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Streptokinase variants from Streptococcus pyogenes isolates display altered plasminogen activation characteristics - implications for pathogenesis

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Abstract

Streptococcus pyogenes (group A streptococcus, GAS) secretes streptokinase, a potent plasminogen activating protein. Among GAS isolates, streptokinase gene sequences (ska) are polymorphic and can be grouped into two distinct sequence clusters (termed cluster type-1 and cluster type-2) with cluster type-2 being further divided into sub-clusters type-2a and type-2b. In this study, far-UV circular dichroism spectroscopy indicated that purified streptokinase variants of each type displayed similar secondary structure. Type-2b streptokinase variants could not generate an active site in Glu-plasminogen through non-proteolytic mechanisms while all other variants had this capability. Furthermore, when compared with other streptokinase variants, type-2b variants displayed a 29- to 35-fold reduction in affinity for Glu-plasminogen. All SK variants could activate Glu-plasminogen when an activator complex was preformed with plasmin; however, type-2b and type-1 complexes were inhibited by α2-antiplasmin. Exchanging skatype-2a in the M1T1 GAS strain 5448 with skatype-2b caused a reduction in virulence while exchanging skatype-2a with skatype-1 into 5448 produced an increase in virulence when using a mouse model of invasive disease. These findings suggest that streptokinase variants produced by GAS isolates utilize distinct plasminogen activation pathways, which directly affects the pathogenesis of this organism.

Keywords

isolates, altered, pyogenes, plasminogen, streptococcus, activation, variants, characteristics, implications, pathogenesis, streptokinase, display, CMMB

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Title: Streptokinase variants from *Streptococcus pyogenes* isolates display altered plasminogen activation characteristics – implications for pathogenesis.

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Short title: Streptokinase variation affects pathogenesis

Key words: Streptokinase, *Streptococcus pyogenes*, plasminogen, pathogenesis

Summary

Streptococcus pyogenes (group A streptococcus, GAS) secretes streptokinase, a potent plasminogen activating protein. Among GAS isolates, streptokinase gene sequences (*ska*) are polymorphic and can be grouped into two distinct sequence clusters (termed cluster type-1 and cluster type-2) with cluster type-2 being further divided into sub-clusters type-2a and type-2b. In this study, far-UV circular dichroism spectroscopy indicated that purified streptokinase variants of each type displayed similar secondary structure. Type-2b streptokinase variants could not generate an active site in Glu-plasminogen through non-proteolytic mechanisms while all other variants had this capability. Furthermore, when compared to other streptokinase variants, type-2b variants displayed a 29-35 fold reduction in affinity for Glu-plasminogen. All SK variants could activate Glu-plasminogen when an activator complex was preformed with plasmin; however type-2b and type-1 complexes were inhibited by α_2-antiplasmin. Exchanging $ska_{\text{type-2a}}$ in the M1T1 GAS strain 5448 with $ska_{\text{type-2b}}$ caused a reduction in virulence while exchanging $ska_{\text{type-2a}}$ with $ska_{\text{type-1}}$ into 5448 produced an increase in virulence when using a mouse model of invasive disease. These findings suggest that streptokinase variants produced by GAS isolates utilise distinct plasminogen activation pathways which directly affects the pathogenesis of this organism.

Introduction

Group A streptococcus (GAS; *Streptococcus pyogenes*) is a human specific pathogen responsible for a diverse range of diseases which have a major impact on global morbidity and mortality rates (Carapetis *et al.*, 2005). GAS readily colonize skin and pharyngeal tissue producing mild superficial infections such as pyoderma, impetigo and pharyngitis. However, GAS may also produce life-threatening systemic (streptococcal toxic shock syndrome) and invasive infections (necrotizing fasciitis). Additionally, post-infection sequelae can occur, which include post streptococcal glomerulonephritis and acute rheumatic fever (Cunningham, 2000).

To cause this diverse range of diseases, GAS employ a broad range of virulence factors that facilitate bacterial colonization, evasion of the immune response and systemic dissemination (Tart *et al.*, 2007, Musser & Shelburne, 2009, Olsen *et al.*, 2010, Cole *et al.*, 2011). Virulence factor expression is exquisitely controlled by 13 two-component regulatory

systems and 30 transcriptional regulators allowing GAS to adapt to the dynamic physiological conditions encountered during the infection process (Kreikemeyer *et al.*, 2003, Sumby *et al.*, 2006). Many of the virulence factors produced by GAS interact specifically with human plasma proteins including fibrinogen, plasmin(ogen), IgG, α_2-macroglobulin, albumin and numerous complement factors (Cunningham, 2000, Walker *et al.*, 2005). GAS can encounter plasma proteins during invasive systemic dissemination through the vasculature, but may also be exposed to plasma constituents at the site of infection through vascular leakage produced during the inflammatory response induced in the host (Herwald *et al.*, 2004).

The interaction of GAS with the plasminogen activation system of the host is a virulence mechanism critical for the invasive pathogenesis of this organism (Sun *et al.*, 2004, Khil *et al.*, 2003, Walker *et al.*, 2005, Sun *et al.*, 2012). Plasminogen (Plg), a single-chain glycoprotein zymogen of the serine protease plasmin, is a key component of the fibrinolytic system and is found in plasma and extracellular fluids. GAS can bind plasmin(ogen) to the cell surface via numerous cell wall associated proteins such as M proteins (PAM, Prp), glyceraldehyde-3-phosphate (GAPDH) and streptococcal enolase (SEN) (Lahteenmaki *et al.*, 2001, Walker *et al.*, 2005, Cole *et al.*, 2011). GAS also secrete a Plg activating protein, designated streptokinase (SK), which facilitates the production of both soluble and cell-bound plasmin activity. The generation of plasmin activity at the site of infection may result in the activation of host matrix metalloproteinases, degradation of extracellular matrix and/or tissue barriers and degradation of fibrin networks produced by the host to confine the initial infection (Walker *et al.*, 2005). These processes allow bacteria to spread to other, normally sterile, sites of the body.

