Role of the N-terminal region of staphylokinase (SAK): evidence for the participation of the N-terminal region of SAK in the enzyme–substrate complex formation

G. Rajamohan, Kanak L. Dikshit*

Division of Molecular Biology, Institute of Microbial Technology, Sector 39A, Chandigarh 160036, India

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Abstract Staphylokinase (SAK) forms an inactive 1:1 complex with plasminogen (PG), which requires both the conversion of PG to plasmin (Pm) to expose an active site in PG–SAK activator complex and the amino-terminal processing of SAK to expose the positively charged (Lys-11) amino-terminus after removal of the 10 N-terminal amino acid residues from the full length protein. The mechanism by which the N-terminal segment of SAK affects its PG activation capability was investigated by generating SAK mutants, blocked in the native amino-terminal processing site of SAK, and carrying an alteration in the placement of the positively charged amino acid residue, Lys-11, and further studying their interaction with PG, Pm, miniplasmin and kringle structures. A ternary complex formation between PG–SAK PG was observed when an immobilized PG–SAK binary complex interacted with free radiolabelled PG in a sandwich binding experiment. Formation of this ternary complex was inhibited by a lysine analog, 6-aminocaproic acid (EACA), in a concentration dependent manner, suggesting the involvement of lysine binding site(s) in this process. In contrast, EACA did not significantly affect the formation of binary complex formed by native SAK or its mutant derivatives. Furthermore, the binary (activator) complex formed between PG and SAK mutant, PRM3, lacking the N-terminal lysine 11, exhibited 3–4-fold reduced binding with PG, Pm or miniplasmin substrate during ternary complex formation as compared to native SAK. Additionally, activator complex formed with PRM3 failed to activate miniplasminogen and exhibited highly diminished activation of substrate PG. Protein binding studies indicated that it has 3–5-fold reduction in ternary complex formation with miniplasmin but not with the kringle structure. In aggregate, these observations provide experimental evidence for the participation of the N-terminal region of SAK in accession and processing of substrate by the SAK–Pm activator complex to potentiate the PG activation by enhancing and/or stabilizing the interaction of free PG.

Key words: Staphylokinase; Site-directed mutagenesis; Mutant; Plasminogen; Plasmin; Kringle domain

1. Introduction

Staphylokinase (SAK), a profibrinolytic 16 kDa protein secreted by several strains of *Staphylococcus aureus*, has recently been identified as a potent fibrin-selective plasminogen (PG) activator [1–3] with immense therapeutic potential for coronary thrombolysis in patients with acute myocardial infarction. It forms a 1:1 stoichiometric complex with PG or plasmin (Pm) to generate an activator complex for the PG activation [4–6] very similar to streptokinase (SK). However, certain key differences have been found in the basic mechanism of PG activation by these two PG activators of bacterial origin. In striking contrast with the PG–SK complex, the PG–SAK complex remains inactive and requires conversion to the SAK–Pm form to generate an active site displaying high specificity for the PG activation. Also, the generation of PG activation potential in SAK proceeds via Pm-mediated removal of the 10 amino-terminal amino acid residues [7]. The three-dimensional structures of SAK and SAK–microplasmin complex have recently been elucidated by X-ray diffraction studies [8,9], which indicated that the amino-terminus of SAK covering the first 17 amino acid residues acquires a flexible, disordered structure and remains surface-exposed. It has been shown that amino-terminal proteolysis of SAK to generate a low molecular form of SAK is essential for the PG activation and may occur in vivo presumably by traces of Pm. The low molecular form of SAK, lacking its first 10 N-terminal amino acid residues, carries full potential for its activation function but its PG activation capability is significantly reduced when Lys-11 is replaced with another amino acid residue or its position is altered, indicating that Lys-11 plays a crucial role in its protein function. Recent experimental evidences suggest that the positively charged amino-terminal region of SAK is obligatory for SAK to achieve full PG activation potential in the enzyme complex formed between SAK and Pm [7]. However, at this stage, a detailed understanding of the molecular mechanism of its action is missing. The aim of this study is to probe the role(s) of the amino-terminal region of SAK in the PG activation process through site-directed mutagenesis of the amino-terminal region of SAK and characterization of these mutants for their functional characteristics during protein–protein interaction. In the present work, the function of the amino-terminal region of SAK has been elucidated by site-directed mutagenesis of the N-terminal processing site of SAK and studying the interaction of these SAK mutants during binary and ternary complex formation in the process of PG activation.

2. Materials and methods

2.1. Bacterial strains, plasmids and reagents

S. aureus (local isolate, designated as SAK11) was used for retrieving the gene (*sak*) encoding SAK. Plasmid vectors, Bluescript KS$^+$

*Corresponding author. Fax: (91)-172-690585/690632.
E-mail: kanak@lion.imtech.ernet.in

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(Stratagene, Germany) and pET9b (Promega, WI, USA) were utilized for cloning and gene expression purposes. All restriction and DNA modifying enzymes were obtained from Promega (WI, USA) or New England Biolabs (Beverly, USA). Lys-Sepharose, CNBr-activated Sepharose CL-4B, DEAE-Sepharose CL-4B and phenyl-Sepharose CL-4B were obtained from Pharmacia Biotech. Chromozyme PL and human PG were purchased from Boehringer Mannheim (Germany).

