The β-domain of streptokinase affects several functionalities, including specific/proteolytic activity kinetics

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Keywords
fibrinolysis; plasminogen activation; protein domains; streptococci clusters; streptokinase; α2-antiplasmin resistance

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(Received 23 February 2019, revised 14 April 2019, accepted 13 May 2019)
doi:10.1002/2211-5463.12657

Streptokinase (SK) is a plasminogen activator which converts inactive plasminogen (Pg) to active plasmin (Pm), which cleaves fibrin clots. SK secreted by groups A, C, and G Streptococcus (SKA/SKC/SKG) is composed of three domains: SKα, SKβ and SKγ. Previous domain-swapping studies between SK1/SK2b-cluster variants revealed that SKβ plays a major role in the activation of human Pg. Here, we carried out domain-swapping between skcg-SK/SK2-cluster variants to determine the involvement of SKβ in several SK functionalities, including specific/proteolytic activity kinetics, fibrinogen-bound Pg activation and α2-antiplasmin resistance. Our results indicate that SKβ has a minor to determining role in these diverse functionalities for skcg-SK and SK2b variants, which might potentially be accompanied by few critical residues acting as hot spots. Our findings enhance our understanding of the roles of SKβ and hot spots in different functional characteristics of SK clusters and may aid in the engineering of fibrin-specific variants of SK for breaking down blood clots with potentially higher efficacy and safety.

Streptokinase (SK), a plasminogen activator (PA) secreted by groups A, C and G streptococci (GAS, GCS and GGS, respectively), converts the inactive plasminogen (Pg) to the active plasmin (Pm) which cleaves the fibrin clots. Despite being considered as a virulence factor (especially in GAS pathogenesis), traditionally, a nonfibrin-specific SK, isolated from the less virulent GCS (H46A or ATCC9542), was widely used as a fibrinolytic drug [1,2]. PA activity of SK is accomplished in two pathways. First, it binds to Pg and forms a 1:1 binary SK-Pg* activator (amidolytic) complex, which converts the free Pg substrate to Pm (nonfibrin-specific pathway I). Subsequently, the generated Pm binds SK to form SK-Pm proteolytic activator complex which converts Pg molecules to Pm (fibrin-specific pathway II) [2,3].

The 414 amino acid, SK, is composed of three distinct structural domains: α, β and γ spanning residues: 1–146, 147–290 and 291–414, respectively. Protein engineering studies indicated the importance of all three domains and the potential role of several critical amino acids (hot spots), such as Ile1 [3], Lys256, Lys257 [4] and recently Ile33, Asn228 and Phe287 [5], for SK functionality. The attained information was used to improve the fibrinolytic characteristics of SK for enhanced PA potency, fibrin specificity and resistance to the inhibitory effect of plasma α2-antiplasmin (α2-AP). Concurrently, heterogeneity of SKs at the

Abbreviations
Fg, fibrinogen; PA, plasminogen activator; PAM, plasminogen-binding group A streptococcal M-like protein; Pg, plasminogen; Pm, plasmin; SA*, specific activity; SK, streptokinase; SKA/SKC/SKG, respectively, groups A, C and G Streptococcus; α2-AP, alpha 2-antiplasmin.
gene (sk) and protein (SK) levels in different strains (even the same group) of streptococci (specifically for GAS) and its relation to functional differences was shown [2]. Studies indicated the highest sequence diversity of β-domain compared to α and γ, particularly in a distinct hypervariable region (sk-V1; residues 147–218). Accordingly, the sk-V1 was suggested as the main source of sk allelic variations, and consequently, phylogenetic analysis of the sk-V1 nucleotide sequences was used to classify the GAS-SK (sk/a) alleles into two main clusters; SK1 and SK2, in which SK2 was further subdivided into subclusters SK2a and SK2b [6,7]. These clusters successfully classified GAS strains into those that contain (a) a Pg/Pm direct binding M-like protein, ‘PAM’ and usually induce invasive skin infections (SK2b), (b) a fibrinogen (Fg) binding M1 protein that does not directly interact with Pg and usually induce upper respiratory tracts (UTR) infections (SK2a) and (c) a M protein that does not interact with either Pg or Fg (SK1) and optimally activates Pg in solution. Although presence of Fg generally enhances the PA activity of all SK types, the specific PA activation rate (2-AP) than SK1 or SK2b. Moreover, complexes of Pm with SK2a and skcg-SK display higher resistance to inhibition by α2-AP than SK1 or SK2b [1]. Therefore, skcg-SKs display some characteristics specific for either SK1 or SK2a clusters and are thus interesting candidates for comparative studies.

