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(54) **Title**: PROTEIN FUSION CONSTRUCTS POSSESSING THROMBOLYTIC AND ANTICOAGULANT PROPERTIES

(57) **Abstract**: The present invention discloses novel hybrid proteins that have both plasminogen activator and anti-thrombotic properties, including clot specific action, that renders these as highly advantageous for the treatment of circulatory disorders involving fibrin clot formation due to underlying tissue damage in the blood vesels leading to myocardial infarction, strokes etc. Also disclosed are new proteins, and methods of obtaining the same, that help to dissolve blood clots by activating plasminogen in a plasmin or thrombin dependent manner and also inhibit both the activity and generation of thrombin through the intrinsic pathway of blood coagulation.

PROTEIN FUSION CONSTRUCTS POSSESSING THROMBOLYTIC AND ANTI-COAGULANT PROPERTIES

FIELD OF THE INVENTION

The present invention relates to thrombolytic drugs which include chimeric fusion proteins comprising a thrombolytic protein and the 4, 5, 6 epidermal growth factor-like domains (EGF 4,5,6) of thrombomodulin.The inventive fusion proteins possess plasminogen activation, thrombin inhibition, and anticoagulant protein C pathway activation activity. The inventive fusion proteins have therapeutic potential to break up thrombi and prevent reocclusion at the site of the clot.

BACKGROUND OF THE INVENTION

Thrombosis, the formation and development of a blood clot or thrombus within the vascular system, while a life saving process when it occurs during a hemorrhage, can be life threatening when it occurs at any other time. The thrombus can block a vessel and stop blood supply to an organ or other body part. If detached, the thrombus can become an embolus and occlude a vessel distant from the original site. Thrombotic disorders now constitute one of the major causes of mortality in both developing and developed countries world-wide. Although still the most preferred emergency ("SOS") medication against thrombotic circulatory disorders, the plasminogen activator protein drugs, such as streptokinase (SK), staphylokinase (SAK) and tissue plasminogen activator (tPA), are slowly losing their supremacy to emergency cardiac interventions such as stenting and bypass surgeries in the affluent countries because of bleeding risks associated with their use, and also the often-encountered problem of clot reformation at the same site of vascular injury due to thrombin activity and/or fresh generation of thrombin. Thus,

there is an acute need to develop smarter and more effective thrombolytic drugs with additional features such as clot specificity and anti-thrombotic properties.

Blood clot formation is an end-result of a complex set of cascade reactions wherein several biochemical events cause the sealing, or repair, at the site of injury (Butenas and Mann 2002). On the basis of initiation of the blood coagulation cascade, pathways have been divided into extrinsic and intrinsic pathway, wherein the extrinsic pathway is initiated by the exposure of tissue factor (TF), whereas the intrinsic pathway is initiated by factor XII (Hageman factor), high molecular weight kininogen (HK), or prekallikrain. However, both cascade pathways ultimately join at the point of factor Xa generation, and subsequently follow common thrombin mediated fibrin generation(Cannon and Tracy 1995).

Thrombin generation is thecentral step of the blood coagulation process in vertebrates. During the thrombin generation process small amount of thrombin gets incorporated into the fibrin clot/network as it expands, and the catalytic site of this protease, being free in this absorbed thrombin, is able to amplify the clot growth (Liu, Nossel et al. 1979; Vali and Scheraga 1988). Thrombin interacts with diverse substrates and activates several clot promoting factors e.g. (a) it causes the activation of platelets by cleaving their cognate receptors (b) causes feed-back activation of factor V, VIII and XI (c) cause the activation of transglutminase, factor XIII and (d) converts fibrinogen into fibrin. Once the clot forms and stabilizes by inter-strand cross-linking, under the pathological conditions, it impedes the normal blood flow in vessels and leads to blockage of arteries and veins.

One of the most common, and preferred, medications against circulatory disorders emanating from pathological thrombus formation in animals/humans such as myocardial infarction, is the intravenous infusion of thrombolytic agents(Lijnen and Collen 1988; Collen and Lijnen 1990;Francis and Marder 1991). Available thrombolytics such as streptokinase (SK), urokinase (UK) and tissue type plasminogen activator (tPA) essentially operate through the same, plasmin-

dependent mechanism (since these are plasminogen activators), where these cleave the scissile peptide between residues 561 and 562 of plasminogen and convert it into its proteolytically activated form, plasmin. Tissue type plasminogen activator and urokinase are proteases that specifically recognize the scissile peptide bond in human plasminogen (direct activators), whereas streptokinase and staphylokinase (which are protein 'co-factors' rather than proteases and thus 'indirect' activators; (see: (De Renzo, Boggiano et al. 1967; Buck, Hummel et al. 1968; McClintock and Bell 1971) first make tight 1:1 complexes with plasmin or plasminogen, and the resultant proteolytically active complex(es) cleave the scissile peptide bond of other, 'free' plasminogen molecules and exponentially generate plasmin (Reviewed by:(Wohl, Summaria et al. 1978; Castellino and Powell 1981; Wohl, Sinio et al. 1983; Davidson, Higgins et al. 1990). Of all the currently available clot-busters, that is, plasminogen activator protein drugs, streptokinase exhibits the highest thrombolytic power, although, being (like SAK) of bacterial origin, it has the limitation of engendering immune reactions in a small minority of patients. Nevertheless, it is widely used as an affordable thrombolytic because of its relatively low cost as compared to tPA and UK.

Successful thrombolytic therapy helps maintain normal blood flow and improves the survival in a significant number of patients(Verstraete 1990), but early re-occlusion or re-thrombosis, often at the same site, has continued to limit the successful application of thrombolytic drugs. Several studies demonstrate that early reocclusion occurs in upto 30% of patients after thrombolytic therapy (Ohman, Califf et al. 1990). The cause of rethrombosis or early reocclusion is explained by plasmin activity which increases the hyper coagulability of blood(Eisenberg, Miletich et al. 1988); it is proposed that plasmin activates contact factors(Ewald and Eisenberg 1995), factor V (Lee and Mann 1989) and likely also prothrombin(Seitz*et al.*, 1993). Another suggested reason is the exposure of clot-bound thrombinsubsequent to the latter's dissolution, which, in turn, generates more thrombin, with fibrin–bound thrombin being relatively resistant to anti-thrombin

inhibitor/s (Hogg and Jackson 1989; Weitz, Hudoba et al. 1990); the 'released' thrombin again locally activates the procoagulant activity and starts to activate platelets (Kumar, Beguin et al. 1994; Puri, Kumar et al. 1995), thereby promoting a cycle of biochemical events leading to rethrombosis. Thus, inhibition of the thrombin at the site of injury both directly, and at the 'secondary' level of its procoagulant activity, should greatly thwart the above-described, unwanted chain of events. If such a property is integrated successfully in the same molecule as the thrombolytic drug, the advantages in terms of lives saved are obvious.

Plasminogen activators are a family of proteases which characteristically catalyse the enzymatic conversion of plasminogen to plasmin. TPA the enzymatic conversion of plasminogen to plasmin through the hydrolysis of a single Arginine-Valine bond.

Tissue plasminogen activator (also known as fibrinokinase, extrinsic plasminogen activator, t-PA or TPA) is a glycoprotein and has an approximate molecular weight (MW) of about 70,000 Daltons (68,000 Daltons). It is a serine protease which catalyses the enzymatic conversion of pro-enzyme plasminogen to active enzyme plasmin through the hydrolysis of a single Arginine-Valine bond. The catalytic site of t-PA is composed of amino acids His-322, Asp-371 and Ser-478. t-PA is a poor plasminogen activator in the absence of fibrin. The amino-terminal region is composed of several domains, which are homologous to other proteins. These distinct domains are involved in several functions of the enzyme, including binding to fibrin, fibrin-specific plasminogen activation, binding to endothelial cell receptors and rapid clearance in vivo. One such domain, comprising amino acid residues 50 to 87 (E domain) is homologous to Human Epidermal Growth Factor and seems to be involved in fibrin binding, fibrin affinity and in vivo clearance. The t-PA cDNA was cloned and subsequently expressed in Chinese hamster ovary (CHO) cells.

Tissue plasminogen activator (t-PA) is a component of the mammalian fibrinolytic system responsible for the specific activation of plasminogen associated with fibrin clots (i.e. it is capable of dissolving blood clots). Tissue plasminogen activator-

mediated clot dissolution shows increased level of fibrinopeptide-A in plasma, which is a direct marker of clot-bound thrombin (Weitz, Leslie et al. 1998). A combination of tissue plasminogen activator and currently known anti-thrombin drugs such as heparin and hirudinis often used in medication but this anti-thrombin acts only on free thrombin, and the clot-bound thrombin is resistant towards heparin and other the inhibitors due to their low affinity. Besides, these drugs do not affect the indirect promoters of further thrombin generation (e.g. Factor V and Factor VIII). Hence, new chimeric proteins need to be designed which activate plasminogen along with inhibition of clot bound thrombin as well as the indirect promoters of thrombin, such as Factor V and Factor VIII.

Work on post-thrombolytic plasmin activity and consequent thrombin generation clearly suggests that there are potent factors which lead to the re-activation of the coagulation pathway even when the pathological clot has been cleared by a clot-buster drug. In addition, the generated thrombin is itself a potent coagulation pathway activator. It is remarkable that thrombin also performs an anti-coagulant function, which is an elegant example of the "self-limiting" control mechanisms of the hemostasis system such that there is no 'run away' coagulation throughout the vasculature. Free thrombin makes a 1:1 high-affinity, non-covalent complex with thrombomodulin, a cell surface protein(Kurosawa, Galvin et al. 1987). Once the thrombin-thrombomodulin complex is formed its substrate specificity is redirected from a pro-coagulant mode to an anti-coagulant one, whereby it activates the Protein C anticoagulant pathway. Thus, even though thrombin alone can activate protein C but once it complexes with thrombomodulin it accelerates protein C activation by nearly a 1000-fold (Esmon and Owen 1981; Owen and Esmon 1981)).

Mature thrombomodulin contains different domains responsible for different functions, namely thrombin inhibition and protein C activation which reside in the epidermal growth factor-like domains of this large protein. Epidermal growth factor-like (EGF) domains 5 and 6 are responsible mainly for thrombin affinity and

EGF 4, 5 and 6 domains, , activate protein C together (Kurosawa, Stearns et al. 1988; Stearns, Kurosawa et al. 1989).Thrombomodulin, or its isolated EGF domains 4, 5 and 6, are known to activate the protein C-centered anticoagulant pathway, and also directly inhibit thrombin's activity, but cannot dissolve the fibrin clots by themselves. For this, a thrombolytic agent is necessary. Both types of agents can, independently of each other, be used during cardiac thrombotic maladies, but the advantages of a single drug with both types of attributes (which has not been demonstrated so far) are obvious.

The term 'hemostasis' refers to the balance between anticoagulant and procoagulant activities in the blood/vascular system, wherein normally, the blood components, particularly platelets, do not interact abnormally with the blood vessels' inner lining. In case of injury or disease condition, platelets tend to adhere, and as a result, blood coagulation factors start to accumulate and get activated at the site of this injury, which, although a response to initiate repair at the site of injury, results in blood occlusion, thereby often precipitating thrombotic crises.

Blood coagulation and dissolution of a clot, inside blood vessels, are both necessary physiological processes for normal hemostasis. Clot formation and dissolution mechanisms inside the blood vessel are well known in the literature, and the role of different pro-coagulant proteins (clot promoting) and anti-coagulant proteins are now fairly also well-studied. Clot formation is the result of a complex set of reactions wherein thrombin plays a central role in initiation as well ascoagulation cascade acceleration, with the end-result appearing in the form of a stable fibrin mesh.

Every modern thrombolytic/fibrinolytic therapy usually combines an anti-thrombotic medication as well (such as aspirin, heparin etc) which is required for the maintenance of normal equilibrium between the pro-coagulants and anti-coagulants during the clot lysis. During lysis, the transiently released thrombin initiates a self-generation loop to amplify clot growth; sometimes thisresults in

ashift of the equilibrium towards the reformation of clot, or activation of the pro-coagulants/clot promoters.

Thrombolytics dissolve pathological clots by activating intrinsic plasminogen in the circulatory system. Among the availableand therapeutically useful thrombolytics, streptokinase exhibits the highest thrombolytic potential but sometimes it leads to hemorrhage during the medication because of a lack of clot specificity. This problem is relatively less associated with other thrombolytics(tPA and SAK) because of their relatively increased fibrin clot specificity, but other shortcomings of these thrombolytics are lesser fibrinolytic potential and shorter *in vivo*half life. All the thrombolytics have essentially same mechanism of thrombolysis (plasminogen activation), but share a common problem, in that that after thrombolysis the generated thrombin often further amplifies thrombin generation by a feed-back mechanism controlled by blood coagulation factor Va and factor VIIIa. In the thrombolysis process, the clot-bound thrombin is also released into the circulation which is relatively resistant to in-built anti-thrombin and other externally provided thrombin inhibitory drugs. This clot-bound thrombin, present at the near vicinity of the 'original' injury also helps to generate even more thrombin in the vicinity of the clot/site of injury, particularly after incomplete removal of clot, which leads to the clinically grave, early re-occlusion problem.

Thombin generation during the lysis and activation of other intrinsic pro-coagulants is a normal part of the hemostasis equilibrium. Anti-thrombin agents like heparin, hirudin and chemically synthesized drugs can inhibit thrombin and prevent its subsequent transient effects, like suppression of hyper-responsive platelet formation process, inhibition of fibrinogen to fibrin conversion and other clot promoting activities which induces hyper-coagulibility in blood. Notably, however, all these direct inhibitors act on transiently generated thrombin, but not on those factors which actually speed up thrombin generation and play key roles in the procoagulant pathway. Thus, none of these drugs acts as efficaciously as desired on

blood coagulation factors V and VIII, which play crucial role in thrombin generation and early re-occlusion.

