

α Domain Deletion Converts Streptokinase into a Fibrin-dependent Plasminogen Activator through Mechanisms Akin to Staphylokinase and Tissue Plasminogen Activator*

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The mechanism of action of plasminogen (Pg) activators may affect their therapeutic properties in humans. Streptokinase (SK) is a robust Pg activator in physiologic fluids in the absence of fibrin. Deletion of a “catalytic switch” (SK residues 1–59), alters the conformation of the SK α domain and converts SK Δ 59 into a *fibrin-dependent* Pg activator through unknown mechanisms. We show that the SK α domain binds avidly to the Pg kringle domains that maintain Glu-Pg in a tightly folded conformation. By virtue of deletion of SK residues 1–59, SK Δ 59 loses the ability to unfold Glu-Pg during complex formation and becomes incapable of nonproteolytic active site formation. In this manner, SK Δ 59 behaves more like staphylokinase than like SK; it requires plasmin to form a functional activator complex, and in this complex SK Δ 59 does not protect plasmin from inhibition by α_2 -antiplasmin. At the same time, SK Δ 59 is unlike staphylokinase or SK and is more like tissue Pg activator, because it is a poor activator of the tightly folded form of Glu-Pg in physiologic solutions. SK Δ 59 can only activate Glu-Pg when it was unfolded by fibrin interactions or by Cl^- -deficient buffers. Taken together, these studies indicate that an intact α domain confers on SK the ability to nonproteolytically activate Glu-Pg, to unfold and process Glu-Pg substrate in physiologic solutions, and to alter the substrate-inhibitor interactions of plasmin in the activator complex. The loss of an intact α domain makes SK Δ 59 activate Pg through classical “fibrin-dependent mechanisms” (akin to both staphylokinase and tissue Pg activator) that include: 1) a marked preference for a fibrin-bound or unfolded Glu-Pg substrate, 2) a requirement for plasmin in the activator complex, and 3) the creation of an activator complex with plasmin that is readily inhibited by α_2 -antiplasmin.

The enzyme plasmin plays a central role in degrading fibrin, the protein matrix of blood clots or thrombi. Plasmin is gener-

ated when the Arg⁵⁶¹-Val⁵⁶² peptide bond of plasminogen (Pg)¹ zymogen is cleaved by a class of proteins known as Pg activators (1). Pg activators are used therapeutically to initiate the dissolution of thrombi in humans (2). The mechanism of action of the Pg activators has important effects on their properties as blood clot dissolving agents. Mammalian Pg activators (*e.g.* urokinase and tissue Pg activator (TPA)) are serine proteases that directly cleave Pg to produce plasmin (3, 4). Bacterial Pg activators (*e.g.* streptokinase (SK), staphylokinase, and *Streptococcus uberis* Pg activator) are cofactors that form an activator complex with plasmin, changing the substrate specificity of plasmin from fibrin to Pg (5). In addition, SK has the singular ability to productively restructure the latent active site in Pg molecule without proteolytic cleavage of the Arg⁵⁶¹-Val⁵⁶² peptide bond to generate the enzyme Pg* (6–8). Currently, SK and TPA are widely used in humans for the treatment of thrombotic diseases, although they differ markedly in their requirement for fibrin as a cofactor for efficient Pg activation.

When compared with TPA, SK explosively activates Pg in the absence of fibrin, and this property may reduce the therapeutic efficacy of SK. We have previously reported that the NH₂ terminus of SK (residues 1–59) contains a “catalytic switch” that allows Pg activation in the presence or absence of fibrin (9). Removal of this switch region alters the structure of the remaining α domain (α_2 subdomain, residues 60–146) and converts the resulting molecule (SK Δ 59) to a *fibrin-dependent* Pg activator like TPA that selectively activates the physiologic form of Pg (Glu-Pg) only in the presence of fibrin (9–11). *In vitro*, SK Δ 59 behaves more like TPA than the SK parent molecule because it can promote more extensive blood clot lysis without the destruction of circulating fibrinogen or plasminogen (9, 12).

Fibrin-dependent Pg activation is thought to be an important determinant of the success of Pg activators as treatments for human thrombotic diseases (13). Several different mechanisms of fibrin-dependent Pg activation have been described. Staphylokinase, a bacterial protein like SK, forms an activator complex with plasmin that efficiently cleaves Pg substrate in the presence or absence of fibrin (14, 15). Staphylokinase is considered to have a fibrin-dependent mechanism in human plasma because α_2 -antiplasmin, a plasmin inhibitor, rapidly

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¹ The abbreviations used are: Pg, plasminogen; Glu-Pg, native plasminogen with amino-terminal Glu; Lys-Pg, plasmin-modified plasminogen with amino-terminal Lys; SK, streptokinase; (DD)E, complex of fibrin D-dimer noncovalently associated with fragment E; MBP, maltose-binding protein; EACA, ϵ -amino-*n*-caproic acid; Ab, antibody; S-2251, H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride; TPA, tissue Pg activator.

inhibits the staphylokinase-plasmin complex unless it is bound to fibrin (16, 17). TPA displays at least two other mechanisms of fibrin-dependent Pg activation. First, the single chain TPA zymogen acquires greater Pg activation efficiency when it is cleaved by plasmin (typically on the fibrin surface) to the two chain form of TPA (18, 19). Second, in the presence of fibrin, TPA acquires greater substrate affinity by forming a ternary TPA-Pg-fibrin complex that more efficiently cleaves the Arg⁵⁶¹-Val⁵⁶² bond of Pg (3). Another example of fibrin-dependent pro-enzyme activation occurs with pro-urokinase, which remains inactive until cleaved by plasmin on fibrin (or other binding sites) to a functional Pg activator (20, 21).

