Function of the 90-loop (Thr^{90}–Glu^{100}) region of staphylokinase in plasminogen activation probed through site-directed mutagenesis and loop deletion

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Staphylokinsae (SAK) forms a bimolecular complex with human plasmin(ogen) and changes its substrate specificity by exposing new exosites that enhances accession of substrate plasminogen (PG) to the plasmin (Pm) active site. Protein modelling studies indicated the crucial role of a loop in SAK (SAK 90-loop; Thr^{90}–Glu^{100}) for the docking of the substrate PG to the SAK–Pm complex. Function of SAK 90-loop was studied by site-directed mutagenesis and loop deletion. Deletion of nine amino acid residues (Tyr^{92}–Glu^{100}) from the SAK 90-loop, resulted in $\approx 60\,\%$ reduction in the PG activation, but it retained the ability to generate an active site within the complex of loop mutant of SAK (SAKΔ90) and Pm. The preformed activator complex of SAKΔ90 with Pm, however, displayed a 50–60 % reduction in substrate PG activation that remained unaffected in the presence of kringle domains (K1+K2+K3+K4) of PG, whereas PG activation by SAK–Pm complex displayed $\sim 50\,\%$ reduction in the presence of kringles, suggesting the involvement of the kringle domains in modulating the PG activation by native SAK but not by SAKΔ90. Lysine residues (Lys^{94}, Lys^{96}, Lys^{97} and Lys^{98}) of the SAK 90-loop were individually mutated into alanine and, among these four SAK loop mutants, SAK_{K97A} and SAK_{K98A} exhibited specific activities about one-third and one-quarter respectively of the native SAK. The kinetic parameters of PG activation of their 1:1 complex with Pm indicated that the K_m values of PG towards the activator complex of these two SAK mutants were 4–6-fold higher, suggesting the decreased accessibility of the substrate PG to the activator complex formed by these SAK mutants. These results demonstrated the involvement of the Lys^{97} and Lys^{98} residues of the SAK 90-loop in assisting the interaction with substrate PG. These interactions of SAK–Pm activator complex via the SAK 90-loop may provide additional anchorage site(s) to the substrate PG that, in turn, may promote the overall process of SAK-mediated PG activation.

Key words: kringle domain, molecular modelling, site-directed mutagenesis.

INTRODUCTION

Staphylokinase (SAK), a 16 kDa profibrinolytic protein from the *Staphylococcus aureu*s, has been demonstrated to induce highly fibrin-specific thrombolysis in both human plasma [1,2] and in limited clinical trials [3,4,5]. Recent studies on the thrombolytic potential of recombinant SAK in achieving early perfusion in myocardial infarction and in the dissolution of platelet-rich clot [4,6] have clearly established its immense utility in clinical medicine as a thrombolytic agent and suggested that it can be developed as a potent clot-dissolving agent. Unlike some other plasminogen (PG) activators, e.g. tissue PG activator and urokinase, SAK has no proteolytic properties by itself, but acts by forming a 1:1 stoichiometric complex with plasmin (Pm), which, in turn, activates other molecules [7–11]. In this bimolecular complex, SAK acts as a cofactor and provides substrate-binding exosites for docking and enhanced presentation of the PG 'substrate' to the Pm active site.

Pm is an 85 kDa serine protease that plays a central role in haemostasis by degrading fibrin clots into soluble products. In human plasma, it circulates as a proenzyme, PG, which is activated by proteolytic cleavage of the Arg^{561}–Val^{562} peptide bond [12]. Human PG contains five kringle domains that serve as functional binding loci for other plasma proteins [13]. The activation of truncated PG derivatives, miniplasminogen (miniPG, carrying only kringle 5 and protease domain) and microplasminogen (μPG, carrying only protease domain) is slower than for the full-length PG, suggesting a role for kringle domains in the PG activation process [14]. The molecular mechanism by which these kringle structures modulate overall processing of PG by various PG activators is not very well known at present. The three-dimensional structures of SAK and the SAK–μPm complex have been elucidated by X-ray-diffraction studies [11,15]. In the heterotrimer complex, consisting of two μPm molecules and one SAK molecule [15], SAK binds one μPm molecule in the proximity of its active site. Formation of this bimolecular complex presents a slightly concave surface on to which the second molecule of μPm docks in a substrate-like manner. Of the amino acids in SAK, 30 % are charged, and these amino acids, including 20 lysine residues, are critical for its PG-activator activity. A number of SAK mutants [16–18] have been generated in order to investigate the determinants of SAK for PG binding and activation. It has been shown that the positively charged N-terminal region of SAK is necessary if it is to achieve its full activation function. Recent studies conducted in our laboratory [19] using site-directed mutagenesis of SAK have indicated that the positively charged N-terminal region of SAK may be involved in the interaction and/or stabilization of substrate PG during the process of PG activation. Previous studies [16] on alanine scanning mutagenesis of SAK have indicated that four clustered charged segments of SAK are important for the functional properties of SAK. Apart from the positively charged N-terminus, two discrete segments of SAK, spanning Glu^{44}–Lys^{50} and Glu^{65}–Asp^{69}, form the core region of SAK and may be

Abbreviations used: SAK, staphylokinase; SK, streptokinase; PG, plasminogen; miniPG, miniplasminogen; μPG, microplasminogen; Pm, plasmin; SAK–μPm, SAK, staphylokinase–microplasmin complex; SKα, α-domain of streptokinase; SAK–Pm complex, staphylokinase–plasmin complex; NPGB, *p*-nitrophenyl *p*′-guanidinobenzoate; SAKΔ90, loop mutant of SAK; SAK_{K97A} (etc.), a mutant of SAK in which Lys^{97} has been mutated to alanine.

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involved in PG binding and activation. Another highly charged region of SAK (Lys^{96}–Lys^{98}) that significantly affect the functionality of SAK, is a part of its surface-exposed loop structure. The three-dimensional structure of the SAK–μPm complex [15] has indicated that this charged loop region of SAK is away from the interaction sites of SAK–Pm activator complex and substrate μPG. Computer-based modelling studies with kringle domain of PG indicate that the surface-exposed positively charged residues of the SAK 90-loop may provide potential docking sites for the kringle domains of the substrate PG. The aim of the present study was to probe the functional role of the positively charged loop region of SAK in the PG activation process through site-directed and deletion mutagenesis. In the present work we have shown that a SAK mutant deleted in the charged loop region retains its native SAK-like capability for generating a bimolecular complex with PG that exhibits highly reduced capability for substrate PG processing. Structural modelling, supported by biochemical studies reported in the present work, have provided the experimental evidence that the 90-loop region of SAK may interact with the kringle domains of substrate which, in turn, may potentiate/stabilize the interaction of substrate PG with the SAK–Pm enzyme complex and facilitate the PG activation process.

MATERIALS AND METHODS

Bacterial strains, plasmids and reagents

S. aureus (local isolate, designated as SAK 11), was used for retrieving the gene (*sak*) encoding SAK as described previously [19]. Bluescript KS^+ expression plasmid, pET9b (Promega) and *Escherichia coli* host strains JM109 and BL21DE3 (Promega) were routinely utilized for cloning and expression of recombinant genes. All restriction and DNA-modifying enzymes were obtained from Promega or New England Biolabs. Chromozym PL and human PG were from Boehringer Mannheim. Pm was obtained from KabiVitrum. All biochemicals were of the highest grade commercially available.

