A Fast-acting, Modular-structured Staphylokinase Fusion with Kringle-1 from Human Plasminogen as the Fibrin-targeting Domain Offers Improved Clot Lysis Efficacy

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To develop a fast-acting clot dissolving agent, a clot-targeting domain derived from the Kringle-1 domain in human plasminogen was fused to the C-terminal end of staphylokinase with a linker sequence in between. Production of this fusion protein in Bacillus subtilis and Pichia pastoris was examined. The Kringle domain in the fusion protein produced from B. subtilis was improperly folded because of its complicated disulfide-bond profile, whereas the staphylokinase domain produced from P. pastoris was only partially active because of an N-linked glycosylation. A change of the glycosylation residue, Thr-30, to alanine resulted in a non-glycosylated biologically active fusion. The resulting mutein, designated SAKM3-L-K1, was overproduced in P. pastoris. Each domain in SAKM3-L-K1 was functional, and this fusion showed fibrin binding ability by binding directly to plasmin-digested clots. In vitro fibrin clot lysis in a static environment and plasma clot lysis in a flow-cell system demonstrated that the engineered fusion outperformed the non-fused staphylokinase. The time required for 50% clot lysis was reduced by 20 to 500% under different conditions. Faster clot lysis can potentially reduce the degree of damage to occluded heart tissues.

Thrombolysis (1–4) is one of the well established treatments for patients with acute myocardial infarction (commonly known as heart attack). Blood clot-dissolving agents currently approved for thrombolytic therapy include tissue plasminogen activator (tPA), urokinase, streptokinase, and their derivatives (3, 4). Although the treatment can reduce mortality, several large scale clinical trials indicate that these blood clot-dissolving agents, which provide a rapid, complete, and sustained reperfusion with minimal side effects, are needed.

Staphylokinase (SAK), a 136-amino acid protein from certain lysogenic Staphylococcus aureus strains, is a plasminogen activator and a promising blood clot-dissolving agent with clinical potency that is at least as good as tPA (9, 10). In addition, it has some desirable features that are superior to tPA (11). Notably, SAK mediates the lysis of platelet-rich and retracted clots efficiently (12, 13) and shows exceptional fibrin specificity (9, 10, 14, 15). These properties can help minimize reocclusion and bleeding complications.

Much has been studied on the action of SAK in vivo. To function as the plasminogen activator, SAK first forms a complex with plasminogen (16). Complex formation is followed by SAK processing in which the N-terminal peptide containing the first 10 amino acids from SAK is removed by cleaving at a twin lysine site between residues 10 and 11 (Fig. 1A). This processing step is essential for attaining an active form of SAK (17–19). The SAK-plasminogen complex then forms a ternary complex with another molecule of plasminogen and converts this plasminogen to plasmin. When the SAK-plasmin complex is not fibrin-bound, it can be inhibited by the natural plasmin inhibitor, α2-antiplasmin, present in plasma. In contrast, the fibrin-bound plasminogen activator complex is much more resistant to α2-antiplasmin-mediated inhibition (15). The result is a preferential plasminogen activation by SAK at the fibrin surface that contributes to the fibrin specificity of SAK in a plasma milieu. This fibrin specificity is made even stronger by the preferential binding of SAK to plasminogen that is fibrin-bound (14). The fibrin-specific property of SAK underlies an interesting observation in clinical trials. The fibrinogen levels in patients treated with SAK remain close to 100%, whereas patients treated with tPA have 32% of fibrinogen cleaved and degraded in their plasma (9, 10).

Although SAK is a fibrin-specific thrombolytic agent, it has no fibrin binding ability by itself. It binds to fibrin clots only indirectly through the interaction with any clot-bound plasminogen. It would be of interest to determine whether the clot lysis efficacy of SAK can be improved by engineering it with direct fibrin binding ability. Faster clot lysis will restore blood flow in a more timely manner and reduce damage to heart tissues. Furthermore, if less SAK is required to achieve the