Attempts to address the role of β-domain heterogeneity for functional characteristics of SK clusters/subclusters started with a study on exchange and swapping the major polymorphic regions between SK1 β-domain (SK1β) and SK2aβ [10]. However, apparently due to the similar PA potencies of the used SK1 and SK2a variants, this study failed to uncover any effect on Pg activation kinetics of the chimeric and parental SKs. Recently, two other studies addressing the domain-exchange strategies between a SK1 (with high PA activation rate) and SK2b indicated the major role of the β-domain in the PA activity, which might be further assisted by α-domain [11]. But how β-domain exchange might alter the kinetics of the amidolytic/proteolytic pathways, Fg-bound-Pg activation or the resistance to inhibition by α2-AP, especially between skcg-SK and SK2b, are other concerns that never addressed. Recently, SK from a newly isolated GGS (SKG88) with high PA activity was introduced [12]. In the present report, using SKG88 and two other SKs belonging to SK2a and SK2b and employing domain-exchange approaches, these concerns are addressed.

Materials and methods

Bacterial strains and reassessment of the SK clusters

The skcg-SK of GGS (G88) with high PA activities [12], which was supposed to be clustered as SK2a [6], was used for β-domain exchange between SK of two GAS strains; STAB902 containing SK2a with very low PA activities [13] and ALAB49 (gifted by Mc. Arthur, University of Wollongong, Australia), containing a well-known SK2b with barely detectable PA activities in culture supernatants [7]. The gene accession numbers are as follows: HM390000.1, CP007041.1 and AY234134, respectively. Sequence alignment and phylogenetic analysis of skβ-V1 region for these three SKs and six other well-known SK clusters [6,7] were accomplished by Molecular Evolutionary Genetics Analysis, MEGA6 [14].

Construction of the parental and β-domain-exchanged SK-encoding plasmids

The detailed steps for cloning of sk into pET26b vector to construct the parental plasmids (pET26b-SKG88, pET26b-SK_ALAB49 and pET26b-SK_STAB902) are illustrated in Fig. S2.

For construction of the β-domain-exchanged SKs, the region corresponding to nucleotides 375–699 (residues 125–233) from the parental vectors was digested by BsiWI/BstEII restriction enzymes and the digested fragments (327 bp) were cross ligated between SKG88 and two other SKs (SK_ALAB49 and SK_STAB902) (Fig. 1B). All the molecular methods were based on the standard protocols [15].

Expression, purification and characterization of SK proteins

Escherichia coli Rosetta (Novagen, USA) was used for protein expression via IPTG induction, and expressed SKs were purified under native conditions using nickel-nitriloacetic acid (Ni-NTA) affinity chromatography (Qiagen, USA) according to the manufacturer’s protocols (QIAexpressionist 2002; Qiagen). Protein concentrations were determined by Bradford assay. Expression and the purity of the purified SKs were assessed by 12% (w/v) SDS/PAGE and confirmed by western blotting. Protein characterizations assays are described in Figs S5 and S6.
Determination of SK-specific activity (SA*)

For evaluation of the SA* in the presence/absence of Fg, the standard colorimetric assay using the chromogenic substrate (S-2251; Sigma, USA) was used throughout this study, as previously described [7]. The detailed procedure for the assay, construction of the calibration curve and calculation of the SA* are provided in Fig. S7, Figs 2 and 3.

Determination of kinetic constants for amidolytic and proteolytic activities

For analysing amidolytic kinetics, first stoichiometric concentrations of Pg and SK (5.5 µM SK and 5 µM Pg) were mixed and incubated for 5 min to produce the SK-Pg* activator complex. Subsequently, an aliquot of the complex (100 nm) was transferred to the assay buffer along with various concentrations of S2251 (0.1–1.5 mM) in a total volume of 100 µL [12].

For analysing proteolytic kinetics, 100 nm of SK was added to assay buffer containing ‘0.1 mM S2251 and varying concentrations of Pg (0.3–5.0 µM)’ and changes in absorbance at 405 nm were monitored for 30 min. The data were plotted as velocity/substrate concentration, and kinetic parameters of Pg activation were determined from Michaelis–Menten (V vs S) and inverse (1/V vs 1/S) Lineweaver–Burk plots using GRAPHPAD PRISM 6 (GraphPad Software, La Jolla, CA, USA) [12].