Structural analysis and molecular modelling of the SAK–μPm complex and kringle domain

Co-ordinates of the SAK–μPm ternary complex, as well as those of the kringle 5 domain of human PG, were retrieved from the Protein Data Bank (Research Collaboratory for Structural Bioinformatics Consortium). Molecular surfaces of both the sets of co-ordinates were calculated by using GRASP [20] with a probe radius of 0.14 nm (1.4 Å). Electrostatic calculations were then performed with default parameters in GRASP, and the potentials were mapped on to the respective surfaces. Residues occurring on distinctly positive regions of SAK and distinctly negative regions of kringle domain were identified as putative interaction sites. Kringle 5 domain, taken as a representative structure of kringle domains of human PG, was manually docked on to the SAK–Pm ternary complex using Insight II package.

Construction of loop-deletion and site-directed mutants of SAK

Recombinant plasmid, pRM1, encoding wild type SAK in *E. coli* [19] was utilized for generating various SAK mutants. The SAK 90-loop deletion mutant, carrying deletion of nine amino acid residues (Tyr^{92}–Glu^{100}) of SAK were generated by an overlap-PCR method [21] using the following sets of oligonucleotide primers:

(1) 5′-GCAGGAGGATCCACGAAGTCTTTCCCTATAA-CAGAA-3′

(2) 5′-TCGCGTGGATCCTCCTGCAGTGACT TCGATCTT-3′

The specific primers for generating site-directed mutants of SAK are as follows:

SAK_{K94A}: 5′-TATTATGATGCGAATAAGAAAAAAGAAG-AAACGAAG-3′

SAK_{K96A}: 5′-TATGATAAGAATGCGAAAAAAGAAGAAA-CGAAGTCT-3′

SAK_{K97A}: 5′-GATAAGAATAAGGCGAAAGAAGAAACG-AAGTCT-3′

SAK_{K98A}: 5′-GATAAGAATAAGAAAGCGGAAGAAACG-AAGTCT-3′

Incorporation of mutation in the *sak* gene was confirmed by DNA sequencing. All SAK mutants were cloned in pET9b and expressed in *E. coli* under the T7 promoter.

Purification of SAK mutants

E. coli cells carrying recombinant plasmids for the expression of respective target genes were induced with 0.1 mM isopropyl β-D-thiogalactoside during the mid-exponential phase of growth [attenuance (D_{600}) 0.6] and harvested after 7–10 h of further incubation at 37 °C. A two-step purification protocol, involving an ion-exchange [sulphopropyl (SP)-Sepharose] and hydrophobic-interaction chromatography (Phenyl-Sepharose), was used to obtain a purified protein preparation essentially as described elsewhere [22]. The specific PG activator activity of a purified preparation of recombinant SAK was found to be comparable (160000 units/mg of protein) with the standard SAK preparation isolated from its natural host (a gift from Professor Patrick J. Gaffney, National Institute of Biological Standards and Control, South Mimms, Herts., U.K.).

PG purification and preparation of kringle domains and miniPm

Human PG was isolated from fresh frozen plasma using lysine–Sepharose affinity chromatography following the published procedure [23]. Separation of kringle domains (K1 + K2 + K3 + K4) and miniPm (kringle 5 and protease domain) from the intact PG moiety was achieved by limited digestion of full-length PG with pancreatic elastase in 0.2 M Tris/HCl, pH 8.8, for 10 min at 37 °C using the enzyme/substrate ratios 1:50–1:100 as described elsewhere [24,25].

SAK assay and PG activation reaction

The specific PG activator activity of native and mutant SAK was determined by using previously published PG-coupled chromogenic substrate assay [26,27]. Briefly, purified SAK or SAK mutant protein (5 nM) was added to PG (1.5 μM) in a quartz cuvette carrying 1.0 mM Chromozym PL (a chromogenic substrate from Boehringer-Mannheim) in 0.1 M phosphate buffer, pH 7.5, containing 0.1 % BSA and 0.01 % Tween 80. The change in A_{405} was then measured as a function of time in a Shimadzu (UV-1601) spectrophotometer at 25 °C. Purified native SAK (obtained from Professor Patrick J. Gaffney) was used as standard to determine PG activator activity in the unknown protein samples. PG activation and processing of substrate PG by SAK–Pm complex was monitored following the published procedure [28,29]. The molar concentration of full-length SAK and SAK loop-deletion mutant was calculated by taking their molecular masses as 16.2 kDa and 15.1 kDa respectively. Briefly, equimolar amounts of SAK or SAK mutant and PG (1.5 μM each) were combined in 100 mM Tris/HCl, pH 7.5, and incubated

at 25 °C. After 5 min, a 7.5 μl aliquot was removed and subjected to SDS/12 %-(w/v)-PAGE for the cleavage of substrate PG. The kinetic constants of PG activation by SAK–Pm complexes were calculated from Lineweaver–Burk plots [30,31]. To construct these plots, equimolar mixtures of SAK or SAK mutant and Pm were preincubated for 5 min at 37 °C in 100 mM phosphate buffer, pH 7.4, containing 0.01 % Tween 80 and left on ice. These preformed complexes (5 nM) were mixed with PG (1.0–10 μM) and the generation of Pm was measured by the ΔA_{405} with Chromozym PL as substrate.

Complex-formation of SAK mutants with Pm and generation of active site within the SAK–PG (Pm) bimolecular complex

The time course of active-site generation in equimolar complexes of Pm with wild-type SAK or SAK mutants was monitored by titration with *p*-nitrophenyl *p*′-guanidinobenzoate (NPGB) at room temperature, as described previously [8,32]. Concentrated stock solutions of PG and SAK–Pm complex were diluted in 0.1 M veronal buffer, pH 8.3, containing 0.1 M arginine, to final concentrations of 4.0 μM and 4.0 μM respectively. At different time points (0–20 min) after mixing of PG and SAK–Pm or loop mutant of SAK (SAKΔ90)–Pm complex, NPGB was added to a final concentration of 100 μM. The concentration of active site was determined from the 'burst' of *p*-nitrophenol, using a molar absorption coefficient of 16700 $M^{-1} \cdot cm^{-1}$ [32].

PG activation by catalytic amounts of preformed equimolar complexes of SAK or SAK mutants with Pm

Equimolar mixtures of SAK or SAK mutants and Pm were preincubated in 0.1 M phosphate buffer, pH 7.5, containing 0.1 % BSA and 0.01 % Tween 80 at 37 °C for 5 min to generate the SAK–Pm bimolecular complex. These preformed activator complexes (5 nM) were then mixed with PG (1.5 μM), and generation of Pm was measured at 25 °C from ΔA_{405} with Chromozym PL.

Measurement of PG binding

Purified protein preparation of native SAK or SAKΔ90 (1.5 ng) was suspended in binding buffer (50 mM Na_2CO_3/$NaHCO_3$, pH 9.5) and was immobilized on the microtitre plates or nitrocellulose strips. Blotted protein was air-dried and blocked with 1 % BSA for 1 h at 4 °C. Different concentrations of ^{125}I-Pm, prepared by the Iodogen method [33], were then added to the immobilized SAK proteins and incubated at 4 °C for 1 h. Excess radioactivity was removed by repeated washing with the same buffer. The amount of labelled Pm, bound with the SAK, was estimated by the amount of radioactivity (measured by using a γ-radiation counter) retained on the blot. BSA was used in place of SAK to check the background level of non-specific binding that was subtracted from the total level in order to determine specific Pm binding.

