The specific functions of the amino acid residues in the streptokinase (SK) γ-domain were analyzed by studying the interactions of human plasminogen (HPlg) and SK mutants prepared by charge-to-alanine mutagenesis. SK with mutations of groups of amino acids outside the coiled coil region of SK γ-domain, SK_K278A,K279A,E281A,K282A and SK_D360A,R363A had similar HPlg activator activities as wild-type SK. However, significant changes of the functions of SK with mutations within the coiled coil region were observed. Both SK_D322A,R324A,D325A and SK_R330A,D331A,K332A,K334A had decreased amounts of complex formation with microplasminogen and failed to activate HPlg. SK_D322A,R330A had a 21-fold reduced catalytic efficiency for HPlg activation. The studies of SK with single amino acid mutagenesis to Ala demonstrated that Arg324, Asp325, Lys332, and Lys334 play important roles in the formation of a HPlg:SK complex. On the other hand, amino acid residues Asp322, Asp328, and Arg330 of SK are involved in the virgin enzyme induction. Potential contact between Lys322 of SK and Glu629 of human microplasmin and strong interactions between Asp322 and Lys339, Asp334 and Lys354, and Asp322 and Lys334 of SK are noticed. These interactions are important in maintaining a coiled coil conformation. Therefore, we conclude that the coiled coil region of SK γ-domain, SK.Leu314-Ala342, plays very important roles in HPlg activation by participating in virgin enzyme induction and stabilizing the activating complex.

Streptokinase (SK), a potent human plasminogen (HPlg) activator, is a single chain secretory protein produced by strains of β-hemolytic Streptococcus (1–3). The HPlg and SK catalyzes the hydrolysis of fibrin and dissolution of blood clots. SK, therefore, has been used as a thrombolytic agent in treatment of thromboembolic blockages in the blood vessels such as acute myocardial infarction.

The NMR spectra of SK and the crystal structure of the catalytic domain of HPlm (μPlm) complexed with SK demonstrate three structurally autonomous domains in SK (4, 5). Based on the functional studies of truncated SK peptides, we demonstrated that SK-(158–414) in complex with equimolar HPlg could form amidolytically active virgin enzyme (6). The NH₂-terminal domain of SK, SK-(16–251), could interact with substrate HPlg and render it to be activated by HPlg:SK activator complex more effectively (7). We also demonstrate that HPlg that binds to the COOH-terminal domains of SK functions as an enzyme to catalyze the conversion of substrate HPlg that binds to the NH₂-terminal domain of SK to HPlm (7). The binding of HPlg and SK is very complex since HPlg consists of five kringle domains and a catalytic domain which have different affinity for SK (7). The crystal structure of SK and μPlm complex revealed that SK consists of three independent domains, α, β, and γ, starting from the NH₂ terminus of SK (5). Many interaction sites are proposed based on the crystal structure of SK:μPlm complex. Extensive charged and hydrophobic interactions between the SK γ-domain especially the major coiled coil region and the strands of β₁ and β₂ with μPlm are observed (5). Direct assistance by the SK β-domain in the docking and processing of substrate HPlg by the activator complex have been suggested (8).

The activation mechanism of HPlg by SK involving multiple interaction steps was suggested (7, 9–15). SK forms a stable one to one complex with HPlg and induces a conformational change of HPlg to become a catalytically active enzyme that is named virgin enzyme. The HPlg:SK virgin enzyme complex is converted to HPlm:SK that functions as an enzyme activator to catalyze the activation of substrate HPlg to HPlm. To be an effective HPlg activator, HPlg and SK must form a stable activator complex, which can bind to the substrate HPlg and catalyze its conversion to HPlm. Based on the studies of the truncated SK peptides, we propose that the COOH-terminal half of SK is essential for maintaining a stable HPlg:SK or HPlm:SK complex and induction of virgin enzyme activity (6, 7). On the other hand, recent studies on the mechanism by which SK forms a virgin enzyme in HPlg have led to some controversial conclusions (16, 17). The experiments with deletion of Ile⁸ of SK (ΔIle⁸-SK) and mutations of Ile⁸ of SK have demonstrated that Ile⁸ of SK is required for the nonproteolytic activation of HPlg by SK (16, 17). In this “molecular sexuality hypothesis” model, the NH₂ terminus, Ile⁸, of SK is proposed to form a salt bridge with Asp⁷⁴⁰ of HPlg, which triggers a conformational change to produce an active site in the HPlg...
moiety, and thereby plays an essential role in the induction of virgin enzyme activity. It is most likely that multiple binding or contact sites between SK and HPlg are required for the induction of virgin enzyme activity as proposed by the “binding activation” model of HPlg activation (6, 7, 18). The elimination of one of the possible binding sites, such as ΔLe1-SK, may impair the induction of virgin enzyme activity (16). In this study, we identified possible binding sites, such as HPlg activation (6, 7, 18). The elimination of one of the

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\text{FIG. 1. Activation of HPlg by SK mutants. HPlg (0.5 μM) was}
\text{activated by incubation with a catalytic amount (0.005 μM each) of}
\text{native SK, wild-type SK (16–378), SK(16–378), SK-282Ala, SK(278–303Ala),}
\text{SK(278–303Ala) or SK(278–303Ala) at 37 °C in 50 mM Tris, pH 7.4, containing 0.5}
\text{mM S-2251 as a substrate. The change in absorbance at 405 nm was}
\text{monitored with a Hitachi 330 spectrophotometer.}
\]

\[
\text{TABLE I}
\]

