Biosci. Biotechnol. Biochem., **71** (5), 1122–1129, 2007

Staphylokinase-Annexin XI Chimera Exhibited Efficient *in Vitro* Thrombolytic Activities

Jeng-Fong CHIOU,[1,4,*] Ming-Dar WOON,[1,*] Shin-Nan CHENG,[2] Chih-Hsueng HSU,[4] Shiou-Chi CHERNG,[3] Feng-Ken HSIEH,[4] Shou-Ming LIN,[4] and Chia-Yang SHIAU[4,†]

[1]*Cancer Center and Department of Radiation Oncology, Taipei Medical University Hospital, 252, Wu-Hsing Street, Taipei, Taiwan, R.O.C.*
[2]*Department of Pediatrics, Tri-Service General Hospital, Taipei, Taiwan, R.O.C.*
[3]*Department of Nuclear Medicine, Tri-Service General Hospital, Taipei, Taiwan, R.O.C.*
[4]*Graduate Institute of Medical Sciences, National Defense Medical Centre, PO BOX 90048-518, Taipei, Taiwan, R.O.C.*

Received May 18, 2006; Accepted January 25, 2007; Online Publication, May 7, 2007
[doi:10.1271/bbb.60279]

Annexins (ANXs) are a family of calcium dependent phospholipid binding proteins. Phospholipids such as phosphatidylserine are rapidly exposed on the surfaces of injured endothelial cells, activated platelets, and apoptotic cells in a large number of disorders. In this study, annexin V and XI (ANXV and ANXXI) were individually fused to the C-terminal of staphylokinase (SAK), a fibrin-selective thrombolytic protein, to form chimeras for evaluation of their *in-vitro* thrombolytic activities. The two chimeras were found to have plasminogen activation activity of comparable efficiency. When the chimeras were challenged under higher concentrations of plasmin for 1 h, hydrolysis of them into moieties was not seen on SDS–PAGE. In two thrombolytic assays, SAK-ANXXI was found to resolve both platelet rich plasma (PRP) clots and platelet poor plasma (PPP) clots with an efficiency similar to that of SAK. However, SAK-ANXV showed significantly reduced efficiency. With regard to anticoagulation ability, SAK-ANXXI was also found to have a stronger effect on dose-dependent extension of clotting time among the four tested proteins. The unique long N-terminal tail of ANXXI, composed of 202 residues, in contrast to the 16 residues of ANXV, probably served successfully to dispatch two moieties to function properly in a complicated microenvironment. Hence, a new option other than the most committed ANXV for the ANX based chimera without elaboration of linker construction is presented.

Key words: annexin; chimera; staphylokinase; thrombolytic

Acute myocardial infarction and ischemic strokes are still the two leading causes of death in the modern world.[1,2] Thrombolytic therapy has been an important treatment for myocardial infarction as well as ischemic strokes over the last decade,[2] however, the available thrombolytic agents have certain limitations,[3–5] such as unexpected bleeding due to systemic exhaustion of α-antiplasmin and other clotting components. The resistance of secondary clots, which are rich in platelets, to the effect of plasminogen activators is another concern.[1,6] This has led to many efforts to improve the potency and safety of thrombolytic agents.[5,7,8]

Following injury to the endothelium and vessel wall, platelets adhere rapidly to the sub-endothelial matrix, then become activated and form the primary hemostatic plug.[9,10] In the meanwhile, phospholipids such as phosphatidylserine (PS) are rapidly exposed on the membrane surfaces of activated platelets.[11–14] The activated platelets provide anionic surfaces for the assembly and catalysis of intrinsic tenase (VIIIa/IXa), prothrombinase (Va/Xa), and XIa complexes, leading to explosive generation of thrombin and consolidation of the fibrin-platelet plug.[15–17] Thus, rapid exposure of PS on the cell surfaces of platelets is the key thrombogenic stimulus initiating and propagating the clotting cascade.[9,10,17]

Annexins (ANXs) are a family of calcium-dependent phospholipid binding proteins.[18,19] Structurally, ANXs are characterized by a highly α-helical and tightly packed protein core domain of four to eight conserved repeats plus the N-terminal region, which is variable both in length and in sequence.[20,21] It has been found

* These two authors contributed equally to this article.
† To whom correspondence should be addressed. Tel/Fax: +886-2-87924857; E-mail: cys@ndmctsgh.edu.tw

Abbreviations: ANX, annexin; PS, phosphatidylserine; PRP, platelet rich plasma; PPP, platelet poor plasma; SAK, staphylokinase; tPA, tissue plasminogen activator

that a stimulated platelet can contain nearly 200,000 annexin V (ANXV) binding sites, while this is minimal in quiescent status.[22] In contrast, only about 25,000 glycoprotein IIb/IIIa on an activated platelet can be bound by antibodies.[23–25] The most studied member of the ANXs is ANXV. ANXV, with a high affinity constant of 7 nM binding to PS, has been utilized in tracing apoptotic cells and activated platelets.[26–29] Many efforts have been made to construct ANXV based chimeras for evaluation of their targeting effect at a potential thrombus.[6,10,30,31] Staphylokinase (SAK) is one of the promising blood clot dissolving agents.[1,6,32–34] Although the thrombolytic potency of SAK has been found to be comparable to that of tPA, clinical trials have indicated that SAK has a superior fibrin specificity.[1,35,36] Hence, SAK has been chosen as the clot dissolving component in several chimeras.[6,37,38] Although a chimera composed of SAK and an ANX might exert both fibrin selective plasminogen activation and platelet targeting anticoagulation, no such chimera lacking a linker in-between has been investigated so far.

Annexin XI (ANXXI) has an unique long N-terminal tail composed of more than 202 amino acids, in contrast to the16 residues of the N-terminal tail of ANXV.[39] Only limited studies of the structure and function of ANXXI have been reported.[40] It was postulated that the long N-terminal tail of ANXXI might be able to function as a natural linker to segregate moieties of ANXXI-based chimeras. Here we report the preparation of two chimeras, SAK-ANXV and SAK-ANXXI, without an inserted linker, and evaluation of their *in vitro* thrombolytic related activities. It was found that SAK-ANXXI preserved good *in vitro* thrombolytic activity and had a better anticoagulation effect at low doses.