About 60% of the treated patients have their blood flow restored 90 min after the onset of treatment (5). Some of the treated patients will suffer from reocclusion or bleeding complications such as hemorrhagic strokes (2, 3, 6). Furthermore, the reoccluded clots are usually platelet-rich and are more resistant to tPA-mediated clot lysis (7, 8). Hence, choices of more potent blood clot-dissolving agents, which provide a rapid, complete, and sustained reperfusion with minimal side effects, are needed.
same degree of clot lysis, the risk for side effects can be minimized. Plasminogen binds to fibrin clots via its kringle domains at the N-terminal regions (20). These kringle domains are ~80 amino acids long; some of them possess the lysine binding capability (21–23). Thus, plasminogen binds abundantly to fibrin clots during clot lysis since more C-terminal lysine residues are generated through the action of plasmin, a trypsin-like protease that cuts after lysine and arginine residues. Among the characterized kringles from human plasminogen (24–28) and tPA (29), Kringle-1 from human plasminogen has the highest lysine binding affinity. Therefore, Kringle-1 was selected as the fibrin targeting domain and fused to the C-terminal end of SAK (Fig. 1). To ensure that each domain within the fusion can fold independently and that each has sufficient space to interact with its target, a 20-amino acid linker is inserted between these domains. The resulting fusion (designated SAK-L-K1) and its three site-specific muteins (SAKM1-L-K1, SAKM2-L-K1, and SAKM3-L-K1) were produced via secretion from Bacillus subtilis and Pichia pastoris. Biochemical properties, clot targeting ability, and clot lysis activity of the purified SAK-L-K1 and its derivatives were determined. In comparison with SAK, the engineered SAKM3-L-K1 consistently mediated faster lysis of both fibrin and plasma clots in vitro.

EXPERIMENTAL PROCEDURES

Construction of B. subtilis Expression Vectors for SAK-L-K1 and Its Derivatives—pSAKLK1 is a pUB18-based B. subtilis plasmid (30) for secretory production of SAK-L-K1 under control of the B. subtilis P43 promoter for transcription and the levansucrase signal sequence (SacB SP) for secretion. To introduce the linker sequence between SAK and the Kringle-1 domain from human plasminogen, the nucleotide sequence of the expression cassette (P43-SacB-SBP-SAK-L-K1) was split into two portions. The first portion covered from the P43 promoter to the middle half of the linker sequence. The sequence was generated by PCR using pSAK (31) as template. The primers used for this amplification were P43F (5′-GGGATCCGAGCTCAGATTATG-3′) and SAKL5 (5′-CTTCTGACCAACACAGAAGTACTCTTACTTCTTCTTAAACAATTTTG-3′). The resulting product was an 895-bp EcoRI-SalI fragment. After digestion with both EcoRI and SalI, the fragment was inserted into pUB18 (30) at the corresponding sites located in the polylinker region of the vector to generate pUB18-SAKLF. The second half of the expression cassette carried a sequence encoding the C-terminal half of the linker and the Kringle-1 domain. This sequence was generated by PCR using a cDNA clone of human plasminogen as the template. This cDNA clone was kindly provided by Dr. Ross T. A. MacGillivray at the Department of Biochemistry and Molecular Biology, University of British Columbia. The forward primer LK1F (5′-GACTCGAAGGATCCGACCTTCGACAAAGG-3′) was used as the template for PCR amplification with AFAC-SAKF (5′-GACTCGAAGGATCCGACCTTCGACAAAGG-3′) as the forward primer and SAKKLICPB (5′-GAGGGCGCCGCTTAAACACTCAAGAATGGTGGCAGATTAG-3′) as the backward primer. A 730-bp PCR product was generated with Xhol at the 5′ end and Nol at the 3′ end. The amplified sequence was digested with Xhol and Nol and ligated to the similarly digested pPICZαA to generate pIFCSAKKLK1. The IFCSAKKLK1 was transformed to E. coli Top10F′ and selected for resistance to zeocin (25 μg/ml) (Invitrogen). pIFCSAKM2LK1 and pIFCSAKM3LK1 were also generated using the same approach with pSAKLK1 and pBSKM3LK1 as the PCR template, respectively. The information of p. pastoris expression cassette (Fig. 2) was linearized with Pmel and transformed to P. pastoris X-33 (a wild-type strain, Invitrogen) using EasyComp method according to the manufacturer’s protocol. Transformsants were selected on zeocin (600 and 1000 μg/ml) and screened for secretory production of SAKM2-L-K1 or SAKM3-L-K1. X-33 cells with pPICZαA and pPICSKALK1 integrated were similarly prepared to serve as the negative and wild-type controls, respectively.

Production and Affinity Purification of SAKM3-L-K1—For protein purification, a Pichia transformant showing the highest expression level of SAKM3-L-K1 was cultured for 16–18 h at 28–30 °C in buffered glycerol-complex medium (34). Cells were pelleted at 3,000 × g for 5 min and resuspended to 100 Klett units with a Klett-Summerson photometric colorimeter (Klett-Mfg. Co.) in buffered medium-complex medium (34). Growth continued at 28–30 °C in a shake flask. Production of SAKM3-L-K1 was induced by methanol (0.5% final concentration) administered every 12 h during the entire culture period. After 28–30 h of culture, the culture supernatant was collected by centrifuging the cells at 3,000 × g for 5 min and applied to a lysine-agarose column (Sigma) equilibrated with column buffer (50 mM Tris-HCl, 50 mM NaCl, pH 7.5). After washing the column with 3–5 bed volumes of the binding buffer, SAKM3-L-K1 was eluted with 0.15 M e-amino-N-caproic acid (EACA) (Sigma). Eluants were analyzed to determine yield and purity of SAKM3-L-K1 by SDS-PAGE and Coomassie Blue staining. The fractions selected were pooled, dialyzed against the binding buffer by ultrafiltration, and concentrated by ultrafiltration (Centricon-10, Pierce). Purified SAKM3-L-K1 was quantified spectrophotometrically at 280 nm using a molar extinction coefficient of 35,350 M−1 cm−1 (35).

