

Published in final edited form as:

Biochem Biophys Res Commun. 2014 February 21; 444(4): 595–598. doi:10.1016/j.bbrc.2014.01.109.

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

Yueling Zhang^{1,2}, Jeffrey Mayfield¹, Victoria A. Ploplis¹, and Francis J. Castellino¹

¹W.M. Keck Center for Transgene Research and Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN 46556, USA

²Institute of Animal Sciences, Heilongjiang Academy of Agricultural Sciences, Harbin, 150086, China

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 α 1a2 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.

Keywords

Streptokinase; *Streptococcus pyogenes*; bacterial virulence; human plasminogen; protein domains

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, Arg⁵⁶¹-Val⁵⁶², 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].

© 2014 Elsevier Inc. All rights reserved.

Corresponding author: Francis J. Castellino, W.M. Keck Center for Transgene Research, University of Notre Dame, Notre Dame, IN 46556, USA, Telephone-574.631.8996; Telefax-574.631.8017; fcastell@nd.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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 protein [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 $\alpha 1a2$ 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) 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 Glu¹-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 Glu¹-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 (50:1, m:m, hPg/rSK) were employed to accelerate the activation of hPg. For these assays, 200 μ l of a solution containing 0.2 μ M hPg/ \pm 0.5 μ 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 μ l of 0.5 μ 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 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 $A_{405\text{nm}}$ 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 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 ($\alpha 1a2$) 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 show 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 $\alpha 2b$, $\beta 2b$ 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 μ M 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. 1 A–C, where, in the absence of rPAM_{AP53}, SK2b_{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 Figure 1 A–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 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 extents of stimulation by cell-expressed PAM are slightly different from that of rPAM_{AP53} protein, the general principles remain the same. In addition, use of the cell line lacking PAM, AP53/ γ pam, only shows a 1.5 fold stimulation 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-fold, 21-fold, and 26-fold, respectively.

In conclusion, our results show that 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.

Acknowledgments

This work was supported, in whole or in part, by National Institutes of Health Grant HL013423.

References

1. Castellino, FJ.; Ploplis, VA. Plasminogen Structure, activation, and regulation. Kluwer Academic/Plenum Publishers; 2003. Human Plasminogen: Structure, activation, and function; p. 3-17.
2. Castellino, FJ.; Ploplis, VA. Plasminogen and streptokinase. In: Bachmann, F., editor. Handbook of Pharmacology. Springer-Verlag; 2001. p. 25-56.