Materials and Methods

Gene cloning and construction of expression vectors. The coding region (411 bps) of the SAK gene of Staphylococcus aureus subsp. aureus N315, listed as A17531 at the NCBI web site, plus sequences for restriction by *Nde I* and *XhoI*, was chemically synthesized and provided by MDBio, Inc., Taipei, Taiwan. The synthesized DNA product was inserted into the *NdeI* and *XhoI* sites of pET24a(+) (Novagen, Madison, WI) to afford pET24-SAK, which was then transformed into JM109 following the standard molecular biology method. The right clone was selected by gene sequencing.

Genes of ANXV and ANXXI were cloned from MOLT-4 cells, a human T-cell leukemia cell line. RNA was isolated using Trizole (Invitrogen, Carlsbad, CA) following the manufacturer's manual. cDNAs were acquired by reverse transcription and amplification using the Superscript™ II RT-PCR system (Invitrogen), following the manufacturer's manual. The primers used for ANXV were sense 5′-GGAATTCCATATGGCACAGGTTCTCAGA-3′ and antisense 5′-CCGCTCGAGGTCATCTTCTCCACAGAG-3′; the primers for ANXXI were sense 5′-GGAATTCCATATGAGCTACCCTGGCTAT and antisense 5′-CCGCTCGAGGTCATTGCCACCACAGAT. The *NdeI* and *XhoI* sites (underlined) were incorporated into the sense and antisense primers respectively for both cDNAs. The PCR products were inserted into the *NdeI* and *XhoI* sites of pET24a(+) to afford pET24-ANXV and pET24-ANXXI. By gene sequencing, the inserted genes were confirmed to be identical to that of the ANXV cDNA listed as NM001154 (GI:4809273) and that of the ANXXI cDNA listed as NM145869 (GI:22165432) at the NCBI web site.

PCR was performed on pET24-SAK to introduce the *NdeI* site (underlined) at the 5′ and 3′ ends of the SAK gene with platinum Taq DNA polymerase (Invitrogen) using sense primer 5′-GGAATTCCATATGTCAAGTTCATTCGAC-3′ and antisense primer 5′-GGAATTCCATATGTTTCTTTTCTATAAC-3′. The PCR product was inserted into the *NdeI* site of pET24-ANXV to afford pET24-SAK-ANXV or of pET24-ANXXI to afford pET24-SAK-ANXXI. The direction of the inserted SAK and its sequence were confirmed by gene sequencing.

Expression and purification of SAK and chimeras. The genes inserted in pET24a(+) were expressed using BL21 (DE3) in 100 ml LB. Induction was performed by the addition of IPTG (1 mM) at 37 °C for 3 h. Cell lysis was carried out in lysis buffer (containing lysozyme 1 mg/ml) by ultrasonication. Purification of the expressed protein was conducted using a Ni-NTA affinity column (Qiagene, Hilden, Germany) following the manufacturer's manual. The buffer used for lysing BL21 and for washing the NTA column contained sodium phosphate (50 mM, pH 8.0), sodium chloride (300 mM), Triton X-100 (0.1%), mercaptoethanol (20 mM), imidazole (10 mM) and glycerol (10%). The desired protein was eluted using 250 mM imidazole in the above lysis buffer. The eluted protein solutions were stored in −20 °C. The purified protein was diluted to the desired concentration in HBS before it was used for clot lysis assay. Protein assay was done as described by Bradford.[41] Concentration determinations were referred to a standard curve established using bovine serum albumin (66 kD).

Plasminogen activation reaction. To monitor the activation of plasminogen activity of fractions during purification of the expressed protein,[38,42] a reaction mixture containing 1 μM human plasminogen (Chromogenix, Milano, Italy), and the protein fraction (3 nM) in 100 mM Tris–HCl, pH 7.5, was incubated at 37 °C. At 2, 4, and 6 min, 5-μl aliquots were removed and diluted with 35 μl of buffer (700 mM NaCl, 100 mM Tris–HCl, pH 7.5).[38] When all of the aliquots had been collected, the chromogenic substrate *N-p*-tosyl-Gly-Pro-Lys nitroanilide (Chromogenix) was added to a final concentration of 1 mM. Cleavage of the substrate by the plasmin produced was monitored using a plate reader (Sunrise, Tecan, Austria) by measuring the increase in absorbance

at 405 nm over a 10-min period at room temperature. Plasmin activity (represented by the increase in absorbance at 405 nm per min) was plotted *versus* the activation time.

For kinetic analysis,[35,43,44] an equimolar mixture of a chimera and plasminogen (approximate final concentration, 5 μM) with a 5% excess of plasminogen were incubated at 37 °C for 10 min in 0.1 M sodium phosphate, pH 7.4, containing 25% glycerol; the mixture were then stored on ice. The preformed equimolar complex (final concentration, 5 nM) was incubated with plasminogen (2–48 μM for SAK-ANXV, 1–32 μM for SAK-ANXXI, 1–24 μM for SAK) at 37 °C in 0.1 M sodium phosphate, pH 7.4. The plasmin generated was measured at different time intervals (0–5 min) with S-2251 (final concentration, 1 mM) after 30-fold dilution in 0.1 M sodium phosphate containing 0.1 M NaCl. Initial activation rates were obtained from plots of the concentration of generated plasmin *versus* time.