Unlike mammalian Plg activators that activate Plg by limited proteolytic cleavage, SK binds to Plg inducing conformational changes in the molecule that results 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 molecules of Plg and proteolytically convert those to plasmin (Boxrud *et al.*, 2000, Boxrud *et al.*, 2004). Plasmin (which has a higher affinity for SK than Plg) rapidly displaces Plg in the SK-Plg* complex to produce an irreversibly activated SK-plasmin complex that is the main catalyst responsible for the full conversion of Plg to plasmin. (known as ‘Direct Proteolytic Activation Pathway’ or ‘Pathway II’) (Boxrud *et al.*, 2000, Boxrud *et al.*, 2004). These SK-plasmin activator complexes are

also resistant to inhibition by host plasma inhibitors (α_2-antiplasmin (α_2-AP) and α_2-macroglobulin), thereby allowing complexes to sequester and activate substrate Plg while bypassing host protease regulation mechanisms (Parry *et al.*, 2000).

SK is a single chain, 414 amino acid protein, composed of three distinct domains; α (aa 1-150), β (aa 151-287) and γ (aa 288-414) (Wang *et al.*, 1998). The three domains of SK are separated by two coiled-coil regions while the N- and C- termini of the protein have disordered flexible structures (Wang *et al.*, 1998). Despite extensive research, the exact role each domain plays in the activation of human Glu-plasminogen (Glu-Plg) is still not completely understood. While the majority of structural and functional studies conducted to date have used the therapeutic form of SK (originally isolated from the group C streptococcal isolate H46A) (Christensen, 1945), SK proteins from group A streptococcal isolates display considerable variability and have not been well characterized (Kalia & Bessen, 2004, McArthur *et al.*, 2008, Kapur *et al.*, 1995). Phylogenetic studies of the most divergent *ska* sequences have revealed two main sequence clusters (cluster type-1 and 2) with evidence of smaller sub-clusters observed in cluster type-2 sequences (cluster type-2a and 2b) (Kalia & Bessen, 2004, McArthur *et al.*, 2008). While some phenotypic differences displayed by GAS type-1 and type-2 SK variants have been identified (McArthur *et al.*, 2008), further research is required to determine if these variants play differing roles in pathogenesis. In this study, we found that SK variants produced by GAS isolates display different mechanisms of Plg activation and that this process directly affects GAS pathogenesis.

Results

Expression and secondary structural analysis of recombinant SK proteins

To characterize the phenotypic differences displayed by SK proteins from different group A streptococcal isolates, a cluster type-1 SK protein (SK_{NZ131}), two cluster type-2a SK proteins (SK_{5448} and SK_{NS696}) and two cluster type-2b SK proteins (SK_{ALAB49} and $SK_{NS88.2}$) were cloned, sequenced and expressed as recombinant proteins. SK from the group C streptococcal isolate H46A (SK_{H46A}) was also produced as a recombinant protein using the same methodology for use as a positive control. From the alignment of the deduced amino acid sequences (Fig. 1), the type-1 SK protein had the most divergent β domain sequence (66% identity). The β domains from type-2b SK proteins and type-2a SK were more conserved displaying 85% and 91% identity respectively. The type-1 SK protein also had the most

conserved α and γ domains (94% and 97% identity) while these domains in type-2a and type-2b proteins were less conserved with identities ranging from 83% to 87% for α domains and 88% to 90% for γ domains.

Recombinant SK proteins were analyzed for size and purity by SDS-PAGE (Fig. 2A). Proteins ranged in size from 44 to 49 kDa, which is similar to the sizes observed for native streptokinase proteins present in GAS culture supernatants (McArthur *et al.*, 2008). All protein preparations were free from contaminating proteins. Far-UV circular dichroism spectroscopy was utilized to compare the secondary structure of all recombinant SK proteins. Despite significant differences in amino acid composition, variant SK proteins had similar molar residue ellipticity spectra across the full range of wavelengths measured. The output spectra observed was typical of that expected for an amalgamation of α helical (minima at ~ 210 nm maxima at ~190 nm) and anti-parallel β sheet (minima at ~215 nm and maxima at ~195 nm) spectra indicative of the known structure of SK (Wang *et al.*, 1998) (Fig. 2B).

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 4-methylumbelliferyl p-guanidinobenzoate (MUGB). This allowed generation of SK-Plg* (conformational activation, Pathway I) to be measured directly. SK_{NZ131} (Type 1) displayed the fastest rate of conformational activation of Glu-Plg, followed by SK_{H46A} (Group C) (Fig. 3A). SK_{NS696} and SK_{5448} (type-2a variants) both displayed very slow rates of Glu-Plg activation while SK_{ALAB49} and $SK_{NS88.2}$ (type-2b variants) failed to induce an active site in Glu-Plg (Fig. 3A). As SK mediated Glu-Plg activation is known to be affected by the conformation of Plg, experiments were conducted in the absence of Cl^- ions to compare the effect of 'open' Plg conformation on non-proteolytic active site generation (McCance & Castellino, 1995). Under these conditions SK_{NZ131} and SK_{H46A} displayed increased rates of active site generation that were very similar (Fig. 3B). The rate of active site generation by type-2a variants (SK_{NS696} and SK_{5448}) was also significantly enhanced, but was less than that observed for SK_{NZ131} and SK_{H46A} (Fig. 3B). Interestingly, type-2b variants (SK_{ALAB49} and $SK_{NS88.2}$) both failed to generate an active site in open Glu-Plg (Fig. 3B). Taken together, these data indicate that type-2b SK variants cannot conformationally induce an active site in open or closed forms of Glu-Plg.

Binding affinity of SK variants to human Glu-Plg and plasmin

To determine if SK variants display differences in affinity for human Glu-Plg and plasmin, the binding of each SK variant to immobilized Plg and plasmin was assessed using surface plasmon resonance (SPR) analysis (Fig. S1-2). Group C SK (SK_{H46A}), type-1 SK (SK_{NZ131}) and type-2a SK (SK_{5448} and SK_{NS696}) variants all displayed high affinity for human Glu-Plg (K_D ranging from 62 – 88 nM). In contrast, type-2b SK variants had lower affinities for human Glu-Plg with SK_{ALAB49} and $SK_{NS88.2}$ displaying a 29 and 35-fold reduction respectively (Table 1). All SK variants had increased (69 - 347 fold) affinity for plasmin over Glu-Plg. Group C SK (SK_{H46A}), type-1 SK (SK_{NZ131}) and type-2a SK (SK_{5448} and SK_{NS696}) had K_D ranging from 0.37 – 1.03 nM, while type-2b (SK_{ALAB49} and $SK_{NS88.2}$) displayed slightly lower affinities for plasmin with K_D values of 11.5 nM and 6.2 nM respectively (Table 1).

