise the preactivation peptide, which may be cleaved by plasmin at Lys $^{77}$, thereby converting native Glu-plasminogen to Lys-plasminogen. Five kringle domains follow, each of which is approximately 90 amino acids in length, ending at position 542. These regions mediate the binding of plasminogen to other proteins, such as fibrin, by binding to lysine residues presented in the appropriate orientation. Cleavage of the Arg $^{176}$-Val $^{176}$ peptide bond by plasminogen activators such as tissue-plasminogen activator or urokinase results in the generation of the active enzyme plasmin, whose enzymatically active site is comprised of residues His $^{683}$, Asp $^{684}$, and Ser $^{741}$.

Actin, one of the most abundant cellular proteins, is found in all eukaryotic cells (4). It has also been found in micromolar concentrations in the circulation of healthy subjects (5-7) and in the blood of huans and animals experiencing various types of tissue injury (8-13). Extracellular actin is presumed to be released from dying cells, but the observation that certain isoforms of actin are released by viable cultured myoblasts (14) suggests that a fraction of the actin in the circulation may be purposely secreted by healthy cells. Introduction of actin into plasma results in its binding to two high affinity actin-binding proteins, vitamin D-binding protein (also known as Gc globulin) and plasma gelsolin (15, 16). Together, these proteins constitute a defense system designed to shorten actin filaments and clear actin from the circulation, thereby protecting the host from actin's deleterious effects upon the microvasculature (17-20).

Because it seems likely that fibrin clots formed at sites of tissue injury would contain trapped actin filaments, we studied the effects of actin upon clot lysis (21). We observed that actin inhibited clot lysis, whether initiated by the addition of t-PA and plasminogen or the direct addition of plasmin. Further analysis revealed that actin binds to the kringle regions of plasmin, thereby inhibiting its cleavage of both small peptide substrates and large proteins. Given these observations, we were interested in studying the effect of actin upon plasmin generation by t-PA, a protease that itself contains two kringle regions (22).

EXPERIMENTAL PROCEDURES

Materials—$\epsilon$-amino caproic acid (e-ACA), carboxypeptidase B, acetic anhydride, and CNBr were obtained from Sigma, aprotinin from Mobay Chemical Corp. (FBA Pharmaceuticals, New York), S-2251, plasmin, and S-2288 from KabiVitrum (Franklin, OH), soluble fibrin, Lys-plasminogen, and single-chain t-PA from American Diagnostica (Greenwich, CT), thrombin from Parke-Davis (Morris Plains, NJ), and CNBr-activated Sepharose from Pharmacia LKB Biotechnology Inc. The concentration of enzymatically active sites in the plasmin preparation was determined by titration with methyl-umbelliferyl guanidinobenzoate. Rabbit anti-plasminogen antibody was obtained from Dako (Santa Barbara, CA) and goat anti-fibrinogen antibody from Atlantic Antibodies (Scarborough, ME).

Protein Preparations—Rabbit skeletal muscle F-actin was used in all experiments. Its preparation and modification by acetic anhydride, as well as the isolation of Glu-plasminogen and fibrinogen, were performed as previously described (21). Plasmin-treated actin was prepared by incubating 100 μl of F-actin (10 mg/ml) with 10 μl of plasmin (1.8 μM) for 60 min at 21 °C, followed by the addition of 10 μl of aprotinin (10,000 KIU/ml). CNBr fragments of fibrinogen were prepared by the method of Verheijen et al. (23). Fresh frozen citrated
plasma, prepared by the blood transfusion service, was thawed, aliquoted, and stored at -70 °C. A fresh aliquot was thawed immediately before each experiment.