Clot lysis assays. Citrated platelet rich plasma (PRP) from healthy donors was provided by Tri-Service General Hospital, Taipei, Taiwan. Platelet pool plasma (PPP) was prepared by centrifugation of PRP at 5,000 × *g* for 5 min. Once prepared, PPP was stored at 4 °C. All plasma was used within one week of blood collection. Clotting was initiated by adding human thrombin (0.8 NIH units/ml, Sigma, St. Louis, MI) and $CaCl_2$ (20 mM) in Hepes-buffered saline (HBS, 20 mM HEPES, 130 mM NaCl, pH 7.4). Immediately after mixing, aliquots of 100 μl of the clotting mixture were transferred to the wells of a microtiter plate and incubated for 2 h at room temperature to form PRP and PPP clots. Clot lysis assays were performed at room temperature by the addition of the tested protein at 200 nM and human plasminogen (1 μM) in HBS-$CaCl_2$ (100 μl). Clot lysis as reflected by reduction in turbidity was monitored using a microtiter plate reader at 405 nm. The solution on top of the PRP clot was displaced before measurement of the OD value and in-placed afterward for further lysis reaction. The percentage of remaining turbidity was plotted over time. Separately, clots were treated with the tested protein (500 nM) in HBS-$CaCl_2$ with no added plasminogen for 1 h. The protein solution was removed, and each clot was washed twice with HBS (100 μl). Then plasminogen (1 μM) in HBS-$CaCl_2$ (100 μl) was added for incubation at 37 °C. All the controls were done as tests except for the addition of the tested protein. Variations in turbidity were also monitored following the addition of thrombin (0.8 NIH units/ml) to the above clotting mixture containing the tested protein at a final concentration of 100, 150, or 200 nM.

Clotting time. A mechanical coagulometer (ST-4; Diagnostica Stago, Parsippany, NJ) was used to monitoring clotting time following the addition of human PRP (120 μl, 3.2% citrated) to a mixture to form a solution of 150 μl containing various concentrations of the tested proteins (50, 100, 150, and 200 nM), thrombin (2 NIH units/ml), and $CaCl_2$ (20 mM) in HBS, pH 7.4.

Stability of chimeras against action of plasmin. To determine the stability of the fusion protein in the presence of plasmin, the method of Ueshima *et al.*, used to process SAK derivatives, was adopted and modified as follows.[45] Equimolar mixtures of plasminogen and a chimera at 6 μM were incubated in Tris–HCl, (100 mM, pH 7.5) at 37 °C for various periods for about 1 h. They were incubated for 8 min when SAK was used in place of a chimera. The processed mixture was incubated with an equal volume of 2 × sample loading buffer containing DTT (0.5 mM) for 5 min in an ice bath. Then it was loaded on a sodium dodecyl sulfate–16% polyacrylamide gel, and separated at 100 V for 190 min.

Results and Discussion

All the expressed proteins contained a 6-His-tag at the C-terminal and were purified to high purity as shown on SDS–PAGE (Fig. 1) using a Ni-NTA column. The concentrated protein, eluted with buffer containing Triton X-100 (0.1%) and glycerol (10%), remained soluble for about 6 months otherwise tended to precipitate after a few days. The arrangement to fuse SAK at the N-terminal of the ANXs was to avoid cleavage of chimeras around the fusion site since plasmin tends to attack around sequences within the first 10 N-terminal residues of SAK.[38] Also, the first 10 N-terminal residues of staphylokinase must be removed with plasmin to expose the positively charged lysine residue at position 11, which is essential for the plasminogen activation activity of SAK.[38,41,46–48]

Plasminogen activation assay was routinely used to monitor active fractions during the expression of the expressed His-tagged proteins, in which amidolytic activity on the chromogenic peptide substrate of plasmin which was generated in the presence of a catalytic amount of the tested protein was measured. Both chimeras were found to be virtually void of amidolytic activity in the absence of plasminogen. Kinetic analysis of chimeras afforded comparable catalytic efficiency (k_{cat}/K_m) of 0.019 $\mu M^{-1} S^{-1}$ for SAK-ANXV and 0.020 $\mu M^{-1} S^{-1}$ for SAK-ANXXI, in comparison with 0.028 $\mu M^{-1} S^{-1}$ for SAK (mean of two independent determinations with $R > 0.99$, 0.97, and 0.99 respectively). The liability of chimera to hydrolysis was checked by challenge under equimolar reaction of plasminogen and chimera at 6 μM.[38] We found that it is difficult to see both SAK and a clear separation of the formed plasmin from the remaining plasminogen under the non-reducing condition in the same gel of SDS–PAGE (data not shown). For clear separation (Fig. 1), DTT was applied to cleave the β-chain (29 kD) of plasmin.[38] It was found that most plasminogen was converted into plasmin in about 10 min by either chimera, as seen in Fig. 1. In contrast, over 1 h of reaction, no hydrolysis of either

