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Allelic variants of streptokinase from Streptococcus pyogenes display functional differences in plasminogen activation.

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Abstract

A common mammalian defence mechanism employed to prevent systemic dissemination of invasive bacteria involves occlusion of local microvasculature and encapsulation of bacteria within fibrin networks. Acquisition of plasmin activity at the bacterial cell surface circumvents this defence mechanism allowing invasive disease initiation. To facilitate this process, S. pyogenes secrete streptokinase, a plasminogen activating protein. Streptokinase polymorphism exhibited by S. pyogenes isolates is well characterised. However, the functional differences displayed by these variants and the biological significance of this variation has not been elucidated. Phylogenetic analysis of ska sequences from 28 S. pyogenes isolates revealed two main sequence clusters (clusters 1 and 2). All strains secreted streptokinase as determined by western blotting and were capable of acquiring cell-surface plasmin activity after incubation in human plasma. Whereas culture supernatants from strains containing cluster 1 ska alleles also displayed soluble plasminogen activation activity, supernatants from strains containing cluster 2 ska alleles did not. Furthermore, plasminogen activation activity in culture supernatants from strains containing cluster 2 ska alleles could only be detected when plasminogen was pre-bound with fibrinogen. This study indicates that variant streptokinase proteins secreted by S. pyogenes isolates display differing plasminogen activation characteristics and may therefore play distinct roles in disease pathogenesis.

Keywords

Streptococcus pyogenes, Streptokinase, Plasminogen, CMMB

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

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Title: Allelic variants of streptokinase from *Streptococcus pyogenes* display functional differences in plasminogen activation

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Short title: Variation in *S. pyogenes* streptokinase

Abstract

A common mammalian defence mechanism employed to prevent systemic dissemination of invasive bacteria involves occlusion of local microvasculature and encapsulation of bacteria within fibrin networks. Acquisition of plasmin activity at the bacterial cell surface circumvents this defence mechanism allowing invasive disease initiation. To facilitate this process, *S. pyogenes* secrete streptokinase, a plasminogen activating protein. Streptokinase polymorphism exhibited by *S. pyogenes* isolates is well characterised. However, the functional differences displayed by these variants and the biological significance of this variation has not been elucidated. Phylogenetic analysis of *ska* sequences from 28 *S. pyogenes* isolates revealed two main sequence clusters (clusters 1 and 2). All strains secreted streptokinase as determined by western blotting and were capable of acquiring cell-surface plasmin activity after incubation in human plasma. Whereas culture supernatants from strains containing cluster 1 *ska* alleles also displayed soluble plasminogen activation activity, supernatants from strains containing cluster 2 *ska* alleles did not. Furthermore, plasminogen activation activity in culture supernatants from strains containing cluster 2 *ska* alleles could only be detected when plasminogen was pre-bound with fibrinogen. This study indicates that variant streptokinase proteins secreted by *S. pyogenes* isolates display differing plasminogen activation characteristics and may therefore play distinct roles in disease pathogenesis.

Key words; bacterial pathogenesis, virulence, invasive disease.

Introduction

Group A streptococcus (*Streptococcus pyogenes*; GAS) is a common bacterial pathogen whose virulence has been honed through the process of evolution with its singular human host. GAS employ numerous pathogenic mechanisms that interact specifically with human proteins which enable evasion of host defences and promote bacterial colonisation, proliferation and dissemination. As an early mammalian defence mechanism, invading bacterial pathogens such as GAS are confined at sites of infection through the deposition of fibrin networks, thereby allowing a directed inflammatory immune response to specifically target this area (1). To circumvent this defence mechanism, GAS subvert components of the host fibrinolytic system to degrade fibrin and thus spread to other areas of the body (2).

A key component of the fibrinolytic system is plasminogen. Plasminogen is a 92 kDa glycoprotein found in plasma and extracellular fluids (3). Cleavage of the plasminogen activation bond (i.e. Arg_{561}-Val_{562}) by the specific host plasminogen activators, urokinase (uPA) and tissue plasminogen activator (tPA), results in the formation of the serine protease, plasmin (4). Plasmin has a broad substrate spectrum that includes fibrin clots and the extracellular matrix (4). GAS secrete streptokinase, a human specific plasminogen activating protein. Unlike host plasminogen activators, streptokinase lacks any intrinsic enzymatic activity and activates plasminogen by forming a stable 1:1 stoichiometric complex with either plasminogen or plasmin (5). Both complexes display the broad-spectrum protease activity of plasmin but this activity is unique in that it cannot be regulated by the normal plasma inhibitors (i.e. α_2-antiplasmin and α_2-macroglobulin) and can also activate other molecules of plasminogen (6).

Plasminogen contains several distinct structural domains, consisting of the amino-terminal peptide, followed by Kringle domains 1–5 (K1-5) and the carboxy-terminal serine protease domain. K1, K4 and K5 contain lysine-binding motifs that are responsible for binding to fibrinogen and to plasminogen receptors (7). The circulating, soluble form of plasminogen (glu-plasminogen) is maintained in a closed form through lysine-dependent interaction/s between the amino-terminal peptide and K5. Upon binding to mammalian or bacterial receptors, a conformational change is induced in glu-plasminogen producing an open, activation-susceptible form (6, 7).