Casein/PG overlay and radial caseinolytic assay

Bacterial colonies overexpressing SAK were detected by overlaying 0.8 % agarose/1 % skim milk/human PG (500 μg/plate)/150 mM NaCl/50 mM Tris/HCl, pH 8.0, on top of the agar plates carrying recombinant *E. coli* colonies as described previously [34]. The plates were incubated at 37 °C for 2–6 h. SAK-positive clones indicated a clearing zone around the colonies. Functional activity of purified SAK or mutant SAK protein was also estimated by radial caseinolytic assay. Petri dishes containing 1.2 % agarose, 1 % skim milk and 10 μg/ml PG were prepared. On this solidified agarose plate, wells of equal diameter were bored and an equal quantity of purified native SAK or mutant SAK protein was added and kept at 37 °C for 6–7 h. The diameter of the halo around the well was measured to check the functional activity of SAK proteins.

Sandwich binding assay

The formation of ternary complex between Pm and native SAK or SAKΔ90 was checked by means of the sandwich binding assay described previously [19,35]. Briefly, 0.01–0.5 ml of Pm (10 μg/ml in 50 mM Tris/HCl, pH 7.5) was immobilized on nitrocellulose strips or microtitre plates, air-dried and blocked with 1 % BSA in PBS for 1–2 h. Native SAK or SAKΔ90 protein was added in excess to the immobilized Pm and incubated for 30–60 min at 4 °C. Unbound SAK was removed after washing with 0.05 M Tris/HCl, pH 7.4, containing 0.1 % BSA and 0.01 % Tween 80. Thereafter ^{125}I-PG was added at various concentrations and incubated further for another 30–60 min at 4 °C. Excess radioactivity was removed by means of repeated washing with the same buffer, and the amount of substrate PG bound to immobilized Pm–SAK complex was determined by the level of radioactivity incorporated (measured using a γ-radiation counter). Non-specific binding was determined by using BSA instead of SAK protein. This was subtracted from the total radioactivity to obtain a value for specific protein binding.

RESULTS

Characteristics of 90-loop region (amino acids Thr^{90}–Glu^{100}) of SAK

SAK is a single-domain PG activator that is folded into a compact and flattened structure that consists of a five-stranded β-sheet packed on a single 12-residue α-helix [11]. It exhibits significant three-dimensional structural similarity with the α-domain of streptokinase (SKα), including similar β-grasp folding characteristics [15,36]. Structure-based sequence alignment of SAK with SKα indicates that SAK 90-loop region, carrying several surface-exposed charged residues, is unique in SAK structure. Protein modelling and structural overlay of SAK and SKα domain indicates that SAK 90-loop is extended out as compared with the corresponding SKα loop region and probably can be deleted without major structural perturbations. SAK 90-loop is hydrophilic in nature and contains four positively charged residues (Lys^{94}, Lys^{96}, Lys^{97} and Lys^{98}) at the tip and three negatively charged (Asp^{93}, Glu^{99} and Glu^{100}) residues at the stem, along with two aromatic residues, Tyr^{92} and Tyr^{93}. The crystal structure of the μPm–SAK–μPm ternary complex suggests that this loop region of SAK is away from the interface between SAK and μPm in the SAK–Pm enzyme complex and does not have any direct structural interaction with the substrate μPG (Figure 1, upper panel). The orientation of the substrate μPm on the SAK–Pm enzyme complex suggests that it may be possible for the kringle domains of substrate PG to make contact with this surface-exposed loop structure. Since the crystal structure of ternary complex of SAK and μPm provides only a partial picture of protein–protein interaction, owing to a lack of kringle domains in μPm, computer modelling, based on the SAK–μPm crystal structure and other available information, including the position of substrate μPm relative to bimolecular complex of SAK–Pm, docking of kringle on SAK 90-loop, etc., was carried out to look for the possible targets of the SAK 90-loop. The result (Figure 1, lower panel) indicates that it is feasible to dock kringle 5 of human PG on the SAK 90-loop. A search for geometric and electrostatic complementary regions of both the protein struc-

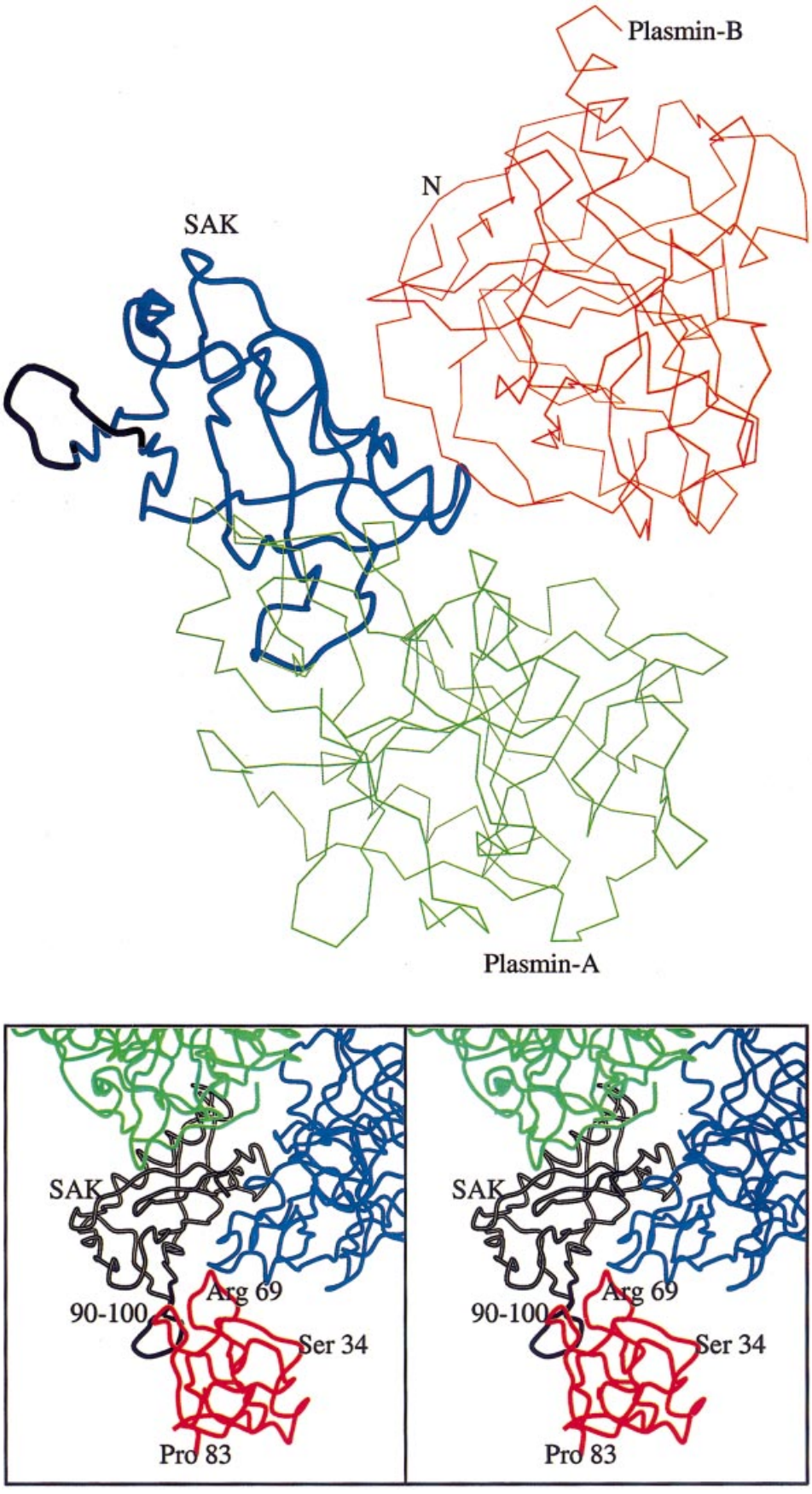

Figure 1 For legend see facing page.

