Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 8879–8883, August 1999
Biochemistry

A catalytic switch and the conversion of streptokinase to a fibrin-targeted plasminogen activator

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Communicated by Gregory A. Petsko, Brandeis University, Waltham, MA, June 11, 1999 (received for review January 18, 1999)

ABSTRACT Plasminogen (Pg) activators such as streptokinase (SK) save lives by generating plasmin to dissolve blood clots. Some believe that the unique ability of SK to activate Pg in the absence of fibrin limits its therapeutic utility. We have found that SK contains an unusual NH_2-terminal "catalytic switch" that allows Pg activation through both fibrin-independent and fibrin-dependent mechanisms. Unlike SK, a mutant (rSKΔ59) fusion protein lacking the 59 NH_2-terminal residues was no longer capable of fibrin-independent Pg activation (k_{cat}/K_m decreased by >600-fold). This activity was restored by coincubation with equimolar amounts of the NH_2-terminal peptide rSK1–59. Deletion of the NH_2 terminus made rSKΔ59 a Pg activator that requires fibrin, but not fibrinogen, for efficient catalytic function. The fibrin-dependence of the rSKΔ59 activator complex apparently resulted from selective catalytic processing of fibrin-bound Pg substrates in preference to other Pg forms. Consistent with these observations, the presence (rSK) or absence (rSKΔ59) of the SK NH_2-terminal peptide markedly altered fibrinolysis of human clots suspended in plasma. Like native SK, rSK produced incomplete clot lysis and complete destruction of plasma fibrinogen; in contrast, rSKΔ59 produced total clot lysis and minimal fibrinogen degradation. These studies indicate that structural elements in the NH_2 terminus are responsible for SK's unique mechanism of fibrin-independent Pg activation. Because deletion of the NH_2 terminus alters SK's mechanism of action and targets Pg activation to fibrin, there is the potential to improve SK's therapeutic efficacy.

Plasmin is an enzyme that dissolves fibrin, the protein matrix of blood clots (1, 2). Plasminogen (Pg) activators generate plasmin by cleaving Pg, an inert zymogen (1, 2). By virtue of their ability to induce the dissolution of blood clots, Pg activators save the lives of people who are having heart attacks (3).

A key mechanistic distinction among Pg activators is their dependence on fibrin as a cofactor during the process of Pg activation (4–7). Indeed, many authorities believe that this property of fibrin dependence is a critical determinant of the therapeutic efficacy of Pg activators (6, 7). The contrast between the two most widely used agents, streptokinase (SK) and tissue Pg activator (TPA), exemplifies this mechanistic distinction. SK does not require fibrin for efficient Pg activation in the blood (3). It is a catalytically inert, bacterial protein that has no structural homologues (8, 9). SK can form an activator complex with plasmin, which contains a functional active site, or with Pg, in which it nonproteolytically induces formation of a functional active site (8–11). In contrast, TPA is a serine protease whose cleavage of Pg is amplified markedly by interactions with fibrin (1, 2, 4, 5, 12–15).

Although the activator complex formed by SK is the most efficient activator of Pg (16), its success as a therapeutic agent seems to be influenced by its unique mechanism of fibrin-independent Pg activation. When given to humans, the SK activator complex rapidly generates plasmin in the circulating blood at sites distant from culprit fibrin clots. This mechanism depletes Pg substrate and squanders plasmin's clot-dissolving (or fibrinolytic) potential (17, 18). This rapid generation of plasmin also accelerates the production of bradykinin, which can dangerously lower blood pressure (19, 20). To avoid these effects, SK is given as a slow intravenous infusion, which in turn probably reduces its lifesaving effects, as patient survival correlates strongly with the rapidity of Pg activator therapy (3). The recent observation that mortality among SK-treated heart-attack patients (7.2%) is higher than that among TPA-treated patients (6.3%) has prompted many U.S. physicians to choose TPA, even though it is 8–9 times more expensive than SK (21, 22). Although the reasons for the differences in cost are multifactorial, this therapeutic choice has enormous public health implications, given the projection that cardiovascular disease will soon become the major cause of death worldwide (23). Because many of these therapeutic properties of SK can be ascribed to its unregulated or fibrin-independent mode of activating Pg and because an understanding of this property would illuminate the mechanisms of fibrin targeting, we looked for structural elements responsible for SK's unique mechanism of action.

