Mapping of the plasminogen binding site of streptokinase with short synthetic peptides

DEEPAK NIHALANI, G.P.S. RAGHAVA, AND GIRISH SAHNI

Institute of Microbial Technology, Sector 39-A, Chandigarh-160036, India

Abstract

Although several recent studies employing various truncated fragments of streptokinase (SK) have demonstrated that the high-affinity interactions of this protein with human plasminogen (HPG) to form the activator complex (SK-HPG) are located in the central region of SK, the exact location and nature of such HPG interacting site(s) is still unclear. In order to locate the "core" HPG binding ability in SK, we focused on the primary structure of a tryptic fragment of SK derived from the central region (SK143–293) that could bind as well as activate HPG, albeit at reduced levels in comparison to the activity of the native, full-length protein. Because this fragment was refractory to further controlled proteolysis, we took recourse to a synthetic peptide approach wherein the HPG interacting properties of 16 overlapping 20-mer peptides derived from this region of SK were examined systematically. Only four peptides from this set, viz., SK234–253, SK254–273, SK274–293, and SK263–282, together representing the contiguous sequence SK234–293, displayed HPG binding ability. This was established by a specific HPG-binding ELISA as well as by dot blot assay using ^{125}I-labeled HPG. These results showed that the minimal sequence with HPG binding function resided between residues 234 and 293. None of the synthetic SK peptides was found to activate HPG, either individually or in combination, but, in competition experiments where each of the peptides was added prior to complex formation between SK and HPG, three of the HPG binding peptides (SK234–253, SK254–273, and SK274–293) inhibited strongly the generation of a functional activator complex by SK and HPG. This indicated that residues 234–293 in SK participate directly in intermolecular contact formation with HPG during the formation of the 1:1 SK-HPG complex. Two of the three peptides (SK234–253 and SK274–293), apart from interfering in SK-HPG complex formation, also showed inhibition of the amidolytic activity of free HPN by increasing the K_m by approximately fivefold. A similar increase in K_m for amidolysis by HPN as a result of complexation with SK has been interpreted previously to arise from the steric hindrance at or near the active site due to the binding of SK in this region. Thus, our results suggest that SK234–253 and SK274–293 also, like SK, bound close to the active site of HPN, an event that was reflected in the observed alteration in its substrate accessibility. By contrast, whereas the intervening peptide (SK254–273) could not inhibit amidolysis by free HPN, it showed a marked inhibition of the activation of "substrate" PG (human or bovine plasminogen) by activator complex, indicating that this particular region is intimately involved in interaction of the SK-HPG activator complex with substrate plasminogen during the catalytic cycle. This finding provides a rational explanation for one of the most intriguing aspects of SK action, i.e., the ability of the SK-HPG complex to catalyze selectively the activation of substrate molecules of PG to PN, whereas free HPN alone cannot do so. Taken together, the results presented in this paper strongly support a model of SK action in which the segment 234–293 of SK, by virtue of the epitopes present in residues 234–253 and 274–293, binds close to the active center of HPN (or, a cryptic active site, in the case of HPG) during the intermolecular association of the two proteins to form the equimolar activator complex; the segment SK254–273 present in the center of the core region then imparts an ability to the activator complex to interact selectively with substrate PG molecules during each PG activation cycle.

Keywords: active site; peptide walking; plasminogen; plasminogen binding; streptokinase; streptokinase-derived peptides

Streptokinase is a secretory protein of 414 residues produced by selected species of the genus *Streptococcus* that is used often as a thrombolytic drug for the treatment of several circulatory disorders, including myocardial infarction (ISIS-3, 1992), wherein it has been demonstrated to be virtually as efficacious as its more expensive alternatives, namely UK and TPA. In contrast to UK and TPA, relatively much less is known of the structural basis for the biochemical action of SK. This protein, which has been studied by CD, Raman, and IR spectroscopies, as well as by NMR at relatively low resolution, has been shown to possess at least three flexible domains (Radek & Castellino, 1989; Fabian et al., 1992; Misselwitz et al., 1992; Welfle et al., 1992; Teuten et al., 1993). Detailed three-dimensional information on SK, such as by high-resolution NMR or X-ray diffraction, is not yet available. However, at least at the biochemical level, its mechanism of action has been the subject of detailed investigations (reviewed in Castellino, 1981). A fundamental difference between SK, on the one hand, and UK and TPA, on the other, lies in the fact that, unlike the latter two, which possess intrinsic protease activity and, hence, directly act as HPG activators, SK is an "indirect" HPG activator. SK possesses no enzymatic activity of its own, but forms an enzymatically active, stoichiometric, noncovalent complex with either HPG or HPN, which then activates other "substrate" molecules of PG to PN by selective cleavage of the Arg_{560}-Val_{561} peptide bond (Markus & Werkheiser, 1964; McClintock & Bell, 1971; Reddy & Markus, 1972). There is now a compelling need to understand the structural basis of SK action in greater detail in order to engineer therapeutically improved SK derivatives (Marder, 1993).

In the past, most attempts at elucidating structure–activity relationships in SK have centered chiefly on examining the HPG activating and/or binding properties of truncated fragments after proteolysis of the native protein, or expression of SK genes carrying defined deletions (Siefring & Castellino, 1976; Malke et al., 1987; Rodriguez et al., 1994, 1995; Shi et al., 1994; Nihalani & Sahni, 1995; Reed et al., 1995; Young et al., 1995; Parrado et al., 1996). These studies collectively have revealed that progressive deletions from either the C- or N-termini of SK beyond roughly 30–40 residues drastically compromise the HPG activation property of the molecule. By contrast, the relatively more "primal" property of HPG binding is preserved even in relatively short polypeptide fragments derived from SK. In the recent past, the binding of HPG has been demonstrated with different truncated fragments of SK viz., SK1–253 (Reed et al., 1993), SK60–233 (Shi et al., 1994), SK120–352, SK244–352 (Reed et al., 1995), SK143–293 (Rodriguez et al., 1994; Nihalani & Sahni, 1995), SK220–414 (Young et al., 1995), SK64–380 and SK147–380 (Parrado et al., 1996). So far, the smallest independently HPG activating SK fragments have been shown to be 147–380 (Parrado et al., 1996) and 143–293 (Rodriguez et al., 1994; Nihalani & Sahni, 1995). Even though these studies, taken together, do successfully narrow down the overall length of the sequence to be examined from the 414 residues of the full-length native polypeptide to roughly the central 150–200 residues, they do not readily permit one to either identify conclusively the minimal-length sequence(s) involved in interaction with HPG (nor the exact boundaries of such sites, if indeed multiple sites exist), or cross-correlate these "primal" HPG binding functions with the "higher" HPG modulation functions. This is an issue of central importance in designing mutants of SK with altered HPG interacting properties. Recently, we have reported the presence of two independent high-affinity HPG binding sites in SK (Nihalani & Sahni, 1995). One, detected in an

