**STREPTOCOCCUS UBERIS PLASMINOGEN ACTIVATOR (SUPA) ACTIVATES HUMAN PLASMINOGEN THROUGH NOVEL SPECIES-SPECIFIC & FIBRIN-TARGETED MECHANISMS**

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Running Head: SUPA & Fibrin-targeted Human Plasminogen Activation

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**Background:** Bacterial plasminogen activators like SUPA subvert mammalian plasminogen activation to facilitate infection in a species-restricted manner.

**Results:** Fibrin blood clots permit SUPA to activate human plasminogen thereby overriding species-restriction.

**Conclusion:** Fibrin provides a source of enzyme cofactor and enhances SUPA’s catalytic efficiency.

**Significance:** Molecular interactions that override species-restricted plasminogen activation may be used to modulate infections or create new human therapeutics.

**SUMMARY**

Bacterial plasminogen activators generate plasmin to degrade fibrin blood clots and other proteins that modulate the pathogenesis of infection. Yet, despite strong homology between mammalian plasminogens, the activity of bacterial plasminogen activators is thought to be restricted to the plasminogen of their host mammalian species. Thus, we find that *Streptococcus uberis* plasminogen activator, isolated from a *Strep sp.* that infects cows but not humans, robustly activates bovine but not human plasminogen in purified systems and in plasma. Consistent with this, *Streptococcus uberis* plasminogen activator forms a higher avidity complex (118-fold) with bovine than human plasminogen and non-proteolytically activates bovine but not human plasminogen. Surprisingly however, the presence of human fibrin overrides the species-restricted action of *Streptococcus uberis* plasminogen activator. First, human fibrin enhances the binding avidity of *Streptococcus uberis* plasminogen activator for human plasminogen by 4-8-fold, in the presence and absence of chloride ion (a negative regulator). Second, although *Streptococcus uberis* plasminogen activator does not protect plasmin from inactivation by α2-antiplasmin, fibrin does protect human plasmin, which forms a 31-fold higher avidity complex with *Streptococcus uberis* plasminogen activator than plasminogen. Third, fibrin significantly enhances plasminogen activation by reducing the Kₘ (4-fold) and improving the catalytic efficiency of the *Streptococcus uberis* plasminogen activator complex (6-fold). Taken together these data suggest that indirect molecular interactions may override the species-restricted activity of bacterial plasminogen activators; this may affect the pathogenesis of infections or may be exploited to facilitate the design of new blood clot-dissolving drugs.

The plasminogen (Pg) and plasmin system play key roles in physiologic processes such as fibrin blood clot dissolution, wound healing, cell migration and the pathogenesis of certain types of bacterial infections. Pg activators convert Pg into plasmin, a proteolytic enzyme that cleaves fibrin polymers into soluble degradation products (1). Both mammals and bacteria produce Pg activators but they are markedly different in structure and mechanisms of action. The mammalian Pg activators, tissue Pg activator (TPA) and urinary-type Pg activator, are serine proteases that directly cleave a broad spectrum of Pgs from different animal species (2-4). In contrast, the bacterial Pg activators lack inherent protease activity and form complexes to cleave a restricted spectrum of mammalian Pgs (5-7).
The mechanisms responsible for the species-restricted action of bacterial Pg activators are not fully understood but they affect the consideration of bacterial Pg activators as potential therapies for human clotting or thrombotic diseases such as heart attacks and strokes. For example, the prototypical bacterial Pg activator streptokinase (SK), which was isolated from bacteria that infect humans, preferentially activates human but not bovine Pg. In a similar fashion, SUPA (PauA), a Pg activator purified from Streptococcus uberis, a bacteria that infects cows, has been shown to activate bovine Pg but not human Pg (8-11). These differences are remarkable considering that both Pg activators function through similar mechanisms. Both SK and SUPA non-proteolytically activate their cognate Pgs, form activator complexes with plasmins, reduce the susceptibility of plasmin to inhibition by α2-antiplasmin and alter the substrate specificity of plasmin (10,12).