Plasminogen activation by variant SK-plasmin activator complexes

The ability of variant SK-plasmin complexes to sequester and activate substrate Plg was determined by mixing preformed, stoichiometric SK-plasmin activator complexes (5 nM) with an excess of substrate Glu-Plg and monitoring the generation of plasmin activity using the chromogenic substrate, S2251. All variant SK-plasmin complexes examined in this study were capable of efficient substrate Plg activation (Fig 4A). The plasmin activity displayed by the plasmin-SK activator complex is known to be resistant to the major physiological plasmin inhibitor α_2-AP (Cederholm-Williams, 1979). In this study, we observed that complexes of plasmin with SK_{H46A} or type-2a SK (SK_{5448} and SK_{NS696}) variants were also resistant to inhibition by α_2-AP (Fig. 4B). Interestingly, complexes of plasmin with type-2b SK variants (SK_{ALAB49} and $SK_{NS88.2}$) or type-1 SK (SK_{NZ131}) were susceptible to inhibition by α_2-AP, displaying IC_{50} values of 20 nM, 35 nM and 7 nM respectively (Fig. 4B).

Role of SK variation in GAS pathogenesis

In vitro characterization of the isogenic mutants 5448Δ*ska*, 5448::ska_{ALAB49} and 5448::ska_{NZ131} indicated all strains maintained similar growth rates and expressed similar amounts of hyaluronic capsule (Fig. S3A-B). Additionally, 5448Δ*ska* did not produce SK while 5448::ska_{ALAB49} and 5448::ska_{NZ131} both secreted the exchanged variant of SK (Fig. S3C). Utilizing the humanized Plg transgenic mouse line *AlbPLG1*, the virulence of the wild-type 5448 and the isogenic mutant GAS strains, 5448Δ*ska*, 5448::ska_{ALAB49} and 5448::ska_{NZ131} was assessed. The virulence of GAS strain 5448 has been well characterized and has previously been shown to be virulent in this mouse model (Fig. 5) (Maamary *et al.*,

2010, Walker *et al.*, 2007). In comparison to the wild-type 5448 strain, the virulence of 5448Δ*ska* and 5448::ska_{ALAB49} was significantly reduced ($P < 0.05$; 50% vs. 10% mortality) (Fig. 5). Conversely, the virulence of 5448::$ska_{NZ131,}$ is increased when compared to the wild-type 5448 strain, although these data were not statistically significant ($P > 0.05$; 100% vs. 50% mortality) (Fig. 5). Taken together, these data suggest that the unique Plg activation kinetics/properties displayed by the different SK variants affect the pathogenesis of GAS.

Discussion

GAS is a versatile human pathogen capable of causing a wide range of human diseases. The broad pathogenicity of GAS is underpinned by the genetic diversity displayed by clinical isolates of the species. There is a large amount of evidence in the literature describing how the absence or presence of virulence genes or changes in the complex regulatory mechanisms controlling the expression of these genes can alter the pathogenicity of a particular GAS isolate (Sumby *et al.*, 2006, Walker *et al.*, 2007, Kreikemeyer *et al.*, 2003, Musser & Shelburne, 2009, Cole *et al.*, 2011). Similarly, allelic variation of specific virulence genes may also influence the pathogenicity of GAS isolates however, these changes may be more subtle and therefore more difficult to characterize.

Allelic variation of the *ska* gene has been well characterized (Kalia & Bessen, 2004, Kapur *et al.*, 1995, McArthur *et al.*, 2008). Bioinformatical analyses of predicted SK protein sequences suggested that SK variants maintain similar secondary structure despite differences in the amino acid sequences (Kapur *et al.*, 1995). In this study, the six SK proteins were specifically chosen as representatives of divergent sequence clusters and despite significant amino acid sequence differences, all recombinant SK proteins displayed similar secondary structures as indicated by individual CD spectra. This data further supports the hypothesis that selection pressure may be placing structural constraints on SK molecules (Kapur *et al.*, 1995). However, biochemical analysis of the SK variants presented in this study clearly demonstrates that these variants do display a number of different phenotypic properties, which alter the ability of these molecules to interact with and activate human Glu-Plg.

GAS SK variants display significant differences in ability to non-proteolytically generate an active site in Glu-Plg. Type-2b SK variants could not induce the formation of an active site in Glu-Plg. Additionally, type-2b SK variants have a 25 fold less affinity for Glu-Plg when

compared to other SK variants which all displayed similar high affinities (Table 1). Therefore, the inability of type-2b SK variants to produce an active site in Glu-Plg may be the result of the type-2b variants failing to interact with Glu-Plg. However, type-1 SK, group-C SK and type-2a SK molecules which all bind to Glu-Plg with similar, high affinity, also displayed different rates of active site formation in Glu-Plg. These data suggest that while the formation of the nascent SK-Plg complex plays a role in the generation of a conformationally re-arranged active site in Glu-Plg, other protein specific changes may also be affecting this process.

SK_{H46A} mutants with deletions or site directed amino acid changes within the α domain, also form complexes with Glu-Plg that display delays in the generation of amidolytic activity (Fay & Bokka, 1998, Wang *et al.*, 1999, Rodríguez *et al.*, 1995, Young *et al.*, 1995). In particular, residues 1-59 of the SK α domain have been shown to be critical for SK mediated Plg activation (Young *et al.*, 1995, Reed *et al.*, 1999, Mundada *et al.*, 2003) SK mutants (such as α domain truncation mutants and numerous amino terminal site directed mutants) also display reduced amidolytic activity, reduced Plg affinity and increased susceptibility to α_2-AP inhibition (Fay & Bokka, 1998, Sazonova *et al.*, 2004, Rodríguez *et al.*, 1995, Boxrud *et al.*, 2000, Mundada *et al.*, 2003). Upon mixing of SK and Glu-Plg, SK is rapidly cleaved at the Lys_{59}-Ser_{60} peptide bond once bound to Plg (Shi *et al.*, 1994). The N-terminal peptide remains associated (non-covalently) with the SK-Plg complex and is required for non-proteolytic active site induction and stabilization of the activator complex (Parrado *et al.*, 1996, Young *et al.*, 1995, Wang *et al.*, 1999, Mundada *et al.*, 2003). For SK to induce an active site in Plg, the SK Ile_1 residue must be positioned within the Plg molecule so that it can form a salt bridge with Asp_{740} of Plg (Wang *et al.*, 1999). SK mutants lacking this residue cannot induce an active site in Plg through non-proteolytic mechanisms (Wang *et al.*, 1999, Mundada *et al.*, 2003). While all SK variants examined in this study had an N-terminal Ile residue, cluster type-2 SK proteins contain over 11 amino acid changes in the 1-59 region (Fig. 1). These changes may prevent the correct positioning of the N-terminal fragment within the SK-Plg complex thereby preventing (or slowing) non-proteolytic active site formation in Glu-Plg.