N-terminal fragment (residues 1–59), could be further localized to residues 37–51 by secondary proteolysis. The other binding site has been shown to lie inside the central portion of the molecule, spanning approximately 150 residues; the binding site(s) in this "core" region could not be narrowed down by further fragmentation due to its refractoriness to several proteases (Misselwitz et al., 1992; Rodriguez et al., 1994, 1995). We therefore decided to identify the core HPG binding region, or regions, resident within this central portion of the SK polypeptide by taking recourse to a synthetic peptide approach ("peptide walking"), wherein a battery of short overlapping peptides of defined sequence, together spanning the region of interest, were investigated for their ability to interact with HPG as evidenced by direct binding experiments, and indirectly, by their ability to inhibit productive complex formation between SK and HPG. "Peptide walking" is a relatively recent approach (Joseph & Pick, 1995) that offers a complementary option to conventional recombinant DNA-based site-directed mutagenic and deletion techniques for the detection and identification of regions of primary structure involved in protein–protein or protein–ligand interactions. In this communication, we report the identity of the minimal SK sequence(s) necessary in the binding site for HPG that is located in the central region of the protein. We also show that synthetic peptide fragments derived from this "core" sequence (about 50–60 residues) competitively inhibit the functionally productive interaction of SK with HPG. In addition, our results suggest that, by binding in the immediate vicinity of the active site in HPG/HPN, a part of this core region is able to impart the characteristic substrate PG recognition ability to the activator complex.

Results

Limited proteolysis of SK by trypsin produces three major HPG-binding fragments viz., SK 1–59, SK60–293, and SK143–293 (Nihalani & Sahni, 1995). In order to examine whether these possessed any activator activity, each fragment, after purification, was subjected to standard SK assays for measuring the activation of HPG to HPN. To examine even very low activities accurately, relatively high molar concentrations of the fragments were employed in the assays, and the activation reactions were followed over a relatively extended period of time (see Materials and methods for details). Although SK1–59 alone did not possess any detectable HPG-activating ability, fragments SK143–293 and SK60–293 showed low, albeit clearly detectable HPG activator activities (data not shown). Moreover, the HPG activating ability of these fragments could be enhanced significantly in the presence of SK1–59 in a dose-dependent manner. The activator activity of SK under these conditions, however, was unaffected by the presence of SK1–59 (up to several hundred-fold molar excess over SK), indicating that the low activities of SK fragments (SK60–293 and SK143–293) are their intrinsic activities and not due to small amounts of contaminating SK. Similar observations with proteolytic fragments of SK have also been made by other workers previously (Shi et al., 1994; Parrado et al., 1996). Our results with the presence of HPG activating ability and its augmentation by SK1–59 in SK60–293 and SK143–293 show clearly that the "core" residues in SK participating with HPG in the formation of a HPG activator site are resident in the region 143–293 of the protein (the sequence common to SK60–293 and SK143–293).

In order to narrow down a minimum-length sequence in the smaller of the two SK fragments capable of SK binding and acti-

vation (SK143–293), we synthesized a series of 16 overlapping peptides, each of 20 residues, altogether encompassing the entire sequence between residues 134 and 302 of SK. The HPG binding ability of each peptide was then checked qualitatively by a highly sensitive dot blot method involving immobilization onto nitrocellulose membranes followed by probing with ^{125}I-radiolabeled HPG. In parallel, the binding isotherms of the peptides were determined by a quantitative ELISA developed especially for HPG binding of SK fragments (Nihalani & Sahni, 1995). The results of these binding experiments are presented in Figure 1A and B and Table 1. The dot blots reveal that, of the 16 peptides screened, only 4 possessed detectable binding with HPG; this was also confirmed by the results of the ELISA, wherein only the same 4 peptides showing

Table 1. *Relative affinities of SK peptides for HPG*

SK Peptide	Apparent dissociation constant with HPG[a] (mol/L)
SK (native)	$4.8 \pm 0.4 \times 10^{-9}$
SK60–293	$5.0 \pm 0.5 \times 10^{-7}$
SK143–293	$4.0 \pm 0.7 \times 10^{-7}$
SK234–253	$4.0 \pm 0.8 \times 10^{-4}$
SK254–273	$5.0 \pm 0.2 \times 10^{-5}$
SK274–293	$4.5 \pm 0.3 \times 10^{-5}$
SK263–282	$1.9 \pm 0.5 \times 10^{-4}$
SK243–262[b]	—
SK223–242	ND[c]
SK283–302	ND