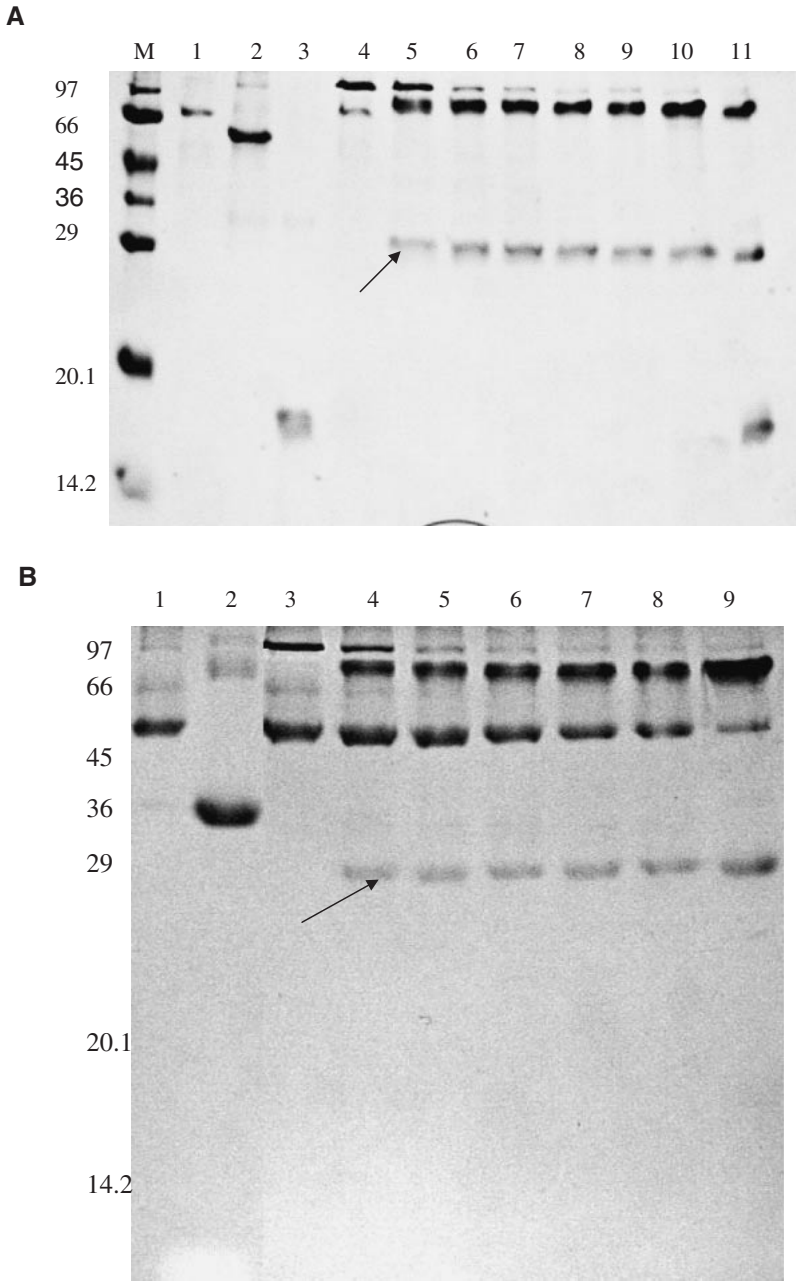

Fig. 1. SDS–PAGE Monitoring the Stability of Chimeras under the Equimolar Reaction with Plasminogen (6 μM).

Samples were loaded with sample loading buffer containing DTT (0.5 mM). A, time course reaction of plasminogen (90.5 kD) and SAK-ANXXI (70 kD), from lanes 4 to 10 for 0, 2, 4, 8, 16, 32, and 64 min; for comparison, lane 1, unreacted SAK-ANXXI; lane 2, ANXXI (54 kD); lane 3 unreacted SAK (15.5 kD); lane 11, reaction of plasminogen and SAK for 10 min; M, marker. B, time course reaction of plasminogen and SAK-ANXV, lanes 3 to 9 for 0, 2, 4, 8, 16, 32, and 64 min; for comparison, lane 1, unreacted SAK-ANXV (51.5 kD); lane 2 ANXV (36 kD). Arrow indicates β-chain of plasmin.

chimera into the corresponding moiety, *e.g.*, SAK, ANXV, or ANXXI, was seen on the gel. Hence, the observed plasminogen activation activities of chimeras are believed to derive mainly from the action of chimeras, not from that of the suspected hydrolysis product SAK. Similarly, most plasminogen was converted into plasmin in 10 min by reaction with SAK (Fig. 1A, lane 11) under the same equimolar condition. The reported structure of SAK indicated that both the N-terminus and the C-terminus were directed away from the protein core.[49] This arrangement might explain why extension of the protein sequence of SAK was tolerated.

After several attempts, clot lysis assay run at 200 nM for convenience of comparison though SAK at a lower dose (75 nM) for test has been reported.[6] It was found that both chimeras preserved the clot lysis activity to different extent (Fig. 2). SAK-ANXXI exhibited better efficiency in hydrolyzing PPP and PRP clots though it was a bit less efficient than SAK. Clot lysis by SAK-ANXV was apparently inefficient. It has been reported

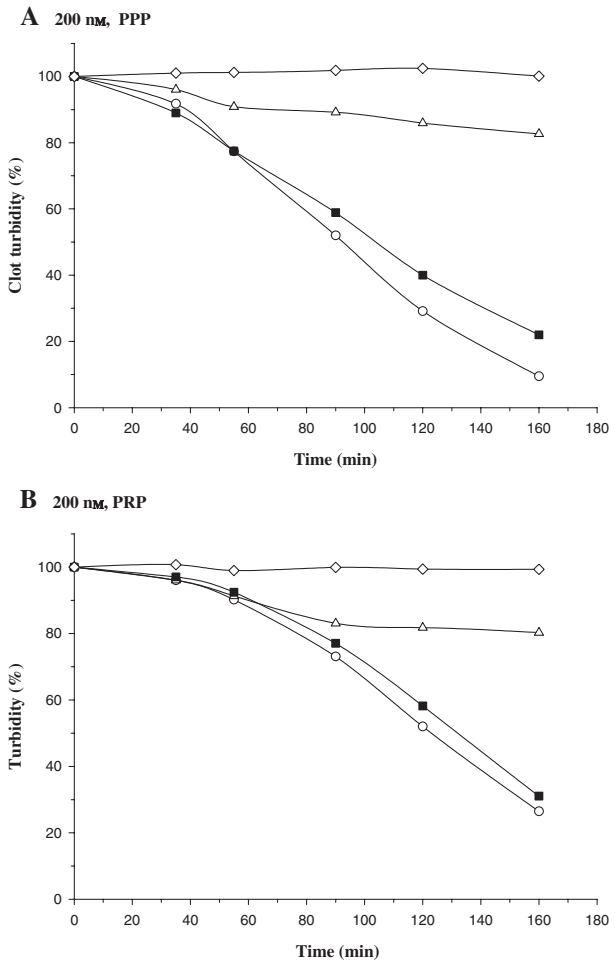

Fig. 2. Plasma Clot Lysis Mediated by SAK and Chimeras.
The clot was treated with a solution containing the protein at 200 nM, plasminogen (1 μM), $CaCl_2$ (20 mM) in HBS (SAK, white circle; SAK-ANXV, white triangle; SAK-ANXXI, black square) or the same solution without the protein as a control for clot stability (diamond). A set of representative results of three independent experiments is presented here. A, platelet poor plasma (PPP) clot; B, platelet rich plasma (PRP) clot.