Inhibition by α2-antiplasmin

Stoichiometric complexes of SK-Pm (400 nm SK and 200 nm Pm) were incubated for 5 min. The complex was
diluted to 20 nM in assay buffer containing γ2-AP (final concentration: 100–400 nM). The mixtures were incubated for 15 min, then S-2251 (500 µM) was added to the reaction, and residual activity of complex was measured by change in absorbance at 405 nm [1].

Statistical analyses

Unpaired, two-tailed Student’s t test with 95% confidence intervals was used for analysis of SK-PA activities and kinetics using SPSS software version 22.0 (SPSS Inc., USA). All linear regressions were by GRAPHPAD PRISM 6, and P-values < 0.05 were considered significant.

Results and Discussion

Confirmation of the SK clusters and production of SK proteins

Sequence alignment of skβ-V1 region for G88, STAB902 and ALAB49 and six other well-known SK clusters [6,7] that were used for construction of the phylogenetic tree is provided in Fig. S1. Phylogenetic analysis (Fig. 1A) identified the skg-encoded-SKG88 as SK2a (SK2aG88) which is in complete agreement with prior reports on clustering of skg alleles [6]. Accordingly, STAB902 and ALAB49 SKs were
subclustered as SK2a (SK2aSTAB902) and SK2b (SK2bALAB49), respectively [7,13]. PCR amplification of all three parental SKs produced the expected 1250-bp amplicon (Fig. S3). Cloning steps for insertion of parental SKs (SK2aG88, SK2bALAB49, SK2aSTAB902) into pET26b vector and exchange of the 327-bp β-domain (BstEII/BsiWI) fragments between SK2aG88 and SK2bALAB49 (hereafter intracluster chimeric constructs; SKC1 (2bALAB2aG88) and SKC2 (2bALAB2aG882bALAB) denote the intracluster chimeras. SKC3 (2aSTAB2bALAB2aG88) and SKC4 (2aSTAB2aG882aSTAB) denote the intra-subcluster chimeras.

The SA* of SK2aG88 (760.82 × 10^3 IU·mg^−1) was about 28-fold and 22-fold higher than that of SK2bALAB49 (26.64 × 10^3 IU·mg^−1) and SK2aSTAB902 (36.50 × 10^3 IU·mg^−1), respectively. Prior studies reported over 10-fold higher PA activity for SK1 compared to SK2b [8,11] which further supports the similarity of SK1 and skcg-SK (SK2aG88) for optimal PA activity in solution [11]. Indeed, SK1β is the most divergent among all SK clusters, and the divergence between SK1 and SK2b might even exceed 40% [6,7], which might further support the determining role of β-domain for functional characteristics between SK1 and SK2b clusters [11,16]. But sequence alignments (Fig. S8 and Table 3) indicated that the exchanged β-domains between SK2aG88 and SK2bALAB49 (intracluster; Fig. 1B) were 89% similar, while α- and γ-domains exhibited 82% and 86% similarity, respectively. Therefore α- and γ-domains might have more contribution in functional characteristics of the skcg-SK2b domain-exchanged SKs in our study (SKC1/SKC2) than that of SK1/SK2b in the prior report [11]. In contrast, exchanging the SK2aG88β and SK2aSTAB902β, for making the two intra-subcluster constructs (SKC3: 2aG882aSTAB2aG88 and SKC4: 2aSTAB2aG882aSTAB; Fig. 1B) led to less alterations in the SA* values for SKC3/SKC4 compared to SK2aG88 and SK2bALAB49 (Fig. 2D and Table 1). Thus, our results, consistent with a prior study on SK1β and SK2β exchanged domains, could not uncover any major effects on PA potencies [10]. However, in the prior study, despite sharing less than 50% identity between exchanged SK1β

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**Fig. 3.** The time-course activity profiles (the change in absorbance at 405 nm as a function of time) of SKs. For measuring the specific activity (IU·mg^−1) of the SKs, the slopes of the linear portion of the curve obtained from plotting absorbance (OD) at 405 nm vs time (Fig. 2A,B) were used. Serial dilutions of Streptase® (CSL) were used as reference for preparation of the standard curve (Fig. S7) and calibration of international units·mg^−1 protein (specific activity) in the samples. SK2aG88, SK2bALAB49 and SK2aSTAB902 are parental constructs. SKC1 (2aG882bALAB2aG88) and SKC2 (2bALAB2aG882bALAB) denote the intracluster chimeras. SKC3 (2aSTAB2bALAB2aG88) and SKC4 (2aSTAB2aG882aSTAB) denote the intra-subcluster chimeras.