[a] Values were computed through analysis of noncompetitive ELISA curves as described in Materials and methods, and are means of four independent experiments.
[b] Insolubility of this peptide under assay conditions precluded determination of HPG binding ability.
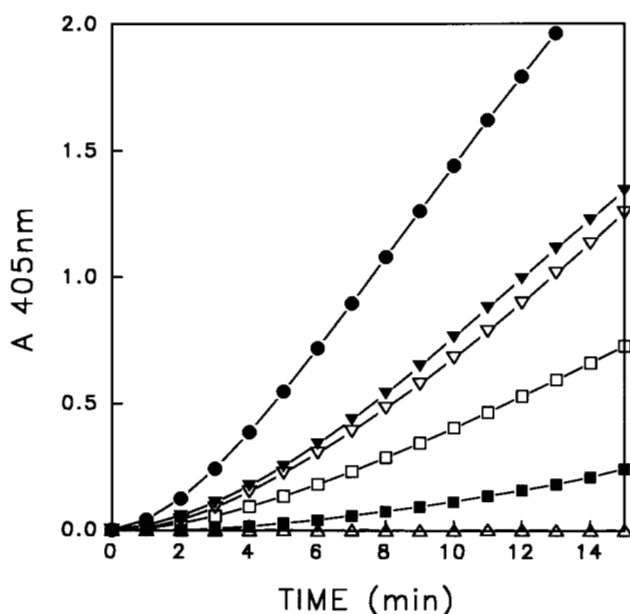[c] ND, HPG binding could not be detected.
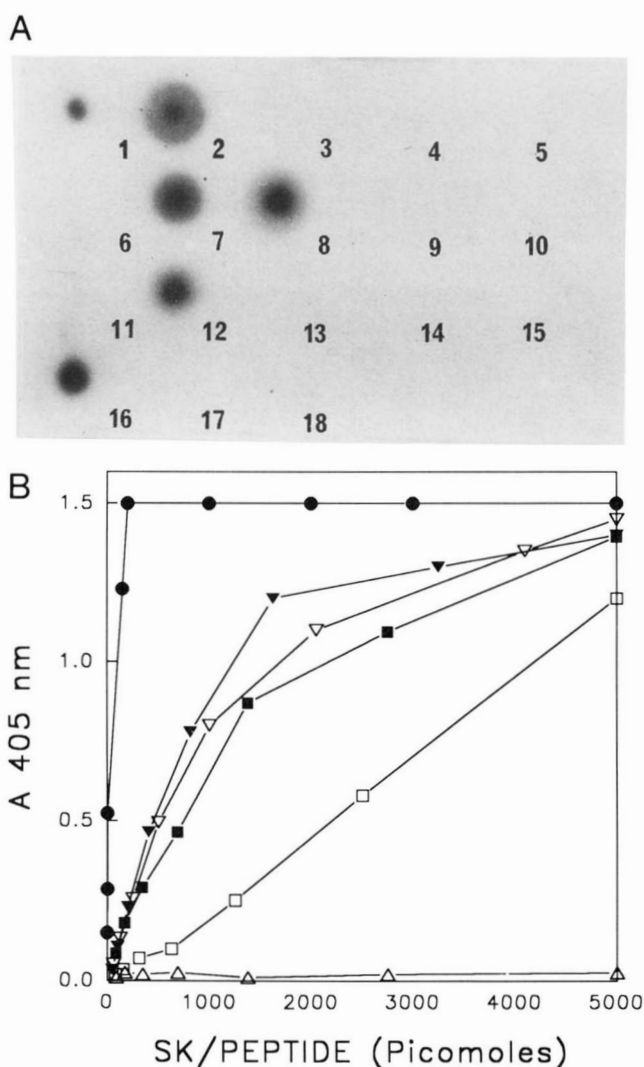
Fig. 1. Plasminogen binding analyses of synthetic peptides by dot blot and ELISA. **A:** Dot blots showing binding of radiolabeled HPG to SK peptides. Positions marked 1 and 2 correspond to SK (1 μg) and SK143–293 (5 μg), respectively, whereas 7, 8, 12, and 16 represent SK234–253, SK254–273, SK274–293, and SK263–282, respectively. **B:** Quantitative ELISA for HPG binding. Plots show binding of either SK (closed circles) or SK peptides viz., SK234–253 (open inverted triangles), SK254–273 (closed triangles), SK274–293 (closed squares), and SK263–282 (open squares), with HPG. Control reaction where SK or SK-peptides were omitted is depicted with open triangles.

positive HPG binding by dot blot (SK234–253, SK254–273, SK274–293, and SK263–282) were found to interact with HPG (Fig. 1B). The HPG reactivity of one of the peptides (SK243–262), with a sequence overlap with that of the other four peptides with detectable HPG binding ability, could not be studied further because of its very low solubility at neutral pH. The apparent dissociation constants of the peptides for HPG were determined through analyses of the noncompetetive ELISA curves (Beatty et al., 1987; Nihalani & Sahni, 1995), and are shown in Table 1. For comparison, the relative affinities of SK and SK143–293, determined in parallel, are also provided. From these data, it was clear that the region 234–293 in SK (the native sequence representing the peptides showing positive HPG binding) encompasses a site for interaction with HPG. It should be noted that the apparent affinities of these peptides for HPG are generally 2–3 orders lower in comparison to the affinity of SK143–293, their "parent" sequence. The affinity of the latter for HPG, in turn, is much lower compared with that of SK. Thus, there was a progressive loss in the HPG affinity of SK upon truncation of the full-length native protein to, first, SK60–293 and SK143–293, and, subsequently, to even shorter fragments. Such a decrease in HPG binding by truncation of SK has been shown previously also (Rodriguez et al., 1995) and likely is related to decreasing conformational stability of the resulting fragments.

We next examined the ability of the short, HPG-binding peptides to activate HPG. The synthetic peptides derived from the region SK234–293 viz., SK234–253, SK254–273, SK274–293, and SK263–282, were pre-incubated individually with HPG, and the appearance of HPN (amidolytic) activity was then determined periodically (see Materials and methods). Whereas the activity of both SK and SK143–293 could be determined easily by this method, no activator activity was detectable even at several thousand-fold molar excess of each peptide over that of HPG when tested for relatively extended durations (2–3 h). Similar assays performed with equimolar mixtures of all three contiguous peptides viz., SK234–253, SK254–273, and SK274–293, at up to several hundred-fold molar excess of each over HPG also failed to show the generation of any detectable plasmin activity (data not shown).