The well-known cell surface molecule thrombomodulin, which makes 1:1 complexes with thrombin and directs its pro-coagulant function into a potent anti-coagulant, namely protein C activator. Both activated protein C and protein S degrade activated factor V and factor VIII (Esmon 1989).

Although all available thrombolytics do possess clot dissolving capability and their mechanism is well evident in literature,butduring clot lysis, remnants of clot and plasmin lead to thrombin generation; at the same time, since these agents cannot inactivate the thrombin which makes a transient appearance during thelysis of the clot, the result is that reocclusion is a commom problem subsequent to thrombolytic therapy. To date, no anti-thrombin drugs are available with a combination of thromolytic and anti-thrombin properties, especially the pro-coagulant activity so as to inactivate the'real culprit' which is chiefly responsible for re-thrombosis.

SUMMARY OF THE INVENTION

The presentinvention relates to the design of improved thrombolytic drugs, which, apart from being capable of plasminogen activation, also exhibit capabilities to inhibit thrombin generation. The presence of these dual properties in the same molecule is potentially of great benefit clinically since this helps to minimize the early re-occlusion problem after thrombolytic therapy. The invention discloses strategically designed chimeric polypeptides, where domains/segments with antithrombin-properties, derived from thrombomodulin, are fused with various thrombolytic proteins such that the resultant fusion/chimeric polypeptides exhibit clot dissolving (thrombolytic) as well as anti-thrombin properties at the same time. These novel fusion constructs have the ability to activate the thrombolytic system in a plasmin and/or thrombin dependent manner (which makes the constructs clot-specific, as fibrin clots are plasmin- as well as thrombin-rich, whereas free plasmin

and thrombin are short-lived in the circulation, being rapidly inactivated by Serpins such as alpha-2 antiplasmin, anti-thrombin etc). These constructs also simultaneously inhibit the transiently generated thrombin released during clot lysis, and also initiate the in-built (intrinsic) anticoagulant Protein C pathway during the medication with these agents in the event of thrombotic disorders such as stroke, myocardial infarction, deep vein thrombosis, etc.

Unlike the currently available thrombolytics, the present invention discloses new thrombolytic constructs which are capable of targeting factors Va and VIIIa of the coagulation cascade (besides the capability to activate the plasmin-dependent thrombolytic pathway) and also the intrinsically in-built anticoagulant Protein C pathway; this combinatorial presence of several desirable capabilities in the same drug molecule improves their overall efficacy, such that these can dissolve the pathological blood clots while minimizing one of the most potentially life-threatening, post-thrombolytic events, namely the relapse of the clotting process.

The approach is to design, test, and validate the creation of fusion proteins with both anti-thrombin and thrombolytic properties. The successful "functional" fusion of EGF 4,5,6into a thrombolytic molecule can potentially target the latter towards thrombin-rich clots due to the former's intrinsic thrombin affinity. At the same time, if the said fusion, apart from retaining the two originally 'independent' parent biological activities (i.e. anti-thrombin and plasminogen activator activities), confers a pro-drug nature into the hybrid molecule in that it preferentially gets activated in the vicinity of the clot and not systemically, this would be another very useful and advantageous feature due to its targeted nature of action.

In the present invention, several hybrid protein molecules with a combination of thrombolytic property with thrombin inhibiting as well as protein C activation capability has been successfully functionalized by effective integration in the same molecule/s in order to vastly improve the efficacy of presently available thrombolytic drugs and help prevent the re-thrombosis phenomenon, since the

presence of all these properties integrated into the same agent/molecule will impart both real-time (temporal) and spatial (*in situ*) beneficial action at the occluding fibrin blood clot in the various thrombotic pathological syndromes.

In the present invention, streptokinase, tissue plasminogena activator and staphylokinase have been fused with EGF 4,5,6, to generate chimeric molecules having both the properties of initiation of plasmin dependent thrombolytic system and thrombin mediated Protein C anticoagulant pathway. Moreover, in some of embodiments of the present invention, the successful integration of thrombolysis, clot specificity,and antithrombin activities — which has not been reported so far is also achieved.

In different fusion constructs, the fusion junctions between the thrombolytic agents and the EGF domains were carefully designed using various preferred amino acid residues in the linkers, and different thrombin and/or plasmin cleavable sites were introduced, which gave different time-dependent plasminogen activation kinetics *in vitro* due to the proteolytic release of EGF 4,5,6 from the chimeras and consequent activation of the thrombolytic component. The presence of different thrombin cleavable site/s at the junction of thrombolytics and EGF 4,5,6 allows these two to separate from each other at the near-vicinity of thrombin rich clots by locally resident enzymes (thrombin or plasmin); thus after cleavage, each domain performs its function independently. Moreover, in some fusion constructs, transglutaminase-recognizable, cross-linking sequences have been introduced at strategic locations in the chimeric polypeptides so that the activated domains would bind covalently to the remnants of clot,in or around the epithelial tissues (whose damage led to the clot generation in the first place), due to the action of blood transglutminase, and thus continue to give the localized effect in terms of both plasminogen activation and thrombin inhibition long after the initial clot has dissolved. This would clearly lead to a 'healing' effect of the drug, an attribute that is unavailable in any thrombolytic protein drug currently being used.

BRIEF DESCRIPTION OF FIGURES

Fig. 1 is a schematic representation of different in-frame gene fusion constructs, where EGF 4,5,6 domains of thrombomodulin were fused with either DNA encoding for Streptokinase, Tissue plasminogen activator or Staphylokinase along with in-frame fused sequences coding for inter-domain linkers including ones with triple-Glycine (GGG), or transglutaminase-recognition signals (TG) to facilitate Coagulation Factor XIII- catalyzed covalent cross-linking of the hybrid protein with other tissues or the fibrin clot, as well as thrombin-cleavable sequence (TCS).

Fig. 1A is a schematic design A represents the DNA sequences encoding for EGF 4,5,6in-fusion at the N-terminal end of SK (streptokinase).

Fig. 1B is a schematic design B represents the EGF 4,5,6fusion at the C-terminal end of SK through an appropriate linker containing flexibility promoting residues (e.g., a triple-glycine stretch, GGG).

Fig. 1C is a schematic design C represents the EGF fusion simultaneously at N & C- terminii of SK along with a transglutaminase recognition site/stretch of residues.

Fig. 1D is a schematic design D represents the EGF 4,5,6 domains' fusion at the C-terminal end of SK, wherein 5 amino acids of SK were deleted.

Fig. 1E is a schematic design Erepresents the simultaneous fusion of EGF 4,5,6 at the N and C- termini of SK, where a thrombin recognition and cleavable sequence (TCS) is present at the junction of EGF and SK.

Fig. 1F is a schematic design Frepresents the simultaneous fusion of EGF 4,5,6 at N & C- termini of SK, where a thrombin recognition sequence is present at the junction of EGF and SK and a transglutaminase recognition sequence is present at the initial part (N-terminal end) of EGF.

Fig. 1G is a schematic design G represents the fusion of EGF domains at the junction of α and β domains of SK.

Fig. 1H is a schematic design H represents the fusion of EGF domains as indicated at the junction of β and γ domains of SK.

Fig. 1I is a schematic design I represents the EGF domains' fusion simultaneously at the N & C- terminii oftPA (tissue plasminogen activator). In all figures and anywhere such terms occur in the specification, "egf" refers to the EGF-like domains of tPA, while "EGF456" refers to the EGF 4,5,6 domains of thrombomodulin.

Fig. 1J is a schematic design J represents the EGF 4,5,6 domains fusion at the C-terminal end of tPA.

Fig. 1K is a schematic design K represents the EGF 4,5,6 domain fusion at the N-terminal end of tPA.

Fig. 1L is a schematic design L represents the EGF 4,5,6 domains' fusion at both N & C- termini of tPA (tissue plasminogen activator), where intrinsic egf of tPA is deleted.

Fig. 1M is a schematic design M represents the EGF 4,5,6 fusion at the N-terminal end of tPA, where intrinsic egf of tPA is deleted.

Fig. 1N is a schematic design N represents the EGF 4,5,6 fusion at the N-terminal end of tPA, where intrinsic egf and kringle 1 of tPA are deleted.

Fig. 1O is a schematic design O represents the EGF fusion at the C-terminal end of tPA, where intrinsic egf of tPA is deleted.

Fig. 1P is a schematic design P represents the EGF domain 4,5,6 fusion at the C-terminal end of tPA, where intrinsic egf and kringle 1 of tPA are deleted.

Fig. 1Q is a schematic design Q represents the EGF 4,5,6 domains fusion with tPA, where intrinsic egf of tPA are replaced by EGF 4,5,6 domains.

Fig. 1R is a schematic design R represents the EGF fusion with tPA, where intrinsic egf and kringle 1 of tPA are replaced by EGF 4,5,6 domains.

Fig. 1S is a schematic design S represents the fusion of EGF domains at the C-terminal end of SAK.

Fig. 1T is a schematic design T represents the fusion of EGFdomains at the N-terminal end of SAK.

Fig. 2. Is a schematic representation ofPCR methods and cloning schemes used in the preparation of different fusion constructs.

Fig. 2A is a schematic design showing the PCR, restriction digestion and cloning strategies used in the construction of N_EGF_SK fusion gene block.

Fig. 2B is a schematic design showing the PCR, restriction digestion and cloning strategies used in the making of SK_EGF fusion gene block.

Fig. 2C is a schematic design showing the restriction digestion and cloning strategies used in the making of N_EGF_SK_EGF fusion gene block.

Fig. 2D is a schematic design showing the PCR, restriction digestion and cloning strategies used in the making of Inter-domain SK_EGF (EGF 4,5,6 fused between the α and β domains of SK) fusion gene block.

Fig. 2E is a schematic design showing the PCR, restriction digestion and cloning strategies used in the making of interdomain SK_EGF (i.e. EGF 4,5,6 fused between the β and γ domains of SK) fusion gene block.

Fig. 2F is a schematic design showing the PCR, restriction digestion and cloning strategies used in the making of tPA gene block.

Fig. 2G is a schematic design showing the restriction digestion and cloning strategies used in the making of N_EGF_tPA and tPA_EGF fusion gene block.

Fig. 2H is a schematic design showing the PCR, restriction digestion and cloning strategies used in the making of tPA_EGF fusion gene block, where intrinsic egf and kringle 1 domains of tPA are selectively deleted.

Fig. 2I is a schematic design showing the PCR, restriction digestion and cloning strategies used in the making of N_EGF_tPA fusion gene block, where intrinsic egf and kringle 1 domain of tPA is deleted.

Fig. 2J is a schematic design showing the PCR, restriction digestion and cloning strategies used in the making of tPA_EGF fusion gene block, where intrinsic egf domain of tPA is replaced with EGF 4, 5,6 domains.

Fig. 2K is a schematic design showing the PCR, restriction digestion and cloning strategies used in the making of tPA_EGF fusion gene block, where intrinsic egf and kringle 1 domain of tPA is replaced with EGF 4, 5, 6 domains.

Fig. 3 Spectrophotometrically scans of plasminogen activation (Abs 405 nm) showing delayed plasminogen activation by N-terminal fused EGF 4,5,6 and SK constructs and decrease in the lag in presence of trace amounts of plasmin (see 'general methods used in examples' for details). Note that native SK does not show any significant lag in plasminogen activation.

Fig. 4. Representation of the increased thrombin clotting time assay by varying amounts (60-180 nm) of different fusion constructs and controls as indicated.

Fig. 5. Protein C activation profiles (see 'general methods' for details) in presence of different SK and EGF fusion constructs and suitable controls as indicated.

Fig. 6. Clotting time assay with different tPA fusion constructs (equimolar; 2 nm) and controls as indicated.

Fig. 7. Plasminogen activation in presence and absence of soluble fibrin with various tPA fusion constructs along with control as indicated.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the design and preparation of new thrombolytic fusion polypeptides that exhibit distinct functional advantages over the available forms available presently, namely tissue plasminogen activator, SK and SAK or their derivatives/modified forms because the currently available forms only dissolve the fibrin clots through plasminogen activation and cannot prevent post thrombolytic consequences which lead to the early re-occlusion problem during thrombolytic therapy essentially due to thrombin generation.

The present invention discloses methods of making fusion constructs with direct and indirect plasminogen activators fused with the 4th, 5th, and 6thEGF domains ofthrombomodulin which retain the capabilitynot only to activate plasminogen bit also inhibit thrombin directly as well as retain the ability for protein C activation through the anticoagulant pathway mediated by thrombin.

The terms "variant", "homolog", "derivative", "fragment" or "analog" in relation to the amino acid sequence of a protein include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acid from or to the sequence providing the resultant protein or (poly)peptide has activity equivalent to that of the unmodified protein. In particular, the term "homolog" covers homology with respect to structure and/or function. With respect to sequence homology, preferably there is at least 70%, more preferably at least 80%, even more preferably at least 85% homology to the sequence of the unmodified

protein. Preferably there is at least 90%, more preferably at least 95%, most preferably at least 98% homology to the sequence of the unmodified protein.