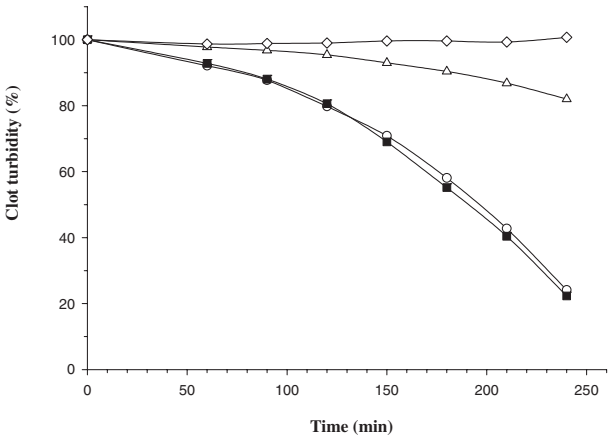

Fig. 3. PRP Clot Lysis Mediated by the Bound Thrombolytic Protein.
A platelet rich plasma clot was treated with a solution containing the thrombolytic protein at 500 nM and $CaCl_2$ (20 mM) in HBS (SAK, white circle; SAK-ANXV, white triangle; SAK-ANXXI, black square) or the same solution without the protein as a control for clot stability (diamond) for 1 h. Then the solution was removed and the clot was washed twice with HBS, followed by the addition of a solution containing plasminogen (1 μM) and $CaCl_2$ (20 mM) in HBS. A set of representative results of three independent experiments is presented here.

that it is the surface assembly of the thin layer complex of clot fibrin•plasma plasmin(ogen)•SAK following distribution of plasma plasminogen to exert the most capability of clot lysis activity plus that derived from plasmin produced by the interaction of plasminogen and SAK in bulky solution above the clot.[36,50] As shown in Fig. 3, the protein tested was allowed to pre-incubate with clots without plasmin(ogen) for 1 h, then the unbound protein was removed and plasminogen was added for further incubation. As reported, a plasminogen activator like SAK does not specifically bind to and lyses clot without the involvement of plasminogen. Only in the case of SAK and SAK-ANXXI up to 500 nM, residual clot lysis activity was seen and the clot lasted much longer than that shown in Fig. 2. Nearly absence of activity was seen for SAK-ANXV. The residual clot lysis activity probably derived from sparse binding of the SAK moiety to plasminogen bound inside when the clot was formed from plasma. It appears likely that the effect of the ANX moiety of a chimera works better to compete against the formation of a PRP clot than to resolve the formed clot. Hence, further comparison was carried out based on anticoagulation activity. The variation in turbidity of the clotting mixture containing SAK or a chimera after the addition of thrombin was monitored. A typical set of data is presented in Fig. 4. The variation in turbidity reflected by the OD value indicates that a clot was forming and lysing although it might not have been concrete enough to be detected using a tip. A distinct variation pattern of turbidity during clot formation was found among chimeras and SAK, probably indicating an integral effect of the unhydrolyzed chimera. In the case of SAK (Fig. 4A), a clot apparently formed. The formed clot was soon lysed. The OD value (turbidity) varied accordingly. In the case of SAK-ANXXI (Fig. 4B), it was apparent that PRP clot formation was retarded at 100 nM since no sound gel type polymer but at most viscous liquid was seen (discerned using a tip). The later increase in the OD value was probably caused by slow formation of a small white aggregate of unknown components in the center of the well, followed by fast movement of it aside to the well wall, resulting in a steep drop in the OD value. In the case of SAK-ANXV at 200 nM (Fig. 4C), PRP clot formation was a bit similar to the case of SAK-ANXXI. At a lower concentration of 150 nM or 100 nM, a clot was formed and was not resolved within the measuring period. Furthermore, a mechanical coagulometer was applied to determine dose-dependent effect on extension of clotting time (Fig. 5).[31] For convenience, thrombin was increased to 2 NIH units per ml. ANXXI were