The identification of peptides that demonstrated positive binding to HPG without any detectable HPG-activating ability raised the question whether they would be able to inhibit the formation of an active complex between HPG and SK. In order to test this, the generation of amidolytic activity by an equimolar mixture of SK and HPG was conducted in the absence or presence of varying amounts of the HPG binding peptides. Controls where the effect of the peptides were evaluated on pre-formed SK-HPG complexes were also included. Apart from the HPG binding peptides, we also tested the other 12 peptides not showing overt HPG binding by dot blot and ELISA. The results demonstrated that only SK234–253, SK254–273, and SK274–293 could inhibit the activation of HPG to HPN by SK in a dose-dependent manner (representative data from one such experiment showing the inhibition observed with SK234–253 is depicted in Fig. 2). In the case of SK234–253, SK254–273, and SK274–293, approximately 50% inhibition was observed in the range of 40–100 μM of peptide (Table 2). It was significant that the inhibition was not observed when the peptides were added into reactions where SK and HPG had been pre-incubated together, thereby demonstrating that the intermolecular complexation of the two proteins was being inhibited by the peptides rather than the amidolytic activity of the complex per se. A synthetic peptide corresponding to SK37–51, which has been shown to possess independent HPG binding ability (Nihalani & Sahni, 1995), was also tested in the inhibition assay, and found to be ineffective up to the highest concentration of 300 μM tried (data not shown). Equimolar combinations of two or more contiguous peptides simultaneously (up to a maximum of three peptides at one time) were also tried, but a synergistic influence was not observed

Table 2. *Inhibition of activator complex formation of SK and HPG by SK peptides*

Sample[a]	Concentration[b] corresponding to 50% inhibition (M)
SK143–293	$1.50 \pm 0.5 \times 10^{-7}$
SK234–253	$3.75 \pm 0.6 \times 10^{-5}$
SK254–273	$1.00 \pm 0.2 \times 10^{-4}$
SK274–293	$1.10 \pm 0.3 \times 10^{-4}$

[a]All 16 synthetic SK peptides, as also the fragment SK143–293, were screened for the inhibition of activator complex formation by SK and HPG, up to a maximum concentration of 300 μM. Only the three SK peptides, shown above, were found to inhibit the activation of HPG by SK (see text for details).
[b]Corresponds to the concentration of peptide that caused 50% inhibition in the rate of a standard reaction containing equimolar amounts of SK and HPG. The values reported are the means of four independent experiments.

(data not shown). In conclusion, the results with the synthetic peptides indicated that the sequence 234–293 in SK represents the primary site of high-affinity contact with HPG during activator complex formation.

Even though the synthetic peptides derived from the HPG binding site in SK143–293 were unable, unlike the parent contiguous fragment, to activate the zymogen (HPG) independently to its active form (HPN), it was of interest to examine if these short peptides could alter or modulate the proteolytic specificity of the pre-formed active site of HPN. The binding of SK with HPN results in the alteration of its substrate specificity, which can be tested experimentally, especially by the ability of SK-HPN to activate BPG; neither free HPN nor SK alone are able to activate BPG directly, but, once SK combines with either HPN or HPG, the complex is able to activate BPG. To test whether even a small proportion of this native-like property of SK was present in any of the synthetic peptides, we incubated these with HPN at up to several hundred molar excess and then measured the ability of these mixtures to activate BPG. Although SK143–293 and HPN together could activate BPG slowly, in the case of the short peptides, even after 24 h of incubation, no BPG activation could be observed (data not shown). Interestingly, in the control reactions where BPG was not added, it was observed that two of the peptides viz., SK234–253 and SK274–293, caused marked inhibition of the basal amidolytic activity of HPN in a concentration-dependent manner. This property was then tested systematically with all of the synthetic peptides, including ones with overt HPG binding ability. The results (Fig. 3A) showed a strong inhibition of plasmin activity by two peptides viz., SK234–253 and SK274–293. However, the intervening segment, SK254–273, which had shown an ability to bind with HPG by dot blot and ELISA as well as inhibit the complexation of SK and HPG, did not display any inhibition of the amidolytic activity of free HPN. It is known that the kinetic parameters for amidolysis by plasmin are altered upon complexation with SK, and this has been interpreted to result from the binding of SK in the close vicinity of the protease active site (Robbins et al., 1981; Wohl, 1984). With this possibility in mind, we investigated if such an alteration in the steady-state kinetic parameters could be contributing to the observed inhibition of the amidolytic activity of HPN by the two peptides. The results (Fig. 3B) showed clearly that, in the presence of either SK234–253

Fig. 2. Inhibition of SK-HPG activator complex formation by SK peptides. The peptide SK234–253, derived from the core HPG binding region of SK, was pre-incubated at different concentrations with constant amounts of HPG (8 nM), and the mixtures were then assayed for generation of amidolytic activity in the presence of SK (10 nM). Closed circles, no peptide added; closed triangles, 20 μM; open inverted triangles, 40 μM; open squares, 80 μM; closed squares, 300 μM. A control containing all components except SK/SK-peptide is depicted with open triangles. Essentially identical inhibition curves were obtained with the peptides SK254–273 and SK274–293.

Fig. 3. Dose-dependent inhibition of plasmin activity by HPG binding peptides. **A:** Amidolytic activity of fixed amounts of HPN (0.4 μM) was measured in the presence of varying concentrations of synthetic SK peptides (0–50 μM), and the percent survival of HPN activity was then quantitated. Different reactions contained either SK234–253 (open triangles), SK254–273 (closed triangles), or SK274–293 (closed circles). **B:** Effect of SK and SK peptides on the kinetic parameters of HPN for amidolysis of G-P-L-pNA. SK (closed circles) (0.12 μM), SK143–293 (open circles) (0.5 μM), and SK peptides (80 μM) viz., SK234–253 (open squares) and SK274–293 (closed inverted triangles), were incubated with HPN and the amidolytic activity in the presence of varying concentrations of G-P-L-pNA (0.06–0.5 mM) were then estimated. Lineweaver–Burke plots of the data are shown. HPN alone (closed triangles) was taken as control.