The aim of these studies was to identify mechanisms through which SKΔ59 acquires a fibrin-dependent mode of Pg activation. Surprisingly, unlike the parent SK molecule, SKΔ59 employs several fibrin-dependent mechanisms, similar to both TPA and staphylokinase.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of Recombinant Proteins—Wild type SK, a deletion SK mutant (SKΔ59) lacking the first 59 amino acids, SK1–59, and SK1–59(L42A) were cloned as described (9, 11, 22). Tryptophan 6 was mutated to alanine (W6A) in the SK1–59 and SK1–59(L42A) by PCR to reduce intrinsic fluorescence. The following primers were used: 5'-gatatcgctggacgtgagctctgtagaccgtccatc (sense) and 5'-gctctagatcatcttggacttaagccttgcctgtc (antisense). Individual SK domains were cloned by PCR using primers: α domain (residues 1–148), gatatcgctggacgtgagctgtagaccgtccatc and aactgcagctattcttatatggc; β domain (residues 149–293), gcctttaaaccaatacaaaacgaagc and aactgcagctattcttaagtgactgcg, and, γ domain (residues 294–414), catcagctgttcacatcaaatagctgtt and aaagctttatgtcgttaggg (Sigma-Genosys, Woodlands, TX). Staphylokinase was cloned from *Staphylococcus sp.* (ATCC 29123) genomic DNA by PCR using primers: 5'-aaggatcatcgaaggtaggtcaagttcattcagacaagg and gctcgcatgctgctgcagttattttctataaacact. The sequences of all clones were confirmed by sequencing both DNA strands. These cDNAs were ligated into the pMalc vector for expression in bacteria as fusion proteins with maltose-binding protein (MBP) (New England Biolabs, Beverly, MA) and were purified by ion exchange chromatography and/or affinity chromatography on amylose resin (New England Biolabs) as described in detail (9, 22). Recombinant SK proteins were cleaved from maltose-binding protein by Factor Xa (New England Biolabs) for 12 h at room temperature in 200 mM Tris-HCl buffer, pH 8.0, 100 mM NaCl, 2 mM CaCl₂. The cleaved fusion proteins SK, SKΔ59, staphylokinase, SK1–59, SK1–59(W6A), and SK1–59(W6A,L42A) were analyzed by SDS-PAGE.

The SK1–59 peptides were separated from maltose-binding protein by ultrafiltration (Centricon Centrifugal filter, YM-10, Millipore). The purity of SK1–59(W6A) and SK1–59(W6A,L42A) were confirmed by silver staining of 20% SDS-PAGE gels and by a wavelength scan (220–300 nm, Beckman DU 640 spectrophotometer). The peptide contained a maximum at 257 nm corresponding to the presence of a phenylalanine residue. The peptides were subjected to fluorescence spectrum analysis (excitation, 282 nm; emission, 340 nm) in a Hitachi 2500 spectrofluorimeter and were found to lack intrinsic fluorescence.

Micro-Pg (residues 530–791 of human Pg) and micro-Pg mutant (R561A) were cloned in pET11d (Stratagene, La Jolla, CA), expressed in bacteria, and purified as described in detail (9, 23). Recombinant mini-Pg that spanned residues 455–791 of human Pg was cloned by PCR from human liver cDNA into pCR II (Invitrogen) using these primers with a 5' extension: 5'-ggatccatcagggtagggctgagactcttcgaagaagctg and 5'-ggatcccccgtccaatttaattattc. The product was subcloned into pProex HTB through BamHI sites. After induction, the mini-Pg was solubilized from inclusion bodies with 6 M guanidine HCl and 10 mM dithiothreitol in TE buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8.0) and refolded at 30 μg/ml in buffer containing 55 mM Tris-HCl, pH 8.2, 10.56 mM NaCl, 0.44 mM KCl, 0.055% (w/v) polyethylene glycol 3350, 2.2 mM MgCl₂, 2.2 mM CaCl₂, 550 mM L-arginine. The mini-Pg was concentrated to 300 μg/ml and dialyzed against TE buffer. The isolated mini-Pg migration was analyzed by a nonreduced SDS-PAGE and Western blot analysis using a monoclonal antibody directed against the protease domain. Recombinant Pg kringles and (DD)E were obtained as described (24–26).

Protein Labeling—Human Glu-Pg (≥95% Glu-Pg; American Hematology) was radioiodinated by the Iodogen method (27), and the specific radioactivity was determined as described previously (28).

Active Site Titration—The molar quantity of active sites in various plasmins was determined as described (22) in a Hitachi 2500 fluorescence spectrophotometer. To examine the effect of SK1–59 on active site formation, SKΔ59 (1 μM) was preincubated with SK1–59 (2 μM) in 4 °C for 2 h. Human Glu-Pg (100 nM) was added to a cuvette containing 2 μM fluorescent active site titrant 4-methylumbelliferyl *p*-guanidinobenzoate (Sigma) in filtered buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) at 25 °C. After 5 min 200 nM preincubated mixture of SKΔ59 and SK1–59 was added. The development of fluorescence was monitored continuously with excitation at 365 nm and emission at 445 nm as described (29).

Plasminogen Activator Assays—Pg activators SK, SKΔ59, or staphylokinase (5–20 nM) were added to a microtiter plates containing 0.5 mM of S-2251 (*H*-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride; Chromogenix, Sweden), and 300 nM of plasminogen (Lys-Pg, Glu-Pg, mini-Pg, and micro-Pg) at 37 °C in a total volume of 100 μl of assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4). The cleavage of S-2251 was monitored at 405 nm for 180 min in a microplate reader (Synergy HT, Bio-Tek). The initial reaction rates were obtained from the first part of activation curve by plotting A_{405}/time^2 . Soluble (DD)E fragment (0–4 μM), active plasmin (0–5 nM), NaCl (0–100 mM), and MBP (0–4 μM) fusion protein (as a control) were added to stimulate Pg activation. The apparent Michaelis and catalytic constants of Glu-Pg activation by SKΔ59 were calculated as described (30).

The effect of chloride ions and EACA on Glu-Pg activation was also examined. Various concentrations of NaCl (0–100 mM) were added to cuvettes containing Glu-Pg (540 nM) in 37 °C HEPES buffer (pH 7.4) with S-2251 (0.5 mM). After 10 min of incubation plasminogen activation was initiated by SKΔ59 (10 nM) and monitored as above. To examine the effects of EACA, Glu-Pg (540 nM) was added to cuvettes with assay buffer (50 mM Tris, pH 7.4) containing 100 mM NaCl, S-2251 (0.5 mM), and 1 μM (DD)E (as a positive control) or various amounts of EACA (0–3 mM) without (DD)E. After 10 min of incubation Pg activation was initiated by adding the preformed Glu-Pg-SKΔ59 activator complex (10 nM) and was detected as described above. To avoid the potential inhibitory effects of EACA on complex formation, the Glu-Pg-SKΔ59 activator complex was preformed for 3 h at 4 °C in the absence of EACA. In a parallel series of experiments, we also examined the effects of EACA (0–3 mM) on plasminogen activation by staphylokinase (10 nM) in 37 °C assay buffer under the same conditions.

Amidolytic Kinetics—The amidase kinetic parameters of human plasmin (10 nM) and various stoichiometric SK or staphylokinase activator complexes formed with plasmin (10 nM) were studied as previously described (30). The ability of SKΔ59 to generate active sites in the micro-Pg molecule was examined using a recombinant noncleavable micro-Pg(R561A) mutant that can only be activated through nonproteolytic mechanisms (31). Active site development in the equimolar activator complex SKΔ59-micro-Pg(R561A) (500 nM) in the presence of SK1–59(W6A) or SK1–59(W6A,L42A) was monitored at 37 °C for 120 min.

