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The β-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 β-domain of SK2b with the same module from SK1 reduces the dependency of SK2b on PAM, and the converse is true when the β-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.

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^{561}–Val^{562}, 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 α, β, or γ 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].

$rPAM_{AP53}$ (residues 42–392, lacking the C-terminal membrane insertion domain) was expressed and purified as described [8].

Human full-length recombinant Glu^1-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^1-hPg in solution

Activation rates of rhPg were continuously monitored in 96-well Corning NBS non-binding microwell plates in Cl^- 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 μl of a solution containing 0.2 μM hPg/± 0.5 μM 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 μl of 0.5 μM 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 $A_{600nm} \sim 0.6$ and collected by centrifugation. The cells were washed with 10 mM Hepes/150 mM NaCl, pH 7.4, and then resuspensed in this buffer to $A_{600nm} \sim 1.0$. For hPg activation assays, 20 μl ($\sim 1 \times 10^7$ cfu) of cells was added to wells of a 96-well Corning NBS non-binding microwell plate, followed by 180 μl of 0.22 μM hPg/0.28 mM S2251 (to reach final concentrations of 0.2 μM and 0.25 mM, respectively) in the same buffer. Finally, 5 nM rSK (final concentration) was added and the A_{405nm} 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 (a1a2) within PAM, enhances its activation rate. Binding of hPm to this same protein protects this enzyme from inhibition by its natural plasma inhibitor, α_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 β_{2b} domain is a major determinant for PAM-dependency while α_{2b}, γ_{2b} play synergistic roles

Three domains exist in the 414-amino-acid SK, *viz.*, an amino-terminal α-domain (residues 1–146), a central β-domain (residues 147–290), and a carboxyl terminal γ-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 μM $rPAM_{AP53}$, a concentration that saturates its effect on hPg activation by all SKs employed (not shown), along with $rSK1_{NS931}$ and $rSK2b_{NS88.2}$, prototypical SK1 and SK2b subtypes [5,9].

The large differences in activation rate of hPg in the absence of $rPAM_{AP53}$ between $rSK1_{NS931}$ ($\alpha_1\beta_1\gamma_1$) and $rSK2b_{NS88.2}$ ($\alpha_{2b}\beta_{2b}\gamma_{2b}$) are shown in Fig. 1A–C, where, in the absence of $rPAM_{AP53}$, $rSK2b_{NS88.2}$ is 35-fold lower than $rSK1_{NS931}$ in the hPg activation activity, in agreement with our earlier data [8]. Upon addition of $rPAM_{AP53}$, $rSK1_{NS931}$ is stimulated by 2–3-fold, whereas $rSK2b_{NS88.2}$ is stimulated by 19-fold, again in agreement with our earlier data [8]. This confirms that SK2b is much more highly stimulated by $rPAM_{AP53}$, as compared to SK1. The data of Fig. 1A–C also demonstrate that substitution of the entire α_1 (in rSK_{M2}) or γ_1 domains (in rSK_{M6}) in place of the corresponding α_{2b} and γ_{2b} domains in $rSK2b_{NS88.2}$, 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 $rPAM_{AP53}$ significantly, compared to $rSK2b_{NS88.2}$, yet still raised the stimulation by $rPAM_{AP53}$ by 46-fold for rSK_{M2} and 43-fold for rSK_{M6}. Conversely, replacing the complete β_1 domain in rSK_{NS931} with the β_{2b} module from $rSK2b_{NS88.2}$, generating chimera $\alpha_1\beta_{2b}\gamma_1$ (rSK_{M3}), resulted in diminished hPg activator activity of this variant in the absence of $rPAM_{AP53}$ to a value nearly equal to that of $rSK2b_{NS88.2}$, and also led to stimulation by

