Introduction

The crystal structure of ternary complex of SAK with microplasmin (μPm) and binary complex of SK with μPm (Wang et al., 1998, Parry et al., 1998) were solved almost simultaneously. Comparison of these structures (Fig. 7.1) reveals that SK essentially surrounds the catalytic domain of Pm whereas much smaller SAK contacts only one side. Due to multi-domain nature of SK, its interactions with μPm are more extensive and complex in comparison to SAK. However, SAK shares a close structural homology with SK (Parry et al., 2000), specifically α domain of SK and even the contacts of the two with microplasmin (μPm) are similar (Rabijns et al., 1997, Parry et al., 1998, Wang et al., 1998). In addition, SAK also shares the same β-grasp folding with SK α and β domains.

Despite these similarities in their structural aspects, there are certain key differences between SAK and SK in their mechanism of action for Pg activation. Unlike to SAK, SK can form a functional activator complex with Pg by modifying its active site without any proteolytic cleavage in Pg molecule while SAK requires the conversion of SAK-Pg complex to SAK-Pm to form a functional activator complex (Grella and Castellino, 1997). It has been observed that the single domain SAK binds with lower affinity to Pm (Lijnen et al., 1994, Sakharov et al., 1996) and SAK-Pm activator complex possesses a relatively lower catalytic efficiency as compared to SK-Pg/Pm complex (Cederholm-Williams et al., 1979). Additionally, SAK-Pm complex, but not the SK-Pg/Pm complex is inactivated by α2-antiplasmin (Sakai et al., 1989, Lijnen et al., 1991b, Lijnen et al., 1992, Silence et al., 1993b, Silence et al., 1993a) and Pg binding to fibrin degradation products dramatically enhances SAK binding and Pg activation (Sakharov et al., 1996).

Various molecular modelling and site directed mutagenesis have been undertaken to unravel the role of different domains in SK and various residues in SK or SAK crucial for cofactor mediated Pg activation process. In a model generated for the SK-μPm complex, Loy et al. (Loy et al., 2001) suggested that a distinct function can be assigned to each domain of SK. Whereas, SAK and SKα domain assist in proper substrate pre-orientation and presentation to the active site of the enzyme, SKβ domain provides a further substrate anchoring site that also modulates the interaction of Pm with macromolecular inhibitors. SKγ domain has been proposed to participate in the binding and activation upon complex formation.
Fig 7.1: Ribbon diagram of SAK-μPm structure (left) and SK-μPm structure (right). μPm is shown in blue while α helix is shown in white. Single domain SAK is shown in green while three domain SK is shown in green and yellow.
Another two domain Pg activator of bacterial origin (*Streptococcus uberis*) i.e. SUPA has been reported (Johnsen *et al*., 1999). Interestingly Pg activation potential of these bacterial cofactors increases with increase in the number of domains from SAK to SK. But unlike to SUPA and SK, SAK being a single domain molecule, can efficiently activate Pg molecules. α domain of SK binds Pg with 10 fold higher affinity but displays several fold reduced Pg activation capability (Loy *et al*., 2001). Pg activation capability of two-domain SUPA is intermediate of SK and SAK, suggesting that there is domain cooperation in the Pg activation process. Thus, it becomes logical to compare the Pg activation characteristics of individual domains of SK with SAK and to test function of various SK domains in modulating the Pg activator activity of SAK. Earlier studies conducted in our lab have shown the effect of various SK domains on the Pg activation property of SAK (Dahiya *et al*., 2005). The catalytic efficiency of SAK-Pm bimolecular complex increases up to 60% in the presence of SKβ or βγ domains. In the present study, chimera of SAK with β domain of SK was generated to see its effect on the cofactor activity of SAK. In addition, linkers of different length and amino acid composition were engineered for attachment of β domain at the C-terminus of SAK in an attempt to improve its Pg activation property to develop it as a better Pg activator.