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**Contribution of the SKβ in specific activity (SA*)**

For calculation of the SA*, the time-course activity profiles (the change in absorbance at 405 nm as a function of time) were measured (Fig. 2A,B), and subsequently, SA* was calculated (Fig. 2C,D and Table 1), from the slope of the linear portion of the curve (Fig. 3) in which serial dilutions of commercial/standard SK (Streptase) were used as the reference for calibration (Fig. S7). As shown in Table 1, the SA* of SK2aG88 (760.82 × 10^3 IU·mg^−1) was about 28-fold and 22-fold higher than that of SK2bALAB49 (26.64 × 10^3 IU·mg^−1) and SK2aSTAB902 (36.50 × 10^3 IU·mg^−1), respectively. Prior studies reported over 10-fold higher PA activity for SK1 compared to SK2b [8,11] which further supports the similarity of SK1 and skcg-SK (SK2aG88) for optimal PA activity in solution [11]. Indeed, SK1β is the most divergent among all SK clusters, and the divergence between SK1 and SK2b might even exceed 40% [6,7], which might further support the determining role of β-domain for functional characteristics between SK1 and SK2b clusters [11,16]. But sequence alignments (Fig. S8 and Table 3) indicated that the exchanged β-domains between SK2aG88 and SK2bALAB49 (intracluster; Fig. 1B) were 89% similar, while α- and γ-domains exhibited 82% and 86% similarity, respectively. Therefore α- and γ-domains might have more contribution in functional characteristics of the skcg-SK2b domain-exchanged SKs in our study (SKC1/SKC2) than that of SK1/SK2b in the prior report [11]. In contrast, exchanging the SK2aG88β and SK2aSTAB902β, for making the two intra-subcluster constructs (SKC3: 2aG882aSTAB2aG88 and SKC4: 2aSTAB2aG882aSTAB; Fig. 1B) led to less alterations in the SA* values for SKC3/SKC4 compared to SK2aG88 and SK2bALAB49 (Fig. 2D and Table 1). Thus, our results, consistent with a prior study on SK1β and SK2β exchanged domains, could not uncover any major effects on PA potencies [10]. However, in the prior study, despite sharing less than 50% identity between exchanged SK1β
and SK2αβ, the parental SKs had relatively similar SA* [10], while despite clustering as SK2α, the SK2αG88 and SK2αSTAB902 in our study show highly different SA* (Table 1). Indeed, the exchanged β-domains (residues 128–233) of SK2αG88 and SK2αSTAB902 were around 97% identical (corresponding to only three residue substitutions out of 108; K138S, I151V, E161K; Table 3) while their α- and γ-domains exhibited 85% and 88% similarity, respectively (Fig. S8). Therefore, it might be the presence of only few scattered residues acting as hot spots rather than accumulated altered residues in a specific domain that counts for the highly different SA* activities, as recently claimed [5]. Having shown that β-domain exchange between SK2α intra-subclusters (SKC3/SKc4) had little contribution to SA*, the rest of the experiments were only performed for SKC1/SKc2.

**Contribution of the SKβ in the kinetics of amidolytic/proteolytic activity**

Amidolytic/proteolytic activity of the SKs was studied by measuring the steady-state kinetic constants of the S225I hydrolysis including substrate affinity (Km), catalytic activity (Kcat) and the constant of catalytic efficiency (Kcat/Km; efficiency of the Pg conversion into Pm). As shown in Table 2, Km and Kcat values did not alter significantly between SKC1 and SK2αG88 (0.39 mM and 1.39 s⁻¹ vs 0.41 mM and 1.39 s⁻¹, respectively) leading to almost similar catalytic efficiency (Kcat/Km).

For SKC2 compared to SK2bALAB49, the Kcat raised by 2.5% (0.88 vs 0.86 s⁻¹) and the Km reduced by 17% (0.34 vs 0.41 mM) leading to an overall 24% increase in catalytic efficiency (2.59 × 10³ vs 2.10 × 10³ s⁻¹.M⁻¹) (Table 2). Interestingly, evaluation of the kinetic parameters for proteolytic activity indicated that the catalytic efficiency of SKC1 declined by 47% compared to SK2αG88 (229.27 × 10³ vs 428.57 × 10³ s⁻¹.M⁻¹), which was mainly due to threefold increase in Km value (0.77 vs 2.05 μM). For SKC2 compared to SK2bALAB49, the Km declined by 57% (3.45 vs 7.92 μM) and the Kcat increased by 40% (0.22 vs 0.16 s⁻¹) leading to more than threefold augmented values for catalytic efficiency (63.77 × 10³ vs 20.20 × 10³ s⁻¹.M⁻¹). These results indicated the determining role of the skcgβ (SK2αG88β) on enhancement of proteolytic activity (Table 2), mainly due to the augmentation of the Km values (increased substrate affinity) which is in accordance with the SA* results (Table 1). Our results are consistent with a prior report on the importance of the SKβ for strong binding of Pg substrate to the SK-Pm proteolytic complex and its efficient conversion to Pm [17].