2.2. Cloning and expression of recombinant SAK and its mutants in Escherichia coli

The structural gene (*sak*), encoding SAK, was retrieved from the genomic DNA of *S. aureus* (SAK11) through PCR amplification using oligonucleotide primer pair (1) 5′-GATATATACATATGTCAAGTTCATTCGACAA-3′ and (2) 5′-AAGCTTTCCACTTTTTATCCTAACTGATTTCTCCCCATAAGT-3′, designed on the basis of the known nucleotide sequence of the *sak* gene [10], and it was cloned on the plasmid vector Bluescript KS$^+$. The authenticity of the cloned gene was established through nucleotide sequencing, which matched perfectly with the published *sak* gene sequence [10]. Overexpression of SAK in *E. coli* was achieved by cloning the *sak* gene on the expression plasmid, pET9b, at the *Nde*I–*Bam*HI site. For the cloning and expression of SAK mutants carrying an N-terminal modification, PCR primers (1) 5′-GTAGCCATGAAAGGAGCATATGCAGCAGGAGAC-3 and (2) 5′-GTAGCCATGGCTGGAGATGACGCGAGT-3′ were utilized for the generation of SAK mutants, PRM2 and PRM3, respectively. Incorporation of a mutation in the *sak* gene was confirmed by DNA sequencing.

2.3. Purification of recombinant SAK

For the production and purification of wild type or mutant SAK proteins, the *E. coli* cells carrying recombinant plasmids for the expression of the respective target gene were induced with 1 mM IPTG during mid log phase ($OD_{600} = 0.6$) and harvested after 7–10 h of further incubation at 37°C. The cells were collected after centrifugation and lysed either by sonication or through a French press. After centrifugation of the cell lysate at 14,000 rpm, clear supernatant was used for the recovery of SAK. A two step purification protocol involving an ion-exchange (DEAE-Sepharose) and hydrophobic chromatography (phenyl-Sepharose) was utilized for obtaining a purified protein preparation essentially as described previously [11]. The specific functional activity of the purified preparation of recombinant SAK was compared with a native protein preparation obtained from *S. aureus* (a gift from Prof. Patrick J. Gaffney, WHO, UK) which exhibited 160,000 U/mg of protein. The specific PG-activating capacity of the different SAK proteins was defined as the amount of Pm (μmol) converted by 1 μmol SAK protein from an excess of human PG within 1 min. Therefore, 2.5 nM SAK was incubated with 1.5 μM human PG in 0.1 M sodium phosphate, pH 7.4, at 37°C for 30 min. The generation of Pm was followed by monitoring the change in the absorbance at 405 nm/min caused by hydrolysis of the synthetic Pm substrate chromozyme PL (Boehringer Mannheim, Germany) used at a final concentration of 1 mM.

2.4. PG purification and miniPG preparation

Human PG was isolated from fresh frozen plasma using Lys-Sepharose affinity chromatography following the established procedure [12]. Separation of the kringle structure (K1+K2+K3+K4) and miniPG (kringle 5 and protease domain of PG) from the intact PG was achieved by limited digestion of full length PG with pancreatic elastase (Sigma) in 0.2 M Tris–Cl (pH 8.8) for 10 min at 37°C using the enzyme substrate ratio 1:100 as described elsewhere [13,14].

2.5. SAK assay and PG activation reaction

The specific PG-activating activities of SAK and its mutants were determined with a PG-coupled chromogenic assay [15]. Kinetic analysis of PG activation was done following the procedure of Arai et al. [16] with slight modification. Briefly, a mixture of PG (1.5 μM) and SAK or its mutants (final concentration 0.8 μM) was incubated at 37°C for 5 min in 0.1 M phosphate buffer (pH 7.5) containing 0.1% bovine serum albumin (BSA) and 0.01% Tween 80. The mixture was diluted 10-fold and the change in absorption at 405 nm was recorded at different time intervals after the addition of chromozyme PL. The kinetic constants of PG activation were derived from Lineweaver–Burk plots.

2.6. Casein–PG overlay

Bacterial colonies overexpressing SAK were detected by overlaying a mixture of 0.8% agarose, 1% skim milk, 500 μg human PG, 150 mM NaCl and 50 mM Tris–Cl (pH 8.0) on top of the agar plates carrying recombinant *E. coli* colonies as mentioned previously [17]. The plates were incubated at 37°C for 2–6 h. SAK positive colonies indicated a clearing zone around the colonies. Zymographic analysis of the protein was done by separating the protein on SDS–PAGE (15%) and placing the protein gel on top of the solidified agarose bed carrying a mixture of casein (1%) and human PG (25 μg/ml). The SAK protein band was identified after the incubation of protein gel at 37°C for 2–6 h [17].

2.7. Sandwich binding assay

Formation of ternary complex between SAK and PG or its truncated fragment carrying kringle domain or miniplasmin was checked through sandwich binding assay as described by Young et al. [18] with slight modification. Briefly, 0.01–0.05 ml of PG (10 μg/ml in 50 mM Tris–Cl, pH 7.5) was immobilized on the nitrocellulose strips or microtiter plates, air-dried and blocked with 1% BSA for 1–2 h. Wild type or mutant SAK was added in excess to the immobilized PG and incubated for 30–60 min at 4°C. Unbound SAK was removed after washing with 0.05 M Tris–Cl (pH 7.4) containing 0.1% BSA and 0.01% Tween 80. Thereafter, ^{125}I-labelled PG, miniPG or kringle structures, at various concentrations, were added separately and incubated further for another 30–60 min at 4°C. Excess radioactivity was removed through repeated washings with the same buffer and the amount of labelled PG bound with the immobilized PG–SAK binary complex was determined by the level of radioactivity incorporated using a γ-counter. BSA was replaced with SAK to check the level of non-specific binding and the background was subtracted from the total level to determine the specific PG binding.

