

Role of the amino-terminal region of streptokinase in the generation of a fully functional plasminogen activator complex probed with synthetic peptides

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Abstract

The mechanism whereby fragments of streptokinase (SK) derived from its N terminus (e.g., SK1–59 or SK1–63) enhance the low plasminogen (PG)-activating ability of other fragments, namely SK64–386, SK60–414, SK60–387, and SK60–333 (reported previously), has been investigated using a synthetic peptide approach. The addition of either natural SK1–59, or chemically synthesized SK16–59, at saturation (about 500-fold molar excess) generated amidolytic and PG activation capabilities in equimolar mixtures of human plasminogen (HPG) and its complementary fragment (either SK60–414 or SK56–414, prepared by expression of truncated SK gene fragments in *Escherichia coli*) that were approximately 1.2- and 2.5-fold, respectively, of that generated by equimolar mixtures of native SK and HPG. Although in the absence of SK1–59 equimolar mixtures of SK56–414 and HPG could generate almost 80% of amidolytic activity, albeit slowly, less than 2% level of PG activation could be observed under the same conditions, indicating that the contribution of the N-terminal region lay mainly in imparting in SK56–414 an enhanced ability for PG activation. The ability of various synthetic peptides derived from the amino-terminal region (SK16–51, SK16–45, SK37–59, SK1–36, SK16–36, and SK37–51) to (1) complement equimolar mixtures of SK56–414 and HPG for the generation of amidolytic and PG activation functions, (2) inhibit the potentiation of SK56–414 and HPG by SK16–59, and (3) directly inhibit PG activation by the 1:1 SK-HPG activator complex was tested. Apart from SK16–59, SK16–51, and 16–45, the ability to rapidly generate amidolytic potential in HPG in the presence of SK56–414 survived even in the smaller SK-peptides, viz., SK37–59 and SK37–51. However, this ability was abolished upon specifically mutating the sequence -LTSRP-, present at position 42–46 in native SK. Although SK16–51 retained virtually complete ability for potentiation of PG activation in comparison to SK16–59 or SK1–59, this ability was reduced by approximately fourfold in the case of SK16–45, and completely abolished upon further truncation of the C-terminal residues to SK16–36 or SK1–36. Remarkably, however, these peptides not only displayed ability to bind PG, but also showed strong inhibition of PG activation by the native activator complex in the micromolar range of concentration; the observed inhibition, however, could be competitively relieved by increasing the concentration of substrate PG in the reaction, suggesting that this region in SK contains a site directed specifically toward interaction with substrate PG. This conclusion was substantiated by the observation that the potentiation of PG activating ability was found to be considerably reduced in a peptide (SK25–59) in which the sequence corresponding to this putative locus (residues 16–36) was truncated at the middle. On the other hand, fragments SK37–51 and SK37–59 did not show any inhibition of the PG activation by native activator complex. Taken together, these findings strongly support a model of SK action wherein the HPG binding site resident in the region 37–51 helps in anchoring the N-terminal domain to the strong intermolecular complex formed between HPG and the region 60–414. In contrast, the site located between residues 16 and 36 is qualitatively more similar to the previously reported PG interacting site (SK254–273) present in the core region of SK, in being involved in the relatively low-affinity enzyme–substrate interactions of the activator complex with PG during the catalytic cycle.

Keywords: amidolytic activity; fragment complementation; N-terminal region of streptokinase; plasminogen; plasminogen activation; streptokinase; substrate plasminogen

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Abbreviations: SK, *Streptococcus equisimilis* streptokinase; PG, plasminogen (either human or bovine); PN, plasmin (either human or bovine); HPG, human plasminogen; HPN, human plasmin; BPG, bovine plasminogen; BPN, bovine plasmin; UK, urokinase; TPA, tissue plasminogen activator; SK-HPG, equimolar complex of streptokinase and human plasminogen; SK-HPN, equimolar complex of streptokinase and human plasmin; SK56–414-HPG, equimolar complex of N-terminally truncated derivative of streptokinase and human plasminogen; NPGb, *p*-nitrophenyl *p*-guanidinobenzoate; G-P-L-pNA, tosyl-gly-pro-lys-*p*-nitroanilide; IPTG, isopropyl-1-thio- β -D-galactopyranoside; TFA, trifluoroacetic acid; RP-HPLC, reverse-phase HPLC; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin.

Streptokinase is a monomeric, multidomain protein of 47 kDa produced by several beta-hemolytic streptococci. Because of its potent PG activation property, SK is widely used as a thrombolytic drug for the treatment of several circulatory disorders, including myocardial infarction (ISIS-3, 1992). However, unlike other PG activators such as TPA and UK, a unique feature of SK action is that it has no proteolytic activity of its own, but forms a high-affinity equimolar complex with human plasminogen, which results in the exposure of the cryptic active site in HPG (reviewed by Castellino, 1981). As a consequence, the active site in HPG develops amidolytic and esterolytic potential even before the introduction of any proteolytic cleavage. This "virgin" enzyme complex is then converted rapidly to fully formed activator (either intact SK-HPG, as in the early phase after complex formation, or later, SK-HPN), which develops the capability to convert other "substrate" plasminogen molecules to plasmin by selective cleavage at the Arg₅₆₀-Val₅₆₁ peptide bond (Markus & Werkheiser, 1964; McClintock & Bell, 1971; Reddy & Markus, 1972). Thus, the most intriguing aspects of the interaction of SK with HPG/HPN are (1) the structural basis of the activation of the cryptic active site in the zymogen, and (2) the conversion of this "nascent" activity to a highly specific proteolytic activity directed toward the scissile peptide bond in substrate PG. These issues are dramatically illustrated by the observation that, although HPN alone cannot activate PG to PN, its 1:1 complexation with SK converts the active site in HPN (an enzyme, essentially with a trypsin-like specificity) to one displaying a highly specific substrate preference. Clearly, an understanding of the roles of various regions of SK in conferring these distinct biochemical functions into plasminogen/plasmin is important for the successful design of novel, second-generation SK-based thrombolytic drugs (Marder, 1993).

Although the exact interplay of different domains in SK during the various stages of PG activation are far from clear at this stage, several recent studies employing truncated fragments of SK have focused on the regions in this protein that participate in the primary event in SK action, namely its avid binding to HPG. A high-affinity HPG binding site has been shown to be associated with the central, "core" region of SK, comprising approximately 150–200 of the total 414 residues of the full-length polypeptide (Nihalani & Sahni, 1995; Reed et al., 1995; Rodriguez et al., 1995). Several partial-length fragments containing this core region, e.g., SK60–333, SK147–380, SK64–380, have been observed to possess only very low levels of intrinsic PG activation ability despite the presence of significant amounts of native-like structure in the isolated fragments (Shi et al., 1994; Parrado et al., 1996). Thus, the exact manner in which the core HPG binding property of SK is structurally translated into PG activation and/or PN modulating functions still remains largely obscure. Interestingly, recent studies have demonstrated that a fragment corresponding to the N-terminal 59 residues of SK could complement the low "PG activating activity" of polypeptide fragments derived from the remainder of the molecule, such as 60–387 and 64–380, to regenerate significant levels of the lost activator activity of the native protein (Shi et al., 1994; Young et al., 1995; Parrado et al., 1996). However, it was noted that a large molar excess of the N-terminal fragment over the complementary fragment was required for optimal potentiation, indicating that the affinity between the two complementing partners, even in the presence of HPG, was relatively low (Shi et al., 1994; Parrado et al., 1996).

Complementation between different fragments of a protein, leading to the regeneration of biologically active, native-like com-

plexes, has been demonstrated in selected systems in the past, most notably in the case of pancreatic ribonuclease and staphylococcal nuclease (reviewed in Anfinsen & Scheraga, 1975). The study of such "fragment complementing" systems provided fundamental insights regarding protein structural and functional aspects, particularly as it also allows the development of semisynthetic proteins where one of the complementing partners is a chemically prepared mutant (Chaiken, 1981; Offord, 1987). It is notable, however, that most of the well-studied systems are comprised of enzymes wherein the optimal spatial configuration of key catalytic residues resident in the two different complementing partners could be brought about by native-like cooperative interactions between the complementing fragments, usually at equimolar proportions, despite the presence of a discontinuity in the polypeptide backbone and/or overlap at the junctions of the fragments (Anfinsen & Scheraga, 1975; Blackburn & Moore, 1982). To draw a direct analogy with the case of SK, although no proteolytic activity is directly associated with the protein, the reconstitution of PG activating capability by two complementary fragments could conceivably result from a native-like re-orientation of two (or more) independent PG binding sites present in the fragments. This possibility appeared particularly alluring in view of our observations that a high-affinity HPG binding site exists in and around residues 230–290 of SK, and another, albeit with relatively lower affinity, is present in the region 37–51 of SK (Nihalani & Sahni, 1995; Nihalani et al., 1997). Keeping these considerations in mind, we sought to examine the nature of the contribution of the N-terminal region, particularly the HPG binding site present in the region 37–51, toward the generation of the fully functional activator complex. For this purpose, we prepared various truncated derivatives of the N-terminal region of SK through synthesis, and then examined their activation potential with HPG and a complementary fragment derived from the rest of the SK polypeptide. A similar approach, utilizing short synthetic peptides, has been used recently to map the main HPG binding site in SK (Nihalani et al., 1997). In the present investigation, we demonstrate a direct role of the N-terminal region in promoting substrate PG recognition by the activator complex.