The species-restricted activation of Pg by bacterial Pg activators is in part due to key sequence differences that alter the intermolecular complementarity between the Pg and the Pg activator (6,13,14). In the present study we show that the species-restricted activation of human Pg activation by SUPA is profoundly affected by fibrin and other molecules that interact with Pg. These findings provide new insights into the regulation of the activity of the bacterial Pg activators in different mammalian hosts that can be used to modulate blood clot-dissolving activity or to regulate their function during bacterial infection.

**EXPERIMENTAL PROCEDURES**

**Protein and Reagents.** Reagents were purchased from the following sources: human Glu-Pg, bovine-Pg, human Pg-depleted fibrinogen and cyanogen bromide (CNBr) digested fibrinogen (American Diagnostica Inc., Stamford, CT); α2-antiplasmin (Calbiochem, La Jolla, CA); citrated frozen human and bovine plasma (Lampire Biological Laboratories, Pipersville, PA); tissue Pg activator (Baxter Biotechnology, Hayward, CA); 125I-fibrinogen (Perkin-Elmer, Waltham, MA); all the other reagents if not specified (Sigma, St. Louis, MO).

**Cloning, Expression and Purification of Recombinant Protein.** SK, staphylokinase and Streptococcus uberis Pg activator (SUPA) were cloned, expressed and purified as described (10,12,15). Human micro-Pg and the cleavage resistant R561A mutant micro-Pg (micro-PgR561A) were prepared as described previously (16). Protein concentrations were determined by BCA protein kit (Thermo Fisher Scientific, Rockford, IL) and the active concentration of each protein was determined by active site titration as described below.

**Active Site Titration.** Non-proteolytic active site generation by Glu-Pg and SK complex or bovine-Pg and SUPA complex was determined at 25°C in a Multi-Detection Microplate Reader (Bio-Tek Instruments, Winooski, VT) by active site titration with the fluorogenic substrate 4-methylumbelliferyl p-guanidinobenzoate (MUGB) (15,17,18). Briefly, 400 nM Pg or micro-Pg was added into microplate well containing 1 μM MUGB in filtered HEPES assay buffer (10 mM HEPES, pH 7.2) at 25°C. After 10 min, SK or SUPA (200-800 nM) or buffer alone (control) were added into test well. In certain runs, complex of Pg and Pg activator, which was pre-formed on ice by mixing both proteins for 10-20 min, was added directly into the assay buffer. The development of fluorescence was monitored continuously with excitation at 360 nm and emission at 460 nm. The fluorescence increase was used to calculate the active site generation by using 4-methylumbelliferone (MUB) as a control.

**Binding Assay.** SUPA and Pg or plasmin binding assays were performed in microtiter plates coated with 100nM SUPA for 1hr at room temperature. Wells were washed and nonspecific binding sites were blocked with 1% bovine serum albumin. After that, varying concentrations of Pg or aprotinin-inactivated plasmin were added for 1hr. After washing, mouse anti-plasmin(ogen) monoclonal antibody was added for 1hr, followed by washing, and the addition of horseradish peroxidase conjugated anti-mouse IgG for 1hr. The bound antibody were detected by TMB substrate and the reaction was monitored at A370nm within the dynamic range of the microplate reader. Binding constants were calculated using Graphpad Prism Software (La Jolla, CA).

**Plasminogen Activation.** The activation of human Glu-Pg/bovine-Pg/human micro-Pg by various amounts of Pg activator (SK, SUPA, staphylokinase) was examined at 37°C in either HEPES buffer (10mM HEPES, with or without 150mM
NaOAc or NaCl, pH 7.4) or Tris-NaCl buffer (50mM Tris-HCl, 100mM NaCl, pH 7.4). Pgs were pretreated with aprotinin-agarose beads for four hours at 4°C to remove contaminating plasmin. Pg was added to assay buffer with the presence of 500 µM S2251 (Chromogenix-Instrumentation Laboratory, Lexington, MA) (19) followed by Pg activator (final volume of 100 µl). The change in absorption at 405 nm was continuously recorded in a Thermomax microtiter plate reader. To examine the effect of fibrinogen or CNBr-fibrin fragments on Pg activation, Pg was pre-incubated with each reagent in the assay buffer for 10 min before the addition of Pg activator. Pg activation parameters were calculated using amido-lytic parameters for plasmin as described by Wohl, et al. (20).