The physiological inhibitor of plasmin, α_2-AP, is a member of the serpin family and tightly regulates the activity of plasmin in plasma (Aoki *et al.*, 1993). When plasmin reacts with α_2-AP, the serpin is cleaved resulting in a covalently bound complex of plasmin and α_2-AP that is inactive (Shieh & Travis, 1987). Activator complexes consisting of Plg/plasmin and

therapeutic SK (SK_{H46A}) are known to be resistant to α_2-AP inhibition (Cederholm-Williams, 1979). Similarly, we found complexes of plasmin and type-2a SK (SK_{5448} and SK_{NS696}) variants were also resistant to inhibition by α_2-AP, while complexes of plasmin and type-2b SK variants (SK_{ALAB49} and $SK_{NS88.2}$) or type-1 SK (SK_{NZ131}) were not (Fig. 4B). The susceptibility of type-2b SK for inhibition by α_2-AP and inability of this variant to non-proteolytically generate in active site in Glu-Plg suggests that GAS isolates expressing a type-2b SK molecule have evolved novel mechanisms to control SK mediated Plg activation. The apparent requirement of plasmin for type-2b SK mediated activation of Plg also suggests that Plg activation may be restricted to areas where there is free plasmin (i.e. at the sites of fibrinolysis). Alternatively, SK variants may require additional cofactors (either host or bacterial in origin) to facilitate successful Plg activation, such as: fibrinogen, SEN, GAPDH or PAM (McArthur *et al.*, 2008, Gaffney *et al.*, 1988, Lahteenmaki *et al.*, 2001).

Subversion of the host Plg activation system is a well documented pathogenic mechanism used by GAS and other bacterial pathogens to cause disease (Boyle & Lottenberg, 1997, Coleman & Benach, 1999, Cole *et al.*, 2011). SK mediated Plg activation has been shown to play a critical role in the invasive pathogenesis of GAS (Sun *et al.*, 2004, Cole *et al.*, 2011). M1T1 GAS strains are considered a highly virulent clone capable of causing serve invasive disease of humans. Consequently, the M1T1 GAS strain 5448 used in this study (which contains a type-2a *ska* allele) is highly virulent for the human Plg transgenic mouse strain that was used in these experiments (Cole *et al.*, 2011, Maamary *et al.*, 2010, Walker *et al.*, 2007). In this study, the acquisition of the type-1 *ska* allele increased M1T1 virulence. Similarly, GAS strain NZ131 (type-1 SK) has previously been shown to be virulent in other mouse models of GAS infection. (Kuo *et al.*, 1998, Tsao *et al.*, 2001, Li *et al.*, 2011). When the type-2a *ska* allele of the M1T1 strain 5448 is replaced with a type-2b allele (ska_{ALAB49}), the invasive pathogenesis of this strain is reduced, similar to a level seen for the isogenic *ska* deletion mutant (5448Δ*ska*) (Fig. 5). Previous studies conducted in our laboratory have shown that wild-type ALAB49 is avirulent in this mouse model (Maamary *et al.*, 2010). This result indicates that type-2b SK cannot reproduce the *in vivo* function of a type-2a SK in a M1T1 genetic background and suggests the requirement of plasmin by type-2b SK to form an efficient activator complex affects the invasive pathogenesis of GAS. Previous studies have shown a strong association between cluster type-2b alleles and skin tropic *emm* pattern D strains containing the high affinity Plg binding M-protein PAM (Kalia & Bessen, 2004). Taken together, these observations suggest that type-2b SK proteins require bacterial co-

289 factors present only in a subset of strains for efficient plasmin acquisition or that
290 reduced/restricted Plg activation kinetics produced by type-2b SK *in vivo* may be beneficial
291 for successful long term skin infections such as impetigo. Similarly, the Plg activator
292 staphylokinase from the ubiquitous skin colonizer, *Staphylococcus aureus*, also requires
293 plasmin for efficient Plg activation, which suggests this mechanism of Plg activation may be
294 advantageous for skin colonization (Grella & Castellino, 1997). Additionally, Plg acquisition
295 and activation mechanisms have been documented for commensal strains of oral streptococci,
296 which also suggests this process may also be involved in maintaining long term infections
297 (Kinnby *et al.*, 2008).

298 This study confirms the importance of SK mediated Plg activation in GAS pathogenesis and
299 highlights a mechanism whereby variability in this important virulence factor can influence
300 the pathogenesis of this organism. Characterizing GAS SK variants represents a novel
301 approach to elucidate the mechanism of SK-mediated Plg activation. Therefore, future
302 comparative studies that characterize GAS SK variants in more detail will help to identify
303 critical residues involved in SK function and could assist the rational design of new drugs
304 targeting this specific interaction which may be useful in treating GAS infections.
305

306 ## Experimental Procedures

307

308 *Bacterial strains and reagents used in this study*

309 GAS isolates NZ131 (Simon & Ferretti, 1991), ALAB49 (Svensson *et al.*, 2000), NS88.2
310 (McKay *et al.*, 2004), NS696 (McKay *et al.*, 2004), 5448 (Aziz *et al.*, 2004) and
311 *S. equisimilis* strain H46A (Christensen, 1945) were used in this study. All streptococci
312 strains were routinely cultured at 37°C on horse-blood agar (Biomerieux, Sydney, NSW,
313 Australia) or in static liquid cultures of Todd-Hewitt broth (BD, Sydney, NSW, Australia)
314 supplemented with 1% (w/v) yeast extract (Oxoid, Adelaide, SA, Australia) (THY medium).
315 *Escherichia coli* strains JM109 and M15[pREP4], were used as hosts for plasmid
316 construction and protein expression respectively and were cultured at 37°C in Luria-Bertani
317 broth. Where appropriate, antibiotics were used for selection at the following concentrations:
318 chloramphenicol, 2 μg mL^{-1} for streptococci and 100 μg mL^{-1} for *E. coli*; erythromycin, 2 μg
319 mL^{-1} for streptococci and 200 μg mL^{-1} for *E. coli*; kanamycin, 50 μg mL^{-1}; and ampicillin,
320 100 μg mL^{-1} for *E. coli*. Glu-Plg and α_2-AP were purchased from Haematologic

Technologies, Essex Junction, VT, USA. Chromogenic substrate H-D-Val-Leu-Lys-pNA·2HCl (S-2251) was from Chromogenix, Mölndal, Sweden.