Matrix-assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry—Purified SAKM3-L-K1 in deionized water was mixed with the matrix solution of sinapinic acid and analyzed on an Applied Biosystems Voyager-DE P50 mass spectrometer calibrated by trypsinogen (m/z 23,981). The instrument operated in the linear mode with an acquisition range 10,000–35,000 Da. This analysis was performed at the Alberta Peptide Institute, Edmonton, Alberta, Canada.

Isothermal Titration Calorimetry—Purified SAKM3-L-K1 was extensively dialyzed against 150 mM sodium phosphate, pH 7.4, and quantified spectrophotometrically at 280 nm as previously described. Titrations were performed using a Microcal VP-ITC calorimeter at 25 °C in 150 mM sodium phosphate, pH 7.4, following the manufacturer’s guidelines and using Microcal Origin for data analysis. EACA (Sigma) was used as the ligand. The incremental heat change accompanying binding was corrected for the corresponding heat of dilution of EACA into buffer that was obtained in a separate experiment by titrating EACA into the sample cell containing buffer only.

Fibrin Binding Study—An ELISA method was used to assess the fibrin binding ability of SAKM3-L-K1 and SAK. Cross-linked fibrin was formed on the wells of a Nunc-ImmuNo MaxiSorp module (Nalge Nunc International Corp.) using a procedure described by Wu et al. (36). Formation of cross-linked fibrin was confirmed by SDS-PAGE (36). The fibrin was digested with plasmin (Roche Applied Science) at 4 milliunits/well (~1 pmol/well) at room temperature. At different time points, the unbound plasmin was removed immediately by washing 4 times with PBST (0.1 mM sodium phosphate, 0.15 mM sodium chloride, pH 7.2, 1× PBS, 0.05% Tween 20). Purified SAKM3-L-K1 or SAK retained on the well was probed with polyclonal antibodies against SAK (31) followed by horseradish peroxidase-conjugated anti-mouse secondary antibodies. The amount of bound fibrin was assessed using 3,3′,5,5′-Tetramethylbenzidine (TMB) (Pierce) as the horseradish peroxidase substrate according to the manufacturer’s instructions. Color development at end point was determined at 450 nm using a microplate reader (CERES 900, Bio-Tek Instruments, Inc.). The experiment was repeated three times.
Fibrin Clot Lysis Study—Fibrin clots were formed by adding human thrombin (to 0.6 NIH units/ml) (1 NIH unit = 0.324 ± 0.075 μg thrombin) and CaCl₂ (to 20 mM) to human fibrinogen (1 mg/ml, final concentration) in HEPES-buffered saline (HBS; 0.01 M HEPES, 0.13 M NaCl, pH 7.4). Both thrombin and fibrinogen were highly purified materials from Sigma. Immediately after mixing, 100-μl aliquots of the polymerizing fibrin solution were pipetted to the wells of a microtiter plate (Falcon 3912 flat-bottom polyvinyl chloride plate, BD Biosciences). Clot formation was allowed to proceed for 3 h at room temperature. The surface of the clots was washed with HBS, and excess fluid was care-

omm. Platelet-poor plasma (PPP) was prepared from the pooled citrated plasma was transferred to the flow cell (Hellma Cells, model 178.710, Beckman Instruments) operated as a duplex on the SDS-

platelet poor plasma (PPP) was prepared from the pooled citrated plasma was transferred to the flow cell (Hellma Cells, model 178.710-BS) to fill up the optical chamber (80 μl) and the inlet and outlet ports. Clotting was allowed to proceed for 3 h at room temperature. The clot was then perfused with PPP for 20 min at 20 μl/min, followed by perfusion with SAKM3-L-K1 or SAK (200 μl in PPP, 20 μl/min) with the flow rate controlled by a Bio-Rad peristaltic pump. The change in absorbance at 600 nm at 37 °C was continuously monitored using a Beckman DU-65 spectrophotometer (Beckman Instruments) operated in the kinetic mode. After 30 min the surface of each clot was gently washed with HBS three times to remove any unbound SAKM3-L-K1 or SAK. 100-μl aliquots of plasminogen (1.5 μM, Sigma) and varied concentrations of purified SAKM3-L-K1 or SAK in each experiment, and the experiment was repeated three times. As a control, some clots were layered only with PBS. Duplicate wells were prepared for each concentration of SAKM3-L-K1 or SAK in each experiment, and the experiment was repeated three times. As a control, some clots were layered only with PBS but treated otherwise the same.