Plasma Fibrinogenolysis. Human or bovine plasma fibrinogenolysis was determined by incubating 180 µl plasma with 20 µl assay buffer containing varying concentrations of SUPA or no SUPA (control) at 37°C. Samples were collected after two hours and mixed with 200 kallikrein inhibitor units (KIU) aprotinin per ml plasma to quench plasmin-mediated lysis. Residual fibrinogen concentrations were determined by the sodium sulfite method (21).

Plasma Fibrinolysis. Human or bovine plasma fibrinolysis was determined by simultaneous mixing 180 µl plasma with various amounts of SUPA in the presence of 1 mM Ca²⁺, 1 UI/ml thrombin and trace amounts of ¹²⁵I-fibrinogen (final volume 200 µl). The total radioactivity of the sample was monitored using Cobra II gamma counter (Perkin-Elmer –Packard BioScience, Waltham, MA). After incubating samples at 37°C for two hours, 200 KIU aprotinin was added to each ml plasma clot to inhibit further lysis. The radioactivity of the clot and supernatant plasma were monitored separately. The percentage of fibrinolysis was defined as the difference between initial and residual clot radioactivity divided by the initial clot radioactivity.

SDS-PAGE and Immunoblotting. The generation of plasmin by SUPA was detected using SDS-PAGE assay under denaturing conditions. The effect of SUPA or SK on the inhibition of plasmin by α2-antiplasmin was examined. Plasmin (0.25 µM) was mixed with varying concentration of SK or SUPA (0.25 – 2.5 µM) in HEPES-NaOAc buffer pH 7.4 on ice for 20 mins, followed by addition of 0.25 µM α2-antiplasmin. After 10 min. samples were analyzed by non-reduced SDS-PAGE followed by immunoblotting with a monoclonal antibody against α2-antiplasmin (RWR, (22)).

RESULTS

Species restricted Pg activation—To confirm that SUPA was restricted in its ability to activate different mammalian Pgs, we examined its action on human and bovine Pg. SUPA rapidly and efficiently activated purified bovine Pg (Fig. 1A) but did not efficiently activate purified human Pg (Fig. 1B). In contrast, as a control, SK had no activity against bovine Pg (Fig. 1A) but it rapidly and efficiently activated human Pg (Fig. 1B).

Pg activation with purified reagents in vitro is quite different from Pg activation in complex biologic solutions like plasma or serum that contain molecules (e.g., fibrinogen, fibrin, α2-antiplasmin, etc.) that modify the activity of both Pg or plasmin. In plasma or serum, the plasmin generated by Pg activators degrades target substrates such as fibrinogen or fibrin. When SUPA was added to bovine plasma (Fig. 2A) it triggered the degradation of fibrinogen in a potent dose-related process with an EC50 of 185 nM. In a similar fashion, when added to bovine serum containing fibrin clots, there was potent degradation of fibrin with a comparable EC50 of 153 nM. When SUPA was added to human plasma (Fig. 2B) it triggered fibrinogen degradation with a potency that was markedly lower (estimated EC50 ~ 1000 nM) than in bovine plasma. In contrast, when SUPA was added to human serum containing fibrin clots, there was potent degradation of fibrin with a comparable EC50 of 153 nM. When SUPA was added to human plasma (Fig. 2B) it triggered fibrinogen degradation with a similar potency (EC50 = 154 nM) to that seen in bovine plasma and serum. These results indicate that fibrin, and to a much lesser extent fibrinogen, significantly alter SUPA’s species-restricted mechanism of human Pg activation.

SUPA-Pg complexes—Plasminogen activation and subsequent degradation of fibrinogen and fibrin by SUPA requires initial formation of SUPA-Pg complexes. Consistent with this notion, in the presence of physiologic levels of chloride ion, bovine Pg bound well to SUPA (K_D = 6.9 ± 0.5 nM). However, the relative binding avidity of SUPA to human Pg was 118-fold less (K_D = 811 ± 356 nM) in the same chloride containing buffer (Table 1). Chloride ion is known to regulate the confor-
formation of Pg and its interactions with other molecules. Indeed, in the absence of chloride ion, there was enhanced binding (4-fold, $K_D = 194 \pm 22 \text{ nM}$) of SUPA to human Pg. In the presence of chloride ion, SUPA binding was also enhanced by fibrin (8-fold, $K_D = 99 \pm 50 \text{ nM}$).