3. Results

3.1. Characteristics of SAK variants altered in N-terminal processing (Lys-10–Lys-11) site

Cleavage of the Lys-10–Lys-11 peptide bond and exposure of Lys-11 in the SAK moiety are the obligatory steps in the SAK-mediated PG activation process. To unravel how this effect is manifested, site-directed mutants of SAK, altered in its N-terminal processing site, were generated (Table 1). PRM2 lacks its native N-terminal processing site (Lys-10-Ala-Lys-11-Ala) but retains a lysine residue at the sixth position. This mutation extends the length of the N-terminus by

Table 1
Characteristics of SAK mutants, altered in the N-terminal domain

Name	N-terminal sequence of SAK variants															Specific activity (U/mg)
	1	2	3	4	5	6	7	8	9	10 ↓	11	12	13	14	15	
PRM1 (native SAK)	S	S	S	F	D	K	G	K	Y	K	K	G	D	D	A	166,000
PRM2					M	K	G	A	Y	A	A	G	D	D	A	35,000
PRM3										M	A	G	D	D	A	75,000

The arrow indicates the proteolysis site in SAK.

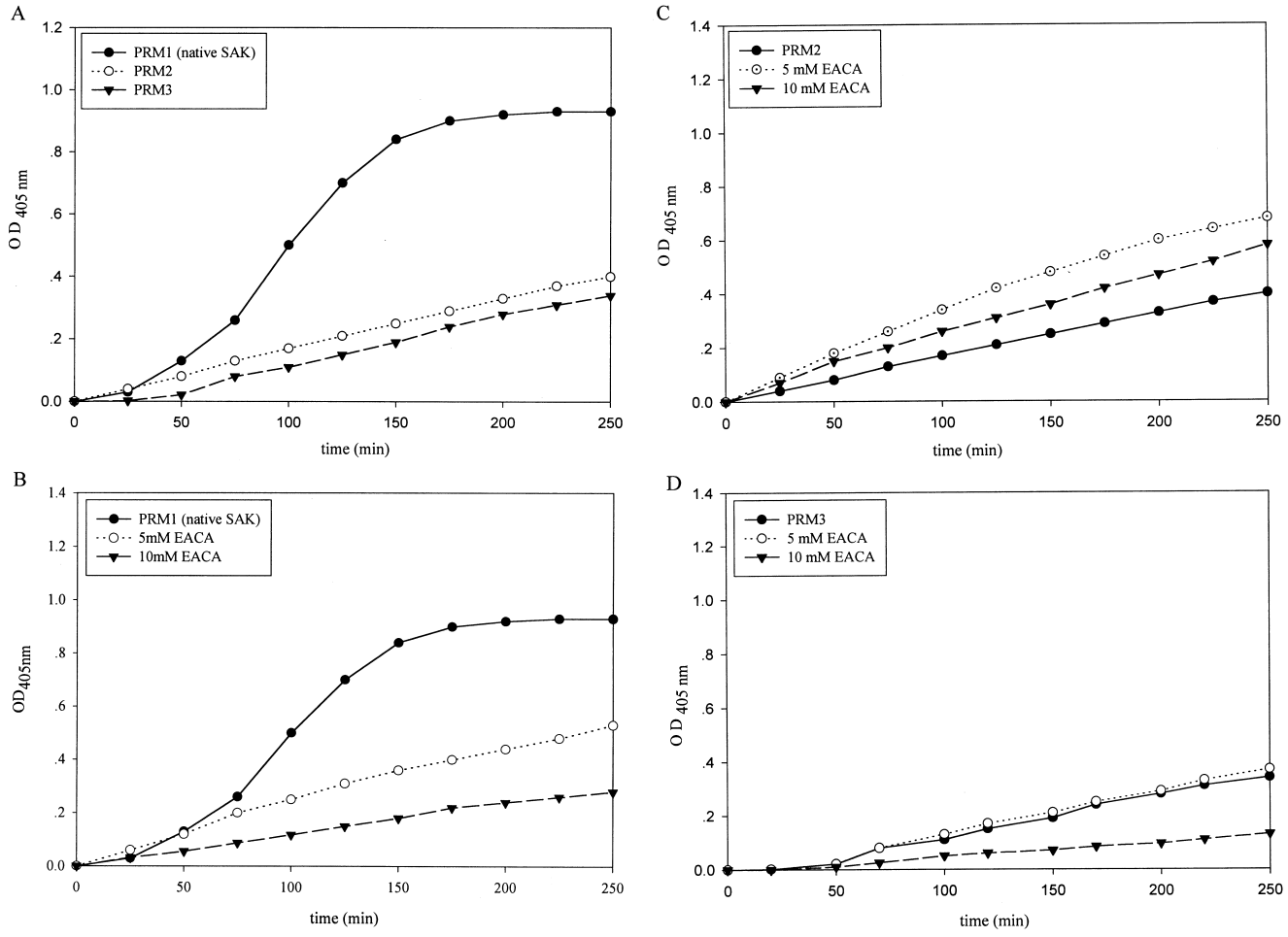

Fig. 1. Pattern of PG activation by recombinant SAK and its mutants (A). Effect of EACA on PG activation by native SAK (B), PRM2 (C) and PRM3 (D). Human PG (1.5 μM) was incubated with SAK or SAK mutants (0.5 μM) in the presence of 1.2 mM chromozyme PL at 25°C in 0.5 M Tris–Cl, 0.1 M NaCl (pH 7.4) containing 0.1% BSA and 0.01% Tween 80. The rate of Pm generation was followed by monitoring the absorption at 405 nm at different time intervals. EACA at 5 and 10 nM concentrations was added in the reaction mixture to observe its effect.