3. Smeesters PR, McMillan DJ, Sriprakash KS. The streptococcal M protein: a highly versatile molecule. *Trends Microbiol.* 2010; 18:275–82. [PubMed: 20347595]
4. Berge A, Sjobring U. PAM, a novel plasminogen-binding protein from *Streptococcus pyogenes*. *J Biol Chem.* 1993; 268:25417–24. [PubMed: 8244975]
5. McArthur JD, McKay FC, Ramachandran V, et al. Allelic variants of streptokinase from *Streptococcus pyogenes* display functional differences in plasminogen activation. *FASEB J.* 2008; 22:3146–53. [PubMed: 18511548]
6. Kalia A, Bessen DE. Natural selection and evolution of streptococcal virulence genes involved in tissue-specific adaptations. *J Bacteriol.* 2004; 186:110–21. [PubMed: 14679231]
7. Walker MJ, McArthur JD, McKay F, Ranson M. Is plasminogen deployed as a *Streptococcus pyogenes* virulence factor? *Trends Microbiol.* 2005; 13:308–13. [PubMed: 15936195]
8. Zhang Y, Liang Z, Hsueh HT, Ploplis VA, Castellino FJ. Characterization of streptokinases from Group A Streptococci reveals a strong functional relationship that supports the coinheritance of plasminogen-binding M protein and cluster 2b streptokinase. *J Biol Chem.* 2012; 287:42093–103. [PubMed: 23086939]
9. Zhang Y, Liang Z, Ginton K, Ploplis VA, Castellino FJ. Functional differences between *Streptococcus pyogenes* cluster 1 and cluster 2b streptokinases are determined by their beta-domains. *FEBS Lett.* 2013; 587:1304–9. [PubMed: 23474243]
10. Nilsen SL, Castellino FJ. Expression of human plasminogen in *Drosophila Schneider* S2 cells. *Protein Expr Purif.* 1999; 16:136–43. [PubMed: 10336871]
11. Boyle MD, Lottenberg R. Plasminogen activation by invasive human pathogens. *Thromb Haemost.* 1997; 77:1–10. [PubMed: 9031440]
12. Sun H, Ringdahl U, Homeister JW, et al. Plasminogen is a critical host pathogenicity factor for group A streptococcal infection. *Science.* 2004; 305:1283–6. [PubMed: 15333838]
13. Sanderson-Smith ML, Dinkla K, Cole JN, et al. M protein-mediated plasminogen binding is essential for the virulence of an invasive *Streptococcus pyogenes* isolate. *FASEB J.* 2008; 22:2715–22. [PubMed: 18467595]
14. Wong AP, Cortez SL, Baricos WH. Role of plasmin and gelatinase in extracellular matrix degradation by cultured rat mesangial cells. *Am J Physiol.* 1992; 263:F1112–8. [PubMed: 1481887]
15. Zwijsen A, van Grunsven LA, Bosman EA, et al. Transforming growth factor beta signalling in vitro and in vivo: activin ligand-receptor interaction, Smad5 in vasculogenesis, and repression of target genes by the deltaEF1/ZEB-related SIP1 in the vertebrate embryo. *Mol Cell Endocrinol.* 2001; 180:13–24. [PubMed: 11451567]
16. Ringdahl U, Svensson M, Wistedt AC, et al. Molecular co-operation between protein PAM and streptokinase for plasmin acquisition by *Streptococcus pyogenes*. *J Biol Chem.* 1998; 273:6424–30. [PubMed: 9497374]
17. Lottenberg R, DesJardin LE, Wang H, Boyle MD. Streptokinase-producing streptococci grown in human plasma acquire unregulated cell-associated plasmin activity. *J Infect Dis.* 1992; 166:436–40. [PubMed: 1386099]
18. Liang Z, Zhang Y, Agrahari G, et al. A natural inactivating mutation in the CovS component of the CovRS regulatory operon in a pattern D *Streptococcal pyogenes* strain influences virulence-associated genes. *J Biol Chem.* 2013; 288:6561–73. [PubMed: 23316057]
19. Ringdahl U, Svensson HG, Kotarsky H, et al. A role for the fibrinogen-binding regions of streptococcal M proteins in phagocytosis resistance. *Mol Microbiol.* 2000; 37:1318–26. [PubMed: 10998165]
20. Macheboeuf P, Buffalo C, Fu CY, et al. Streptococcal M1 protein constructs a pathological host fibrinogen network. *Nature.* 2011; 472:64–8. [PubMed: 21475196]

- Domains were switched between cluster 1 (SK1) and cluster 2b (SK2b) streptokinases
- The dependency on PAM protein of the resulting streptokinase variants was assessed
- The β domain of SK2b is the determinate domain for the dependency of SK2b on PAM
- The α and γ domains play synergistic roles in dictating the properties of SK

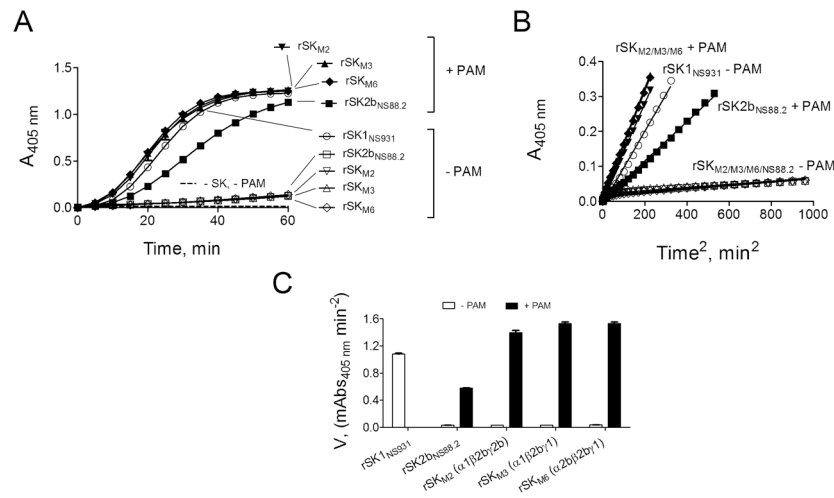


Figure 1.

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 absent 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 ($A_{405\text{ nm}}$) versus time at 37 °C using 0.25 mM (final concentration) S2251 (H-D-Val-Leu-Lys-pNA). (B). $A_{405\text{ nm}}$ 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 $\text{m(illi)A}_{405\text{ nm}}$ versus time^2 .

