



# The $\beta$ -domain of cluster 2b streptokinase is a major determinant for the regulation of its plasminogen activation activity by cellular plasminogen receptors



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## ABSTRACT

Cluster 2b streptokinase (SK2b), secreted by invasive skin-trophic strains of *Streptococcus pyogenes* (GAS), is a human plasminogen (hPg) activator that optimally functions when human plasma hPg is bound, via its kringle-2 domain, to cognizant bacterial cells through the a1a2 domain of the major cellular hPg receptor, Plasminogen-binding group A streptococcal M-like protein (PAM). Another class of streptokinases (SK1), secreted primarily by GAS strains that possess affinity for pharyngeal infections, does not require PAM-bound hPg for optimal activity. We find herein that replacement of the central  $\beta$ -domain of SK2b with the same module from SK1 reduces the dependency of SK2b on PAM, and the converse is true when the  $\beta$ -domain of SK1 is replaced with this same region of SK2b. These data suggest that simple evolutionary shuttling of protein domains in GAS can be employed by GAS to rapidly generate strains that differ in tissue tropism and invasive capability and allow the bacteria to survive different challenges by the host.

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## 1. Introduction

The conversion of the inactive single-chain 791-amino acid residue protein, human plasma plasminogen (hPg), to the disulfide-linked two-chain serine protease, plasmin (hPm), occurs upon mandatory cleavage of a single peptide bond, Arg<sup>561</sup>-Val<sup>562</sup>, in the hPg zymogen [1]. Thus, physiological mammalian hPg activators are themselves serine proteases with very limited specificity for this peptide bond. On the other hand, hPg is also efficiently converted to hPm by a secreted 414-amino acid residue streptococcal protein, streptokinase (SK), that possesses no inherent proteolytic activity. This process occurs via complexes of SK and hPg that specifically generate highly specific enzymatic activity initiated through conformational alterations in these SK/hPg complexes, ultimately leading to a robust hPg activator [2].

*Streptococcus pyogenes* (GAS) is a strict human pathogen, and >250 serotypes have been identified through polymorphic and immunologic differences in surface-exposed M- and M-like proteins [3]. These strains are pathologically distinguished based on their dermal or pharyngeal specificity, as well as their virulence and ability to disseminate in the human host. Most of the ~700 M

GAS infections reported annually are of a superficial nature and are readily treated by antibiotics. However, several strains of GAS have been isolated from patients with highly invasive infections, such as necrotizing fasciitis and streptococcal toxic shock syndrome. In the strains that have been characterized, e.g., AP53 and NS88.2, those that result in highly invasive skin infections contain the hPg/hPm direct binding M-like protein PAM [4], and also secrete a specific type of SK needed to activate PAM-bound hPg, cluster 2b streptokinase (SK2b) [5,6]. Several pharyngeal-specific strains of GAS, e.g., SF370, contain a distinct M-protein, M1, encoded by the *emm1* gene, in place of PAM that does not directly interact with hPg, but with fibrinogen (Fg), which then interacts with hPg. This strain secretes a variant SK (SK2a) that optimally activates hPg bound to cells via Fg [5,7]. Lastly, many strains of GAS possess a M-protein that does not interact with hPg or Fg, e.g., NS931, and also produce a variant of SK (SK1) that optimally activates hPg in solution [8]. While highly homologous to each other, critical differences between PAM and M1 reside in a small amino-terminal ~30 amino acid region, which in PAM contains the a1a2 locus that is fully responsible for the tight interaction between hPg/hPm and PAM. This form of M-protein, viz., PAM, appears to be coinherited in strains that produce SK2b [6,8].

In the current study, we have exchanged each of the three known domains of SK2b into SK1 in order to identify the region(s)

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of this protein that confers PAM-dependent SK2b. The results of this investigation are reported herein.

## 2. Materials and methods

### 2.1. Proteins and cell lines

The recombinant (r) SK chimeric mutants, with  $\alpha$ ,  $\beta$ , or  $\gamma$  domain exchanges between SK1 from GAS strain NS931 and SK2b from GAS strain NS88.2, were expressed and purified from cDNA clones as described [9].

rPAM<sub>AP53</sub> (residues 42–392, lacking the C-terminal membrane insertion domain) was expressed and purified as described [8].