Plasma Clot Lysis Study (Clot Perfusion Model)—Blood was drawn by venipuncture from healthy adult donors who had taken no medicine within 2 weeks (to 0.6 NIH units/ml) and CaCl₂ (to 20 mM) to fresh thawed PPP. 200 μl of the polymerizing plasma was transferred to the flow cell (Hellma Cells, model 178.710-QS) to fill up the optical chamber (80 μl) and the inlet and outlet ports. Clotting was allowed to proceed for 3 h at room temperature. The clot was then perfused with PPP for 20 min at 20 μl/min, followed by perfusion with SAKM3-L-K1 or SAK (200 μl in PPP, 20 μl/min) with the flow rate controlled by a Bio-Rad peristaltic pump. The change in absorbance at 600 nm at 37 °C was continuously monitored using a Beckman DU-65 spectrophotometer (Beckman Instruments) operated with a Kinetics Soft-Pac module. The experiment was performed three times for both SAKM3-L-K1 and SAK.

Plasmogen Caseinolytic Assay—Caseinolytic activity was determined via secretion. Al-

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Effects of recombinant SAKM2-L-K1 and SAKM3-L-K1 from 30 zeocin-resistant clones of P. pastoris transformants was analyzed in a time course study ranging from 15 to 60 h of culture. For all the clones, optimal conditions (high secretion yield with little degradation) were achieved with 28–30 h of cultures. On the other hand, the level of expression varied considerably across the clones. Clones showing the highest level of expression of SAKM2-L-K1 and SAKM3-L-K1 were designated PM2 and PM3, respectively, and were used to produce these muteins for all subsequent experiments. Using known amounts of pure SAKM3-L-K1 as the standard, the secretory levels of these muteins from PM2 and PM3 were estimated to be around 100 and 150 mg/liter of culture, respectively. These amounts exceed those secreted by B. subtilis (Fig. 2A, lanes 3 and 4 versus lane 5). SAK-L-K1 produced from P. pastoris migrated as a duplex on the SDS-polyacrylamide gel. More than 80% of this protein had a slower mobility and represented the glycosylated form. In contrast,
FIG. 2. Production of SAK-L-K1 and its derivatives in P. pastoris. A. Coomassie Blue-stained gel showing SAK-L-K1 and its muteins in the culture supernatants of P. pastoris. Culture supernatants were collected at 19 h of culture. 24 μl of culture supernatant from each sample was loaded. M, molecular weight marker. Lane 1, P. pastoris X-33[pPICZaA] (this serves as the negative control); lane 2, X-33[pSAKM2-L-K1]; lane 3, X-33[pSAKM2-L-K1]; lane 4, X-33[pSAKM3-L-K1]; lane 5, B. subtilis WB800[pSAKM3-L-K1]. 24 μl of culture supernatant was loaded. B, radial caseinolyis assay comparing activities of equimolar amounts of the different muteins. Numbers correspond to samples collected from the culture supernatants as designated by the different lanes in panel A.

Both muteins (SAKM2-L-K1 and SAKM3-L-K1) produced from P. pastoris showed a faster migration (Fig. 2A, lanes 3 and 4) with mobility similar to the non-glycosylated SAK-L-K1 from P. pastoris and SAKM3-L-K1 produced from B. subtilis. The absence of N-linked glycosylation with these muteins was further confirmed by Western blotting probed with concanavalin A-conjugated peroxidase. Strong concanavalin A binding activity was observed only with the P. pastoris-produced SAK-L-K1 but not with SAKM2-L-K1 or SAKM3-L-K1 (data not shown). Thus, changing either Asn-28 or Thr-30 to Ala did successfully eliminate N-linked glycosylation of SAK in P. pastoris. In terms of plasminogen activation activity, SAKM3-L-K1 produced by P. pastoris and S. subtilis showed comparable activity (Fig. 2B). SAK-L-K1 and SAKM2-L-K1 from P. pastoris, on the other hand, demonstrated weaker activity as judged by the smaller haloes. Because SAKM3-L-K1 was produced at a much higher yield with P. pastoris, this mutein was purified from P. pastoris for further characterization.