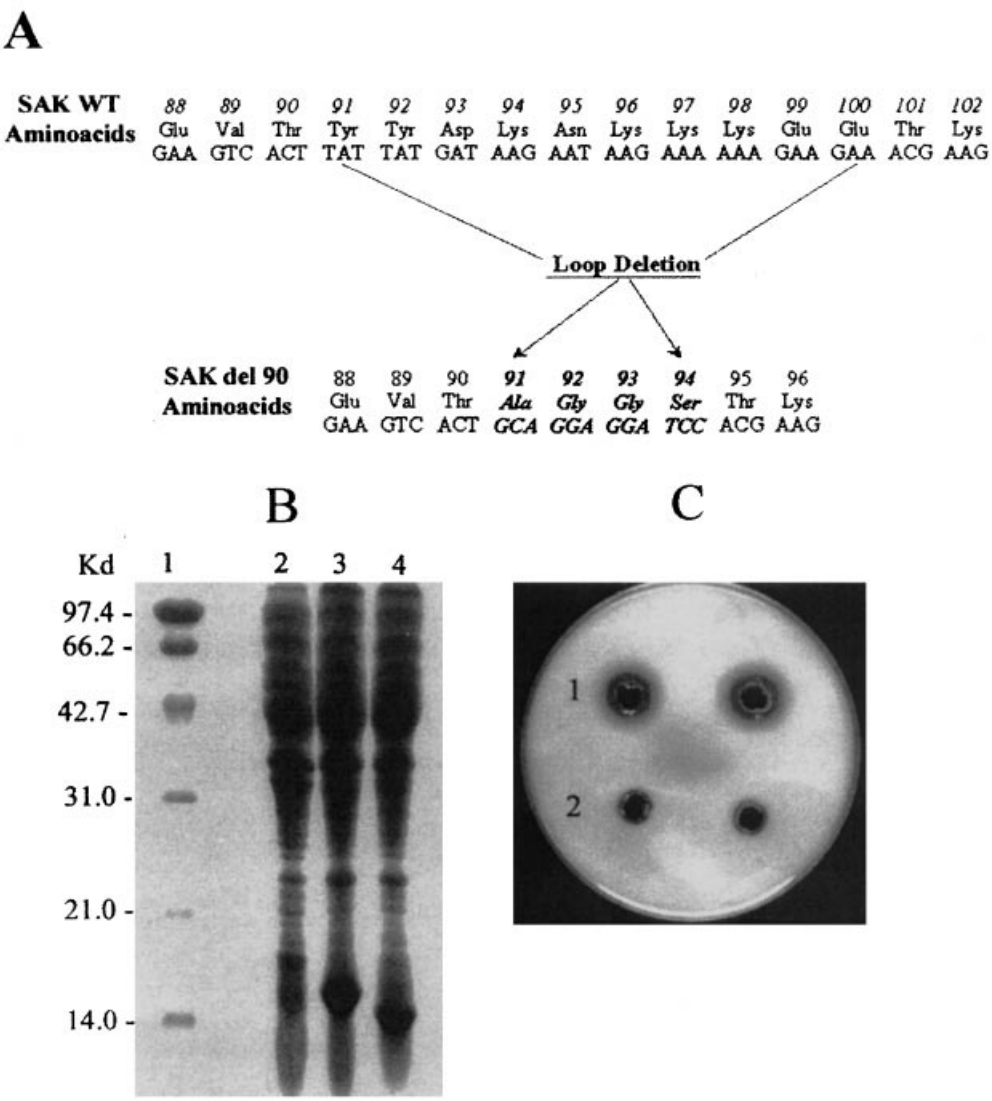

Figure 2 Characteristics of loop-deletion mutant (SAKΔ90) of SAK

(**A**) Sequence of 90-loop region of SAK ('SAK WT') and SAKΔ90 ('SAK.del 90'). Amino acid residues and nucleotide sequence of the SAK 90-loop region are shown. Amino acid residues from Tyr^{92} to Glu^{100} have been deleted from the SAKΔ90 and a small linker (shown in **bold** letters) has been incorporated to facilitate the folding of the loop. Amino acid sequence of deleted region of SAKΔ90 is shown. (**B**) Expression of SAKΔ90 in *E. coli*. Recombinant plasmids, pRM1 and pSAKΔ90, encoding native SAK and SAKΔ90, respectively, were transformed in *E. coli* BL21DE3 for the overexpression of recombinant SAK proteins. A 1% inoculum of overnight-grown culture of these cells were inoculated into 50 ml of Luria broth and grown at 37 °C for 3 h at 180 rev./min; after that, 0.1 mM isopropyl β-D-thiogalactoside was added to the flasks and cells were further grown for another 6 h. Cells were then harvested by centrifugation and subjected to SDS/15%-PAGE. Lane 1, molecular-mass markers ('Kd' = kDa); lane 2, control *E. coli* cells; lane 3, *E. coli* expressing native SAK; lane 4, *E. coli* expressing SAKΔ90. (**C**) PG activation activity of SAKΔ90 as determined by the radial caseinolysis assay. A 50 ng portion each of native and mutant SAK purified proteins in the individual wells were added and plates were incubated at 37 °C for 6 h. 1, Wild-type SAK; 2, SAKΔ90.

tures indicated that Lys^{96}, Lys^{97} and Lys^{98} of SAK 90-loop align and orient towards the cationic centres of kringle 5 and may be the probable target for the kringle interaction. Considering the similarity between the three-dimensional structure and the basic conformation of the anionic centre of the lysine-binding sites of kringle domains 1–5 of human PG [14], it may be envisaged that

Figure 1 The ternary complex of SAK and μPm (upper panel) and stereoscopic view of the manually docked complex of kringle 5 domain with the ternary complex of SAK and μPm (lower panel)

Upper panel: this was generated by using the co-ordinates of the ternary complex of SAK and μPm submitted by Parry et al. [15] to the Protein Data Bank. The position of the SAK 90-loop with respect to the SAK–Pm enzyme complex [SAK in blue, Pm ('Plasmin-A') in green] and substrate μPm ('Plasmin-B', in red) is shown in black. It is clearly seen that this loop makes no direct interaction with the substrate μPm. Orientation of the N-terminus of the substrate μPm is indicated. Lower panel: the anionic centre of the kringle 5 domain (red) carrying negatively charged patches docks well with positively charged residues on the SAK 90-loop which contributes to the major positively charged surface of the SAK molecule. The position of the specific region of kringle 5 with the SAK 90-loop is shown. The grey structure represents SAK, whereas green and blue structures represent partner and substrate plasmin respectively.