Human full-length recombinant Glu<sup>1</sup>-plasminogen (hPg) was expressed in *Drosophila Schneider* S2 cells and purified as described [10].

GAS strain AP53 was obtained from M.J. Walker (Queensland, Australia).

### 2.2. Activation of Glu<sup>1</sup>-hPg in solution

Activation rates of rhPg were continuously monitored in 96-well Corning NBS non-binding microwell plates in Cl<sup>-</sup> buffer to maintain the closed (tight) conformation of hPg. Catalytic levels of rSK (40:1, *m:m*, hPg/rSK) were employed to accelerate the activation of hPg. For these assays, 200  $\mu$ l of a solution containing 0.2  $\mu$ M hPg/± 0.5  $\mu$ M rPAM/0.25 mM S2251 (final concentrations) in 10 mM Hepes/150 mM NaCl, pH 7.4, was added to the wells, followed by addition of 2  $\mu$ l of 0.5  $\mu$ M rSK (5 nM, final concentration). The amidolytic activity of the generated plasmin (hPm) was monitored by the absorbance (A) at 405 nm from release by plasminolysis of p-nitroanilide (pNA) from the chromogenic substrate, S2251 (H-D-Val-Leu-Lys-pNA; Chromogenix, Milan, Italy) [8].

### 2.3. Activation of hPg on GAS cells

GAS AP53 cells were grown in THY medium to  $A_{600\text{nm}} \sim 0.6$  and collected by centrifugation. The cells were washed with 10 mM Hepes/150 mM NaCl, pH 7.4, and then resuspended in this buffer to  $A_{600\text{nm}} \sim 1.0$ . For hPg activation assays, 20  $\mu$ l ( $\sim 1 \times 10^7$  cfu) of cells was added to wells of a 96-well Corning NBS non-binding microwell plate, followed by 180  $\mu$ l of 0.22  $\mu$ M hPg/0.28 mM S2251 (to reach final concentrations of 0.2  $\mu$ M and 0.25 mM, respectively) in the same buffer. Finally, 5 nM rSK (final concentration) was added and the  $A_{405\text{nm}}$  was continually measured as above [8].

## 3. Results and discussion

### 3.1. hPg activation ability of SK likely evolved along with the M-protein serotype

It is clear that host hPg/hPm is a necessary invasive GAS pathogenicity factor [11–13]. Thus, one important virulence mechanism employed by GAS involves the secretion of SK which then specifically activates the host fibrinolytic system, producing the extracellular serine protease, hPm, thereby allowing the microorganism to acquire extracellular protease activity. hPm possesses activities which are important to bacterial virulence, such as dissolution of fibrin that encapsulates the bacteria and digestion of extracellular matrix components and basement membrane, e.g., laminin, fibronectin, either directly or indirectly via activation of matrix metalloproteinases [14,15].

Some highly invasive strains of GAS, such as skin tropic AP53, possess hPg/hPm cell surface receptors. One such receptor of paramount importance is PAM [16]. Binding of hPg to a small

N-terminal region ( $\alpha_1\alpha_2$ ) within PAM, enhances its activation rate. Binding of hPm to this same protein protects this enzyme from inhibition by its natural plasma inhibitor,  $\alpha_2$ -antiplasmin (AP) [17], thus, in a coherent manner utilizing the host to acquire proteolytic activity to combat one feature of its own innate immune response. Other less invasive GAS strains that do not possess PAM, e.g., pharyngeal-tropic NS931, also do not bind hPg/hPm strongly [18], a feature that appears to be general. Another group of GAS strains, e.g., SF370, possess a M-protein, that interacts strongly with Fg, which in-turn interacts with hPg, and also shows enhanced virulence.