Results

In order to define a suitable two-fragment complementing system for SK that could be readily amenable to analysis employing chemically synthesized N-terminal fragments or their variants, we first studied natural SK1–59 prepared by limited tryptic digestion of *Streptococcus equisimilis* SK (Nihalani et al., 1997) and its exact complementary fragment (SK60–414). The latter was prepared by expression of a truncated SK gene lacking the codons for the first 59 residues in *Escherichia coli* (see the Materials and methods for details). However, the level of the expression of SK60–414 was poor (less than 5% of soluble intracellular protein). Despite this, we could purify SK60–414 in small quantities using HPG-agarose affinity chromatography (Rodriguez et al., 1992), allowing us to confirm that SK1–59, when added in excess over its complementing partner, could successfully potentiate the low basal PG activating ability of SK60–414 (Fig. 1A), as reported earlier (Young et al., 1995). However, for undertaking detailed studies, we required much larger amounts of the complementing fragment. In our attempts to enhance the expression of SK60–414, it was observed that by merely extending four SK codons at the 5'-end of the open reading frame encoding SK60–414 (i.e., to obtain SK56–414), the level of expression of the polypeptide was increased

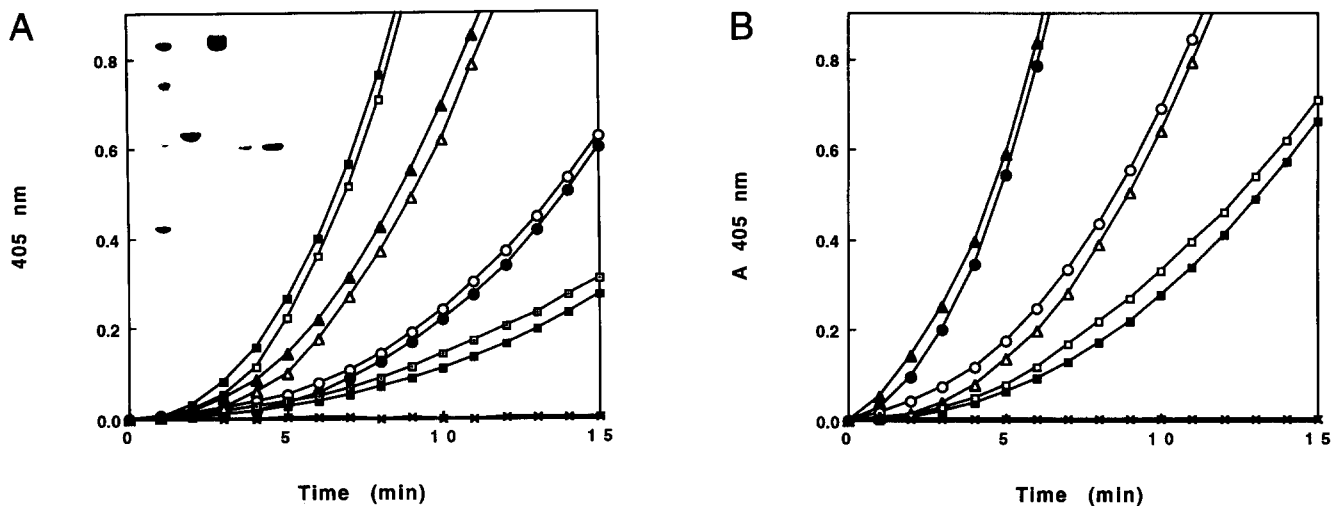


Fig. 1. A: HPG activating abilities of SK60-414 and SK56-414 in the absence and presence of SK1-59. The basal HPG-activating ability of recombinant SK-fragments (viz., SK56-414 or SK60-414), i.e., in the absence of SK1-59, was assayed by adding different concentrations (ranging from 0.1 to 0.5 μM) of these fragments to excess HPG (2 μM) in assay buffer containing chromogenic substrate in spectrophotometric cuvette as detailed in the Materials and methods. Activation of HPG to HPN was monitored at 405 nm. HPG activation in the presence of varying concentrations of SK1-59 (ranging from 0.1 to 2.5 μM) was also assayed similarly, by adding HPG (2 μM final concentration) to the mixture of SK1-59 with either SK60-414 or SK56-414 (25 nM final concentration) and then measuring the PG activation as described above. Typical progress curves obtained from two different concentrations of SK60-414 (100 nM, cross in square; 200 nM, closed circles) or SK56-414 (100 nM, dot in square; 200 nM, open circles) in the absence of SK1-59 are shown. Also are shown the curves obtained as a result of potentiation of the basal activities of fixed concentration (25 nM in each case) of either SK60-414 (open triangles and open squares using 0.5 and 1.0 μM of SK1-59, respectively) or SK56-414 (closed triangles and closed squares using 0.5 and 1.0 μM of SK1-59, respectively). Control reaction in which no SK-fragment was added is depicted with crosses. All the values represent means obtained from four independent experiments. Inset: SDS-PAGE gel of the reagents used in the experiments. From left to right: lane 1, standard molecular weight markers, top to bottom: phosphorylase b (97.4 kDa), BSA (66.2 kDa), ovalbumin (42.7 kDa), and trypsin inhibitor (21.5 kDa); lane 2, purified native SK; lane 3, purified human Glu-plasminogen; lane 4, purified SK60-414; lane 5, purified SK56-414. **B:** Potentiation of the PG activator activity of SK56-414 by SK1-59 and SK16-59. The assay was performed under similar conditions as described above, except that varying concentrations (ranging from 2.5 to 10 μM) of either SK1-59 or SK16-59 were added to fixed amounts of SK56-414 (0.1 μM). The typical curves obtained using 0.1 μM of SK56-414 and 2.5 μM of SK16-59 (closed squares), 2.5 μM SK1-59 (open squares), 5 μM SK16-59 (open circles), 5 μM SK1-59 (open triangles), 10 μM SK16-59 (closed circles), and 10 μM SK1-59 (closed triangles) are shown in the figure. Control reaction in which SK56-414 was omitted is depicted with crosses. All the curves are averages of at least four independent experiments (SD values, not depicted, were less than $\pm 5\%$).

dramatically, to approximately 30% of soluble intracellular protein, thereby considerably facilitating the preparation of this fragment in quantities suitable for our studies. Natural SK1-59 could successfully potentiate the PG activating property of SK56-414, despite the three-residue overlap between the two fragments, in a manner indistinguishable from that observed with SK60-414 (Fig. 1A). This result was not surprising, particularly because it has become clear from recent studies that the junction region between the two fragments probably exists in an inherently flexible state, a phenomenon that is reflected in its facile propensity to undergo selective proteolysis by different enzymes, and the ready isolation of fragments such as 1-59 and 1-63, all of which could complement core fragments such as 64-380, 60-387, 60-293, or 60-414 during PG activation (Shi et al., 1994; Nihalani & Sahni, 1995; Young et al., 1995; Parrado et al., 1996). Therefore, in all subsequent experiments, the complementation observed with this two-fragment system (viz., SK1-59 and SK56-414) was used to compare and contrast the effects of controlled deletions/mutations in the N-terminal region of SK.

The fragment SK56-414 was able to form an equimolar complex with HPG as observed by gel filtration (Fig. 2), which is in accord with the known presence of a high-affinity HPG binding site in the region 230-290 of SK (Nihalani et al., 1997). Likewise,

SK1-59, which also contains an independent HPG binding site, albeit of lower affinity (Nihalani & Sahni, 1995), could also be seen to associate with HPG by gel filtration (Fig. 2). However, the association of SK1-59 with either SK60-414 or SK56-414, if any, could not be detected by this technique at a 1:1 molar ratio (Fig. 2, profile f). From these results, it appears that a ternary complex forms between SK1-59, SK56-414, and HPG during the potentiation reaction.