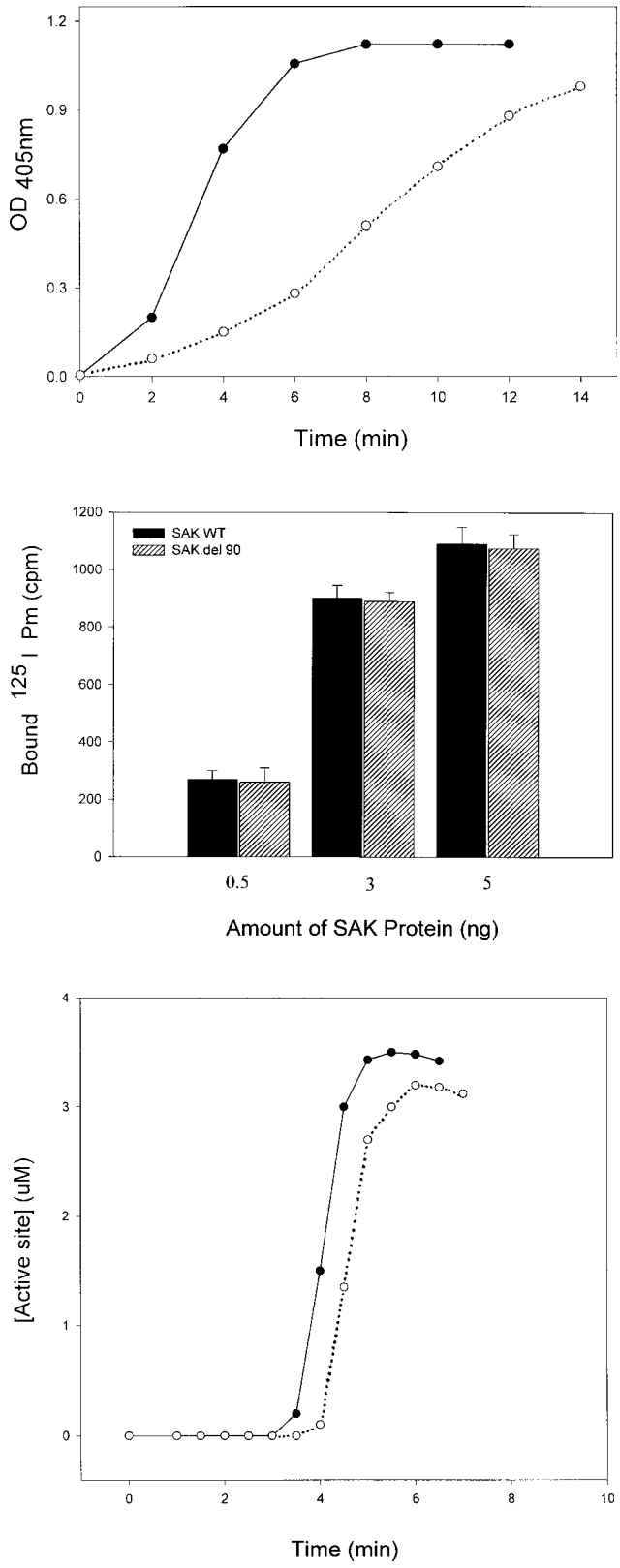

Figure 3 PG activation and binding profile of SAKΔ90

Upper panel: PG activation by SAKΔ90 (○) and native SAK (●). Human PG (1.5 μM) was incubated with native SAK or SAKΔ90 (0.05 μM) in the presence of 1.2 mM Chromozym PL (Boehringer-Mannheim) at 25 °C in 0.05 M Tris/HCl/0.1M NaCl, pH 7.4, containing 0.1% BSA and 0.01% Tween 80. The rate of Pm generation was monitored at 405 nm at different time intervals. Middle panel: pattern of binary-complex formation with PG by SAKΔ90. Native ('SAK WT') or mutant SAK (SAKΔ90; 'SAK.del 90') proteins (1.5 ng) were immobilized on nitrocellulose strips. Non-specific protein-binding sites were blocked with skimmed milk (1%). Unbound proteins were removed by repeated washing with 0.05 M Tris/HCl, pH 7.5, containing 0.1% BSA and 0.01% Tween 80. Immobilized protein was probed with ^{125}I-human PG and the level of PG binding was determined in a γ-radiation counter. Each binding result represents the mean of three independent determinations. Bottom panel: time-course acylation of Pm active site in equimolar mixtures of PG with wild-type SAKΔ–Pm (●) and SAKΔ90–Pm (○) complexes. Concentrated stock solutions of PG and SAK–Pm binary complexes were diluted in 0.1 M Veronal buffer, pH 8.3, containing 0.1 M arginine, both to a final concentration of 4.0 μM. At different time points after mixing of PG and SAK–Pm or SAKΔ90–Pm (0–20 min), NPGB was added to a final concentration of 100 μM.

any one of these modular structures of the substrate PG could make possible contact with the SAK 90-loop and facilitate optimal substrate positioning for processing by the SAK–Pm activator complex.

Deletion of 90-loop does not abolish the PG activation function of SAK

Structural analysis of SAK and its superimposition on SKα, indicated that SAK 90-loop region may be deleted without any major structural perturbation in overall conformation of SAK. To check this probability experimentally, nine amino acid residues covering Tyr^{92}–Glu^{100} of SAK were deleted and the resulting SAK mutant, SAKΔ90, cloned and overexpressed in *E. coli*. The sequence of the deleted region of SAKΔ90 is given in Figure 2(A). On SDS/PAGE, SAKΔ90 migrated more quickly than native SAK, supporting the corresponding truncation in the SAK loop-deletion mutant (Figure 2B). A PG-coupled radial caseinolytic assay (Figure 2C) of SAKΔ90 revealed that it is able to activate PG; however, the diameter of its clearance zone indicated that its PG activation capability is relatively lower than that of the native SAK.

PG binding and activation properties of SAKΔ90

To examine the effect of loop deletion on the functional properties of SAK, the pattern of PG binding and activation by SAKΔ90 was compared with that of native SAK. SAKΔ90 exhibited more than 60% decrease (64 units/μg) in specific PG activation activity as compared with full-length SAK (160 units/μg). The catalytic amount of native SAK (5 nM) induced rapid activation of PG to Pm, resulting in nearly 90% of PG activation within 6 min. In contrast, SAKΔ90 activated PG into Pm slowly and reached the level achieved by native SAK in about 15 min (Figure 3, upper panel). The pattern of radiolabelled-PG binding with immobilized native SAK and SAKΔ90 indicated that concentration-dependent binding of ^{125}I-PG with SAK and SAKΔ90 has a similar trend in the formation of SAK–PG binary complex. These results suggested that the ability of SAKΔ90 to generate stoichiometric complex with its partner PG is similar to that of native SAK (Figure 3, middle panel). To check further that the bimolecular complex of SAKΔ90 and Pm had not altered their active-site geometry, we tested the ability of this mutant to expose its active site by monitoring the burst of *p*-nitrophenol release with equimolar mixtures of native SAK or SAKΔ90 after reaction with the active-site acylating agent [32]. Active-site exposure in the mixture of PG and SAK–Pm occurred exponentially, with an initial lag of 3 min, whereas, in the case of SAKΔ90–Pm, the corresponding value was about 4 min, resulting in 98% of active-site exposure (Figure 3, bottom panel). Overall, results thus revealed no perceptible alteration in the functional properties of SAKΔ90 with respect to its ability to form a native-

