Site-restricted plasminogen activation mediated by group A streptococcal streptokinase variants

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SK (streptokinase) is a secreted plasminogen activator and virulence factor of GAS (group A *Streptococcus*). Among GAS isolates, SK gene sequences are polymorphic and are grouped into two sequence clusters (cluster type-1 and cluster type-2) with cluster type-2 being further classified into subclusters (type-2a and type-2b). In the present study, we examined the role of bacterial and host-derived cofactors in SK-mediated plasminogen activation. All SK variants, apart from type-2b, can form an activator complex with Glu-Plg (Glu-plasminogen). Specific ligand-binding-induced conformational changes in Glu-Plg mediated by fibrinogen, PAM (plasminogen-binding group A streptococcal M protein), fibrinogen fragment D or fibrin, were required for type-2b SK to form a functional activator complex with Glu-Plg. In contrast with type-1 and type-2a SK, type-2b SK activator complexes were inhibited by α_2-antiplasmin unless bound to fibrin or to the GAS cell-surface via PAM in combination with fibrinogen. Taken together, these data suggest that type-2b SK plasminogen activation may be restricted to specific microenvironments within the host such as fibrin deposits or the bacterial cell surface through the action of α_2-antiplasmin. We conclude that phenotypic SK variation functionally underpins a pathogenic mechanism whereby SK variants differentially focus plasminogen activation, leading to specific niche adaption within the host.

Key words: fibrinogen, plasminogen, *Streptococcus pyogenes*, streptokinase, thrombolytic therapy.

INTRODUCTION

SK (streptokinase) is a Plg (plasminogen) activator secreted by β-haemolytic streptococcal species. SK from *Streptococcus equisimilis* isolate H46A (SKc) is the most characterized SK protein because of its widespread use as a thrombolytic therapeutic since approval in 1977 [1]. Unlike host Plg activators that activate Plg by limited proteolytic cleavage, SKc binds to Plg inducing conformational changes in the protein that result in the formation of an active site and the production of an enzymatically active complex, termed SK–Plg* (known as the 'conformational activation pathway' or 'Pathway I'). The conformationally activated SK–Plg* complex can then sequester substrate Plg and proteolytically convert these molecules into the broad-spectrum serine protease plasmin via cleavage at the Arg^{561}–Val^{562} peptide bond [2,3]. Plasmin, which has a higher affinity than Plg has for SK, rapidly displaces Plg in the SK–Plg* complex to produce an activated SK–plasmin complex that is the main catalyst responsible for the full conversion of Plg into plasmin (known as 'direct proteolytic activation pathway' or 'Pathway II') [2,3].

The process of Plg activation in a healthy individual is strictly controlled. Multiple regulatory mechanisms function in a co-ordinated manner to restrict Plg activation to specific locations within the host where the potent protease activity of plasmin is required. Plg activation is greatly influenced by the conformation of the Glu-Plg molecule. Plg contains several structural domains, consisting of the N-terminal PAp (PAN/Apple) domain [also known as the NTP (N-terminal peptide) domain and PAN module domain], followed by five kringle domains (K1–K5) and the C-terminal serine protease catalytic domain [4–8]. Intramolecular binding between lysine residues and the lysine-binding sites of these kringle domains maintains circulating Glu-Plg in a 'closed' conformation that is highly resistant to activation. Upon binding to specific cell-surface receptors or ligands such as fibrin, Glu-Plg adopts an 'open' conformation that becomes more susceptible to activation [9]. Whereas soluble plasmin is readily inhibited by the circulating inhibitor α_2-AP (α_2-antiplasmin), plasmin that remains bound to receptors/ligands may be resistant to inhibition, thereby restricting plasmin activity to specific foci such as the cell surface or fibrin thrombi [10].

Plg activation by SKc is not affected by these regulatory mechanisms as the closed conformation of Glu-Plg does not prevent activation and because plasmin activation activity displayed by the SKc-Plg* activator complex is not inhibited by α_2-AP [6,11]. Thus SKc-mediated Glu-Plg activation can rapidly generate high levels of soluble plasmin activity as activator complexes sequester and activate substrate Plg while bypassing host regulation mechanisms [12]. On the basis of these observations, it is now a widely held view that the human specific pathogen *Streptococcus pyogenes* [GAS (group A *Streptococcus*)] hijacks the host Plg activation system by generating unregulated soluble and cell-bound plasmin activity that is used to overcome immune defences and break down tissue barriers to facilitate dissemination [13–15]. However, recent research that has characterized SK variants from different GAS isolates suggests that these variants may play differing roles in the pathogenesis process [16,17]. SK produced by GAS displays considerable genetic and phenotypic diversity [16–20]. Phylogenetic studies of *ska* sequences from GAS isolates have revealed two main sequence clusters (cluster type-1 and -2) with cluster type-2 sequences being further subdivided (cluster type-2a and -2b) [18,19]. Epidemiological studies have shown the type-2b *ska* linage to be largely restricted to skin-tropic *pam*-positive GAS

Abbreviations: α_2-AP, α_2-antiplasmin; Fg, fibrinogen; FgD, Fg fragment D; FgE, Fg fragment E; FU, fluorescence unit(s); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GAS, group A *Streptococcus*; MUGB, 4-methylumbelliferyl *p*-guanidinobenzoate; PAM, plasminogen-binding group A streptococcal M protein; Plg, plasminogen; Prp, PAM-related protein; SEN, streptococcal enolase; SK, streptokinase; THY medium, Todd–Hewitt broth supplemented with 1 % (w/v) yeast extract.

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isolates expressing the high-affinity PAM (plasminogen-binding group A streptococcal M protein) [18].

Despite significant sequence variation, SKc and type-1 and type-2a SK variants display several important phenotypic similarities, such as a high affinity for Glu-Plg and an ability to conformationally activate Glu-Plg via Pathway I. Conversely, type-2b SK variants have low affinity for Glu-Plg and cannot induce an active site in this protein through conformational rearrangement [16,17]. In addition, the exchange of *ska* allelic variants in a virulent M1T1 GAS isolate (strain 5448; cluster type-2a) alters pathogenesis in a murine model of invasive infection [16]. Although these observations confirm the importance of Plg activation in GAS pathogenesis, the rationale and mechanisms defining how SK variants influence virulence are yet to be determined.

In the present study, we examined the role of bacterial and host-derived cofactors in Glu-Plg activation mediated by different SK variants with a view to determine how these interactions may influence GAS pathogenesis. Our data suggest that Glu-Plg activation by type-2b SK variants is restricted by ligand-induced conformational changes in the Glu-Plg molecule and that soluble type-2b SK–Plg* activator complexes are inhibited by α_2-AP. Taking the data together, we propose that the activation of Glu-Plg by type-2b SK variants is restricted to specific microenvironments at the infection foci which may promote long-term skin colonization.