Cloning, expression and purification of recombinant SK proteins

The SK encoding gene (*ska*) from each GAS strain was amplified from genomic DNA by polymerase chain reaction (PCR). PCR primers were designed to incorporate *Bam*H1 and *Pst*I restriction sites at the 5' and 3' ends of the fragment respectively. This allowed cloning into pQE-30 (Qiagen, Valencia, CA, USA) for expression of recombinant SK as a poly-histidine tagged fusion protein. A Factor Xa recognition site was incorporated at the 5' end of sense primers to facilitate removal of the poly-histidine tag after purification and expose the functional Ile_1 N-terminal residue of SK. The following primers were used: Type-1, type-2a and type-2b *ska* sense (5'-GTGGATCCATCGAGGGAAGGATTGCTGGGTATGAATGG CTG-3'). H46A *ska* sense (5'-GTGGATCCATCGAGGGAAGGATTGCTGGACCTGAG TGGCTG-3'). Type-1, type-2a and type-2b (NS88.2) *ska* antisense (5'-TGCTGCAGTTA TTTGTCTTTAGGGTTATC-3'). H46A and type-2b (ALAB49) *ska* antisense (5'-TGCTG CAGTTATTTGTCGTTAGGGTTATC-3') (Sigma-Aldrich, Sydney, NSW, Australia). The cloned PCR products were sequenced in entirety and no sequence errors were detected. Recombinant SK protein expression in transformed M15[pREP4] was induced by addition of 1 mM isopropyl-1-thio-b-D-galactopyranoside to log-phase cells. Three hours later, cells were harvested by centrifugation, lysed using an EmulsiFlex-C5 (Avestin Inc., Ottawa, ON, Canada) and purified under native conditions using nickel-nitriloacetic acid affinity chromatography. Native recombinant SK proteins were cleaved from the poly-histidine tag by incubation with Factor Xa (Sigma-Aldrich, Sydney, NSW, Australia) for 12-36 h at 4°C. Post Factor Xa treatment a secondary truncation product was observed for all SK preparations during sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Nanoelectrospray-ionization mass spectrometry was undertaken to determine the lower molecular weight product was the result of non-specific proteolysis occurring at Arg_{401} in the C-terminus of recombinant SK molecules (data not shown). To separate full-length recombinant SK from truncation products, a secondary purification step using anion exchange chromatography was undertaken. This resulted in the retrieval of full-length recombinant SK, free of contaminating proteins.

Far-UV circular dichroism (CD) spectroscopy

CD spectroscopy was performed using a J-810 spectropolarimeter (Jasco, Tokyo, Japan) at room temperature. Samples were prepared in 10 mM phosphate buffer (pH 7.4) to a final concentration of 100-300 µg mL^{-1}. Spectra representing the average of six scans were collected from 190 nm to 250 nm at 1 nm intervals, with a path length of 1 mm. Molar residue ellipticity (Θ) was calculated using the following formula: $[\Theta] = \Theta$ x 100 x molecular weight (kDa)/concentration (mg mL^{-1}) x path length x number of amino acids.

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 titrant 4-methylumbelliferyl p-guanidinobenzoate (MUGB) (Marker Gene Technologies, Inc., Eugene, OR, USA) in a POLARstar Omega fluorescence spectrophotometer (BMG LABTECH, Ortenberg, HE, GER). Glu-Plg (200 nM) was added to a black 96-well micro-plate containing MUGB (1 µM) in assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) 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. Data was normalised by subtracting a control reaction of 200 nM Glu-Plg 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.

Surface plasmon resonance

Binding of SK variants to Glu-Plg and plasmin were examined *via* Biacore T200 (Biacore AB, Uppsala, Sweden) at 25ºC. Ligand Glu-Plg and plasmin were immobilized on a Series S Sensor Chip CM4 (Biacore AB, Uppsala, Sweden) via primary amino acids using an amine coupling kit according to the manufacturer's instructions (Biacore AB, Uppsala, Sweden). Briefly, the chip was activated with a 1:1 mixture of 0.2 M *N*-ethyl-*N*'-(3-dimethylaminopropyl)carbodiimide and 0.05 M *N*-hydroxysuccimide. Glu-Plg and plasmin were coated onto the chip at 40 µg mL^{-1} in 10 mM sodium acetate (pH 4) to a level of ~1500 and ~250 response units respectively. Unoccupied binding sites were blocked using 1 M ethanolamine, pH 8.5. A blank immobilized CM4 cell was used as a reference. Analytes were diluted into running buffer (10 mM HEPES, 150 mM NaCl, 0.005% P-20, pH 7.4) and kinetic assays were performed by injecting recombinant SK proteins at varying concentrations (0-1600 nM), for 200-300 s at a flow rate of 20 µL min^{-1} with a 600 s

dissociation period. Regeneration of the ligand surface was achieved with 6 M urea in running buffer for 15 s at 20 µL min^{-1}. Glu-Plg interaction biosensorgram data were prepared for analysis using Scrubber2 (BioLogic Software, Campbell, ACT, Australia) and data was analyzed manually using a two-component heterogeneous surface model with data curves fit using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). For Glu-Plg interactions with each SK variant, one binding component accounting for ~30-90% of the total response showed relatively fast association rate constants (k_a values) and dissociation rate constants (k_d values), with k_a showing a linear dependence on [SK], while the other showed slow on and off rates independent of [SK]; we chose to ignore this second non-specific component and determined equilibrium binding constants (K_D) from the ratio of k_d and k_a for the specific binding component. For plasmin interactions, k_a, k_d and K_D were calculated from sensorgrams by non-linear fitting of the association and dissociation curves according to a 1:1 Langmuir binding model using the Biacore T200 evaluation software supplied by the manufacturer (Biacore AB, Uppsala, Sweden).