Influence of SKΔ59, SK, and Staphylokinase on the Human Plasmin/ α_2 -Antiplasmin Reaction—Inhibition reaction kinetic measurements were carried out in a cuvette at 25 °C using a Carry 100-Bio spectrophotometer. Human active plasmin (14 nM) was added to cuvettes containing S-2251 (0.5 mM) and SKΔ59 (0–3 μM), staphylokinase (0–3 μM), SK (0–300 nM), or MBP fusion protein (0–3 μM) in filtered assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4). The change in absorbance at 405 nm was recorded for 60 s prior to the addition of human α_2 -antiplasmin (Chromogenix; 90 nM). The residual enzyme activity was calculated by the first derivative (dA/dt) of the curve before 60% of the enzyme was inactivated. The apparent rate constant ($k_{1,app}$) of the inhibition of the enzyme activity by α_2 -antiplasmin in the presence of various concentration SKΔ59, SK, staphylokinase, or MBP was calculated as described (32). The percentage of change in the apparent rate constant was plotted versus the concentration of potential inhibitors.

Intrinsic Fluorescence Measurement—The change in conformation of Pg was examined by monitoring alterations in the intrinsic fluorescence of the molecule as described (33, 34). Fluorescence emission spectra (excitation at 282, emission at 340 nm) were recorded for Glu-Pg, Lys-Pg, or mini-Pg in filtered Tris-buffer (50 mM Tris-HCl, 100 mM NaCl, 20% glycerol, pH 7.4) in the presence or absence of SK1–59(W6A) and SK1–59(W6A,L42A). Control spectra alone for the SK1–59 peptides and EACA were also recorded. In other experiments Pg (50 nM) and SKΔ59 (50 nM) were preincubated in Tris-buffer (50 mM Tris-HCl, 100 mM NaCl, 20% glycerol, pH 7.4) for 5 min at 37 °C. The intrinsic fluorescence of the equimolar complex was monitored for 10 min until a stable background was obtained. Then SK1–59(W6A) and SK1–59(W6A,L42A) were added (80–300 nM), and the change in fluorescence was determined.

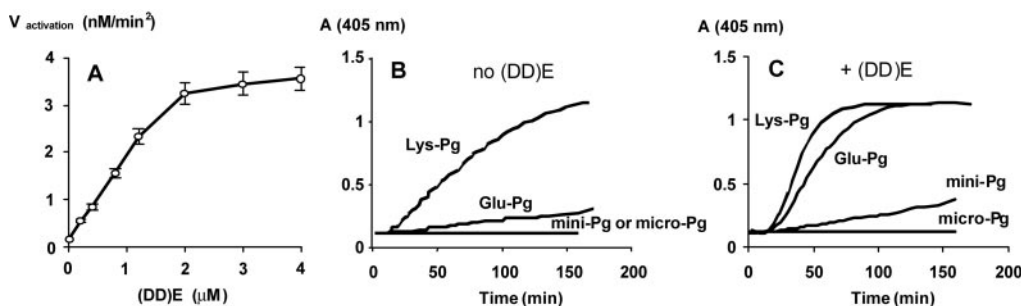


FIG. 1. **Fibrin-dependent Pg activation by SKΔ59.** A, dose-dependent Pg activation by SKΔ59. Various amounts of (DD)E fragment (0–4 μM) were added to cuvettes containing Glu-Pg (300 nM) in 37 °C assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) with S-2251 (0.5 mM). After 10 min of incubation Pg activation was initiated by SKΔ59 (20 nM) and detected by continuously monitoring the change in absorbance at 405 nm. The initial reaction rates were obtained from the first 10–15 min by plotting A_{405}/time^2 . B and C, role of plasminogen kringle forms in (DD)E-stimulated activation by SKΔ59. Glu-Pg, Lys-Pg, mini-Pg, or micro-Pg (300 nM) were preincubated without (DD)E (B) or with (DD)E (B) (2 μM) in 37 °C assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4), containing S-2251 (0.5 mM) for 30 min. Pg activation was initiated by SKΔ59 (20 nM) and monitored at 405 nm in a spectrophotometer.

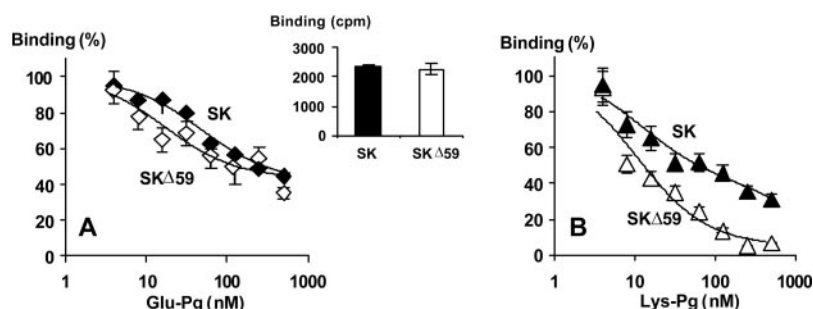


FIG. 2. **Pg substrate binding to SK-Glu-Pg and SKΔ59-Glu-Pg complexes.** Glu-Pg was separated from trace amounts of plasmin by preincubation with aprotinin-Sepharose for 4 h at 4 °C. Microtiter plates were coated with 50 μl of Glu-Pg (50 μg/ml) or no protein (control). Nonspecific binding was blocked with 1% bovine serum albumin. Then SK and SKΔ59 were added for 1 h in concentrations that gave equivalent binding to Glu-Pg (Insert) according to a standard calibration curve. After washing, ¹²⁵I Glu-Pg substrate (4 nM) was added in the presence of various amount of unlabeled Glu-Pg (A) or Lys-Pg (B) (0–500 nM). The amount of bound ¹²⁵I Glu-Pg substrate was determined after washing and γ-counting. The percentage of bound ¹²⁵I Glu-Pg was calculated by reference to samples containing no Pg competitor (defined as 100% bound) and wells containing no activator complex (defined as 0% specific bound). The data represent the means ± S.E., and the binding curves were determined by nonlinear regression as described under "Experimental Procedures."

Binding Assays—The binding of various Pgs or Pg kringle domains to SK domains was examined in microtiter plates. The wells were coated with 50 μl of various Pgs (5 μg/ml) or Pg kringle domains (5 μg/ml) or no protein (control). Nonspecific binding was blocked with 1% bovine serum albumin. After washing various SKs or MBP alone (50 μl) were added in different concentrations (0–50 μg/ml). One hour later the wells were washed, and 50 μl of mouse anti-MBP Ab was added for 1 h. After washing, bound Ab was detected by 50 μl of ¹²⁵I goat anti-mouse Ab (50,000 cpm) followed by γ-counting.