Results

7.1 Structural and functional comparison of SAK with SK domains

Sequence homology between individual SK domains and SAK is beyond recognition using conventional sequence alignment methods. However, SAK shares a considerable structural homology with α and β domains (Fig. 7.2) of SK. (Wang *et al*., 1998). The validity of this structural alignment is reinforced by its correlation with the functions of theses domains, *i.e.* SKα and SAK performs similar functions in Pg activator complex while SK β does not. Comparison of SAK-μPm bimolecular interface with that of SK-μPm shows similar type of interactions at the interface for the respective binary activator complexes (Wang *et al*., 1998, Parry *et al*., 1998). For example SAKGlu46 and SKαGlu39 are found to be critical for interacting with PgArg719 in the complex formation. A pair of His43-Tyr44 of SAK plays an important role in generating the specificity switch with μPm. The structurally equivalent position in SK to SAK is occupied by a Lys36-Phe37 pair to generate intermolecular interactions with μPm in a similar fashion. Although, individual α and β domains of SK were structurally
Fig 7.2: Ribbon diagram representation of SAK and three domains of SK. Despite the low sequence identity, the four domains show considerable structural homology. (Figure adapted from Parry et al. 2001).
homologous to SAK but their Pg activation potential is very low. But in full length SKα, β and γ domains show synergistic effect and display very high Pg activation activity. Such a close structural and functional equivalence between α domain of SK and SAK prompted us to characterize the effect of β and γ domains of SK on Pg activation properties of SAK.

7.2 Generation of chimeric SAK carrying β and γ domains of SK

The presence of SKβ and βγ domains separately facilitated the Pg activation property of SAK, (Dahiya et al., 2005) indicating that various SK domains can be fused with SAK to generate a better Pg activator molecule. To experimentally check this hypothesis, chimeric proteins were constructed in which SK β domain was fused with SAK.

7.3 Cloning, expression and purification of SAKβ fusion proteins

Overlap PCR was used for in frame fusion of SK β encoding region of sk gene with sak gene as described in material and methods. The chimeric DNA was cloned in pET9b vector and SAKβ fusion proteins were expressed in E. coli BL21 DE3 under IPTG inducible T7 promoter. The proteins were purified in a single step purification using ion (anion) exchange chromatography on DEAE-sepharose column. Column was equilibrated with 20 mM Tris.HCl, pH 7.5 and protein was eluted in 50-70 mM NaCl in 20 mM Tris.HCl, pH 7.5 (Fig. 7.3 A,B) that showed the presence of a single band on 15% SDS PAGE. Initially, β domain of SK along with naturally present linker between SKα and β domain was fused with C-terminus of SAK to mimic the similar protein folding as in native SK (SAKβ).

7.4 Catalytic efficiency of preformed SAK fusion–Pm complex

To calculate the steady state kinetic constants of Pg activation by SAKβ, equimolar mixtures of SAK fusions (0.5 μM) and Pg (0.5 μM) 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 activator complex. These preformed activator complexes (5 nM) were mixed with different concentrations of Pg (0.5 to 5 μM) and 1.0 mM Chromozym PL and change in absorbance at 405 nm was measured as a function of time. The overall activation pattern obeyed Michaelis-Menton kinetics and kinetic constants are given in
Fig 7.3: SDS PAGE gel showing A) expression profile of β and SAKβ B) expression and purification of β and SAKβ.
Table 7.1. Catalytic efficiency of SAK was reduced upon fusion of SK domains as indicated by $K_{cat}$ values in Table 7.1.

<table>
<thead>
<tr>
<th>Bimolecular complex</th>
<th>$K_m$ (μM)</th>
<th>$K_{cat}$ (s$^{-1}$)</th>
<th>$K_{cat}/K_m$ (s$^{-1}$ μM$^{-1}$)</th>
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<tbody>
<tr>
<td>SAK-Pm</td>
<td>0.8 ± 0.09</td>
<td>0.44 ± 0.06</td>
<td>0.55</td>
</tr>
<tr>
<td>SAKβ-Pm</td>
<td>0.5 ± 0.08</td>
<td>0.25 ± 0.04</td>
<td>0.50</td>
</tr>
<tr>
<td>SAKLβ-Pm</td>
<td>0.48 ± 0.06</td>
<td>0.28 ± 0.03</td>
<td>0.58</td>
</tr>
<tr>
<td>SAKHLβ-Pm</td>
<td>0.52 ± 0.06</td>
<td>0.24 ± 0.02</td>
<td>0.46</td>
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Table 7.1: Kinetic constants for the Pg activation properties of equimolar complexes of SAK fusion proteins and Pm. The results are means±S.E.M. of three determinations.