Figure 3 shows the time-course of PG activation, as well as the amidolytic activity generated, by equimolar mixtures of SK or SK56-414 with HPG. In the case of native SK, both amidolytic and plasminogenolytic functions are generated rapidly, followed by a slow decline, presumably as a result of plasmin-mediated degradation. On the other hand, SK devoid of the N-terminal region is a poor PG activator when mixed with the zymogen. This fragment, however, is able to generate significant (75-80%) amidolytic activity in HPG, although slowly compared with full-length SK. It may be noted that the delay in the development of amidolytic activity in the mixture of SK56-414 and HPG was not due to the delay in the complex formation because the association between the two proteins could be visualized easily through gel-filtration after a brief preincubation of only 5 min (Fig. 2). Thus, the removal of the N-terminal region selectively affects PG acti-

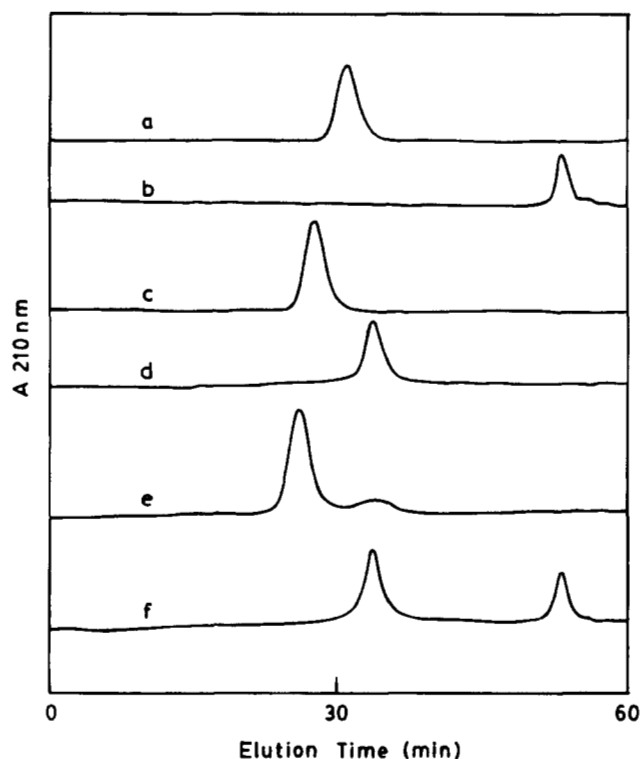


Fig. 2. Binding of HPG to SK1-59 and SK56-414 as demonstrated by size-exclusion HPLC. The chromatography was performed on a TSK G2000 SWXL (0.78 × 30 cm) column as described in the Materials and methods. Mixtures of HPG with either SK1-59 or SK56-414 and SK1-59 with SK56-414 were applied onto the column equilibrated in 0.1 M sodium phosphate buffer, pH 6.8, containing 10 mM 6-aminohexanoic acid. The column was run at 250 μ L/min and the absorbance of the eluant was monitored continuously at 210 nm as a function of time. Elution profiles a, b, c, d, e, and f show positions of free HPG, free SK1-59, mixture of HPG and SK1-59, free SK56-414, mixture of HPG and SK56-414, and mixture of SK1-59 and SK56-414, respectively.

vation, without a corresponding influence on the amidolytic capability of the molecule.

With the objective of defining possible cross-correlations between primary structural elements in the N-terminal region of SK and its PG activation and amidolytic potentiating properties, we required a facile system whereby we could generate several truncated versions of SK1-59, and then examine their ability to potentiate the basal PG activation and amidolytic capability of equimolar mixtures of SK56-414 and HPG. It is known that the first 15 residues of SK can be truncated without adversely affecting its PG activating properties (Malke et al., 1987) because these residues play a role in the secretion of this protein from the host cell (Pratap et al., 1996). This observation encouraged us to explore the synthetic peptide route, especially because moderate-length peptides can be routinely synthesized chemically with relatively high fidelity. This avenue became quite attractive when the synthetic peptide SK16-59 (lacking 15 residues from the N-terminal of SK1-59 observed to be nonessential in native SK) was found to be able to potentiate the HPG activating ability of SK56-414 essentially in a manner identical to that of SK1-59 (Fig. 1B). We then prepared several truncated peptides derived from the 16-59 region of SK, viz., SK16-51, SK16-45, and

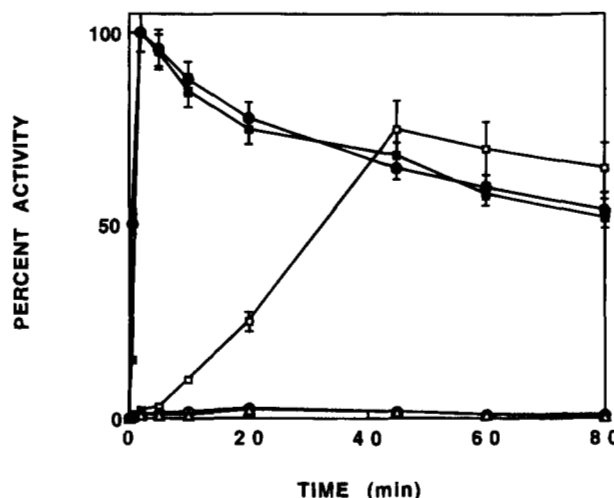


Fig. 3. Generation of amidolytic and activator activities in HPG upon complexation with SK or SK56-414. An equimolar mixture of SK or truncated SK-fragment SK56-414 with HPG (500 nM each) was prepared, and the amidolytic activity as well as the activator activity generated in the complex was assayed periodically. The increase in activator and amidolytic activities of SK-HPG complex as a function of preincubation are shown by closed circles and closed squares, respectively, and those of SK56-414-HPG are represented by open triangles and open squares, respectively. The activity generated by equimolar SK56-414 and HPG was compared with the maximum activity generated by the (SK-HPG) native activator complex (taken as 100%) to calculate the percent increase in activity. The control reaction, without SK or SK56-414, is depicted with open circles. Mean values from four independent experiments are shown. SDs are shown by vertical error bars.

SK16-36 with the objective of specifically examining the effect of truncation of SK16-59 in the region of its C terminus, where a proposed HPG binding sequence (LTSRPA) is present (position 42-46) (Gonzalez-Gronow et al., 1993; Nihalani & Sahni, 1995). We also synthesized the peptide SK37-59 derived from this region, as well as its shorter derivative (SK37-51), which has been shown previously to bind to HPG (Nihalani & Sahni, 1995), in order to closely examine the significance of this site in SK-HPG interaction.

The potentiation of the amidolytic and PG activator activities of SK56-414 by truncated N-terminal peptides are presented in Figures 4 and 5, respectively. These data reveal that the presence of saturating concentrations of either SK16-59 or SK16-51 (which display essentially identical potentiating properties) could reverse the loss of PG activator activity of SK brought about by the removal of the N-terminal domain. Surprisingly, the PG activator activity of SK56-414 could be raised twofold over that of native SK in the presence of a saturating level of SK16-59 (Fig. 5); the exact basis for this "superactivation" is presently unclear, but is probably not a phenomenon unique to the two-fragment system, because the addition of the N-terminal fragment to the native SK-HPG complex also resulted in the enhancement of the maximal activator activity attained (see Fig. 5). Because the addition of a similar excess of natural SK1-59 to native SK also caused "superactivation" (Fig. 5), it is clear that this phenomenon is not an offshoot of the use of synthetic peptides. In the case of amidolysis, where the effect of truncation of the N-terminal residues resulted not so much in a loss of activity (approximately 75% compared with SK) as a delayed attainment of full amidolytic potential (Fig. 3), the kinetics of the generation of amidolytic function was

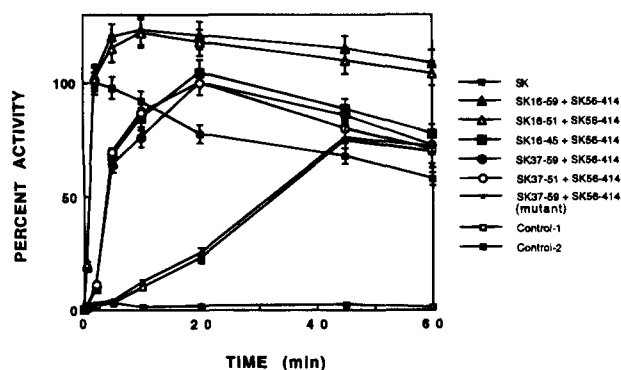


Fig. 4. Enhancement of the amidolytic activity of equimolar SK56-414 and HPG by N-terminal SK-peptides. Various concentrations of each SK N-terminal peptides (ranging from 0 to 500 μM) were added to SK56-414 (500 nM) and equimolar amounts of HPG. Amidolytic activity generated over time in SK56-414-HPG complex was then followed spectrophotometrically. Amidolytic activity generated (using saturating concentration of peptide that showed maximum increase in the activity) was compared with that generated in equimolar HPG by native SK (closed small squares; indicated by "SK" in the legend), taken as 100%, to calculate the percent activity in each case. Various SK-peptides used in the reaction, as indicated in the legend, were: SK16-59 (100 μM), closed triangles; SK16-51 (100 μM), small open triangles; SK16-45 (300 μM), closed large squares; SK37-59 (300 μM), closed circles; and SK37-51 (350 μM), open circles. Control-1, where no SK-peptide was added, is shown as open squares, whereas control-2, in which neither SK-peptide nor SK56-414 was added, is depicted with dot in square. Amidolytic activity of HPG in the presence of SK56-414, and a mutant peptide derived from the SK37-59 fragment (see text for details) is shown as dots. All the values are averages of four independent measurements.