GAS capture and facilitate plasminogen activation to plasmin, conferring cell-bound proteolytic activity, by several pathways. Plasminogen bound directly to GAS via cell-surface receptors such as the plasminogen-binding group A streptococcal M-like proteins (PAM) (8-10), glyceraldehye-3-phosphate dehydrogenase (GAPDH) (11) or streptococcal enolase (SEN) (12), can be activated by streptokinase:plasminogen/plasmin complexes, or by host activators such as tissue plasminogen activator (13). Alternatively, plasmin formed in solution may subsequently bind to GAS cell-surface receptors (14, 15). GAS may also acquire cell-surface plasmin activity by binding a tri-molecular complex of plasminogen, human fibrinogen and streptokinase (16, 17).

Streptokinase alleles from GAS are polymorphic (18). Previous studies have linked streptokinase polymorphism with strains associated with acute post-streptococcal glomerulonephritis (19, 20) and with strains that exhibit skin tissue tropism (21). However, the functional significance of streptokinase polymorphism has not been elucidated. With the majority of structural and functional studies on streptokinase having been performed using the therapeutic streptokinase from group C streptococcus, the phenotypic differences displayed by

GAS streptokinase proteins has yet to be explored. This report investigates streptokinase polymorphism in a number of clinical GAS isolates and demonstrates that variant streptokinase proteins display differing plasminogen activation capacities.

Materials and Methods

GAS isolates and culture

The 28 GAS isolates from the Northern Territory of Australia used in this study were collected from patients between 1990 and 1998 and their clinical source, *emm* type, *pam* genotype and plasminogen binding characteristics have been described in detail (17). This strain set was selected, as the region suffers endemic GAS infections, and the strains examined were epidemiologically diverse. *Pam*-positive isolates were defined as those positive for *pam* by Southern hybridisation and containing *pam* encoding A1 and A2 repeat regions with greater than 50% amino acid sequence homology to that of the prototype PAM sequence (8). The invasive GAS isolate 5448 (M type 1), the North American *emm53* impetigo isolate ALAB49 and its isogenic streptokinase deletion mutant have been described previously (22-26). GAS were cultured on defibrinated horse blood agar plates (Biomerieux, Sydney, NSW, Australia) or in Todd Hewitt broth (BD, Sydney, NSW, Australia) supplemented with 1% (w/v) yeast extract (Oxoid, Adelaide, SA, AUstralia; THY) at 37°C as stationary cultures.

Streptokinase expression

For analysis of streptokinase expression, aliquots of overnight GAS cultures were washed twice by centrifugation and subsequent resuspension with an equal volumes of fresh THY before being used to inoculate new cultures. When GAS cultures had reached mid-log phase

(A_{600} = 0.6), supernatants were harvested by centrifugation, filtered using a 0.2 µm PVDF syringe filter (Millipore, Sydney, NSW, Australia) and stored at -70°C until analysis.

Thawed GAS culture supernatants were then concentrated 18 fold by 10% trichloroacetic acid precipitation prior to SDS-PAGE and Western transfer to PVDF membrane (Millipore). For detection of streptokinase, the membranes were immunoblotted using rabbit immune serum raised against commercial group C streptokinase (Sigma-Aldrich, Sydney, NSW, Australia) as described previously (27).

Streptokinase activity

An indirect plasminogen activation assay was used to measure streptokinase activity in GAS culture supernatants using the plasmin amidolytic substrate Spectrozyme® PL (American Diagnostica, Stamford, CT, USA), as previously described (28). Briefly, supernatants were defrosted on ice and a 20 µl aliquot incubated at 37°C with 100 µl 50 mM Tris, pH 7.5, containing 20 µg/ml glu-plasminogen (Haematologic Technologies, Essex Junction, Vermont USA) for 15 min. Spectrozyme® PL (20 µl of 2.5 mM) was added and absorbance at 405 nm was measured every 3 min for 60 min. Absorbance was plotted against time and activity rates were determined from the linear portion of the curve. The amount of streptokinase activity in each bacterial culture supernatant was converted to units/ml using a standard curve of group C streptokinase (Sigma-Aldrich) serially diluted in THY medium using Softmax® Pro software. All reactions were performed in duplicate in the presence and absence of plasminogen to confirm that proteolytic activity of supernatants was attributable to plasminogen activation. Inter-assay variation was corrected for using an internal positive control sample (250 units/ml of purified group C streptokinase; Sigma-Aldrich) in each assay. In assays examining the effects of plasminogen binding ligands on streptokinase mediated plasminogen activation,

human fibrinogen (Sigma), recombinant streptococcal enolase (SEN) (29) or recombinant PAM (30) were mixed in 1:1 stoichiometric ratios with plasminogen and pre-incubated at 37°C for 15 min prior to the addition of bacterial culture supernatants.