SAKM3-L-K1 Secreted by P. pastoris Folds Properly and Binds Efficiently to Lysine-Agarose—To show that Kringle-1 domain in SAKM3-L-K1 is functional, the culture supernatant from X33[pPICSAKM3LK1] was loaded to a lysine-agarose column. Approximately 95% of SAKM3-L-K1 could be retained on the column and recovered by EACA elution (Fig. 3A). This indicates that the majority of SAKM3-L-K1 produced by P. pastoris retained lysine binding capability. To further examine the formation of disulfide bond in the Kringle-1 domain of SAKM3-L-K1, culture supernatants from both X33[pPICSAKM3LK1] (Fig. 3B, lanes 1 and 3) and WB800[pSAKM3LK1] (lanes 5 and 7) either in the presence or absence of reducing agent were analyzed by Western blotting. In both hosts, SAKM3-L-K1 migrated faster under the non-reducing condition (lanes 3 and 7) presumably because of a more compact conformation with disulfide bond formation. When run under the reducing conditions, protein from either host migrated as a sharp major band with an apparent molecular size around 33 kDa (lanes 1 and 5). Electrophoresis of proteins under the non-reducing conditions differentiated the nature of SAKM3-L-K1 produced by the two hosts. Whereas in P. pastoris, majority of the protein migrated at the fastest migration position (lane 3), the opposite held true for B. subtilis (lane 7). This observation suggests that the majority of SAKM3-L-K1 produced by P. pastoris forms disulfide bonds and retains a compact structure, whereas only a tiny portion of SAKM3-L-K1 produced by B. subtilis achieves the proper folding configuration. This offers a possible explanation for the observed difference in the ability of SAKM3-L-K1 produced by either host to bind to lysine-agarose.

Each Domain in the Affinity-purified SAKM3-L-K1 from P. pastoris Is Highly Functional—To confirm that fusion of SAK and Kringle-1 in SAKM3-L-K1 does not interfere with the function of each domain, SAKM3-L-K1 from P. pastoris was purified on a lysine-agarose column and used for functional evaluations. As shown in Fig. 4A, with equimolar amounts of both proteins the rate of plasminogen activation by SAKM3-L-K1 was found to be around 96% that by SAK. To determine the functionality of the Kringle-1 domain in SAKM3-L-K1, the affinity of this domain in SAKM3-L-K1 to EACA, a lysine analog, was determined by isothermal titration calorimetry. A typical example of heat changes accompanying binding of EACA to the Kringle-1 domain of SAKM3-L-K1 was shown in
A Staphylokinase Fusion with Direct Clot Binding Ability

Based on the average of duplicate experiments, $K_a$ for the interaction between SAKM3-L-K1 and EACA was found to be 15 μM. As a reference, the binding affinity of Kringle-1 domain and its derivative carrying the N-terminal proactivation peptide of human plasminogen to EACA, expressed in terms of the dissociation constant, has been determined to be 17 μM by equilibrium dialysis (24), 12 μM by isothermal titration calorimetry (25), and 13.7 μM by fluorescence study (42). Thus, the $K_a$ obtained with SAKM3-L-K1 is within the range of the reported values.

**Molecular Mass Determination of SAKM3-L-K1 from P. pastoris**—Because the apparent molecular mass of SAKM3-L-K1 on SDS gel (33 kDa) differs from that calculated (28,169 Da), purified SAKM3-L-K1 was subjected to both N-terminal sequencing and MALDI-TOF mass spectrometry analyses. Sequencing of the first six amino acid residues from SAKM3-L-K1 (SSSFDK) matched exactly with the predicted mature SAK sequence (Fig. 1A). Therefore, the α-factor signal peptide was properly processed by the P. pastoris signal peptidase. Results from mass spectrometry analysis determined the molecular mass of SAKM3-L-K1 to be 26,320.99 which is in close agreement with the calculated value. This value confirmed the absence of N-linked glycosylation in SAKM3-L-K1 since the molecular mass of the N-linked oligosaccharide unit would clearly be larger than 153 Da.

**Activated SAKM3-L-K1 Is Resistant to Plasmin Digestion**—Because the SAKM3-L-K1 fusion is designed to introduce a fibrin targeting capability to SAK via the Kringle-1 domain, it is essential that the Kringle-1 domain in SAKM3-L-K1 should not be cleaved off in the presence of plasmin. Otherwise, the full impact of this fusion in clot lysis will be significantly weakened. The last two C-terminal residues in SAK are known to be lysine (Fig. 1A). If plasmin, a trypsin-like protease, cleaves SAK or its fusions at this site, any domain introduced C-terminally to SAK will be cleaved off. To examine the stability of SAKM3-L-K1 in the presence of plasmin, purified SAKM3-L-K1 was mixed with plasminogen at a 1:1 molar ratio, and the stability of SAKM3-L-K1 was monitored at different time points by SDS-PAGE under either reducing or non-reducing condition. As shown in Fig. 5A (non-reducing condition), 5 min after mixing SAKM3-L-K1 with plasminogen (lane 3), SAKM3-L-K1 was completely converted from the original intact form with an apparent molecular mass of 29 kDa to a smaller form (28 kDa). This slight change in size reflects the removal of the first 10 amino acid residues (Fig. 1A) of SAKM3-L-K1 by plasmin during the activation event. However, none of the processed SAKM3-L-K1 showed apparent molecular masses in the range around 16 kDa, which corresponds to that of SAK even after SAKM3-L-K1 had been mixed with plasminogen for 1 h (Fig. 5A, lane 5). This suggests that plasmin did not cleave the twin lysine site located naturally at the C-terminal end of SAK.