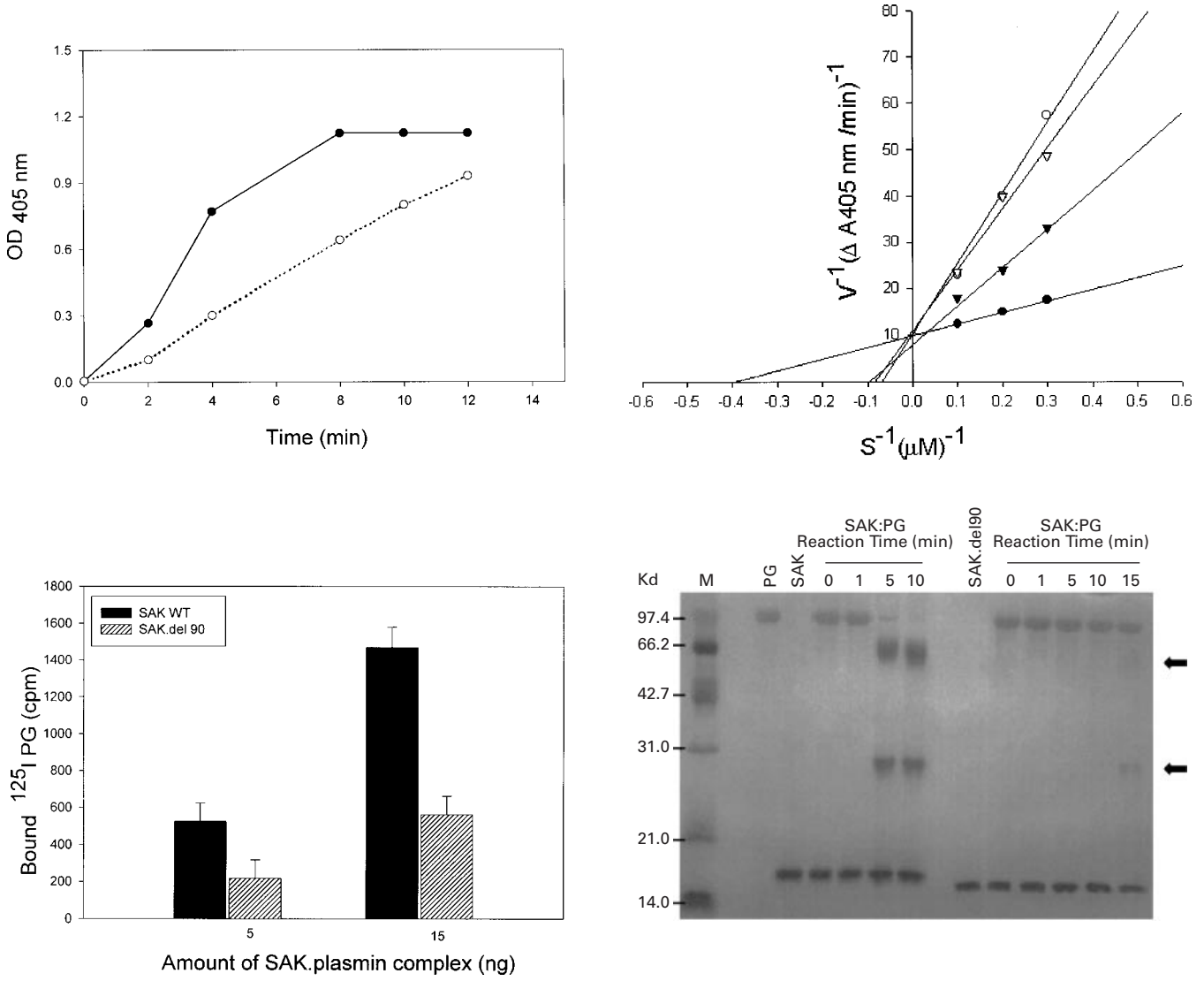

Figure 4 Substrate PG binding and activation by preformed complex of Pm with native SAK and SAKΔ90

Top left panel: activation of substrate PG by preformed complex of Pm with native SAK (●) and SAKΔ90 (○). Pm (1.5 μM) and SAK (1.5 μM) were preincubated for 10 min at 37 °C in Tris/HCl, pH 7.4, containing 0.1 M NaCl, 0.1% BSA and 0.01% Tween 80. This complex was subsequently diluted (final concentration 5 nM) and incubated with PG (1.5 μM) and generated Pm activity was measured at different time intervals in the presence of Chromozym PL. Top right panel: Lineweaver–Burk plots of amidolysis by SAK and SAK mutants. Various concentrations (1.0–10 μM) of substrate PG were mixed with the activator complex (5 nM) formed between Pm and SAK (●) or SAK mutants (○, SAKΔ90; ▼, SAK_{K97A}; ▽, SAK_{K98A}) in a quartz cuvette containing assay buffer. The absorption at 405 nm was continuously recorded for 60 min to obtain velocities. Lineweaver–Burk plots of $1/v$ ('V^{-1}') [$(\Delta A_{405}/\text{min})^{-1}$] against $1/s$ ('s^{-1}'; μM^{-1}) from typical experiments are shown. Bottom left panel: formation of ternary complex (PG–SAK–Pm) with substrate PG by immobilized binary complex formed with native SAK ('SAK WT') or SAKΔ90 ('SAK.del 90') with Pm. Different concentrations of human Pm were immobilized and non-specific sites were blocked with BSA (1%) as described in the Materials and methods section. Native or mutant SAK (SAKΔ90) proteins (2 μM) were added in 2-fold excess to form binary complex with the bound Pm. After repeated washing with 0.05 M Tris/HCl, pH 7.4, containing 0.1% BSA and 0.01% Tween 80, ^{125}I-PG (2–3 μM) was added. Unbound PG was removed after three to five washings with the same buffer and the level of bound PG was determined in a γ-radiation counter. Each binding result represents the mean of three independent measurements. Lower-right panel: cleavage of PG by equimolar complex of Pm with native SAK and SAKΔ90 ('SAK.del 90'). Equimolar amounts of SAK or SAKΔ90 and PG (1.5 μM each) were mixed in 100 mM Tris/HCl, pH 7.5, and incubated at 25 °C for 15 min. At each time point, a 7.5 μl aliquot was removed and analysed for the conversion of PG into Pm by SDS/12.5%-PAGE. Lane M, Molecular-mass markers (Kd = kDa); lane 1, PG; lane 2, purified SAK; lanes 3–6, PG–SAK ('SAK:PG') reaction mixtures incubated for 0, 1, 5 and 10 min respectively; lane 7, purified SAKΔ90; lanes 8–12 are PG–SAKΔ90 ('SAK.del 90:PG') reaction mixtures incubated for 0, 1, 5, 10 and 15 min respectively. The arrow indicates the cleavage products of PG to Pm.

SAK-like bimolecular complex with Pm and exposure of the active site within the activator complex.