Sequence of PCR primers used for the construction of SK mutants

\[
\begin{array}{|c|c|c|}
\hline
\text{Mutant} & \text{Abbreviation} & \text{Primer sequence} & \text{Restriction} \\
\hline
\text{SK(278–282Ala) } & \text{SK(278–282Ala)} & s:5'GTCCTTGCTGAGGAGGCAGGCTTGAATGATCCCTTTG3' & \text{PstI} \\
\text{SK(360–363Ala)} & \text{SK(360–363Ala)} & a:5'CTTCTTGCTGCAAGGCTGTAATTTTCCTC3' & \text{BamHI} \\
\text{SK(322–325Ala)} & \text{SK(322–325Ala)} & s:5'TTAAAGCTGGGAAGTCTCTGCTGCTG3' & \text{BamHI} \\
\text{SK(328–330Ala)} & \text{SK(328–330Ala)} & a:5'GTATAATGAGCTGCTGCTGCTGCTG3' & \text{PstI} \\
\text{SK(330–334Ala)} & \text{SK(330–334Ala)} & s:5'TTAAAGCTGGGAAGTCTCTGCTGCTG3' & \text{BamHI} \\
\text{SK(330–334Ala) } & \text{SK(330–334Ala)} & a:5'GTATAATGAGCTGCTGCTGCTGCTG3' & \text{PstI} \\
\end{array}
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\[
\text{TABLE II}
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Steady state kinetic parameters of the activation of HPlg by native SK and SK mutants

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\text{Values are the mean ± S.E. of three experiments.}
\]

\[
\begin{array}{|c|c|c|c|}
\hline
\text{Activator} & \text{Activator parameters} & \text{Activator parameters} & \text{Activator parameters} \\
\hline
\text{K_{plg}} & \text{h_{plg}} & \text{h_{plg}}/K_{plg} \\
\hline
\text{μM} & \text{min}^{-1} & \text{μM} & \text{min}^{-1} \\
\hline
\text{Native SK} & 0.21 ± 0.02 & 17.32 ± 0.88 & 82.5 \\
\text{SK(16–378)} & 0.14 ± 0.01 & 11.93 ± 0.23 & 85.2 \\
\text{SK(278–282Ala)} & 0.14 ± 0.03 & 22.90 ± 1.04 & 163.6 \\
\text{SK(360–363Ala)} & 0.12 ± 0.02 & 11.55 ± 0.94 & 96.3 \\
\text{SK(328–330Ala)} & 0.16 ± 0.32 & 4.79 ± 0.53 & 4.1 \\
\text{SK(360–363Ala)} & 0.08 ± 0.01 & 49.03 ± 0.43 & 6.1 \\
\text{SK(328–330Ala)} & 0.05 ± 0.23 & 3.68 ± 0.54 & 16.0 \\
\text{SK(330–334Ala) } & 0.06 ± 0.02 & 5.17 ± 0.15 & 5.9 \\
\text{SK(328–330Ala) } & 0.05 ± 0.23 & 4.72 ± 0.70 & 57.1 \\
\text{SK(330–334Ala) } & 0.15 ± 0.03 & 6.17 ± 0.78 & 52.2 \\
\text{SK(330–334Ala) } & 0.15 ± 0.03 & 7.14 ± 0.36 & 58.8 \\
\end{array}
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EXPERIMENTAL PROCEDURES

Materials—Enzymes used in DNA manipulation were purchased from Roche Molecular Biochemicals, New England Biolabs, Stratagene, or Promega Laboratories and were used according to the Cold Spring Harbor Manual (19) or the recommendations by the suppliers. The full-length SK gene was obtained from Streptococcus equisimilis H46A (ATCC 12449) by the amplification with polymerase chain reaction as previously reported (6). Lys-Septahrose and CNBr-activated Sepharose 4B were from Amersham Pharmacia Biotech; Macro-Prep High Q was from Bio-Rad; NH2-D-Val-Leu-Lys-p-nitroanilide (S-2251) and p-nitrophenyl-guanidinobenzoate (NPGB) were obtained from Sigma. Bis(sulfosuccinimidyl) suberate (BS3) was purchased from Pierce. Aprotinin was purchased from Roche Molecular Biochemicals. All chemicals were of the highest grade commercially available. SK antiserum was prepared in our laboratory from mice.

Preparation of HPlg, μPlg, and Native SK—HPlg was prepared from pooled human plasma as described (6, 15). Form 1 and 2 of native HPlg were separated by chromatography on Lys-Septahrose column (20, 21). HPlg was passed through an aprotinin-substituted Sepharose 4B column to remove possible trace amounts of HPlm contamination. The method of preparing aprotinin-substituted Sepharose 4B was described in a previous paper (15). Form 2 of HPlg was used throughout the experiment. Human μPlg was made by incubating HPlg with HPlm in

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an alkaline solution and purified by Lys-Sepharose and soybean trypsin inhibitor-Sepharose 4B as described in previous reports (22–24). Native SK (Behringwerke AG, Marburg, Germany) was further purified by passing it through a Blue-Sepharose CL 6B column to remove serum albumin (25).