or SK274–293, the K_m value of HPN for its amidolytic substrate was increased nearly fivefold (from approximately 0.2 mM to 1.0 mM) relative to free HPN, with little detectable change in V_{max}. This property, i.e., little or no alteration of the V_{max} values, but a significant increase in K_m, was comparable to that observed with

SK as well as the truncated fragment SK143–293. Thus, in terms of the property of K_m alteration, the segments SK234–253 and SK274–293 could mimic completely the native, full-length protein. It should be mentioned here that the inhibitory effect was not due to a competition by the peptides with the amidolytic substrate for hydrolysis by HPN, as could be argued, particularly because the peptides also contained potentially HPN-sensitive Arg- and Lys-containing peptide bonds. However, the observation that this effect was encountered only in the case of SK234–253 and SK274–293 among the 16-peptide set, many of which also contained internal Arg and Lys peptide bonds, argued against such a possibility. We confirmed experimentally that this indeed was the case by RP-HPLC analysis of the test mixtures, which showed a complete absence of degradation of the peptide(s) during the duration of the kinetic measurements (data not shown).

Apart from the high-affinity 1:1 complexation of SK and HPG to form the activator complex, another important facet of SK-HPG interaction is the generation of a functionality possessing affinity specifically for "substrate" PG, a property that might be imparted by a region, or regions, in the SK moiety in the complex. This, indeed, could be responsible for the (transient) formation of a ternary complex between SK-HPG and substrate PG, envisioned as a key intermediate in the catalytic conversion of PG to PN by the activator complex. Once the region playing a dominant role in 1:1 SK-HPG complexation was localized in and around residues 230–290, we were tempted to explore whether the putative substrate PG recognition property could also be detected in any of these core segments. To test this possibility, the regular PG activator assay, where a high concentration of substrate PG (>1 μM) is employed normally, was modified to contain relatively lower amounts of substrate PG (0.06 μM), and the time of activation was extended (see Materials and methods for details) so that the assay could become sensitive to even low degrees of inhibition of the activation of substrate PG (by a fixed quantity of pre-formed 1:1 SK-HPG complex) by any fragment competing with the interaction of the complex with substrate PG. Besides HPG as the substrate, this assay was also performed with BPG because it is not activated by SK alone, but only by the SK-HPG or SK-HPN activator complexes, thus becoming an optimal substrate for the measurement of SK-HPG activator activity (Radek et al., 1993). Before testing the synthetic peptides, however, we wanted to evaluate the validity of this approach by testing if the fragment SK143–293 showed any ability to selectively inhibit substrate PG activation by the pre-formed SK-HPG complex. Remarkably, SK143–293 could easily inhibit the activation of substrate BPG in a concentration-dependent manner [50% inhibition (IC_{50}) at approximately 1.2 μM]. We next tested each of the 16 peptides representing the region SK134–302 in the above assay up to a maximum concentration of 300 μM. However, of these, only SK254–273 was found to inhibit significantly the activation of BPG by activator complex (IC_{50} = approximately 65 μM), with one other peptide (SK274–293) showing relatively marginal inhibition (Fig. 4A). In order to further explore the basis of this inhibition, the steady-state kinetic parameters for the activation of BPG by SK-HPG were also determined in the presence of inhibitory concentrations of SK254–273 and SK143–293. Whereas no significant change was observed in the values of k_{cat} for BPG activation in both cases (around 3.8 min^{-1}/ng of SK-HPG used), the values of K_m in the presence of SK143–293 and SK254–273 were increased significantly (approximately to 0.82 μM) compared with uninhibited controls (around 0.2 μM) (Fig. 4B, inset). This indicated clearly that it was the apparent
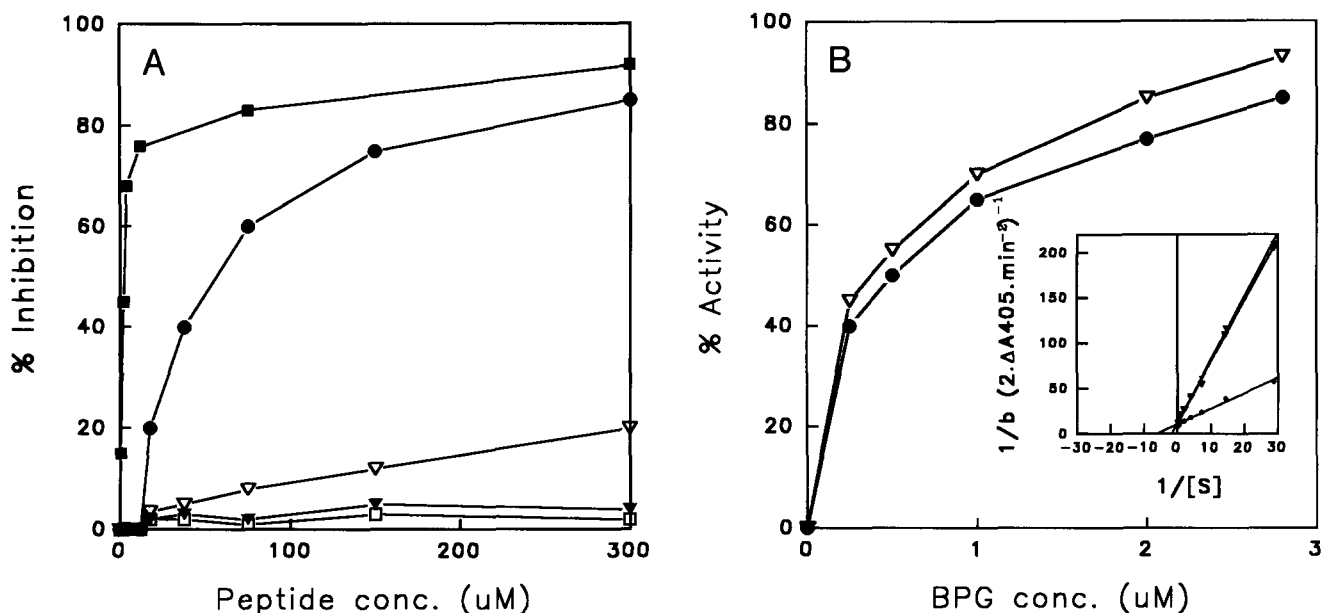
Fig. 4. Inhibition by SK254–273 of "substrate" PG activation by the pre-formed activator complex. **A:** Fixed amounts of pre-formed equimolar complex of SK and HPG (20 nM each) were added to BPG (1 μM) in assay buffer containing chromogenic substrate as well as varying concentrations of different SK peptides (0–300 μM). Plasmin activity generated was then measured spectrophotometrically. Different reactions contained either SK143–293 (closed squares), SK254–273 (closed circles), SK234–253 (closed inverted triangles), SK274–293 (open inverted triangles), or SK283–302 (open squares). Percent inhibition was calculated from the rates of plasmin activation in the presence of the peptides compared with those in controls (taken as 100%) without any peptide. **B:** Reversal of the inhibition of activator activity of SK-HPG by SK254–273 (closed circles) and SK143–293 (open triangles) in the presence of increasing concentrations of BPG. Percent activity represents the increase in substrate PG activating activity with increase in BPG concentration relative to the corresponding control reaction (100%) in which no peptide/fragment had been added. Inset: Lineweaver–Burke plot for the determination of kinetic parameters for BPG activation by SK-HPG complex in the presence of SK peptides. The experiment was performed under conditions identical to those mentioned above, except that the concentration of BPG was varied from 0.035 to 3 μM in the reaction. The control reaction where no peptide was added is depicted with closed circles, whereas reactions where either SK254–273 or SK143–293 was included are shown as open triangles and closed triangles, respectively.