Typically, for the variant, homolog, derivative, fragment or analog of the present invention, the types of amino acid substitutions that could be made should maintain the hydrophobicity/hydrophilicity of the amino acid sequence. Amino acid substitutions may be made, for example from 1, 2 or 3 to 10, 20 or 30 substitutions provided that the modified sequence retains the ability to act in accordance with the present invention. Amino acid substitutions may include the use of non-naturally occurring analogues, for example to increase blood plasma half-life of a therapeutically administered polypeptide.

Conservative substitutions may be made, for example, as shown below. Amino acids in the same line may be substituted for each other:

ALIPHATIC Non-polar G A P I L V

Polar - uncharged C S T M N Q

Polar - charged D E K R AROMATIC H F W Y

As indicated above, proteins of the invention are typically made by recombinant means, for example as described herein, and/or by using synthetic means using techniques well known to the skilled person such as solid phase synthesis.

As used herein a "deletion" is defined as a change in either nucleotide or amino acid sequence in which one or more nucleotides or amino acid residues, respectively, are absent.

As used herein an "insertion" or "addition" is a change in a nucleotide or amino acid sequence, which has resulted in the addition of one or more nucleotides or amino acid residues, respectively, as compared to the naturally occurring protein.

As used herein "substitution" results from the replacement of one or more nucleotides or amino acids by different nucleotides or amino acids, respectively.

As used herein, the terms "4, 5, and 6 epidermal growth factor-like domains" or "EGF 4,5,6" can be any one of the EGF 4, 5, or 6 domains of thrombomodulin, or can refer to all three EGF 4, 5, and 6 domains of thrombomodulin covalently bonded together as a peptide or peptide fragment. Each of the 4, 5, and 6 epidermal growth factor-like domainshave homology to one or more domains of the epidermal growth factor (EGF) protein.

The present invention discloses a chimeric protein construct comprising the 4, 5, and 6 epidermal growth factor-like domains (EGF 4,5,6) of thrombomodulin fused to a thrombolytic protein selected from the group consisting of a streptokinase, a tissue plasminogen activator, a staphylokinase, a urokinase, and derivatives and analogs thereof.

In an embodiment of the present inventions is disclosed a chimeric protein construct , where thrombomodulin EGF 4,5,6 domains are fused to said thrombolytic protein, or derivative or analog thereof at the N-terminus, C-terminus, or both N- and C-termini of said thrombolytic protein, or derivative or analog thereof.

The present invention also discloses the design of genetic constructs with minimum essential antithrombotic parts of EGF fused with a thrombolytic protein (SK, tPA or SAK) at the portion encoding the N-terminal end of the thrombolytic protein, wherein translation (in the resultant 'chimeric' ORF) begins with the 4th domain of EGF followed by the portion encoding for thrombolytic polypeptide synthesis. Thus, the mature polypeptide or folded protein contains not only the two different types of proteins but both the functionalities as well. These constructs with the thrombolytic protein are termed as EGF N-terminal fusion constructs.

In another embodiment,we have defined the design principle, and methods of construction, of EGF domains fused at the C-terminal side of the thrombolytic

protein, by first fusing ofthe oligonucleotides blocks encoding the relevant portions (thrombolytic-EGF 4,5,6) such that the translation begins with the thrombolytic protein and terminates at the 6th domain of EGF, and then expressing the oligonucleotide hybrid block in an expression system, purifying the hybrid and testing it for plasminogen activation as well as thrombin inhibition etc. Here, the translated polypeptide starts with a functionally active thrombolytic protein and has the EGF domains at the C-terminal end, thus also possessingantithrombin and protein C activation capabilities. Designs that lead to the preparation of functional polypeptides have also been disclosed wherein the chimeric polypeptide is inactive initially but attains its plasminogen activation capability upon proteolytic truncation with either plasmin or thrombin.

In another embodiment of the present invention is disclosed a thrombolytic protein comprisinf a streptokinase with one or more amino acid substitutions, insertions, deletions, or truncations, and wherein the construct possesses plasminogen activation, thrombin inhibition, and anticoagulant protein C pathway activation activity.

Another embodiment contains method of making N & C- terminal fusion constructs, where the thrombolytic protein contains the three EGF domains at the N- and C-termini of thrombolytic protein at the same time. These constructs were designed with the help of expression of oligonucleotide blocks with N-terminal and C-terminal EGF 4,5,6 fusion constructs joined via common restriction site and expression thereof using established methods of gene cloning etc.

In yet another embodiment,the design of intra-protein fusion carrying EGF domainswithin a thrombolytic such as streptokinase and tissue plasminogen activator, are disclosed.

In another embodiment, the hybrid/ fusion construct had tissue plasminogen activator wherein the EGF 4,5,6 domainsreplaced the kringle 1 domain of tPA. This constructexhibited good plasminogen activity as well as antithrombin properties.

Another embodiment discloses the oxidation resistant forms of EGF domains where the methionine/s of EGF 4,5,6 were replaced with valine, alanine or glutamine amino acid residues in either the N-terminal,C-terminal and N& C-terminal (simultaneous) fusion constructs with thrombolytic (SK, tPA and SAK), described in the foregoing sections, via established methods of site directed mutagenesis.

In yet another embodiment of the invention is disclosed the thrombomodulin EGF 4,5,6 domains which are fused between the alpha and beta or beta and gamma domains of said streptokinase or between the alpha and beta or beta and gamma domains of a streptokinase derivative or analog, wherein the streptokinase derivative or analog comprises one or more mutations, additions, insertions, or truncations, and wherein said construct activates plasminogen, inhibits thrombin, and activates the anticoagulant protein C pathway.

In another embodiment of the present invention is disclosed a construct where the EGF 4,5,6 domains are fused in-frame to streptokinase, or a streptokinase derivative or analog, at one or more positions selected from the streptokinase N-terminus, streptokinase C-terminus, both N- and C- streptokinase termini, or at an inter-domain position of streptokinase.

In yet another embodiment of the present invention is disclosed a chimeric protein construct wherein the streptokinase derivative spans residues 5-383 or 5-414.

In further embodiment of the present invention is disclosed a chimeric protein construct wherein the streptokinase derivativespans residues 16-383.

In yet another embodiment of the present invention is disclosed a chimeric protein construct where the EGF 4,5,6 domains are fused in-frame at one or more positions

selected from the streptokinase N-terminus, streptokinase C-terminus, or both N- and C- streptokinase termini, wherein the Met 41 of the EGF 4,5,6 domains is replaced by either valine, alanine, or glutamine; or the C-terminal Met 435 is replaced by valine, alanine, or glutamine; or in the simultaneous N- and C-terminal fusion constructs, Met 41 and Met 435 are independently replaced by either valine, alanine, or glutamine.

In another embodiment of the present invention is disclosed a chimeric protein construct further comprising transglutaminase recognition sequences.

In further embodiment of the present invention is disclosed a chimeric protein construct further comprising one or more thrombin cleavable sequences at the junction of the EGF 4,5,6 domain and the thrombolytic protein, or derivative or analog thereof.

In another embodiment, the hybrid/ fusion constructs comprise a thrombin cleavable site at the junction of EGF 4,5,6 and their oxidation resistant forms.

In another embodiment, the hybrid/ fusion construct contained EGF 4,5,6 domains are mutated to possess oxidation resistancedue to methionine oxidation and consequent loss of activity during their preparation.

In another embodiment of the present invention is disclosed a chimeric protein construct comprising the EGF 4,5, 6 domain of thrombomodulin fused to a tissue plasminogen activator (tPA) derivative, analog, or fragment at the N-terminus of the tPA derivative, analog, or fragment, at the C-terminus of the tPA derivative, analog, or fragment, at both termini of the tPA derivative, analog, or fragment, or internally within the tPA derivative, analog, or fragment.

In yet another embodiment of the present invention is disclosed a chimeric protein construct wherein the fusion between the EGF 4,5,6 domain and the tPA derivative, analog, or fragment further comprises one or more linker fragments.

In further embodiment of the present invention is disclosed a chimeric protein construct wherein the one or more linker fragments comprise one or more amino acids that promote flexibility of said construct.

In yet another embodiment of the present invention is disclosed a chimeric protein construct wherein the one or more amino acids ischosen fromthe group consisting of Gly, Asn, Pro, Ser, Gln, Arg and Lys.

In another embodiment of the present invention is disclosed a chimeric protein construct wherein the tPA derivative, analog or fragment comprisesone or more mutations, additions, insertions, or truncations, and wherein said tPA derivative, analog or fragment activates plasminogen, inhibits thrombin, and activates anticoagulant protein C.

In yet another embodiment of the present invention is disclosed a chimeric protein construct comprising a fragment of tissue plasminogen activator (tPA), or a truncated or modified form thereof, fused to one or morethrombomodulin EGF 4,5,6 domains, such that the EGF domains of tPA are replaced by the one or more thrombomodulin EGF 4,5,6 domains, and wherein the construct has both antithrombin and plasminogen activation activity.

In further embodiment of the present invention is disclosed a chimeric protein construct comprising a fragment of tissue plasminogen activator (tPA), or a truncated or modified form thereof, fused to one or morethrombomodulin EGF 4,5,6 domains, such that the kringle 1 and EGF domains of tPA are replaced by the one or morethrombomodulin EGF 4,5,6 domains, and wherein the construct has both antithrombin and plasminogen activation activity.

In yet another embodiment of the present invention is disclosed a chimeric protein construct wherein the tPA EGF domains and kringle 1 domain are replaced by one or more thrombomodulin EGF 4,5,6 domains at the N-terminus or C-terminus of the tPA fragment.

In another embodiment of the present invention is disclosed a chimeric protein construct further comprising one or more linker fragments between the tPA fragment or truncated or modified form thereof and the thrombomodulin EGF 4,5,6domains,the linker fragments comprisingamino acid residues that promote flexibility of the constructso thatthe construct has thrombin inhibition, protein C activation,and plasminogen activation capabilities.

In yet another embodiment of the present invention is disclosed a chimeric protein construct wherein the methionine 41 of the EGF 4,5,6 component is replaced by either alanine, valine or glutamine.

In further embodiment of the present invention is disclosed a chimeric protein construct comprising an EGF 4,5,6 domain of thrombomodulin fused in-frame to the N-terminal or C-terminal end of staphylokinase (SAK).

In yet another embodiment of the present invention is disclosed a chimeric protein construct wherein the methionine 41 of the thrombomodulin EGF 4,5,6 domain is replaced by either alanine, valine or glutamine.

In another embodiment of the present invention is disclosed a chimeric protein construct further comprising thrombin cleavable sequencesbetween the SAK and EGF 4,5,6 domain regions.

In yet another embodiment of the present invention is disclosed a chimeric protein construct further comprising transglutaminase cross-linking sequences.

In another embodiment of the present invention is disclosed a chimeric protein construct wherein said constructs are soluble in aqueous or saline solution.

In this invention, in another embodiment the recombinant chimeric proteins are expressed by plasmids under the control of tightly regulated promoters.

In an embodiment of this invention the various chimeric constructs were expressed through recombinant DNA technology in a suitable hosts such as bacteria, fungi, yeast or animal cells.

In another embodiment of the present invention is disclosed a chimeric protein construct which is secreted into an extracellular medium.

In another embodiment, tissue plasminogen activator and EGF 4,5,6 fusion constructs were expressed in prokaryotic and eukaryotic hosts from which the polypeptides were harvested to obtain the active hybrid constructs in pure form/s.

In another embodiment, various tissue plasminogen activator fusions with EGF constructs were also expressed in eukaryotic systems like animal cell lines, yeast expression system and plant cells where expression cassettewas either integrated into host genome or remained in the cytoplasm as an episomal body.The expressed hybrid proteins may be glycosylated or non-glycosylated depending upon the host using for expression.

In another embodiment of the present invention is disclosed a method of treating thrombosis in a mammal, comprising administering to said mammal in need of treatment a therapeutically effective amount of a chimeric protein construct.

In another embodiment of the present invention is disclosed a method of inhibiting thrombin comprising the use of a chimeric protein construct.

In further embodiment of the present invention is disclosed a method of activating protein C comprising the use of a chimeric protein construct.

In yet another embodiment of the present invention is disclosed a method of providing both antithrombin and plasminogen activation comprising the use of a chimeric protein construct.

In another mbodiment of the present invention is disclosed a pharmaceutical formulation comprising a pharmaceutically effective amount of a chimeric protein construct.

In another embodiment of the present invention is disclosed a method of thrombolysis in a mammal comprising administering to said mammal in need thereof a therapeutically effective amount of a chimeric protein construct.

In another embodiment, encoding DNA sequences of the hybrid constructs were optimized for the suitable expression host.

In another embodiment, a nucleic acid sequence encoding the chimeric protein construct mentioned above.

In another embodiment, a vector comprising a nucleic acid sequence..

In another embodiment, a host cell comprising a vector.

In another embodiment, a chimeric protein construct is prepared by expression of a nucleic acid sequence encoding said protein in a host cell expression system.

In another embodiment ,a method of preparing a chimeric protein construct by expression of a nucleic acid sequence encoding said protein in a host cell expression system.

In another embodiment,the method in which the host cell expression system is a eukaryotic expression system.

In another embodiment,the method in which the host cell expression system is a bacterial expression system.

In another embodiment,the method, in which the bacteria is E. coli.

In another embodiment, the method in which the eukaryotic expression system is an animal cell, or a yeast cell.

In another embodiment,the method in which the yeast is Pischia pastoris.

In another embodiment,the method, wherein the chimeric protein construct is secreted from the host cell into an extracellular medium.

In another embodiment, the purified constructs are useful as therapeutic agents and in pharmaceutical compositions for treatment of mammals having various thrombotic conditions. Mammals may be humans, rodents, or domesticated animals.

In another embodiment, the purified constructs were used as therapeutic agents to alleviate various circulatory disorders with or without additional stabilizing agents and excipients.