Although naturally present linker between α and β domains is sufficient for independent folding of fusion protein domains, SAK fusion showed reduced activity. Therefore, an attempt was made to alter the linker region. A stretch of twenty residues (STSGG)$_5$ was introduced at the site of fusion by a series of PCR to minimize inter-domain interference during protein folding. SAK fusion proteins with increased linker length (SAKLβ) were purified and characterized for their Pg activation properties. In another attempt, a linker already reported for generated functional SAK fusion chimera at C-terminus (SAKKLβ) (Wu et al., 2003), was used and checked for Pg activation ability of chimera. None of these fusion chimeras was able to regain the full Pg activation activity as that of SAK.

Discussion

Bacterial Pg activators display three distinct types of domain organization i.e. single domain, two domain and three domain structure, where each domain carries out distinct function in Pg activation process. SAK is the only known, single domain Pg activator that can activate human Pg. Although SAK is a small protein, but it is capable of interacting with two molecules of Pg/Pm, one as an enzyme partner and the other as a substrate for its efficient catalysis. In contrast, other bacterial Pg activators e.g. SUPA and SK require the participation of β and βγ domains respectively for activating Pg. (Wang et al., 1998). Unlike SAK, SKα domain is not fully functional as a Pg activator.
and requires the integration of both \( \beta \) and \( \gamma \) domains to induce optimal Pg activation capability in full length SK molecule (Loy et al., 2001, Sundram et al., 2003). Structural elements required for SAK versus SK mechanism of Pg activation and contribution of domain interactions in bringing distinct functional differences between these two Pg activators are not clearly understood at present. Isolated \( \beta \) or \( \beta\gamma \) domain of SK, bring dramatic increase (up to 60%) in Pg activation potentiality of SAK, whereas, \( \gamma \) domain alone has no effect on SAK mediated Pg activation. Since \( \beta \) domain of SK carries high affinity binding site for Pg that does not overlap with SAK, it is possible that \( \beta \) domain may interact with the partner Pm along with SAK and restructure the activation complex to a conformation best suited for the Pg activation resulting in its enhanced catalytic activity.

The structural homology between SK\( \alpha \) domain and SAK provided an opportunity to determine the effect of SK\( \beta \) and \( \gamma \) domains on Pg activation by SAK. Increased Pg activation activity of SAK by the presence of SK\( \beta \) and \( \gamma \) domains prompted us to generate SAK fusion proteins with SK\( \beta \) or \( \gamma \) domains. Moreover, two domain Pg activator i.e. SUPA is fully capable of activating Pg which also suggests the possibility of generating a SAK chimera with enhanced Pg activation property. Surprisingly, in contrast to isolated domains of SK, fusion of \( \beta \) or \( \beta\gamma \) domain with SAK reduced its Pg activation potential suggesting that integration of SK domains generates some constrain in the fusion protein due to which SAK is not able to display its cofactor activity efficiently. Overall biochemical characterization of SAK fusion proteins showed that instead of showing enhanced activity, SAK even failed to fully retain its inherent Pg activation activity upon fusion of SK domains. Linkers of different length and composition were used for generating SAK chimeras but cofactor activity of SAK could not be enhanced.

It is reported that carboxy terminus fusion of SAK with hirudin (Szarka et al., 1999) and kringle 1 of Pg (Wu et al., 2003) showed no alteration in Pg activation property of SAK. In the present study we observed that although addition of individual purified SK domains facilitated Pg activation by SAK, but carboxy terminus fusion of SAK with SK domains caused severe reduction in Pg activation property of SAK. When functional properties of SK\( \beta \) domain were compared with hirudin and kringle 1, unlike SK\( \beta \) or \( \beta\gamma \) domains, neither hirudin nor kringle 1 interacts directly with Pg. SK\( \beta \) or \( \beta\gamma \)
domains make extensive contacts with enzyme and/or substrate Pg. Fusion of SAK with protein domains having high affinity for the Pg might interfere with correct positioning of SAK in the binary and/or ternary complex of SAK fusion protein with Pg and inhibit SAK from efficiently displaying its functional properties. Attempts are underway to engineer the linker region between SAK and SK domains in SAK fusion proteins to check whether it is possible to transfer the enhanced cofactor activity of SAK imparted by the presence of isolated SK domains to SAK fusion proteins.