Construction of SK Mutant Genes—Thirteen SK mutants were constructed in which clusters of two to four charged residues and a specific single charged residue in the SK molecule between residues 278 and 363 were converted to Ala. Since we have found previously that truncated wild-type SK-(16–378) could activate HPlg as efficiently as the authentic SK (6), all the SK mutants were made with the length from residues 16 to 378. Primers SK-S16, SK-AS378, and pairs of mutagenic primers (as shown in Table I) were custom synthesized by Pan Asia Hospital Supply Co. (Taiwan, Republic of China). SK-S16 and SK-AS378 were a pair of DNA primers covering the coding region of SK gene encoding amino acid residues from 16 to 378. Their specific sequences were as shown in Sequences 1 and 2,

**SEQUENCE 1**

SK-S16: 5′ TCTGTCGGATCC AG CCAATTAGTTG 3′

**SEQUENCE 2**

SK-AS378: 5′ AATGGGATCC AG CATTCTCTCCTTCTGGGTCGC 3′

For cloning convenience, BamHI recognition sequences (underlined) were created. The mutagenic sense primer (as shown in Table I), which contained a specific restriction site, and SK-AS378 primer, were used to amplify a part of SK DNA covering the mutated position to 1134 of the coding region of SK gene by a PCR technique. The mutagenic antisense primer and SK-S16 were used to amplify another part of SK DNA covering nucleotide positions 48 to mutated point of coding region of SK.
gene. These two amplified DNA fragments were purified by agarose gel electrophoresis. The mutated SK gene was constructed from these two pieces of DNA by denaturation, annealing, and extension. SK R330A,D331A,K332A,K334A (abbreviated as SK330–334Ala) gene was constructed by Exo− PCR-based Site-directed Mutagenesis kit (Stratagene). The 1.1-kilobase DNA fragment was then ligated to PCR-Scripta pl vector (Stratagene) and transformed into E. coli XL10-Gold™ Kan ultracompetent cells. The mutations were confirmed by the sequencing of the complete coding region using dye-dye sequencing techniques (26). Both strands of each mutated gene were completely sequenced to make sure no other PCR-initiated mutations had been introduced.

Expression and Purification of SK Mutants—Mutated SK gene fragments were subcloned in-frame into the overproducing plasmid pET-3a (Novagen) at the BamHI site. Transformed bacteria cells, E. coli BL21(DE3)pLysS, were grown to mid-log phase, and target gene expression was induced by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside. After 3 h, the expressing cells were harvested, washed, and disintegrated. Then the target proteins were concentrated by ammonium sulfate precipitation and purified to homogeneity by a high Q anion exchange column (27). The expressed wild-type SK (16–378) and all the SK mutant proteins were sequenced, and the results showed that an additional fusion peptide of 14 amino acid residues derived from the pET-3 plasmid and BamHI restriction site was attached at their NH₂ termini.

**Protein Concentration**—The protein concentrations were determined spectrophotometrically using the following ε values and molecular weights, respectively: HPlg, 17.0 and 94,000; μPlg, 16.0 and 28,617; SK-(16–387), 9.5 and 43,000.

**Steady State Kinetic Parameters of Activation of HPlg by SK Mutants**—A one-stage assay as described previously was used to measure HPlg activation by SK mutants (28, 29). Briefly, HPlg at final concentrations ranging from 0.04 to 4 μM was incubated with 0.5 mM S-2251 in an assay cuvette containing 150 μL of 0.05 M Tris buffer, pH 7.4, and 0.1 M NaCl. Activation was initiated by adding an SK mutant of fixed concentration, and the change in absorbance at 405 nm was monitored at 37 °C with a Hitachi 330 spectrophotometer. The increments of absorbance between 10 and 300 s after addition of SK samples were used to measure the initial rate of HPlg activation. Initial reaction rates were determined from the slopes of plots of absorbance versus t², and double-reciprocal plots were then constructed. HPlg activation parameters, Kₚ (the apparent Michaelis constant for the HPlg substrate) and kₚ (the catalytic rate constant of activation), were calculated as described by Wohl et al. (28). The ε₄₀₅ at 405 nm employed for p-nitroanilide was 9559.

**Active Site Titration**—The generation of active sites by SK mutants in HPlg was determined by active site titration using the fluorogenic substrate 4-methylumbelliferyl β-guadinobenzoate (MUGB, Fluaka) in a Hitachi 850 fluorescence spectrophotometer as described (9, 30–32). Briefly, HPlg (100 nM) was added to a cuvette containing 1 μM of MUGB in 50 mM Tris-HCl, 0.15 mM NaCl, pH 7.4, at 25 °C. After 2 min, native SK (100 nM) or SK mutants (100 nM) or buffer alone was added, and the increment of fluorescence was monitored at extinction wavelength 365 nm and emission wavelength 445 nm. The stock solution of MUGB (1 mM) was prepared in NN-dimethylformamide before use and was diluted to 1 mM with the titration buffer.

**Discontinuous Assays of Amidolytic Activity for Determination of the Maximal Formation of HPlg-SK Complex**—The HPlm-free HPlg samples used in this experiment were prepared either by passing HPlg samples through an aprotinin column or by pretreatment with NPGB immediately before experiments as described previously (10) to remove possible trace amounts of HPlm contamination. Incubations of equimolar HPlg and SK mutant proteins (final concentration, 2 μM) were carried out at 25 °C in 10 mM Hepes/NaOH, pH 7.4. Aliquots were removed at various intervals to assay the amidolytic activity and also for SDS-PAGE analysis. The amidolytic activity was measured by adding aliquots of the HPlg-SK proteins (final concentration, 0.2 μM) in an assay cuvette containing 0.5 mM S-2251 in 0.05 M Tris buffer, pH 7.4, and 0.1 mM NaCl. The absorbance at 405 nm was monitored between 10 and 80 s. The initial reaction rate was calculated, and the duration to achieve maximal amidolytic activity was determined as described by Chibber et al. (35).