four residues as compared to native SAK which gets processed at Lys-11 after PG (Pm) binding [7]. The second SAK mutant, PRM3 lacks its first 10 N-terminal residues and Lys-11 was substituted with alanine (Table 1). Recombinant SAK and its mutants were purified from *E. coli*, BL21DE3, transformed with the respective plasmid constructs. PRM2 and PRM3 exhibited around 70% and 40% reduction, respectively, in their functional activity as compared to the native SAK (Table 1). Additionally, activation of PG in the presence of PRM2 and PRM3 occurred slowly with a marked lag phase as opposed to native SAK (PRM1) where PG activation occurred exponentially with time (Fig. 1A). Zymographic analysis of SAK mutants exhibited the similar pattern (data not presented). These results indicated that PRM2 and PRM3 are significantly impaired in their capability of PG activation.

Cleavage of SAK at the Lys-10–Lys-11 junction exposes Lys-11 in the processed SAK, which is an obligatory step for the optimal function of SAK. Lysine analog, 6-aminocaproic acid (EACA), binds to PG via the lysine binding sites in the kringle structure. Therefore, to check that the N-terminal region of SAK mediates its effect via interaction with the lysine binding site, the pattern of PG activation by SAK and its mutants (Fig. 1B–D) was studied in the presence of varying amounts of EACA. Activation of PG in the presence of SAK occurred progressively with a brief lag phase followed by an exponential increase in PG activation. Under these experimental conditions, neither the PG nor SAK alone was able to activate PG and generate Pm activity. In the presence of EACA (10 mM), the PG activation rate via native SAK (PRM1) was inhibited more than 60%. In contrast, a low concentration of EACA (5 mM) increased PG activation twice by PRM2 as compared to control (Fig. 1C). PRM3 exhibited only marginal (10–20%) inhibition in the presence of EACA (Fig. 1D). These observations provided the evidence that the lysine binding site(s) resident in the kringle structure of PG may be playing a crucial role in SAK-mediated PG activation.

3.2. Pattern of PG activation and SAK–PG binary complex formation by SAK mutants

To evaluate that the impairment in PG activation capability of SAK mutants, PRM2 and PRM3, is due to alteration in their interaction with PG during the formation of SAK–PG (Pm) activator (enzyme) complex and to check whether the N-terminal lysine has any role in this process, the capability of SAK and its mutants to generate complex with PG has been

checked by studying the complex formation between radiolabelled PG and SAK immobilized on nitrocellulose strips or microtiter plates. The concentration dependent binding of ^{125}I-labelled PG with SAK and its mutants was observed. Fig. 2A shows an increasing trend in the formation of SAK–PG binary complex in a dose dependent manner. No binding of ^{125}I-PG was observed when BSA was used in place of SAK. A similar pattern of SAK–PG binary complex formation has been observed by SAK variants, PRM2 and PRM3, indicating that the alteration in the amino-terminal region of SAK does not affect the capability of SAK for the formation of activator complex with PG. Addition of EACA did not exhibit any significant effect on the formation this binary complex of PG with native or mutant SAK (Fig. 2B).

3.3. Substrate PG binding by the SAK–PG binary complex

Interaction of SAK–PG (Pm) enzyme complex with the free substrate (PG), during PG activation, may form a transient ternary structure in which two PG (Pm) molecules may associate with one SAK moiety. A sandwich binding assay was performed to compare the capability of ternary complex formation with the activator complex formed with PG and wild type SAK or its mutants, PRM2 and PRM3. In this experiment, a varying amount of PG was coated on the microtiter plate or immobilized on the nitrocellulose strips and then allowed to react with the saturating amounts of native or mutant SAK to develop a SAK–PG complex. Excess SAK was removed by repeated washing. PG–SAK binary complex thus created was then challenged with increasing amounts of ^{125}I-labelled PG. The amount of ^{125}I bound to a PG–SAK-coated plate or nitrocellulose strips, which represented the PG–SAK–PG ternary complex, was determined by measuring the level of radioactivity incorporated (Fig. 3A). The level of ternary complex formation increased with an increasing concentration of immobilized PG–SAK binary complex. Negligible binding of labelled PG was observed when BSA was used as a control in place of SAK. Immobilized SAK–PG complex, formed with SAK mutants, PRM2, exhibited about

