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Specificity Role of the Streptokinase C-Terminal Domain in Plasminogen Activation

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Several pathogenic bacteria secrete plasminogen activator proteins. Streptokinase (SKe) produced by *Streptococcus equisimilis* and staphylokinase secreted from *Staphylococcus aureus* are human plasminogen activators and streptokinase (SKu), produced by *Streptococcus uberis,* is a bovine plasminogen activator. Thus, the fusion proteins among these activators can explain the function of each domain of SKe. Replacement of the SKα domain with staphylokinase donated the staphylokinase-like activation activity to SKe, and the SK$\beta\gamma$ domain played a role of nonproteolytic activation of plasminogen. Recombinant SKu also activated human plasminogen by staphylokinase-like activation mode. Because SKu has homology with SKe, the bovine plasminogen activation activities of SKe fragments were checked. SKe$\beta\gamma$ among them had activation activity with bovine plasminogen. This means that the C-terminal domain (γ-domain) of streptokinase determines plasminogen species necessary for activation and converses the ability of substrate recognition to human species. © 2002 Elsevier Science

Key Words: **streptokinase; staphylokinase; plasminogen; plasmin; activation; fusion; specificity; domain; α_2-antiplasmin; fibrin.**

Activation of human plasminogen have been found to be present in a variety of sources. Because the urokinase-type plasminogen activator and tissue-type plasminogen activator are serine proteases that act directly on the peptide bond of Arg560–Val561 of plasminogen, they activate all plasminogens of mammalian species (1). In contrast, bacteria-originated activators show selective scope in activation of mammalian plasminogens (2). The species specificity of staphylokinase (SAK) in plasminogen activation is similar to that of streptokinase (SKe) (414 residues) secreted by *Streptococcus equisimilis* (3). The size of SKe is three times larger than that of SAK (136 residues) and SKe has three independent domains (SKα, SKβ, and SKγ domain). Moreover, the three-dimensional structure of SAK is similar to that of SKα domain (4, 5). Thus it has been thought that the different features of SAK are due to the lack of two domains ($\beta\gamma$ domain) of SKe.

Recently, a plasminogen activator (SKu) secreted from *Streptococcus uberis* was purified and cloned (6, 7). The plasminogen activator gene encodes a protein consisting of 286 amino acids including a signal peptide of 25 amino acids. The amino acid sequence of the protein showed weak homology (23.5–28%) to SKe (6). Additionally, The molecular size of SKu (261 residues) is about two times larger than that of SAK and two-third of SKe. Especially, the major differences between SKe and SKu are apparently the deletion of the C-terminal domain possessed by SKe and the lack of identity in the N-terminal part (residues 1 to 64 of SKe) (6).

In summary, three activators show a tendency to increase in their molecular size by one domain unit of SKe from SAK to SKe via SKu. These features suggest that a common ancestor gene, whose product activated all plasminogen molecules, has evolved into three or more differentiated activators. To prove this hypothesis, in first, the fusion proteins were constructed. The crystal structures of SKe and SAK deduced many common features between the two activators (8). Thus, the replacement of α domain of SKe with SAK resulted in the rational fusion proteins. In second, recombinant SKu and the gene fragment encoding the $\alpha\beta$ domain of SKe were produced, and the activities of plasminogen activation of them were checked. This work may explain the role of the α and γ domain of SKe in species specificity and the possibility of evolutionary relationship between bacterial plasminogen activators.

MATERIALS AND METHODS

Materials. For expression of bacterial activators, pKK-*ompA* was developed for secretion of gene products into the periplasm of *Esch-*

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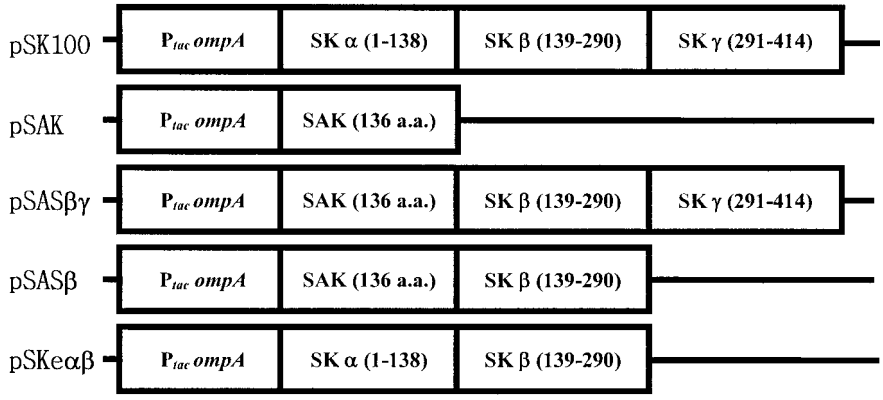