**Contribution of the SKβ on Fg-bound-Pg activation**

The Pg activation rate of various SKs in the presence/absence of Fg was measured by monitoring the absorbance at 450 nm and calculated by linear regression

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**Table 1. Specific activities of SK variants.**

| Parental SKa         | Specific activity (x 10¹² IU·mg⁻¹) | Chimeric SKa      | Specific activity (x 10¹² IU·mg⁻¹) |
|----------------------|-------------------------------------|------------------|-------------------------------------|
| SK2αG88 | SK2βALAB49 | x2αβ2y2α            | 760.82 ± 13.63 | Intracluster | SKC1 (2αG88-2bALAB-2αG88) | 329.24 ± 7.07 |
| SK2βALAB49 | x2αβ2y2b            | 26.64 ± 1.79 | SKC3 (2αG88-2bALAB-2αG88) | 83.24 ± 1.28 |
| SK2αSTAB902 | x2αβ2y2α            | 36.50 ± 1.02 | SKC4 (2αG88-2bALAB-2αG88) | 713.54 ± 2.64 |
| SK2αSTAB902 | x2αβ2y2α         | 36.50 ± 1.02 | SKC4 (2αβSTAB-2αG88-2αSTAB) | 70.84 ± 0.89 |

*All measured P-values were less than 0.05 (P < 0.05) considered significant.

*aParental and chimeric SK denote the three originally isolated SKs and four SKβ-exchanged SKs. Please see the text for complete explanations.

**Table 2. Kinetic parameters for amidolytic/proteolytic activities of SK variants.**

| SK variants | Amidolytic constants | Proteolytic constants |
|-------------|----------------------|-----------------------|
|             | Km x 10⁻³ (μM)       | Kcat (s⁻¹)            | Kcat/Km x 10³ (s⁻¹.M⁻¹) |
| SK2αG88     | 0.41 ± 0.006         | 1.39 ± 0.031          | 3.39                   |
|             | 0.77 ± 0.14          | 0.33 ± 0.082          | 428.57                 |
| SK2βALAB49  | 0.41 ± 0.048         | 0.86 ± 0.122          | 2.10                   |
|             | 7.92 ± 0.16          | 0.16 ± 0.004          | 20.20                  |
| SKC1        | 0.39 ± 0.062         | 1.39 ± 0.092          | 3.56                   |
|             | 2.05 ± 0.753         | 0.47 ± 0.237          | 229.27                 |
| SKC2        | 0.34 ± 0.062         | 0.88 ± 0.090          | 2.59                   |
|             | 3.45 ± 0.23          | 0.22 ± 0.018          | 63.77                  |

*All measured P-values were less than 0.05 (P < 0.05) considered significant.
As shown in Figs 4A and 5, generally, in the presence of Fg, the activation rate \( (\Delta OD_{405}/t) \) of all SKs (SK2aG88, SKC1, SKC2, SK2bALAB49) raised significantly (2.00, 2.00, 0.83, 0.92 \times 10^{-2} \) vs (0.57, 0.19, 0.14, 0.10 \times 10^{-2} \) respectively), but the effect is more}

from the linear regions of the plots (OD_{405}/time). As shown in Figs 4A and 5, generally, in the presence of Fg, the activation rate \( (\Delta OD_{405}/t) \) of all SKs (SK2aG88, SKC1, SKC2, SK2bALAB49) raised significantly (2.00, 2.00, 0.83, 0.92 \times 10^{-2} \) vs (0.57, 0.19, 0.14, 0.10 \times 10^{-2} \) respectively), but the effect is more
Table 3. The altered residues in the exchanged SKβ domain of SK2aG88 compared to SK2aSTAB902 and SK2bALAB49. Conserved (identical) residues are indicated by dots.