In a crystal structure of the activator complex published recently (24), SK was found to contain three domains (α, spanning residues 12–150; β, spanning 151–287; and γ, spanning 288–372) that contacted the catalytic domain of plasmin. Beyond these domains, the NH_2- and COOH-terminal residues were found to be disordered. During Pg activation *in vitro* or in human plasma, SK is cleaved within seconds to minutes at its NH_2 terminus to yield the Ile^1-Lys^{59} peptide fragment, a stable core fragment spanning Ser^{60}-Lys^{386} and a COOH-terminal peptide fragment (24–31). Although the COOH-terminal peptide fragment does not seem to be necessary for efficient Pg activation (32), the Ile^1–Lys^{59} peptide remains tightly associated with the SK core fragment in the activator complex after cleavage (24, 28, 29, 31). It seems that the Ile^1–Lys^{59} peptide plays a role in Pg activation, although there are significant disagreements about the magnitude of the peptide's effects and the stoichiometry between it and the core fragment (27–31).

MATERIALS AND METHODS

Cloning, Expression, and Purification of Recombinant Proteins. The SK gene was cloned from *Streptococcus equisimilis*,

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Abbreviations: Pg, plasminogen; SK, streptokinase; TPA, tissue Pg activator; r, recombinant; n, native; μPg, micro-Pg; S2251, *H*-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride; PPACK, D-Phe-Pro-Arg chloromethylketone.

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expressed as a factor Xaa-cleavable fusion protein in bacteria via the pMALc vector (New England Biolabs), cleaved, and purified as described in detail (33). The recombinant (r) deletion mutant rSKΔ13 was formed by restriction digestion of the SK gene with *Hinc*II. rSKs lacking the first 23 (rSKΔ23) and 59 (rSKΔ59) amino acids were created by PCR with primers containing introduced *Eco*RV sites (for rSKΔ23, 5′-GCGAT ATC GGT ACT GTT GAG GG; for rSKΔ59, 5′-GCG GAT ATC TCA AAA CCA TTT GCT AC). The rSK1–59 cDNA was created by PCR with the malE primer (New England Biolabs) and an antisense primer with an *Xba*I site (5′GC TCT AGA TCA CTT TGG ACT TAA GCC TTG CTC TGT C). The correct cDNA sequence was confirmed after cloning. The purity of the rSK proteins was assessed by SDS/PAGE as described (33).

Recombinant micro-Pg (μPg) that spanned residues 530–791 of human Pg was cloned and ligated into pET11d (Stratagene) through the *Nco*I and *Bam*HI sites. To avoid incorrect disulfide bond formation during renaturation from bacterial inclusion bodies, the unpaired Cys-536 and Cys-540 residues were mutated to Ala and Ser, respectively. The μPg was solubilized from inclusion bodies with 6 M guanidine HCl and 10 mM DTT in TE buffer (50 mM Tris·HCl/10 mM EDTA, pH 8.0) and diluted into a refolding buffer containing 3.5 M guanidine, 5 mM L-cysteine, and 0.05 mM L-cystine in TE buffer to a concentration of 35 μg/ml. The μPg was then progressively dialyzed into TE buffer alone. The isolated μPg migrated as a single band on a nonreduced SDS/PAGE gel and underwent active-site titration as indicated below.

Enzymatic Assays. *Active-site titration.* The molar quantity of active sites generated by the various rSK–Pg activator complexes was determined at 25°C in a Hitachi (Tokyo) 2000 fluorescence spectrophotometer by active-site titration with the fluorogenic substrate 4-methylumbelliferyl *p*-guanidinobenzoate (Sigma) as described (33).

Kinetic assays of the rSK-Pg activator complex. The amidase kinetic parameters of the activator complexes formed by rSK, rSKΔ13, and rSKΔ23 were studied by using a paranitroanilide substrate (S2251 or H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride, Chromogenix, Molndal, Sweden) in a manner similar to that described (33). The rSK (20 nM) and Glu-Pg (10 nM; Hepar, Franklin, OH) were mixed together and incubated at 37°C for 5 min to construct the SK-Pg activator complex. The mixture was then transferred to a thermostatically regulated (37°C) quartz cuvette containing assay buffer (50 mM Tris/100 mM NaCl, pH 7.4) and various concentrations of S2251 (100–800 μM) in a total volume of 600 μl. The change in absorbance was monitored at 404 nm for 10 min at 37°C in a Hewlett–Packard 8451 A diode array spectrophotometer in a total volume of 600 μl. The data were plotted as velocity over substrate concentration and analyzed with the aid of a hyperbolic curve-fitting program as described (33).