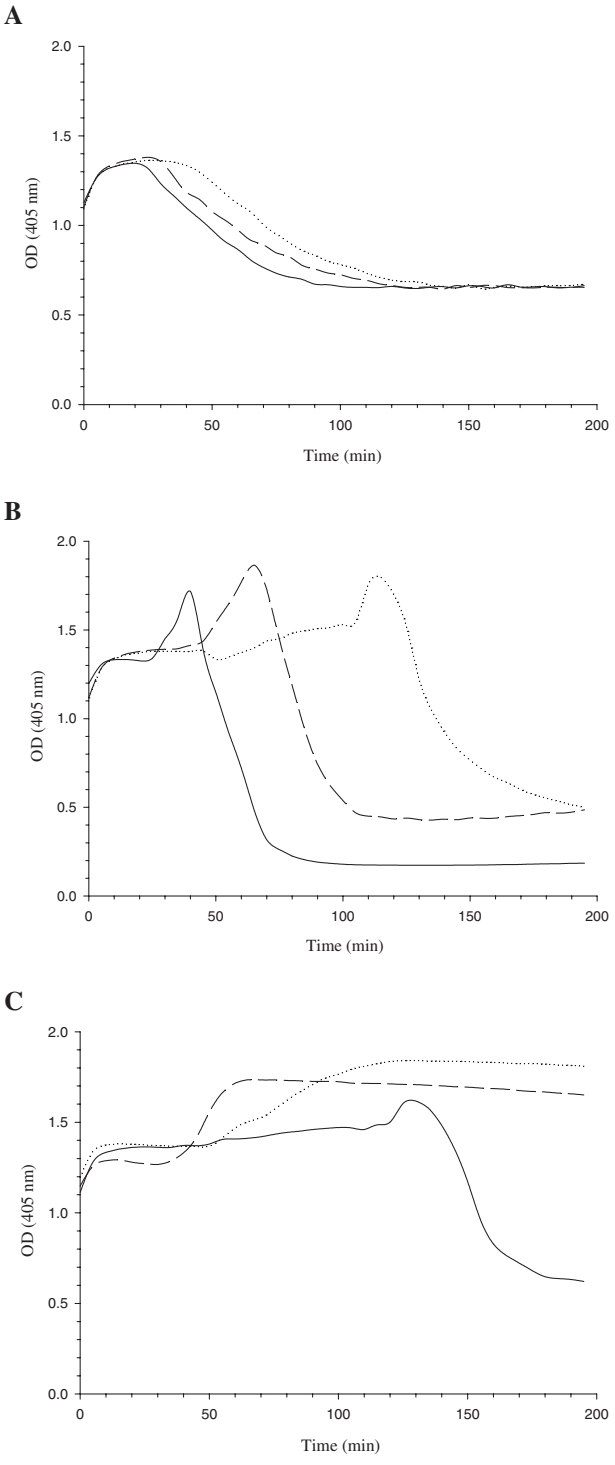

Fig. 4. Retardation of Clot Formation.

PRP was mixed with a solution containing a chimera or SAK at various concentrations and $CaCl_2$ (20 mM) in HBS immediately after the addition of thrombin. Variations in the OD value at 405 nm were recorded. A, SAK; B, SAK-ANXXI; C, SAK-ANXV; Solid line, 200 nM; long dashed line, 150 nM; dotted line, 100 nM. A set of typical data is presented here.

found to have a stronger effect on the extension of clotting time than ANXV. Chimeras were found to have a stronger effect than unfused ANXs. It is apparent that

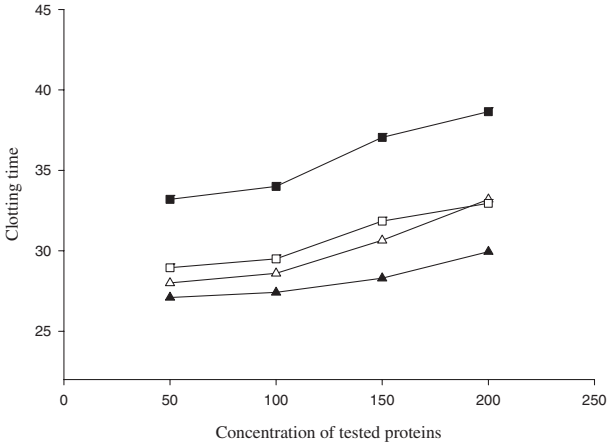

Fig. 5. Clotting Time.

Clotting time was measured with a mechanical coagulometer (ST-4; Diagnostica Stago, Parsippany, NJ) following the addition of human PRP (120 µl, 3.2% citrated) to a mixture to form a solution of 150 µl containing the tested protein (50, 100, 150, and 200 nM respectively), thrombin (2 NIH units/ml), and $CaCl_2$ (20 mM) in HBS, pH 7.4; (white triangle, SAK-ANXV; black square, SAK-ANXXI; black triangle, ANXV; white square, ANXXI). A typical set of data of the average of three experiments is presented.

SAK-ANXXI had a much stronger effect than the other three proteins tested. SAK did not have a dose-dependent effect (data not shown) probably due to lysis of the clot at a higher dose when it was forming, as shown in Fig. 4A.

Interaction between plasmin(ogen) and SAK can be various depending on the presence and status of fibrin.[35,51] It has been reported that SAK has much weaker affinity to plasminogen in the absence of fibrin, *i.e.*, plasminogen activation assay and SAK has the strongest affinity to partially degraded fibrin bound plasmin(ogen) when fibrinolysis has just begun.[50] SAK tends to undergo surface assembly of a thin layer complex with fibrin bound plasma plasmin(ogen) on the clot. It is known that ANX protein tends to polymerize on anionic membrane surfaces,[52] thus making the anionic phospholipids unavailable for assembly of coagulation enzyme complexes on platelets.[53,54] Whether the ANX moiety after it is fused with SAK can still polymerize to the highest degree on activated platelets and in the meanwhile whether the fused SAK moiety can interact properly with partially degraded fibrin bound plasmin(ogen) is a major concern in the application of an ANX chimera. It was postulated that the long N-terminal sequence of ANXXI probably serves as a natural linker to dispatch two moieties to function properly in a complicated microenvironment when they are still linked. Thus, a new option other than the most committed ANXV and without the need for elaborate work on construction of the complicated linker when one considers the ANX based chimera for a good targeting effect at activated platelets or apoptotic cells is presented here.

Acknowledgments

We thank the National Defense Medical Center of Taiwan (DOD-92-79) and the Teh-Tzer Study Group for Human Medical Research Foundation (A932084) for financial support. We also thank Miss H. Y. Huang for help in preparing cDNA of ANXXI.