MATERIALS AND METHODS

Ethical approval

Permission to collect human blood was obtained from the University of Wollongong Human Ethics Committee (HE08/250). Blood was taken from healthy adult volunteers, who provided informed written consent.

Bacterial strain, culture conditions and reagents

Escherichia coli strain M15 [pREP4] was used as a host for protein expression and was cultured at 37 °C in LB broth. GAS isolates ALAB49 [21], ALAB49Δ*pam*, NS88.2 [22] and NS88.2*prp* [23] were used in the present study. All streptococcal strains were routinely cultured at 37 °C on horse-blood agar (Biomerieux) or in static liquid cultures of Todd–Hewitt broth (BD) supplemented with 1 % (w/v) yeast extract (Oxoid) (THY medium). Where appropriate, 50 μg/ml kanamycin and 100 μg/ml ampicillin were used for selection. Glu-Plg, plasmin, Fg (fibrinogen) and α_2-AP were purchased from Haematologic Technologies. The chromogenic substrate H-D-Val-Leu-Lys-*p*-nitroaniline · 2HCl (S-2251) was obtained from Chromogenix. Thrombin from human plasma and 4-methylumbelliferone was purchased from Sigma–Aldrich. MUGB (4-methylumbelliferyl *p*-guanidinobenzoate) was from Marker Gene Technologies.

Cloning, expression and purification of recombinant proteins

Recombinant SK variants, PAM, SEN (streptococcal enolase) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were cloned, expressed in *E. coli*, purified and characterized as described previously [16,24–26].

Non-proteolytic active-site generation in Glu-Plg

Non-proteolytic active-site generation in Glu-Plg by SK variants was examined using the fluorescent active site MUGB in a POLARstar Omega fluorescence spectrophotometer (BMG Labtech). Glu-Plg (200 nM) was added to a black 96-well microtitre plate containing 1 μM MUGB in assay buffer 1 (50 mM Tris/HCl, pH 7.4, and 100 mM NaCl) at 37 °C. To initiate the reaction, SK was added to a final concentration of 400 nM in a total volume of 100 μl and the development of fluorescence was monitored continuously with excitation at 355 nm and emission at 460 nm. To test the effect of various Plg ligands and cofactors on non-proteolytic active-site generation in Glu-Plg by SK variants, each of these molecules were incubated in various combinations for 10 min at 37 °C before the addition of MUGB, at 200 nM: SEN, GAPDH, PAM, Fg, FgD (Fg fragment D) and FgE (Fg fragment E). For active-site experiments in the presence of plasma, SK was added to a 1:10 dilution of pooled EDTA-treated human plasma and MUGB (1 μM). Data were normalized by subtracting a control reaction of all protein species without the addition of SK and 1 μM MUGB. This accounted for intrinsic fluorescence associated with buffer and protein species, as well as non-specific hydrolysis of MUGB over the course of reactions. Fluorescence measurements were expressed as the fractional change in the initial fluorescence $(F_{obs} - F_0)/F_0 = \Delta F/F_0$. Values for rates of active-site generation were calculated using the initial linear portion of the reactions as $(\Delta F \cdot {F_0}^{-1}) \cdot \min^{-1}$. This is subsequently referred to in the simplified form as $\Delta\text{FU} \cdot \min^{-1}$, where FU are fluorescence units. To calibrate relative fluorescence units and convert these measurements into SK–Plg* concentrations, standard curves of known concentrations of the fluorescent product 4-methylumbelliferone were constructed. The fluorescence measurements were made in assay buffer 1 at 37 °C containing 400 nM SK and 200 nM Glu-Plg, with the same instrument settings as MUGB experiments. Values for rates of active-site generation were calculated using the initial linear portion of the reactions as pmol of SK–Plg* $\cdot \min^{-1}$. Initial rates of active-site generation were compared using a two-tailed unpaired Student's *t* test using GraphPad Prism 5.

Plasminogen activation by variant SK–Plg complexes

The capacity of variant SK–Plg complexes to activate substrate Glu-Plg was studied by the addition of SK (final concentration 5 nM) to assay buffer 2 (10 mM Hepes, pH 7.4, 150 mM NaCl and 0.01 % Tween 20) containing a large excess of Glu-Plg (500 nM) and S-2251 (500 μM) in a total volume of 100 μl. The parabolic generation of plasmin was monitored by the change in absorbance at 405 nm measured over 30 min at 37 °C using a SpectraMax Plus 384 spectrophotometer (Molecular Devices). To assess the role of cofactors and ligands, each was pre-incubated with Plg for 10 min at 37 °C at the following concentrations: Fg (500 nM), PAM (500 nM) and α_2-AP (50 nM). For quantitative comparison of ligand effects, the change in $A_{405,}$ which is a function of S-2251 substrate cleavage by plasmin generated during the activation of Glu-Plg by SK, was plotted against t^2. The velocities of these reactions were then calculated from the gradient of A_{405} against t^2. All data transformation and linear regressions were performed in GraphPad Prism 5.

To examine the role of Plg binding to the GAS cell surface of type-2b-expressing strains, whole GAS cells were used as an exogenous source of the cell receptors PAM/Prp (PAM-related protein) to assess the effect on Plg activation, with PAM- and Prp-knockout strains used as controls. For these experiments, a single colony of each strain (NS88.2, NS88.2*prp*, ALAB49 and ALAB49Δ*pam*) was inoculated in THY medium for growth overnight at 37 °C. The overnight culture (D_{600} of 1.1–1.3) was diluted (10 %, v/v) in pre-warmed THY medium and grown to a D_{600} of 0.5. The cells were collected by centrifugation at 5000 ***g*** for 10 min and washed twice with sterile PBS

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(3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, pH 7.4, 1.3 mM KCl and 135 mM NaCl) followed by resuspension of 10^8 CFU (colony-forming units) of each GAS strain per replicate in 90 μl of assay buffer 2 containing 500 nM Glu-Plg and repeated in the presence or absence of 500 nM α_2-AP and/or 500 nM Fg. Protein mixtures were incubated for 10 min at 37 °C in a 96-well plate to bind Plg to the cell surface. To initiate Plg activation, SK_{ALAB49} (GenBank® accession number AY234134.1) or $SK_{NS88.2}$ (GenBank® accession number JQ650490) and S-2251 were added to the wells at 5 nM and 250 μM respectively in a final volume of 100 μl. The exponential generation of plasmin was monitored by the change in absorbance at 405 nm measured over 30 min at 37 °C using a SpectraMax Plus 384 spectrophotometer with shaking between measurements.