Pg activation assays. The kinetics of Pg activation by rSK and rSK mutants were also studied as described (33). Glu–Pg and rSK (2–6.7 nM, depending on the mutant) were mixed in stoichiometric ratios and preincubated at 37°C to form an activator complex as described above. They were then added to a quartz cuvette containing assay buffer (50 mM Tris·HCl/100 mM NaCl, pH 7.4), 500 μM S2251, and various concentrations of Glu–Pg ($0.3 \times K_m$ to $5 \times K_m$ for SK). The change in absorbance at 405 nm was monitored at 37°C in a Hewlett–Packard 8451 A diode array spectrophotometer equipped with a thermocycler (VWR Scientific, model 1136). Initial reaction rates were obtained from the first 300 s by plotting A_{405}/min^2, and the apparent Michaelis constants and catalytic rate constants were calculated by fitting the data to a hyperbolic curve as described (33). In other experiments, purified fibrinogen or soluble fibrin fragments (desAA fibrinogen X or desafib X; American Diagnostica, Greenwich, CT) were used in various amounts (0–2 μM) to stimulate Pg activation by 10 nM stoichiometric complexes of rSKΔ59 and Glu-Pg.

Fibrinolysis Assays. Pooled citrated human plasma (45 μl) obtained from the Massachusetts General Hospital Blood Bank was mixed with trace amounts of ^{125}I-fibrinogen and clotted with calcium (5 mM) and thrombin (0.1 units) for 1 h at 37°C as described (34). After a wash with Tris-buffered saline (pH 7.4) containing the thrombin inhibitor PPACK (10 μM, D-Phe-Pro-Arg chloromethylketone, Calbiochem), the clots were suspended in 2 ml of plasma containing 10 μM PPACK. Identical quantities (0–50 nM) of rSK and rSKΔ59 that had undergone active-site titration were added to the plasma, and, after 6 h, the amount of fibrinolysis was determined by measuring the release of soluble ^{125}I-fibrin degradation products (34). The plasma supernatants also were promptly isolated, mixed with aprotinin (100 units) to inhibit plasmin activity, and precipitated with sodium sulfite to determine fibrinogen concentrations, as described (34).

RESULTS AND DISCUSSION

To establish unambiguously the role of SK's NH_2-terminal peptide (residues 1–59) in fibrin-independent Pg activation, we studied a series of rSKs with altered NH_2 termini. Because some NH_2-terminal SK mutants are difficult to express, we constructed the rSKs as fusion proteins in a manner that does not affect SK activity (33, 35). The proteins included rSK, containing the full sequence of native (n) SK (from group C streptococci), and mutants lacking 13, 23, and 59 amino acids: rSKΔ13, rSKΔ23, and rSKΔ59, respectively. Deletion mutant rSKΔ13 was selected because of a naturally occurring restriction site; rSKΔ23 was chosen, because Gly-24 has been reported to be critical to SK function (36).

All rSK fragments except rSK1–59 formed an activator complex with Glu-Pg, (the physiologic form of Pg; ref. 37) and induced in the molecule a site that was able to undergo active-site titration (data not shown). The rates of Pg activation induced by nSK and rSK were similar (Fig. 1*A* and Table 1), confirming earlier reports that expressing rSK as a fusion protein does not affect its catalytic function significantly (33, 35, 38). Deletion of the first 13 NH_2-terminal amino acids (rSKΔ13) had a minimal effect on Pg activation. Deletion of the first 23 amino acids of the NH_2 terminus (rSKΔ23) also minimally affected the K_m (2-fold increase) but reduced the k_{cat} (6-fold decrease) relative to rSK. Deletion of residues 1–59 (rSKΔ59) did not affect the K_m grossly but markedly reduced the k_{cat} (>600-fold decrease relative to rSK), effectively eliminating Pg activation. This finding indicates that the rSK1–59 peptide, particularly residues 24–59, is critical for Pg activation and that the peptide plays a more important role than is generally appreciated (27–31).