According to another embodiment of the invention, the purified constructs are formulated into one or more pharmaceutical compositions. The pharmaceutical compositions with purified constructs, whether one or more, can be formulated in both solid form (i.e. freeze-dried in vials to later be reconstituted in a suitable solution) or also in liquid form.

In one particular embodiment, these compositions with the purified constructs constitute a kit for thrombolytic therapy or treatment which may optionally include other components, such as: containers with solutions for reconstituting the active ingredients, cannulas, drip bags with physiological serum for intravenous application and instructions for use, etc.

The pharmaceutical combinations may be formulated and used either in combination form (i.e. wherein all the active ingredients are combined into one formulation) or in individual form (i.e. wherein the active ingredients are not combined (or not all combined) into one formulation) as tablets, capsules or elixirs

for oral administration; suppositories for rectal administration; sterile solutions, suspensions for injectable administration; and the like. The dose and method of administration can be tailored to achieve optimal efficacy but will depend on such factors as patient weight, diet, concurrent medication and other factors which those skilled in the medical arts will recognise.

Generally, with respect to the purified constructs (including uses, methods, pharmaceutical compositions and products) of the present invention, an amount between 0.1 to 1000 mg is administered (as a single dose or on a multi-dose, as-needed basis), dependent upon the potency of the purified construct used.

Preferred embodiments encompass pharmaceutical compositions prepared for storage and subsequent administration which comprise a therapeutically effective amount of the purified constructs or an enriched composition of the purified constructs, as described herein in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985).

Preservatives, stabilisers, dyes and even flavouring agents may be provided in the pharmaceutical composition. For example, sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid may be added as preservatives. In addition, antioxidants and suspending agents may be used.

In employing the purified constructs or their pharmaceutical compositions or products in a combination therapy in vivo, the compositions/products can be administered to the mammal in a variety of ways, including parenterally (e.g. intravenously, subcutaneously, intramuscularly, colonically, rectally, nasally, buccal, transdermally, vaginally or intraperitoneally), employing a variety of dosage forms.

As will be readily apparent to one skilled in the art, the useful in vivo dosage to be administered and the particular mode of administration will vary depending upon the mammalian species treated, the particular composition employed, and the specific use for which these compositions are employed. The determination of effective dosage levels, that is the dosage levels necessary to achieve the desired result, will be within the ambit of one skilled in the art. Typically, applications of compositions are commenced at lower dosage levels, with dosage level being increased until the desired effect is achieved.

Generally, in carrying out the methods of this invention, the dosage for the purified constructs or pharmaceutical compositions can range broadly depending upon the desired effects and the therapeutic indication. Typically, suitable dosages of the purified constructs will be between about 0.1 and 1000 mg, preferably between about 10 and 500 mg, more preferably between about 10 and 150 mg, most preferably between about 10 and 120 mg. Administration is preferably parenteral, such as intravenous. Administration is also preferably as a single dose or on a multi-dose, as-needed basis. Administration is preferably parenteral, such as intravenous. Administration is also preferably as a single dose or on a multi-dose, as-needed basis.

Injectables can be prepared in conventional forms either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable excipients are, for example, water/saline, dextrose, mannitol, lactose, lecithin, albumin, sodium glutamate, cysteine hydrochloride or the like. In addition, if desired, the injectable pharmaceutical compositions may contain minor amounts of non-toxic auxiliary substances, such as wetting agents, pH buffering agents, and the like. If desired, absorption enhancing preparations (e.g. liposomes) may be utilised.

For parenteral administration, solutions of a pharmaceutically active agent used in accordance with this invention in either sesame or peanut oil or in aqueous

propylene glycol may be employed. The aqueous solutions may be suitably buffered (preferably between pH 4 to pH 9) if necessary and the liquid diluent first rendered isotonic. For example, NIF is used in aqueous solution at a pH of around 7. However, NIF is stable in aqueous solution down to about pH 4. The preferred "partner compound", t-PA (or variants thereof, is generally used in aqueous solution at around pH 5. These aqueous solutions are suitable for intravenous injection purposes. The oily solutions are suitable for intra-articular, intramuscular and subcutaneous injection purposes. The preparation of all these solutions under sterile conditions is readily accomplished by standard pharmaceutical techniques well known to those skilled in the art.

It should be noted that the pharmaceutical compositions and products of the present invention may be lyophilised for storage, prior to reconstitution and thereafter administration using methods well known to those skilled in the art. Whether stored as lyophile(s) or otherwise, the active components of the combinations of the present invention may be mixed together before lyophilisation or after reconstitution (for later co-administration) or stored individually (for later simultaneous, separate or sequential administration).

In one particular embodiment, administration is parenteral, for example by intravenous injection, or administered locally by catheterization for in situ administration in the near vicinity of the clot.

The quantities of purified constructs which may be present in the compositions of the pharmaceutical combination provided by this invention may vary within a broad range, but always in therapeutically effective quantities.

The dosage for each thrombolytic treatment protocol with the compositions of the pharmaceutical combination of this invention will depend on numerous factors, including the patient's age, condition, the severity of the clinical condition to be

treated, the route and frequency of administration and of the purified construct which is going to be administered in each case.

In one aspect, this invention relates to a pharmaceutical combination or a kit of the invention, as have already been described, for thrombolytic therapy.

In another additional aspect, the invention also relates to a method of treatment and thrombolytic therapy consisting of administering to the patient a therapeutically effective quantity of one or more purified constructs. In this connection, the compositions according to the present invention are particularly eligible for treating ischemic heart disease and its complications, as well as ischemic cerebral strokes, rheumatoid diseases and other pathologies, in whose pathogenesis there take place an inflammatory reaction, ischemia of tissues, disturbances of hemorheology and vascular microcirculation because of thrombosis.

The present invention discloses fusion constructs of tissue plasminogen activator with EGF 4,5,6 domains of thrombomodulin with a six amino acid containing linker sequence. This linker sequence length and amino acid sequence order can be changed or modulated for further optimizing the functions of both the properties. Initially designed constructs with no linker sequence compared with linker-designed construct showed only a small fraction, roughly 20-25 %, of the protein C activity in the different constructs containing optimized linkers.

In another embodiment, invention discloses the making of non natural fusion protein encoding gene blocks and their variants through the standard methods of genetic engineering.

Further, the inter-protein fusion linker sequences preferentially contained 3 or more glycine amino acid residues at the junction sites of the EGF 4,5,6 domains with the plasminogen activator component so as to provide the necessary flexibility to the 4^{th} domain of EGF which plays key role in Protein C binding and activation. Similarly, linkers were put just after the 6^{th} domain of EGF when these were fused

at the N-terminal region of tPAso that it could facilitate thrombin binding. This was especially so in case where EGF4,5,6 was introduced between the ordered structures/domain of the tissue plasminogen activator. Thus the role of linkers was evident when two partners were fused to obtain preservation of the functionalities of both partner proteins.

The linker sequence was chosen in such a way wherein one positively charged amino acid (Lysine or Arginine) was placed in tandem with a Val residue but before the two or three glycine residuesso that it could be cleaved by plasmin in the close vicinity of the clot where the chances of transient thrombin formation is very high during the lysis and both the domains could perform their work independently at the site of injury.

The Linker also contains one proline amino acid which actually helps to change the orientation of EGF 4,5,6 domains so that they can work optimally while binding to thrombin and activation protein C.

In another embodiment, the designed construct linker length can be increased or decreased as well as sequences of amino acids can be altered with natural and non natural amino acids in order to effect the rates of activation.

In another embodiment, thrombin cleavable sequence, transglutaminase recognition sequence and other blood protease susceptible sites can be introduced in the linkers without affecting fibrinolytic and antithrombotic function.

In another embodiment, invention discloses the expression and purification methods of various fusion polypeptides and their better variants.

In another embodiment, invention discloses various non natural polypeptides which show combined properties of thrombolytics and anti thrombin as well as protein C anticoagulant properties.

In another embodiment, oxidation resistant forms of EGF4,5,6 were fused with the functionally improved variants of tissue plasminogen activator which possess mutational variations compared with native tPA(e.g. DNA SEQ ID 20).

In another embodiment, oxidation resistant forms of EGF4,5,6 were fused with a plasmin resistant form of streptokinase.

In another embodiment, oxidation resistant forms of EGF 4,5,6were fused with the thrombolytic protein where one or more cysteine residues were free.

In another embodiment, expressed fusion polypeptide areused for the treatment of cardio vascular diseases.

In another embodiment, suitable pharmaceutical composition possess expressed fusion polypeptide/s with FDA approved chemical stabilizer like mannitol, human serum albumin (HSA), etc and solubilizing agents.

Expressed fusion construct may formulate for the intravenous administration to human being may contains FDA approved stabilizers.

In another embodiment, EGF 4,5,6 and its variants can be fused with the urokinase type of plasminogen activator.

In another embodiment, different EGF fusion constructs can be made with those proteins which show 75-100% homology with SK, tPA, andSAK and showing 50-100% plasminogen activation potential compared to their native protein/s.

In another embodiment, variants of EGF4,5,6 fused with the SK, tPA and SAK where EGF 4,5.6 variants show 75-100% homology/similarity and 50-100% antithrombin and protein C activity as compare to independently expressed native EGF 4,5,6 domains.

REAGENTS

GENETIC CONSTRUCTS:

EGF 4,5,6 domain sequences were that of human thrombomodulincDNAwhich wascommercially custom-synthesized (GeneScript Inc., USA) for optimal expressionin the yeast, *Pichia pastoris*. The synthetic gene segments were ligated in the correct order and cloned in a bacterial plasmid. The fusion constructs between EGF 4,5,6 domains of thrombomodulin, on the one hand, and a thrombolytic eg SAK, SK or tPA (or their derivatives/mutants) was carried out essentially by a combination of (a) custom-gene synthesis using chemical means, and (b) using the well established PCR technology for obtaining selected gene segments using specifically designed primersfrom the appropriate plasmids (see Methods used in Examples, below), followed by isolating the PCR-generated gene-segment blocks and 'fusing' them in-frame using specialized PCR methods such as Overlap Extension PCR etc. Generally, the regular PCR was done with a suitable, high processivitythermostable DNA polymerase (*Pfu* DNA polymerase from FermantasInc., or Stratagene Inc.), or high fidelity*pfu* turbo (Stratagene Inc.) enzyme.The hybrid DNA constructs were cloned/expressed in the T7 RNA polymerase promoter-based expression vector, pET-23d by transformation into the appropriate *Escherichia coli* strain--XL-Blue for routine plasmid sub-cloning without concomitant protein expression, and strain BL21 (DE3) strain for protein expression under the T7 RNA Pol promoter-- which were procured from Novagen Inc. (Madison, WI, USA), and also expressed in yeast (*Pichia pastoris*) under the methanol inducing promoter having in-frame α-mating factor signal sequence in the vector, pPIC-9K, and *GS115* cells were used for expression (Invitrogen Life Technologies, California, USA). The various restriction endonucleases, T4 DNA ligase and other DNA modifying enzymes were acquired from New England Biolabs (Beverly, MA). Oligonucleotide primers were supplied by Biobasic, Inc., Canada. Purifications of DNA and extraction of PCR amplified products from agarose gels

were performed using kits available from Qiagen GmbH (Germany). Automated DNA sequencing using fluorescent dyes was done on an Applied Biosystems 3130 xl genetic analyzer equipped with a 16-capillary set-up. Glu-plasminogen was either purchased from Roche Diagnostics GmbH (Penzberg, Germany) or purified from human plasma by affinity chromatography (Deutsch and Mertz, 1970). Human protein C, thrombin, hirudin were purchased from Calbiochem., USA, and standard thrombomodulin of rabbit, and recombinant thrombomodulin were both purchased from American Diagnostica Inc., USA. The N-terminal gas-phase amino acid sequencing was done with an Applied Biosystems sequencer, Model 491. Urokinase, EACA, sodium cyanoborohydride, and L-Lysine were purchased from Sigma Chemical Co., St. Louis, USA. Phenyl Agarose 6XL and DEAE Sepharose (Fast-Flow) were procured from GE-Amersham, Uppsala, Sweden, while Ni-NTA beads were from Qiagen. All other reagents were of the highest analytical grade available.

GENERAL METHODS USED IN EXAMPLES:

*1. Recombinant DNA fusion methods:*various methods collectively referred to as Recombinant DNA technology are now well known in the field of molecular biology. Several techniques, standard protocols and their modified forms were have been very well described in several reference books, like Sambrook et al., Molecular Cloning: A laboratory Manual (IInd edition, Cold Spring Harbor Press, New York., 1989; McPherson, M. J., Quirke, P., and Taylor, G. R., [Ed.] PCR : A practical approach., IRL Press, Oxford., 1991). But several modifications were required in the designing of fusion construct where different publications from the published literature are cited for specific applications. In this invention, where we fused two genes,we took recourse the method known as the Ovelap Extension PCR of Ho et al.,(Ho, Hunt et al. 1989), and Mehta and Singh (Mehta and Singh 1999). For small constructs amplification upto 2Kb, *pfu* DNA polymerase (Fermentas Inc., USA) were used, and for longer PCR where high fidelity of polymerase action was required for the point mutation construction*pfu* turbo (Stratagene) was used for the

amplification of 6-7 Kb long fragment amplification and site directed mutagenesis(Wang and Malcolm 1999).