**Kinetic Analysis of Plasmin Generation and t-PA Activity**—Kinetic measurements were performed at 21 °C in wells of polystyrene microtiter plates (1.0 × 0.6 cm) using a Bio-Tek Instruments enzyme-linked immunosorbent assay plate reader to monitor liberation of p-nitroaniline from S-2288 (H-D-Ile-Pro-Arg-pNA) or S-2251 (H-D-Val-Leu-Lys-pNA) at 405 nm. Conversion of plasminogen to plasmin was monitored in a two-stage assay similar to that described by Hoylaerts et al. (24) except that 10 mM e-ACA was included in the second stage to prevent inhibition of the plasmin generated by actin (21). In the first stage, Glu-plasminogen, t-PA, and varying amounts of ϵ-ACA, actin, CNBr cleavage products of fibrinogen, or soluble fibrin were incubated in 40 μl of a solution containing 20 mM Tris, 150 mM NaCl, pH 7.4 (TBS), at 37 °C. In the second stage, 5-μl aliquots of the incubation mixture were added to wells of a microtiter plate that contained 95 ml of a TBS solution containing S-2251 (0.2 mM) and e-ACA (10 mM), thereby diluting the primary reactants by a factor of 20. The initial rate of change of the absorbance of the solution at 405 nm/min of the stage I incubation time was used to determine the rate of plasmin generation by using a standard curve constructed by determining the rate of S-2251 hydrolysis by known quantities of plasmin.

**Electrophoretic Analysis of Plasmin Generation**—Conversion of plasminogen to plasmin by t-PA, in the presence or absence of actin or plasmin-treated actin, was determined by incubating mixtures of these proteins at 37 °C for varying periods of time, after which they were boiled in gel sample buffer (25) and electrophoresed in 5-15% SDS-polyacrylamide gels under reducing conditions.

**Binding Studies**—Binding of actin to plasminogen and/or t-PA was assessed by centrifuging 12-20 ml of a TBS solution containing 10-18 μg of actin (or plasmin-treated actin) and either 10 μg of t-PA or 10 μg of plasminogen (Glu- or Lys-) at 150,000 × g for 30 min at 21 °C in a Beckman airfuge. In some cases 10 mM e-ACA was included. The resulting supernatant fluid and pellet were separated, boiled in gel sample buffer in the presence of a reducing agent, and electrophoresed on 5-15% polyacrylamide gels.

Binding of actin to t-PA was also studied by adding actin (5 μg) to t-PA (1.5-7.5 μg) in TBS (total volume, 100 μl). The resulting mixture was either assayed for t-PA activity or centrifuged at 150,000 × g for 45 min and the pellet and supernatant fluid separated. The t-PA activity of both the uncentrifuged mixture and the supernatant fluid was measured by adding 90 μl of either to wells of a microtiter plate that contained TBS (5 μl) and S-2288 (5 μl, final concentration, 0.5 mM) and monitoring the initial rate of change of absorbance of the solution at 405 nm. These measurements were used as a measure of the total and free t-PA, respectively. The dissociation constant was determined by Scatchard analysis.

**Thrombin Time Measurements**—The thrombin time of plasma samples containing varying amounts of actin and t-PA were measured with a fibrometer (Baltimore Biological Laboratories, Baltimore, MD). Citrated plasma was diluted 50% by the addition of varying quantities of known accelerators of plasminogen activation was examined. Actin was added to solutions of t-PA and Glu-plasminogen containing either CNBr-cleaved fibrinogen fragments or shown). The reaction obeyed Michaelis-Menten kinetics, as demonstrated when the data were examined with a Lineeweaver-Burk plot (Fig. 2). Micromolar concentrations of actin progressively lowered the $K_m$ for activation but had little effect upon $k_{cat}$ (Table I).

The effect of actin upon plasmin generation in the presence of known accelerators of plasminogen activation was examined. Actin was added to solutions of t-PA and Glu-plasminogen containing either CNBr-cleaved fibrinogen fragments or
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| [Actin] μM | K_m μM | k_cat s^{-1} | k_cat/K_m s^{-1} μM^{-1} |
|------------|--------|--------------|--------------------------|
| 0          | >100   |              |                          |
| 2.9        | 28     | 0.1816       | 0.0065                   |
| 5.7        | 19     | 0.1885       | 0.0099                   |
| 8.6        | 12     | 0.1772       | 0.0148                   |

### Table 1: Actin accelerates plasmin generation by t-PA

### FIG. 3. Stimulation of t-PA-mediated plasmin generation by actin and fibrinogen fragments. Panel A, t-PA (22 nM) and Glu-plasminogen (2.7 μM) were incubated at 37 °C in the absence (■) or presence of 10 μM actin (●), 1.7 mg/ml CNBr fibrinogen fragments (●), or both actin and CNBr fibrinogen fragments (●). 5-μl aliquots were removed and assayed for plasmin activity as described in the legend to Fig. 2. Plasmin activity is expressed as the change in absorbance units/min.