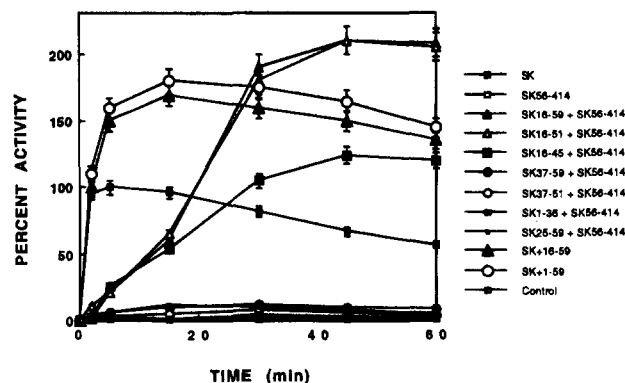


Fig. 5. Potentiation of HPG activator activity of equimolar SK56-414 and HPG by N-terminal SK-peptides. The increase in HPG activator activity of SK56-414-HPG complex with time was measured by adding different N-terminal SK-peptides (saturating concentrations of each peptide was used) to SK56-414 (500 nM) and equimolar amounts of HPG and monitoring the activation reaction spectrophotometrically. The percent activity in each case is shown relative to the maximum activator activity generated in equimolar HPG by native SK (depicted with closed squares; indicated by "SK" in the legend), taken as 100%. The various N-terminal SK-peptides, as indicated in the legend, added to the potentiation reaction containing equimolar HPG and SK56-414, were: SK16-59 (100 μM), closed triangles; SK16-51 (100 μM), small open triangles; SK16-45 (300 μM), closed large squares; SK37-59 (300 μM), closed circles; SK37-51 (350 μM), small open circles; and SK1-36 (500 μM), cross in square. Control reaction in which SK56-414 was excluded is represented by dots in squares. Enhancement in the activator activity of the SK-HPG complex by 5 μM of either SK16-59 (large closed triangles) or SK1-59 (large open circles) is also shown. The HPG activator activity of SK56-414 in the presence of SK-peptide SK25-59 (in which the region SK16-36 was truncated) is represented by dots. Data shown are the means and SDs of quadruplicate determinations.

brought about to virtually the same level as that shown by full-length SK. However, in the presence of saturating concentrations of either SK16-59 or 16-51, the peak activity attained was approximately 20% above that of native SK. A kinetic analysis revealed that this could be ascribed to an enhanced rate of catalysis (Table 1) without a concomitant change in the apparent affinity for the amidolytic substrate (the K_m of SK56-414 in the absence and presence of SK16-59 was essentially unchanged). On the other hand, as shown in Table 2, the intrinsically low catalytic efficiency (k_{plg}/K_{plg}) of SK56-414 for PG activation was enhanced by the presence of the complementing fragment by nearly 1,400-fold, but only about 3-fold in terms of amidolytic functioning (cf. Table 1). The increase in PG activation rates due to the presence of saturating concentrations of SK16-59 was reflected both in terms of a decreased K_m for HPG as well as increased V_{max} for catalysis of HPG to HPN. From these data, it can be concluded that the

Table 1. Steady-state kinetics parameters for the amidolytic activity of HPG with SK and SK-fragments

SK/SK-fragment	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \text{mM}^{-1}$)
SK	0.61 \pm 0.05	374 \pm 10	613
SK56-414	0.92 \pm 0.08	173 \pm 12	188
SK56-414 + SK16-59	0.55 \pm 0.05	374 \pm 18	680

N-terminal region of SK (residues 16-59) plays a major role in augmenting the low PG activator activity associated with the rest of the molecule.

The results of the experiments with the PG activator and amidolysis enhancing potential of the truncated derivatives of SK16-59 showed that shortening of SK16-59 to SK16-51 had virtually no effect on either amidolytic or PG activating capabilities of the N-terminal fragment. However, further deletion at the C-terminal end, to obtain SK16-45, resulted in a very perceptible alteration in both of these properties. The kinetics of amidolysis, as well as PG activation, were considerably slower with SK16-45; in quantitative terms, 60-70% reduction in the potentiation of amidolytic and

Table 2. Steady-state kinetics parameters for the activation of HPG by SK and SK-fragments

Activator	K_{plg} (μM)	k_{plg} (min^{-1})	k_{plg}/K_{plg} ($\text{min}^{-1} \mu\text{M}^{-1}$)
SK-HPG	0.23 \pm 0.01	4.40 \pm 0.20	19.0
SK56-414-HPG	1.00 \pm 0.05	0.05 \pm 0.005	0.05
+ SK16-59	0.15 \pm 0.01	7.00 \pm 0.30	46.7
SK-HPG+SK16-59	0.11 \pm 0.01	4.56 \pm 0.22	41.5

PG activator activities at saturation, in comparison to the full-length fragment, was observed. The effect of deletion of these particular residues was also reflected in the HPG binding ability of these N-terminal SK-peptides. This binding was performed by specific assays, viz., dot-blot and ELISA (Fig. 6A,B). Although the dot blots served as a qualitative screening procedure, the relative affinities of the different peptides with HPG were determined by ELISA. In the case of SK16-59 and SK16-51, their binding with HPG was found to be almost similar ($K_d = 3.8 \pm 0.5 \times 10^{-6}$) and thus comparable to that of SK1-59, reported previously through analyses of the ELISA binding isotherms (Nihalani & Sahni, 1995). However, the truncation of SK16-59 to SK16-45 resulted in approximately 10-fold reduction in HPG binding of this fragment ($K_d = 5.0 \pm 0.2 \times 10^{-5}$).

Upon complete truncation of the HPG binding region, as in the fragment 16-36, the ability to potentiate PG activation in SK56-414 was completely lost [no detectable increase was observed even at a concentration of 500 μ M, compared to the control, viz., equimolar (500 nM) mix of SK56-414 and HPG]. This fragment was also inactive with respect to enhancement in the kinetics/extent of potentiation of amidolysis by SK56-414. However, the HPG binding curves (Fig. 6A,B) showed that SK16-36 possessed HPG binding ability ($K_d = 2.5 \pm 0.5 \times 10^{-5}$) comparable to that displayed by SK37-51 (Nihalani & Sahni, 1995). In order to test whether the

observed loss in potentiating ability in SK16-36 was due to conformational destabilization brought about by reduction to a very short length rather than an actual truncation of an epitope per se, we synthesized SK1-36, with an extended length at the N-terminal side. However, this fragment also could not potentiate amidolysis or PG activation by SK56-414. Derivatives of SK16-59 with truncation at the N-terminal end, but with the C-terminal sequences preserved, viz., SK37-59 and SK37-51, also could not enhance the PG activation by SK56-414 beyond the basal 3-4% levels compared to that of native SK. By contrast, these fragments were efficient in promoting nearly full potentiation of amidolysis by SK56-414 (Fig. 4), although at relatively higher concentrations compared with SK16-59 or SK16-51. Moreover, in the presence of the saturating concentrations of these peptides (i.e., SK37-59 and SK37-51), the generation of full amidolytic activity in SK56-414 was rapid (i.e., 10-20 min) compared with SK56-414 alone, which was able to generate 75% amidolytic activity in HPG after 45 min of preincubation (Fig. 4). The role of this segment (residues SK37-59) in enhancing amidolysis was then tested by examining a mutant peptide (SK37-59), altered specifically in primary structure at a proposed HPG interacting site (Gonzalez-Gronow et al., 1993; Nihalani & Sahni, 1995) by altering the sequence LTSRP, at position 42-46, to AAAAA. This peptide displayed no amidolytic enhancing ability (Fig. 4), demonstrating the involvement of this

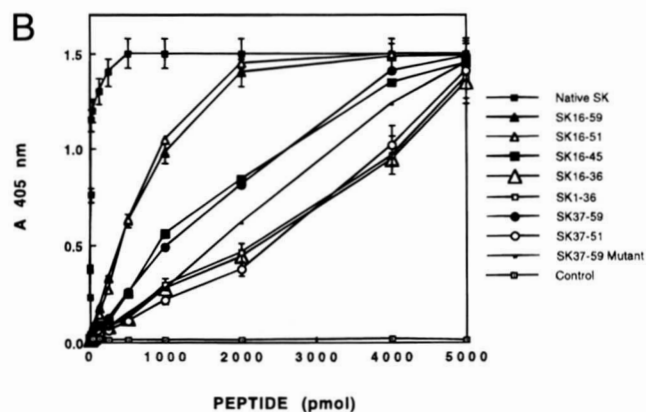
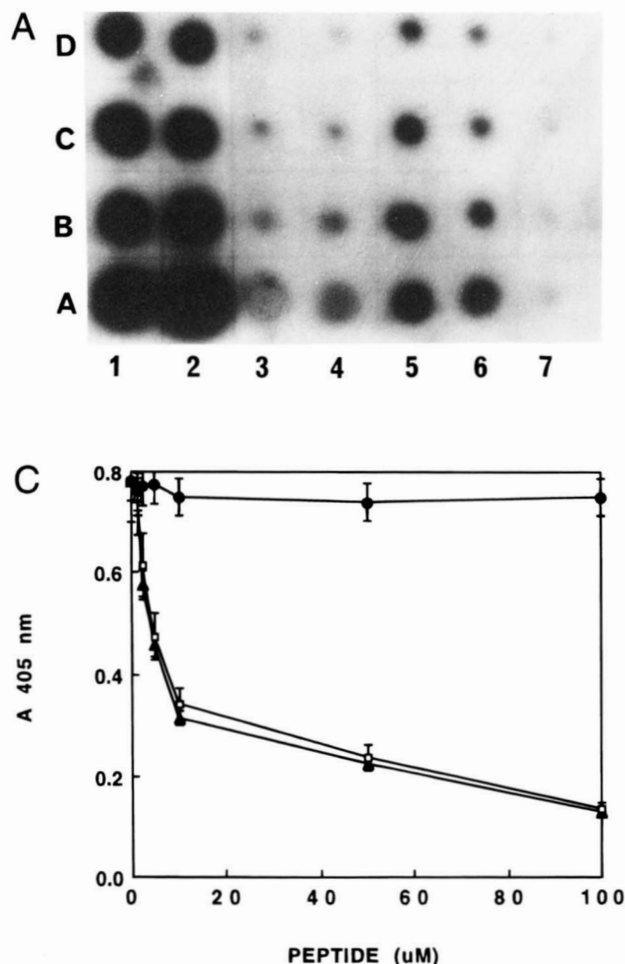