Glu-Plg activation assays

The Plg activation potential of SK variants were studied by the addition of stoichiometric SK-plasmin activator complexes (final concentration 5 nM) that had been preformed for 5 min at 37°C, to assay buffer (10 mM HEPES, 150 mM NaCl, 0.01% Tween-20, pH 7.4) containing Glu-Plg (500 nM) and S-2251 (500 µM) in a total volume of 100 µL. The exponential generation of plasmin was monitored by change in absorbance at 405 nm and measured for 30 min using a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 37°C.

Inhibition of amidolysis by α_2-antiplasmin

Stoichiometric complexes of SK-plasmin were formed by mixing SK (400 nM) and plasmin (200 nM) for 5min at 37°C. Complexes were diluted to 20 nM in assay buffer in the presence of increasing α_2-AP concentration (0-400 nM) and incubated at 37°C for 15 min. The reactions were initiated by the addition of S-2251 (final concentration 500 µM) and change in absorbance at 405 nm was measured at 37°C. IC_{50} values were determined by plotting percentage of residual activity (V_{max}) versus log α_2-AP concentration and fit to a sigmoidal dose response curve using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA)

Allelic exchange mutagenesis

Isogenic mutants of GAS strain 5448 were produced by replacing the parental *ska*$_{5448}$ with either *ska*$_{ALAB49}$, *ska*$_{NZ131}$ or with the chloramphenicol acetyltransferase (*cat*) gene via precise, allelic replacement using a modified protocol to Buchanan *et al.* (2006). To construct plasmids for allelic exchange, *ska*$_{ALAB49}$ and *ska*$_{NZ131}$ were amplified from genomic DNA by PCR using sense primer: (5'-TTCTTCCTGTCTGTTTATGTACCCGCAGCTACTTGA TACC-3') and antisense primer: (5'-TTGTCCTCTTCTGTTTTGGCTACCAAGAACGCTT GATTG-3') (Sigma-Aldrich, Sydney, NSW, Australia), to include GAS chromosomal flanking regions 834 bp upstream and 853 bp downstream of *ska,* in addition to regions homologous to the temperature sensitive, erythromycin resistant shuttle vector pHY304-LIC. For knockout plasmid construct p5448Δska, upstream (369 bp) and downstream (538 bp) *ska* DNA fragments containing regions homologous to pHY304-LIC and *cat* were amplified using sense primer (5'- TTCTTCCTGTCTGTTTAGATGAGGGCCTACTTGCATC-3') and antisense (5'-GTGGCTTTTTTCTCCATACGGTCTGGTAGCCATCCAT-3') for the upstream homology region and sense primer (5'-GTGGCTGGGCGGGGCGTAAAA GCTTACAGCTACCTGCGT-3') and antisense (5'-TTGTCCTCTTCTGTTTCGGAC CAATGGCTAAGAAAG-3') for the downstream homology region. The *cat* gene was amplified using the sense primer (5'-GGAGAAAAAAGCCACTGGATATACCACC-3') and antisense primer (5'-ACGCCCCGCCCAGCCACTCATCGCAATACTGTT-3'). Single-strand overhangs were created on all PCR products and *Pme*I pHY304-LIC shuttle vector by T4 DNA polymerase treatment at 22°C for 30 min. Treated pHY304-LIC was combined with equal concentrations of *ska* upstream/downstream regions and the *cat* gene to create the Δ*ska* knock-out construct, or with either *ska*$_{ALAB49}$ or *ska*$_{NZ131}$ gene fragments to create allelic exchange constructs. Complementary sequences were allowed to anneal on ice for 30 min before transformation into chemically competent *E. coli*. The purified plasmid constructs were confirmed by DNA sequencing analysis and transformed into GAS strain 5448. Erythromycin resistant transformants were grown at the permissive temperature for plasmid replication (30°C). Single-crossover chromosomal insertions were selected by shifting to the non-permissive temperature (37°C) while maintaining erythromycin selection. Single cross-over mutants were incubated overnight at 30°C to allow for looping out of the inserted plasmid and then patched onto both THY agar and THY agar containing erythromycin and incubated at 37°C. This allowed selection of double crossover mutants encoding in-frame allelic exchanges and was confirmed using DNA sequence analysis. The allelic exchange mutant strains were designated 5448::*ska*$_{ALAB49}$, 5448::*ska*$_{NZ131}$ and 5448Δ*ska*.

Transgenic murine infection model

Humanized Plg transgenic *AlbPLG1* mice, heterozygous for the human Plg gene (Sun *et al.*, 2004), were used as the animal model for determining GAS invasive potential as previously described (Walker *et al.*, 2007). Briefly, GAS isolates were grown in THY medium at 37°C to logarithmic phase (OD_{600} = 0.6), washed with sterile 0.7% (w/v) NaCl and appropriately diluted to prepare the inoculum. Cohorts of 9-10 mice were infected with a 100 µL intradermal injection containing 5448 (Maamary *et al.*, 2010) (3.9 x 10^7 CFU/dose), 5448::Δ*ska* (3.7 x 10^7 CFU/dose), 5448::ska_{ALAB49} (4.6 x 10^7 CFU/dose), or 5448::ska_{NZ131} (3.7 x 10^7 CFU/dose) and mortality was recorded over a 10-day period.

Statistical analyses

Differences in survival of humanized plasminogen transgenic mice infected with GAS strains 5448, 5448::Δ*ska*, 5448::ska_{ALAB49}, or 5448::ska_{NZ131} were determined by the log-rank test. Differences were considered statistically significant at $P < 0.05$. All statistical tests were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

Ethics permissions

Permission to undertake animal experiments was obtained from the University of Wollongong Animal Ethics Committee.

Accession numbers

The nucleotide sequences of the *ska* genes used in this study are deposited in the GenBank database: H46A: K02986.1, NZ131: CP000829.1, 5448: JQ650489, NS696: JQ650488, ALAB49: AY234134.1 and NS88.2: JQ650490. Details of data submission can be found at: GenBank: www.ncbi.nlm.nih.gov.

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635 **Table 1.** Association (k_{on}) and dissociation (k_{off}) rate constants and apparent equilibrium dissociation (K_D) constants for the interaction of SK
636 variants with immobilized Plg and plasmin by SPR.