The competitive binding of Glu-Pg substrate to SK and SKΔ59 activator complexes was examined. A calibration curve was constructed to determine the amounts of SK and SKΔ59 that gave equivalent binding to Glu-Pg. Glu-Pg (50 μl, 5 μg/ml) was coated on microtiter plates for 1 h. Nonspecific binding was blocked with 1% bovine serum albumin. After washing, various concentrations of SK or SKΔ59 (50 μl; 3–500 nM) were added for 1 h in buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, pH 7.4). The conformation-independent anti-SK monoclonal Ab 8F5 (22) was added for 1 h to detect bound SK and SKΔ59. After washing bound Ab was detected by ¹²⁵I goat anti-mouse Ab (50,000 cpm/50 μl) by γ-counting. Concentrations of SK and SKΔ59 samples that gave equivalent binding were added to microtiter plates containing immobilized Glu-Pg. After washing, ¹²⁵I Glu-Pg substrate (4 nM) was added in the presence of various amounts of unlabeled Glu-Pg or Lys-Pg (0–500 nM) as competitors. The amount of bound ¹²⁵I Glu-Pg substrate was determined after washing and γ-counting. The percentage of bound ¹²⁵I Glu-Pg was calculated by reference to samples containing no Pg competitor (defined as 100% bound) and wells containing no activator complex (defined as 0% specific bound). The binding competition curves were analyzed by nonlinear regression analysis to obtain the best statistical fits (35).

RESULTS

Influence of Fibrin on Pg Activation by SKΔ59—SK and staphylokinase form activator complexes that efficiently acti-

vate Glu-Pg in the absence of fibrin. In contrast, the SKΔ59 mutant that lacks the SK NH₂ terminus (residues 1–59) has little capacity to activate Glu-Pg efficiently in the absence of fibrin (Fig. 1) (9). Soluble fibrin fragments containing the (DD)E domain, which are known to amplify the activity of TPA (36, 37), markedly increased the activator efficiency of the SKΔ59 complex with Glu-Pg (Fig. 1A). The amplifying effects of the fibrin (DD)E fragments on Glu-Pg activation induced by SKΔ59 were dose-dependent and saturable (Fig. 1A) at a concentration of about 2 μM. Because fibrin interacts with the kringle of Pg, we examined whether fibrin differentially promoted activation of different Pg fragments including: 1) Glu-Pg, the physiologic, tightly folded form of the molecule that contains an NH₂-terminal peptide, five kringle, and a protease domain; 2) Lys-Pg, which lacks the NH₂-terminal peptide of Glu-Pg and has an unfolded conformation; 3) mini-Pg, which contains one kringle domain (kringle 5); and 4) micro-Pg, which contains only the protease domain and has no kringle. In the absence of fibrin, SKΔ59 induced significantly more activation of Lys-Pg than of Glu-Pg or mini-Pg (Fig. 1B). No activation of micro-Pg was detected (Fig. 1B). Although the presence of fibrin (DD)E fragments markedly affected the ability of SKΔ59 to activate Glu-Pg (Fig. 1C), it had relatively less effect on the activation of Lys-Pg (Fig. 1C) or mini-Pg (Fig. 1C) and no effect on the activation of micro-Pg (Fig. 1C). In the absence of fibrin, SKΔ59 was most efficient at activating Lys-Pg, which is more unfolded than Glu-Pg (Fig. 1B). In the presence of fibrin, the activation of Glu-Pg and Lys-Pg were nearly identical (Fig. 1C).

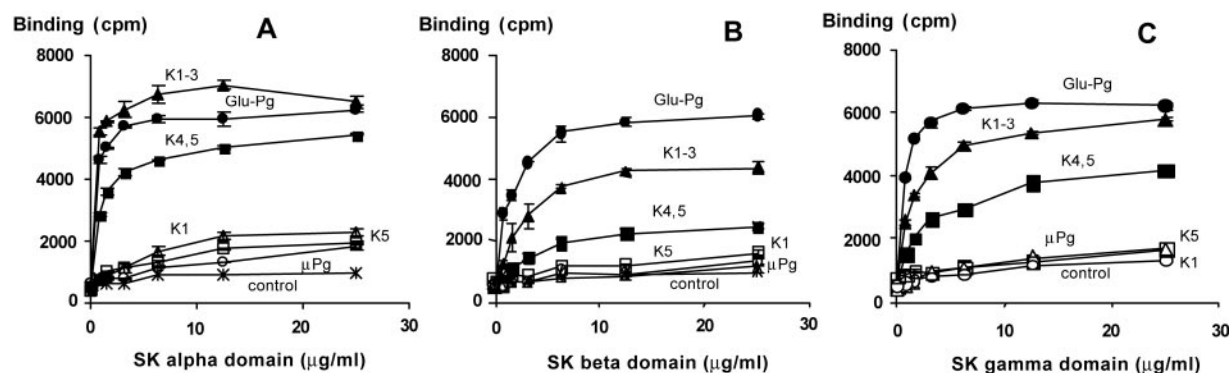


FIG. 3. **Binding interactions between SK and Pg domains.** Microtiter plates were coated with Glu-Pg (closed circles), micro-Pg (open circles), kringles 1–3 (K1–3) (closed triangles), kringle 1 (K1, open triangles), kringles 4–5 (K4,5, closed squares), kringle 5 (K5, open triangles) (50 μ l, 5 μ g/ml) or no protein (asterisks) for 1 h. After washing, nonspecific binding was blocked by 1% bovine serum albumin. After 1 h of incubation and washing, various concentrations (0–25 μ g/ml) of MBP-SK domains α (A), β (B), or γ (C) were added for 1 h. After washing, anti-MBP monoclonal antibody was added, and the amount of bound anti-MBP Ab was determined by 125 I goat anti-mouse Ab (50,000 cpm/50 μ l) followed by γ -counting. The means \pm S.E. are shown.

TABLE I
Binding of SK domains to various Pg structures

Analysis of direct binding curves in Fig. 3 where $B = B_{\max}/(K_{D,app} + [SK_{\text{domain}}])$. B represents the amount bound, B_{\max} is the maximal binding on saturation, $K_{D,app}$ is the concentration of SK domain that gives $B_{\max}/2$.

Pg/kringle	$K_{D,app} \pm$ S.E.		
	SK α	SK β	SK γ
	nM		
Glu-Pg	18 \pm 4	71 \pm 13	29 \pm 5
Kringles 1–3	13 \pm 5	140 \pm 32	74 \pm 12
Kringles 4–5	48 \pm 8	160 \pm 45	121 \pm 31
Kringle 5	NA ^a	NA	NA
Kringle 1	190 \pm 73	110 \pm 89	187 \pm 76
Micro-Pg	NA	NA	NA

^a NA, minimal binding was observed under these conditions.