However, when a combination of rSK1–59 and rSKΔ59 was incubated with Pg, functional molecular complementation occurred (Fig. 1*B*) and efficient Pg activation was revived. Similar results were obtained when human plasmin was used instead of human Pg in activator complexes with the rSK fragments: only the combination of rSKΔ59 and rSK1–59 created a functional activator complex with plasmin (not shown). In kinetic assays, a stoichiometric combination of rSK1–59 and rSKΔ59 restored Pg activation measurements to levels similar to those obtained with nSK (Table 1). These experiments indicated that the NH_2 terminal peptide (particularly residues 24–59) acts as a catalytic switch that gives the rSKΔ59 activator complex the capacity to activate Pg, regardless of whether the complex contains Pg or plasmin.

In solution, Pg assumes a closed conformation that is thought to change significantly when it binds to fibrin through its kringle domains (1, 2, 4, 5). This fact prompted us to determine whether the rSK1–59 peptide was also necessary for Pg activation in the presence of fibrin. Purified, soluble fibrin

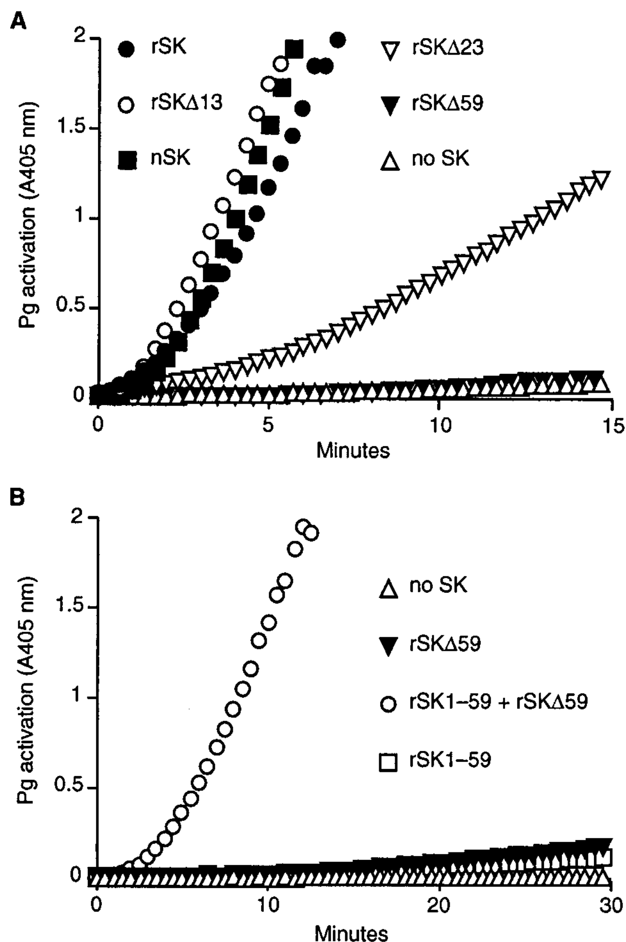

FIG. 1. (*A*) Fibrin-independent Pg activation by different rSKs and Glu-Pg as a function of time. The various rSKs (10 nM) were added to a quartz cuvette containing human Glu-Pg (300 nM) and S2251 (see Table 1) in 50 mM Tris·HCl/100 mM NaCl, pH 7.4 at 37°C. The rate of substrate cleavage was monitored by continuously recording the change in absorption at 405 nm. (*B*) Restoration of fibrin-independent Pg activation to rSKΔ59 by rSK1–59. rSKΔ59 (10 nM), rSK1–59 (10 nM), rSK1–59 (10 nM) + rSKΔ59 (10 nM), or no SK was mixed with Glu-Pg (300 nM) and S2251 substrate (0.5 mM), and the rate of substrate cleavage was monitored as above.

fragments, which have been used widely to study TPA activity (40), induced a dose-dependent increase in Pg activation by the rSKΔ59 activator complex (Fig. 2). Fibrin-dependent Pg activation was also observed when the maltose-binding protein fusion partner was separated from rSKΔ59 (Fig. 2 *Inset*), indicating that the maltose-binding protein did not mediate this fibrin-dependent process. In contrast, fibrinogen (the precursor to fibrin) had little effect on Pg activation (Fig. 2). This experiment indicated that the rSKΔ59 activator complex is a fibrin-dependent enzyme, much like TPA. To confirm the physiologic relevance of these findings, we studied the ability of the rSKΔ59 activator complex to initiate the dissolution of fibrin clots suspended in plasma. In these assays, the fibrinolytic potency of the rSKΔ59 activator complex was greater than that of rSK (Fig. 3*A*). These results led to the surprising conclusion that the NH_2 terminus of SK, although necessary for Pg activation in the absence of fibrin, was not necessary for efficient fibrinolysis.