**SUPA-Plasmin complexes**—By comparison to human Pg, human plasmin bound with greater avidity to SUPA. In the presence of chloride ion, the $K_D$ for SUPA-plasmin binding was 31-fold lower ($K_D = 26 \pm 4 \text{ nM}$, Table 1). In contrast to Pg, the binding of human plasmin was minimally, if at all, affected by the absence of chloride ion ($K_D = 33 \pm 3 \text{ nM}$) or the presence of fibrin ($K_D = 26 \pm 1 \text{ nM}$).

**Fibrin enhances activation of human Pg by SUPA**—After formation, the SUPA activator complex interacts with Pg substrate to catalyze the production of plasmin. In fluids containing physiologic concentrations of chloride ion, human Glu-Pg adopts a folded conformation that protects it from activation (24,25). Fibrin opposes the effects of chloride ion and unfolds the Glu-Pg substrate which may modulate the ability of SUPA activator complex to activate human Glu-Pg substrate (26,27). Although chloride did not significantly affect the formation of the SUPA-plasmin activator complex (see below and Table 1), increasing chloride concentrations inhibited SUPA-induced Pg activation in a concentration-dependent manner (Fig. 3A). In the presence of chloride ion, fibrinogen modestly enhanced Pg activation by SUPA, when compared to BSA alone (as a non-specific protein control, Fig. 3C). However, Pg activation by SUPA (Fig. 3C) was enhanced more markedly by cyanogen bromide-digested fibrin fragments (CNBr-Fx), a widely-used soluble form of fibrin (28,29). Direct analysis of these reactions by SDS-PAGE (Fig. 4) confirmed that fibrin fragments enhanced human Pg activation by SUPA when compared to Pg activation in the absence of fibrin. Kinetic analysis of Pg activation by SUPA in HEPES buffer containing 150 mM chloride ion indicated that the presence of 0.1 mg/ml CNBr-Fx decreases $K_{pg}$ (4.2-fold), increases $k_{pg}$ by 1.4-fold and enhances the catalytic efficiency ($k_{pg}/K_{pg}$) by 6.1-fold (Table 2).

**SUPA is unable to activate Pg through non-proteolytic mechanisms**—In part, SK efficiently activates human Pg in plasma through a non-proteolytic mechanism which does not require cleavage of the Arg561-Val bond to form plasmin. In a similar fashion, SUPA can non-proteolytically activate bovine Pg (10,11). Since plasmin is a trace contaminant of Pg preparations, we examined whether SUPA can non-proteolytically activate human Pg using micro-Pg$_{R561A}$; this contains an activation loop mutation of arginine 561 to alanine that prevents it from forming plasmin (16,23). Consistent with its ability to non-proteolytically activate Pg, SK generated active complexes with both micro-Pg and mutant micro-Pg$_{R561A}$ (Fig. 5A). In contrast, staphylokinase, which can’t non-proteolytically activate human Pg, activated micro-Pg, but not micro-Pg$_{R561A}$ (Fig. 5B). Similar to staphylokinase, SUPA activated micro-Pg, but not micro-Pg$_{R561A}$ (Fig. 5C). These observations confirm that human Pg activation by staphylokinase or SUPA requires the presence of micro-plasmin which is a trace contaminant of micro-Pg but not micro-Pg$_{R561A}$ (16,23). Thus, SUPA appears to activate human Pg in plasma through a mechanism akin to staphylokinase—by forming an activator complex with plasmin.

**SUPA does not prevent the formation of plasmin-a2-antiplasmin complexes**—By forming covalent plasmin-a2-antiplasmin complexes, a2-antiplasmin rapidly inactivates plasmin in plasma. The binding of plasmin to fibrin partially protects from inactivation by a2-antiplasmin (30,31). Thus, as expected, when mixed with plasmin, a2-antiplasmin formed inactive plasmin-a2-antiplasmin complexes (Fig. 6). SK protected against the formation of inactive human plasmin-a2-antiplasmin complexes. However, SUPA did not prevent the formation of human plasmin-a2-antiplasmin complexes (Fig. 6). Thus, in contrast to its interactions with bovine plasmin, SUPA is unable to protect human plasmin from in activation by a2-antiplasmin.