| Residue position | SK2aG88 | SK2aSTAB902 | SK2bALAB49 |
|------------------|---------|-------------|------------|
| 132              | V       |             | I          |
| 134              | E       | S           | Q          |
| 138              | K       | V           | R          |
| 151              | I       | .           | T          |
| 153              | N       | .           | P          |
| 154              | Q       | .           | I          |
| 160              | V       | .           | R          |
| 161              | E       | K           | K          |
| 176              | R       | G           | T          |
| 178              | G       | K           |           |
| 209              | K       |             |            |
| 210              | T       |             | D          |
| 213              |         |             |            |

reflective for SK2bβ containing constructs (SKC1,SK2bALAB49) than SK2aG88/SKC2 (10.80/9.20 vs 3.51/5.93-fold enhancement of activation rates, respectively). These results are consistent with prior reports for higher influence of Fg on enhancement of SK2b activity compared to SK1 [7,8] and that of the SK2a compared to a skc-SK [9], indicating more similarity of the skcg (SK2aG88) to SK1 variants for activation of Fg-bound-Pm. As shown in Fig. 4A and consistent with a recent study [12], SK2aG88 showed high intrinsic Fg-bound-Pg activation. This higher Fg-bound-Pg activation (twofold higher than SK2bALAB49, 2.00 vs 0.92) is completely retained in SKC1, while in the absence of Fg, PA potency of SKC1 is three times lower than the parental SK2aG88 (0.19 vs 0.57). Of note, these characteristics of SKC1 might be of interest for development of a fibrin-specific version of SK for targeted fibrinolysis [3]. Interestingly, SKC2 retained the same (and low) activity as SK2bALAB49 in the absence of Fg, while its Fg-bound-Pg activation showed 42% (0.83 vs 2.00) and 90% (0.83 vs 0.92) of the parental activity (SK2aG88 and SK2bALAB49, respectively). Collectively, while these results support the major contribution of the SKβ for the Fg-bound-Pg, but in agreement with prior reports also implies the potential contribution of other domains for this characteristic [3].

Contribution of the SKβ on resistance to α2-AP inhibition

As shown in Fig. 4B, all four SK-Pm complexes resisted the inhibitory effect of α2-AP (400 nM) by retaining more than 50% of their activity. This observation is consistent with the long-known phenomena for resistance of the SK-Pm complex to the major physiological plasmin inhibitor ‘α2-AP’ [18]. However, for SK2aG88 and SKC2 (containing SK2aG88β), retained activity was about 80%, while for that of SK2bALAB49 and SKC1 (containing SK2bALAB49β), it was about 50% (Fig. 4B). Thus, our results indicated that skcg-SK was more resistant to inhibition by α2-AP than SK2b, which is in agreement with recent findings for Pm-complexed with either SK-H46A (skc) or SK2a variants [1]. Therefore, our results indicated the resemblance of skcg-SK to SK2a variants for ‘α2-AP resistance’ and the determining role of SKβ in these characteristic. Although this finding is consistent with prior suggestion on the contribution of SKβ to the interaction of SK with inhibitors [19], the role of other domains, specially residue 1–59 of α-domain for resistance to α2-AP, was also suggested [3].

Potential contribution of the substituted residues (hot spots) in SK functionalities

As emphasized earlier, a recent study on a new isolate of skcg-SK (GGS-132) indicated that presence of only three altered residues (Ile33Phe, Asn228Lys and Phe287Ile) that probably acted in a synergic mode as hot spots might induce enhanced proteolytic/Fg-bound-Pg activation compared to a SKC (GCS-SKC9542) [5,12]. Accordingly, it was also shown that SK2aG88, in the present study, exhibited enhanced proteolytic/Fg-bound-Pg activation compared to the same SKC, while only seven residues scattered within domains of the two SKs were substituted (98% similarity) [12]. As shown in Table 3, the exchanged segments between SK2aG88β and SK2bALAB49β differ by 12 residues. Among these altered residues, ‘V160I, E161R and K209E’, consistent with a recent report [17], might have potentially acted as hot spots for the induced functionalities of the domain-swapped SKs (SKC1/SKC2). The precise insight on the effect of these substitutions might be gained via site-directed mutagenesis experiments.