Analyte	Ligand Plg			Ligand Pm		
	k_a ($M^{-1} s^{-1}$)	k_d (s^{-1})	K_D (nM)	k_a ($M^{-1} s^{-1}$)	k_d (s^{-1})	K_D (nM)
SK_{H46A}	$2.9 \pm 0.5 \times 10^{4}$	$1.8 \pm 0.3 \times 10^{-3}$	62 ± 19	$8.6 \pm 0.2 \times 10^{5}$	$3.1 \pm 0.5 \times 10^{-4}$	0.37 ± 0.04
SK_{NZ131}	$2.8 \pm 0.7 \times 10^{3}$	$2.5 \pm 0.6 \times 10^{-4}$	88 ± 12	$1.1 \pm 0.8 \times 10^{6}$	$1.1 \pm 0.4 \times 10^{-3}$	0.76 ± 0.09
SK_{5448}	$1.1 \pm 0.2 \times 10^{6}$	$72.6 \pm 1.4 \times 10^{-3}$	66 ± 7	$1.0 \pm 0.6 \times 10^{6}$	$8.3 \pm 0.7 \times 10^{-4}$	0.96 ± 0.39
SK_{NS696}	$1.2 \pm 0.2 \times 10^{6}$	$84.1 \pm 3.4 \times 10^{-3}$	70 ± 17	$1.2 \pm 0.8 \times 10^{6}$	$9.4 \pm 0.1 \times 10^{-4}$	1.03 ± 0.47
SK_{ALAB49}	$4.6 \pm 0.4 \times 10^{4}$	$81.3 \pm 0.8 \times 10^{-3}$	1767 ± 199	$4.2 \pm 0.4 \times 10^{5}$	$4.3 \pm 0.3 \times 10^{-3}$	11.55 ± 3.03
$SK_{NS88.2}$	$7.9 \pm 0.5 \times 10^{4}$	$170.0 \pm 3.0 \times 10^{-3}$	2153 ± 512	$4.4 \pm 0.3 \times 10^{5}$	$2.2 \pm 0.6 \times 10^{-3}$	6.22 ± 2.70

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638 Glu- Plg binding data was prepared for analysis using Srubber2 (BioLogic Software, Campbell, ACT, Australia) and analyzed by fitting data to a
639 heterogeneous ligand population model using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA) as described under 'Experimental
640 Procedures'. Values represent the mean ± SD. Plasmin binding data were calculated by non-linear fitting of the association and dissociation
641 curves according to a 1:1 Langmuir binding model using the Biacore T200 evaluation software (Biacore AB, Uppsala, Sweden)

Figure Legends

Fig. 1. ClustalW Multiple alignment of deduced amino acid sequences of the SK protein variants used in this study. Amino acid residues identical to those in SK_{H46A} are indicated with a dot while amino acid changes have been indicated. Non-conserved amino acid changes are highlighted in grey.

Fig. 2. SDS-PAGE and Far-UV circular dichroism spectroscopy analysis of recombinant SK proteins used in this study. (A) Variant SK_{H46A} (lane 1), SK_{NZ131} (lane 2), SK_{5448} (lane 3), SK_{NS696} (lane 4), SK_{ALAB49} (lane 5) and $SK_{NS88.2}$ (lane 6) were separated by 10% SDS-PAGE under reducing conditions. Molecular weight standards in kDa are indicated. (B) All SK variants have similar molar residue ellipticity values and CD out-put spectrum structure across the full range of wavelengths indicating no differences in secondary structure.

Fig. 3. Non-proteolytic active site generation in Glu-Plg by SK variants and influence of Glu-Plg conformation on SK-Plg* generation. Glu-Plg (200 nM) was added to 1 µM MUGB in (A) Cl^- present assay buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.4) or (B) chloride Cl^- absent buffer (50 mM Tris pH 7.4) 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.

Fig. 4. Plg activation by SK-plasmin variant activator complexes and inhibition of variant SK-plasmin complexes by α_2-antiplasmin. (A) SK was preformed with plasmin for 5 min at 37°C (5 nM), then added to assay buffer (10 mM HEPES, 150 mM NaCl, 0.01% Tween-20, pH 7.4) containing Glu-Plg (500 nM) and S-2251 (500 µM). The generation of plasminogen activation activity was monitored at an absorbance of 405 nm. (**B)** Stoichiometric complexes of SK-plasmin (20 nM) were preformed for 5 min at 37°C in assay buffer (10 mM HEPES, 150 mM NaCl, 0.01 % Tween-20, pH 7.4) and subsequently incubated for 15 min at 37°C with α_2-antiplasmin (final concentration 0 – 400 nM). The reactions were initiated by addition of S-2251 (500 µM) to complex mixtures and the percentage of residual activity of the SK-plasmin complexes was determined by measuring the absorbance at 405 nm.