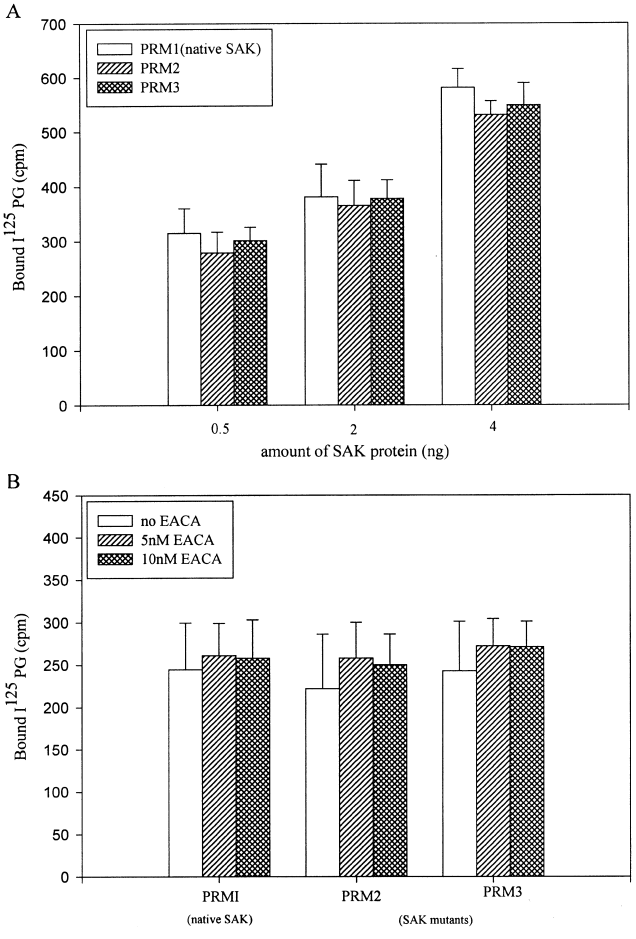

Fig. 2. Pattern of SAK–PG binary (activator) complex formation by recombinant native SAK (PRM1) and its mutants (PRM2 and PRM3) in the absence (A) and presence of EACA (B). Native or mutant SAK proteins (1.5 ng) were immobilized on nitrocellulose strips. Non-specific protein binding sites were blocked with skim milk (1%). Unbound proteins were removed by repeated washing with 0.5 M Tris–Cl (pH 7.5) carrying 0.1% BSA and 0.01% Tween 80. Immobilized protein was probed with ^{125}I-labelled human PG and the level of PG binding was determined by a γ-counter. Each binding result represents the mean of three independent determinations.

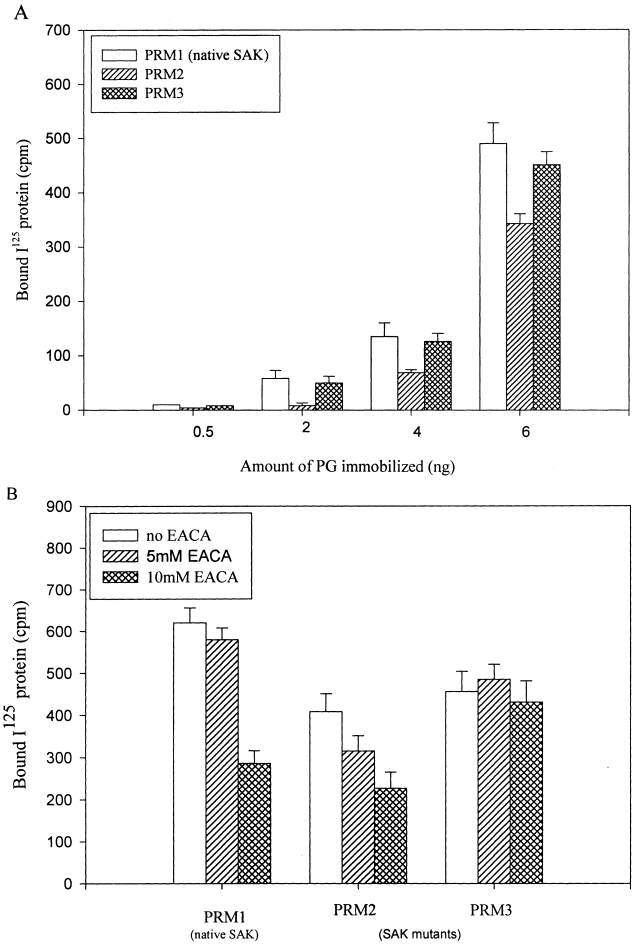

Fig. 3. Formation of ternary complex (PG–SAK–PG) with substrate PG by immobilized binary PG–SAK complex formed with native SAK and its mutants in the absence (A) and presence of EACA (B). Different concentrations of human PG were immobilized and non-specific sites were blocked as mentioned under Section 2. Native or mutant SAK proteins were added (2 μM) to form binary complex with the bound PG. After washing with 0.5 M Tris–Cl (pH 7.4) containing 0.1% BSA and 0.01% Tween 80, ^{125}I-labelled PG (2–3 μM) was added. Unbound PG was removed after repeated washing with the same buffer and the level of bound PG was determined by a γ-counter.

40% reduction in the interaction with the substrate PG in the formation of ternary complex as compared to PRM1. The K_m values for the substrate PG binding by the preformed activator complex formed with PRM2 and PRM3 were 26 ± 2 μM and 11 ± 1.8 μM, respectively, as compared to 3.4 ± 1.2 μM in the case of native SAK suggesting 4–8-fold reduction in the affinity for the substrate PG in SAK mutants. These results strongly indicated that structural features of the N-terminal region of SAK are specifically involved in the interaction of SAK–Pm activator complex with substrate PG.

When radiolabelled PG was challenged with the binary complex formed with PG and native SAK in the presence of lysine analog, EACA (5 mM and 10 mM), the level of ternary complex was significantly reduced as compared to control (Fig. 3B). More than 50% reduction in the formation of ternary complex has been observed in the presence of 10 mM EACA. PRM2 exhibited a more or less similar pattern with respect to substrate PG binding. In contrast, binary complex formed with PG and PRM3, lacking N-terminal lysine, had little effect on the overall level of association with radiolabelled PG. These observations suggested that interaction of activator (binary complex between SAK–PG) complex with the substrate (free) PG may involve kringle structure(s) carrying lysine binding site(s) of PG and the N-terminal Lys-11 residue may play a crucial role in this process.