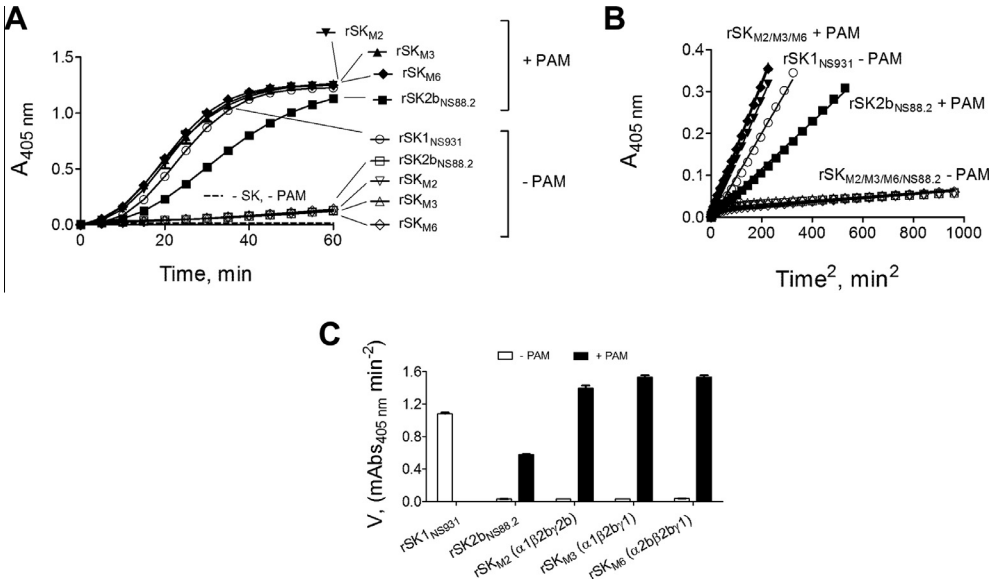

Fig. 1. Activation of rhPg by purified rSK and rSK variants in the absence and presence of rPAM$_{AP53}$. (A) Activation of 200 nM of hPg by 5 nM of purified rSKs, as labeled in Panel A, without PAM (−PAM) and with 0.5 μM rPAM$_{AP53}$ (+PAM). The unfilled symbols are assays performed in the absence of PAM, and filled circles are assays in the presence of 0.5 μM rPAM$_{AP53}$. The control (dashed line) was performed under the same conditions, except without the addition of rSK and rPAM$_{AP53}$. The generation of amidolytic activity was monitored continuously by the absorbance at 405 nm (A_{405nm}) *versus* time at 37 °C using 0.25 mM (final concentration) S2251 (H-D-Val-Leu-Lys-pNA). (B) A_{405nm} *versus* time^2 transformed from Panel A with linear regression of the linearized region. The symbols are as in Panel A. (C) The initial velocities calculated from the linear regions of plots in Panel B and shown as m(illi) A_{405nm} *versus* time^2.

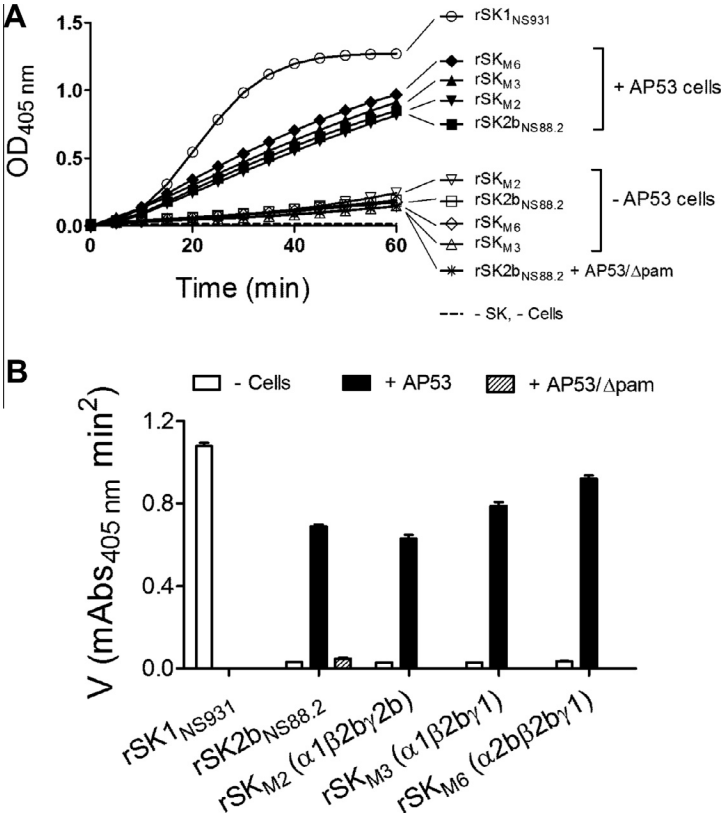

Fig. 2. Activation of rhPg by purified rSK and rSK mutants in the absence and the presence of GAS WT-AP53 cells or AP53/Δ*pam* cells. (A) Activation of 200 nM of hPg by 5 nM (final concentrations) of purified rSKs, as labeled in Panel A. The unfilled symbols are assays absent cells, and the filled circles are assays in the presence of 20 μl of GAS WT-AP53 cells (A_{600nm}, 1.0). The control (dashed line) was performed under the same conditions, except without the addition of rSK and cells. The generation of amidolytic activity was monitored continuously by the absorbance at 405 nm (A_{405nm}) *versus* time at 37 °C using 0.25 mM (final concentration) of S2251 as the hPm substrate. (B) The data from A were replotted as initial velocities of activation (as in Fig. 1B).

$rPAM_{AP53}$ (43-fold) to a value similar to those of rSK_{M2} and rSK_{M6}. Thus, variants that contain β_{2b} in rSK possess rSK2b-type activity, whereas the α_1 and γ_1 domains do not generate this property. Undoubtedly, however, the α- and γ-domains play a synergistic role with the β-domain in dictating the properties of SK, since each of the chimeric variants of Fig. 1 are more effectively stimulated by $rPAM_{AP53}$ than the native $rSK2b_{NS88.2}$.

The above results have been generally reproduced using the same rSKs, with a PAM-producing cell line, GAS-AP53, from which the $rPAM_{AP53}$ has been cloned, as compared to $rPAM_{AP53}$ (Fig. 2). While the extent of stimulation by cell-expressed PAM is slightly different from that of $rPAM_{AP53}$ protein, the general principles remain the same. In addition, use of the cell line lacking PAM, AP53/Δ*pam*, only shows a 1.5-fold stimulation of hPg activation by rSK2b, suggesting a minor importance in this regard of cellular receptors other than PAM, whereas stimulations of hPg activation activity by WT-AP53 cells with rSK_{M2}, rSK_{M3}, and rSK_{M6} are 22-, 21-, and 26-fold, respectively.

In conclusion, our results show that the primary structural differences in the β-domains dictate hPg activation differences between SK1 and SK2b, while sequence variations in the SK α- and γ-domains result in more minor and synergistic effects on the β-domain. This report on the primary structure–functional relationships between naturally-occurring SK1 and SK2b sheds light on the different manners in which GAS adapts to host factors to enhance its virulence.

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