Cell surface plasmin activity

Plasmin acquisition by *S. pyogenes* isolates after incubation in human plasma was determined essentially as described previously (27). Frozen plasma was purchased from the Red Cross Blood Bank (Sydney, NSW, Australia), defrosted on ice and pooled. Aliquots of pooled plasma were depleted of plasminogen by incubation at 4°C on ice with excess lysine-sepharose® 4B (Amersham Biosciences, Sydney, NSW, Australia) for 1–2 h with gentle agitation. GAS were cultured overnight, washed once in PBS, pH 7.4, and resuspended to A_{600} = 0.7. Aliquots of this suspension were pelleted by centrifugation (1500 x *g* for 10 min) and resuspended in an equal volume of 100% human plasma or plasminogen-depleted plasma at 37°C. GAS were incubated in plasma for 3 h at 37°C, pelleted by centrifugation and washed twice with 1 volume of ice-cold 0.01 M EDTA, 0.1% gelatin in PBS, pH 7.4. GAS were resuspended in 0.1% gelatin in PBS, pH 7.4 to A_{600} = 0.75. Aliquots (100 µl) of this suspension were incubated in triplicate in the presence and absence of 20 µl Spectrozyme® PL (2.5 mM) at 37°C for 60 min in a 96-well plate. The reaction was quenched with 80 µl of 1.75 M acetic acid, the plates centrifuged and A_{405} of supernatants determined.

Plasmin activity was determined as the difference between A_{405} in the presence and absence of substrate. Isolate NS931 was included as an internal control in every experiment. Each isolate was assayed in at least 3 independent experiments. Plasmin equivalents and the linear range of the assay (A_{405} = 0 – 0.6) were determined using a standard curve of purified plasmin (Roche Diagnostics, Sydney, NSW, Australia).

Streptokinase genotype

Chromosomal DNA was extracted from GAS strains using a commercially available system (DNeasy Kit, Qiagen, Melbourne, Vic, Australia). The variable region of the streptokinase gene was PCR-amplified using previously described primers (31), sequenced using the BigDye Terminator cycle sequencing kit and analysed using ABI Prism Autoassembler software (Applied Biosystems, Melbourne, Vic, Australia). A phylogenetic tree was constructed for the 423 bp nucleotide sequence encoding the variable β-domain of the streptokinase protein of the 29 NT isolates using MEGA (32) as previously described (21). The Genbank accession numbers for the 29 partial *ska* sequences are EU352612 to EU352641.

Statistical analyses

Differences of enzyme activities between isolates were determined using unpaired, two tailed Student's t-test with 95% confidence intervals. The effect of pre-binding ligands to plasminogen on streptokinase activation was analysed using a one way ANOVA and a Newman-Keuls multi-comparison post test. All statistical analyses were carried out using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA).

Results

Phylogenetic analysis of streptokinase β-domain gene sequences

The relatedness of streptokinase alleles from 5448, ALAB49 and 28 NT GAS isolates were investigated by phylogenetic analysis of nucleotide sequences encoding the highly variable β-domain of streptokinase (Figure 1). The β-domain sequences were found to cluster into two major groups (clusters 1 and 2) with evidence of several smaller subclusters present. This

phylogeny is similar to that seen in previous studies (21). The *ska* alleles of all *pam*-negative isolates fell within cluster 1, with the exceptions of the two *emm1* isolates NS696 and 5448 which fell within a smaller subcluster of cluster 2 alleles termed cluster 2a. The majority of *pam*-positive isolates (12/13) possessed a cluster 2 allele that was termed cluster 2b. A notable exception was the *pam*-positive isolate NS53, which possessed a cluster 1 *ska* allele. This association between cluster 2b *ska* alleles and *pam* genotype has been previously observed (21).

Activity and expression of streptokinase allelic variants

Streptokinase activity was determined in supernatants collected from cultures at mid-log phase for the 31 GAS isolates. The results demonstrate a striking difference in soluble plasminogen activation capacities displayed by the different streptokinase variants produced by each isolate. For all strains harbouring a cluster 1 *ska* allele, there were high levels of streptokinase activity present in culture supernatants (Figure 2A). These ranged from 9 to 132 units/ml. For strains harbouring a cluster 2 *ska* allele, no streptokinase activity was detected in the culture supernatants (Figure 2A). The *pam*-positive strain, NS53, which contains a cluster 1 *ska* allele produced measurable streptokinase activity (33 units/ml) in culture supernatants (Figure 2A).

We next investigated whether the lack of soluble streptokinase activity seen in culture supernatants from strains containing a cluster 2 *ska* allele was due to a lack of streptokinase protein in culture supernatants. Western immunoblotting experiments using anti-SpeB polyclonal sera demonstrated that the cysteine protease SpeB, which is known to degrade streptokinase (33) was not present in supernatants harvested at this phase of growth (data not shown). This was corroborated by Western-immunoblotting performed using anti-

streptokinase polyclonal sera as it detected an immunoreactive band ranging from approximately 46 to 49 kDa in culture supernatants for all GAS isolates, including those harbouring a cluster 2 *ska* allele (Figure 2B). This corresponds to the approximate size of streptokinase (28). The ALAB49Δ*ska* mutant strain lacked this 46 to 49 kDa immunoreactive band, instead producing a 27 kDa band that is consistent with the expected size of the truncated streptokinase protein produced by this strain (25). These results clearly show the lack of soluble streptokinase activity in culture supernatants from isolates with a cluster 2 *ska* allele was not attributable to a lack of streptokinase protein being present in these samples.