**Activation of HPlg by Catalytic Amounts of SK Proteins**—A one-stage assay as described previously was used to measure HPlg activation by SK proteins (28, 29). Human Glu-plasminogen (Glu-Plg) (0.5 μM) was activated by incubation with a catalytic amount of SK protein (0.005 μM) at 37 °C in 50 mM Tris, 0.1 mM NaCl, pH 7.4, containing 0.5 mM S-2251 as a substrate. The change in absorbance at 405 nm was monitored with the Hitachi 330 spectrophotometer. For the assay of activation of HPlg by a catalytic amount of HPlm-SK complex, equimolar HPlm and SK (0.125 μM each) were premixed at 4 °C for 2 min. HPlg (0.5 μM) was activated by incubation with a catalytic amount of the preformed HPlm-SK complex (0.005 μM) at 37 °C in 50 mM Tris, 0.1 mM NaCl, pH 7.4, containing 0.5 mM S-2251 as a substrate. The change in absorbance at 405 nm was monitored as described.

**SDS-PAGE Analysis**—Protein samples were subjected to SDS-PAGE according to the method of Laemmli (34).

**Amino Acid Sequence Analysis**—The amino acid sequences of the SK fragments were determined by Edman degradation in an Applied Biosystems Sequencer (model 477A).
protein at a concentration of 5 μM were mixed at room temperature for 2 min and followed by reaction with 0.1 mM BS3 as cross-link reagent. After 60 min, the reaction was stopped by adding ethanolamine (8 mM), N-ethylmaleimide (3 mM) in sodium phosphate buffer (8 mM, pH 7.4). Parallel samples containing either μPlg or SK were used as controls. The samples were then subjected to SDS-PAGE in 10% acrylamide according to the method of Laemmli (34). The electrophoresed proteins on the gel were transferred onto Immobilon-P transfer membrane (Millipore), stained with Amido Black and Western blotting with SK antisera (35). The relative amount of the cross-linked product was determined by scanning the Amido Black-stained protein bands by a Bio-Imaging analyzer (Fuji, Japan) with a computer program MacBAS version 2.4.

RESULTS

Thirteen mutants of recombinant SK in which clusters of two to four charged residues and a specific single charged residue were converted to Ala were prepared using the primers shown in Table I. All the SK mutants were made with the length from residues 16 to 378 since wild-type SK (16–378) could activate HPlg as efficiently as the authentic SK (6). DNA sequencing confirmed the expected nucleotide sequence changes. Homogeneous SK proteins were obtained after ammonium sulfate precipitation and high Q column chromatography. All the SK mutant proteins had the expected molecular mass of 43 kDa as analyzed by SDS-PAGE.