**DISCUSSION**

The restricted ability of bacterial Pg activators to activate different mammalian Pgs has long been considered a fundamental distinction between mammalian and bacterial plasminogen activators (6). Thus, in plasma, or in purified systems with physiologic concentrations of chloride, SUPA efficiently activates bovine Pg but not human Pg. However, in the presence of fibrin, SUPA acquires the ability to efficiently activate human
Pg. This fibrin-targeted activity of SUPA with human Pg is the result of several different mechanisms.

First, fibrin modifies the avidity of binding interactions between SUPA and human Pg. In plasma and fluids that contain physiologic concentrations of chloride ion (e.g. ~ 120-150 mM), the binding interactions between SUPA and human Pg are of much lower avidity ($K_D$ 118-fold higher) than the binding interactions between SUPA and bovine Pg. Fibrin significantly enhances the binding interactions between SUPA and human Pg ($K_D$ = 8-fold lower) as does the absence of chloride ion ($K_D$ = 4-fold lower). Fibrin has a minimal effect on the binding interactions between SUPA and human plasmin which are already much higher avidity (31-fold lower $K_D$) than the binding interactions with human Pg.

Second, fibrin provides a protective environment for the existence of plasmin, which permits the formation of the SUPA-plasmin complex. This is critical because SUPA can’t non-proteolytically activate human Pg as it does bovine Pg. Similar to staphylokinase, SUPA does not protect human plasmin from inhibition by $\alpha_2$-antiplasmin, fibrin does (30,31). This effectively limits Pg activation by the SUPA-plasmin complex to the fibrin surface where it is partially protected from inhibitors.

Third, fibrin enhances the catalytic efficiency of the SUPA-plasmin complex with human Pg substrate by 6-fold. Since fibrin does not significantly affect the formation of the SUPA-plasmin complex (Table 1), this enhanced catalytic efficiency is likely to reflect the known effects of fibrin on unfolding the Pg substrate conformation (26,27). In a similar fashion, the negative effects of chloride ion on the catalytic efficiency of the SUPA-plasmin complex may well be attributed the effect chloride ion has on promoting a folded, less readily activated Pg substrate.

Species-restricted activation of Pg by bacterial Pg activators has been noted for decades but the mechanisms responsible for it are still poorly understood. This species restriction occurs despite homology between Pgs from different species and, between different SK-like bacterial Pg activators. For example, the alpha and beta domains of SUPA show considerable sequence identity (30.9% and 27.4%) and structural similarity to SK; and, significant but less sequence identity (11.9% and 15.6%) and structural similarity to staphylokinase (10, 11). Species restriction also occurs despite evidence of binding interactions between different SK-like molecules and different species Pgs (13,33). One key determinant of species-restriction is the requirement of intermolecular complementarity at sites necessary for function (6,14). Alteration of key loop residues in human Pg makes it susceptible to activation by SUPA (6). In a complementary fashion, replacing the SK $\beta$ domain with the SUPA $\beta$ domain creates an SK chimera that activates horse Pg, a non-cognate substrate of native SK (6). Still, it has not been appreciated that Pg-interacting molecules may also regulate species-specific Pg activation by altering the interactions, conformation, etc. of Pg. This study shows that human fibrin, which binds and alters the conformation of Pg, improves the substrate binding and processing of human Pg by SUPA.

The Pg-plasmin system has been exploited by bacteria to facilitate infection (6). For example, Group A streptococci producing SK (which doesn’t activate mouse Pg) can’t infect normal mice but they can infect mice carrying a transgene for human Pg (36). In a similar fashion, vaccines generated against SUPA (PauA) have been shown to reduce infection by *Strep. iberis* (37). Both Pg and fibrinogen are key modulators of the pathogenesis and inflammatory response to bacteria producing Pg activators such as *Yersinia pestis* (38). The action of bacterial Pg activators on the host Pg system can degrade extracellular matrix to facilitate the spread and invasion of bacteria (39). In addition to Pg activators, bacteria also produce plasmin binding molecules that have been implicated in the pathogenesis of infection because 1) they protect plasmin from inhibition by serpins like $\alpha_2$-antiplasmin (40) and, 2) they modify the activity of the Pg activator complexes. It has also recently been shown that bacteria that lack intrinsic fibrinolytic activity produce a molecule (Skizzle) which can enhance Pg activation by either urinary-type or tissue type Pg activator (33).