Acquisition of cell-surface plasmin by GAS isolates in plasma

To determine whether the secreted streptokinase protein encoded by cluster 2 *ska* alleles was inactive, an indirect assay measuring the acquisition of cell-surface bound plasmin after incubation in human plasma was performed for all isolates (Figure 3). Despite the apparent lack of streptokinase activity in solution, isolates containing a cluster 2 *ska* allele acquired significantly higher levels of plasmin activity at the cell surface compared to the isolates containing a cluster 1 *ska* allele (Figure 3; $p = 0.0018$). As might be expected, the isogenic streptokinase-negative mutant ALAB49Δ*ska* acquired significantly less cell-surface plasmin activity than wild-type ALAB49 (Figure 3; $p = 0.019$). As secreted cluster 2 type streptokinase appears to contribute to cell-surface plasmin acquisition, these observations suggest that this type of streptokinase is active but only in the presence of cell surface receptors and/or human plasma.

Effects of plasminogen binding receptors on plasminogen activation by streptokinase

Fibrinogen can contribute to the acquisition of cell surface plasmin activity by binding streptokinase-plasminogen complexes to M-proteins located on the bacterial cell surface (16).

The effect of pre-binding plasminogen to fibrinogen on plasminogen activation by group A streptococcal streptokinase variants was thus examined (Figure 4). Fibrinogen significantly increased the streptokinase activity detected in GAS culture supernatants from isolates harbouring cluster 1 *ska* alleles when compared to activities detected in the absence of fibrinogen (Figure 4A; $p<0.001$). Importantly, streptokinase activity was also detected in GAS culture supernatants from isolates harbouring the cluster 2 *ska* alleles when plasminogen was pre-bound with fibrinogen (Figure 4A).

The binding of glu-plasminogen to mammalian or bacterial receptors induces a conformational change that can affect the activation of this zymogen (6, 7). When plasminogen was pre-bound to PAM, a significant increase in streptokinase activity in GAS culture supernatants from isolates harbouring cluster 1 and cluster 2a *ska* alleles was observed when compared to activities detected in the absence of PAM (Figure 4B; $p<0.01$). However, this enhanced streptokinase activity is not biologically relevant as the majority of cluster 1 *ska* allele GAS strains do not express PAM. GAS culture supernatants from *pam*-positive isolates harbouring a cluster 2b *ska* allele did not produce detectable streptokinase activity when plasminogen was pre-bound to PAM (Figure 2B). When plasminogen was pre-bound to SEN, streptokinase activity in all GAS culture supernatants was similar to levels detected in the absence of SEN (Figure 4C; $p>0.05$). When plasminogen was pre-bound to a combination of fibrinogen and PAM or fibrinogen and SEN, streptokinase activity was increased to levels similar to those seen for fibrinogen alone suggesting that the presence of PAM or SEN does not confer an additional benefit (Figure 4D and E). For all reaction conditions, ALAB49Δ*ska* did not exhibit streptokinase activity in culture supernatants (Figure 4). These data demonstrate that, for cluster 2 *ska* alleles, the presence of fibrinogen is a prerequisite for the cluster 2 streptokinase-plasminogen complex to gain functional activity.

Discussion

Activating plasminogen and acquiring plasmin activity to the bacterial cell surface has been recognised as a pathogenic mechanism for a variety of bacterial species that can cause invasive infections (34, 35). A long standing question in group A streptococcal research has been the correlation of high level cell surface plasminogen binding capacity with the absence of detectable streptokinase activity (2, 28). In this report, we demonstrate for the first time that allelic variants of streptokinase produced by different GAS isolates display unique plasminogen activation properties which confer on all GAS isolates the potential to harness plasmin activity onto the bacterial cell surface.

Plasminogen receptors interact with different regions of the plasminogen protein. The GAS plasminogen-binding M protein, PAM, binds to plasminogen via interaction with K2 (36), whereas fibrinogen and the GAS plasminogen receptors SEN and GAPDH bind via lysine dependant interactions with K1, K4 and K5 (7). Streptokinase encoded by cluster 2b *ska* alleles failed to produce any soluble plasminogen activation activity in the absence of fibrinogen. Pre-binding of the plasminogen receptors PAM or SEN to plasminogen did not enhance plasminogen activation by cluster 2b allelic variants of streptokinase. This is consistent with previous studies that have noted specific isolates of *S. pyogenes* fail to activate plasminogen (28). All the GAS isolates used in this current study expressed and secreted streptokinase into culture supernatants indicating that the lack of plasminogen activation activity was not due to lack of *ska* expression. Streptokinase encoded by cluster 2b *ska* alleles was able to activate plasminogen when pre-bound to fibrinogen or to cells pre-incubated in human plasma. Cluster 2b streptokinase expression by the PAM-positive strain ALAB49, was required for cell-surface plasmin acquisition during incubation in human plasma as deletion of

the *ska* gene in ALAB49Δ*ska* abolished surface plasmin acquisition. Streptokinase encoded by cluster 2a *ska* alleles also readily activated plasminogen to produce plasmin activity in the presence of fibrinogen. Taken together, these data suggest cluster 2 streptokinase requires the formation of a tri-molecular complex with plasminogen and fibrinogen in order to display plasminogen activating capability.