3.4. Interaction of the kringle structure (K1+K2+K3+K4) of PG and miniplasmin with SAK–PG activator complex

Since the PG binding and activation pattern with native and SAK mutants suggested the participation of the N-terminal region of SAK and kringle structure of PG in the SAK-mediated PG activation process, we evaluated the interaction of kringle (K1+K2+K3+K4) and miniplasmin (K5+serine protease domain), separately, with the activator complex formed between PG and SAK or its mutants, PRM2 and PRM3. The kringle structures were able to bind with SAK–PG complex formed with the native or SAK mutant, PRM2, with a more or less similar efficiency. As opposed to native SAK (PRM1) and PRM2, binary complex formed with PG and PRM3 exhibited about 30% reduction in the formation of ternary complex with kringle domain of PG (Fig. 4). Additionally, miniPG, carrying the protease domain of PG and kringle 5, exhibited more than 50% reduction in the formation of ternary complex with the immobilized PG–SAK enzyme complex formed the SAK mutant, PRM3, as compared to PRM2 and wild type (Fig. 4). These observations suggested that the presence of N-terminal lysine might be important for

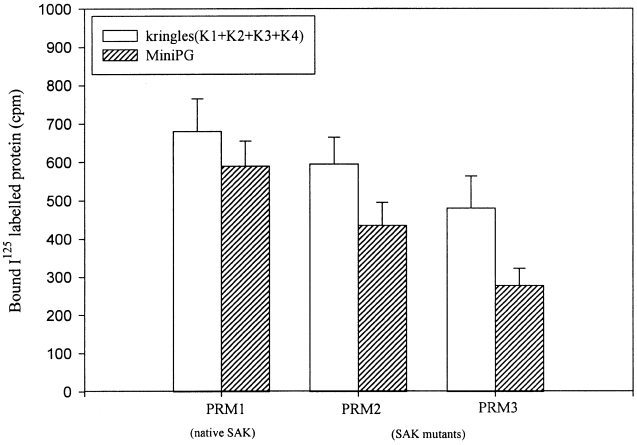

Fig. 4. Formation of ternary complex by kringle structure (A) and miniplasmin (B) by native and mutant SAK proteins. Binary complex formed with PG and native SAK or its mutants (see Section 2) was challenged with 2 ng of ^{125}I-labelled kringle (K1+K2+K3+K4) structure (A) and 2 ng of ^{125}I-labelled miniplasmin (B) essentially as described in Section 2 for the intact PG and binding of radiolabelled protein was checked by a counter. Non-specific binding was determined using BSA (1 ng) and that level was subtracted from the total binding to determine specific binding of each protein.

the interaction with the substrate PG probably through the involvement of kringle 5. Although PRM2 interacts more or less similar to that of wild type SAK, its PG activation capability is significantly reduced. It is probable that apart from the N-terminal lysine residue, precise structural characteristics of the N-terminal domain of SAK may also be required for its optimal functioning. To further clarify the role of kringle structures and the N-terminal region of SAK in the PG activation process, competition for binding between kringle structure, miniplasmin, and PG with the binary (enzyme) complex formed with PG along with native SAK was checked. Both kringle structures and miniplasmin compete effectively with intact PG for binding with PG–SAK binary complex, whereas binding of kringles did not change significantly in the presence of miniPG (Table 2). To further assess the functional role of the amino-terminal region of SAK, the kinetics of PG and miniPG activation by preformed activator complex, generated by native SAK (PRM1) or its mutants (PRM2 and PRM3), was examined. As compared to PG, progression of Pm generation was significantly slower when miniPG was used as a substrate in the presence of the activator complex formed with Pm and native SAK. The kinetic constants obtained for the miniPG activation were about 3-fold higher (K_m, 12.5 μM) as

Table 2
Interaction of binary complex formed with PG and native SAK or its mutants with various substrates

Substrates	Percentage of ^{125}I-labelled PG binding with SAK–PG binary complex		
	PRM1 (native SAK)	PRM2	PRM3
^{125}I-PG	100	85	56
^{125}I-PG+kringle (K1+K2+K3+K4) (2 μM)	64	59	28
^{125}I-PG+kringle (K1+K2+K3+K4) (4 μM)	48	41	15
^{125}I-PG+miniPG (2 μM)	71	67	48
^{125}I-PG+miniPG (4 μM)	56	36	41

6–8 ng of human PG was immobilized on a microtiter or nitrocellulose strips and native (PRM1) or mutant SAK (PRM2, PRM3) protein was added in excess individually and incubated for 3–4 h at 4°C to develop a binary complex between SAK and PG. Excess SAK proteins were removed by repeated washing with phosphate-buffered saline containing 0.05% Tween 20. Competition between ^{125}I-labelled PG and kringle structure or miniplasmin to form a tertiary complex was determined by adding excess unlabelled kringle structure (20–40 ng) or miniplasmin (40 ng) along with ^{125}I-labelled PG.