In conclusion, to the best of our knowledge, we reported the first domain-exchange study for skcg and cluster 2-ska alleles to elucidate the contribution of SKβ for a broad range of functional characteristic including kinetics of specific proteolytic activity, fibrinogen-bound Pg activation and α2-antiplasmin resistance. Results pointed to the ‘minor to determining’ contribution of SKβ in these functionalities which might be potentially accompanied by a few critical residues acting as hot spots. Our findings indicated the (a) similarity of coclustered, skcgβ and SK2aβ
variants (only three residues alteration) and minor contribution of their SKβ for highly different SA* between these two alleles; (b) similarity of skcg to cluster 1-ska alleles (SK1) for optimal PA activity in solution and activation of Fg-bound-Pm compared to that of the SK2 variants and major contribution of SKβ in this characteristic; (c) major role of the SKβ on enhancement of proteolytic activity between skcg-SK and SK2b that is mainly due to the augmentation of the $K_m$ values (increased substrate affinity); and (d) similarity of skcg-SK to SK2a variants for ‘$\alpha$-2-AP resistance’ and the determining role of SKβ in this characteristics. These findings might assist in better understanding of the roles displayed by SKβ and hot spots for different functional characteristic of SK clusters and engineering fibrin-specific versions of SK.

**Acknowledgements**

This study was financially supported by Pasteur Institute of Iran (BP-8922) in partial fulfilment of the Ph.D thesis of MR in medical biotechnology programme.

**Conflict of interest**

The authors declare no conflict of interest.

**Author contributions**

MR, the Ph.D candidate, did most of the experiments and prepared the primary draft of the manuscript. MK, the 1st advisor, assisted in cloning and kinetics analysis and design of the study. MMA, the 2nd supervisor, assisted in strain isolation and microbiology assays, and design of the study. AA, the 2nd advisor, assisted in protein expression and purification assays. FR, the 1st supervisor, designed and supervised the study and prepared the final manuscript for submission.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Multiple DNA Sequence alignment of skβ-V1 fragments. Multiple Sequence alignment of skβ-V1 fragments (hypervariable region of the β-domain) from nucleotide “445 to 655” of sk gene, corresponding to the amino acid residues “147 to 218” of the SK of the strains used in this study (G88, STAB902 and ALAB49; Genbank Accession numbers: HM390000.1, CP007041.1 and NY234134, respectively) together with the well-known strains that were employed for construction of phylogenetic tree in Fig. 1A (Genbank Accession numbers: NS10; EU352637.1, NS32; EU352630.1, 5448; CP008776.1, NS414; EU352621.1, NS501; EU352616.1, NZ131; CP000829.1). The alignment was created using MEGA software [14]. Conserved (identical) nucleotides are indicated by dots.

Fig. S2. Schematic illustration for construction of the parental SK-encoding plasmids. For construction of the three parental constructs, the genomic DNA from culture of the three targeted streptococci (G88, STAB902 and ALAB49) grown in BHI (brain heart infusion) broth, were isolated by DNA extraction kit (Qiagen, USA) according to the manufacturer’s protocols. The extracted genomic DNA was used as template for PCR-based amplification of the coding region of sk genes (lacking the signal peptide sequence) using a pair of primers with inserted restriction sites (NdeI-XhoI) for direct cloning into pET26b vector (forward primer; NdeI-SKf: GACGAGACATATGATTGCT GGACCTGAGTG-3; reverse primer; XhoI-SKr 5-GA CACTCGAGTTTCTGCATTAGGTTACG-3). The sequences corresponding to restriction sites are underlined. The resulting amplified fragments were digested with NdeI and XhoI and cloned into the same sites of pET26b expression downstream of T7 promoter, in tandem with the fused C-terminus 6XHis-tag to provide the three parental SK-encoding vectors (pET26b-SK_G88, pET26b-SK_ALAB49 and pET26b-SK_STAB902). All cloning steps were performed according to standard procedures [15]. ATG stands for vector-derived, translation-start codon; MCS, multiple cloning sites; 6His-tag is the tag derived from the vector.

Fig. S3. Analysis of the PCR-amplified sk genes by agarose gel (1%) electrophoresis. The coding region of sk gene (lacking the signal peptide sequence) was amplified by PCR using Skf and Skr primers. PCR reactions resulted in a single band of the expected length (1250bp) of sk genes. Lane1: DNA Marker 1kb (Thermo scientific SM0311), Lane2 and 3: PCR products of skg88 and skstab902 gene from genomic DNA. The corresponding bands were indicated by arrows. The sizes of the bands of DNA marker are illustrated on the right.

Fig. S4. Agarose gel (1%) electrophoresis of the restriction enzyme analysis of the recombinant vector pET26b-SKG88. Lane 1: Digested pET26b-SKG88 by BsiEI, produced two bands with the approximate size of 1400 and 5100 bp. Lane 2: Digested pET26b-SKG88 by NdeI-XhoI, yielded 5230 and 1250 bp fragments corresponding to vector and PCR fragments, respectively. The corresponding bands were indicated by arrows. Lane 3: DNA Marker 1kb (Thermo scientific SM0311). The same analysis for the other two parental constructs pET26b-SK_ALAB49 and pET26b-SK_STAB902 produced the same results (not shown).