Fig. 6. HPG binding of SK or SK-peptides as determined by dot blot and ELISA. **A:** Dot blot showing binding of different amounts of each N-terminal SK-peptides (20 μ g to 2.5 μ g) to 125 I-labeled HPG. Positions 1-6 correspond to SK-peptides, viz., SK16-59, SK16-51, SK16-45, SK16-36, SK37-59, and SK37-51, respectively, whereas the position marked 7 represents a control peptide derived from the primary structure of SK16-36, but in which the sequence was randomly scrambled. Positions A, B, C, and D correspond to 20, 10, 5, and 2.5 μ g of each peptide blotted onto nitrocellulose by serial dilution. **B:** Quantitative ELISA for HPG binding. The figure shows binding of SK (closed squares), SK16-59 (closed triangles), SK16-51 (small open triangles), SK16-45 (closed large squares), SK16-36 (large open triangles), SK1-36 (small open squares), SK37-59 (closed circles), SK37-59 mutant peptide (dots), and SK37-51 (open circles) with HPG as determined by ELISA using polyclonal antisera specific for HPG. Control reaction where SK or SK-peptide was omitted is shown as dot in square. Data is representative of four independent experiments. **C:** Competition of SK1-36 peptide with either SK37-59 or SK254-273 for HPG binding. SK1-36 (100 μ M per well) was covalently immobilized onto ELISA plate. The ability of varying amounts (0-100 μ M) of either SK254-273 (closed circles) or SK16-59 (closed triangles) to inhibit the binding of immobilized SK1-36 to HPG was then tested. The competition of SK16-59 with SK37-59 (open squares) for HPG binding is also shown. Three independent experiments gave the same result and the typical one is shown here.

region in the generation of amidolysis in HPG by SK. Thus, two distinct epitopes/structural units in the N-terminal region seem to be responsible for the potentiation of the activity of the central domain of SK mediated by the N-terminal domain. The epitope assisting the potentiation of amidolysis essentially could be isolated to a small segment (SK37–51). The potentiation of PG activation by SK16–59, however, required both this C-terminal segment as well as sequences contained in the region 16–36, because peptides devoid of this region were also, on their own, unable to potentiate the PG activation effectively. When the peptides representing these two regions, i.e., SK16–36 and SK37–59 (in equimolar amounts), were added simultaneously to equimolar mixtures of HPG and SK56–414, no further enhancement in HPG activation (beyond a basal 4% relative to SK-HPG) was observed even at very high molar excess of SK1–36 and SK37–59, indicating that the continuity in the peptide backbone is also required between the two segments to bring about fruitful complementation with SK56–414 (data not shown).

Although the fragment SK16–36 was inactive in the promotion of PG activation by the complementary fragment, it was of interest to examine if it could inhibit the potentiation displayed by the fragments SK16–59 or SK16–51 (both with relatively high PG activating abilities). Results of potentiation–inhibition experiments performed with these and other truncated fragments derived from the segment 16–59 of SK indicated that only SK16–36 (as also SK1–36) was highly efficient in showing this inhibition (the inhibitory concentration of peptide causing 50% inhibition, i.e., $IC_{50} = 13 \pm 1 \mu\text{M}$), with marginal inhibition also shown by SK16–45 (data not shown). Remarkably, both of these peptides could also effectively inhibit the PG activation by the SK-HPG activator complex ($IC_{50} = 15 \pm 2 \mu\text{M}$ for SK16–36 and $25 \pm 2 \mu\text{M}$ for SK16–45) (Fig. 7). A kinetic analysis of this inhibition indicated that it arose specifically from a fourfold alteration of the K_m of the SK-HPG activator complex for substrate PG (raised from $0.2 \mu\text{M}$ to $0.8 \mu\text{M}$) in the presence of inhibitory concentrations ($20 \mu\text{M}$) of SK16–36 and SK16–45 ($50 \mu\text{M}$) (Fig. 7, inset). As a corollary to this observation, the inhibition by a fixed concentration of either SK16–36 or SK16–45 could be smoothly reversed by simply increasing the substrate PG in the reaction (data not shown). These results strongly pointed to the presence of an epitope/conformation in the region 16–36 of the activator complex that interacted specifically with molecules of substrate PG. The importance of this putative region in substrate PG activation was further established by synthesizing a peptide, SK25–59, which lacks the first nine residues of SK16–36. The ability of this peptide to potentiate the activator activity of SK56–414-HPG complex was reduced to barely 5–10% compared with the SK-HPG complex (Fig. 5). However, as expected from the presence of the region 37–59 in this peptide, the amidolysis enhancing potential was fully preserved in this peptide (data not shown).

Recently, we have reported the presence of a region in the high-affinity core PG binding site of SK (identified as residues 234–293 by Peptide Walking) that displays specific binding to substrate PG molecules (Nihalani et al., 1997). A synthetic peptide based on this sequence (viz., SK254–273) was found, in a manner similar to that observed here with SK1–36/SK16–36, to competitively inhibit PG activation by SK-HPG, and also to increase the K_m of the activator complex for PG (Nihalani et al., 1997). In order to examine if this substrate PG-binding epitope present in the N-terminal region of SK displayed antagonism/additivity/synergy with the substrate PG-binding site present in the core region of the protein, we performed

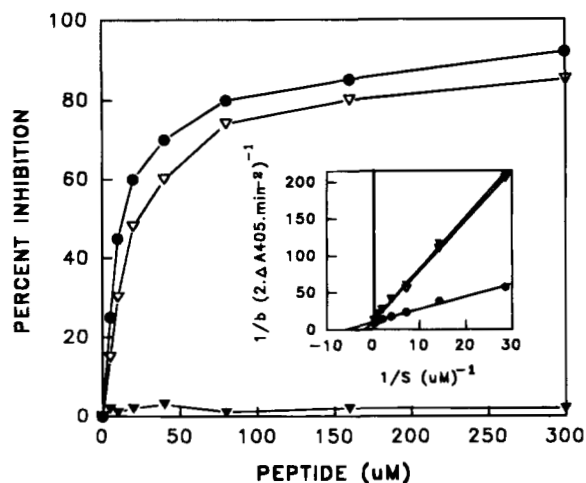


Fig. 7. Inhibition by N-terminal SK-peptides of substrate PG activation by SK-HPG activator complex. Preformed equimolar SK-HPG complex (10 nM) was added to HPG ($1 \mu\text{M}$) in the assay buffer containing various concentrations of N-terminal SK-peptides ($0\text{--}300 \mu\text{M}$). HPG activation was measured spectrophotometrically. Percent inhibition due to either SK16–36 (closed circles) or SK16–45 (open triangles) was calculated from the rates of plasmin generated in the presence of peptides compared with that in the activation reaction (taken as 100%) where no peptide was added. The control reaction where no SK was added is depicted with closed triangles. Inset: Lineweaver–Burke plot for the determination of kinetic parameters for HPG activation by SK-HPG complex in the presence of N-terminal SK-peptides. The experiment was performed under identical conditions as mentioned above, except that the concentration of HPG in the reaction was varied from 0.035 to $3.0 \mu\text{M}$. The control reaction, in which no peptide was added, is depicted with closed circles, whereas reactions where either SK16–36 or SK16–45 were included are shown as closed triangles and open triangles, respectively. Qualitatively similar results were obtained when BPG instead of HPG was used as a substrate. Data are representative of four independent experiments performed in duplicates.

inhibition experiments using mixtures of the two peptides, which, when used individually, brought about a certain defined level of inhibition (25–50%) in PG activation by SK-HPG. The results (Table 3) clearly showed that the inhibitory influence of both these peptides was additive. To further examine if these two sites bound with HPG independently of each other, competitive ELISA was performed. The results presented in Figure 6C show that, unlike SK-peptide SK16–59, SK254–273 was unable to compete with SK16–36 for HPG binding. This indicated that the binding of the region 16–36 of SK with HPG occurred at a site distinct from that of the region 254–273, present at the core of the high-affinity HPG binding site.