Effect of fibrin on the amidolytic activity of variant SK–Plg complexes

To study the amidolytic activity of SK–Plg complexes bound to insoluble fibrin matrices, fibrin-coated microtitre plate wells (96 wells/plate) were prepared by incubating Fg (3 μM) and thrombin (0.8 NIH unit/ml) in 50 μl of sample buffer (0.05 M Tris/HCl, pH 7.75, 0.02 M $CaCl_2$ and 0.1 % Tween 20) at room temperature (23 °C) for 4 h. Wells were blocked for 1 h with 3 % (w/v) BSA and washed five times with washing buffer (0.05 M Tris/HCl, pH 7.75, and 0.1 % Tween 20). Glu-Plg (20 nM) in 100 μl of assay buffer 2 was bound to the fibrin clots for 1 h. Wells were washed five times with assay buffer 2. To measure the amidolytic activity of complexes, each SK variant (40 nM) and S-2251 (500 μM) was added to wells, in the presence or absence of α_2-AP (250 nM) and the change in absorbance at 405 nm was measured over 30 min at 37 °C using a SpectraMax Plus 384 spectrophotometer.

Plasma clot lysis assay

Cross-linked plasma clots were prepared using human plasma pooled from healthy donors, which had been collected using potassium EDTA BD vacutainers. Clotting was initiated by adding human thrombin (0.8 NIH unit/ml) and $CaCl_2$ (20 mM) to plasma at room temperature. Immediately after mixing, 50 μl of the polymerizing plasma was transferred to a 96-well microtitre plate. The plasma clots were formed at room temperature for 2 h and washed once with 50 μl of plasma. Clot lysis was performed by adding 50 μl of pooled plasma containing variant SK (50 nM) to the clot at 37 °C. The clot lysis process was monitored by the change in turbidity at 340 nm using a SpectraMax Plus 384 spectrophotometer. As a control, plasma clots were incubated with 50 μl of plasma without addition of SK to monitor stability during the course of the assay.

Plasma Fg depletion by SK variant plasminogen activation

The level of plasma Fg depletion by each SK variant was determined by a modified version of the sodium sulfite precipitation method [27]. Briefly, SK variants (50 nM, final concentration) were added to 100 μl of pooled EDTA-treated human plasma and incubated at 37 °C for 30 min. Each aliquot was immediately mixed with aprotinin (20 μM) to inhibit plasmin activity, and precipitated with 900 μl of 10.5 % (w/v) sodium sulfite by incubation in a 37 °C water bath for 15 min. Precipitant Fg was cleared from solution by centrifugation at 1500 **g** for 15 min. The supernatant was carefully decanted and the cleared Fg was washed with 500 μl of 10.5 % (w/v) sodium sulfite and re-centrifuged, and the supernatant was removed. To dissolve the collected Fg, 1 ml of 4 M urea in 0.1 M NaOH was added to each tube and incubated at 100 °C for 15 min. Finally, the A_{280} of each solution was measured using a NanoDrop 2000c spectrophotometer (Thermo Fischer Scientific) and the remaining Fg concentration was determined by comparison with control plasma aliquots prepared without addition of SK. Statistical data analysis was conducted via one-way ANOVA with Tukey's multiple-comparison test. Datasets were considered significantly different at $P < 0.05$. All analysis was performed using GraphPad Prism 5.

RESULTS

Non-proteolytic active-site generation in Glu-Plg by variant SK

Active-site generation in Glu-Plg by SK variants (SK–Plg*) was examined using the fluorescent active-site titrant MUGB. This allowed generation of SK–Plg* (conformational activation, Pathway I) to be measured directly. To calibrate relative fluorescence units and convert these measurements into SK–Plg* concentrations, standard curves of known concentrations of the fluorescent product 4-methylumbelliferone were constructed. The fluorescence measurements were made in assay buffer 1 at 37 °C containing 400 nM SK and 200 nM Glu-Plg, with the same instrument settings as MUGB experiments. We concluded that 1.0 mol of 4-methylumbelliferone/mol of SK–Glu-Plg* was formed. It was identified previously that SK_{NZ131} (GenBank® accession number CP000829.1) (type-1) displays the highest rate (3.2 pmol of SK–Plg* $\cdot$ min^{-1}) of conformational activation of Glu-Plg, followed by SKc (GenBank® accession number K02986.1) (1.9 pmol of SK–Plg* $\cdot$ min^{-1}). SK_{NS696} (GenBank® accession number JQ650488) and SK_{5448} (GenBank® accession number JQ650489) (type-2a variants) both display very low rates of active-site generation (0.34 and 0.27 pmol of SK–Plg* $\cdot$ min^{-1} respectively), whereas SK_{ALAB49} and $SK_{NS88.2}$ (type-2b variants) fail to induce an active site in Glu-Plg [16] (Figure 1A). As SK-mediated Glu-Plg activation is affected by the conformation of Glu-Plg, experiments were conducted in the presence of Fg, FgD and FgE to assess the effect on conformational rearrangement of Plg by these molecules. In the presence of Fg and FgD, SK_{NZ131} (type-1) and SKc displayed increased rates of active-site generation (Fg = 0.35 and 0.33 pmol of SK–Plg* $\cdot$ min^{-1} respectively, and FgD = 0.36 and 0.33 pmol of SK–Plg* $\cdot$ min^{-1} respectively) (Figures 1B and 1C). The rate of active-site generation by type-2a variants (SK_{NS696} and SK_{5448}) was also significantly enhanced (1.9–2.0- and 2.6–3.0-fold respectively), but the rates were less than those observed for SK_{NZ131} and SKc (Figures 1B and 1C). The type-2b variants (SK_{ALAB49} and $SK_{NS88.2}$) could generate an active site in Glu-Plg when combined with Fg or FgD; however, this occurred at a low rate of 0.12–0.19 pmol of SK–Plg* $\cdot$ min^{-1} for both variants with each ligand (Figures 1B and 1C). FgE had a minimal effect on the rate of active-site generation for the SK variants and did not allow type-2b SK to generate an active site in Glu-Plg (Figure 1D). This indicated that binding of Glu-Plg to Fg or FgD, but not FgE, results in a conformational change in the Glu-Plg molecule that readily allows non-proteolytic active-site generation (Pathway I) by SK variants.

Effect of GAS cell-surface Plg receptors on non-proteolytic active-site generation in Glu-Plg by variant SK

The binding of plasmin(ogen) to the bacterial cell surface via cell-wall-associated proteins such as PAM and Prp, GAPDH and SEN has been well documented [9,13,28]. To assess the effect of these bacterial Plg receptors on SK-mediated conformational activation