HPlg activation assay by a catalytic amount of SK mutants was performed to determine which mutation might affect the ability of SK to form a functional HPlg activator complex with HPlg (Fig. 1). Five SK mutants, SKD276RA,K279A,K281A,K282A (abbreviated as SK(275–282Ala)), SKD360A,R363A (SK(360–363Ala)), SKD331A, SKK332A, and SKK334A induced rapid activation of HPlg to HPlm as efficiently as the wild-type SK (16–378). Kinetic assays revealed that the catalytic efficiencies (kplg/Kplg) for HPlg activation of complexes of HPlg with SK(278–282Ala), SK(360–363Ala), SKD331A, SKK332A, SKK334A, and SKK334A were comparable with wild-type SK (16–378). In contrast, kplg/Kplg values of SK(328–330Ala), SKD325A, and SKD330A were 14–21-fold lower than that of wild-type SK (16–378), whereas kplg/Kplg value of SKD325A was 5-fold lower (Table II). In complex with SK, HPlg undergoes a conformational change and become catalytically active without cleavage of the activating peptide bond, which is the so-called virgin enzyme. The rate of HPlg activation could be slowed down by lowering the reaction temperature to 25 °C, and the virgin enzyme could be observed. To determine whether these SK mutants could form a virgin enzyme in the HPlg:SK complex, the active site generation was monitored in the presence of an acylating agent MUGB that could also inhibit any trace amounts of HPlm, as originally described by McClintock and Bell (9). Wild-type SK-(16–378) could generate an active site in HPlg in a one-to-one equimolar complex at 25 °C (Fig. 2A). Native SK could generate an active site in HPlg more rapidly than wild-type SK (16–378) (Fig. 2A). In contrast, no active site was titrated in equimolar mixtures of HPlg with SK(322–325Ala), SK(328–330Ala), SKD322A, SKD325A, and SKD330A (Fig. 2A). SKD323A, SKK331A, SKK332A, and SKK334A in reaction with equimolar HPlg induced rapid formation of enzyme active sites, whereas SKR324A and SKD325A showed a greater delay than wild-type SK (16–378) in the generation of active sites (Fig. 2A). To investigate further the interaction of HPlg and SK mutants, amidolytic activity measurement (Fig. 2B) and SDS-PAGE analysis (Fig. 2C) were performed to characterize the reaction products of equimolar incubation of HPlg and SK in the absence of acylating agent MUGB. HPlg samples used in this experiment were prepared by either passing through an aprotinin column or pretreatment with NPGB to remove trace amounts of HPlm as described, and the HPlg sample prepared with a lag phase slightly longer than that of the wild-type SK (16–378) (Fig. 1). Parallel samples containing either M of SKD322A (to make a HPlg:SKD322A ratio of 2:1) or with 1 μM of SKD322A (to make a HPlg:SKD322A ratio of 2:1) at 25 °C in 50 mM Tris, 0.1 mM CaCl2, pH 7.4, using S-2251 at a final concentration of 0.5 mM (C). HPlg:SKD322A ratio of 1:2 preincubated at 25 °C for 2 min and followed by reaction with 0.1 mM BS3 as cross-link reagent. After 60 min, the reaction was stopped by adding ethanolamine (8 mM), N-ethylmaleimide (3 mM) in sodium phosphate buffer (8 mM, pH 7.4). Parallel samples of A were preincubated at 25 °C for 2 min and shifted to 37 °C for further incubation and for SDS-PAGE analysis (A). Parallel samples of A were preincubated at 25 °C for 2, 4, and 7 min, and aliquots (10-fold dilution) were removed for assay of amidolytic activity at 37 °C in 50 mM Tris, 0.1 mM CaCl2, pH 7.4, using S-2251 at a final concentration of 0.5 mM (C). HPlg:SKD322A ratio of 1:2 preincubated at 25 °C for 2 min (○); 4 min (□); and 7 min (△). HPlg:SKD322A ratio of 2:1 preincubated at 25 °C for 2 min (●); 4 min (▲); and 7 min (▲).
by the two methods gave the similar result on the amidolytic activity measurement. Wild-type SK-(16–378) could form an amidolytically active enzyme with HPlg in an equimolar complex at 25 °C (Fig. 2B). No cleavage of the activating peptide bond, Arg351–Val362 was observed at up to 4 min determined at intervals in a parallel experiment as shown by SDS-PAGE analysis, but hydrolysis of S-2251 was observed (Fig. 2, B and C). After reaction for more than 7 min, some HPlg was hydrolyzed, and heavy and light chains of HPlm were detected, and some SK-(16–378) was degraded to a 36-kDa peptide (Fig. 2C). The 36-kDa fragment of SK was SK-(60–378) as determined by NH2-terminal amino acid sequence analysis and our previous finding (15). Native SK could develop an amidolytically active complex with HPlg more rapidly than wild-type SK-(16–378) (Fig. 2B). However, no enzymatic activity was observed in equimolar mixtures of HPlg with SK(322–325Ala), SK(328–330Ala), SKD322A, SKD325A, and SKD323A (Fig. 2B), and no cleavage of either HPlg or SK was observed as shown in SDS-PAGE analysis (Fig. 2C). Amidolytic activity could be observed rapidly in equimolar mixtures of HPlg with SKP324A, and HPlg was converted to HPlm (Fig. 2, B and C). At the same time, SKK332A, and SKD325A were degraded to peptide fragments of molecular masses less than 36 kDa as HPlm appeared (Fig. 2C). These two SK mutants were extensively degraded to smaller peptide fragments at longer incubation periods (Fig. 2C). The SK peptide fragments with molecular masses less than 30 kDa had very little or no HPlg activator activity (15). As a consequence, low HPlg activator activities of SKP324A, and SKD325A were observed (Fig. 1). HPlg was converted to HPlm, and amidolytic activity was observed as soon as HPlm was formed in equimolar mixtures of HPlg with SKD322A, SKD323A, and SK(330–334Ala) (Fig. 2, B and C). Less degradation of these SK mutants was observed, and the major SK degradation product was a 36-kDa fragment (Fig. 2C). SKP321A, SKK326A, and SKD344A in reaction with equimolar HPlg induced rapid formation of amidolytic activity similar to that of SKD325A and SKD322A (Fig. 2B). However, the patterns of SK degradation products were different. Less degradation of SKD321A, SKK326A, and SKD344A with the major SK degradation product of 36-kDa fragment was observed (Fig. 2C).

The stability HPlg and SK complex is essential for it to be an effective HPlg activator, since the effective concentration of the activator complex is dependent on the stability of the complex. Dissociation of SK from the complex might render it to be degraded by HPlm and consequently lose HPlg activator activity. The affinity and stability of the equimolar µPlg-SK complex could be analyzed by the cross-linked products of the one-to-one µPlg and SK mixture after reaction with BS and separated by SDS-PAGE. A protein band of 70 kDa corresponding to µPlg-SK cross-linked product was observed in the incubations of µPlg with wild-type SK (16–378), SK(328–330Ala), SKD322A, SKD325A, and SKD323A (Fig. 3, A–C). Reduced amount of the 70-kDa cross-linked product was observed in SKD321A (−67% of the wild-type), SKD322A, and SKD323A (−40% of the wild-type), and SK(322–325Ala), SK(330–334Ala), SKD324A, and SKD326A (≤20% of the wild-type) (Fig. 3, A–C). SKK326A and SKD325A were extensively degraded in the incubations with µPlg as shown in the Western blot analysis (Fig. 3B).

SKD322A could not form virgin enzyme with HPlg; however, it induced rapid HPlg activation (Figs. 1 and 2A). To determine the mechanism responsible for the observation, we performed an experiment in which HPlg was incubated with either 2-fold (to make a HPlg:SK ratio of 1:2) or ½-fold (to make a HPlg:SK ratio of 2:1) concentration of SKD322A, and the amidolytic activities and SDS-PAGE of the incubations were determined. A HPlg:SK ratio of 1:2 is to ensure the formation of a one-to-one HPlg-SK complex, and no free HPlg existed, whereas a HPlg:SK ratio of 2:1 is to increase the chance for the formation of a HPlg-SK-HPlg ternary complex. No cleavage of either HPlg or SK was observed as shown in SDS-PAGE analysis (Fig. 4C). Amidolytic activity could be observed rapidly in equimolar mixtures of HPlg with SKP324A, and HPlg was converted to HPlm (Fig. 2, B and C). However, the patterns of SK degradation products were different. Less degradation of these SK mutants was observed, and the major SK degradation product was a 36-kDa fragment (Fig. 2C). SKP321A, SKK326A, and SKD344A in reaction with equimolar HPlg induced rapid formation of amidolytic activity similar to that of SKD325A and SKD322A (Fig. 2B). However, the patterns of SK degradation products were different. Less degradation of SKD321A, SKK326A, and SKD344A with the major SK degradation product of 36-kDa fragment was observed (Fig. 2C).