Binding of SK-Glu-Pg and SK Δ 59-Glu-Pg Complexes to Pg Substrate—The inability of SK Δ 59 to activate Glu-Pg (Fig. 1) suggested that, by comparison with SK, the SK Δ 59 activator complex may not bind Glu-Pg as a substrate in the absence of fibrin. The binding of Pg substrates to immobilized SK-Glu-Pg and SK Δ 59-Glu-Pg complexes was assessed in competition binding experiments using 125 I Glu-Pg substrate (4 nM) and various amount of unlabeled Pg competitor (Fig. 2). All of the studies were performed in the presence of phenylmethylsulfonyl fluoride to inhibit protease activity of activator complexes. Synchronous experiments verified that the amounts of immobilized SK-Glu-Pg and SK Δ 59-Glu-Pg complexes were equivalent (Fig. 2, inset).

The binding competition curves were analyzed by nonlinear regression analysis to obtain the best statistical fits. Activator complexes containing SK Δ 59 and SK bound Glu-Pg substrate through a single binding site with similar avidities (EC_{50} of 17 nM versus 46 nM; Fig. 2A) that were statistically indistinguishable ($p > 0.05$). The SK Δ 59-Pg complex bound Lys-Pg substrate at a single site (EC_{50} 13 nM), whereas the SK-Pg complex bound Lys-Pg through two different binding sites (EC_{50} of 12 nM and EC_{50} of 1.10 μ M).

Binding of Streptokinase Domains to Pg and Its Kringles—The finding that SK Δ 59 cannot efficiently activate kringle-less micro-Pg substrates (Fig. 1) and that substrate binding involves interactions at more than one site (Fig. 2) suggested that kringles may play a critical role the interactions of SK with substrate Pg molecules. Consequently we examined the direct binding of the SK domains to Glu-Pg and various kringles (Fig. 3). The binding of SK domains to different Pg fragments was saturable and specific (as compared with controls lacking Pg).

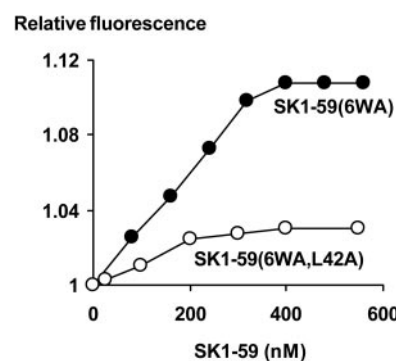


FIG. 4. **SK requires an intact α domain to alter the conformation of Glu-Pg.** Glu-Pg (50 nM) and SK Δ 59 (50 nM) were preincubated in Tris buffer (50 mM Tris-HCl, 100 mM NaCl, 20% glycerol, pH 7.4) for 5 min at 37 $^{\circ}$ C. The equimolar complex was added to quartz cuvette (final concentration, 50 nM), and the intrinsic fluorescence was monitored at excitation 282 nm and emission 340 nm for 10 min until a stable background was obtained. Then SK1–59(W6A) or SK1–59(W6A,L42A) mutant were added, and the change in fluorescence was monitored.

The maximal binding of all three SK domains to whole Glu-Pg were comparable and was significantly greater than the binding of the SK domains to kringle-less micro-Pg (Fig. 3). All of the SK domains bound with higher relative avidity to kringles 1–3 and kringles 4 and 5 than to individual kringle 1 or 5 or to micro-Pg. Among the kringle domains, the SK α domain bound to kringles 1–3 and kringles 4 and 5 with 3–6-fold greater avidity than the other SK domains (Table I).

SK Δ 59 and Glu-Pg Conformation—The tight conformation of Glu-Pg is mediated by kringle interactions (38, 39). Ligands that interact with kringles unfold Glu-Pg, inducing large positive changes in the intrinsic protein fluorescence of the molecule (4, 33, 40, 41). The finding that the SK α domain interacts with the kringle domains suggested that it may modulate the activity of the SK-Pg activator complex by unfolding the Pg molecule. Consistent with this notion, when SK interacts with Glu-Pg, it induces a conformation change that unfolds the molecule (42, 43). SK Δ 59 alone did not alter Glu-Pg conformation. When SK1–59 interacts with the remaining fragment of the α domain of SK Δ 59, it reconstitutes the native α domain structure (11, 44). To determine the effect of SK1–59 on Glu-Pg conformation, we ablated the intrinsic fluorescence of SK through mutation of the single tryptophan (residue 6) to alanine (W6A). When SK1–59(W6A) was added to the equimolar SK Δ 59-Glu-Pg complex, SK1–59(W6A) induced significant changes (10%) in the intrinsic fluorescence consistent with an

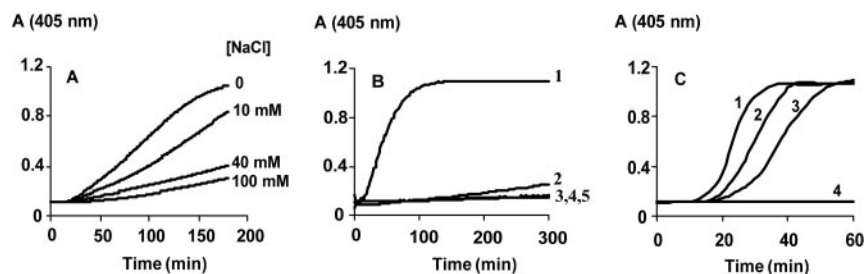


FIG. 5. **Role of chloride ions and EACA in Glu-Pg activation by SK Δ 59 and staphylokinase.** A, effect of chloride ions on activation by SK Δ 59. Various concentrations of NaCl (0–100 mM) were added to cuvettes containing Glu-Pg (540 nM) in 37 °C HEPES buffer (pH. 7.4) with S-2251 (0.5 mM). Pg activation was initiated by SK Δ 59 (10 nM) and was detected by continuously monitoring the change in absorbance at 405 nm. B, effect of EACA on activation by SK Δ 59. Glu-Pg (540 nM) was added to cuvettes with assay buffer (50 mM Tris, pH 7.4) containing 100 mM NaCl, S-2251 (0.5 mM) with 1 μ M (DD)E (spectrum 1) or without (DD)E in the presence of various amounts of EACA (0 mM (spectrum 2), 1 mM (spectrum 3), and 3 mM (spectrum 4)). After 10 min of incubation, Pg activation was initiated by adding the preformed Glu-Pg-SK Δ 59 activator complex (10 nM) and was detected as described above. The amidolysis of the preformed Glu-Pg-SK Δ 59 complex without Glu-Pg substrate was used as a control (5). C, effect of EACA on activation by staphylokinase. Glu-Pg (540 nM) was activated by staphylokinase (10 nM) in 37 °C assay buffer (50 mM Tris, pH 7.4), containing 100 mM NaCl, S-2251 (0.5 mM) with 3 mM (spectrum 1), 1 mM (spectrum 2), or no (spectrum 3) EACA. Glu-Pg without staphylokinase was used as a control (spectrum 4).