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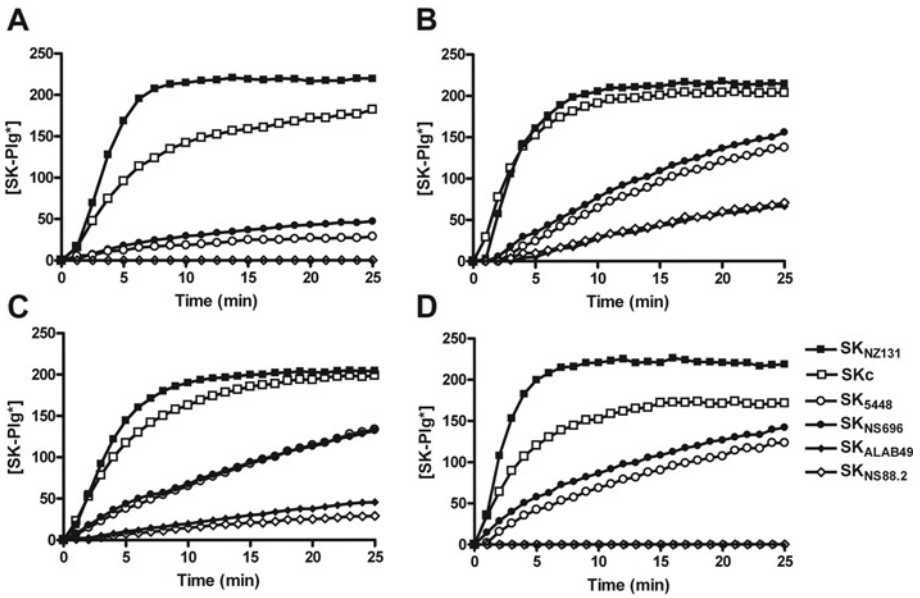

Figure 1 Effect of Fg and Fg fragments on non-proteolytic active-site generation in Glu-Plg and plasminogen activation by SK variants

Glu-Plg alone (200 nM) (**A**) or in the presence of 200 nM Fg (**B**), FgD (**C**) or FgE (**D**) was added to 1 μM MUGB in assay buffer 1 at 37 °C to pre-form for 10 min. To initiate the reaction, SK was added to a final concentration of 400 nM in a total volume of 100 μl and the development of fluorescence was monitored continuously with excitation at 355 nm and emission at 460 nm. For comparison, (**A**) is reproduced from [16] Cook, S.M., Skora, A., Gillen, C.M., Walker, M.J. and McArthur, J.D. (2012) Streptokinase variants from *Streptococcus pyogenes* isolates display altered plasminogen activation characteristics: implications for pathogenesis. Mol. Microbiol. **86**, 1052–1062 with permission. Results are representative of a minimum of two independent experiments in duplicate.

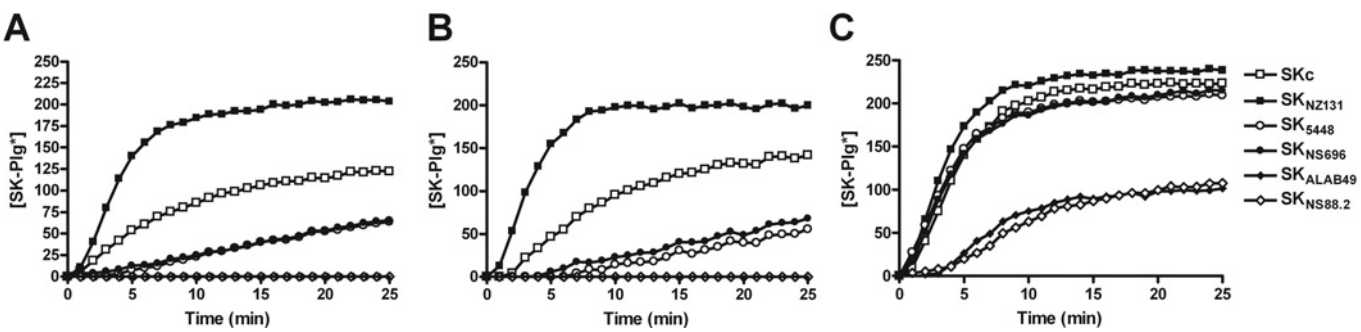

Figure 2 Effect of GAS cell-surface Plg receptors on non-proteolytic active-site generation in Glu-Plg and plasminogen activation by SK variants

Glu-Plg (200 nM) in the presence of 200 nM SEN (**A**), GAPDH (**B**) or PAM (**C**) was added to 1 μM MUGB in assay buffer 1 at 37 °C to pre-form for 10 min. To initiate the reaction, SK was added to a final concentration of 400 nM in a total volume of 100 μl, and the development of fluorescence was monitored continuously with excitation at 355 nm and emission at 460 nm. Results are representative of a minimum of two independent experiments in duplicate.

of Glu-Plg, active-site-generation assays were performed in the presence of recombinant GAPDH, SEN and PAM. Pre-binding Glu-Plg with either SEN or GAPDH had no effect on active-site generation in this molecule by any of the SK variants (Figures 2A and 2B). Interestingly, when Glu-Plg was pre-bound with PAM, type-2b variants (SK$_{ALAB49}$ and SK$_{NS88.2}$) were able to non-proteolytically induce an active site in Glu-Plg. Furthermore, the rate of active-site generation mediated by SKc, and type-2a variants (SK$_{NS696}$ and SK$_{5448}$) were all enhanced, producing activation rates similar to type-1 SK (SK$_{NZ131}$), which was not affected by any of the bacterial cofactors (Figure 2C).

Non-proteolytic active-site generation in human plasma by variant SK

To assess Glu-Plg activation under conditions that are more physiologically relevant, active-site-generation experiments were repeated in the presence of EDTA-treated human plasma pooled from several donors. When added directly to plasma, SKc displayed the highest rate of conformational activation of Glu-Plg, followed closely by SK$_{NZ131}$ (type-1) (0.033 and 0.025 $\Delta FU \cdot min^{-1}$ respectively) (Figure 3A). SK$_{NS696}$ and SK$_{5448}$ (type-2a variants) both displayed low rates of Glu-Plg activation (0.004 and 0.002 $\Delta FU \cdot min^{-1}$), whereas SK$_{ALAB49}$ and SK$_{NS88.2}$ (type-2b variants) failed to induce an active site in Glu-Plg (Figure 3A). This result indicates that type-2b SK variants do not form active complexes with Glu-Plg when circulating in plasma despite the presence of Fg. When this experiment was repeated in the presence of PAM, type-2b variants (SK$_{ALAB49}$ and SK$_{NS88.2}$) were able to induce an active site in Glu-Plg (Figure 3B), indicating that the presence of PAM is required for type-2b SK variants to generate plasmin activity through conformational activation of Glu-Plg in plasma. Given that Fg and α_2-AP are present at significant concentrations in plasma, these results suggest that active type-2b SK–Glu-Plg* complexes formed in the presence of plasma Fg are not resistant to inhibition by α_2-AP unless PAM is also available.