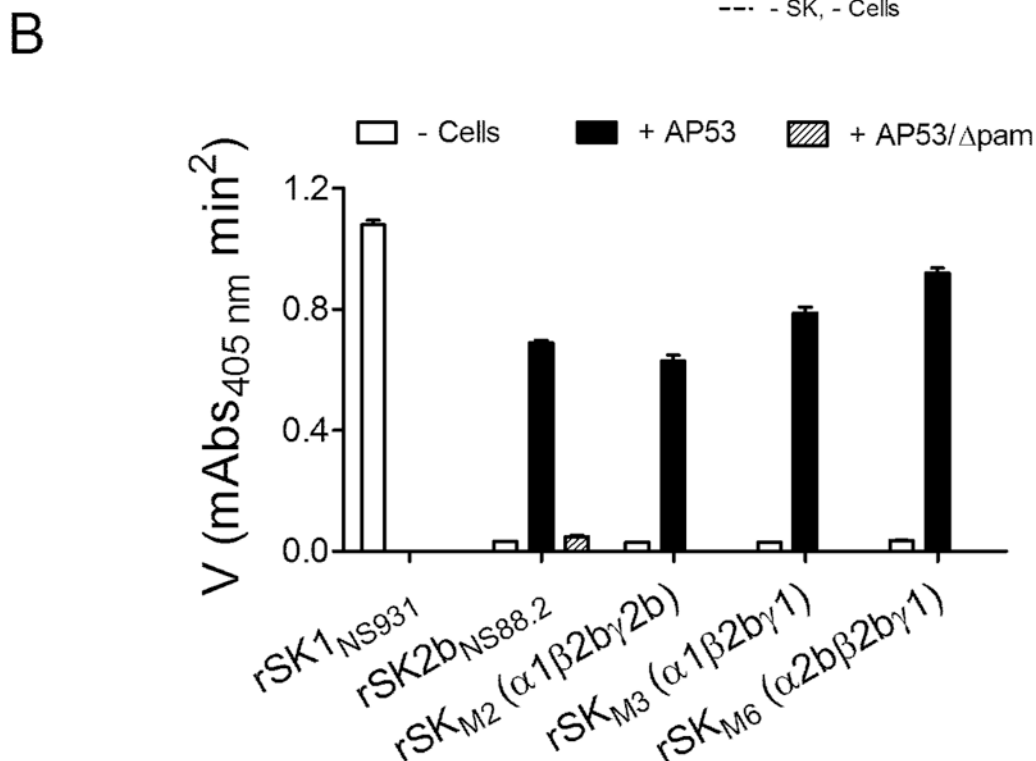
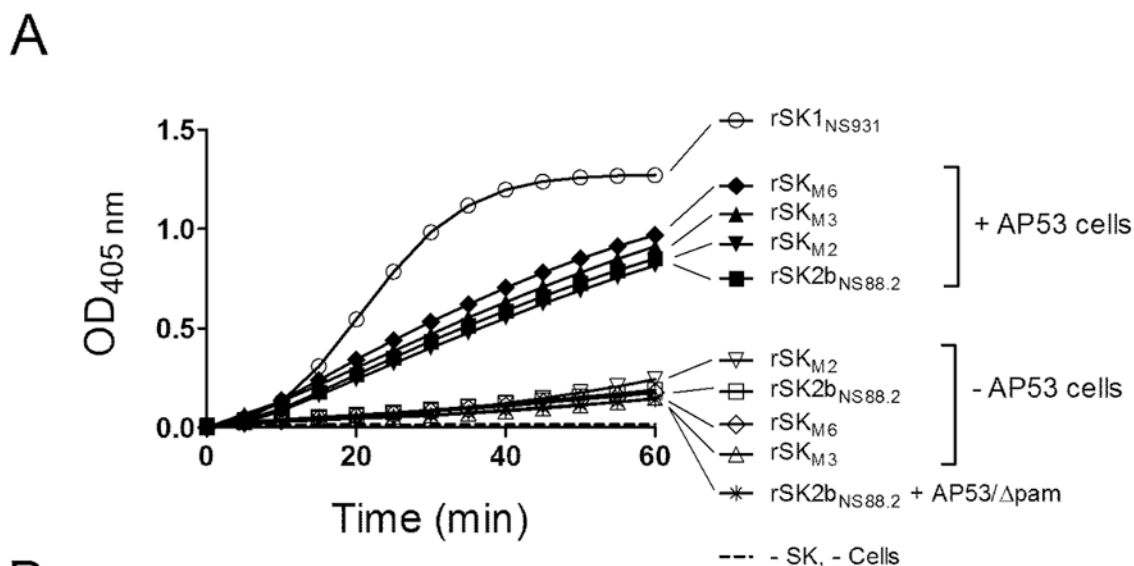


Figure 2.

Activation of rhPg by purified rSK and rSK mutants in absence and in presence of GAS WT-AP53 cells or AP53/Δpam 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 ($A_{600 \text{ nm}}$, 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 ($A_{405 \text{ nm}}$) 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 Figure 1B).