It is well known that the singular ability of nSK to activate Pg in the absence of fibrin depletes Pg and degrades fibrinogen. The finding (Fig. 2) that fibrin, but not fibrinogen, effectively amplifies Pg activation by the rSKΔ59 activator complex implied that the mutant would not induce fibrinogen degra-

Table 1. Pg activation parameters for rSKs

	Pg activation parameters		
Type of SK	K_m, μM	k_{cat}, min^{-1}	k_{cat}/K_m, μM$^{-1}\cdot$min^{-1}
nSK	0.33 ± 0.05	1.4 ± 0.16	4.2
rSK	0.24 ± 0.06	2.3 ± 0.41	9.8
rSKΔ13	0.28 ± 0.06	1.7 ± 0.27	6.1
rSKΔ23	0.45 ± 0.07	0.4 ± 0.03	0.9
rSKΔ59*	0.19 ± 0.14	0.003 ± 0.0001	0.016
rSKΔ59 + rSK1–59	0.27 ± 0.07	1.2 ± 0.15	4.4

Pg activation was performed under conditions similar to those described for SK (38, 39), which were sufficient for all rSKs to achieve maximal stable function. Glu–Pg (>99% Glu type; ref. 38) and various SKs were mixed together for 5 min at 37°C to make a stoichiometric complex. Then 2–6.7 nM activator complex was added to a cuvette containing various concentrations of Glu–Pg (0.3 × K_m to 5 × K_m for SK; ref. 39) and S2251 substrate (0.5 mM) at 37°C. The change in absorbance at 405 nm was continuously monitored at 37°C. Initial reaction rates were obtained from the first 300 s, and the apparent Michaelis constants and catalytic rate constants were calculated as described (38, 39). The means ± SD are shown.

*The activity of the rSKΔ59–Pg complex was so low that the parameters must be regarded as estimates.

dation. We examined this possibility by measuring the relative amount of fibrinogen degradation induced by rSKΔ59 during the lysis of fibrin clots suspended in plasma. As expected, the rSK activator complex caused complete degradation of the fibrinogen in the plasma (Fig. 3*B*) but only incomplete lysis of the fibrin in the clot (Fig. 3*A*). In contrast, the rSKΔ59 activator complex caused minimal fibrinogen degradation (Fig. 3*B*), even though it lysed the clot almost completely (Fig. 3*A*). As judged by its requirement of fibrin for Pg activation and by its sparing of fibrinogen during fibrinolysis, the activator complex formed by rSKΔ59 is more akin to TPA than to its parent molecule SK.

For both TPA and the rSKΔ59 activator complex, fibrin amplifies the catalytic efficiency of Pg activation by about 500- to 1,000-fold (4–7), whereas fibrinogen, the precursor to fibrin, has minimal amplifying effects (Fig. 2; refs. 4 and 5). The specificity of this fibrin-vs.-fibrinogen cofactor effect is also

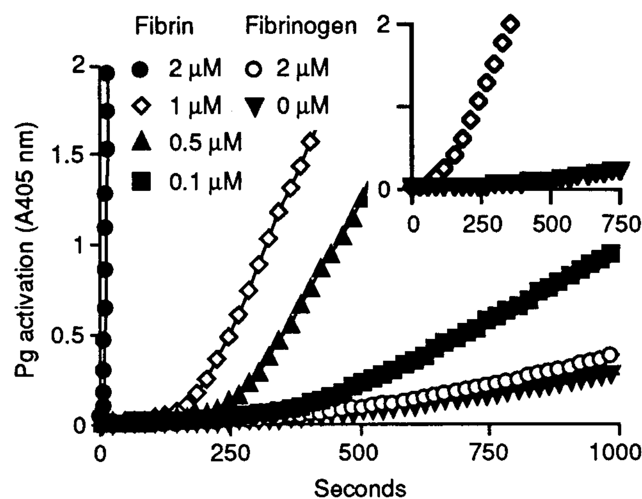

FIG. 2. The effect of fibrinogen and soluble fibrin fragments (desafib X) on the activation of Glu–Pg by the activator complex formed by rSKΔ59 or by rSKΔ59 after removal of the maltose binding protein fusion partner (*Inset*). Glu-Pg (300 nM) was mixed with 10 nM rSKΔ59 and added to cuvettes containing various amounts of human fibrinogen (0 and 2 μM) or fibrin fragments (0–2 μM) and 0.5 mM of S2251 (final) in assay buffer (50 mM Tris·HCl/100 mM NaCl, pH 7.4) at 37°C. Pg activation was detected by monitoring the change in absorbance at 405 nm in a spectrophotometer.