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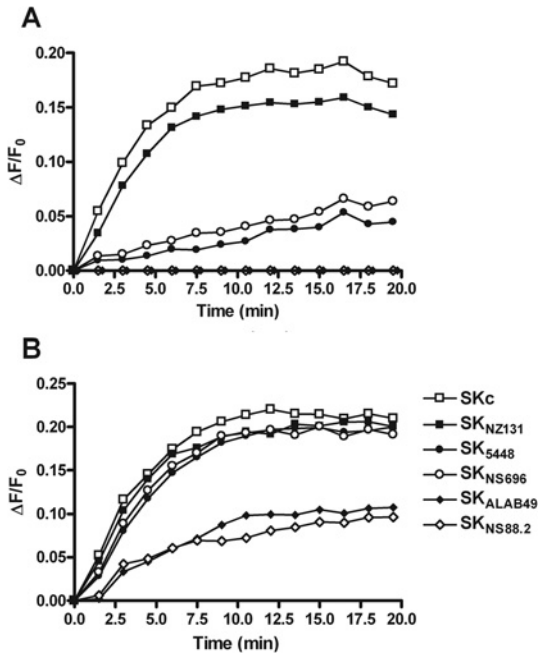

Figure 3 Non-proteolytic active-site generation in Glu-Plg and plasminogen activation by SK variants in human plasma

Pooled human plasma was added (1:10 dilution) to 1 μM MUGB in assay buffer 1 at 37 °C in the absence (**A**) or presence (**B**) of 200 nM PAM. To initiate the reaction, SK was added to a final concentration of 400 nM in a total volume of 100 μl and the development of fluorescence was monitored continuously with excitation at 355 nm and emission at 460 nm. Results are representative of a minimum of two independent experiments in duplicate.

Additionally, in the presence of PAM, SK_{NS696} and SK_{5448} (type-2a variants) displayed 7- and 9.6-fold increases ($P < 0.01$) in active-site generation, whereas reaction rates produced by SKc and type-1 SK_{NZ131} were not affected (Figure 3B). This suggests that interaction of Glu-Plg with PAM via kringle 2 produces a conformation that is favourable for activation by cluster 2 SK variants.

Regulation of soluble Plg activation activity of type-2b SK–Plg variants by host α_2-AP

To investigate how Plg ligands affect the inhibition of SK–Plg activator complexes by α_2-AP, a catalytic amount of SK (5 nM) was added to a 100-fold excess of substrate Glu-Plg, with various combinations of Fg and PAM in the presence or absence of α_2-AP (Figure 4). As expected, SKc could efficiently activate Glu-Plg substrate ($v = 6.9$ milli-absorbance units at 405 nm $\cdot$ min^{-2}) and the initial velocity for rate of activation was partially reduced by the presence of α_2-AP (2.1 milli-absorbance units at 405 nm $\cdot$ min^{-2}) (Figure 4A). As type-2b variants cannot generate an active site in Glu-Plg, no Plg activation activity was observed in the reaction with SK_{ALAB49} (Figure 4A). In the presence of either Fg or PAM, the initial velocity of SKc-mediated Plg activation was enhanced (2.2- and 2.6-fold respectively) and was not inhibited by α_2-AP (Figures 4B and 4C). As type-2b SK variants can non-proteolytically generate an active site in the presence of Fg (Figure 1B) and PAM (Figure 2C), type-2b SK activator complexes could subsequently activate Glu-Plg substrate in the presence of either of these ligands ($v = 1.75$ and 0.6 milli-absorbance units at 405 nm $\cdot$ min^{-2} respectively) (Figures 4B and 4C). However, the activity displayed by these activator complexes was not resistant to inhibition by α_2-AP (Figures 4B and 4C). When SK was added in the presence of both PAM and Fg together, both SKc and type-2b SK (SK_{ALAB49}) could efficiently activate substrate Plg at an initial velocity of 30 milli-absorbance units at 405 nm $\cdot$ min^{-2} and this activity was not inhibited by α_2-AP (Figure 4D).

To determine whether similar results would be observed on the bacterial cell surface, whole GAS cells were used as an exogenous source of Plg receptors (PAM/Prp) in Glu-Plg activation assays. In the presence of wild-type GAS cells (ALAB49 and NS88.2), type-2b SK variants (SK_{ALAB49} and $SK_{NS88.2}$) could readily initiate substrate Glu-Plg activation. In the presence of isogenic PAM/Prp-knockout strains (ALAB49Δ*pam* and NS88.2*prp*), type-2b SK variants could not initiate substrate Glu-Plg activation confirming the essential role for PAM/Prp in type-2b SK-mediated Glu-Plg activation (Figure 4E). α_2-AP was able to inhibit Plg activation at the cell surface in these assays (Figure 4E). As expected, in the presence of purified Fg, type-2b SK variants could initiate Glu-Plg activation in the presence of both wild-type and knockout GAS strains (Figure 4F). Additionally, in the presence of Fg and wild-type GAS cells, cell-surface Plg activation activity was not inhibited by α_2-AP, whereas in the presence of Fg and ALAB49Δ*pam* and NS88.2Δ*prp* cells, Glu-Plg activation activity was inhibited (Figure 4F). These data demonstrate a critical role for both PAM/Prp and Fg as synergistic cofactors in the generation of unregulated Plg activation activity on the surface of GAS strains expressing type-2b SK.

The effect of fibrin on the activity of type-2b SK variants

Therapeutic SK is considered a fibrin-independent agent because it rapidly generates plasmin in the blood at sites distant from fibrin clots. Although type-2b SK variants do not readily activate circulating Glu-Plg, we investigated whether this SK variant could activate conformationally modified Glu-Plg when bound to fibrin. Fibrin clots were produced *in vitro* by the addition of thrombin and $CaCl_2$ to purified human Fg. Glu-Plg was then bound to fibrin and activated by the addition of SK variants (Figure 5). In the absence or presence of fibrin, SKc could readily activate Glu-Plg, whereas type-2b variants (SK_{ALAB49} and $SK_{NS88.2}$) could only activate Glu-Plg that was bound to fibrin (Figures 5A and 5B) and furthermore this activity was not regulated in the presence of α_2-AP (Figure 5C). As type-2b SK variants were able to generate Plg activation activity in the presence of fibrin, we compared the capacity of SKc and type-2b SK variants to lyse plasma clots produced from human plasma. As expected, SKc displayed efficient fibrinolytic activity (Figure 6A). Both type-2b SK proteins (SK_{ALAB49} and $SK_{NS88.2}$) displayed fibrinolytic activity with potency similar to that of SKc (Figure 6A). Additionally, plasma Fg depletion during these activation experiments in plasma was high in SKc-mediated lysis reactions (~99 %), but was significantly reduced in both SK_{ALAB49} ($P < 0.05$) and $SK_{NS88.2}$ ($P < 0.01$) -mediated lysis reactions (~40 % and ~19 % respectively) (Figure 6B). Taken together, these data indicate that the plasmin activity generated by type-2b SK variants is restricted to the surface of the fibrin clot.