### FIG. 4. Binding of native and plasmin-treated actin to plasminogen and t-PA. Panel A, binding of native actin to Glu- and Lys-plasminogen. 12-μl solutions containing 18 μg of native actin and 10 μg of Lys- or Glu-plasminogen (lanes 1 and 2) or Glu-plasminogen (lanes 3 and 4) were centrifuged at 150,000 × g for 30 min. The resulting supernatant fluids (lanes 1 and 2) and pellets (lanes 2 and 4) were separated and electrophoresed on 5–15% SDS-polyacrylamide gels under reducing conditions. Panel B, effect of ε-ALA upon the binding of t-PA to actin. 20-μl solutions containing t-PA (10 μg) and actin (10 μg) were centrifuged at 150,000 × g for 30 min in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 10 mM ε-ALA. The resulting supernatants (lanes 1 and 3) and pellets (lanes 2 and 4) were subjected to electrophoresis on 5–15% polyacrylamide gels, as described above. Panel C, plasmin-treated actin (18 μg) was substituted for native actin in the experiment shown in panel A. All gels were stained with Coomassie Blue. HC, plasmin heavy chain.

### FIG. 2. Plasmin activity is expressed as the change in absorbance units/min.
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FIG. 5. Binding of t-PA to native actin. 100-μl aliquots of TBS containing actin (1.15 μM) and varying amounts of t-PA (0.18–2.7 μM) were centrifuged at 150,000 × g for 40 min. The amount of t-PA that bound to actin was determined by a functional assay for t-PA that was unaffected by the presence of actin. Scatchard analysis indicates that $K_d = 0.55$ mM. The line fit the data with $R^2 = 0.94$.

this system. Overall, these experiments indicate that actin's stimulatory effects upon plasmin generation are due to a positive feedback mechanism, mediated by plasmin's cleavage of either actin or plasminogen.

Plasmin-treated actin appears to differ from native actin largely because it binds Glu-plasminogen with higher affinity (Fig. 4C). Plasmin-treated actin did not bind t-PA to any greater extent than native actin (data not shown) nor did it directly stimulate t-PA hydrolytic activity (data not shown). Glu-plasminogen did not sediment with plasmin-treated actin that was treated with carboxypeptidase B (data not shown), indicating that Glu-plasminogen binds to a newly created COOH-terminal lysine, probably that formed by plasmin's cleavage of actin at Lys$^{275}$, Cys$^{276}$ (27). Glu-plasminogen did not displace t-PA from plasmin-treated actin filaments when solutions containing all three molecules were centrifuged (data not shown). These results suggest that plasmin-treated actin's effect is due to its ability to simultaneously bind t-PA and plasminogen, thereby increasing their local concentrations and promoting plasmin generation.

Actin Accelerates Plasminogen Activation in Plasma—To determine whether actin might stimulate plasmin generation under more physiological conditions, actin and t-PA were added to citrated plasma and incubated at 37 °C. As shown in Fig. 7, actin prolonged the thrombin time of plasma containing various amounts of t-PA in a time- and dose-dependent manner, suggesting that significant quantities of plasmin and fibrinogen split products had been generated.

Plasma samples containing actin and t-PA were subsequently studied by immunoblotting. As shown in Fig. 8, actin promoted the generation of both plasmin (detected as plasmin-α2-anti-plasmin complexes) and fibrinogen breakdown products, despite the presence of plasma actin-binding proteins and plasma protease inhibitors.

**DISCUSSION**

Actin has not previously been reported to interact with t-PA or plasminogen, although these interactions were anticipated by the determination that actin binds to plasmin's kringle regions (21). Because the binding of actin to plasmin results in a loss of plasmin's enzymatic activity, a property of the distal part of the plasmin molecule, it seemed possible that actin might also inhibit t-PA cleavage of plasminogen's Arg$^{606}$-Val$^{607}$ peptide bond. The experiments reported here indicate that, in fact, actin accelerates plasmin generation by t-PA.

Actin is not alone in stimulating plasmin generation by t-PA, for fibrin (28), fibrinogen (24), CNBr cleavage fragments of fibrinogen (23), polylysine (29), and a number of denatured proteins (30) also do so. This phenomenon appears to require one or more appropriately oriented lysine residues (31, 32), which likely exert their effects by interacting with the kringle regions of plasminogen and/or t-PA.