Our results suggest that a number of different bacterial Pg activators, once thought to be specific for cognate mammalians Pgs can be induced to become activators of human Pg. Further insights into the mechanisms of action of Pg activators may result in new therapeutic agents targeted to human clots as well as novel approaches for ablat-
ing the contribution of bacterial Pg activators to the pathogenesis of infections.

REFERENCES


FOOTNOTES
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The abbreviations used are: Pg, plasminogen; SUPA, Streptococcus uberis plasminogen activator; TPA, tissue plasminogen activator; SK, streptokinase; MUB, 4-methylumbelliferone; CNBr, cyanogen bromide; KIU, kallikrein inhibitor units.

FIGURE LEGENDS

Fig. 1. Species-restricted Pg activation by SUPA. Bovine Pg (A) or human Glu-Pg (B) (100nM) was activated by 40 nM SK or SUPA in Tris-NaCl (50mM Tris-HCl, 100mM NaCl, pH 7.4) assay buffer at 37°C. The production of plasmin was monitored using S2251 substrate (500 µM) as described in the Methods. The results shown are representative of at least three experiments.

Fig. 2. Fibrin-targeted activation of human but not bovine Pg by SUPA. Effect of SUPA-mediated Pg activation on fibrinolysis and fibrinogenolysis was examined in (A) bovine and (B) human plasma. The results shown are representative of two independent experiments. Mean ± SD are shown.

Fig. 3. Fibrin and chloride ions modulate human Glu-Pg activation by SUPA. (A) Effect of chloride on Pg activation. Human Glu-Pg (100 nM) mixed with HEPES buffer containing various amounts of NaCl (0-150 mM) and 500 µM S2251. The total ionic strength of the buffers was maintained at 150 mM by the addition of NaOAc to buffers with chloride concentrations less than 150 mM. After incubating at 37°C for 10 min, SUPA (40 nM) was added at t=0 and the absorbance at 405 nm was monitored to detect the generation of plasmin. Results are representative set of two experiments. (B) and (C) Fibrin fragments enhance Glu-Pg activation by SUPA. Human Glu-Pg (100 nM) was added to Tris-NaCl buffer containing 500 µM S2251 at 37°C in the absence (Ctrl) or presence of varying concentration of CNBr-fibrin fragments, fibrinogen, or BSA. After 10 min, Pg activation was initiated by the addition of 0 (B) or 20 nM SUPA (C) and the absorbance at 405 nm was monitored. The results shown are representative of two independent experiments.

Fig. 4. Analysis of human Glu-Pg activation by SUPA in the presence of fibrin. Human Glu-Pg (500 nM) was mixed with 40 nM SUPA in Tris-NaCl buffer, in the absence or presence of 0.1 mg/ml CNBr-fibrin fragments, for half hour at 37°C. Samples were collected, reduced and analyzed by SDS-PAGE. Lanes 1-4, reagents alone at t=0 (Glu-Pg- lane 1; human plasmin- lane 2; Fx- lane 3; SUPA, cut from fusion partner maltose binding protein (MBP) - lane 4). Lanes 5-9, reagents after incubation at 37°C for half hour (Glu-Pg- lane 5; human plasmin- lane 6; Glu-Pg in the presence of Fx- lane 7; Glu-Pg activated by SUPA- lane 8; Glu-Pg activated by SUPA in the presence of Fx- lane 9). Lane 10, protein standards and relative molecular mass (kDa).