674 **Fig. 5.** Survival curves after subcutaneous infection of humanized Plg transgenic *AlbPLG1*
675 mice with GAS strain 5448 and isogenic mutants. 5448 (3.9 x 10^7 CFU/dose; *n* = 10)
676 (Maamary *et al.*, 2010), 5448::*ska*$_{ALAB49}$ (4.6 x 10^7 CFU/dose; *n* = 10), 5448Δ*ska* (3.7 x 10^7
677 CFU/dose; *n* = 10) or 5448::*ska*$_{NZ131}$ (3.7 x 10^7 CFU/dose; *n* = 9).
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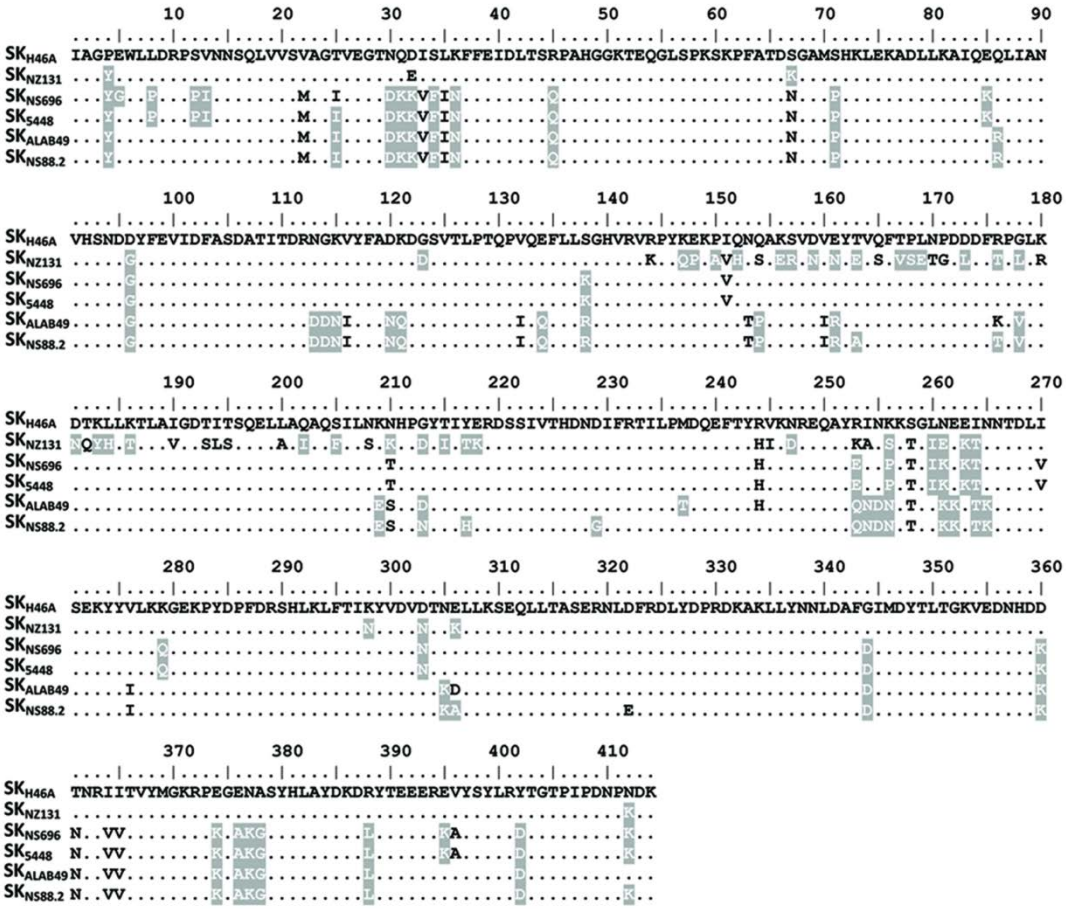

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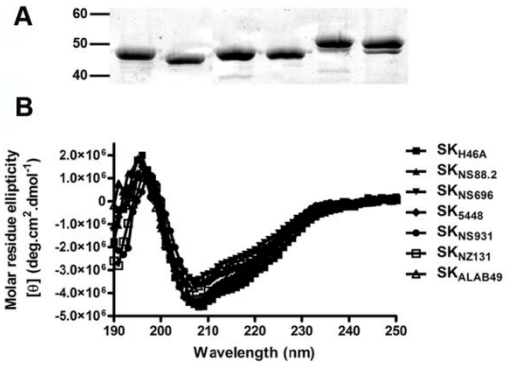

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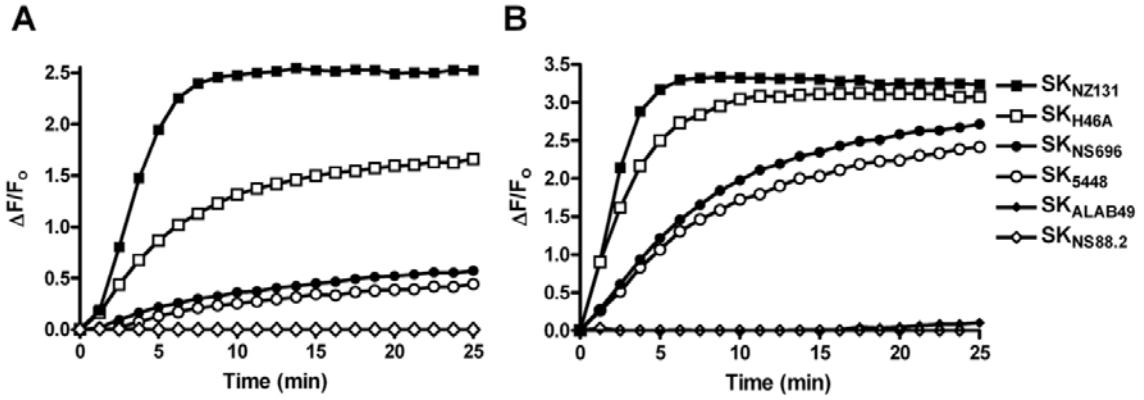

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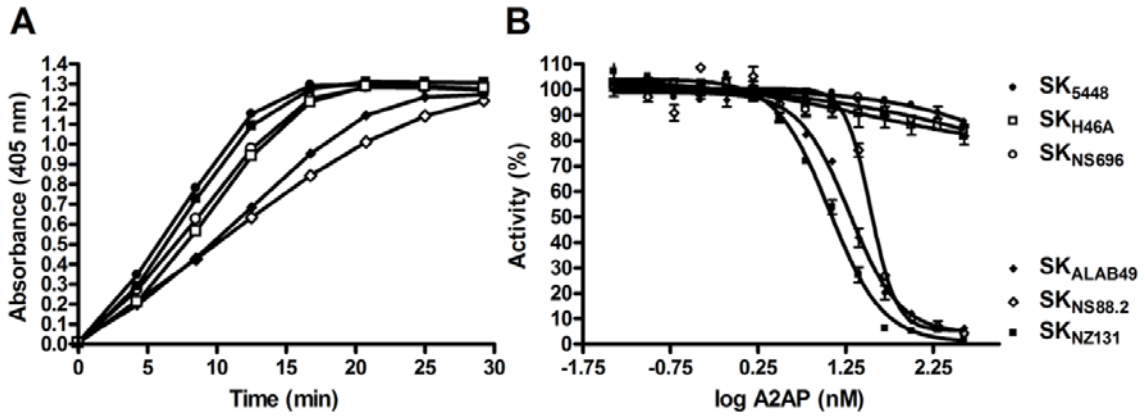
A
Absorbance (405 nm)
Time (min)
B
Activity (%)
log A2AP (nM)
SK5448
SKH46A
SKNS696
SKALAB49
SKNS88.2
SKNZ131

691

692

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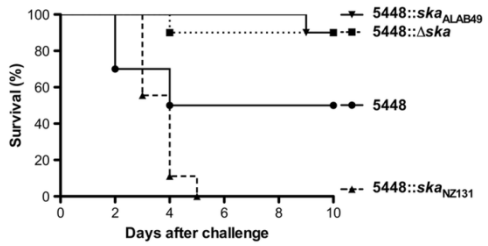

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