These different receptor bound and free forms of host hPg encountered by GAS would benefit the virulence of the microorganism by GAS providing a hPg activator that optimized activation under the circumstances presented. Indeed this seems to be the case. SK, not being a protease, is not inactivated by host protease inhibitors, and is an optimal activator for hPg. This protein is secreted by all GAS strains thus-far encountered and indeed several general variants of SK are produced. Non-invasive strains of GAS, e.g., NS931, produce SK cluster 1 (SK1) [5], which optimally activates hPg in solution. The hPm thus produced would be systemic in this case and would be rapidly inactivated by AP. These conditions are not optimal for promoting dissemination. On the other hand, skin-tropic invasive strains of GAS, e.g., NS88.2, AP53, secrete a different SK, subcluster SK2b [5], which has very low activity toward hPg in solution and much higher activity with hPg bound to PAM [8]. Lastly, SK subcluster 2a (SK2a) is produced in strains in which the M-protein (M1) interacts with hPg through assembly of Fg [19,20]. One known GAS strain of this type SF370 is not highly virulent, but a clonal variant of SF370, M1T1, is virulent and depends on the presence of hPg.

### 3.2. The $\beta_{2b}$ domain is a major determinant for PAM-dependency while $\alpha_{2b}$ , $\gamma_{2b}$ play synergistic roles

Three domains exist in the 414-amino-acid SK, viz., an amino-terminal  $\alpha$ -domain (residues 1–146), a central  $\beta$ -domain (residues 147–290), and a carboxyl terminal  $\gamma$ -domain (residues 291–414). In order to attempt to understand the molecular evolution of SK in GAS, we exchanged each of the domains in SK1 and SK2b and assessed whether PAM dependence could be incorporated into SK1, or eliminated from SK2b, by limited exchanges between SK1 and SK2b. In this study, we employed 0.5  $\mu$ M rPAM<sub>AP53</sub>, a concentration that saturates its effect on hPg activation by all SKs employed (not shown), along with rSK1<sub>NS931</sub> and rSK2b<sub>NS88.2</sub>, prototypical SK1 and SK2b subtypes [5,9].

The large differences in activation rate of hPg in the absence of rPAM<sub>AP53</sub> between rSK1<sub>NS931</sub> ( $\alpha_1\beta_1\gamma_1$ ) and rSK2b<sub>NS88.2</sub> ( $\alpha_{2b}\beta_{2b}\gamma_{2b}$ ) are shown in Fig. 1A–C, where, in the absence of rPAM<sub>AP53</sub>, rSK2b<sub>NS88.2</sub> is 35-fold lower than rSK1<sub>NS931</sub> in the hPg activation activity, in agreement with our earlier data [8]. Upon addition of rPAM<sub>AP53</sub>, rSK1<sub>NS931</sub> is stimulated by 2–3-fold, whereas rSK2b<sub>NS88.2</sub> is stimulated by 19-fold, again in agreement with our earlier data [8]. This confirms that SK2b is much more highly stimulated by rPAM<sub>AP53</sub>, as compared to SK1. The data of Fig. 1A–C also demonstrate that substitution of the entire  $\alpha_1$  (in rSK<sub>M2</sub>) or  $\gamma_1$  domains (in rSK<sub>M6</sub>) in place of the corresponding  $\alpha_{2b}$  and  $\gamma_{2b}$  domains in rSK2b<sub>NS88.2</sub>, thus creating  $\alpha_1\beta_{2b}\gamma_{2b}$  and  $\alpha_{2b}\beta_{2b}\gamma_1$  rSK domain chimeras, respectively, did not elevate the hPg activator activity in the absence of rPAM<sub>AP53</sub> significantly, compared to rSK2b<sub>NS88.2</sub>, yet still raised the stimulation by rPAM<sub>AP53</sub> by 46-fold for rSK<sub>M2</sub> and 43-fold for rSK<sub>M6</sub>. Conversely, replacing the complete  $\beta_1$  domain in rSK<sub>NS931</sub> with the  $\beta_{2b}$  module from rSK2b<sub>NS88.2</sub>, generating chimera  $\alpha_1\beta_{2b}\gamma_1$  (rSK<sub>M3</sub>), resulted in diminished hPg activator activity of this variant in the absence of rPAM<sub>AP53</sub> to a value nearly equal to that of rSK2b<sub>NS88.2</sub>, and also led to stimulation by

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