The stability HPlg and SK complex is essential for it to be an effective HPlg activator, since the effective concentration of the activator complex is dependent on the stability of the complex. Dissociation of SK from the complex might render it to be degraded by HPlm and consequently lose HPlg activator activity. The affinity and stability of the equimolar µPlg-SK complex could be analyzed by the cross-linked products of the one-to-one µPlg and SK mixture after reaction with BS and separated by SDS-PAGE. A protein band of 70 kDa corresponding to µPlg-SK cross-linked product was observed in the incubations of µPlg with wild-type SK (16–378), SK(328–330Ala), SKD322A, SKD325A, and SKD323A (Fig. 3, A–C). Reduced amount of the 70-kDa cross-linked product was observed in SKD321A (−67% of the wild-type), SKD322A, and SKD323A (−40% of the wild-type), and SK(322–325Ala), SK(330–334Ala), SKD324A, and SKD326A (≤20% of the wild-type) (Fig. 3, A–C). SKK326A and SKD325A were extensively degraded in the incubations with µPlg as shown in the Western blot analysis (Fig. 3B).
The distances between oxygen atom of Lys334 are 2.14 and 2.06 Å, respectively. The pairs of charged amino acid residues are within the distance of potential hydrogen bond formation. Therefore, HPlm

FIG. 6. Ribbon diagram of SK-μPlm binary complex. A, overall structure of the complex. The α-helix, β-strands, and loop region of μPlm are shown in red, blue, and white, respectively. The SK is shown in yellow, and the residues 314–342 of the coiled coil region in SK γ domain are shown. B, potential interactions between Lys332 of SK and Glu623 of μPlm and intramolecular interactions among the residues in the coiled coil region of SK γ-domain are shown. Lys332 of SK forms potential hydrogen bond with Glu623 of μPlm. The distance between the ε-amino group of Lys332 and the γ-carboxyl group of Glu623 is 2.03 Å. The distances between oxygen atom of γ-carboxyl group of Asp328 and nitrogen atom of α-amino group of Arg330 and those between Asp331 and Lys334 are 2.14 and 2.06 Å, respectively. The pairs of charged amino acid residues are within the distance of potential hydrogen bond formation. C, interaction between Asp331 and Lys334 is shown. The distance between oxygen atom of γ-carboxyl group of Asp328 and nitrogen atom of α-amino group of Lys334 is 1.92 Å (adapted from Wang et al. (5)).

(16–378) and SK_D322A (Fig. 5B). The amidolytic activity of HPlm-SK_K332A increased compared with that of HPlm only (Fig. 5B). Therefore, HPlm-SK_D322A can serve as a HPlg activator even though SK_D322A cannot generate an active site in HPlg-SK complex.

The interactions of the coiled coil region in the γ-domain of SK and that with HPlg are very important in the activation of HPlg by SK. The coiled coil region of SK is intercalated between the calcium-binding loop and the activation loop of HPlg (Fig. 6A). Potential hydrogen bonds between side chains of SK Asp328 and Arg330, and between side chains of SK Asp331 and Lys334 could affect the stability of the coiled coil structure of SK and the activator activity (Fig. 6B). SK Lys334 also forms hydrogen bond with SK Asp322 (Fig. 6C). SK Lys332 is involved in potential hydrogen bond interaction with Glu623 of μPlm, which may play an important role in the formation of HPlg and SK complex (Fig. 6D). Thus, mutations of these amino acids to Ala will disrupt the potential hydrogen bonds or possible contacts and may cause instability of the coiled coil conformation of SK and also interfere with the interaction with HPlg.

DISCUSSION

In the present study, the amino acid residues in the SK γ-domain, which are involved in interaction with HPlg, were investigated by construction of mutants in which clusters of two to four charged amino acids and a specific single charged residue were mutagenized to Ala. The formation of the HPlg and SK complex, the ability to induce virgin enzyme activity, and the HPlg activator activity of the SK mutants were evaluated. This kind of approach has been used to study the structure-function relationships in other HPlg activators, e.g. staphylokinase (36), tissue-type plasminogen (Plg) activator (37), and urokinase-type Plg activator (38).

The interactions of the coiled coil region in the SK γ-domain with HPlg are very important in the activation of HPlg by SK. The coiled coil region of SK is intercalated between the calcium-binding loop and the activation loop of HPlg (Fig. 6A). Potential hydrogen bonds between side chains of SK Asp328 and Arg330, and between side chains of SK Asp331 and Lys334 could affect the stability of the coiled coil structure of SK and the activator activity (Fig. 6B). SK Lys334 also forms hydrogen bond with SK Asp322 (Fig. 6C). SK Lys332 is involved in potential hydrogen bond interaction with Glu623 of μPlm, which may play an important role in the formation of HPlg and SK complex (Fig. 6D). Thus, mutations of these amino acids to Ala will disrupt the potential hydrogen bonds or possible contacts and may cause instability of the coiled coil conformation of SK and also interfere with the interaction with HPlg.