SDS-PAGE of a duplicated set of samples run under the reducing condition shows that plasminogen was completely converted to plasmin with the appearance of a heavy chain (70 kDa) and a light chain (29 kDa) within 20 min after mixing with SAKM3-L-K1 (Fig. 5B, lane 4). Western blotting of an equivalent gel probed with SAK-specific antibodies confirmed that the processed SAKM3-L-K1 remained stable for at least an hour in the presence of plasmin (Fig. 5C). The same result was observed on mixing SAKM3-L-K1 with plasminogen at a 1:3 molar ratio (data not shown). A faint band with an apparent molecular mass of 22 kDa was observed after 20 min (Fig. 5C, lanes 4 and 5). The size of this faint band suggests a low level of proteolytic cleavage of SAKM3-L-K1, possibly close to the N-terminal region of the Kringle-1 domain.
SAKM3-L-K1 binds to partially digested fibrin—Because exposed lysine residues are needed for effective binding of the Kringle-1 domain, fibrin coated on the microtiter plate in the ELISA experiment had to be partially digested for the binding study. Hence, plasmin was used to digest the fibrin with varied time periods to generate the optimal condition for Kringle-1 binding. Fig. 6 shows a typical result demonstrating the distinct difference between SAKM3-L-K1 and SAK in their ability to bind to fibrin. Although SAK showed only a weak fibrin binding for the entire time period, SAKM3-L-K1 bound readily to the fibrin. The fibrin had to be at least slightly digested for any binding to occur. Generation of newly exposed C-terminal lysine residues and increased accessibility of the fibrin to SAKM3-L-K1 could both contribute to the enhanced binding of SAKM3-L-K1 to the partially digested fibrin. In this particular example, at the plateau (which last from 20 to 40 min of fibrin digestion), the amount of SAKM3-L-K1 bound was 21 times that of SAK.

Fibrin Clot Lysis Kinetics: Effects of SAKM3-L-K1 Versus SAK—Whether the enhanced fibrin binding ability by SAKM3-L-K1 could be translated to a faster clot lysis was investigated in a fibrin clot lysis assay using an ELISA plate under two different conditions. Under the first set of experimental condition, the thrombolytic agents were not removed from the clots throughout the entire clot lysis period. Fig. 7 shows the typical changes in clot turbidity that accompany clot lysis mediated by either agent. SAK (closed triangles) mediated clot lysis began with a longer lag phase, and the time required for 50% clot lysis ($T_{\text{50\%}}$) was obviously longer than that by SAKM3-L-K1 (open triangles). Table II summarizes the $T_{\text{50\%}}$ values with different amounts of SAKM3-L-K1 or SAK. $T_{\text{50\%}}$ mediated by SAK was 22–50% longer than that by SAKM3-L-K1. Because SAK in general is introduced to patients by a short infusion and the in vivo half-life of SAK in human is only 3 min (43), the infused SAK is expected to be rapidly depleted from circulation once the infusion process is terminated. To simulate this effect, clot lysis was examined under the second experimental condition in which any unbound agent (SAK or SAKM3-L-K1) was removed by gently washing the clot surface 30 min after incubation with the clots. The difference in $T_{\text{50\%}}$ values between SAK and SAKM3-L-K1 was even more dramatic (Fig. 7, SAK (close circle) versus SAKM3-L-K1 (open circle)). Table II shows that depending on the concentration used in the study, SAK needed 40–80% longer time to achieve 50% clot lysis. Approximately four times the amount for SAK (200 nM) was required to attain the same rate of lysis mediated by 50 nM SAKM3-L-K1. Whether the agents were present throughout the lysis period or not, the enhancement of clot lysis by SAKM3-L-K1 was increasingly obvious at lower dosages of either thrombolytic agent used.