DISCUSSION

Plasmin is a potent protease with many important physiological functions including fibrin clot dissolution, tissue repair/remodelling and cell migration [29]. Regulation of the Plg activation system is essential for the maintenance of homoeostatic function and the conversion of Plg into plasmin (the critical first step involved in this process) is a major regulatory target [9]. The conformation of the Glu-Plg molecule greatly influences

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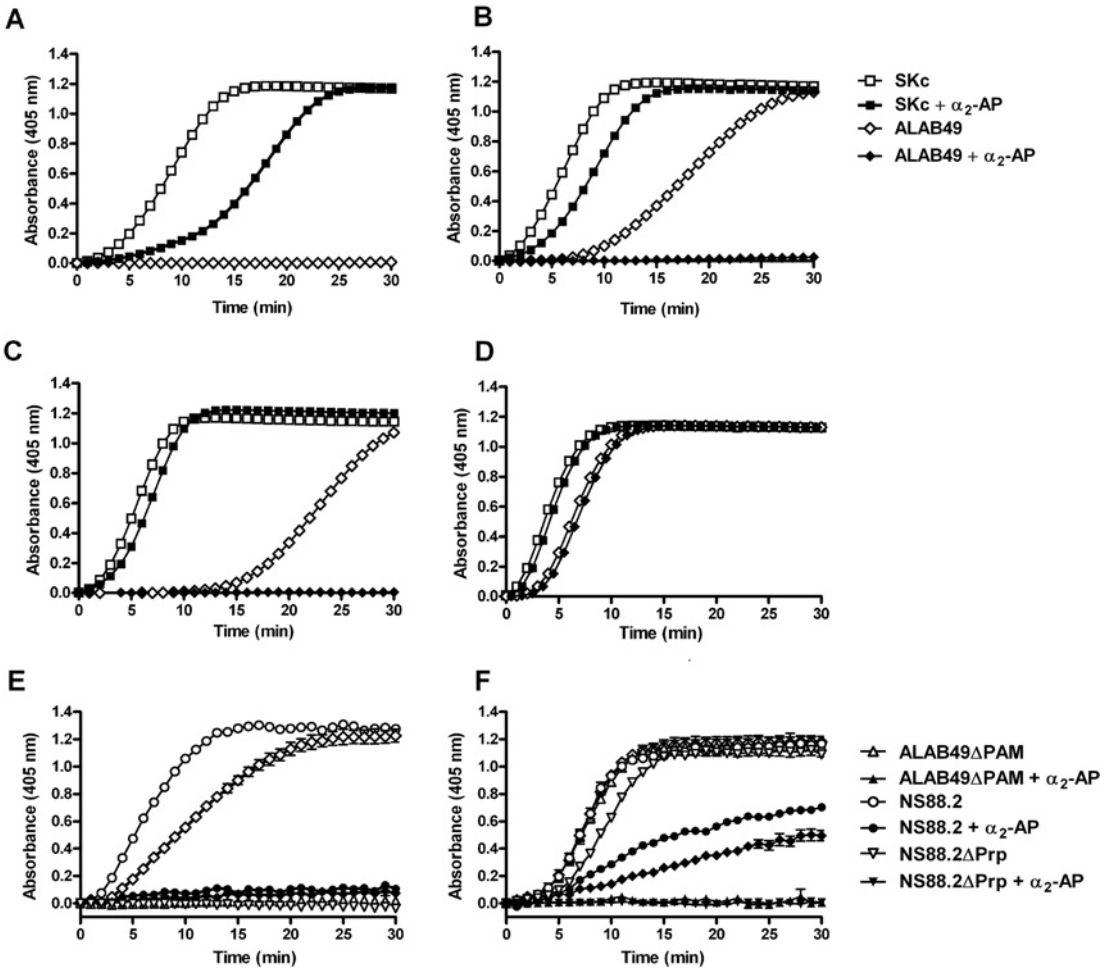

Figure 4 Effect of Fg and PAM on inhibition of SK variant-mediated Plg activation ability by α_2-AP

SK (5 nM final concentration) was added to assay buffer 2 containing S-2251 (500 μM) with combinations of (**A**) Glu-Plg (500 nM), (**B**) Glu-Plg (500 nM) and equimolar Fg, (**C**) Glu-Plg (500 nM) and equimolar PAM, or (**D**) Glu-Plg (500 nM) with equimolar Fg and PAM. (**A**)–(**D**) were tested for resistance to inhibition with α_2-AP (50 nM). Whole-cell GAS experiments were conducted as above with 10^8 cells added as a source of exogenous PAM/Prp with ΔPAM/Prp strains used as controls. To these cells (**E**) Glu-Plg (500 nM) or (**F**) Glu-Plg (500 nM) and equimolar Fg were added. All combinations were tested in the presence or absence of α_2-AP (250 nM). The generation of Plg activation activity was monitored at an absorbance of 405 nm at 37°C. Results are representative of a minimum of two independent experiments in duplicate and are means ± S.E.M.

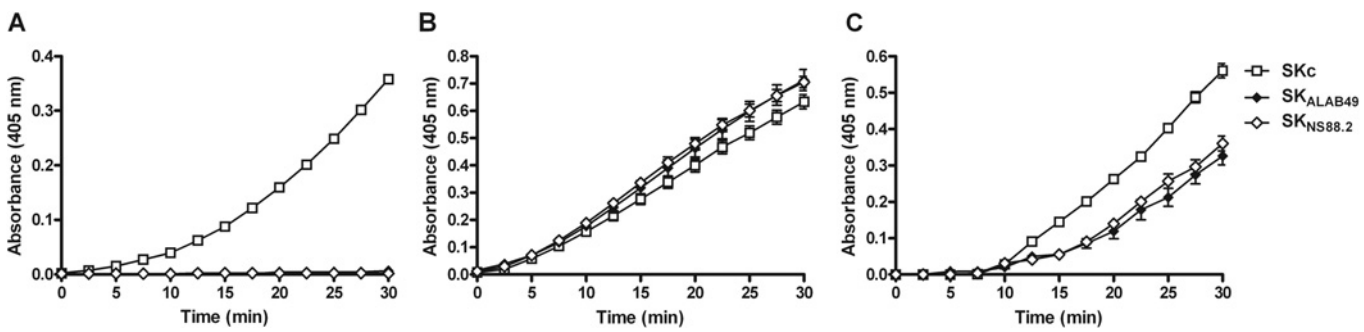

Figure 5 Generation of amidolytic activity and resistance to inhibition of variant SK–Plg complexes formed in the absence or presence of a fibrin clot

(**A**) Complex formation was initiated by addition of variant SK (40 nM) to assay buffer 2 containing Glu-Plg (20 nM) and chromogenic substrate S-2251 (500 μM) then monitored at A_{405}. (**B**) Fibrin clots were formed for 4 h at room temperature in a 96-well plate by the addition of thrombin (0.8 NIH unit/ml) and $CaCl_2$ (20 mM) to Fg (3 μM). Plg (20 nM) was bound to the fibrin clots for 1 h at 37°C. Bound Plg was washed before complex formation was initiated and monitored at A_{405} by addition of variant SK (40 nM) to assay buffer 2 with chromogenic substrate S-2251 (500 μM) at 37°C. (**C**) The ability of SK–Plg complexes formed in the presence of fibrin were also assessed for susceptibility to inhibition by α_2-AP (200 nM). Results are representative of three independent experiments in duplicate and are means ± S.E.M.