affinity of activator complex for hydrolysis of BPG that was affected in the presence of the peptide. Indeed, this conclusion, that there was direct competition between SK254–273 and SK-HPG for binding with substrate PG, was validated when the reversal of inhibition of substrate PG activation by a given concentration of SK254–273 could be achieved by simply increasing the concentration of BPG in the reaction (Fig. 4B).

Discussion

At least two qualitatively distinct types of SK-PG interactions can be envisioned in the generation of PG activator capability by SK and HPG: (1) the intermolecular, high-affinity interactions between SK and HPG to form the 1:1 molar SK-HPG activator complex, and (2) the transient binding of the SK-HPG activator complex with substrate PG molecules (ternary complex) during each catalytic cycle of PG activation. Precise information on the exact location and nature of these PG binding site(s) in SK has not been obtained until now. As a first step toward this goal, we chose to focus on the relatively short fragment, namely SK143–293, which showed both HPG binding and low but intrinsic PG activator activity. In order to identify a minimal-length PG interacting unit (or units, if more than one locus for interaction with PG is involved), we employed short synthetic peptides that spanned the complete length of this fragment viz., residues 134–302, to probe the PG-interacting functions in this region of the molecule. Even

though it is well-recognised that short peptides generally possess overall disordered structures in solution even when these are derived from stably folded segments of native proteins, such fragments display sufficient propensity to adopt their original native-like formats, allowing the "peptide walking" approach to be exploited for deciphering protein–protein interactions in diverse systems (Joseph & Pick, 1995; Bayley et al., 1996; Mohri et al., 1996). In our study, too, this approach could be used successfully to demonstrate the direct involvement of an approximately 60-residue region in SK (SK230–290) that is involved in both binding with HPG to form the equimolar SK-HPG activator complex, as well as in the interaction of this activator complex with substrate PG. The results also indicate that the region in HPG where this binding takes place is probably in the close vicinity of the active site of HPG/HPN.

The complexation of SK with free HPN is manifested in overall reduced accessibility for the active site of the latter by small molecular weight substrates and inhibitors probably as a result of the binding of SK in this region (Wohl, 1984; Dawson et al., 1994). For example, the altered behavior of the SK-HPN complex compared with that of uncomplexed HPN is reflected in altered kinetics of amidolysis of chromogenic substrates, such as a significant increase in the K_m of the SK-HPN complex compared with that of HPN alone (Wohl et al., 1980; Robbins et al., 1981). Likewise, the complexation of SK with HPG results in a measurable change in the kinetics of binding of inhibitors at the cryptic active site of the

zymogen (Wohl, 1984). One reasonable explanation for these phenomena is that the binding of SK occurs in the close vicinity of the active site region, thus creating steric hindrance for the substrate or ligand. Our results on the increased K_m for amidolysis by HPN in the presence of the HPG binding peptides derived from the segment 234–293 of SK indicate that the core residues of this region also bind in the close vicinity of the active site. Because essentially the same results were observed with either SK or its truncated fragment, SK143–293, the inescapable conclusion that follows from this is that the binding of epitope(s) formed by the residues 234–254 and 274–293 of SK with HPN must occur at or near the active site, and this affects the accessibility of the active site for the amidolytic substrate. Thus, even though none of the short peptides was capable of promoting any detectable PG activation, with respect to at least this basal property (binding at or near the active site), two peptides could mimic the full-length native molecule with virtually complete fidelity. At the same time, the inability of the intervening segment viz., the peptide SK254–273, to inhibit the amidolytic activity suggests that this segment, in the SK-HPG activator complex, is either quite flexible or oriented in a manner that does not constrain access of the substrate to the active site sterically.