Streptokinase encoded by cluster 1 *ska* alleles readily activated soluble plasminogen to produce plasmin activity. The addition of fibrinogen to cluster 1 *ska* supernatants resulted in significantly higher levels of streptokinase activity (Figure 4). Similarly, activation of glu-plasminogen by group C streptokinase can be enhanced when plasminogen is pre-bound with fibrinogen (37). Although SEN has been shown to bind to plasminogen (38), this interaction did not affect plasminogen activation mediated by any of the streptokinase variants examined in this study.

Kalia and Bessen (21) demonstrated a strong linkage disequilibrium between skin-tropic GAS strains, cluster 2b *ska* alleles and PAM suggesting the resultant phenotype may contribute to increased bacterial fitness during skin infection. In this study 13 of the 14 *pam*-positive strains contained a cluster 2b *ska* allele. Although PAM was shown to have no effect on plasminogen activation by streptokinase encoded by cluster 2b allele types, we hypothesise that PAM, rather than playing a direct role in plasminogen activation, has evolved to bind to K2 of plasminogen, allowing fibrinogen to interact with K4 and K5 in a non-competitive manner. This allows the plasminogen-fibrinogen-streptokinase tri-molecular complex to bind to the surface of PAM-positive GAS isolates expressing cluster 2b *ska* (Figure 5A). Supporting this hypothesis, Svensson et al. (25) showed that an isogenic *pam* knockout mutant of ALAB49 failed to acquire and activate plasminogen after incubation in human plasma. Other GAS

plasminogen binding proteins such as SEN cannot bind this tri-molecular complex as the plasminogen domains (K 4 and K5) required for interaction with SEN are involved in the interaction with fibrinogen. This hypothesis accounts for the co-selection of PAM and cluster 2b *ska* observed by Kalia and Bessen (21). For cluster 2a GAS, the fibrinogen-binding capacity of M1 protein (39-41) allows attachment of the tri-molecular complex to the bacterial surface (Figure 5B). In contrast, cluster 1 GAS produces a streptokinase molecule that does not require the participation of fibrinogen to activate plasminogen. We hypothesise that anchoring of the cluster 1 streptokinase-plasminogen complex to the GAS cell surface can occur via the interaction of K4 and K5 with SEN or other plasminogen receptors, or alternatively via the interaction of the cluster 1 streptokinase-plasminogen complex with fibrinogen bound to GAS fibrinogen receptors (Figure 5C).

Our results show that allelic variants of streptokinase produced by different GAS strains exhibit differing plasminogen activation capacities. This suggests that variation within the β domain of these proteins may be responsible for the observed differences in plasminogen activation activities. Lizano and Johnston (42) have suggested that sequence polymorphism in the β-domain has little affect on plasminogen activation. The recombinant streptokinase produced from SF13013 (containing a cluster 2a *ska* allele) in that study displayed soluble plasminogen activation activity. In our study, streptokinase produced by two serotype M1 isolates containing cluster 2a alleles displayed no soluble plasminogen activation activity. This indicates that sequence differences outside the β-domain region may also contribute to the variable plasminogen activation activities observed for streptokinase variants produced by GAS.

In this study, we demonstrate that activation of plasminogen by cluster 2 type streptokinase requires the presence of fibrinogen and the formation of a tri-molecular complex between streptokinase, plasminogen and fibrinogen. This requirement underpins the previously observed linkage disequilibrium between PAM and cluster 2b alleles (21) as PAM is critical for harnessing plasmin activity to the bacterial cell surface via binding of the tri-molecular complex. The sequence diversity of streptokinase displayed by GAS may be the result of selection pressures acting on *ska* during the infection process, thereby producing streptokinase proteins that are better adapted to interact with the human fibrinolytic system while avoiding a functional immune system. Therefore, understanding the molecular basis for the phenotypic variations observed in this study could assist the rational design of second generation thrombolytic therapeutics (43) with improved efficacy and safety.

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Figure legends

Figure 1. Phylogenetic tree for a 423 bp variable region encoding the β-domain of streptokinase for the 31 isolates examined in this study. The DNA sequence of the *ska* allele from ALAB49 (AY234134) was obtained from a previous study (21). Bootstrap values of ≥ 90% (500 replicates) are indicated and the scale bar indicates 0.05 substitution per site. PAM-positive GAS isolates are in italics. The Genbank accession numbers for the 29 partial *ska* sequences are EU352612 to EU352641.