2. Restriction digestion and ligation: Restriction digestion and ligation enzymes were obtained from New England Biolabs, USA and 'fast digester' restriction enzymes of FermantasInc. were used according to the manufacturers' protocols. Mentioned with respect to almostall chimeric fusion construction examples, wherein ligation was carried out between the Xho I (tetra base cutter) and Not I (Hexa base cutter) digested vector and the insert/s, maximized the chances of directional cloning.

3.*Electroporation of ligation mixtures in E. coli XL 1B competent cells and transformation of linearized insert containing vector in pPIC -9K:* Ligation mixtures of reaction were electroporated into XL 1B competent cells (Sharma and Schimke 1996). High amount of DNA prepared from the Midi prep kit of Qiagen by following the instruction. In final steps of Midi Preparation DNA was eluted in high salt concentration, here we used 0.3 M sodium acetate of pH 5 at final concentration and 70 % ethanol was used for the removal of excess salt (Sambrook et al., 1989), which may interfere in the digestion reactions. By this process we obtained 50-60 µg of DNA which were subjected for the linearization step, with the help of Bgl II enzyme, and after digestion again subjected to the sodium acetate and ethanol precipitation steps, and finally the dried pellet dissolved in 7-8 µl of autoclaved salt free water, where 1-3 µg/µl of DNA was obtained. Here 7-10 µg of DNA was used for the transformation in electrocompetent cells of His $^{-}$ GS 115 cells of Pichia pastoris, finally plated on minimal dextrose where only His^{s+} transformants were grown on plates. The plasmid pPIC-9k is designed in such a way so that multicopy insertion of desired gene is possible through homologous recombination of with AOX 1promotor (Ramchuran, Mateus et al., 2005). Multicopy target gene insertion can be monitored through the bacterial kanamycin resistance gene (Tn903) through monitoring the genticin resistant population with increasing antibiotic

concentrations. Pichia can tolerate genticin resistance of 0.25 mg/ml as such, but 0.5mg/ml and 4 mg/ml shows 1-2 copy and 7-12 copy insertion, respectively. Genticin resistance directly correlates with the multiple insertion of desired gene. Due to gene dosage effect it can be deduced that the higher secretion of desired gene products corresponds to a higher insertion number (Norden, Agemark et al. 2011).

4. Expression, isolation and refolding of inclusion bodies of fusion constructs. Some constructs such as EGF-SAK an SAK–EGF were expressed under pET 23-d where the chimeric proteins accumulated in the form of inclusion bodies in the host *E. coli* Bl 21- DE 3 cells. Where inclusion bodies were formed maximum amount of o*ver exp*ressed protein was obtained (Misawa and Kumagai 1999; Zhang, Xu et al. 2009). The E. coli BL21 DE3 cell were used for the production of chimeric fusion protein' IBs, where 25ml of LB (Luria Bertani) from overnight grown BL21 DE3 cell culture, was used as primary inoculum, which were transferred to 500 ml LB (secondary culture) media. Once the OD_{600}reached 0.6-0.8 the protein production was induced with the help of 1mM IPT_G. After induction, cells were kept under shaking conditions for 6 hrs at 40°C. After 6 hr incubation, cells were harvested by centrifugation at 6000 rpm for 10 minutes at 4°C. Finally, harvested cells were washed with 50mM tris, 100 mM NaCl and 1mM EDTA solution to remove the media components from cell mass. After washing, cells were suspended in washing solution to a final OD_{600} between 35-40; at this dilution cells were subjected for sonication with pro_{be}-based sonicator where 30 sec on and off cycles were used for 45 minutes. After completion of lysis cycles, cell pellets were harvested by centrifugation at 12,000 rpm for 15 minutes. Obtained pellets were washed twice with 100 mM NaCl, 50 mM Tris Cl pH 7.4, 1 mM EDTA, 0.1% Triton X-100 and 2M urea-containing solution. Again these IBs were re-suspended in the solution and were harvested by centrifugation it 12000 rpm for 15 minutes. Harvested pellet was washed 2x with 100 mM NaCl, 50 mM Tris Cl pH 7.4 and 1mM EDTA solution so that Triton X-100 was removed. Finally pellet was re-suspended in 8M urea (prepared in 20 mM Tris Cl pH 7.4) and 1mM DTT for 2 hrs. Most of the dissolved

inclusion bodies in this solution were separated from pellet by centrifugation at 12000 rpm for 20 minutes, and the final supernatant contained maximum portion of the chimeric fusion proteins. Now this protein fraction was subjected for refolding where it was diluted upto 0.2 mg/ml and refolded under the following conditions: 2M urea, oxidized and reduced glutathione mole- ratio 1.5:0.5 in 50 mM NaCl, 50mM Tris-Cl, 2% glycerol at 4°C for 36 hrs with very gentle stirring. After completion of this refolding step, the mixture was dialyzed against 20 mM Tris Cl pH 7.6, and 50 mM urea for 48 hrs and dialyzed reaction mixture was subjected to purification by tandem chromatographies using hydrophobic and ion exchange chromatography. Disulfide bond formation was monitored by DTNB reaction (Riener, Kada et al. 2002) which is a direct indicator of disulfide bond formation and refolding. Finally, the purified protein was subjected to different activity assays.

5. Casein-plasminogen overlay for the detection of plasminogen activation: In fusion constructs, at the preliminary level, plasminogen activation was screened by this method, where 5% (w/v) skimmed milk is boiled in water containing 15 mM NaCl, and 50 mM Tris along with 1%agarose. After cooling, ~200-400 µg of plasminogen were added in this mixture, and overlaid onto LB ampicillin containing plates with colonies/clones to be screened (Malke and Ferretti 1984). All the fused recombinant protein with plasminogen activation capability were easily detected by the zone of hydrolysis after incubation for periods varying between 1-2 h and upto 18 h, due to the formation of plasmin which degraded the casein, giving rise to easily visualized zones of clearance against a white background.

6. Screening of best produce clones by checking plasminogen activation profiles: After selecting higher copy number containing transformants by the use of genticin resistant marker were subjected for the actual screening of best producer clones. In this method primary culture raised in 2.5 ml of BMGY media (1% yeast extract, 2% peptone, 1X glycerol, 1X yeast nitrogen base without amino acids and 100 mM of potassium phosphate buffer pH 5.5) at 30°C for 16-18hrs. Once the optical density

(OD_{600}) reached upto 3-4 units, we induced the culture by adding 7.5 ml of BMMY media (1% yeast extract, 2% peptone, 1 X Methanol, 1 X yeast nitrogen base without amino acids and 100 mM of potassium phosphate buffer, pH 5.5). These cultures are further induced for the production of recombinant production with final 0.5 v/v % methanol for 5 days, such that the recombinant fusion proteins were produced under the influence of the strong methanol induced promoter.

7. Zymography: Extracellular secreted products and desired band of interest possessing plasminogen activation capability were detected by this method, where 10-12.5 % SDS- page gels were run in non reducing sample buffer. After completion, excess of sodium dodecyl sulphate wasremove by the washing in 2.5 % triton X-100 solution. Then this gel was rinsed by 2-3 times with 50 mMTris, pH 7.4. This washed gel was placed on skimmed milk agarose and plasminogen containing solid surface (agar gel). After incubation for 5-7 hours the development of zones of hydrolysis become visible signifying Plasminogen activating capability in the resolved proteins.

8. Western blotting technique: Detection of desired protein was done with the help of Western blotting. All the fusion constructs that were secreted into extracellular medium from *Pichiapastois* cellswere separated by the help of centrifugation at 6000 rpm and supernatants were taken for the desired product identification. Supernatant were directly taken as such for the western blotting or concentrated first by the 5 KDa or 10 KDa cut off range concentrators (Amicon) or subjected to the tri-chloro acetic acid precipitation, washed with acetone, and loaded onto 10-12.5 % SDS polyacylamide gels. Proteins were transferred on nitrocellulose membrane with the help of Transfer buffer containing 25 mMtris, 175 mM glycine and 20 % mehanol. Gel blotting on membranes were done at 250 mA for 35 minutes. Blotted membranes were soaked on 10% skimmed milk overnight at 4° or incubated at 37° c for 2 hours. This blot was further washed with phosphate buffered saline containing 0.1% tween-20 for three time, which removes the excess

of skimmed milk. After that blot was submerged in primary antibody or polysera for 1 hat recommended dilution and washed with Tween-20 containing PBS, three times. Further this blot was incubated with the HRP conjugated secondary antibody followed by the PBS washing three times. Finally, blots were developed by the addition of DAB (di-amino benzidine) solution.

9. Lysine-agarose chromatography: Various tissue plasminogen activator fusion construct with EGF 4,5,6 contains kringle domains which known for affinity toward the lysine residues (McCance, Menhart et al. 1994; Ye, Rahman et al. 2001). In this method, supernatant obtained from *Pichia pastoris* fermentation, hav*ing tissue plasm*inogen activator fusion polypeptide was dialyzed against the phosphate buffer pH 7.5 for 3-4 hrs and loaded on lysine-Sepaharose column pre-equilibrated with phosphate buffer (purchased from Amersham Biosciences, Uppasala, Sweden) with slow flow rate of approx. 0.5ml/ min. After completion of loading, column was again washed with 4-5 bed volumes of phosphate buffer and protein finally eluted in 0.3 M epsilon amino caproic acid and 100 mM NaCl solution (Qiu, Swartz et al. 1998).

*10. Thrombin affinity chromatography:*Thrombin coupling was done on cyanogen bromide activated bead by previously described procedure (Salem, Maruyama et al. 1984). Here, proteins obtained from different steps of purification were finally subjected to thrombin affinity where columns were equilibrated in 50 mM Tris Cl pH 7.4 and dialyzed against 50 mM Tris Cl, pH 7.4 used for the loading at slow flow rate of approx. 0.5 ml/ min; after completion of loading, the columns were washed with the same buffer. Finally protein was eluted by increasing gradient of NaCl (Salem, Maruyama et al. 1984). Different protein fractions were then tested for the protein C activation capability.

11.Hydrophobic Interaction chromatography: Purification of different fusion gene constructs of EGF –SK, EGF-tPA and EGF-SAK was done by different methods of chromatography, where hydrophobic and ion exchange chromatography was used for frequent purification of different fusion polypeptides (Goyal, Sahoo et al. 2007).

In hydrophobic chromatography, phenyl sepharose (Amersham Biosciences, Uppasala, Sweden) 6% crossed linked beads with average particle size 100-300 μM size diameterwere used for the column preparation. Simple XK16/20 column (Amersham Biosciences, Uppasala, Sweden) was packed with the help of peristaltic pump, approximately 25 ml phenyl Sepharose bed was made and equilibrated with 4-5 bed volumes of 0.3 M Na Cl and 50 mM tris, side by side supernatants obtained during the *Pichia pastoris* fermentation, containing the *desired fusion* constructs, were maintained in the same buffer strength and salt compositions as the equilibration buffer by dialysis. Equilibrated supernatant was loaded on packed column with 40ml/hr flow rate, and after completion of supernatant loading, 4-5 bed volume of equilibration buffer was passed for removal of media components and nonspecifically bound impurities and finally desired fusion polypeptides were eluted in water. Eluted proteins were subjected for the second round of purification (see below), and tested for the plasminogen activation, thrombin inhibition and protein C activation assays.

12.DEAE (diethylaminoethyl) ion-exchange chromatography: In this chromatographic procedure, DEAE Sepharose ™ fast flow were packed in XK 16/20 column (Amersham Biosciences, Uppasala, Sweden) and in general the manufacturer's instructions were followed, where mostly, 20 ml swollen matrix was used for the packing. This column was equilibrated with the 20 mM Tris Cl buffer pH 7.4, and supernatants which was obtained from *pichia pastoris*, after being extensively dialyzed against *the 20 mM Tris* Cl buffer pH 7.4, were loaded on equilibrated columns (in separate runs), or after being purified by the hydrophobic interaction chromatography (above), and with its ionic strength maintained same as that of the equilibration buffer,were loaded on the respective columns. After completion of loading, each column was washed with the 4-5 bed volume of equilibration buffer, which helps in the removal of non-specifically or loosely bound impurities and polypeptides. Elution of protein was done by applying increasing gradient of 1M NaCl over 5 bed-volumes of matrix. Protein quantization done with

the help of the Bradford method (Bradford 1976) and compared with the standard curve of BSA. Finally purified protein was concentrated by 20 K Da cut-off concentrators (Amicon) and used for various analytical and functional assays.

13. Plasminogen activation assays for EFG-thrombolytic fusion constructs: All chimeric fusion polypeptides constructs contained a thrombolytic component (SK, SAK and tPA), so these constructs' capability to activate the plasminogen was measured by the release of color from chromogenic peptide substrate for plasmin. One-stage assays of streptokinase and all fusion constructs were carried out under conditions where 2 μM Human plasminogen, 50mM Tris, 0.05% BSA and 5mM of chromogenic substrate was used, and release of chromogenic substrate is monitored spectrophotometrically as function of time. It followed non linear regression, hence a plot between absorbance and time^{2}followed straight line equation. Streptokinase immediately starts conve^{r}sion of plasminogen into plasmin and shows virtually no delay in activation but in case of some of the fusion constructs, it takes a protracted time to activate the plasminogen into plasmin. This indicated that trace amount of plasmin makes the active complex as the zymogen activation of the plasminogen into plasmin (Pathway I) was not active. This was confirmed, when the progressive addition of trace (nanomolar) amounts of external plasmin progressively reduced the lag time. In case of Plasminogen activation by tissue plasminogen activator, a rapid and simple spectrophotometric method was followed, where simple chromgenic peptide (H-D-Val-Leu-Lys-pNA; S-2251; purchased from Chromogenix Inc.) was used for the detection of plasmin formed by the action of tissue plasminogen activator (Verheijen, Mullaart et al. 1982). This method also was used for validation of enhanced plasminogen activation in presence of fibrin and tissue plasminogen activation (van Zonneveld, Veerman et al. 1986). In this activation assay different purified amount of protein amount in incubated with 2μM plasminogen, 0.05 M Tris Cl ph 7.2, 100 mM NaCl, 0.05% and 0.5 mM of S-2251 used in presence and absence of soluble fibrin (purchased from American

Diagnostics, USA) and without fibrin. Absorbance at 405 monitored upto 2 hr with 1 minute interval time.