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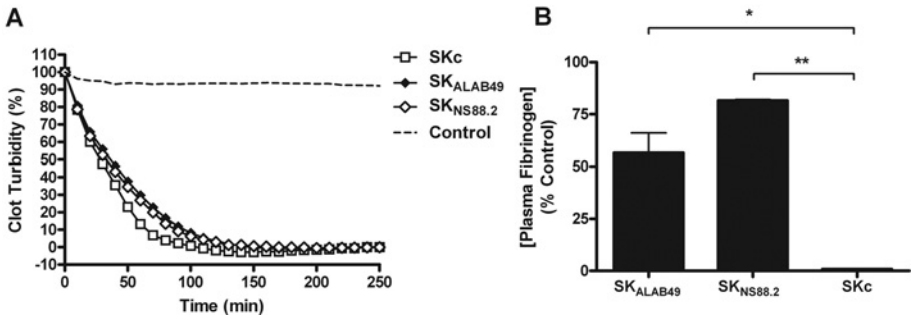

Figure 6 Induction of plasma clot lysis by SK variants and capacity to deplete plasma Fg

(**A**) Fibrin clots were formed in pooled EDTA-treated human plasma by the addition of thrombin (0.8 NIH unit/ml) and $CaCl_2$ (20 mM). Variant SK molecules were then suspended in fresh plasma to a final concentration of 50 nM over the plasma clot, and lysis was monitored by measuring a reduction in turbidity at 340 nm at 37°C. (**B**) Group C SK or type-2b SK variants (50 nM) were added to plasma for 30 min at 37°C. Plasmin activity was inhibited by the addition of aprotinin (20 μM), and plasma Fg concentration was measured using the sodium sulfite precipitation method. Results (means $\pm$ S.E.M.) are standardized to control reactions, which lacked SK, and are representative of three independent experiments in duplicate. *$P < 0.05$ and **$P < 0.01$ (one-way ANOVA).

the regulation of Plg activation. Glu-Plg conformation may be altered upon interaction with ligands, primarily through lysine-binding sites located within the kringle domains, and it is now well recognized that these interactions play a central role in modulating key steps in Plg activation by host and bacterial Plg activators [9,30,31].

Fg is known to enhance activation of human Plg by uPA (urokinase-type plasminogen activator), tPA (tissue-type plasminogen activator) and SK; however, the exact mechanism for this is not defined [32,33]. Whereas full-length Fg is relatively inert in circulation, fibrin(ogen) fragments that enhance plasminogen activation can be generated through numerous physiological processes: Fg proteolysis by thrombin during fibrin deposition, fibrinolysis, and direct Fg proteolysis by SK–Plg activator complexes at the site of bacterial infection [32,34–36]. To differentiate the effect of full-length Fg and Fg fragments on Glu-Plg activation by SK variants, the plasmin-inhibiting fluorogenic active-site titrant MUGB was used to measure SK-Plg* activator complex formation. Our results indicated that full-length Fg and FgD accelerated the formation of SK–Plg* for all SK variants and also permitted type-2b SK to generate an active site in Glu-Plg (Figures 1B, 1C and 4B). Although Glu-Plg and Fg molecules are normally relatively inert in circulation, the interaction between SK, Glu-Plg and Fg must therefore permit a series of conformational modifications that expose binding sites that are not present in native Glu-Plg and Fg molecules. This supports the previously proposed hypothesis that SK, Plg and Fg can interact to form a trimolecular complex [1,37,38].

Bacterial Plg receptors interact with plasmin(ogen) to acquire protease activity at the cell surface [28]. However, the influence of bacterial ligands on Glu-Plg conformation and the subsequent effect on activation kinetics by SK variants has not been assessed. PAM binds to Glu-Plg via interaction with the Plg K2, but this bacterial receptor is restricted to skin-tropic *emm* pattern D GAS strains [39]. SEN and GAPDH are present in all GAS isolates and display weaker Glu-Plg-affinity binding to K1 and K5 through lysine-dependent interactions [40]. SEN and GAPDH do not affect the generation of the SK–Plg* activator complex for any of the SK variants (Figures 2A and 2B). PAM significantly enhanced active-site generation in Glu-Plg by cluster 2 SK variants and, more importantly, allowed the non-proteolytic generation of an active site in Glu-Plg by type-2b SK (Figures 2C and 4C). This suggests that differences in the conformational state adopted by Glu-Plg upon interaction with different ligands affects the potential of these ligands to enhance active-site generation by SK. Therefore it is possible that some bacterial ligands may play a role in Plg activation, whereas others may only function to bind Plg or plasmin. Interestingly, Plg/plasmin bound to the bacterial cell surface has also been shown to function as a bridging molecule, facilitating bacterial adherence and internalization of bacteria into keratinocytes/epithelial cells [41,42]. As this process occurs independently of protease activity, Plg receptors with no role in Plg activation may be useful for such purposes.

Whereas the conformation of Glu-Plg plays an important role in the regulation of plasmin generation, the primary regulator of plasmin activity is α_2-AP. The inhibitor α_2-AP binds to plasmin through an initial interaction with the lysine-binding sites in the kringle domains which is then followed by a slower irreversible covalent modification of the active centre [11,43]. SKc induces an active site in Glu-Plg by the insertion of Ile^{1} of SKc into the Glu-Plg N-terminal binding cleft, which forms a crucial salt linkage with Asp^{740}, in place of Val^{562} of Plg [44]. SKc–Plg* activator complexes are resistant to α_2-AP inhibition which is thought to be the result of SKc occupying or blocking critical lysine-binding sites in Plg and/or through steric shielding of the active site [11]. The inability of type-2b SK to induce an active site in Glu-Plg and the fact that type-2b SK–plasmin activator complexes are inhibited by α_2-AP may suggest that these variants fail to block α_2-AP binding to specific lysine-binding sites in Plg or they are unable to correctly position the N-terminal region of the type-2b SK molecule within Plg [16]. Whereas type-2b SK–Plg* activator complexes that formed in the presence of either Fg or PAM separately can be inhibited by α_2-AP, in the combined presence of PAM and Fg, activator complexes were resistant to α_2-AP inhibition (Figure 4D). This complex of multiple ligands may shield the active site by restricting access for α_2-AP and/or may occupy the lysine-binding sites of Plg that are required for initial α_2-AP binding, functions that cannot be imparted by the presence of each ligand alone. As such, type-2b SK variants require additional ligands to assist with the conformational rearrangement of Glu-Plg to induce a proteolytically active site and further additional ligand combinations are required to protect the newly formed active site from inhibition by α_2-AP. The present study provides the first comprehensive demonstration of the functional relationship between GAS SK variants, bacterial and host Plg-binding ligands and α_2-AP.