FIG. 1. Construction of recombinant plasminogen activators. pSK100 and pSAK are the expression plasmids of SKe and SAK, respectively. The N-terminal amino acids (Ile and Ser in SKe and SAK, respectively) were inserted behind the alanine residue of OmpA signal sequence which is a cleavage site recognized by signal peptidase after translocation into periplasm of *E. coli* JM109.

erichia coli in our laboratory. pSK100 is an SKe expression plasmid in which the *ske* (gene encoding the mature region of SKe) was inserted into the downstream of *ompA* signal sequence of pKK-*ompA* (9). The *ske* was cloned in our laboratory from *Streptococcus equisimilis* ATCC9542 into *E. coli* by short gun cloning (10). The *sak* (gene encoding mature region of SAK) from *Staphylococcus aureus* NCTC10033 was fused with *ompA* signal sequence of pKK-*omp*A at the *Hin*dIII site (11). The recombinant expression plasmid, pSAK (11), was used for expression of SAK in *E. coli* strain JM109 [*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi*Δ*(lac-proAB)*]. The *sku* (gene encoding mature region of SKu) from *Streptococcus uberis* NCTC3858 was amplified by PCR with the primer pair, pSKUNA (5′-GCGATGGCCATAACCGGTTATGATTCCGAC-3′) and pSKUCB (5′-TCCGGATCCTTAAGGTTTATAACTTTTTTT-3′) containing the *Msc*I and *Bam*HI sites, respectively. The *Msc*I- and *Bam*HI-digested gene amplified by PCR was ligated to the linearized pET-22b(+) vector (Novagen, Co., Madison, WI) by *Msc*I and *Bam*HI and the resulting plasmid, pEP10, was delivered to *E. coli* BL21(DE3) [*hsdS gal(*λ*cIts857 ind1 Sam7 nin5 lacUV5-T7 gene1*)] strain for expression of *sku*.

Construction of expression plasmids for staphylokinase-streptokinase fragment fusion proteins. The domain fusions between SKe and SAK were performed. The former SAK fusion partner was amplified from pSAK employing two oligomers, pSAKfu (5′-CATGCA-CCCGGGTTTCTTTTCTATAACAACCTTTGTAAT-3′) having *Xma*I site and pSAKr (5′-TATAATGTGTGGAATTGTGAGCGGATAACA-3′). The amplified SAK partner was subcloned into the *Xma*I–*Eco*RI site of pUC19. The latter SKe$\beta\gamma$ partner, a gene of SKeβ and SKeγ domain, was amplified from pSK100 with two oligomers pSKfuxf (5′-CTAAGCCCCGGGGTGCGCGTTAGACCATATAAAGAAAAA-3′) containing *Xma*I site and pSKfupr (5′-TTTAGAAGACTGCAGTTA-TTTGTCGTTAGGGTT-3′) containing the *Pst*I site. The SKe$\beta\gamma$ partner was fused to the 3′ end of SAK partner in pUC19. Then, the fused DNA was subcloned into pKK223-3 plasmid for the construction of pSAS$\beta\gamma$ (Fig. 1). The fusion of SAK and SKeβ domain was isolated and amplified from pSAS$\beta\gamma$ by two oligomers, pSAKf (5′-TAT-AATGTGTGGAATTGTGAGCGGATAACA-3′) and pSKrb (5′-ATC-AGAATTCTTTTACCCTTTTTTAAGGACGTAATA-3′). PCR product was *Eco*RI-digested and subcloned into pKK223-3. As a result, pSASβ was constructed (Fig. 1). To confirm the accurate construction of the fusion genes, nucleotide sequencing was performed.

Identification and purification of plasminogen activators. *E. coli* cells containing expression plasmids were harvested in 6 h after IPTG induction and the periplasmic fractions were extracted by cold osmotic shock. The harvested cells were resuspended in 1/20 vol of STE buffer (30 mM Tris–Cl (pH 8.0), 1 mM EDTA, and 30% (w/v) sucrose) and incubated on ice for 30 min. After centrifugation, the supernatants were transferred into new tubes, and then 1/100 vol of 50 mg/ml protamine sulfate was added for the removal of other protein contaminants. After centrifugation the supernatants were dialyzed against 20 mM Tris buffer (pH 8.0) and filtrated through a 0.2-μm Minisart CE0297 filter kit (Sartorius, Inc., New York, NY). Then the filtrates were applied to diethylaminoethyl (DEAE)–Sepharose column and Mono-Q column (Amersham–Pharmacia Biotech Korea Ltd., Seoul, Korea) and eluted by salt gradient (12).

The expressed proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using Laemmli's method (13). Comparing absorbance at 750 nm of protein sample with standard curve prepared with bovine serum albumin of Protein Concentration kit (Bio-Rad, Laboratories, Hercules, CA), determined the protein concentration.