Discussion

One of the principal conclusions that emerges from our fragment complementation studies is that the amino-terminal fragment-mediated potentiation of PG activation by SK56–414 and HPG is affected in a much more dramatic manner than that of the potentiation of amidolytic capability. The potentiation of PG activation by SK56–414 is almost completely dependent on the presence of the N-terminal fragment (either SK1–59, SK16–59, or SK16–51). It may be noted that a very large molar excess of the N-terminal SK-fragment was required to generate full HPG activating/amidolytic ability at saturation. This result, although surprising at

Table 3. Inhibition of activator activity of SK-HPG activator complex by combination of SK16–36 and SK254–273 SK-peptides

Percent inhibition ^a by individual SK-peptides ^b		Percent inhibition ^c upon combination of both the SK-peptides
SK16–36	SK254–273	
22	15	40 ± 2
15	35	45 ± 2
40	15	50 ± 1
30	40	65 ± 3

^aDifferent concentrations of the peptides that showed the desired inhibition of substrate PG activation individually were added simultaneously in the activator assay to obtain the additive effect. Percent inhibition in each case was calculated by comparison with the control (taken as 100%) where no peptide was added.

^bIn the negative control reaction, an HPG binding SK-peptide, SK234–253, but which did not show any significant inhibition of substrate PG activation on its own, was chosen (100 μM final concentration). When this peptide was added to either SK16–36 or SK254–273 in the inhibition assays, the inhibitory effect of only SK16–36 or SK254–273 could be observed and no additive effect could be detected.

^cData represent the mean of four independent experiments.

first sight, has also been noted previously (Shi et al., 1994; Parrado et al., 1996). A plausible explanation for this was forwarded by Parrado et al. (1996), based upon their CD and NMR studies of the isolated domains of SK. They suggested that the N-terminal region of SK, when isolated in solution, remains largely in an unstructured conformation. Therefore, we speculate that the majority of the unfolded molecules are unable to associate with the remainder of the SK molecule, whereas only a minority population with a native-like conformation can do so. Thus, in such a situation, a large increase in the overall concentration in the N-terminal fragment would be required to “push” the equilibrium more toward complex formation. This supports our finding that, at a 1:1 molar ratio, the two complementary fragments are unable to associate with each other, whereas, at a fivefold molar excess of the N-terminal fragment, some association could be detected by gel filtration (Conejero-Lara et al., 1996). The fact that several hundred-fold molar excess of the N-terminal fragment is required to obtain a saturating level of PG activating ability even in the potentiation reactions suggests that the inherently weak affinity between the two isolated fragments is not substantially overcome by the presence of HPG, although each of the two complementary fragments possesses independent HPG binding ability.

In contrast to the PG activating ability, the ability to generate nearly 75% of amidolytic capability in HPG, although slower compared with native SK, already exists in SK56–414, despite the truncation of the N-terminal region. Remarkably, the ability to potentiate the amidolytic activity was found to be fully preserved even in the small fragments corresponding to the C-terminal region of SK16–59 (viz., SK37–59 and SK37–51). In contrast, the ability to potentiate the PG activator activity in these fragments (SK37–59 and SK37–51) was very low. Thus, the principal role of the N-terminal region of SK in terms of generating amidolytic potential in the activator complex seemed to be localized in and around residues 37–51 of the polypeptide. Interestingly, a stretch of amino acid sequence present in the segment 37–51 (LTSRPAH), has also

been shown to be present in the PG binding domain of human fibronectin (Gonzalez-Gronow et al., 1993). A Ser residue in this sequence undergoes ATP-mediated phosphorylation through a reaction autocatalyzed by the SK-HPN complex; as a result, the amidolytic activity of the activator complex is decreased significantly (Serrano et al., 1996), strongly indicating the contributory role of this PG binding region in the catalytic functioning of the molecule. Our experiments show that truncation in the 37–51 region in the N-terminal fragment (to obtain SK16–45 or SK16–36) drastically reduces the ability of this region to potentiate PG activation in SK56–414. Thus, our findings that the mutation of the “target” sequence in SK37–59 (LTSRP) to Ala residues (as well as the earlier report that the phosphorylation of Ser-44 in native SK) virtually completely abolished the ability of this region to bring about amidolytic potentiation of SK56–414 and HPG is rationally consistent with the conclusion that the HPG binding site between residues 37 and 51 of SK is important for the functioning of the molecule, particularly in the rate of generation of amidolytic capability by the activator complex.

In contrast to the fragments SK16–59 and SK16–51, fragments SK1–36 or SK16–36 could not themselves bring about any potentiation either of PG activation or amidolysis, showing that, for PG activation as well, the presence of the sequence in and around residues 37–51 of SK is important. It is particularly revealing that neither SK16–36, SK1–36, SK37–59, or SK37–51 individually, nor mixtures of SK1–36 and SK37–59, could effectively potentiate the basal PG activator activity of SK56–414. Nevertheless, SK1–36 or SK16–36 were able to efficiently inhibit PG activation by the native activator complex (SK-HPG) in the micromolar range of concentration. This suggests that an “epitope” existed in the region 16–36 of the N terminus of SK that might be participating in conferring substrate specificity to the activator. The fact that potentiation of PG activation was significantly reduced in SK16–45, in which partial truncation of the SK37–59 HPG binding site had occurred, and completely abolished in SK16–36, points strongly toward the requirement for the simultaneous presence of both sites in one contiguous peptide unit for optimal complementation by the N-terminal region. Indeed, this conclusion is validated by the observation that no potentiation of PG activation could be seen with SK25–59 also (a fragment in which the putative PG interacting site between residues 16 and 36 would have been truncated) but which, nevertheless, retained complete amidolytic potentiating function. Clearly, the presence of a discontinuity between these two epitopes (16–36 and 37–51) would immediately imperil the functioning of the substrate activation reaction because the “substrate gathering” epitope would lose the necessary anchoring with the rest of the activator complex (a property presumably provided by SK37–51). Thus, a peptide such as SK1–36, bearing only the substrate PG-specific function, when added in excess into PG activation reactions catalyzed by SK-HPG, strongly inhibits the reaction, probably by binding with substrate PG and competing with the corresponding epitope in the N-terminal portion of the SK moiety of the activator for binding with substrate, a scenario entirely consistent with our observation that the K_{pg} of SK-HPG is raised severalfold in the presence of SK1–36. On the other hand, the inclusion of SK1–59 or SK16–59 in excess to PG activation reactions catalyzed by SK-HPG results in an apparent “superactivation” of the latter. We propose that SK1–59, which possesses both the “anchoring” and substrate binding functions, provides a transient “bridge” between the activator complex and substrate PG and, hence, enhances the overall rate of PG activation (this scenario presupposes

that a dynamic exchange is possible between the N-terminal region of the native complex and the externally added SK1–59). Although entirely speculative at present, the fact that the K_{plg} values of both native SK-HPG and SK56–414-HPG actually decrease by nearly twofold in the presence of SK16–59 (Table 2) lends support to this possibility. However, additional factors, such as stabilization of the activator complex by the N-terminal region (as proposed by Conejero-Lara et al., 1996; Parrado et al., 1996), or its ability to compensate for the loss of this region from the activator complex and the consequent loss of activity (Siefing & Castellino, 1976) may also play a role in the twofold superactivation phenomenon, particularly if the presence of an excess of the externally added fragment allows, at saturation, a level of PG activator activity to be attained that would not be possible in even the native SK-HPG complex because of the degradation of this region.

It is quite intriguing that at least three clearly demarcated loci in SK are involved in the interaction of this protein with HPG (see Fig. 8). Although the locus present in SK16–37 has been detected for the first time, the PG binding properties of the regions 37–51 and 234–293 of SK have been shown earlier (Nihalani & Sahni 1995; Nihalani et al., 1997). Although the function associated with the region 37–51 is only now becoming clear, the high-affinity HPG binding site present in residues 234–293 recently has been shown to constitute a principal intermolecular contact region between SK and HPG during the formation of the 1:1 SK-HPG activator complex (Nihalani et al., 1997). Remarkably, this region appears to bind in the close vicinity of the active site in HPG/HPN, and contains a site (residues 254–273) that, like the one present in the region 16–36, binds selectively with substrate PG. However, the results obtained in the present study through the competitive ELISA experiments demonstrate that peptides SK16–36 and SK254–273 do not compete with each other for HPG binding, indicating that each is directed at its cognate site in substrate PG that is sterically distinct from the other. It therefore appears quite likely that each of these sites participates independently of the other in interacting with substrate PG. The observations reported earlier, that specific alterations in these two regions, viz., a change of Gly-24 to a bulkier residue, such as Glu or His (Lee et al., 1989), or the modification of critical lysine residues (viz., Lys-256, 257 to alanines) (Lin et al., 1996) substantially reduced the PG activation properties of SK, strongly lend support to our finding that both binding sites are intimately involved in substrate recognition. Such a two-point attachment, indeed, could be the reason why the peptides bearing these two substrate PG-specific loci, viz., SK16–36

and SK254–273, show an additive influence in their inhibition of the PG activation by pre-formed activator complex.