It has long been hypothesized that SK-mediated Plg activation, and subsequent recruitment of plasmin activity to the GAS cell surface, acts to prevent plasmin inhibition by α2-AP. By characterizing this delicate interplay between bacterially derived

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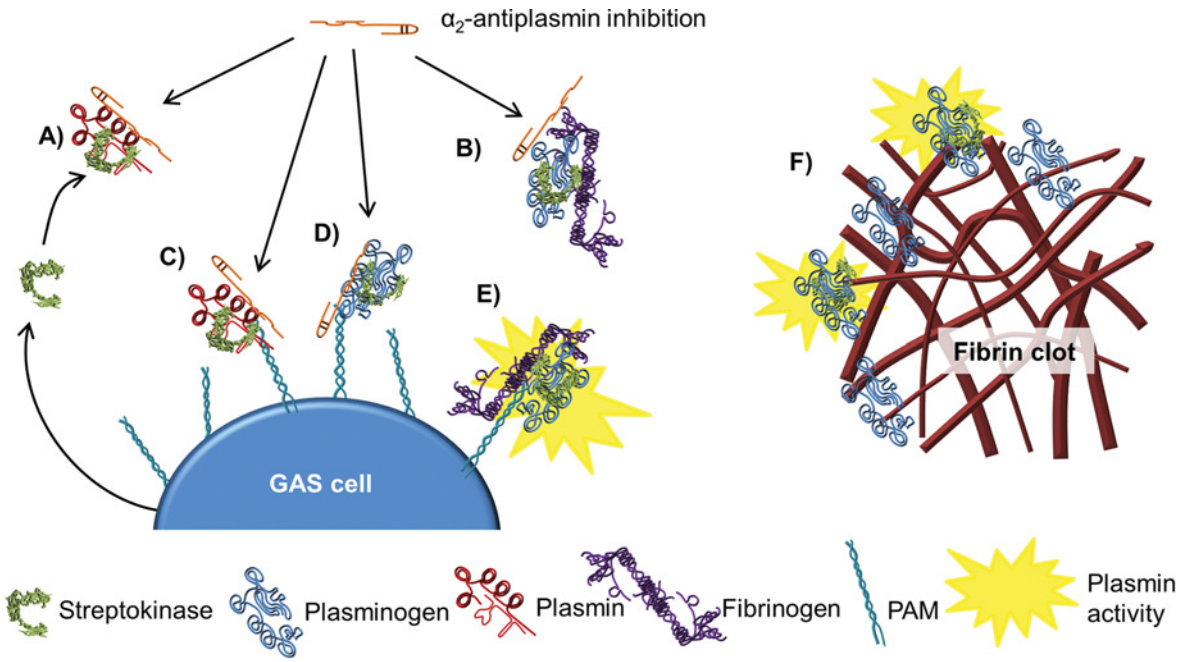

Figure 7 Schematic diagram summarizing the site/surface-restricted activation of plasminogen by type-2b SK-expressing GAS strains

Cluster type-2b SK cannot induce an active site in Glu-Plg. Free complexes of cluster type-2b SK with plasmin (**A**) or Plg/plasmin–Fg (**B**) are inhibited by α_2-AP. Similarly, complexes of cluster type-2b SK with Plg/plasmin with PAM are also inhibited by α_2-AP (**C** and **D**). Cluster type-2b SK can only generate unregulated plasmin activity when a trimolecular complex of SK–Plg–Fg is bound to the cell-surface PAM (**E**) or Plg is bound to fibrin (**F**). Therefore plasmin activity generated by cluster type-2b SK is restricted to the bacterial cell surface or to fibrin thrombi.

SK variants and cell-surface receptors such as SEN, GAPDH, M proteins (PAM and Prp) and host proteins such as Fg, fibrin and α_2-AP, we have been able to define a possible mechanism for how these phenotypic variations influence the pathogenic GAS strains (Figure 7). Whereas type-1 and type-2a SK variants display Plg activation/inhibition mechanisms that are similar to SKc, GAS strains expressing type-2b SK have evolved a distinct and unique system for the interaction with the human Plg activation system that limits soluble plasmin generation. Unlike other SK variants, type-2b SK proteins cannot conformationally induce an active site in Glu-Plg when it has a closed conformation. Type-2b SK can form an activator complex with plasmin and with Plg–PAM or Plg–Fg complexes; however, these complexes are readily inhibited by α_2-AP (Figures 7A–7D). Type-2b SK will form an activator complex that is resistant to α_2-AP inhibition when Glu-Plg is bound by a combination of both PAM and Fg or when Glu-Plg is bound to fibrin (Figures 7E and 7F). As a result, Plg activation by type-2b SK will be limited by the availability of cofactors and will restrict plasmin activity to specific microenvironments such as the bacterial cell surface and to the fibrin clot surface. This Plg activation mechanism displayed by type-2b SK has similarities to the Plg activation activity of staphylokinase produced by *Staphylococcus aureus*. Both activators fail to generate soluble plasmin activity, as fluid-phase activator complexes are readily inhibited by α_2-AP. *S. aureus* is a ubiquitous skin colonizer. Similarly, epidemiological studies have shown the type-2b *ska* lineage to be largely restricted to *pam*-positive *emm* pattern D GAS strains which have a strong tendency to cause skin infections [18]. Therefore these characteristics could limit systemic bacterial dissemination as there would be low activation of metalloproteases and less degradation of extracellular matrix components [45]. Plasmin activity that is restricted to the cell surface could promote bacterial persistence in the skin by facilitating evasion of innate immune defences, producing delayed wound healing and allowing transmission to new hosts [46].

Bacterial activators with 'fibrin-specific' Plg activation activity such as staphylokinase and a truncated form of SKc (SKΔ59) have been proposed as potential thrombolytic therapeutics with improved fibrinolytic activity [47,48]. The fibrin selectivity displayed by these activators results from (i) a requirement of plasmin to generate a functional Plg activation complex, and (ii) free activator complexes being inhibited by α_2-AP, thereby restricting plasmin activity to the fibrin clot environment. Similar to these activators, soluble type-2b SK activator complexes are inhibited by α_2-AP. However, unlike staphylokinase and SKΔ59, type-2b SK is not reliant on trace plasmin present in low quantities at the clot surface to produce an activator complex. Type-2b SK activators are capable of activating fibrin-bound Glu-Plg which is present in much higher quantities when compared with free plasmin. As the fibrinolytic potency of type-2b SK variants was comparable with that of SKc (Figure 6A), but with significantly reduced Fg depletion (Figure 6B), these variants could be useful for thrombolytic therapeutic applications.

AUTHOR CONTRIBUTION

Simon Cook cloned, expressed and purified recombinant SK protein and performed all experiments. Amanda Skora expressed and purified SEN, GAPDH and PAM. Simon Cook and Jason McArthur conceived the study, designed all experiments and prepared the paper. Martina Sanderson-Smith contributed to whole-cell GAS assay experimental design and preparation of the paper. Mark Walker assisted in preparation of the paper. All authors contributed to and have approved the paper.

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