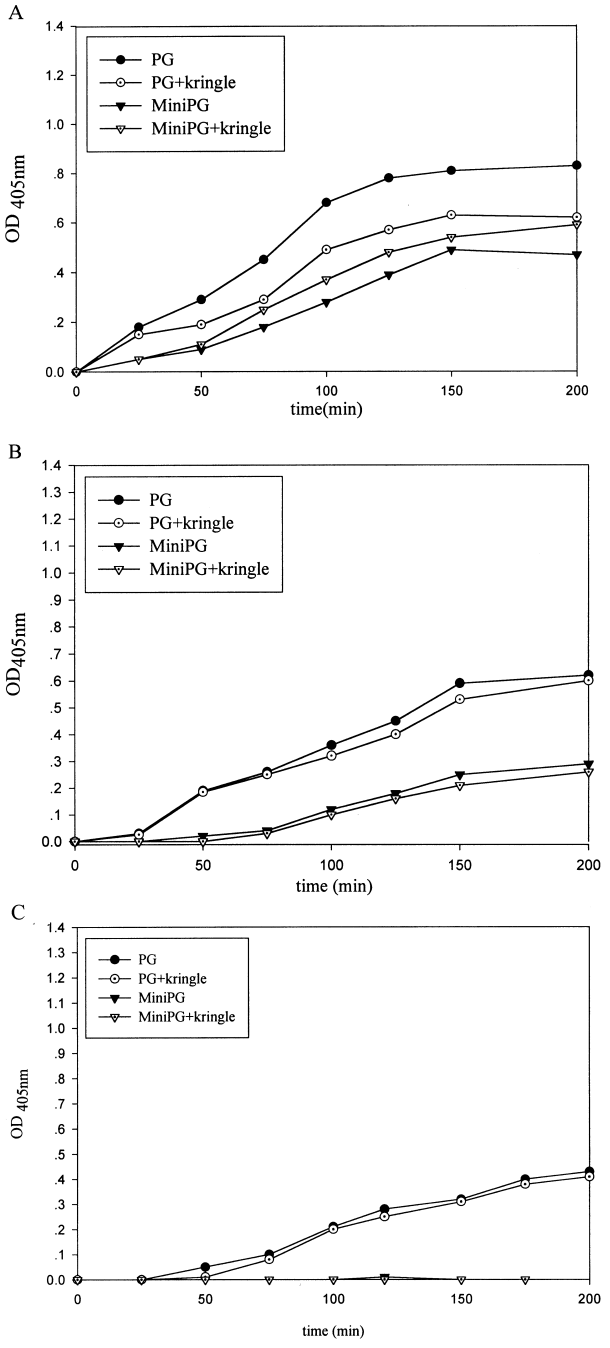

Fig. 5. Effect of kringle structure on preformed Pm–SAK complex catalyzed activation of PG and miniPG. Equimolar mixtures of PG and SAK variants were preincubated for 5 min at 37°C in 0.05 M Tris–Cl (7.4), 0.1 M NaCl containing 0.0% BSA, 0.01% Tween 80 and 25% glycerol and then kept on ice. These preformed activator complexes (final concentration 20 nM) were then mixed with PG or miniPG (10 μM), and generated Pm was measured at 405 nm in the presence of chromogenic substrate chromozyme PL (1 mM). (A) preformed complex with native SAK, PRM1, (B) PRM2 and (C) PRM3.

compared to the substrate PG (K_m, 2.4 μM). These observations indicated a lower affinity of miniPG for the SAK–Pm activator complex as compared to the substrate PG. In the presence of kringle structure (K1+K2+K3+K4, 4 μM), PG activation decreased 20–30%, whereas a 10–15% increase in miniPG activation was observed when activator complex formed with PRM1 was utilized. Inhibition of PG activation by kringle structure in the presence of preformed activator complex clearly suggested that kringles are competing with the substrate for the interaction/binding with the enzyme complex but not with the miniPG. A slight increase in the activation of miniPG in the presence of kringle structure may be due to better accessibility of enzyme complex to the miniPG substrate as miniPG does not compete with kringle structure for binding with the binary complex. Activation of PG or miniPG substrate in the presence of enzyme complex formed with PRM2 followed more or less a similar pattern (Fig. 5B) although the efficiency of PG or miniPG activation was relatively less. In contrast, activator complex formed with PRM3 exhibited a highly diminished overall rate of miniPG activation, suggesting that the replacement of Lys-11 within the N-terminus of SAK has adversely affected the interaction of enzyme complex with the miniplasmin substrate.

In aggregate, these observations suggested that the interaction of miniplasmin and kringle structures with SAK–PG complex may involve independent regions. Additionally, miniplasmin carrying kringle 5 of full length PG may have closer interaction with the N-terminal region of SAK during PG activation.

4. Discussion

The PG activation process via SAK involves several characteristics including protein–protein interaction and complex formation apart from proteolytic cleavage of SAK itself which removes the first 10 N-terminal residues of the full length SAK and exposes Lys-11 in the enzyme (SAK–PG/Pm) complex [7,11]. In the present study, we have demonstrated that the N-terminal regions of SAK modulate the interaction of enzyme with substrate (free PG) but may not have any significant role in the formation of binary complex to generate an initial enzyme complex consisting of SAK and PG. Sandwich binding assay, utilizing preformed immobilized enzyme complex, and radiolabelled PG as a substrate, indicated the formation of ternary complex in the form of PG–SAK–PG. Inhibition of this ternary complex formation in the presence of EACA suggested the involvement of the lysine binding site(s) of kringle domain of PG in this process. These results support the recent proposal that kringle domain of PG may be involved in SAK-mediated PG activation [17].