14.Clotting time experiments with different fusion constructs:In order to see the effect on thrombin-induced clot formation in presence of different chimeric constructs, a standard curve with different thrombin concentrations was made from which a 20second clotting time with 6.6 IU of thrombin was obtained. Same amount of thrombin was then incubated with increasing concentrations of constructs to measure the concentrations where clotting time became double compared to the controls (Lougheed, Bowman et al. 1995).

15.Protein C activation by different fusion/chimeric constructs: Different fusion constructs of tissue plasminogen activator, staphylokinase and streptokinase were subjected for the thrombin mediated protein C activation assays in a dose-dependent manner. In this assay different amount of protein (in the nM range) were incubated with 10 nM of thrombin, in presence of 50 mM Tris-Cl, 5 mM CaCl2, and 0.05% BSA at 37°C for 20 min. After this time 0.5μM of protein C was added into the wells and incubated for 20 minutes at 25°C. After that 0.5 mM hirudin was added to inhibit thrombin and incubation continued for 5 minutes at 25°C and then chomogenic substrate was added at 0.5 mM final concentration, and the release of colored pNA was monitored at 405 nm with respect to time as detailed earlier (Eisenberg, Miletich et al. 1988; Ewald and Eisenberg 1995; Lougheed, Bowman et al. 1995; Meininger, Hunter et al. 1995; Dahlback and Villoutreix 2005).

EXAMPLES

The following examples are given by way of illustration of the present invention and therefore should not be construed to limit the scope of the present invention.

Example 1

PREPARATION OF VARIOUS FUSION GENES BETWEEN STREPTOKINASE AND EGF 4,5,6 DOMAINS:

(i) Hybrid gene constructs for expression of EGF domains fused in-frame upstream to the SK encoding Open-reading-frame (ORF): A double stranded (ds) DNA block, encoding EGF-SK protein fusion, in which the EGF 4,5,6 encoding sequences were fused in-frame at the N-terminal encoding side of the SK ORF, was constructed using primers N_EGF_SKFp 1 and N_EGF_SK Rp 2 (see table 1 for sequence of primers). The design and construction of a bacterial expression vector pET 23-d_SK has been described by Nihalani et al., 1998. It involved the cloning of the SK gene from *Streptococcus equisimilis*H46A in pBR 322 (Pratap*et al.*, 1996), followed by sub-cloning into pET-23d, an expression vector containing a highly efficient ribosome binding site from the phage T7 major capsid protein (Studier and Moffatt, 1986), and further modification of the 5' end of the gene to minimize the propensity for formation of secondary structure. Strerptokinaseexpressed from pET-23-d _SK construct is Met-SK. Further details are described in United States Patent No. 7,163,817. This construct was used as a template for amplification of the SK gene (DNA SEQ ID 1, and corresponding protein SEQ ID 113).

A double-stranded polynucleotide block corresponding to EGF 4,5,6 domains (SEQ ID 2, and corresponding protein SEQ ID 111) was selectively amplified using as template a synthetic gene (DNA polynucleotide) corresponding to sequence ID 2 which was prepared by custom DNA synthesis and validated by automated DNA sequencing to sequence ID 2, after cloning in pET 23-d (Novagen) vector. Primer N_EGF_SK Fp1 also contained (see table 1 for detailed description of primers)aXho I restriction site at its 5' end so that the resultant gene block obtained after PCR could be docked into a yeast expression plasmid. Primer N_EGF_SK Rp 2 contained sequence at the 5' end (besides nucleotides hybridizing with the end of the 6^{th} domainof EGF 4,5,6) and also additional nucleotides which anneal with the 5 side of SK gene ORF. PCR cycle

conditions were as follow: Hot start, complete denaturation at 95°C for 5 minutes; denaturation at 95°C for 45 seconds, followed by annealing at 45°C for 45 seconds, extension at 72°C for 1 minute, with a total no. of 28 cycles, and a final extension of 72°C for 10 minutes for the completion of the amplification of any incomplete PCR products. For the amplification of the SK gene block, the primerN_EGF_SK Fp 3 (upstream) and primer N_EGF_SK_Rp 4 (downstream primer)(see table 1 for detailed description of primers) were used. PCR conditions were as follows: PCR conditions: Hot start, complete denaturation at 95°C for 5 minutes, denaturation at 95°C for 45 seconds, annealing at 45°C for 45 seconds, extension at 72°C for 3 minute, with a total no of 28 cycles and a final extension of 72°C for 10 minutes for the complete amplification of any incomplete PCR products. Both PCR products were gel purified from agarose gels by gel extraction purification kit (Qiagen). These two purified PCR products were subjected to Splice Overlap Extension (SOE) PCR (see 'General Methods used in Examples' section, above, for details) in order to construct a contiguous EGF 4,5,6-SK hybrid gene construct in which EGF 4,5,6 encoding sequences were fused in-frame with SK gene encoding sequence ending with a terminator codon. This gene block was isolated from agarose gel in purified form and digested with Xho I and Not I restriction enzymes (R.E. enzymes) and ligated into similarly cut pET 23-d plasmid vector (refere to fig.1A and Fig.2 A), which was then transformed into *E. coli* XL1B (rec A^- and end A^-) cells in which the polypeptide is not expressed although plasmid DNA will be propagated. This plasmid was subjected to Sanger's di-deoxy method of sequencing in order to validate the DNA sequences of the EGF & SK components fused correctly (SEQ ID NO. 4, and corresponding protein SEQ ID 112).In this construct, Xho I and NotI digested cassette was isolated from agarose gel and 'docked' into pPIC-9K. In this resultant construct, the upstream EGF 4,5,6 sequences are placed in-frame with the α-secretory signal sequences as well as the Kex2 processing site, such that the hybrid gene construct, EG-4,5,6-SK is expressed in *Pichia pastoris*(strain GS115) from the alcohol oxidase promoter located in the vector after its integration into the host genome(Norden, Agemark et al. 2011). The hybrid polypeptide expressed from this expression vector is also efficiently transported across the membrane (see Examples below) and can be isolated there from in pure form.

*(ii) Construction of SK-EFG4,5,6 encoding gene construct, where EGF4,5,6 encoding domains were fused in-frame at the downstream/C-terminal encoding end of SK encoding gene:*Byusing

the set of SK_EGF Fp1 (upstream) and SK_EGF Rp2 (downstream) primers (see table 1 for primer sequence), nucleotide sequences corresponding to SK (DNA SEQ ID 1, , and corresponding protein SEQ ID 114) were amplified using pET23-d-SK plasmid as template (see 'General Methods used in Examples'). Primer SK_EGF Fpl contains a Xho I restriction site at its 5' end, whereas SK_EGF Rp2, at its 5'end, contains SK sequence upto 1149 bp followed by a triple glycine (Gly-Gly-Gly) encoding segment, as well as a transglutaminase recognition encoding sequence, finally ending with sequence overlapping with the 5' end encoding for the 4^{th} domain of EGF4,5,6. The resultant gene block contains the same Xho I restriction site at its 5' end, and downstream (3'-end) contains overlapping nucleotide sequence for the 4^{th} domain. In a second PCR, SK_EGF Fp3 and SK_EGF Rp4 (see table 1 for primer sequence) set of primers were used for the amplification of EGF4,5,6 domains from pET23-d_EGF4,5,6 (containing the Synthetic custom-made gene for EGF4,5,6) as template. Primer SK_EGF_Fp3 contained downstream sequence of SK, upto 1149 bp, followed by a triple glycine codon segment, and transglutaminase recognition site encoding sequence towards its 5' end; the other primer, namely SK_EGF_Rp4 contained partial sequence of the end of the 6^{th} domain of EGF4,5,6 and a Not I restriction site at its 5'end. After PCR, the resultant gene block 2, obtained by use of this set of primers, contains at its 5' end (upstream 1149^{th}bp of SK, followed by a triple glycine-encoding and transglutaminase recognition encoding sequences, and on the other hand, at its 3' end it contains a Not I restriction site to facilitate cloning into the pET-23-d vector. PCR conditions were as follows: Complete denaturation at 95^0C for 5 min, then 28 cycles of: 95^0C for 45 seconds, annealing of primers at 45^0C for 45seconds, and 72^0C for 1 min; finally an extension at 72^0 for 10 min to complete any partial length PCR products.

Both the PCR blocks (PCR block of SK, and PCR block encoding EGF4,5,6 with partially overlapping sequences) were purified from gel by using QIagen gel extraction kit. These purified PCR products were subjected to splice overlap extension PCR in order to construct contiguous SK_GGG_transglutaminase_EGF4,5,6 hybrid gene construct, and finally amplified with the help of SK_EGF Fp1 and SK_EGF Rp4 (see table 1 for primer sequence) set of 'end' primers. Final PCR product was then digested with Xho I and Not I restriction enzymes (refer to Fig. 1 B and Fig.2 B). After purification from gel, finaldigested and purified PCR block was ligated into pET23-d (previously similarly digested with Xho I and Not I, and gel purified). The resultant

plasmid was termed pET23-d_SK_GGG_TG_EGF, which was propagated in *E. coli* XL1B (recA⁻, end A⁻) cells. This plasmid was subjected to Sanger's di-deoxy chain termination method and the complete cloned insert sequence was validated (DNA SEQ ID 6, and corresponding protein SEQ ID 114). From this plasmid construct, by using Xho1 and Not1 enzymes, the hybrid gene construct was isolated and ligated into pPIC-9K where the upstream XhoI site helped the SK-GGG-TG-EG4,5,6 block's in-frame docking into the α-secretory signal sequence upstream of the hybrid gene in the expression plasmid. This hybrid gene-construct was expressed in *Pichiapastoris* (*GS 115*) under the control of the alcohol oxidase promoter, after its integration into the host genome and selection of plasminogen activator-positive clones by functional screening.

*(iii) Construction of DNA hybrid gene for the in-frame insertion of the EGF 4,5,6 domains into the streptokinase encoding gene segment:*Construction of 'inter-domain SK'-EGF constructs in which the sequences for EGF4,5,6 were interspersed *within* the sequences of SK in a translationally in-frame manner, to give rise to hybrid gene construct/s, were also designed, and then made, with the use of suitable primes (see table no 1 for sequence of primers). The EGF sequence was inserted into the region of the SK gene encoding the inter-domain flexible segments (Wang et al., 1998; Yadav &Sahni, 2009), that is, either between the α and β domains, on the one hand, or between the β and γ domains, on the other. For this purpose, amplification of the DNA encoding the α-domain of SK was carried out first, employing the primer set IDα.Fp1 and IDα Rp 2 (for sequence details, please refer to table 1). Primer IDα.Fp1 contains an Xho I restriction site at its 5'end; on the other hand primer IDα Rp 2 contains an overlapping sequence for the 4th domain of EGF at its 5' end. For this reaction, the following PCR conditions were used: Hot start, complete denaturation at 95°C for 5 minutes, followed by 28 cycles of: denaturation at 95°C for 45 seconds, annealing at 45°C for 45 seconds, extension at 72°C for 1.5 minute, and finally an 'extension segment' of 72°C for 10 minutes to allow any incomplete PCR products to be completed. A DNA block encoding EGF 4,5,6 was also amplified separately using the primers set ID EGF Fp3 and IDEGF Rp4 (see table 1 for the sequence of these primers). Using this primer set a DNA block encoding EGF 4,5,6 sequence was obtained in which upstream sequence towards the 5' end partially overlapped with the downstream sequences of α-domain of SK; likewise the 3' end of this block contained sequence which were overlapping with the upstream sequence of the SK β-domain. A third PCR block encoding both β and γ domains of SK was obtained by PCR using

the primer set ID β Fp5 and ID γ Rp6 (see table 1 for sequence of these primers). For this reaction the following PCR conditions were used: Hot start, complete denaturation at 95°C for 5 minutes, followed by denaturation at 95°C for 45 seconds, annealing at 45°C for 45 seconds, extension at 72°C for 1.5 minute, with a total of 28 cycles, and a final extension of 72°C for 10 minutes for the complete amplification of any incomplete PCR products. ID β Fp5 primer was designed such that 5' end of this was homologous with downstream sequences of the 6th domain of EGF, whereas downstream primer ID γRp6 contained a terminator codon followed by a NotI restriction site. All three gene-blocks were purified from agarose gels with the help of gel purification kit (Qiagen) and subjected to a one-pot splice overlap extension reaction, in order to obtain a single contiguous gene product in which the order of gene segments was to be as follows: SKα-EGF 4,5,6-SKβ-SKγ. All three segments were added in 1:1:1 molar ratio in a simple PCR reaction where initial 10 cycles were done without the primers and next 18 cycles were done by adding 0.6 μM final concentration each of IDαFp1 and IDγRp6, in order to further amplify the three-fragment SOE intermediate. For this reaction, the following PCR conditions were used: Hot start, complete denaturation at 95°C for 5 minutes, followed by (in each cycle) denaturation at 95°C for 45 seconds, annealing at 45°C for 45 seconds, extension at 72°C for 3 minutes, and a final extension of 72°C for 10 minutes for the completion of any incomplete PCR products. Final PCR product was gel purified and digested with Xho I and Not I restriction enzymes (refer to Fig 1G and Fig.2 D). This hybrid gene construct was then ligated into pET 23–d vector and transformed into *E. coli* XL1B (*rec A*$^-$ and *end A*$^-$) competent cells, wherein the DNA could propagate without protein expression (since the host did not provide the required the phage-encoded RNA polymerase), which was subjected to Sanger's automated sequencing with the so-called di-deoxy chain termination method (DNA SEQ ID 3, and corresponding protein SEQ ID 116). The results completely validated the hybrid gene construct. After this, the hybrid gene cassette was isolated from the pET-23-d inter-domain SK_EGF plasmid by digesting it with Xho I and Not I enzymes. This cassette was then ligated into pPIC-9K upstream of, and in-frame with, the α-secretory signal sequence, and transformed into *Pichiapastoris* (*GS115*), cloned and selected as before by functional screening for plasminogen activation, the expression being under the influence of the alcohol oxidase promoter of the vector after integration in host genome after methanol induction as detailed in the Methods section.