The presence of a strong inhibitory effect of SK234–253, SK254–273, and SK274–293 on SK-HPG complexation prompted us to examine if these [or indeed any other peptide(s) from the complete set] could selectively inhibit the activation of substrate PG by activator complex as well. Experiments to explore this possibility demonstrated clearly that only the peptide SK254–273 could inhibit the activation of either BPG or HPG by the pre-formed activator complex in a concentration-dependent manner. A kinetic analysis of the inhibition of HPG/BPG activation reaction by SK254–273 revealed that, whereas the V_{max} for PG activation was essentially unaffected, the K_m values were increased significantly in comparison to uninhibited control reactions. Entirely in keeping with this observation, the inhibitory effect at a given peptide level could be reversed by merely increasing the concentration of substrate PG in the reaction. Thus, the enhancement in the K_m values for plasminogen activation by SK-HPG in the presence of SK254–273 is due to a direct competition between the inhibitory peptide and the corresponding sequence in the SK-HPG complex for interaction with the substrate. When these results are gauged together with the findings on the ability of SK234–253 and SK274–293 to inhibit the formation of the SK-HPG complex, one concludes that this core region consists of epitopes/structural units in SK that (1) occupy a position at the interface of the SK-HPG complex and (2) are also proximal to the protease active site in the SK-HPG complex. The segment SK254–273 in this core could then transiently attach to substrate PG during the catalytic cycle of PG activation by the 1:1 SK-HPG complex, and so impart the highly characteristic substrate-discrimination property into the SK-HPG complex by positioning the substrate in the proper orientation for cleavage at the scissile peptide bond. The present study thus offers tantalizing evidence for the first time that a stretch of residues in the core region of SK that is involved in tight binding near the active site of HPG/HPN itself contributes to the generation of this intriguing but fundamental functionality in the activator complex. With the localization of the core sequences involved in interacting with PG to a relatively small segment of primary structure in SK, several powerful experimental approaches, including site-directed mutagenesis, can now be applied in concert toward understanding the molecular basis of SK action with greater precision. It should

be recognized, however, that regions in SK other than the core region identified in our study also contribute to the generation of full-blown activator activity, first in the initial SK-HPG virgin enzyme complex, and, subsequently, in the SK-HPN complex (Young et al., 1995). This is exemplified clearly by the observation that the fragment SK143–293, which contains the core region responsible for both HPG binding during activator complex formation as well as substrate recognition by pre-formed complex, is weakly active at best. The fact that SK1–59, which encompasses an additional HPG binding site but, by itself, is inactive (Shi et al., 1994; Nihalani & Sahni, 1995; Parrado et al., 1996), can considerably enhance the low activity of SK143–293, highlights strongly the role of the N-terminal region of SK in the attainment and/or maintenance of a proper native-like fold in the core region (Young et al., 1995; Parrado et al., 1996). Studies aimed at understanding the mechanism of the complementation of the low intrinsic activity of SK fragments derived from the central portion of the molecule by synthetic peptides based on the N-terminal region, in concert with site-specific mutagenesis of residues of the core region, should now be helpful in revealing the mechanism of SK action with greater clarity.

Materials and methods

Reagents

Glu-plasminogen was purified from human plasma by affinity chromatography (Deutsch & Mertz, 1970) (>98% Glu-form as determined by N-terminal amino acid sequencing), or obtained from Boehringer-Mannheim. Conversion of HPG to HPN was performed by treatment with immobilized UK (100 Plough units/mg of HPG; UK was immobilized on cyanogen bromide activated Sepharose 4B). Highly pure, albumin-free SK from *Streptococcus equisimilis* (strain H46 A) was a kind gift from Dr. D. Gerlach (Jena, Germany) and Dr. R.A.G. Smith (Smith-Kline Beecham, UK). Both preparations showed single bands by SDS-PAGE, and displayed specific activities of 110,000–120,000 I.U./mg protein (Bradford, 1976; Jackson et al., 1981). Antisera to HPG was raised in rabbits and their titers determined by dot blot as well as ELISA. All other reagents used were of the highest purity commercially available.

Preparation and characterization of tryptic fragments of SK

Tryptic fragments of SK, namely SK1–59, SK60–293, and SK143–293, were prepared as described previously (Nihalani & Sahni, 1995). The individual fragments were cross-checked for heterogeneity as well as the possible presence of any residual undigested SK by rechromatography on a narrow-bore C-4 column (2 × 100 mm; Brownlee) (gradient: 5–45% acetonitrile [ACN] containing 0.1% trifluoroacetic acid [TFA] over 120 min at a flow rate of 80 μL/min). The HPLC work was performed on a Perkin Elmer-Applied Biosystems model 172 microbore HPLC system. Control "spiking" experiments showed that even trace quantities of SK could be detected under these conditions of chromatography, which eluted at a position distinctly resolved from either SK60–293 or SK143–293 (data not shown). Moreover, the kinetic parameters of these fragments were found to be quite different from that of SK (four- to fivefold increase in K_m for HPG activation and approximately 1,000-fold decrease in V_{max}), which remained unaltered upon rechromatography. Also the kinetic characteristics of SK sub-

jected to RP-HPLC under these conditions were found to be un-altered, indicating that the low activities of these fragments are not due to denaturation brought about by the isolation procedure. SK fragments were characterized by N-terminal sequence analyses and ion-spray mass spectroscopy, and quantitated by amino acid analysis after PITC derivatization (Findlay & Geisow, 1989). Ly-ophilized polypeptide fragments were stored desiccated at −20 °C in the dark until reconstituted just prior to use.

Assays for studying the activation of HPG by SK and SK fragments

A one-stage assay method was used to measure the kinetics of HPG activation by SK or its truncated fragments (Wohl et al., 1980; Shi et al., 1994). SK (0.5 nM) or individual fragments, viz., SK60–293 (0.2 μM), SK143–293 (0.4 μM), or SK1–59 (1 μM), were reconstituted freshly from the stored state in 50 mM Tris-Cl buffer, pH 7.5, and added to 100 μL-assay cuvette containing HPG (4 μM) in assay buffer [50 mM Tris-Cl buffer, pH 7.5, also con-taining 0.5 mM chromogenic substrate (tosyl-Gly-Pro-Lys-pNA, obtained from Sigma) and 0.1 M NaCl]. The change in absorbance was then measured as a function of time in a Shimadzu UV-160 model spectrophotometer at 22 °C.

Generation of amidolytic activity in HPG by SK60–293 and SK143–293 in the presence of SK1–59

Fragment SK1–59 (0.1–0.8 μM) was added to 0.2 μM of either SK60–293 or SK143–293 and incubated at 22 °C for 5 min. The mixture was then added to the assay cuvette containing assay buffer and 4.0 μM HPG. Generation of amidolytic activity was observed by recording change in absorbance at 405 nm as de-scribed previously. In control reactions, the activity of either SK1–59 alone, or in the presence of SK, was also measured. Under these conditions, SK1–59 did not show any detectable activity up to a concentration of 1 μM, nor did it enhance the activity of SK.