Ten N-terminal residues of SAK are released during conversion of inactive SAK–PG complex into catalytically active SAK–Pm enzyme by hydrolysis of the Lys-10–Lys-11 peptide bond of SAK. Presence of a Lys residue at the N-terminus of SAK is required for achieving its full PG-activating potential [7]. However, it is not yet clear whether proteolysis at this precise position is necessary or occurred only because of susceptibility of this site for proteolytic processing. This problem was addressed by generating two SAK mutants, one carrying blockage in its native processing site at Lys-10–Lys-11, but retaining its N-terminal lysine at the sixth N-terminal position, and another lacking its 10 N-terminal residues as well as Lys-11 substituted with alanine. SAK mutants, PRM2 and PRM3, exhibit a significantly reduced PG activation capability as compared to the native SAK. PRM2 carries blockage at Lys-10–Lys-11 but retains a lysine residue at the sixth N-terminal position. Its capability to form binary complex and interact with the substrate PG compares well with native

SAK. Since SAK mutant, PRM2, exhibits significant reduction in its PG activation capability, it is possible that increasing the length of the N-terminal region of SAK by blocking its native processing site at the Lys-10 residue inhibits full exposure of functionally important residues of the enzyme complex formed between SAK and PG (Pm) and thus affects its PG activation potential. The N-terminal region of SAK remains unstructured and exhibits high conformational variability [7,8] and recent nuclear magnetic resonance solution studies [19] on SAK have indicated that the proteolytic cleavage of a 1–10 amino acid residue peptide from the full length SAK may result in increased exposure to solvent of several regions of the core of the SAK structure. Additionally, the three-dimensional structure of SAK has indicated that the flexible N-terminal region of SAK may form a loose association with the functionally important region of SAK [8]. Thus, PRM2, which has a four residue extension due to the blockage of the native processing site at Lys-10–Lys-11 but retains a positively charged residue, Lys, at the sixth position may not fully expose the functional region of SAK which in turn may affect the catalytic efficiency of SAK–PG (Pm) enzyme complex. PG binding studies have indicated that it interacts well with the substrate PG or miniplasmin as compared to PRM3, which lacks an N-terminal lysine. These observations suggested that the removal of 10 amino acid residues of SAK may be essential for unmasking the functional core of SAK to expose its full PG activation potential. It has been proposed [6] that the exposed amino-terminal lysine of SAK may influence the active site configuration of the enzyme complex via its α-amino group. A precise conformation and length of the amino-terminal region of SAK may be required for this process. Thus, it is quite possible that the increase in the length of the N-terminal region of SAK creates a less effective active center within the enzyme complex by shielding the functionally important region of enzyme complex.

In an attempt to study the functional role of the N-terminal region of SAK, we investigated the interaction of various substrates, e.g. PG, miniplasmin and kringle structure of PG, with the enzyme complex formed with mutant SAK and PG. Preformed binary complex generated with PG and native SAK or its mutant, PRM2, carrying an N-terminal lysine at the sixth position, interacts with radiolabelled substrate PG with a more or less similar efficiency. This interaction is reduced significantly in the presence of lysine analog, EACA. Moreover, activator complex formed with PG and SAK mutant, PRM3, lacking N-terminal lysine and its first 10 N-terminal residues, interacts with the substrate PG with a much lower efficiency. The presence of lysine residue within the N-terminal region of SAK improves the capability of the enzyme complex, formed between SAK–PG, to interact with the substrate. It has been proposed that the N-terminal region of SAK must be located near the active site of Pm (in the PG–(Pm)–SAK complex) to allow the initial cleavage of SAK at Lys-10 [7,8]. This step may bring several changes in the enzyme complex such as exposure of functionally important residues and reconformation of the active site with altered substrate specificity. It is possible that these changes within the SAK–PG binary complex may provide enhanced binding and stabilization of substrate PG, resulting in efficient conversion of PG into Pm. Cocrystallization of SAK and microplasmin complex and modeling of microplasmin and SAK structure have suggested [8] that a newly created N-terminal region of SAK after Lys-10–Lys-11 peptide bond cleavage extends away from the compact ellipsoid structure, running along a depression between the enzyme and the substrate and may interact with the lysine binding site of kringle 5 of a miniplasmin substrate assisting in the fixation of PG substrate. Our observations are in line with the proposed hypothesis and demonstrate that SAK with a terminal lysine residue can interact better with the substrate PG in contrast to the mutant SAK lacking N-terminal lysine. Sandwich binding studies utilizing preformed PG–SAK complex and free PG substrate have clearly indicated that SAK mutant, PRM3, lacking N-terminal lysine forms ternary complex with the substrate PG with less efficiency as compared to native SAK or SAK mutant carrying lysine at the sixth N-terminal position. Competition studies involving interaction of miniplasmin and kringle structure of PG with SAK–PG complex have demonstrated that kringle domain of PG (1–4) and miniplasmin interact independently with the SAK–PG (Pm) enzyme complex.

A role of the amino-terminal region of SAK in achieving proper interaction with the substrate PG has been suggested on the basis of the present study for the PG activation process via native and mutant SAK. This interaction may stabilize the enzyme (SAK–PG (Pm))–substrate (free PG) directly through N-terminal lysine (Lys-11) via one of the lysine binding sites present within the substrate PG. Since kringle 5 of PG resides close to the protease domain of PG in the microplasmin form, it is probable that the N-terminal arm of SAK interacts with K5 via its N-terminal lysine and modulates the interaction of substrate PG. This proposition is consistent with the recent prediction through modeling of SAK with that of microplasmin [8]. Alternatively, removal of 10 amino-terminal residues from the N-terminus of SAK with an exposed Lys residue may reconfigure the enzyme complex and generate an efficient active center which interacts better with the substrate and stabilizes the enzyme substrate complex during PG activation via SAK.

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