*(iv)Construction of hybrid SK-EGF gene in which the SK component is flanked on both sides by the EGF 4,5,6 encoding domains:*Another construct in which the SK encoding sequences were flanked on both sides by in-frame fused EGF 4,5,6 domains, was also constructed by taking pET23-d N_ EGF_SK plasmid construct (see above, sub- sections i and ii), and subjecting it to Xho I and AflII (unique sites in the pET23-d N_EGF_SK construct) digestion, the same treatment also being given to pET -23-d-SK_EGF construct, and both digestion products resolved on agarose gels. The Xho I and Afl-II segment obtained from N_EGF_SK construct was ligated with the larger fragment of SK_EGF digestion, and the result of this gave the N_EGF_SK_EGF (DNA SEQ ID 7, and corresponding protein SEQ ID 115) in the pET23-d vector (refer to Fig.1.C and Fig.2.C). The expression cassette from this plasmid was isolated by Xho I and Not I digestions (this construct being first validated by DNA sequencing, as before) and ligated into pPIC-9K in-frame of the α-secretory signal sequence upstream of the EGF-SK-EGF hybrid cassette and expressed in *pichia pastoris*under the control of the alcohol oxidase promoter after integration in host genome, as before, after functional screening (see below).

*(v)Construction of N_EGF_SK, SK_EGF and N_EGF_SK_EGF encoding gene segments containing sequences encoding oxidation resistant polypeptide segments corresponding to EGF 4,5,6:*In N_EGF_SK, SK_EGF and N_EGF_SK_EGF polypeptides, the methionine residue present at the 41st amino acid residue of EGF 4,5,6 domains, or the 434th amino acid residue of SK_EGF, and 41st and 434th amino acid position N_EGF_SK_EGF, respectively, as part of the EGF4,5,6 domains are known for their oxidation proneness, which hampers the antithrombin activity especially the protein C activation function of these domains. Hence, keeping this in mind, in our design of the various SK-EGF fusion/gene fusion segments, we replaced this methionine with valine/alanine/glutamine amino acid residues at the gene level. This goal was achieved by the use of high fidelity enzyme *pfu* turbo DNA polymerase (Stratagene), as before, and introduction of site specific mutations in different templates using appropriate primers (Wang and Malcolm 1999). In order to make these constructs, three sets of primers were designed which allow the incorporation of either valine, alanine, or glutamine separately in different constructs at the position of the original Met residue in the EGF 4,5,6 domain/segment of the gene construct. The primers sets were named as follows:

i. M rep V Fp, and M rep V Rp (refer: table 1 for sequence of primer)

ii. M rep A Fp, and M rep A Rp (refer: table 1 for sequence of primer)

iii. M rep Q Fp, and M rep Q Rp (refer: table 1 for sequence of primer)

In the next step, pET 23-d _N_EGF_SK and pET 23-d_SK_EGF plasmids were used as template with above mentioned sets of primers. For each set of primers PCR cycle schemes were as follows: complete denaturation at 95°C, followed by denaturation at 95° C for 45 seconds, annealing at 45°C for 45 second, extension at 68°C for 7 minutes for 28 cycles and extension for a further 10 min at 72 °C. Final PCR blocks obtained by this reaction were digested with Dpn I restriction enzyme which cleaves methylated DNA and enhances the probability of getting positive clones after the transformation. This PCR product was transformed in to *E. coli* XL1Blue cells and plated on ampicillin containing LB agar plates and incubated at 37°C for 16-18 h. A few clones were randomly picked and raised into 7-10 ml cultures in amipicillin-containing LB media. Plasmids were isolated and sequenced, to validate the presence of the desired mutation/s. By adopting this procedure, methionine was replaced with valine, alanine or glutamine amino acids at the gene level in both the constructs. These constructs were used to make valine, alanine and glutamine mutations in N_EGF_SK_EGF gene by standard sub-cloning procedures. Finally, these mutants were transferred to pPIC-9K and their expression was checked in GS115 strain of *Pichia pastoris* (see 'general methods used in examples').

(vi)Construction of ΔSK_EGF construct with N-terminal of SK with five amino acid deletion: In this construct, the final objective involved was the removal of five amino acid at the N-terminal region of SK. These 5 amino acids-encoding nucleotides were removed with the help of PCR primers ΔSK_EGF Fp 1 and ΔSK_EGF Rp 2 (referto table 1 for sequence of these primers) using pET 23-d _SK_GGG_TG_EGF (oxidation resistant, where met 41st in EGF 4,5,6 segment was replaced by valine; see above) as a template. The primer Δ SK_EGF Fp1 contains a Xho I restriction site and overlapping sequence of SK started from 16th base pair at its 5' end. ΔSK_EGF Rp 2 contains sequence of 6th domain of EGF 4,5,6 and Not I site at its 5' end. PCR cycles were used for this reaction as follows: complete denaturation at 95°C for 5 minutes, next 28 cycles: denaturation 95°C for 45 seconds, annealing at 55°C for 45 seconds, extension at 72°C for 3 minutes and final extension done at 72°C for 10 minutes for complete amplification of incomplete PCR products. Final PCR product was gel eluted and digested with the Xho I and Not I enzymes and ligated into pET 23-d vector, and sequenced for validation of complete and

correct open reading frame. Finally ΔSK_EGF (refer to Fig. 1D) cassette was ligated in pPIC-9K and checked for its expression by standardized procedures as described earlier.

(vii) In-frame introduction of DNA sequences encoding for a thrombin cleavable site at the junction of oxidation resistant N_EGF_SK and N_EGF_SK_EGF encoding gene blocks: From the expression of N_EGF_SK, purification and kinetic analysis of plasminogen activation, which established a delayed nature of plasminogen activation, it was apparent that unlike native/unmodified SK, this construct could not activate human plasminogen directly, but required the presence of pre-formed plasmin. This automatically confers an adavantage of clot-specific activation in such a construct since the clot in vivo is plasmin rich whereas the plasmin is rapidly inactivated in the general circulation. Similarly, fibrin clots are also thrombin-rich; hence, we mutated suitable sites in the construct to make it thrombin activatable as well. In order to do this, we mutated the inter-domain joining region of EGF and SK by a thrombin cleavable site, the result of which should give a thrombin cleavable activation switch in plasminogen activation. This can be monitored in one-stage assay by adding incrementally small amounts of thrombin and observing (as in the case of a similar plasmin-enrichment experiment described before) a progressive decrease of lag in plasminogen activation in N-terminal EGF4,5,6 fused SK construct. Thrombin cleavable sequence of factor XI was introduced at the junction of EGF and SK gene construct. This was achieved by the overlap extension PCR, where junction primers containing overlapping sequence and thrombin cleavable amino acid encoding nucleotides were used. In first PCR reaction, primers N_EGF_TCS Fp 1 and N_EGF_TCS Rp 2 (refer table 1 for the sequence of these primers) were used for the amplification of EGF 4,5,6 domains. The primer N_EGF_TCS Fp 1 contains a Xho I restriction site and sequence of 4^{th} domain of EGF at its 5' end; on the other hand N_EGF_TCS Rp2 contains 6^{th} domain sequence of EGF followed by thrombin cleavable site encoding sequence followed by upstream part of SK. PCR conditions were as follows: denaturation at 95°C for 5 minutes, in next 28 cycles, denaturation at 95°C for 45 seconds, annealing at 50°C for 45 seconds, extension 72°C for 1 minute and final extension done at 72°C for 10 minutes in order to complete the incomplete PCR fragment. PCR block obtained by this reaction contains a Xho I restriction site at its 5'end and thrombin cleavable encoding nucleotide sequence and overlapping part of SK at 3'end. In next PCR reaction TCS_SK Fp 3 and SK Rp 4 (refer table 1 for the sequence of these primers)were used, where

upstream primer (TCS_SK Fp 3) contains sequence of 6th domain, overlapping part of EGF4,5,6, thrombin cleavable encoding nucleotide sequence, and SK encoding nucleotide sequence in 5' to 3' order; on the other side, SK Rp 4 contains a Not I cleavable site at its 5'end. PCR conditions were as follows: denaturation at 95°C for 5 minutes, in next 28 cycles, denaturation at 95°C for 45 seconds, annealing at 50°C for 45 seconds, extension 72°C for 1 minute and final extension at 72°C for 10 minutes in order to complete the synthesis of any partial-length daughter fragments. The obtained PCR block should contain overlap sequence of 6th domain and thrombin cleavable encoding nucleotides upstream, whereas downstream it should contain a Not I restriction site. Both PCR blocks were gel purified and mixed in 1:1 molar ratio in a single pot reaction, and amplified by the end-primers TCS Fp1 and SK Rp6, which gave the required N_EGF_TCS_SK PCR block (refer to Fig. 1.E) (TCS: thrombin cleavable sequence), which was gel purified and digested with Xho I and Not I enzymes, and finally ligated into similarly digested pET23-d by standard procedures. This construct sequence, after sub-cloning in E. coli XL Blue, was validated with respect to it open reading frame. This established that a Factor XI thrombin cleavable site with the following amino acid sequence at the junction of EGF4,5,6 and SK was created in the gene: Ile-Lys-Pro-Arg-Ile-Val-Gly. In this sequence, the thrombin specificity results in cleavage at the junction of Arginine and Isoleucine such that after thrombin's action, one amino acid was removed from the N-terminal end of SK and also, the second amino acid would be changed to valine (in native SK, the N-terminal residues are Ile-Ala-Gly-). After this, the N_EGF_TCS_SK cassette was ligated into pPIC-9K and its expression was carried out in *Pichia pastoris*. The thrombin cleavage was indeed found to be true in case of the purified construct, after treatment with thrombin, and confirmed by N-terminal protein sequencing.

(viii) EGF4,5,6 domains fused translationally in-frame at the junction of the β and γ domain of SK: For this purpose, amplification of a β- domain was carried out employing primer ID αβ Fp1 & ID αβ Rp2 (refer table 1 for the sequence of these primers) by using pET23-d_SK as template. Primer ID αβ Fp1 contains Xho I restriction site at its 5' end, on the other hand ID αβ Rp2 contains downstream sequence of β domain of SK as well as partly overlapping sequence of 4th domain of EGF4,5,6. PCR block obtained by these set of primer contain Xho I restriction site at upstream sequences, and downstream contain terminal sequences of β-domian and overlapping

sequence of 4th domain of EGF. In next step pcr block 2, which encodes for EGF4,5,6, were isolated by using IDE4Fp3 and IDE6Rp4 (refer table 1 for the sequence of these primers)set of primers. Primer IDE4Fp3 contains downstream sequence of β-domian of SK at its 5'end and IDE6Rp4 contains γ domain upstream overlapping sequence at its 5' end. PCR block 2 obtained by these set of primer contains overlapping sequence of downstream β-domain at 5'end and other end contains upstream sequence of γ domain. PCR conditions for this amplification as follows. Complete denaturation at 95^0c for 5 min. next 27 cycles 95^0c – 45 second, annealing at 45^0C for 45 seconds, extension at 72^0C for 1 minute and final extension done 72^0C – 10 minutes.In next step γ domain of SK was isolated by using IDγFp5 & ID γ Rp6 (refer table 1 for the sequence of these primers) set of primer. Primer ID γ Fp5 designed in such a way, where 5' end of IDγFp5' contains homologous downstream sequence of 6th domain of EGF4,5,6, where as IDγRp6 contains termination codon followed by Not I restriction site. The following pcr scheme used for the amplification of block 3; Hot start for 5 minutes at 95^0C, denaturation at 95^0C for 45 seconds, annealing at 47^0C for 45 seconds and extension done at 72^0 for 1.5 minutes. A total no of 28 cycles carried out, and a final extension of 10 minutes at 72^0C for completion of any incomplete amplified products. All the obtained PCR were gel purified by gel extraction kit (Qiagen) and quantitated by A_{260}spectrophotometrically. In final pct reaction, all the three gene blocks subjected in a single pot for splice overlap extension reaction, in order to get single contiguous gene block in which order of resultant construct as follow: SKα-SKβ- EGF4,5,6-SKγ, all the three pcr mixed in 1:1:1 molar ratio in a polymerase chain reaction, where initial 10 cycles carried out without any primers and next 18 cycles done by adding0.6 μM final concentration of IDαβFp1 & IDγRp6 primers, in order to amplify the three fragment intermediate complete gene block. PCR conditions were as follows: Hot start at 95^0C for 5 minutes, donaturation at 95^0C for 45 second, annealing at 45^0C for 45 seconds, and extension at 72^0c for 3 minutes, a total no. of 28 cycles were carriedout where initial 10 cycle without primers and final amplification done at 72° for 10 minutes. Finally obtained gene block contains Xho I site at its 5'end and Not I site at it 3' end. This gene block digested with Xho I and Not I enzymes and clone to pET 23-d and pPIC 9K. Its expression , purification and characterization checked in *pichia pastoris* This gene block contain EGF 4,5,6 domains between the β and γ domain of SK (DNA SEQ ID 5, and corresponding protein SEQ ID 129; see Fig.1H, and Fig 2E).