The fibrin surface is thought to accelerate plasmin generation by bringing together t-PA and plasminogen (24, 33). Degradation of fibrin by plasmin significantly speeds up the process by generating additional fibrin-binding sites for plasminogen and t-PA (34–39). These sites are created by plasmin's cleavage of fibrin at internal lysine residues, thereby
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Fig. 7. Effects of actin and t-PA upon the thrombin clotting time of plasma. The thrombin time of citrated plasma diluted 50% with varying amounts of actin and t-PA was determined following incubation at 37 °C. Panel A, the thrombin time of plasma samples containing t-PA (500 ng/ml, 7.4 μM) is shown as a function of the time of incubation for different concentrations of added actin: ○, 0; O, 2.5 μM; □, 5 μM; ●, 10 μM. Panel B, the thrombin time of plasma samples incubated at 37 °C for 30 min in the presence of native actin is shown as a function of the concentration of added t-PA (mean ± S.D. of triplicate determinations): ○, 250 ng/ml, 3.7 nM; △, 500 ng/ml, 7.4 nM; ▲, 1000 ng/ml, 14.7 nM; □, 1500 ng/ml, 22.1 nM; ●, 2000 ng/ml, 29.4 nM.

Generating COOH-terminal residues. This change allows these residues, which normally bind only to the low affinity aminohexyl-binding site of kringle 5, to bind to the "lysine-binding sites" of kringles 1–4 that mediate high affinity plasminogen-fibrin binding (40–42).

The experiments reported here show that actin accelerates plasmin generation in a similar manner. Its effects are dependent upon the appropriate three-dimensional presentation of its lysine residues which interact with kringle regions of plasminogen and t-PA. The interaction of the three depends, however, upon the action of the first plasmin molecules generated in the solution, for native actin binds poorly to Glu-plasminogen. Plasmin-treated actin, on the other hand, is able to simultaneously bind Glu-plasminogen and t-PA, thereby promoting plasmin generation, even in the presence of aprotinin. Actin's acceleratory effect appears to be due to its ability to bind both t-PA and plasminogen, thereby increasing their local concentration. It thus behaves in a manner similar to that described for immobilized thrombospondin and histidine-rich glycoprotein, each of which may promote plasmin generation by t-PA, particularly if first treated with plasmin (43, 44).

Actin's stimulatory effect on t-PA-mediated plasmin generation may account in part for the findings of Bicsak and Hsueh (45), who reported that t-PA present in rat oocyte extracts was fully active in the absence of an added t-PA simulator (such as fibrin or polylysine). They attributed this finding to a dissociable factor, not otherwise characterized, which was also present in media conditioned by rat insulinoma cells. The results reported here suggest that actin may be the endogenous stimulator detected by these investigators. The relevance of this interaction remains uncertain, however, until it can be shown that actin and t-PA are present simultaneously in a relevant compartment of the oocyte.
The potential clinical significance of these findings lies in actin’s ubiquity (4). It is found in virtually all eukaryotic cells and is the predominant protein constituent of vertebrate platelets, white blood cells, and most other nucleated cells. Inflammation may cause endothelial cell death and/or Weibel-Palade body secretion, resulting in locally elevated t-PA concentrations in regions where actin is released from injured cells. The simultaneous introduction of both proteins into the extracellular space may overwhelm the defenses provided by plasma protease inhibitors and plasma actin-binding proteins, leading to plasmin generation, which in turn may act upon platelets, endothelial cells, and plasma proteins. Fibrinogen split products, for example, are known to have deleterious effects upon clot formation and platelet function. The release of actin from dying cells may thus amplify the effects upon the clinical and laboratory findings of disseminated intravascular coagulation, a syndrome that frequently accompanies plasma protease inhibitors and plasma actin-binding proteins, platelets, white blood cells, and most other nucleated cells. Extracellular actin may contribute, in some circumstances, to split products, for example, are known to have deleterious effects upon clot formation and platelet function. The release of actin from dying cells may thus amplify the effects of the inflammatory mediators that caused cell death and its release. Extracellular actin may contribute, in some circumstances, to the clinical and laboratory findings of disseminated intravascular coagulation, a syndrome that frequently accompanies sepsis and tissue injury.

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