Determination of plasminogen activation activities. The colonies on agar plate were covered with 5 ml top agar containing 50 mM Tris–Cl (pH 8.0), 150 mM NaCl, 5.0% (w/v) skim milk, 100 μg of human or bovine plasminogen preparation, and 1 mM IPTG for induction of the gene expression by P*tac* promoter. After further incubation, the cells were grown for an additional 6 h at 37°C and the clear zones were analyzed.

Quantitative SAK, SKe, and fusion protein activities with equimolar ratio were determined with following plasminogen-coupled chromogenic substrate assay that is slightly modified Schlott's method (14). Forty microliters of SAK or fusion protein samples (final 10 nM), 40 μl of human plasminogen (final 10 nM) in 20 mM sodium phosphate buffer (pH 7.5), and equimolar human fibrin (Sigma–Aldrich Co., St. Louis, MO; F5386) were mixed and incubated for 25 min at 37°C. Then 20 μl of chromogenic substrate, S-2251 (final 1 mM, Sigma–Aldrich Co, V-0882) was added. Absorbance at 450 nm was measured after 5 minutes by spectrophotometer. Measurement of initial velocity of plasminogen activity was determined by the plasminogen-coupled chromogenic substrate assay (15) with following modification. A mixture containing 1.4 ml of assay buffer [0.1 M potassium phosphate (pH 6.0), 0.1 M NaCl, and 0.01 M L-lysine], 10 μl (final 2 nM) of human or bovine plasminogen, and 60 μl (final 0.02 nM) of plasminogen activator sample was incubated for 3 minutes at 37°C. Then, 30 μl (final 600 nM) of *N*-α-carbobenzoyl-L-lysine-*p*-nitrophenyl ester (CLN, Sigma–Aldrich, Co.), which was dissolved in 50% (v/v) dimethyl formamide, were added to the mixture. Optical density of the mixture was scanned for 1 min at 340 nm with Beckman Model DU-65 spectrophotometer. One CLN unit was defined as a 0.01 increase per minute in the absorbance at 340 nm in the above conditions. The measured CLN units were calculated to the blood clot lysis unit of the World Health Organization (WHO) by the CLN assay of standard streptokinase (S-4645, Sigma–Aldrich, Co.).

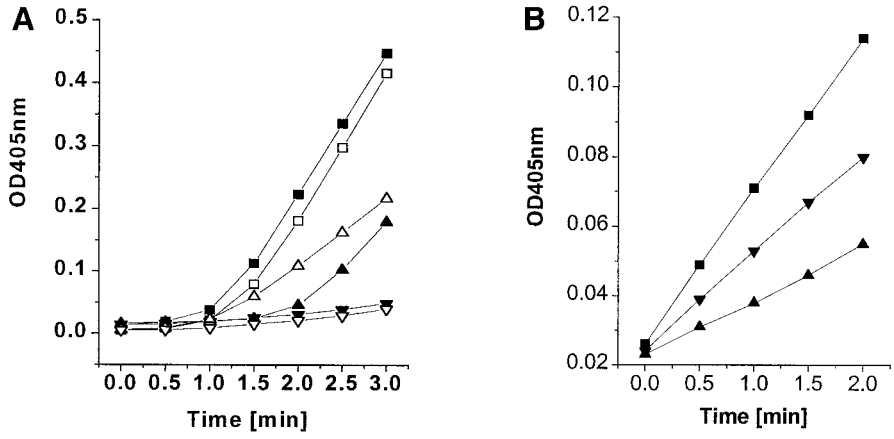

FIG. 2. Activation of human plasminogen by fusion proteins. (A) Equimolar mixture of plasminogen and fusion proteins. Generated plasmin activity was monitored with staphylokinase (squares), staphylokinase-SKβ (up triangles), and staphylokinase-SK$\beta\gamma$ (down triangles) in the absence of fibrin (closed symbols) and in the presence of fibrin (open symbols). (B) Equimolar mixture of plasmin and fusion proteins. Plasmin activity was monitored with staphylokinase (squares), staphylokinase-SKβ (up triangles), and staphylokinase-SK$\beta\gamma$ (down triangles).

RESULTS AND DISCUSSION

Plasminogen Activation Activities of SAK–SKe Fusion Proteins

Streptokinase (SKe) can form a plasminogen activator by two pathways. In the first pathway, SK forms a complex with human plasminogen (16). This initial complex rearranges to form an active complex whose amidolytic activity is generated in the human plasminogen moiety of this complex (17, 18) and the activating peptide bond of Arg560–Val561 is not cleaved (19). Then, the SKe-plasminogen complex catalyzes the hydrolysis of the specific peptide bond of Arg560–Val561 on substrate human plasminogen, resulting in the formation of plasmin (20). In the other pathway, SKe can form a plasminogen activator by complexing directly with human plasmin as staphylokinase(SAK) do (16). Trace amounts of human plasmin are always present in human plasminogen preparations, and human plasmin appears to have a higher affinity for SKe (21).