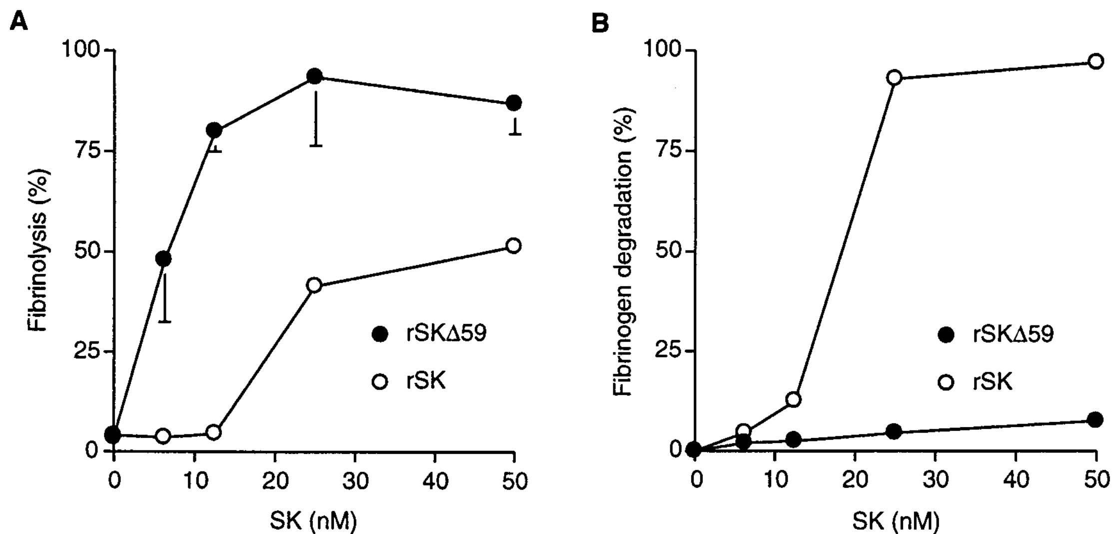

FIG. 3. (*A*) Fibrinolytic effects of rSKs on human plasma clots. Human plasma (45 μl) was mixed with trace amounts of ^{125}I-fibrinogen and clotted with calcium (5 mM) and thrombin (0.1 units) for 1 h at 37°C as described (34). After a wash with Tris-buffered saline (pH 7.4) containing the thrombin inhibitor PPACK (10 μM), the clots were suspended in 2 ml of plasma containing 10 μM PPACK. Various amounts of rSK and rSKΔ59 that had undergone active-site titration (0–50 nM) were added to the plasma, and, after 6 h, the amount of fibrinolysis was determined by measuring the release of soluble ^{125}I-fibrin degradation products (34). The means ± SD are shown (but in some cases the SD are too small to be visible). (*B*) Fibrinogen degradation by rSK activator complexes. At the end of the fibrinolysis experiments presented in *A*, the plasma supernatants were isolated, mixed with aprotinin (100 units) to inhibit plasmin activity, and precipitated with sodium sulfite to determine fibrinogen concentrations, as described (34). The means ± SD are shown (but in some cases the SD are too small to be visible).

indicated by the fact that the rSKΔ59 activator complex (Fig. 3 *A* and *B*) and TPA (6, 7) both selectively induce degradation of fibrin but not fibrinogen during fibrinolysis, when both molecules are present as potential plasmin substrates. This preferential degradation of fibrin indicates that the generation of plasmin by the SKΔ59 activator complex probably occurs selectively on the fibrin surface, as has been described for TPA (6, 7).