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**Fig. 5.** Stability of SAKM3-L-K1 in the presence of plasmin. SAKM3-L-K1 and plasminogen at a 1:1 molar ratio were mixed, and samples were collected at different time points for SDS-PAGE under non-reducing (A) or reducing (B) conditions. C, Western blot probed against polyclonal antibodies against SAK. Samples were prepared under reducing conditions. Lane 1, plasminogen. Lane 2, purified SAKM3-L-K1. Lanes 3–5, plasmin processing of purified SAKM3-L-K1 for 5 min, 20 min, and 1 h, respectively. Lane 6, purified SAK. Lane 7, plasmin standard. M, molecular weight marker. The asterisk marks the position of the N-terminally processed SAKM3-L-K1. A and b refer to the heavy (70 kDa) and light (29 kDa) chains of plasmin, respectively.

**Fig. 6.** A representative ELISA study showing fibrin binding ability of SAK and SAKM3-L-K1. Fibrinogen (5 μg/ml) was coated onto the wells of a Nunc-Immuno MaxiSorp module and left overnight at 4 °C. After blocking the exposed sites with BSA, the wells were incubated with a solution of phosphate-buffered saline containing human thrombin (1 NIH unit/ml) and CaCl$_2$ (20 mM) for 2 h at 37 °C. Cross-linked fibrin clots on the wells were partially digested with plasmin for different time periods. Binding of SAK and its derivative to the partially digested fibrin was monitored as described under “Experimental Procedures.” The amount of SAK-specific antibody retained on the wells was estimated by measuring the activity of bound horseradish peroxidase (HRP). ○, SAKM3-L-K1; •, SAK.

**Fig. 7.** A representative curve showing the time course of fibrin clot lysis. Fibrin clots were formed on the wells of an ELISA plate. SAK or SAKM3-L-K1 was used at 50 nM. The decrease in absorbance at 405 nm with time was used to calculate the relative clot turbidity at different time points. The clots were either incubated with SAK (△) or SAKM3-L-K1 (○) throughout the entire period or incubated with SAK (●) or SAKM3-L-K1 (□) for 30 min and buffer-washed at the time indicated by an open arrowhead. Control clots treated with buffer only (data not shown) showed that the readings were stable throughout the incubation period. See “Experimental Procedures” for experimental details.
**Table II**

**Summary of the time required to lyse 50% of the fibrin clot (T_{50%}) with different amounts of SAK or SAKM3-L-K1**

The clots were either buffer-washed after 30 min of incubation with plasminogen and SAK/SAKM3-L-K1 or left unwashed. Results shown represent the average values and S.D. from 6 clots (n = 6) for each concentration of SAK or SAKM3-L-K1.

| Concentration | Average (min) | S.D. (min) | Ratio | Clot washed |
|---------------|---------------|------------|-------|-------------|
|               |               |            |       | Average (min) | S.D. (min) | Ratio |
| SAK           |               |            |       | 204          | 8.5        | 1.79  |
| 50            | 168           | 6.7        | 1.50  |             |            |       |
| 100           | 128           | 3.5        | 1.33  |             |            |       |
| 200           | 99            | 2.5        | 1.22  |             |            |       |
| SAKM3-L-K1    |               |            |       | 113          | 6.7        | 1.40  |
| 50            | 112           | 3.5        | 1.14  |             |            |       |
| 100           | 97            | 6.9        | 0.98  |             |            |       |
| 200           | 81            | 4.2        | 0.81  |             |            |       |

\* T_{50%} of SAK/T_{50%} of SAKM3-L-K1

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**Fig. 8. A typical time course of plasma clot perfusion study.**

Plasma clot was formed from PPP in an optical flow cell as described under “Experimental Procedures.” The decrease in absorbance at 600 nm with time was used to calculate the relative clot turbidity at different time points. The clot was either perfused with PPP throughout the experiment or at the time marked by an open arrowhead, with PPP containing 200 nM SAK (○) or SAKM3-L-K1 (○). In this example, T_{50%} was 93 min for SAK and 17.5 min for SAKM3-L-K1.

**Plasma Clot Lysis Kinetics: Effects of SAKM3-L-K1 Versus SAK**—To more closely simulate the physiological situation, clot lysis experiment was repeated using human plasma under a constant flow model. Constant plasma flows can supply plasminogen to the clot surface. This can greatly influence clot lysis efficiency. Because human plasma has a strong absorbance at 405 nm, measurement was taken at 600 nm (at which human plasma has negligible absorbance) to ensure a more accurate reflection of clot turbidity. Fig. 8 shows that when SAK was used as the thrombolytic agent, a lag of ~30 min occurred before any obvious clot lysis was detected. In contrary, clot lysis occurred almost instantaneously after the introduction of SAKM3-L-K1. Based on the average of triplicate assays, T_{50%} value was 92 min (S.D. 1.5 min) for SAK and 17 min (S.D. 0.5 min) for SAKM3-L-K1. Thus, SAKM3-L-K1 was at least five times more effective than SAK in mediating plasma clot lysis.