References

1) Collen, D., Staphylokinase: a potent, uniquely fibrin-selective thrombolytic agent. *Nat. Med.*, **4**, 279–284 (1998).
2) Ohman, E. M., Harrington, R. A., Cannon, C. P., Agnelli, G., Cairns, J. A., and Kennedy, J. W., Intravenous thrombolysis in acute myocardial infarction. *Chest*, **119**, 253S–277S (2001).
3) Collen, D., Towards improved thrombolytic therapy. *Lancet*, **342**, 34–36 (1993).
4) Anderson, H. V., and Willerson, J. T., Thrombolysis in acute myocardial infarction. *N. Engl. J. Med.*, **329**, 703–709 (1993).
5) Collen, D., De Bono, D. P., Lijnen, H. R., and Pannekoek, H., Development of novel thrombolytic agents. *J. Intern. Med.*, **236**, 433–437 (1994).
6) Lian, Q., Szarka, S. J., Ng, K. K., and Wong, S. L., Engineering of a staphylokinase-based fibrinolytic agent with antithrombotic activity and targeting capability toward thrombin-rich fibrin and plasma clots. *J. Biol. Chem.*, **278**, 26677–26686 (2003).
7) Hanna, G. P., and Smalling, R. W., Therapy with thrombolytic agents in coronary artery disease. *Curr. Opin. Cardiol.*, **13**, 267–273 (1998).
8) Collen, D., Designing thrombolytic agents: focus on safety and efficacy. *Am. J. Cardiol.*, **69**, 71A–81A (1992).
9) Falati, S., Gross, P., Merrill-Skoloff, G., Furie, B. C., and Furie, B., Real-time *in vivo* imaging of platelets, tissue factor and fibrin during arterial thrombus formation in the mouse. *Nat. Med.*, **8**, 1175–1181 (2002).
10) Chen, H. H., Vicente, C. P., He, L., Tollefsen, D. M., and Wun, T. C., Fusion proteins comprising annexin V and Kunitz protease inhibitors are highly potent thrombogenic site-directed anticoagulants. *Blood*, **105**, 3902–3909 (2005).
11) Martin, S. J., Reutelingsperger, C. P., McGahon, A. J., Rader, J. A., van Schie, R. C., LaFace, D. M., and Green, D. R., Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J. Exp. Med.*, **182**, 1545–1556 (1995).
12) Zwaal, R. F., and Schroit, A. J., Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood*, **89**, 1121–1132 (1997).
13) Emoto, K., Toyama-Sorimachi, N., Karasuyama, H., Inoue, K., and Umeda, M., Exposure of phosphatidylethanolamine on the surface of apoptotic cells. *Exp. Cell Res.*, **232**, 430–434 (1997).
14) Sims, P. J., and Wiedmer, T., Unraveling the mysteries of phospholipid scrambling. *Thromb. Haemost.*, **86**, 266–275 (2001).
15) Dachary-Prigent, J., Toti, F., Satta, N., Pasquet, J. M., Uzan, A., and Freyssinet, J. M., Physiopathological significance of catalytic phospholipids in the generation of thrombin. *Semin. Thromb. Hemost.*, **22**, 157–164 (1996).
16) Morrissey, J. H., Neuenschwander, P. F., Huang, Q., McCallum, C. D., Su, B., and Johnson, A. E., Factor VIIa-tissue factor: functional importance of protein-membrane interactions. *Thromb. Haemost.*, **78**, 112–116 (1997).
17) Zwaal, R. F., Comfurius, P., and Bevers, E. M., Lipid-protein interactions in blood coagulation. *Biochim. Biophys. Acta*, **1376**, 433–453 (1998).
18) Burgoyne, R. D., and Geisow, M. J., The annexin family of calcium-binding proteins. *Cell Calcium*, **10**, 1–10 (1989).
19) Freemont, P. S., A review of studies leading towards a three-dimensional understanding of the annexin family of proteins. *Biochem. Soc. Trans.*, **18**, 1098–1101 (1990).
20) Huber, R., Romisch, J., and Paques, E. P., The crystal and molecular structure of human annexin V, an anticoagulant protein that binds to calcium and membranes. *EMBO J.*, **9**, 3867–3874 (1990).
21) Jost, M., Weber, K., and Gerke, V., Annexin II contains two types of Ca(2+)-binding sites. *Biochem. J.*, **298** (Pt 3), 553–559 (1994).
22) van Heerde, W. L., Poort, S., van't Veer, C., Reutelingsperger, C. P., and de Groot, P. G., Binding of recombinant annexin V to endothelial cells: effect of annexin V binding on endothelial-cell-mediated thrombin formation. *Biochem. J.*, **302** (Pt 1), 305–312 (1994).
23) Shattil, S. J., Hoxie, J. A., Cunningham, M., and Brass, L. F., Changes in the platelet membrane glycoprotein IIb.IIIa complex during platelet activation. *J. Biol. Chem.*, **260**, 11107–11114 (1985).
24) Stratton, J. R., Dewhurst, T. A., Kasina, S., Reno, J. M., Cerqueira, M. D., Baskin, D. G., and Tait, J. F., Selective uptake of radiolabeled annexin V on acute porcine left atrial thrombi. *Circulation*, **92**, 3113–3121 (1995).
25) Shattil, S. J., Brass, L. F., Bennett, J. S., and Pandhi, P., Biochemical and functional consequences of dissociation of the platelet membrane glycoprotein IIb–IIIa complex. *Blood*, **66**, 92–98 (1985).
26) Thiagarajan, P., and Tait, J. F., Binding of annexin V/placental anticoagulant protein I to platelets: evidence for phosphatidylserine exposure in the procoagulant response of activated platelets. *J. Biol. Chem.*, **265**, 17420–17423 (1990).
27) Kingston, J. K., Bayly, W. M., Sellon, D. C., Meyers, K. M., and Wardrop, K. J., Measurement of the activation of equine platelets by use of fluorescent-labeled annexin V, anti-human fibrinogen antibody, and anti-human thrombospondin antibody. *Am. J. Vet. Res.*, **63**, 513–519 (2002).
28) Reutelingsperger, C. P., Kop, J. M., Hornstra, G., and Hemker, H. C., Purification and characterization of a novel protein from bovine aorta that inhibits coagulation: inhibition of the phospholipid-dependent factor-Xa-catalyzed prothrombin activation, through a high-affinity binding of the anticoagulant to the phospholipids. *Eur. J. Biochem.*, **173**, 171–178 (1988).
29) Thiagarajan, P., and Tait, J. F., Collagen-induced exposure of anionic phospholipid in platelets and