Exploration of the HPG binding region (SK134–302) using synthetic peptides for identification of sequences involved in HPG binding

A set of 16 overlapping peptides corresponding to the sequence 134–302 of SK (Malke et al., 1985), containing 20 amino acid residues each, were synthesized by Chiron Mimotopes, Australia. The purity of each peptide was then established through ion-spray mass spectrometry on a Perkin-Elmer Sciex II spectrometer and RP-HPLC. Qualitative analysis of binding of each peptide to HPG was determined by a dot blot assay established earlier to be a sensitive index of PG binding by SK or its proteolytically trun-cated products (Nihalani & Sahni, 1995). This involved, briefly, the adsorption of each peptide onto nitrocellulose sheet, and then probing with ^{125}I-labeled HPG. Quantitative binding of each pep-tide to HPG was performed through an ELISA using polyclonal HPG-specific antiserum, as described previously (Nihalani & Sahni, 1995). The relative binding affinities of HPG binding peptides with reference to SK were computed through noncompetitive ELISA (Beatty et al., 1987). HPG binding of one of the SK peptides, SK243–262, could not be studied because of its very low solubility under the conditions of assay. Efforts were made to solubilize it both at low and high pH, or by inclusion of NaCl or DMF (di-methyl formamide); indeed, with some of the above conditions,

approximately 20–30% solubility was achieved. However, upon reversal to assay conditions by dilution in buffer of near-neutral pH, precipitation was found to reoccur.

Assay for the generation of amidolytic activity in peptide–HPG mixtures

HPG (final concentration 4.0 μM) was incubated with 80 μM each of HPG binding peptides at 22 °C for 10 min. Amidolytic activity was determined by adding a suitable aliquot of the complex to an assay cuvette containing assay buffer in a final volume of 100 μL. The change in absorbance at 405 nm was monitored as previously. Complexes of HPG with SK, SK143–293, and HPG nonbinding peptides were taken as controls. Competition assays were per-formed to determine the effect of HPG binding peptides on the creation of an active site in HPG by SK. Various concentrations of peptides (0–300 μM) were incubated with HPG in 50 mM Tris-Cl buffer, pH 7.5, at 22 °C for 5 min. The mixture was then added to the assay cuvette containing assay buffer and SK. The final con-centrations of SK and HPG in the reaction mixture were 10 nM and 8 nM, respectively. Change in absorbance was measured at 405 nm for 15 min at 22 °C. Assay where no SK or peptide was added was taken as control.

Amidase parameters of HPN in the presence of SK, SK fragments, and SK peptides

The generation of activator activity in peptide–HPN mixtures was determined by incubating 80 μM peptide with 0.4 μM HPN in Tris-Cl buffer, pH 7.5, containing 0.5% (w/v) BSA at 22 °C for 10 min. BPG (2.5 μM final concentration) was then added to the reaction mixture and BPN activity was measured as described previously. Reactions where no BPG was added to the complex (peptide–HPN), or with HPN alone (with no peptide added), were taken as controls. Kinetic parameters for the HPN (amidolytic) activity in the presence of SK (0.12 μM), SK fragment (0.5 μM), or SK peptides (80 μM) were determined by incubating them with 0.1 μM HPN in 50 mM Tris-Cl buffer, pH 7.5, containing 0.5% (w/v) BSA at 22 °C for 5 min. Aliquots from these mixtures were then added to assay cuvettes containing various concentrations of G-P-L-pNA in Tris-Cl buffer, pH 7.5, ranging from 0.06 mM to 0.5 mM. The change in absorbance was monitored spectrophoto-metrically at 405 nm for 10 min. In the control reaction, no SK, SK fragments, or SK peptides were added.

Assay for determining inhibition of PG activator activity of pre-formed SK–HPG complex by peptides

The SK–HPG activator complex was formed by pre-incubating equimolar concentrations (20 nM each) of SK and HPG in a total volume of 50 μL containing 50 mM Tris-Cl buffer, pH 7.5, and 0.5% BSA at 22 °C for 2 min (Radek et al., 1993). A 10-μL aliquot from this reaction mixture was then added to a spectrophotometric cuvette (100-μL capacity) containing various concentrations of individual peptides (0–300 μM) in assay buffer and also 0.06 μM of either BPG or HPG in a total volume of 100 μL. After rapid mixing, the generation of amidolytic activity was measured spec-trophotometrically by recording the increase in rate of release of *p*-nitroanilide from the chromogenic substrate over control reac-tion by the bovine or human plasmin generated as a result of BPG or HPG activation for a period of 10 min at 22 °C. The control

reaction, to measure the basal activity of the activator complex, contained all components except the test peptide. The extent of inhibition of activator activity by different concentrations of a given peptide was then determined and expressed in percentage relative to control reactions (taken as 100%) conducted in the absence of peptide. For the determination of kinetic parameters of BPG/HPG activation by the pre-formed activator complex, the 1:1 SK-HPG complex was formed under identical conditions as described above and a similar aliquot was added to the assay cuvette containing 50 μM concentration of peptide SK254–273 or 1 μM of 143–293 and varying concentrations of either BPG or HPG (0.035–3 μM) in assay buffer and the amidolysis was observed for 5 min at 22 °C. In the control reaction, no peptide was added. The inhibition of substrate PG activation brought about by the peptide (using a fixed concentration that resulted in approximately 50% inhibition in the reactions when 0.5 μM concentration of BPG/ HPG was used) was relieved by using various increasing concentrations (0–3 μM) of BPG/HPG in the above reaction. The kinetic parameters for BPG/HPG activation, K_m (the apparent Michaelis constant for PG as substrate) and k_{cat} (the catalytic rate constant of activation), were calculated by standard methods (Wohl et al., 1980).

Acknowledgments

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