Catalytic efficiency of preformed SAKΔ90–Pm complex

Although SAKΔ90 exhibited native-SAK-like ability to generate activator complex with its partner Pm, its PG activation rate was 7–8-fold slower than that of wild-type SAK. These results prompted us to explore whether this deficiency in PG activation is due to the alteration in the interaction of SAKΔ90–Pm complex with the substrate PG. Therefore we compared the pattern of PG activation by preformed complexes of Pm with native SAK and SAKΔ90 (Figure 4, top-left panel). Activation of PG by preformed SAK–Pm complex occurred progressively, with a brief lag phase followed by an exponential phase. The overall activation pattern obeyed Michaelis–Menton kinetics, as

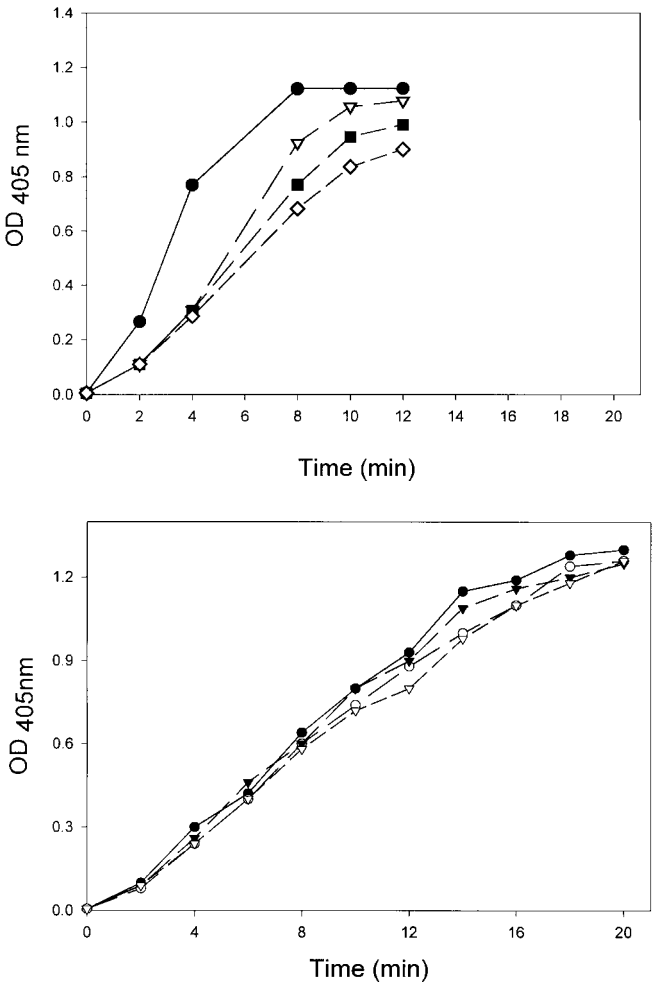

Figure 5 Effect of kringle domains of human PG on PG activation by preformed activator complex formed by Pm with native SAK (upper panel) and SAKΔ90 (lower panel)

Preformed complex of Pm (1.5 μM) with native SAK or SAKΔ90 (1.5 μM) were generated by incubating this mixture at 37 °C for 10 min. The mixture was then diluted 50-fold and incubated with PG (1 μM) in the presence of different concentration of kringles (0–20 μM) and generated Pm activity was measured at different time intervals in the presence of Chromozym PL. Upper panel: ●, SAK wild-type; ▽, SAK wild-type + 5 ng of K(1–4); ■, SAK wild-type + 10 ng of K(1–4); ◇, SAK wild-type + 20 ng of K(1–4). Lower panel: ●, SAKΔ90; ○, SAKΔ90 + 5 ng of K(1–4); ▼, SAKΔ90 + 10 ng of K(1–4); ▽, SAKΔ90 + 20 ng of K(1–4). '$OD_{405\ nm}$' = A_{405}.

revealed by linear double-reciprocal plots of the initial activation rate versus the PG concentration (Figure 4, top-right panel). The catalytic efficiency and K_m of preformed SAKΔ90–Pm complex was about 50 % lower and about 5-fold higher respectively than the preformed SAK–Pm complex (Table 2 below). Figure 4, top-right panel, shows Lineweaver–Burk plots obtained for the activator complex formed by SAK and SAK mutants in enzyme kinetic studies. To further compare the capability of ternary-complex formation with the activator complex formed with Pm and native SAK or SAKΔ90, the interaction of substrate PG with the bimolecular complex formed with SAKΔ90 and Pm, a sandwich-binding assay was performed. Immobilized bimolecular complex formed with SAKΔ90 and Pm exhibited a more-than-40 % decrease in the interaction with the substrate PG in the formation of ternary complex as compared with native SAK (Figure 4, bottom-left panel). Cleavage of substrate PG to Pm by an equimolar complex of Pm with SAK or SAKΔ90 was monitored by SDS/PAGE to further compare the catalytic efficiency of SAKΔ90 with native SAK (Figure 4, bottom-right panel). Densitometric analysis of the protein profile indicated that more than 90 % of the PG is converted into Pm within 5 min by SAK–Pm complex. Under similar conditions, nearly 25 % of the substrate PG was cleaved into Pm in about 15 min by a preformed complex of SAKΔ90 and Pm.

Effect of kringle (K1 + K2 + K3 + K4) domains on the activation of PG by preformed complex of SAKΔ90 and Pm

Since PG activator complex formed with the loop-deletion mutant of SAK (SAKΔ90) exhibited slower processing of substrate PG, and protein-modelling studies indicated possible interaction of kringle domain of substrate PG with the SAK 90-loop region, we studied the pattern of PG activation by preformed SAK–Pm complex in the presence of isolated kringle domains (K1 + K2 + K3 + K4) of human PG. A time-course study on PG activation by pre-formed SAK–Pm complex indicated that, in the presence of 5 μM kringles, activation of PG was inhibited by 30 % which was further increased to about 50 % when the amount of kringles were increased to 20 μM (Figure 5, upper panel), indicating that the kringle domains are competing with full-length substrate PG molecules for the interaction with the SAK–Pm enzyme complex. Interestingly, preformed SAKΔ90–Pm complex exhibited very little difference in the PG activation process and exhibited only 10 % inhibition in the presence of 20 μM of kringles (Figure 5, lower panel), suggesting that the kringle domains are not effectively competing with the substrate PG during activation via SAKΔ90–Pm enzyme complex.

Table 1 Functional properties of SAK mutants carrying alteration within the 90-loop region

		Sequence of the loop region*										
SAK mutant	Position ...	92	93	94	95	96	97	98	99	100	101	Specific activity (units/μg)†
Wild-type		Tyr	Asp	Lys	Asn	Lys	Lys	Lys	Glu	Glu	Thr	160
SAK_{K94A}		Tyr	Asp	Ala	Asn	Lys	Lys	Lys	Glu	Glu	Thr	140
SAK_{K96A}		Tyr	Asp	Lys	Asn	Ala	Lys	Lys	Glu	Glu	Thr	155
SAK_{K97A}		Tyr	Asp	Lys	Asn	Lys	Ala	Lys	Glu	Glu	Thr	49
SAK_{K98A}		Tyr	Asp	Lys	Asn	Lys	Lys	Ala	Glu	Glu	Thr	55
SAKΔ90		Gly	Gly	Ser			Δ‡				Thr	45

* Underlined amino acid residues indicate substitution with alanine.
† Average value of three individual determinations.
‡ Deletion.

Table 2 Kinetic constants for the PG activation properties of equimolar complexes of SAK mutants and Pm

The results are means ± S.E.M. of three determinations.