unfolding of the Glu-Pg molecule (Fig. 4). This effect required SK Δ 59 because SK1–59(W6A) alone had minimal effects on the conformation of Glu-Pg ($\leq 3\%$, not shown). In contrast, an L42A mutant of SK1–59 (SK1–59(W6A,L42A)), which does not reconstitute the native structure of the α domain, could not unfold the Glu-Pg in the equimolar SK Δ 59-Glu-Pg complex (Fig. 4). These studies indicate that a normally folded SK α domain, which requires interactions between SK1–59 and the remaining α domain fragment of SK Δ 59, mediate a conformational change upon binding to Glu-Pg.

These studies suggested that the ability of SK Δ 59 to activate the Lys-Pg but not the Glu-Pg molecule may be due to a failure to unfold the Pg substrate. Glu-Pg is kept in a tightly folded conformation in physiologic solutions by chloride ion (Cl^-) but unfolds in the absence of Cl^- . In the absence of Cl^- SK Δ 59 became an efficient activator of Glu-Pg (Fig. 5A) with a K_m of 229 ± 82 nM and a k_{cat} of $0.018 \pm 0.003 \text{ min}^{-1}$. Cl^- induced a dose-dependent decrease in the activation of Glu-Pg (Fig. 5A) with minimal activation detected at the physiologic $[\text{Cl}^-]$ of 100 mM. Kinetic studies showed that the K_m for activation increased more than 10-fold as the $[\text{Cl}^-]$ was increased to 10 mM ($K_m = 430 \pm 150$ nM) to 40 mM ($K_m = 3090 \pm 1210$ nM). At the same time Cl^- had relatively small effects on the k_{cat} for Pg activation because the $[\text{Cl}^-]$ was increased to 10 mM ($k_{\text{cat}} = 0.018 \pm 0.003 \text{ min}^{-1}$) to 40 mM ($k_{\text{cat}} = 0.037 \pm 0.012 \text{ min}^{-1}$).

In physiologic solutions containing Cl^- , the lysine analog EACA acts to unfold the Glu-Pg molecule (33, 41). In buffers containing 100 mM Cl^- , EACA accelerated the activation of Glu-Pg by staphylokinase (Fig. 5C). However, unlike (DD)E, EACA failed to accelerate the activation of Glu-Pg by the SK Δ 59-Glu-Pg activator complex (Fig. 5B), perhaps because of its known inhibitory effects on SK-Pg complex and substrate interactions (45).

Active Site Generation—One of the qualities that distinguishes SK from staphylokinase and contributes to the fibrin-independent mechanism of SK action is the ability of SK to generate an active site in the Pg zymogen without cleavage of the Arg⁵⁶¹-Val⁵⁶² bond. Nonproteolytic activation is mediated by the NH_2 -terminal Ile¹, as well as conformational interactions mediated by the β and γ domains (23, 30, 31, 46–50). SK Δ 59 in combination with SK1–59(W6A) can nonproteolytically activate a micro-Pg(Arg561Ala) mutant that cannot be cleaved by standard Pg activators (Fig. 6). In contrast, SK Δ 59 alone and the mixture of SK Δ 59 and SK1–59(W6A,L42A), which contains an Leu⁴² mutation that inhibits the interactions of this peptide with SK Δ 59, are incapable of nonproteolytic activation of micro-Pg(R561A) (Fig. 6). Taken together

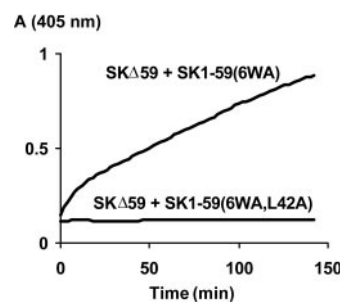


FIG. 6. **Nonproteolytic active site generation in micro-Pg(R561A) by SK Δ 59 and SK1–59.** SK Δ 59 (1000 nM) was preincubated with and without a stoichiometric amount of SK1–59(W6A) or SK1–59(W6A, L42A). Then the incubation mixture was added to a cuvette at 37 °C containing micro-Pg(R561A) mutant (500 nM) and S-2251 (0.5 mM) in assay buffer, and amidolysis was monitored at A405 nm.

these results (Figs. 4 and 6) indicate that SK1–59 acts to both alter the structure of Glu-Pg in the SK-Glu-Pg complex and to generate an active site, largely by interactions mediated through the α domain of SK that are missing in SK Δ 59.

Pg Activation and Amidolysis—If SK Δ 59 is incapable of nonproteolytic activation of Pg, it must be dependent, like staphylokinase, on complex formation with plasmin for Pg activation. As such, trace amounts of plasmin in fibrin may act as an accelerator, thereby decreasing the lag phase for Pg activation by the SK Δ 59 activator complex. The addition of small amounts of plasmin significantly increased the rate of Glu-Pg activation by SK Δ 59 in the presence of (DD)E and proportionally diminished the lag phase in Pg activation (Fig. 7A). In the absence of (DD)E, the same amount of plasmin had no significant effect on the activation of Glu-Pg by SK Δ 59 (Fig. 7B). However, unlike SK Δ 59, the lag phase in activation of Glu-Pg by staphylokinase was diminished by plasmin both in the presence and absence of fibrin. Thus, despite the fact that they require a plasmin moiety for efficient Pg activation, staphylokinase-plasmin and SK Δ 59-plasmin complexes differ because the SK Δ 59-plasmin complex requires fibrin for efficient Pg activation.

Kinetic studies were performed to examine how SK Δ 59 alters substrate processing in the activator complex with human plasmin. The kinetic parameters of stoichiometric complexes of plasmin with SK Δ 59, SK, and staphylokinase are compared in Table II. When wild type SK or the mixture SK Δ 59 and SK1–59 formed a complex with plasmin, they decreased the apparent Michaelis-Menten constant (K_m) for

FIG. 7. The role of active plasmin in fibrin-dependent and fibrin-independent activation of Glu-Pg by staphylokinase and SKΔ59. Human plasmin (0–5 nM) was added to a cuvette containing Glu-Pg (300 nM), S-2251 (0.5 mM) in assay buffer (50 mM Tris-HCl, 0.15 M NaCl, pH 7.4) in the presence (A) or absence (B) of (DD)E fragment (2 μM). Then 20 nM of staphylokinase (open symbol) or SKΔ59 (closed symbol) was added, and plasmin generation was detected at 37 °C for 150 min. The lag phase in the time of Glu-Pg activation was plotted *versus* concentration of added plasmin.