platelet-derived microparticles. *J. Biol. Chem.*, **266**, 24302–24307 (1991).
30) Tait, J. F., Engelhardt, S., Smith, C., and Fujikawa, K., Prourokinase-annexin V chimeras: construction, expression, and characterization of recombinant proteins. *J. Biol. Chem.*, **270**, 21594–21599 (1995).
31) Huang, X., Ding, W. Q., Vaught, J. L., Wolf, R. F., Morrissey, J. H., Harrison, R. G., and Lind, S. E., A soluble tissue factor-annexin V chimeric protein has both procoagulant and anticoagulant properties. *Blood*, **107**, 980–986 (2006).
32) Heymans, S., Vanderschueren, S., Verhaeghe, R., Stockx, L., Lacroix, H., Nevelsteen, A., Laroche, Y., and Collen, D., Outcome and one year follow-up of intra-arterial staphylokinase in 191 patients with peripheral arterial occlusion. *Thromb. Haemost.*, **83**, 666–671 (2000).
33) Szemraj, J., Walkowiak, B., Kawecka, I., Janiszewska, G., Buczko, W., Bartkowiak, J., and Chabielska, E., A new recombinant thrombolytic and antithrombotic agent with higher fibrin affinity: a staphylokinase variant. I. *In vitro* study. *J. Thromb. Haemost.*, **3**, 2156–2165 (2005).
34) Dahiya, M., Rajamohan, G., and Dikshit, K. L., Enhanced plasminogen activation by staphylokinase in the presence of streptokinase beta/betagamma domains: plasminogen kringles play a role. *FEBS Lett.*, **579**, 1565–1572 (2005).
35) Lijnen, H. R., Van Hoef, B., De Cock, F., Okada, K., Ueshima, S., Matsuo, O., and Collen, D., On the mechanism of fibrin-specific plasminogen activation by staphylokinase. *J. Biol. Chem.*, **266**, 11826–11832 (1991).
36) Collen, D., Vanderschueren, S., and Van de Werf, F., Fibrin-selective thrombolytic therapy with recombinant staphylokinase. *Haemostasis*, **26** (Suppl. 4), 294–300 (1996).
37) Wu, S. C., Castellino, F. J., and Wong, S. L., A fast-acting, modular-structured staphylokinase fusion with Kringle-1 from human plasminogen as the fibrin-targeting domain offers improved clot lysis efficacy. *J. Biol. Chem.*, **278**, 18199–18206 (2003).
38) Szarka, S. J., Sihota, E. G., Habibi, H. R., and Wong, S., Staphylokinase as a plasminogen activator component in recombinant fusion proteins. *Appl. Environ. Microbiol.*, **65**, 506–513 (1999).
39) Towle, C. A., and Treadwell, B. V., Identification of a novel mammalian annexin: cDNA cloning, sequence analysis, and ubiquitous expression of the annexin XI gene. *J. Biol. Chem.*, **267**, 5416–5423 (1992).
40) Farnaes, L., and Ditzel, H. J., Dissecting the cellular functions of annexin XI using recombinant human annexin XI-specific autoantibodies cloned by phage display. *J. Biol. Chem.*, **278**, 33120–33126 (2003).
41) Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254 (1976).
42) Schlott, B., Hartmann, M., Guhrs, K. H., Birch-Hirschfeid, E., Pohl, H. D., Vanderschueren, S., Van de Werf, F., Michoel, A., Collen, D., and Behnke, D., High yield production and purification of recombinant staphylokinase for thrombolytic therapy. *Biotechnology* (NY), **12**, 185–189 (1994).
43) Collen, D., Van Hoef, B., Schlott, B., Hartmann, M., Guhrs, K. H., and Lijnen, H. R., Mechanisms of activation of mammalian plasma fibrinolytic systems with streptokinase and with recombinant staphylokinase. *Eur. J. Biochem.*, **216**, 307–314 (1993).
44) Lijnen, H. R., Van Hoef, B., and Collen, D., Interaction of staphylokinase with different molecular forms of plasminogen. *Eur. J. Biochem.*, **211**, 91–97 (1993).
45) Ueshima, S., Silence, K., Collen, D., and Lijnen, H. R., Molecular conversions of recombinant staphylokinase during plasminogen activation in purified systems and in human plasma. *Thromb. Haemost.*, **70**, 495–499 (1993).
46) Gase, A., Hartmann, M., Guhrs, K. H., Rocker, A., Collen, D., Behnke, D., and Schlott, B., Functional significance of NH_2- and COOH-terminal regions of staphylokinase in plasminogen activation. *Thromb. Haemost.*, **76**, 755–760 (1996).
47) Schlott, B., Guhrs, K. H., Hartmann, M., Rocker, A., and Collen, D., Staphylokinase requires NH_2-terminal proteolysis for plasminogen activation. *J. Biol. Chem.*, **272**, 6067–6072 (1997).
48) Schlott, B., Guhrs, K. H., Hartmann, M., Rocker, A., and Collen, D., NH_2-terminal structural motifs in staphylokinase required for plasminogen activation. *J. Biol. Chem.*, **273**, 22346–22350 (1998).
49) Rabijns, A., De Bondt, H. L., and De Ranter, C., Three-dimensional structure of staphylokinase, a plasminogen activator with therapeutic potential. *Nat. Struct. Biol.*, **4**, 357–360 (1997).
50) Sakharov, D. V., Lijnen, H. R., and Rijken, D. C., Interactions between staphylokinase, plasmin(ogen), and fibrin: staphylokinase discriminates between free plasminogen and plasminogen bound to partially degraded fibrin. *J. Biol. Chem.*, **271**, 27912–27918 (1996).
51) Lijnen, H. R., De Cock, F., Van Hoef, B., Schlott, B., and Collen, D., Characterization of the interaction between plasminogen and staphylokinase. *Eur. J. Biochem.*, **224**, 143–149 (1994).
52) Voges, D., Berendes, R., Burger, A., Demange, P., Baumeister, W., and Huber, R., Three-dimensional structure of membrane-bound annexin V: a correlative electron microscopy-X-ray crystallography study. *J. Mol. Biol.*, **238**, 199–213 (1994).
53) Mo, Y., Campos, B., Mealy, T. R., Commodore, L., Head, J. F., Dedman, J. R., and Seaton, B. A., Interfacial basic cluster in annexin V couples phospholipid binding and trimer formation on membrane surfaces. *J. Biol. Chem.*, **278**, 2437–2443 (2003).
54) Oling, F., Bergsma-Schutter, W., and Brisson, A., Trimers, dimers of trimers, and trimers of trimers are common building blocks of annexin a5 two-dimensional crystals. *J. Struct. Biol.*, **133**, 55–63 (2001).