Figure 2. Solution phase streptokinase activity and expression of 31 clinical isolates of GAS. (A) Streptokinase activity in GAS culture supernatants at mid-log phase (A_{600} = 0.6) measured by an indirect assay of plasmin activity in the presence of human plasminogen using the plasmin-specific chromogenic substrate Spectrozyme® PL. Results are presented as the mean of streptokinase activity from duplicate assays, expressed as units/ml using a standard curve of group C streptokinase and corrected for an internal positive control sample in each assay. Error bars represent the range for each point. (B) Western blot of mid-log phase GAS culture supernatants using rabbit immune serum raised against group C streptokinase. GAS isolates containing a cluster 1 *ska* allele are indicated with a solid horizontal bar while isolates containing a cluster 2 *ska* allele are indicated with a dashed horizontal bar. GAS isolates containing a sub-cluster 2a *ska* allele are underlined. PAM-positive GAS isolates are in italics.

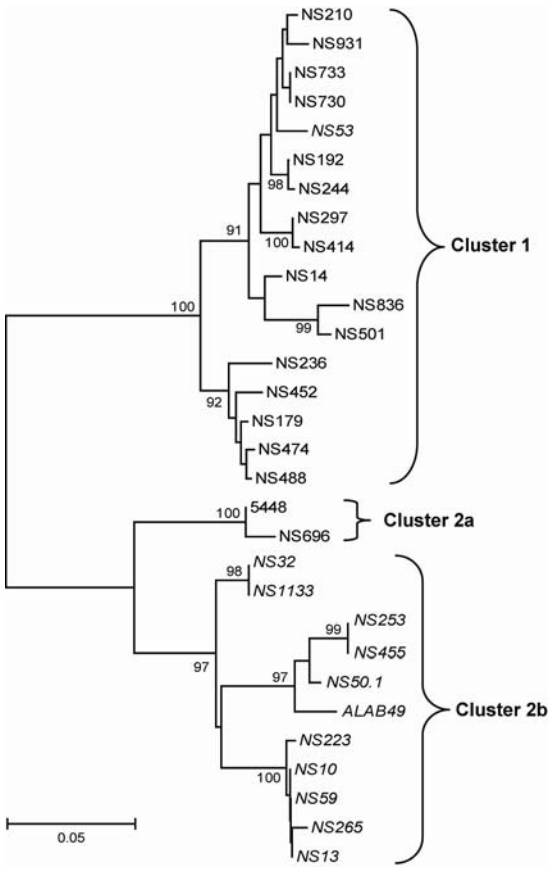
Figure 3. Cell-surface plasmin activity of GAS isolates. Cell surface plasmin activity of 32 clinical isolates of GAS following incubation in human plasma (filled bars) or plasminogen-depleted plasma (unfilled bars) determined by cleavage of Spectrozyme® PL. Data represent mean ± SEM of triplicate cultures of GAS, expressed as A_{405} of cleaved substrate in solution.

Overall, strains containing cluster 2 *ska* alleles acquire more cell-surface plasmin activity compared to Strains containing cluster 1 *ska* alleles ($p = 0.0018$). GAS isolates containing a cluster 1 *ska* allele are indicated with a solid horizontal bar while isolates containing a cluster 2 *ska* allele are indicated with a dashed horizontal bar. GAS isolates containing a sub-cluster 2a *ska* allele are underlined. PAM-positive GAS isolates are in italics.

Figure 4. Streptokinase activity in GAS culture supernatants at mid-log phase ($A_{600} = 0.6$) measured by an indirect assay of plasmin activity in the presence of human plasminogen pre-bound with fibrinogen (A), PAM (B), SEN (C), fibrinogen and PAM (D) or fibrinogen and SEN (E). Results are presented as the mean of streptokinase activity from duplicate assays, expressed as units/ml using a standard curve of group C streptokinase and corrected for an internal positive control sample in each assay. Error bars represent the range for each point. GAS isolates containing a cluster 1 *ska* allele are indicated with a solid horizontal bar while isolates containing a cluster 2 *ska* allele are indicated with a dashed horizontal bar. GAS isolates containing a sub-cluster 2a *ska* allele are underlined. PAM-positive GAS isolates are in italics.

Figure 5. Schematic diagram summarising the hypothesised pathways of cell surface plasmin acquisition by GAS strains expressing different alleles of streptokinase. (A) Cluster 2b streptokinase must combine with plasminogen and fibrinogen to form a tri-molecular complex that exhibits plasmin activity. The tri-molecular complex is bound to the cell surface via the interaction between PAM and kringle 2 of plasminogen. Other GAS plasminogen binding proteins such as SEN cannot bind this tri-molecular complex as the domains K 4 and K5 required for interaction are involved in the interaction with fibrinogen. (B) Cluster 2a streptokinase also must combine with plasminogen and fibrinogen to form a tri-molecular