TABLE II
Kinetic constants for amidolysis by various stoichiometric activator complexes

The amidolytic experiments were carried out at 37 °C as described under "Experimental Procedures." The values represent the means ± S.E.

Enzyme	K_m μM	k_{cat} S ⁻¹	k_{cat}/K_m μM ⁻¹ s ⁻¹
Plasmin ^a	500 ± 90	14.2 ± 0.5	0.028
SK-plasmin ^a	250 ± 9	26.4 ± 0.6	0.106
SKΔ59-plasmin	509 ± 149	16.8 ± 2.1	0.033
Staphylokinase-plasmin	507 ± 98	15.6 ± 3.2	0.032
(SKΔ59 + SK1-59) _{L42A} -plasmin ^b	307 ± 65	34.3 ± 2.3	0.11
(SKΔ59 + SK1-59) _{L42A} -plasmin ^b	459 ± 90	22.9 ± 2.1	0.05

^a The kinetic parameters were determined by Ref. 30.

^b The kinetic parameters were determined by Ref. 11.

amidolysis by plasmin ~ 1.6–2-fold and increased the amidolysis rate constant (k_{cat}) by ~1.8–2.4 fold. When SKΔ59 or staphylokinase formed a complex with plasmin, the K_m did not change, and the k_{cat} was minimally affected (<1.2-fold). The addition of SK1-59(L42A) to SKΔ59, which is impaired in reconstitution of the SK α domain (11), had effects on plasmin amidolysis that were intermediate between SK and SKΔ59; it modestly decreased the K_m (~1.1-fold) and increased the k_{cat} (1.6-fold). Finally, in contrast to SK, which increased the efficiency of amidolysis (k_{cat}/K_m) by ~3.5-fold, neither SKΔ59 nor staphylokinase had any significant effects on amidolytic efficiency.

Effect of SKΔ59 on the Interaction of Plasmin with α₂-Antiplasmin—Another fundamental distinction between the SK and staphylokinase mechanisms of action is the ability of SK to form a tight stable complex with plasmin that protects it from inactivation by its major inhibitor α₂-antiplasmin. In contrast, the staphylokinase-plasmin complex is not resistant to α₂-antiplasmin in solution; it acquires relative resistance only when it is bound to fibrin, a factor that contributes to the fibrin-dependent mechanism of staphylokinase. Increasing concentrations of SK prevented α₂-antiplasmin from inhibiting plasmin (Fig. 8). By comparison SKΔ59 and staphylokinase were much less potent at preventing plasmin inhibition. In these experiments (Fig. 8) SK was ~110-fold more potent than SKΔ59 and ~220-fold more potent than staphylokinase in preventing the inhibition of plasmin (10 nM) by α₂-antiplasmin (30 nM). Thus, lacking an intact α domain, the SKΔ59-plasmin complex does not have the resistance to α₂-antiplasmin typical of the SK-plasmin complex and became more like the staphylokinase-plasmin complex in its vulnerability to α₂-antiplasmin.

DISCUSSION

Members of the SK gene family are remarkable for their ability to activate Pg in the blood in the absence of fibrin. The discovery that SK contains within its NH₂ terminus a structural element that can switch Pg activation between fibrin-dependent and fibrin-independent mechanisms has provided new

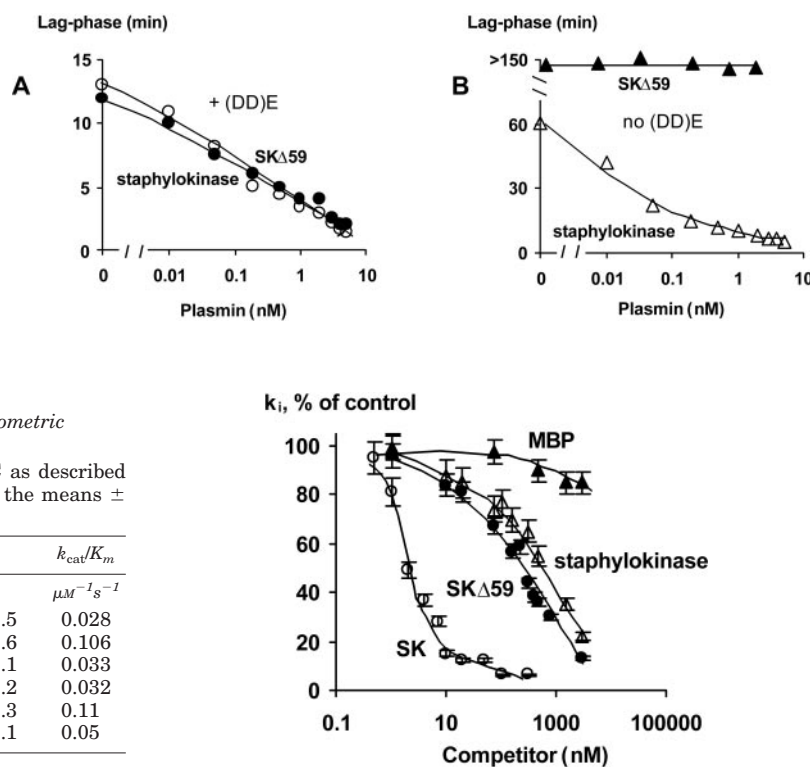


FIG. 8. Influence of SK, SKΔ59, and staphylokinase on the inhibition of human microplasmin by α₂-antiplasmin. Various amounts of SK, SKΔ59, or staphylokinase (0–3000 nM) were mixed with human plasmin (10 nM) and S-2251 (0.5 mM) in assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) at 37 °C. The A₄₀₅ was continuously recorded, and after 90 s human α₂-antiplasmin (30 nM) was added. The residual plasmin activity was determined at different time intervals, and the apparent inhibition rate constant ($k_{i,app}$) was calculated (see "Experimental Procedures"). The data are expressed as percentages of the k_1 value obtained in the presence of a given cofactor concentration *versus* that in the absence of competitor. The data represent the means ± S.E.

insights into the Pg activation process. In the absence of the NH₂ terminus (residues 1–59), SKΔ59 displays several features typical of a staphylokinase-like, fibrin-dependent mechanism of Pg activation. For example, like staphylokinase, and unlike its parent molecule SK, SKΔ59 was unable to generate an active site in Pg through a nonproteolytic mechanism. Consequently the lag phase in activation of Glu-Pg by SKΔ59 was, like staphylokinase, closely linked to the amount of plasmin present for activator complex formation. SKΔ59 was also more similar to staphylokinase than SK in how it modified the proteolytic properties of plasmin in the activator complex; both SKΔ59 and staphylokinase had minimal effects on the catalytic processing of small peptide substrates such as S-2251. In addition, unlike SK, neither SKΔ59 nor staphylokinase form activator complexes that protect plasmin from inhibition by α₂-antiplasmin. Thus, removal of the NH₂ terminus converted SKΔ59 into an activator with properties more akin to staphylokinase than to the parent SK molecule.