The mechanisms by which fibrin amplifies Pg activation are still not known, despite extensive study. For the rSKΔ59 activator complex, three mechanisms seem plausible: (*i*) fibrin may serve as a template that optimally docks the active site of the rSKΔ59 activator complex opposite the substrate Pg cleavage site; (*ii*) fibrin binding may alter the conformation of the Pg substrate to permit efficient processing by the rSKΔ59 activator complex; or (*iii*) fibrin may restructure the activation domain of the rSKΔ59 activator complex to a conformation best suited for Pg activation. To examine these hypotheses we used μPg, which contains only the catalytic B chain of Pg/plasmin and lacks the kringle domains of the A chain that interact with fibrin (1, 2, 4, 5). When the rSKΔ59 activator complex was formed with Glu–Pg, fibrin amplified the activation of a Glu–Pg substrate (Fig. 2) but had little effect on the activation of a μPg substrate (Fig. 4). When the rSKΔ59 activator complex was formed with μPg, fibrin amplified the activation of a Glu–Pg substrate (Fig. 4) but had little effect on a μPg substrate. Thus, the stimulation of Pg activation by fibrin requires that the Pg substrate, but not the rSKΔ59 activator complex, contain the fibrin-interacting kringle domains of the A chain. These data support the second hypothesis: that fibrin amplifies the effect of the rSKΔ59 activator complex by changing the conformation of the Pg substrate. By extension, in nSK, the ability to process the Pg substrate (in the absence of fibrin) would be provided by the NH_2-terminal peptide which, because of the results obtained in structural modeling experiments, is believed to interact with the Pg substrate in the active site of the activator complex (24). In future studies, it

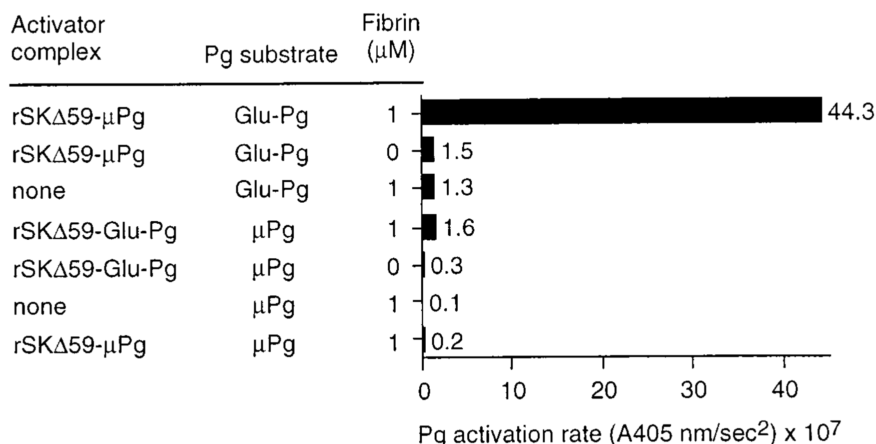

FIG. 4. Effect of fibrin on rSKΔ59-mediated activation of Glu–Pg or μPg. rSKΔ59 (10 nM) or no SK was mixed with stoichiometric amounts of Glu–Pg or μPg for 30 min on ice to form an activator complex, after which it was added to cuvettes containing Glu–Pg or μPg (300 nM) with or without soluble fibrin fragments (1 μM) and 0.5 mM S2251 (final) in assay buffer (50 mM Tris·HCl/100 mM NaCl, pH 7.4) at 37°C. Pg activation was monitored as described in Table 1, and the initial reaction rates were obtained from the first 300 s by plotting A_{405}/min^2.

will be important to determine how the NH_2-terminal peptide functionally interacts with Pg substrate and whether, through these interactions, the NH_2-terminal peptide can also modify Pg activation by fibrin-dependent activators such as TPA.

Our results indicate that the NH_2-terminal peptide (in particular residues 24–59) contains a catalytic switch for Pg activation. This switch gives the SK—Pg activator complex a singular ability to activate Pg efficiently in the absence of fibrin. Removal of this switch profoundly alters the mechanism of Pg activation, toggling it to a fibrin-dependent, TPA-like mode of action. To our knowledge, the ability of the SK activator complex to act through two different mechanisms is unique among the many serine proteases and cofactors that interact to regulate blood clotting and blood-clot dissolution. In a practical sense, altering SK function to create a fibrin-targeted Pg activator would eliminate many of the therapeutic drawbacks of the native molecule and could produce a superior agent for dissolving human blood clots.

We are grateful to Mr. Thomas J. McVarish for editorial assistance and to Drs. Mary E. Russell and Michael N. Margolies for valuable comments. This work was supported in part by National Institutes of Health Grants HL-02348 and HL-57314 to G.L.R., AI-33175 to B.P.-S., and HL-50366 to L.H.

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