The results obtained in the present study, together with those of studies in the recent past (Shi et al., 1994; Nihalani & Sahni, 1995; Parrado et al., 1996; Serrano et al., 1996; Nihalani et al., 1997), now permit us to tentatively propose the following model of SK action, at least in terms of the principal mechanistic events involved. Upon complex formation, the N terminus of SK, which is covalently attached to the main chain, becomes anchored to the rest of the SK-HPG complex through the PG binding site present in the region 37–51. All of these interactions, together with the high-affinity intermolecular interaction of HPG and SK mediated by the core binding region (i.e., in and around residues 234–293) of the latter, as well as the resultant conformational changes in the zymogen, cause the exposure of the active site in the complexed HPG. In the native SK-HPG complex, this rapidly results in the activation of the HPG to HPN. However, the transformation of SK-HPG to SK-HPN, by itself, does not confer PG activation ability onto the activator complex, but requires additional structural inputs to engender the characteristic substrate preference associated with the fully functional activator. Our studies show that this is conferred by the concerted action of the two substrate PG-specific sites resident in the sequences 16–36 and 254–273, both probably acting cooperatively to sequester substrate (PG) selectively at the active site during the catalytic cycle. Thus, it is probably this interplay of the three PG binding loci of SK, rather than a direct change of the enzymatic specificity by interference at the secondary binding subsites of the active site of the complexed plasmin/plasminogen, that brings about the remarkable alteration in substrate preference consequent to SK binding. Of course, this description does not necessarily discount the involvement of other, as yet unidentified, contacts between SK and HPG, and/or between SK-HPG/HPN and substrate PG, that might also play structural roles in the functioning of the activator complex. Virtually all of the recent studies on the mechanism of SK action have focused on identifying primarily the sites in SK that are responsible, first, for the formation of the strong 1:1 equimolar complex with HPG, followed by the highly specific but transient interaction between the activator complex and substrate PG during which the scissile peptide bond in the zymogen is cleaved. However, the relatively more subtle, but nevertheless important questions regarding the dynamics/structural processes responsible for the sequential release of the product (plasmin) at the end of each catalytic cycle deserves close scrutiny as well. These and several other funda-

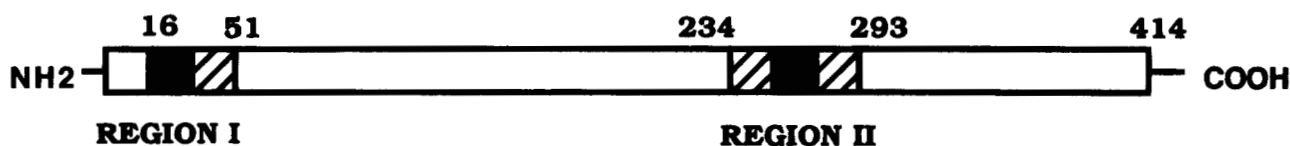


Fig. 8. Schematic representation of the different HPG-interacting sites in SK. The two regions, I and II (spanning residues 16–51 and 234–293), involved in the interaction of SK with PG, are shown. Region I (residues 16–51) has a bifunctional character. The locus resident between SK37–51 (shaded) is important for PG activation because it likely tethers the N-terminal portion of SK with the SK-HPG complex even in the absence of a continuity between peptide bond 59–60; the locus SK16–37 contains an epitope that possesses specificity for substrate PG. Region II (shaded areas) is primarily involved in the formation of the high-affinity equimolar complex with HPG; it encompasses a smaller locus (residues 254–273) that, like the one present in the N-terminal region (SK16–37), imparts additional substrate specificity to the complex. The cooperative action of both the substrate PG specific loci (depicted as dark areas in Regions I and II) in sequestering substrate PG to the active site probably results in the high PG activation rates associated with the native activator complex.

mental issues can now be addressed more precisely by undertaking detailed mutational analyses of the regions in SK identified by physico-chemical approaches to play major roles in its functioning. Investigations along these lines are currently in progress.

Materials and methods

Reagents

Highly pure streptokinase from *S. equisimilis* H46 A was a kind gift from Dr. R.A.G. Smith (Smith-Kline Beecham, U.K.). It displayed a specific activity of 120,000 I.U./mg protein measured by the procedure of Jackson et al. (1981) using chromogenic peptide substrate; protein was determined according to Bradford (1976). The preparation showed a single band on SDS-PAGE (see Fig. 1A, inset). The human Glu-plasminogen used in our kinetics experiments was procured from Boehringer-Mannheim (Germany). Assays using synthetic peptide substrate showed all batches to contain less than 0.01% of free plasmin, which was in accordance with the supplier's specifications. The number of active sites generated in HPG (used for the enzymatic assays) was determined by active-site titration using the acylating agent, *p*-nitrophenyl guanidobenzoate as described for SK by McClintock and Bell (1971). In experiments where stabilizer-free HPG was needed, as in the gel filtration and dot blot experiments, we purified the HPG from fresh citrated human plasma collected in the presence of protease inhibitors by the standard procedure of Deutsch and Mertz (1970), which consistently gave plasminogen with less than 0.02% of plasmin. The chromogenic plasmin substrate and BPG were procured from Sigma Chemical Co. (St. Louis, Missouri). Thermostable DNA polymerase (pfu) for PCR was obtained from Stratagene Inc. (Germany). All other reagents used were of the highest purity available. The expression vector T7 pET-23 (d) was obtained from Novagen, Inc. (Wisconsin). The N-terminal SK-fragment, SK1-59, was prepared by limited proteolysis of SK by trypsin as described (Nihalani & Sahni, 1995). The synthetic N-terminal SK-peptides were custom synthesized by Chiron Mimotopes, Australia. Crude synthetic peptides were purified by RP-HPLC on preparative C-18 Vydac columns (250 mm × 10 mm) using relatively shallow acetonitrile gradients in 0.1% TFA. Their purities (ranging from 70 to 85%) were established through analytical microbore RP-HPLC and ion-spray mass spectroscopy. Quantitation of the peptides was performed by amino acid analysis after PITC derivatization (Findlay & Geisow, 1989).

Construction and purification of truncated SK derivatives

The complete structural gene of streptokinase from *S. equisimilis* H 46 A (as a 2.5-kb *Pst* I fragment), along with its flanking regulatory regions, was first cloned into plasmid vector pBR322 in *E. coli*, essentially according to the procedure of Malke and Ferretti (1984) (see also Pratap et al., 1996 for details regarding construction of SK-containing vectors). The SK gene (as a *Nco* I-*Bam*H I fragment) was then transferred from the vector, pJKD-57, to pET-23d, the T7 promoter-based expression vector (Studier, 1991) and also to pBluescript II KS⁻ (Stratagene Inc.) at the single *Eco*R V site after T4 DNA-polymerase catalyzed fill-in of the insert using standard methodologies (Sambrook et al., 1989). The latter construct (pBluescript containing the SK gene) was used as the template for the generation of truncated SK genes by PCR,

whereas the SK-containing expression vector, pET-23d-SK, was used for the cloning of the PCR products and expression of N-terminally truncated SK polypeptides, viz., SK60-414 and SK56-414. The open reading frame coding for SK60-414 was prepared by, first, pfu DNA polymerase-catalyzed PCR using specific primers (upstream primer: 5'-TCAAACCATTTGCTACTGATAGTGGC-3'; downstream primer: 5'-AATATAGGCTAAATGATAGCTAGCATTTCTCTCC-3'). The cycling conditions for denaturation, annealing, and extension used during PCR reaction were 95 °C for 45 s, 55 °C for 1 min, and 72 °C for 1 min, respectively, and were performed for 25 cycles, after which 10 min at 72 °C were provided before termination of the reaction. This DNA (encoding for residues 60-384 of SK) was then digested with *Bsr*G I, at a unique site resident around nucleotide 1388 (numbered according to Malke et al., 1985) of the sequence of the *Pst* I fragment. This corresponds approximately to residue 164 of the primary structure of mature SK. The digested fragment was agarose gel-purified, and then cloned into pET-23d-SK pre-digested with *Nco* I followed by a fill-in reaction with T4 DNA polymerase, and then treated with *Bsr*G I to make it compatible for ligation with the PCR product (containing a blunt terminus at one end and a cohesive terminus, produced by *Bsr*G I, at the other). The ligation of this fragment with the digested SK-containing pET-23d recreates the original open reading frame of SK in the expression vector, but one that now begins with a deletion corresponding to 59 N-terminal residues. Construction of SK56-414 was performed similarly using an upstream primer (5'-TTAAGTCCAAAATCAAACCATTTGCTACT-3') encoding four additional amino acid residues at the 5' end, and the same downstream primer as in the case of SK60-414, described above. After transformation of *E. coli* strain BL-21 (Studier, 1991) and selection of positive colonies by restriction analysis of plasmid DNA, the inserts were sequenced by the dideoxy method (Sanger et al., 1977). For the production and purification of desired proteins, the bacterial cells containing the target gene were induced with 1 mM IPTG in their mid-log phase of growth and were harvested after 3 h of further incubation at 37 °C. The cells were lysed by sonication, centrifuged, and the supernatant diluted 10-fold with 50 mM Tris-Cl buffer, pH 7.5, containing 100 mM NaCl. The level of expression of SK60-414, as analyzed by SDS-PAGE, was found to be less than 5% of the total intracellular soluble protein, whereas that of SK56-414 was found to be more than 25% (data not shown). In the case of SK60-414, where protein expression was low, single-step purification by affinity chromatography using HPG-agarose was performed (cf. Fig. 1A, inset), essentially as described for SK (Rodriguez et al., 1992). SK56-414 was purified as follows. To the diluted bacterial lysate containing SK56-414, NaCl (to a final concentration 2.0 M) was added, and the solution loaded onto a 25-mL bed-volume phenyl-Sepharose column (Pharmacia Ltd.) pre-equilibrated with buffer (50 mM Tris-Cl, pH 7.5) containing 2 M NaCl. The column was then washed with the equilibration buffer (10 bed-volumes) followed by 50 mM Tris-Cl buffer, pH 7.5 (5 bed-volumes). The bound protein was eluted with sterile distilled water and made 25 mM in Tris-Cl, pH 7.2; it was then loaded onto a 25-mL DEAE-Sepharose Fast Flow (Pharmacia) ion-exchange column pre-equilibrated with the same buffer. Elution was performed by applying a linear gradient of 0-0.3 M NaCl (50 mL each) in buffer. All chromatographic steps were conducted at 4 °C. The fractions containing protein were analyzed on SDS-PAGE along with standard SK and MW markers. Desired fractions were pooled conservatively to obtain the homogenous preparation of SK56-414