Bimolecular complex	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot \mu M^{-1}$)
SAK–Pm	2.5 ± 0.12	2.0 ± 0.09	0.80
SAKΔ90–Pm	14.4 ± 0.20	1.9 ± 0.05	0.14
SAK_{K97A}–Pm	10.4 ± 0.16	2.1 ± 0.10	0.21
SAK_{K98A}–Pm	11.8 ± 0.18	1.8 ± 0.10	0.16

Site-directed mutagenesis of 90-loop region of SAK and functional properties of SAK mutants

With a view to further explore whether the charged residues of SAK 90-loop structure provide any contribution to substrate PG processing and activation, we attempted to delineate the probable target of 90-loop region of SAK by site-directed mutagenesis. Four SAK mutants, namely K94A, K96A, K97A and K98A, were created by individually converting lysine into alanine. Out of these four SAK mutants, SAK_{K94A} and SAK_{K96A} exhibited PG activator activity comparable with that of native SAK, implying that these lysine residues may not be important for modulating the PG activation function of SAK. By contrast, the other two mutants, SAK_{K97A} and SAK_{K98A}, exhibited about a 4–5-fold reduction in their specific activities for PG activation (Table 1). All these mutants retained near-native-SAK-like binding capability for the Pm binding as determined by solid-phase radioassay employing ^{125}I-Pm. Additionally, these SAK mutants exhibited a 4–6-fold reduction in their affinity for the substrate PG (Table 2), which was evident from their higher K_m values for the substrate PG (Figure 4, top-right panel). These observations clearly indicated that mutations in lysine residues of the 90-loop region of SAK had led specifically to a reduction in the ability of these SAK mutants to interact with the substrate PG.

DISCUSSION

Although three-dimensional structures of SAK and its ternary complex with μPG [15] have been resolved, the molecular mechanism whereby SAK brings about structural changes after association with its partner PG or Pm and interacts with free PG to generate Pm activity is still largely unknown. From a mechanistic perspective, two distinct protein–protein interactions occur during the SAK-mediated PG activation process. SAK generates an inactive 1:1 bimolecular complex with Pm which requires removal of a decapeptide from the N-terminus of SAK [17,18] and the conversion of substrate PG into Pm [6,36,37]. SAK does not directly induce conformational changes in the active-site residues, but alters the substrate specificity of Pm indirectly by creating new subsites on to which substrate PG docks for the enhanced presentation of the PG activation loop towards the enzyme [11]. Precise molecular interactions that occur in between the SAK–Pm enzyme complex and the substrate PG are far from clear at present. The crystal structure of the ternary complex μPm–SAK–μPm reveals only a partial picture, owing to the absence of auxillary domains (kringle structures) in μPm as compared with the full-length PG molecule. Recent biochemical studies [38] indicate the participation of kringle structure in SAK-mediated PG activation. In the present study we have demonstrated that the SAK–Pm enzyme complex may make an additional interaction with kringle domains of the substrate PG via a charged loop region of SAK (SAK 90-loop) that may facilitate interaction of substrate PG during PG activation.

The SAK 90-loop region is at a position totally distant from the protein–protein interaction sites in the three-dimensional structure of μPm–SAK–μPm ternary complex (Figure 1, upper panel). Retention of PG activation capability in the SAK mutant, lacking this loop region, suggests that the SAK loop-deletion mutant is still able to fold back and achieve its native-SAK-like conformation. SAK exhibits closest structural similarity with SKα, and it has been suggested that SAK and SK perform similar function(s) in the PG activator complex [15]. Structural overlay and amino acid alignment of SAK and SKα, based on their three-dimensional structural homology, indicate that there are two distinct mobile regions that do not align when the structures of these two protein units are superimposed. One of these region spans positions 44–64 in SKα, and another distinct region is in SAK, covering the 90-loop region that is absent in SKα domain. Involvement of the Asp^{41}–His^{48} region of SK in interaction with the substrate PG has been recently suggested on the basis of site-directed mutagenesis within this mobile region of SK [39]. Deletion of the SAK 90-loop region results in a 5–6-fold decrease in specific activity of SAK and generates significant reduction in the PG activation properties of the molecule. In principle, SAK could modify the specificity of the Pm active site by altering its conformation or by changing substrate PG accession through altered docking of PG on the SAK–Pm enzyme complex. SAKΔ90 forms a 1:1 SAK–Pm complex which displays an amidolytic activity comparable with that of the native SAK–Pm binary complex. Progressive exposure of active site within the SAKΔ90–Pm complex clearly indicated that the SAK loop-deletion mutant is capable of generating activator complex with Pm very similar to that of native SAK. Therefore the slow activation kinetics of PG activation by SAKΔ90 may not be due to alteration in activator complex formation with Pm.

Deletion of the SAK 90-loop led to a perceptible increase in K_m for the substrate PG. The slow kinetics of PG activation by the preformed activator complex of SAKΔ90 and Pm suggested that this bimolecular complex is not able to interact optimally with the substrate PG. These results were further substantiated by slow cleavage of PG into Pm by activator complex of SAKΔ90 with Pm. Furthermore, site-directed mutagenesis within the SAK 90-loop region revealed that substitution of Lys^{97} or Lys^{98} with alanine within this loop region results in similar lesion in substrate PG activation by these SAK mutants. These results provided the evidence that Lys^{97} and Lys^{98} of the SAK 90-loop may be assisting in the sequestering of substrate PG. These results conform with those obtained in previous studies on alanine-clustered charge mutagenesis of SAK [16]. Overall, results thus suggest that the SAK 90-loop may not be very important in maintaining the functional conformation of SAK, but may be involved in accession/interaction of substrate PG via its positively charged residues, Lys^{97} and Lys^{98}.

In an effort to determine the probable target of the SAK 90-loop on substrate PG, we analysed the orientation of substrate PG with respect to the SAK–Pm enzyme complex in a three-dimensional model of the μPm–SAK–μPm complex. It indicated that the SAK 90-loop may provide an ideal docking site for the kringle structure of substrate PG. Free PG binds with the activator complex of SAK–Pm with 3-fold higher affinity compared with miniPG [40], suggesting that the presence of kringle structures are crucial for optimal interaction of substrate PG. In an attempt to evaluate further the probable interaction of kringle structures and the SAK 90-loop, competition between substrate PG and kringle was examined during PG activation by SAKΔ90–Pm activator complex and compared with that of native SAK–Pm complex. Kringle domains of PG (K1 + K2 + K3 + K4) effectively competed with PG for interaction with SAK–Pm activator

complex, whereas the SAKΔ90–Pm complex did not exhibit any significant change in PG activation pattern in the presence of kringles. Conceptually, kringles could modulate the interaction of substrate PG by providing more optimal docking of substrate on SAK–Pm activator complex through additional interaction site(s) via their lysine-binding sites. Biochemical studies on SAK have indicated that the lysine-binding sites in K1 + K2 + K3 + K4 kringle domain of PG may play a role in the activation of PG by SAK–Pm complex [38]. In this process, the SAK 90-loop may be the ideal target for this interaction, owing to the presence of several surface-exposed clustered lysine residues. Molecular docking of kringle 5 on SAK 90-loop indicates that Lys^{97} and Lys^{98} are facing the anionic centre of the kringle. A drastic decrease in the PG activation properties of SAK mutants SAK_{K97A} and SAK_{K98A} supports this structural prediction. Since the presence of kringle structure (K1 + K2 + K3 + K4) effectively compete with full-length PG, any one of these kringle domains may possibly show an interaction with the SAK 90-loop region. The overall basic conformations of the kringle domains are more or less similar, including kringle 5, which we have tried to dock on the SAK 90-loop in our modelling studies.

In conclusion, the present study on site-directed and loop-deletion mutagenesis of SAK has provided novel information on SAK–PG interaction and provided the experimental evidence for the participation of the SAK 90-loop region in facilitating the interaction(s) of substrate PG with the SAK–Pm enzyme complex. Lys^{97} and Lys^{98} of the SAK 90-loop region may be specifically involved in this process. These additional interactions between free PG substrate and bimolecular SAK–Pm complex thus may potentiate the SAK-mediated PG activation process.

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