An additional mechanistic distinction among plasminogen activators is their dependence on fibrin as a cofactor during the process of plasminogen activation. Indeed, many authorities believe that this property of fibrin dependence is a critical determinant of the therapeutic efficacy of plasminogen activators (22). The contrast between two most widely used agents, SKe and tissue-type plasminogen activator, exemplifies this mechanistic distinction. SKe does not require fibrin for efficient plasminogen activation in human blood (23).

From skim milk-plasminogen overlay test, the activities of SASβ and SAS$\beta\gamma$ were similar to that of SK (data not shown). The kinetics of equimolar mixture of human plasminogen or human plasmin with the fusion proteins were examined. When the SASβ and SAS$\beta\gamma$ were incubated with equimolar human plasminogen, they had lag phases in increase of absorbance, which means that their counter part in the binary complex is not human plasminogen but plasmin (Fig. 2A). To prove this hypothesis, kinetics of the equimolar mixtures of a human plasmin with the fusion proteins were examined (Fig. 2B). No lag phase in absorbance increase showed that human plasmin binds to the fusion proteins. From these data, it is clear that the fusion proteins, SASβ and SAS$\beta\gamma$, have SAK-like plasminogen activation mechanism. Also, the activities of SAS$\beta\gamma$ was the lowest, and that of SASβ was lower than that of SAK (Fig. 2A). This shows that SKβ and SKγ domain fused with SAK does not play a role of binding with the counter part in the fusion proteins. Additionally, fibrin enhanced the activity of SASβ (Fig. 2A) which means SASβ is more efficient in plasminogen activation than SAS$\beta\gamma$.

The above results suggest that the fusion proteins activate a human plasminogen in SAK mode and that inhibition of human plasmin by α_2-antiplasmin was not influenced by SKβ and SKγ domain.

Species Specificities in Plasminogen Activation

First, to distinguish the difference between SKu and SKe activity, skim milk overlay test was performed using bovine and human plasminogen (Fig. 3A). As predicted, SKe did not activate bovine plasminogen but SKu did (Fig. 3A). However, unexpectedly, human plasminogen was activated by SKe and SKu (Fig. 3B). This result conflicts with the Leigh's report (24). The fact that activity of SKu in human plasminogen activation was very low and showed lag time in increasing of A_{340} in chromogenic assay (data not shown), means that SKu binds human plasmin for formation of active complex and the plasmin receptor on *S. uberis* surface enhances the activation ability of SKu (25).

In second, the streptokinase fragment, SKe$\alpha\beta$, may provide a clue that it can also activate bovine plasmin-

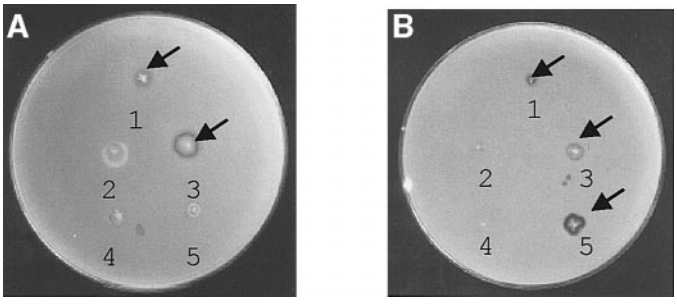

FIG. 3. Plasminogen activation by bovine plasminogen activator and the streptokinase $\alpha\beta$-domain. Skim milk–plasminogen overlay test using bovine (A) and human plasminogen (B). *E. coli* colonies contain plasmids: 2, pET-22b; 3, pEP10; 4, pKK-*ompA;* 5, pSK100. *Streptococcus uberis* NCTC 3858 is 1. Arrows indicate clear zones which mean activation of plasminogens.

ogen. Thus, we constructed the expression vector of SKe$\alpha\beta$ and checked the activation activities of human and bovine plasminogen. In the test using human plasminogen, the increasing rate did not have correspondence to concentration of activator (data not shown). In bovine plasminogen, the increasing rate was proportional to the concentration of activator (data not shown). From these data, it is thought that SKu and SKe$\alpha\beta$ can activate bovine plasminogen and the spectrum of substrate species by SKe is restricted into human plasminogen by γ-domain of SKe.

One of the physiological features among bacterial plasminogen activators is species specificity in recognizing the plasminogen substrate from various mammalian sources. It can be proposed that some regions of γ domain of SKe recognize plasminogen or plasmin species during formation of a binary complex. The plasminogen activation activities of these fusion proteins for human and bovine plasminogen can provide some information involved in the species specificity mechanism between bacterial plasminogen activators. The species specificity in plasminogen activation of SKe is determined by some region in its γ-domain (286–414 residues).

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