Despite these similarities there are important differences between staphylokinase and SKΔ59 in the mechanism of Pg activation. Unlike staphylokinase, but similar to TPA, SKΔ59 activator complex cannot efficiently activate Glu-Pg in the absence of fibrin in solutions containing physiologic amounts of Cl⁻. However both SKΔ59 (Fig. 1) and TPA activate Lys-Pg in the absence of fibrin, which suggests that for both activators, the partial unfolding that occurs in the Lys-Pg molecule, when it is converted from Glu-Pg, is a major requirement for efficient substrate processing (3, 9, 36, 51). The fact that the activation

of Lys-Pg was increased by fibrin suggests that full unfolding of the Pg molecule further enhances substrate interactions by the SK Δ 59 activator complex and by TPA. Consistent with this notion, removal of Cl⁻ from solutions unfolds Glu-Pg and allows efficient activation of the Glu-Pg molecule by SK Δ 59 by markedly reducing the K_m . Thus, it would appear that removal of the NH₂ terminus from SK converts SK Δ 59 into an activator that can only efficiently process unfolded forms of Pg substrates.

In physiologic solutions containing Cl⁻, Glu-Pg can be unfolded by the interactions of COOH-terminal lysines of fibrin fragments such as (DD)E (33, 52). Binding of Glu-Pg to COOH-terminal lysines of fibrin fragments may enhance fibrinolysis by staphylokinase and may explain the ability of (DD)E to accelerate the activation of Glu-Pg by SK Δ 59. EACA mimics the structure of COOH-terminal lysines and is sufficient to unfold Glu-Pg (33, 52). EACA accelerates the activation of Glu-Pg by staphylokinase (Fig. 5C). Interestingly, however, EACA does not amplify the activation of Glu-Pg by SK Δ 59 (Fig. 5B). This likely reflects the special effects EACA has on SK-Pg interactions where it both inhibits the formation of the activator complex and interferes with the binding of Pg substrate (45).

At the same time, kringles 1–4 in the Pg molecule are also important for substrate interactions with the activator complex, because SK Δ 59 does not efficiently activate mini-Pg and micro-Pg in the presence and absence of fibrin. As such the α domain, which is unfolded in SK Δ 59 (11, 53), may play an important role in the binding of kringles during substrate processing. Consistent with this notion, the SK α domain bound in a saturable and specific manner to Glu-Pg as well as to isolated kringle domains 1–3, 4–5, 1, and 5. The highest binding avidity of the SK α , β , and γ domains occurred with preparations containing multiple kringles (*e.g.* $K_{D, app}$ kringles 1–3 < kringles 4–5 < kringle 1 or 5). Thus, in the ternary SK-plasmin-Pg complex, each domain of SK appears capable of multiple different kringle interactions.

How are the promiscuous binding interactions between SK domains and the five kringles in the plasmin moiety of the activator complex and the five kringles in the Pg substrate coordinated to mediate efficient Pg activation? The finding that the kringle-less SK-microplasmin complex is a more efficient Pg activator than other SK-plasmin complexes suggests that kringles are not required in the interactions between SK and the plasmin moiety in the activator complex. In fact, the kringles of the plasmin moiety in the activator complex may compete for binding to SK with the kringles of the Pg substrate (30, 54). At the same time, kringles are clearly important in the Pg substrate for efficient catalytic processing. For example, the SK-microplasmin complex most efficiently activates Lys-Pg, but it is nearly inert with micro-Pg substrate (30, 54).

In the activator complex, the α domain does not simply bind substrate to permit the activation of Glu-Pg in the absence of fibrin. Indeed, assays that simulate substrate binding indicate that SK Δ 59 activator complexes bind to Glu-Pg and Lys-Pg substrates with avidities comparable with the SK activator complex. Recent studies, using different techniques, have also found that SK activator complexes lacking the α domain still bind substrate at levels comparable with native SK activator complexes (55). Thus, in addition to interactions with the protease domain (11), it is likely that the α domain not only binds substrate but specifically alters the conformation/orientation of the Glu-Pg substrate to permit processing by the activator complex (55). Consistent with this notion, SK unfolds the Glu-Pg molecule through a process that requires an intact α domain because it occurs when SK1–59(W6A) is added to

SK Δ 59 but not with the addition of a mutant SK1–59(W6A,L42A), which is unable to reconstitute the normal α domain structure (11). In the absence of an intact α domain, the SK Δ 59-activator complex can only efficiently process unfolded forms of the Pg substrate such as Lys-Pg, Glu-Pg in the presence of fibrin, or Glu-Pg in the absence of Cl⁻.

A number of residues in the SK NH₂ terminus have been identified in structural and biochemical studies that participate in contact interactions with the catalytic domain of Pg or plasmin and mediate nonproteolytic activation of the Pg molecule (9, 11, 12). Although truncation of the first 23 amino acids in the NH₂ terminus (*e.g.* removal of the first 23 residues) eliminates the ability of SK to nonproteolytically activate Pg, it is not until mutations or deletions are made that alter the structure of the α domain that the resulting SK fragment acquires fibrin dependence (9, 11, 12). Without an intact α domain, SK loses its ability to modify the catalytic properties of the plasmin in the activator complex with regard to Pg and to small molecule substrates. It also loses its ability to protect plasmin from inhibition by α_2 -antiplasmin. Given that mutations in the residues that contact the catalytic domain of Pg or plasmin are not responsible for fibrin independence (11), it is likely that interactions of the α domain of SK with kringles in the Pg substrate confer on SK the ability to activate Pg efficiently in the absence of fibrin. Taken together, these findings suggest that the α domain alters the properties of the plasmin moiety in the activator complex and coordinates the binding and unfolding of Pg substrate molecules for efficient Pg activation. Because these functions appear to be mediated by discrete structural elements in the α domain, it should be feasible to design mutant SKs with altered nonproteolytic activation, fibrin dependence, and/or α_2 -antiplasmin resistance to determine how these properties regulate Pg activation and fibrinolysis under physiologic conditions.

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α Domain Deletion Converts Streptokinase into a Fibrin-dependent Plasminogen Activator through Mechanisms Akin to Staphylokinase and Tissue Plasminogen Activator

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