Fig. S5. Analysis of the protein expression and purification by SDS/PAGE (12%). For protein expression, first the transformation of E. coli Rosetta cells (Novagen, USA) with the seven SK-encoding recombinant vectors (three parental vectors: pET26b-SK_G88, pET26b-SK_ALAB49 and pET26b-SK_STAB902 and four domain-exchanged chimeric vectors: pET26b-SK_C1 to pET26b-SK_C4) by standard CaCl2 method was performed. Subsequently, protein
expression was induced at OD$_{600}$ of 0.5–0.6 by isopropyl-$\beta$-D-thio-galactoside (IPTG) to a final concentration of 1 mM for 3 hours at 37°C. Finally, cells were harvested by centrifugation and stored at -20°C for purification steps. Purification of His-tagged SK proteins from induced *E. coli* Rosetta cells was performed under native conditions, using nickel-nitriloacetic acid (Ni-NTA) affinity chromatography and according to manufacturer’s protocol (QIAexpressionist, 2002, Qiagen company website). Briefly, the cell pellets were resuspended in binding buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole) with 0.5 mg/ml lysozyme at 2–5 ml per gram wet weight. After incubation on ice for 30 min, the cells were disrupted by sonication, and supernatant was collected after centrifugation at 10,000 g for 20-30 min at 4°C. After addition of 1ml resin Ni-NTA to the clear lysate, the mixture was shaken at 4°C for 60 minutes, loaded on column and washed 4 times with 4 ml wash buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 20 mM imidazole) then 4 times with 0.5 ml elution buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 250 mM imidazole) (QIAexpressionist 2002). SDS-PAGE analyses were performed according to standard procedures [15]. (A) Analysis of the bacterial crude extracts for expression by pET26b-SK$_{G88}$. Lanes 1-3; un-induced bacterial cells, Lane 4; protein marker 10-180 kDa (SM7012 CinnaGen Co), Lanes 5-8; corresponds to IPTG induced cells with 0.3, 0.5, 0.7, 1 and 1.2 mM IPTG, respectively. (B) Analysis of protein purification steps for pET26b-SK$_{G88}$. Lanes 1- 4 represent the elution E1 to E4, respectively collected after protein extraction under native condition. Lane 5: protein marker 10-180 kDa (SM7012 CinnaGen Co). Arrows indicate the location of 47 kDa (SK). Analysis of other constructs for expression and purification produced the same results (not shown).

**Fig. S6.** Confirmation of the expressed proteins by western blotting. Western blotting was performed according to the standard protocols [15]. Briefly, proteins were transferred from SDS-PAG to the nitrocellulose membrane and the membrane was blocked by 5% BSA. Mouse anti-His monoclonal antibody (Qiagen, USA) was used as the primary antibody and goat anti-mouse IgG conjugated to HRP (Horse Radish peroxidase) (Qiagen, USA) as the secondary (tracking) antibody. Detection of the bands was by 3, 3-diaminobenzidine (DAB) (Qiagen, USA). Western-blot analysis for SK$_{2aG88}$ and SK$_{C1}$ proteins are shown. Lane 1; molecular weight marker (SM7012, Cinnagen Co), lanes 2 and 4; crude lysis of *E. coli* Rosetta cells after induction by IPTG (1mM), expressing SK$_{2aG88}$ and SK$_{C1}$, respectively, lane 3; crude lysis of *E. coli* Rosetta cells before induction (no band was observed). Lanes are spliced together to remove an intervening lane and the vertical dotted line is at the location of the spliced lanes. The arrow indicates the location of 47 kDa (SK).

**Fig. S7.** Calibration curve for standard SK. Serial dilutions of Streptase® (CSL, Behring, Germany), a commercially available standard SK, were used to prepare the standard calibration curve based on Hydrolysis of S-2251 by Pg, as explained in Fig. 2 and Fig. 3.

**Fig. S8.** Amino acid sequence alignment of SK proteins corresponding to reference strain SK$_{9542}$ (*S. equisimilis*, ATCC9542, the commercial source for production of SK), SK$_{2bALAB49}$, SK$_{2aG88}$, and SK$_{2aSTAB902}$. The alignment was created using MEGA6 software [14]. Conserved (identical) amino acids in the alignment are indicated by dots. The exchanged fragments are highlighted.