complex that exhibits plasmin activity. The tri-molecular complex is bound to the cell surface via the interaction between fibrinogen binding receptors (FgR) such as M1 protein and fibrinogen. (C) Cluster 1 type streptokinase will combine with plasminogen to form a complex with plasmin activity. This complex can be bound directly to the bacterial cell surface via plasminogen receptors (PLR) or through an interaction with fibrinogen and fibrinogen receptors.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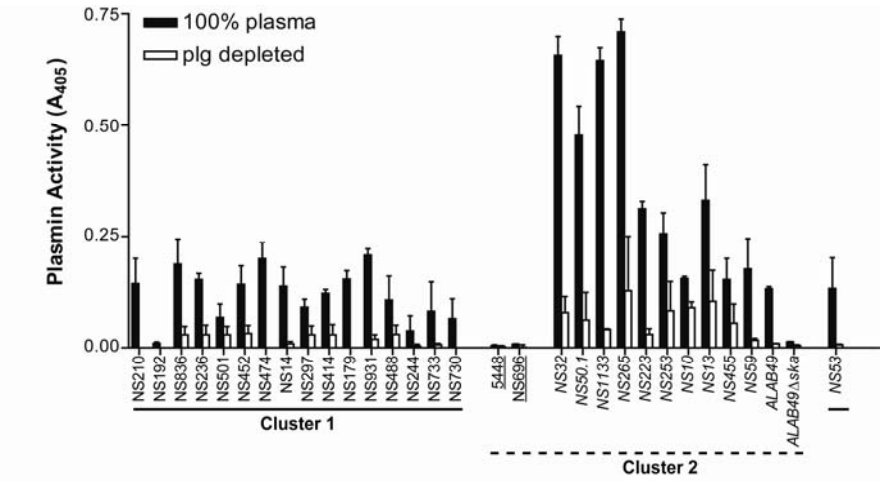
NS474
NS488
5448
NS696
NS32
NS1133
NS253
NS455
NS50.1
ALAB49
NS223
NS10
NS59
NS265
NS13
Cluster 1
Cluster 2a
Cluster 2b
98
91
100
100
99
92
100
98
99
97
97
100
0.05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plg depleted
Plasmin Activity (A_{405})
0.75
0.50
0.25
0.00
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NS192
NS836
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NS501
NS452
NS474
NS14
NS297
NS414
NS179
NS931
NS488
NS244
NS733
NS730
5448
NS696
NS32
NS50.1
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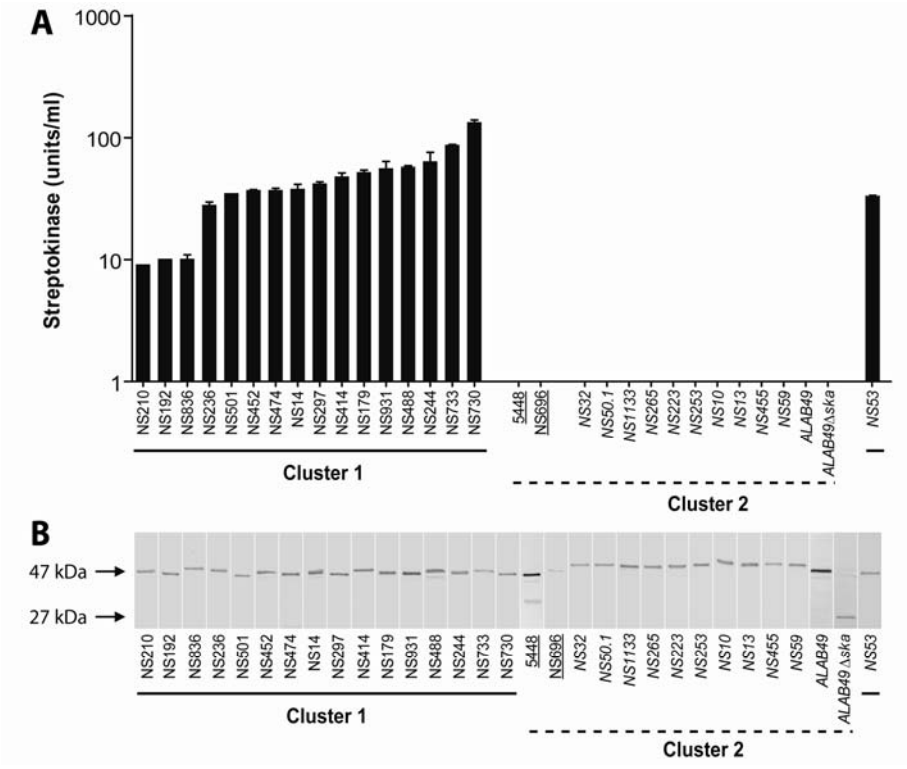
NS455
NS59
ALAB49
ALAB49Δska
NS53
Cluster 1
Cluster 2