Fig. 5. Activation of micro-Pg or micro-Pg R561A by different Pg activators. Human micro-Pg (uPg) or the cleavage resistant mutant micro-Pg R561A (uPg R561A) was added into HEPES buffer containing 500 µM S2251. After incubating for 10 min, (A) 200nM SK, (B) 200nM staphylokinase or (C) 1000nM SUPA or no Pg activators was added into uPg or uPg R561A at an equimolar concentration. Controls consisted of
micro-Pg (uPg, 200 nM) or the cleavage resistant mutant micro-Pg\textsubscript{R561A} (200 nM) in HEPES buffer containing 500 µM S2251 without Pg activator. Results are representative set of two experiments.

Fig. 6. SK-plasmin and SUPA-plasmin resistance to α2-antiplasmin inhibition. Plasmin (0.25 µM) was mixed with SK (0.25 µM) or varying concentrations of SUPA (0.25 or 2.5 µM) in HEPES-NaOAc buffer and incubated on ice for 20 mins, followed by addition of 0.25 µM α2-antiplasmin (α2AP) and incubated for additional 10 min. Samples were analyzed by SDS-PAGE under non-reduced condition using western blotting. The formation of the α2-antiplasmin-plasmin complex was detected by mouse anti-human α2-antiplasmin monoclonal antibody at the appropriate molecular size as indicated. Lane 1, plasmin alone; lane 2, α2-antiplasmin alone; lane 3, plasmin and α2-antiplasmin; lane 4, plasmin, 0.25 µM SK and α2-antiplasmin; lane 5 to 6: plasmin and 0.25 or 2.5 µM SUPA mixed with α2-antiplasmin.
Table 1. Binding constants ($K_D, \times 10^9$M) for SUPA-Pg or SUPA-plasmin complexes

<table>
<thead>
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<th>+ Chloride$^1$</th>
<th>- Chloride</th>
<th>+ Chloride + fibrin$^2$</th>
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<tr>
<td>SUPA-human Pg</td>
<td>811 ± 356</td>
<td>194 ± 22</td>
<td>99 ± 50</td>
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<tr>
<td>SUPA-human plasmin</td>
<td>26 ± 4</td>
<td>33 ± 3</td>
<td>26 ± 1</td>
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$^1$ chloride concentration 150 mM.

$^2$ fibrin concentration 0.1 mg/ml.
Table 2. Effects of chloride and fibrin on activation of human Pg by SUPA.

<table>
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<th>Buffer</th>
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<td>$k_{Pg}/K_{Pg}$ (µM$^{-1}$ min$^{-1}$)</td>
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<td>96 ± 15</td>
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<td>HEPES-NaCl w/ fibrin</td>
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<td>48 ± 7</td>
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<td>HEPES-NaOAc w/ fibrin</td>
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<td>30 ± 2</td>
<td>3.48 ± 0.01</td>
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Activation experiments were carried out at 37°C in a total volume of 100 µl with HEPES-NaCl or HEPES-NaOAc buffer in the absence or presence of 0.1 mg/ml fibrin (CNBr-fibrin fragments). The kinetic analysis of activation parameters is described under Experimental Methods. The values represent the mean ± SD.
Figure 1

A

B

A405nm

Time (min)

Ctrl

SK

SUPA

A405nm

Time (min)

Ctrl

SK

SUPA
Figure 2
Figure 3

A

[Cl]:

0mM
10mM
20mM
40mM
80mM
150mM
Ctrl

B

No SUPA

Ctrl
0.1 mg/ml fibrin
0.6 mg/ml fibrinogen
0.6 mg/ml BSA

C

20nM SUPA

fibrin
0.1mg/ml
0.05mg/ml
fibrinogen
0.6mg/ml
0.1mg/ml
BSA
0.6mg/ml
0.1mg/ml
Ctrl

Time (min)

A 405nm

Time (min)

A 405nm

Time (min)

A 405nm
Figure 4
Figure 5
Figure 6

\[
\begin{array}{c|ccccc}
\text{Plasmin} & + & + & + & + & + \\
\text{\(\alpha\text{-}2\text{-AP}\)} & + & + & + & + & + \\
\text{SK} & + & & & & \\
\text{SUPA} & & + & + & & \\
\end{array}
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Streptococcus uberis plasminogen activator (SUPA) activates human plasminogen through novel species-specific & fibrin-targeted mechanisms
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