**DISCUSSION**

An important genetic manipulation in this study involves the production of properly folded, non-glycosylated SAK-Kringle-1 fusion protein in *P. pastoris*. Glycosylation of SAK in *P. pastoris* has been found to result in a SAK with attenuated plasminogen activator activity (41). The presence of the oligosaccharide moiety is suggested to cause subtle changes in the orientation of plasminogen to the clot surface. This can greatly influence clot lysis efficiency. Two key residues in the glycosylation sites were targeted in this study, Asn-28 and Thr-30. Our site-directed mutagenesis studies suggest that Asn-28 is a critical residue for the function of SAK. Replacement of Asn-28 with Asp or Ala reduced the activity of SAK (Fig. 2B). A crystal structure (16) of the ternary complex of microplasmin-SAKit activator complex-microplasmin (substrate) indicates that Asn-28 of SAK forms hydrogen bonds and other contacts with Gly-174 and Gln-177 of the microplasmin (the one in the active plasminogen activator complex). It also has intra-chain contacts with Met-26, which has been shown to be important for SAK function (45). Missing some or all of these contacts may explain the reduced activity of SAK in SAKM1-L-K1 and SAKM2-L-K1. In contrast, replacement of Thr-30 with Ala is very well tolerated in SAK and results in the production of large amounts of the non-glycosylated, functional SAKM3-L-K1 from *P. pastoris* for biochemical and functional analyses.

Success of thrombolytic therapy heavily relies on the rapid and complete restoration of blood flow in occluded blood vessels. tPA, considered to be one of the best thrombolytic agents available, takes on average 45 min to restore blood flow under an optimized treatment regimen. Furthermore, only about 60% of the treated patients have blood flow restored 90 min after the treatment. Development of more potent and faster-acting thrombolytic agents that can speed up the clot lysis process and have a higher reperfusion rate would be desirable. In this study, by simply equipping staphylokinase with direct clot binding capability, the efficacy of this already potent thrombolytic agent can be improved further. In comparison with SAK, SAKM3-L-K1 resulted in a 5-fold reduction in T_{50%} for plasma clot lysis observed in the flow-cell model or a 4-fold reduction in concentration needed for comparable clot lysis in a static fibrin clot lysis assay.

The observed enhancement of clot lysis by SAKM3-L-K1 appears to be the effect of increasing the local concentration of this plasminogen activator to the clot. Because SAK has no direct fibrin binding ability, the amounts of clot-bound SAK would be strictly dependent on the level of clot-bound plasminogen. As shown in the fibrin binding study (Fig. 6), SAK binds poorly to the plasmin-treated fibrin clot. With 1 pmol of plasmin used in the clot treatment, the theoretical maximum amount of clot-bound SAK is expected to be 1 pmol or less even though 20 pmol of SAK were present in the binding reaction. In contrast, the amount of SAKM3-L-K1 captured on plasmin-treated clots can be 20 times more than that of SAK under the same conditions. This was exactly what was observed in this study.
study (Fig. 6). The high local concentration of clot-bound SAKM3-L-K1 can allow direct interaction of SAKM3-L-K1 with the adjacent clot-bound plasminogen to form functional plasminogen activators or to capture more plasminogen from plasma to the clot surface. Any plasminogen activated is likely to be clot bound and is less susceptible to antiplasmin-mediated inhibition (15).

Although conjugation of fibrin-targeting domains (e.g., fibrinspecific antibody fragments) to either tPA or urokinase has been shown to increase the potency of these agents (46–48), a recent report suggests that introduction of Kringle-2 domain from tPA to SAK did not lead to enhanced clot lysis under in vitro conditions (49). These SAK fusions are K2/SAK/HIR and RGD/K2/SAK/HIR. The failure to observe enhanced clot lysis by these recombinant fusions may possibly be attributed to the positioning of the Kringle domain at the N-terminal end of SAK and the use of Kringle 2 from tPA as the fibrin targeting domain. Because plasmin has to activate SAK by cleaving the N-terminal peptide at the twin lysine site (Fig. 1A), any fibrin-targeting domain fused to the N-terminal end of the circulating SAK fusion can potentially be cleaved off by any plasmin generated during the plasminogen activation process. This may reduce the clot-targeting potential of the fusion protein. This concern is addressed in our design of SAKM3-L-K1 by placing another potential advantage. Because this domain originates from human plasminogen, this concern has been addressed by the recent development of the mutated versions of SAK with lower immunogenicity (51) and the attachment of polyethylene glycol in a site-specific manner to the cysteine substitution variants of recombinant staphylokinase (43). The use of these SAK mutants in combination with the Kringle-1 domain from human plasminogen may offer the potential to develop potent and fast-acting thrombolytic agents with low immunogenicity.

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