A
Streptokinase (units/ml)
1000
100
10
1
NS210
NS192
NS836
NS236
NS501
NS452
NS474
NS14
NS297
NS414
NS179
NS931
NS488
NS244
NS733
NS730
5448
NS696
NS32
NS50.1
NS1133
NS265
NS223
NS253
NS10
NS13
NS455
NS59
ALAB49
ALAB49Δska
NS53
Cluster 1
Cluster 2
B
47 kDa
27 kDa
NS210
NS192
NS836
NS236
NS501
NS452
NS474
NS14
NS297
NS414
NS179
NS931
NS488
NS244
NS733
NS730
5448
NS696
NS32
NS50.1
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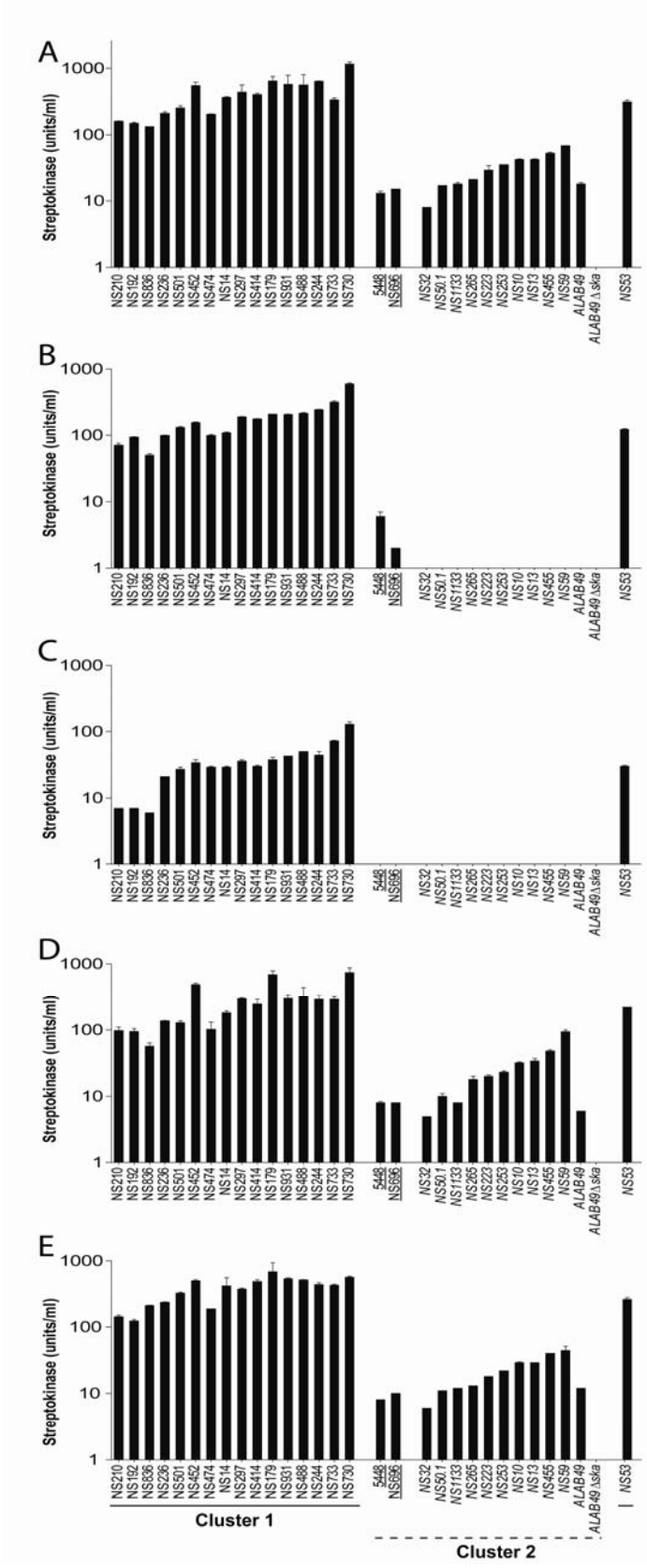
NS13
NS455
NS59
ALAB49
ALAB49 Δska
NS53
Cluster 1
Cluster 2


A
B
C
D
E
Streptokinase (units/ml)
1000
100
10
1
NS210
NS192
NS836
NS226
NS501
NS452
NS474
NS14
NS297
NS414
NS179
NS931
NS488
NS244
NS733
NS730
5448
NS696
NS32
NS501.1
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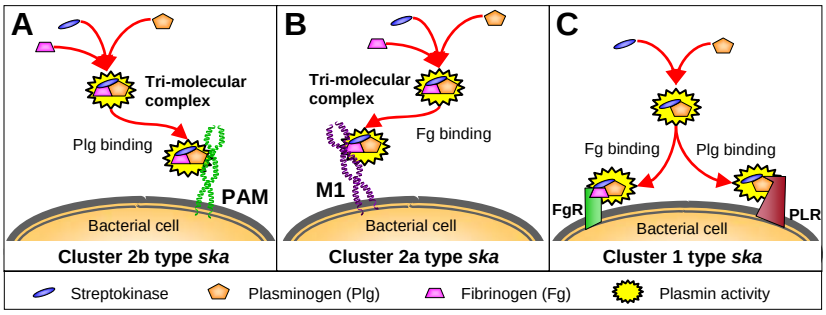
NS455
NS59
ALAB49
ALAB49.Δska
NS53
Cluster 1
Cluster 2


A
Tri-molecular complex
Plg binding
PAM
Bacterial cell
Cluster 2b type ska
B
Tri-molecular complex
Fg binding
M1
Bacterial cell
Cluster 2a type ska
C
Fg binding
Plg binding
FgR
PLR
Bacterial cell
Cluster 1 type ska
Streptokinase
Plasminogen (Plg)
Fibrinogen (Fg)
Plasmin activity