(Fig. 1A, inset). Apart from gene sequencing, the authenticity of SK56–414 and SK60–414 was also established by gas phase N-terminal amino acid sequencing of both proteins on an Applied Biosystems–Perkin Elmer protein sequencer model 476-A.

HPG activator activity of SK56–414 in the absence and presence of either SK1–59 or SK16–59

The HPG activating ability of recombinant SK-fragments (viz., SK60–414 or SK56–414) in the absence of N-terminal SK-peptides was assayed by adding various concentrations of these fragments (ranging from 0.1 to 0.5 μM) to assay cuvette containing assay buffer [50 mM Tris-Cl buffer, pH 7.5, containing 0.5 mM chromogenic substrate (Tosyl-Gly-Pro-Lys-pNA) and 0.1 M NaCl] and excess HPG (2 μM). The activation of HPG to HPN, resulting in the hydrolysis of chromogenic substrate, was monitored by continuously recording the change in absorbance at 405 nm in a Shimadzu UV-160 model spectrophotometer at 22 °C for 10 min. In the control reaction, no SK60–414 or SK56–414 was added. The HPG activating ability of these SK-fragments in the presence of various concentrations of either SK1–59 or SK16–59 (0.1–2.5 μM) was also assayed by employing 25 nM of either SK60–414 or SK56–414 as described above.

Size-exclusion HPLC to demonstrate binding of SK1–59 and SK56–414 to HPG

The chromatography was performed at 22 °C using an ABI-Perkin Elmer model 172 HPLC system fitted with a TSK G2000 SWXL column (0.78 \times 30 cm). The column was equilibrated in 0.1 M sodium phosphate buffer, pH 6.8, containing 10 mM 6-amino-hexanoic acid, and the flow was kept at 250 $\mu\text{L}/\text{min}$. Mixtures of HPG (2.7 μM) with SK1–59 (2.7 μM) or SK56–414 (3.0 μM) were preincubated for 5 min in the presence of 2 mM NPGb and 50 μL were then applied onto the column. Similarly, a mixture of SK1–59 (2.7 μM) with SK56–414 (3.0 μM) was also loaded onto the column after a preincubation of 5 min. The absorbance of the eluant was monitored continuously at 210 nm as a function of time.

Assay for the generation of amidolytic activity in HPG by SK and SK-fragments in the absence and presence of N-terminal SK-peptides

Equimolar complexes of HPG with either SK, SK60–414, or SK56–414 (final concentration, 0.5 μM of each component) in the absence or presence of various concentrations of different N-terminal SK-peptides (ranging from 20 to 500 μM) were incubated at 22 °C for different time intervals (0–120 min) in dilution buffer. Aliquots were transferred periodically to measure the amidolytic activity generated in the complexes by adding them to 100- μL assay cuvette containing assay buffer (final concentration of complex, 0.15 μM). The change in absorbance was measured as described previously. In the control reaction, no SK, SK60–414, or SK56–414 was added.

Amidase parameters of complexes of HPG with SK or SK56–414 in the presence of SK16–59

Equimolar HPG (final concentration 0.5 μM) was added to either SK, SK56–414, or SK56–414 containing 100 μM of SK16–59 in

50 μL of dilution buffer (50 mM Tris-Cl buffer, pH 7.5, containing 0.5% BSA), and the mixture was incubated at 22 °C. Aliquots of 15 μL each from these mixtures were then withdrawn at specified times (2 min for SK and SK56–414 containing SK16–59 and 45 min for SK56–414, depending upon the maturation of the complex, where maximal amidolytic activity was observed) and added to assay cuvette (100- μL total volume) containing various concentrations of chromogenic substrate (0.06–2.0 mM) in assay buffer. The change in absorbance was monitored spectrophotometrically at 405 nm for 10 min. Kinetic parameters for amidolysis were then determined from the Lineweaver–Burke plots (Radek et al., 1993).

Assay for determining the steady-state kinetic constants for HPG activator activity in complexes of HPG with SK or SK56–414 in the presence and absence of SK16–59

HPG was mixed with either SK (500 nM) or SK56–414 (5 μM) in equimolar amounts in dilution buffer and incubated at 22 °C for 2 min and 30 min, respectively. Aliquots from this mixture were then added to the assay cuvette (final concentration 10 nM for the SK-HPG complex, and 0.5 μM for the SK56–414-HPG complex) containing assay buffer and various concentrations of HPG (0.05–3.0 μM) (Radek et al., 1993). The rate of HPG activation was monitored spectrophotometrically by recording the increase in rate of release of *p*-nitroanilide from the chromogenic substrate by the HPN generated as a result of HPG activation. For determining the kinetic constants for HPG activator activity in the presence of N-terminal SK-peptides, 100 μM or 5 μM of SK16–59 was added to SK56–414 or SK, respectively, followed by the addition of equimolar amounts of HPG (final concentration 500 nM with respect to SK or SK56–414) to the mixture. After incubation at 22 °C for 10 min in the case of SK and 30 min for SK56–414, aliquots from these mixtures were added to assay cuvette (final concentration of SK-HPG or SK56–414-HPG complex, 10 nM) containing assay buffer and various concentrations of HPG (ranging from 0.05 to 3.0 μM). The rate of HPG activation was monitored as described above. The kinetic parameters for HPG activation, $K_{p/g}$ (the apparent Michaelis constant for HPG as substrate) and $k_{p/g}$ (the catalytic rate constant of activator) were calculated by the construction of Lineweaver–Burke plots, as described (Wohl et al., 1980).

Potential of the HPG activator activity of SK56–414-HPG complex by N-terminal SK-peptides

Various peptides corresponding to the N-terminal of SK were added at different concentrations (ranging from 0 to 500 μM) to SK56–414 (0.5 μM) in dilution buffer and incubated at 22 °C for 2 min. HPG (final concentration, 0.5 μM) was then added to the mixtures and incubated further at 22 °C for various time intervals (0–60 min). Periodically, aliquots were removed and assayed for HPG activator activity by adding to assay cuvette (final concentration of SK56–414-HPG complex 10 nM) containing assay buffer and 2 μM of HPG. The HPG activation was followed as described previously. In positive-control reactions, either 0.5 μM of equimolar SK-HPG complex or SK-HPG complex containing 5 μM of either SK1–59 or SK16–59 were taken, whereas the negative-control reaction contained SK N-terminal peptides without SK or SK56–414.

Analysis of binding of N-terminal SK-peptides with HPG by dot-blot and ELISA

Dot-blot and ELISA were performed as described previously (Nihalani & Sahni, 1995). Briefly, for dot-blot analyses, varying amounts of N-terminal SK-peptides (prepared by twofold serial dilution, and ranging from 25 to 2.5 μg) were adsorbed onto nitrocellulose and probed with ^{125}I -labeled HPG. The relative binding affinities of each peptide with reference to SK were determined by ELISA using polyclonal HPG-specific antiserum as described previously (Nihalani & Sahni, 1995).

Assay for determining the inhibition of activator activity of SK-HPG complex by N-terminal SK-peptides

The assay was performed essentially as described previously (Nihalani et al., 1997). Briefly, an equimolar SK-HPG complex (500 nM) was formed by preincubating SK and HPG in dilution buffer for 2 min at 22 °C. An aliquot from this mixture was then added to assay cuvette (final concentration of SK-HPG complex, 10 nM) containing various concentrations of individual peptides (0–300 μM) and 1.0 μM of either BPG or HPG in the assay buffer. The activation of PG to PN was monitored as described previously. The extent of inhibition of substrate PG activation by different concentrations of peptides was expressed as a percentage relative to a control reaction (taken as 100%), where no peptide was added. For determination of the kinetic parameters of substrate PG activation by activator complex in the presence of SK-peptides, the assay was performed as described above, except that the concentration of peptides used in the assay cuvette was 20 μM , and varying concentrations of either BPG or HPG (0.25–3.0 μM) were used in the assay buffer. The kinetic parameters for PG activation were calculated by standard methods (Wohl et al., 1980).

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