Thirteen SK mutants were prepared, two of which had the mutations outside the coiled coil region in the SK γ-domain. One mutant, SK(360–363Ala), had the similar HPlg activator activity as wild-type SK, and the other mutant, SK(278–282Ala), had even better activator activity (Table II) than the wild-type SK. These results are consistent with the observations by x-ray crystallography that there is no direct contact by these amino acids with μPlm, which functions as an activator enzyme (5). These amino acid residues may not be involved in the substrate HPlg binding either since the Kplg values of these SK mutants do not show a significant change (Table II).

SK mutants with mutations of charged amino acid residues to Ala in the coiled coil region of the SK γ-domain resulted in significant changes in the functions of SK. Two SK mutants with multiple charged amino acids to Ala mutations in the coiled coil region, including SK(322–325Ala) and SK(330–334Ala), failed to activate HPlg (Fig. 1). This result is consistent with the previous report that the ability of rSK_K332A,K334A to activate or cleave HPlg was reduced by 34-fold (39). Both SK(322–325Ala) and SK(330–334Ala) had reduced capability to form a stable complex with μPlg and had the least HPlg activator activity (Fig. 1 and Fig. 3). On the other hand, SK(328–330Ala) could form a stable complex with μPlg and activate HPlg with a lag time and with the catalytic efficiency 21-fold lower than that of wild-type SK-(16–378) (Fig. 1, Fig. 3, and Table II). It appears that the groups of charged amino acids in the coiled coil region of SK play important but different roles in the activation of HPlg.

We further analyzed the effect of mutation of single charged amino acid to Ala in the SK coiled coil region on the activation of HPlg. The mutants could be classified into three different categories according to their effects on the interactions with HPlg (Table III). The SK mutants, including SK_K332A, SK_D322A, SK_D331A, SK_K332A, and SK_K334A, had reduced capability to form a complex with μPlg (Fig. 3 and Table III). SK_K324A and SK_D325A had a markedly reduced HPlg activator activity (Fig. 1), and the SK molecule in the
one-to-one HPlg:SK mixture was rapidly degraded to smaller peptides with molecular masses less than 30 kDa (Fig. 2C). However, SKK332A, SKK334A, and SKK334A in the one-to-one HPlg:SK mixture were degraded to a 36-kDa peptide that was stable for at least 20 min (Fig. 2C). Arg324 and Asp325 in the SK γ-domain may play important roles in the formation of a stable complex with μPlm, although no direct interaction of these residues with μPlm was observed in the crystal structure of the complex (5). The possibility that mutations of these two residues may disturb the coiled coil structure cannot be excluded. SK mutants that cannot form stable complex with HPlg tend to be degraded fast in the presence of HPlm and lose their activator activity. Mutations at Lys332 or Lys334 also caused decrease of μPlg:SK complex formation. However, in reaction with HPlg, SKK332A or SKK334A was degraded much slower, and the major SK degradation product was a 36-kDa fragment, which still had the HPlg activator activity (15). Mutations at Lys332 or Lys334 would prevent the cleavage of the peptide bonds of 332–333 or 334–335. The stability of the complex of μPlg with SKK332A, or SKK334A was declined; however, they still could activate HPlg because the slow inactivation of SK molecule may compensate for the effect of loss of the stability of the complex. SK(330–334Ala) with combined mutation at these residues will result in a further decrease in the capability of complex formation with HPlg and the loss of activator activity to a greater extent.

Mutations at Asp322 and Arg330 belong to another category. SKD325A, SKR332A, and SK(328–330Ala) formed stable complexes with μPlg as with wild-type SK (Fig. 3). However, no virgin enzyme activity was detected in equimolar mixture of HPlg and SK(328–330Ala) and SKD325A or SKR332A (Fig. 2A), and a delayed amidolytic activity was observed as soon as HPlm was formed in equimolar mixtures of HPlg with SKD325A or SKR332A (Fig. 2B and C). These mutants also had a lag period in initiation as well as lower efficiency in HPlg activation (Fig. 1 and Tables II and III). SK, on binding to HPlm, simply modifies the substrate specificity of the enzyme HPlg (40). HPlm:SK is an efficient Plg activator, although HPlm itself has no such activity. The catalytic activities of HPlm:SK and HPlm:SK, as measured by the rate of hydrolysis of small peptide substrate, were similar and were higher than that of HPlm alone (Fig. 5B). However, the activation of HPlg by SKR332A or preformed HPlm:SK complex was slower than the wild-type SK (Fig. 1 and Fig. 5A). The Kplg of HPlg activation was significantly higher for SKR332A and SK(328–330Ala) (Table II), suggesting that Arg330 may be involved in binding of substrate HPlg. Therefore, mutation at Arg330 will reduce its HPlg activator activity and impair the virgin enzyme induction, although it could form a HPlg:SK complex that was proteolytically resistant. Mutation at Asp328 had similar effects as that of Arg330, although SKD328A had a relatively higher efficiency in HPlg activation.

SKD322A is another very unique SK mutant that can form a stable complex with μPlg with no virgin enzyme activity (Fig. 2 and Fig. 3). However, SKD322A could activate HPlg as efficiently as wild-type SK with a delay of onset of HPlg activation (Fig. 1). The delayed time was reduced if the complex of HPlm and SKD322A was used as the activator (Fig. 5A). The delayed activation was observed in the activation of HPlg by staphylokinase which could not form a virgin enzyme with HPlg, and HPlg-staphylokinase complex cannot activate HPlg (36, 41). Mutation at Asp322 only inhibits the virgin enzyme formation, but the complex stability and activator activity are not compromised. Thus, Asp322 should be very essential for induction of the virgin enzyme activity but is not for the complex stability of SK and μPlg. The result of the 2:1 ratio of HPlg:SK incubation (Fig. 4, B and C) indicated that if HPlg:SKHPlg ternary complex was formed, the HPlg could be converted to HPlm directly without the formation of the virgin enzyme. The observation is consistent with the proposal that the close proximity of two HPlg molecules in the HPlg:SK:μPlg complex may accelerate the autoactivation of HPlg forming HPlm:SK without the prior formation of HPlg-γ-SK virgine enzyme complex (16). The side chain of Asp322 forms a potential hydrogen bond with the α-amino group of Lys334 to form a loop (Fig. 6C). Kinetic analysis of HPlg activation by SK mutants (Table II) showed that both the Kplg and kplg of SKD322A were higher than those of wild-type SK, probably because no virgin enzyme was involved in the activation of HPlg by SKD322A, and the activation might not follow the same reaction steps.

In conclusion, the functional studies of SK mutants demonstrate that amino acid residues in the coiled coil region of SK (Leu314–Ala342) are involved in virgin enzyme induction, stability of the HPlg:SK complex, and the activation reaction. Mutations at Arg324, Asp325, Lys332, and Lys334 to Ala reduced the formation of the HPlg:SK complex. Mutation at Asp322 to Ala abolished virgin enzyme activity but had no effect on HPlg activation. Mutations at Asp328 and Arg330 resulted in impairment of virgin enzyme formation, lower efficiency of HPlg activation without affecting the formation of μPlg:SK complex. Therefore, the amino acid residues Arg324, Asp325, Lys332, and Lys334 to Ala reduced the formation of the HPlg:SK complex. Mutation at Asp322 to Ala abolished virgin enzyme activity but had no effect on HPlg activation. Mutations at Asp328 and Arg330 resulted in impairment of virgin enzyme formation, lower efficiency of HPlg activation without affecting the formation of μPlg:SK complex. Therefore, the amino acid residues Arg324, Asp325, Lys332, and Lys334 to Ala reduced the formation of the HPlg:SK complex. Mutation at Asp322 to Ala abolished virgin enzyme activity but had no effect on HPlg activation. Mutations at Asp328 and Arg330 resulted in impairment of virgin enzyme formation, lower efficiency of HPlg activation without affecting the formation of μPlg:SK complex. Therefore, the amino acid residues Arg324, Asp325, Lys332, and Lys334 to Ala reduced the formation of the HPlg:SK complex. Mutation at Asp322 to Ala abolished virgin enzyme activity but had no effect on HPlg activation. Mutations at Asp328 and Arg330 resulted in impairment of virgin enzyme formation, lower efficiency of HPlg activation without affecting the formation of μPlg:SK complex. Therefore, the amino acid residues Arg324, Asp325, Lys332, and Lys334 to Ala reduced the formation of the HPlg:SK complex. Mutation at Asp322 to Ala abolished virgin enzyme activity but had no effect on HPlg activation. Mutations at Asp328 and Arg330 resulted in impairment of virgin enzyme formation, lower efficiency of HPlg activation without affecting the formation of μPlg:SK complex. Therefore, the amino acid residues Arg324, Asp325, Lys332, and Lys334 to Ala reduced the formation of the HPlg:SK complex. Mutation at Asp322 to Ala abolished virgin enzyme activity but had no effect on HPlg activation. Mutations at Asp328 and Arg330 resulted in impairment of virgin enzyme formation, lower efficiency of HPlg activation without affecting the formation of μPlg:SK complex. Therefore, the amino acid residues Arg324, Asp325, Lys332, and Lys334 to Ala reduced the formation of the HPlg:SK complex. Mutation at Asp322 to Ala abolished virgin enzyme activity but had no effect on HPlg activation. Mutations at Asp328 and Arg330 resulted in impairment of virgin enzyme formation, lower efficiency of HPlg activation without affecting the formation of μPlg:SK complex. Therefore, the amino acid residues Arg324, Asp325, Lys332, and Lys334 to Ala reduced the formation of the HPlg:SK complex. Mutation at Asp322 to Ala abolished virgin enzyme activity but had no effect on HPlg activation. Mutations at Asp328 and Arg330 resulted in impairment of virgin enzyme formation, lower efficiency of HPlg activation without affecting the formation of μPlg:SK complex. Therefore, the amino acid residues Arg324, Asp325, Lys332, and Lys334 to Ala reduced the formation of the HPlg:SK complex. Mutation at Asp322 to Ala abolished virgin enzyme activity but had no effect on HPlg activation. Mutations at Asp328 and Arg330 resulted in impairment of virgin enzyme formation, lower efficiency of HPlg activation without affecting the formation of μPlg:SK complex. Therefore, the amino acid residues Arg324, Asp325, Lys332, and Lys334 to Ala reduced the formation of the HPlg:SK complex. Mutation at Asp322 to Ala abolished virgin enzyme activity but had no effect on HPlg activation.
and Arg^{330}, Asp^{331}, and Lys^{334}, and Asp^{322} and Lys^{334} are also noticed (Fig. 6, B and C). The conformation of the coiled coil region of SK γ-domain is essential in stabilizing the formation of HPlg:SK complex and in inducing the virgin enzyme. Studies of the interaction between SK α- and β-domains and HPlg are in progress. This study will lead to identifying the essential amino acid residues in SK molecule as a Plg activator and better understanding of the reaction mechanism. This knowledge may be applied to create an SK molecule with designed properties for improving the clinical application of SK as a thrombolytic agent.

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