(ix) Incorporation of a transglutaminase recognition sequence at the N-terminal encoding end of N_EGF_TCS_SK hybrid-gene block (containing methionine oxidation resistant mutations): For incorporation of a transglutaminase recognition sequence at the beginning or before the 4th domain of EGF 4,5,6 domains in the EGF-SK constructs, primers TG_ N_EGF_ Fp 1 and Afl II Rp 2(refer table 1for detailed sequence of primer) were designed where TG Fp1 contains a Xho I restriction site, and nucleotide sequence encoding for the blood factor XIII transglutaminase recognition amino acid sequence at its upstream region, while primer Afl II Rp 2 contains nucleotide sequence of that region which contains Afl II restriction digestion site (165 -170th nucleotide of SK contains Afl II restriction site which is situated at the centre of this primer). From these primers, we obtained by PCR a gene block that contains a Xho I restriction site, transglutaminase recognition encoding nucleotides, and sequence of 4th domain of EGF4,5,6 with the downstream part of the block containing an Afl II restriction site. This gene block (oxidation resistant and thrombin cleavable sequence containing) was extracted from gel. Plasmids pET 23-d N_EGF_TCS_SK and pET 23-d N_EGF_TCS_SK_EGF were digested with Xho I and Afl II. Result of this gave the Xho I and Afl II gene block and larger and smaller parts from both the plasmid constructs. The digested gene block was ligated with the larger part of the constructs, and transformed into E. coli XL Blue to obtain the TG_N_EGF_TCS_SK and TG_N_EGF_TCS_SK_EGF(refer to Fig.1F) containing plasmids. These were sequenced and validated for the correct open reading frames i.e. presence of transglutaminase encoding sequence in the context of the EGF-SK constructs described earlier. Both cassette were then ligated in Pichia pastoris and checked for their expression of these constructs (please refer to' methods used in examples').

Table 1. Primers used for the various EGF 4, 5, 6 and streptokinase gene fusion constructs preparations.

S. No.	SEQ ID Nos.	Name of primer	Primers Sequence

1	SEQ ID 21	N_EGF_SK Fp 1	5' GAATATCTCGAGAAAAGAGTGGACCCGTGCTTCA-GAGCCA 3'
2	SEQ ID 22	N_EGF_SK Rp 2	5'GTCTAGCAGCCACTCAGGTCCAGCAATGCCGGAGTCA CAGTCGGTGCC 3'
3	SEQ ID 23	N_EGF_SK Fp3	5'GGCACCGACTGTGACTCCGGCATTGCTGGACCTG AGTGGCTGCTAGAC 3'
4	SEQ ID 24	N_EGF_SK Rp 4	5' CCTATACGCGGCCGCTTATTTGTCGTTAGGGTTAT-CAGGTAT 3'
5	SEQ ID 25	SK_EGF Fp 1	5' GCATATCTCGAGAAAA-GAATTGCTGGACCTGAGTGGCTGCTA 3'
6	SEQ ID 26	SK_EGF Rp 2	5'GAAGCACGGGTCCACCACAATTTGTTGAGCTTGTCCG CCACCGGCTAAATGATAGCTAGC 3'
7	SEQ ID 27	SK_EGF Fp 3	5'GCTAGCTATCATTTAGCCGGTGGCGGACAAGCTCAAC AAATTGTGGTGGACCCGTGCTTC 3'
8	SEQ ID 28	SK_EGF Rp 4	5' CCATATCGCGGCCGCGCCGGAGTCA-CAGTCGGTGCCAAT 3'

9	SEQ ID 29	ID α Fp 1	5 'GGATATCTCGAGAAAA-GAATTGCTGGACCTGAGTGGCTGCTA '3
10	SEQ ID 30	ID α Rp 2	5' AAGCACGGGTCCACGGGCTCA-GATTTCGCTTGGTTTTGTATT 3'
11	SEQ ID 31	ID EGF Fp 3	5'AATACAAAACCAAGCGAAATCTGAGCCCGTGGACCCG TGCTT 3'
12	SEQ ID 32	ID EGF Rp 4	5'TACAGTATATTCCACATCAACGCCGGAGTCACAGTCA GTGCCAA3'
13	SEQ ID 33	ID β Fp 5	5'ATTGGCACTGACTGTGACTCCGGCGTTGATGTGGAAT ATACTGT3'
14	SEQ ID 34	ID γ Rp 6	5' AAATATCGCGGCCGCTTTGTCGTTAGGGTTATCAGG-TATA 3'
15	SEQ ID 35	M repV Fp	5' CACAGGTGTCAGGTGTTTTGCAATCAGACTG 3'
16	SEQ ID 36	M rep V Rp	5' CAGTCTGATTGCAAAACACCTGACACCTGTG 3'
17	SEQ ID 37	M rep A Fp	5'CCGCACAGGTGCCAGGCTTTTTGCAACCAGACTG CTTGTCCA 3'

18	SEQ ID 38	M rep A Rp	5' TGGACAAG-CAGTCTGGTTGCAAAAAGCCTGGCACCTGTGCGG 3'
19	SEQ ID 39	M rep Q Fp	5' CACAGGTGTCAGCAATTTTGCAACCAGA-CAGCCTGT 3'
20	SEQ ID 40	M rep Q Rp	5' ACAGGCTGTCTGGTTGCAAAATTGCTGA-CACCTGTG 3'
21	SEQ ID 41	ΔSK_EGF Fp 1	5' GCATAACTCGAGAAAAGAGAGGCTTGGCTGCTA-GACCGTCCA 3'
22	SEQ ID 42	ΔSK_EGF Rp 2	5' CCATATCGCGGCCGCGCCGGAGTCA-CAGTCGGTGCCAAT 3'
23	SEQ ID 43	N_EGF_TC S Fp 1	5' GCCTAACTCGAGAAAAGA-GAGCCCGTGGACCCGTGCTTCAGA 3'
24	SEQ ID 44	N_EGF_TC S_Rp2	5 'GACAATTCTAGGTTTAATGCCAGAGTCA-CAGTCGGTGCCAAT 3'
25	SEQ ID 45	TCS_SK Fp 3	5' GGCATTAAACCTA-GAATTGTCGGACCTGAGTGGCTGCTAGA 3'

26	SEQ ID 46	SK Rp 4	5' CCTATACGCGGCCGCTTATTTGTCGTTAGGGTTAT-CAGGTAT 3'
27	SEQ ID 47	ID αβ Fp1	5 'GGATATCTCGAGAAAA-GAATTGCTGGACCTGAGTGGCTGCTA '3
28	SEQ ID 48	ID αβ Rp2	5'CTCTGAAGCACGGGTCCACGGGCTCCAAGTGACTGCG ATCAAA 3'
29	SEQ ID 49	IDE4Fp3	5'TTTGATCGCAGTCACTTGGAGCCCGTGGACCCGTGCTT CAGAG 3'
30	SEQ ID 50	IDE6Rp4	5'AACGTATTTGATGGTGAACAGTTTGCCGGAGTCA CAGTCG 3'
31	SEQ ID 51	IDγFp5	5'CGACTGTGACTCCGGCAAACTGTTCACCATCAAATAC GTT 3'
32	SEQ ID 52	ID γ Rp6	5' AAATATCGCGGCCGCTTTGTCGTTAGGGTTATCAGG-TATA 3'
33	SEQ ID 53	TG_ N_EGF_ Fp 1	5'GGTATCCTCGAGAAAAGAGTTCAAGCGCAACAGA TCGTGGAACCCGTGGACCCGTGCTTCAGA3'

34	SEQ ID 54	Afl-II Rp2	5' GGTTTTGATTTTGGACTTAAGCCTTGCTCTGTCT 3'

Example 2

EXPRESSION AND FUNCTIONAL CHARACTERIZATION OF VARIOUS EGF-SK HYBRID POLYPEPTIDES:

The different gene blocks described in Example 1, e.g.N_EGF-SK, SK-EGF and N_EGF_SK_EGF, and another class of inter-domain fusion constructs, like interdomain EGF_α ß SK, interdomain EGF_ß γSK, truncated ΔSK_EGF, and oxidation resistant forms of N_EGF_SK, SK_EGF and N_EGF_SK_EGF, with transglutaminase and thrombin cleavage sites etc incorporated, and all cloned in the expression vector pPIC-9K, and also validated with respect to express functionally active hybrid gene products expected from the sequence of the respective gene-fusions, were then isolated from relatively larger-volume cultures prior to their further characterization. As described in Example 1,*Pichia*clones were routinely tested initially by the casein overlay method for plasminogen activation capability, and several (10-20 each) positives were then grown on BMGY and BMMY media (BMGY=1% yeast extract, 2% peptone, 1X glycerol, 1X yeast nitrogen base without amino acids and 100 mM of potassium phosphate buffer pH 5.5, and BMMY =1% yeast extract, 2% peptone, 1X Methanol, 1X yeast nitrogen base without amino acids and 100 mM of potassium phosphate buffer, pH 5.5) and then inducing with methanol as described earlier under 'Methods', and the cell-free supernatants were used for casein overlay assays (for re-confirmation) as well as plasminogen activation (spectrophotometric) assays in presence of peptide chromogenic substrate. Those found to be relatively high producers were then grown on one- liter level and proteins purified by hydrophobic interaction (e.g.

phenyl-agarose) and ion-exchange (DEAE-agarose) chromatographies in tandem (details are described under the section 'general methods used in examples').The hybrid SK-EGF polypeptides obtained by thesetwo methods of purification were generally 90-95% pure by SDS-PAGE. All the purified constructs were tested for plasminogen activation capability (single-stage assays using human plasminogen) as well as thrombin inhibition assays for measuring clotting time, as well as chromogenic assays for Protein C activation (see 'methods used in examples'). While SK_EGF and oxidation resistant SK_EGF showed kinetics of PG activation closely similar to SK but with a 2-3 minute additional lag in activation kinetics, in case of N_EGF_SK, and oxidation resistant N_EGF_SK, N_EGF_TCS_SK and TG_N_EGF_SK the lag in the activation was highly pronounced (20-25 minutes) after which the plasminogen activation kinetics was native-SK like. This suggested that the initial activation of the PG by the constructs was delayed whereas in the case of unmodified SK, it is virtually instantaneous. However, once the SK construct was activated, albeit after the lag, it exhibited full plasminogen activating characteristics. A slightly greater lag (30-35 minutes) found in the bi-polar EGF fusion protein, namely N_EGF_SK_EGF, oxidation resistant N_EGF_SK_EGF, N_EGF_TCS_SK_EGF and its variant TG_N_EGF_TCS_SK_EGF. However as stated before, after abolishment of lag,therate of plasminogen activation per milligram of protein (specific activity) was similar to that of unmodified SK. The reason for the lag in N_EGF_SK, N_EGF_SK_EGF and similar kind of construct was found to be the abolishment of the self activation mechanism of the zymogen (Bajaj and Castellino 1977; Boxrud, Verhamme et al. 2004;Aneja, Datt et al. 2009). This was established by the observation that there was a progressive lossof lag by slight addition of small quantities (in nanomolar amounts) of Plasmin into the reaction assays. In addition, there was no lag when the pre-formed plasmin complexes of these constructs were used in assays clearly suggesting that instead of an instantaneous plasminogen activation mechanism observed with SK, the fusion constructs with the lag were now capable of plasminogen activation only once they were complexed with plasmin. A similar plasmin dependent activation was also

found in the inter-domain α β EGF_SK construct; however, after activation its overall specific activity was found to be only 40-50% that of unmodified streptokinase. Remarkably, in case of the other inter-domain construct, where the EGF 4,5,6segementswere placed at the junction of the β and γ domain of SK, the purified protein showed less than 5% plasminogen activity compared to unmodified SK. This clearly shows that any fusion of EGF with SK is not automatically functional, but only the correctly designed ones are so. These can be selected out by screening (when the designing has been done either empirically or by virtue of structural assumptions) using the plate/spectrophotometric assays for plasminogen activation, thrombin inhibition etc which can be easily adapted for a high-throughput screening system. Thus, any designed site-specific substitution, deletion or domain-additive mutants of SK, or naturally occurring variants of streptokinase, can be used to get similar SK-EGF proteins by the approach adopted here.

The purified constructs were also tested for the measurement of inhibition of thrombin by the clotting time assay using human blood factors (see Methods used in Examples, previously described). Remarkably all of the constructs showed significant increase in clotting time in these assays (approximately 2.5-3 fold) over that of control assays which did not have any construct, or had native SK, as another control. Moreover, the increase in clotting time was found to follow a linear dose-dependent behavior over the low nano-molar range of concentration, which was closely similar to that observed with EGF4,5,6 domains obtained by expression in *Pichia,* used as a positive control. These results established that the EGF fusion constructs which had plasminogen activation capability (as also the one which had very low activity) also exhibited strong thrombin inhibiting capabilities as well.

Example 3

CONSTRUCTION OF DNAS ENCODING VARIOUS EGF4,5,6 AND TPA HYBRID GENES:

A DNA segment encoding EGF4,5,6 sequence (SEQ ID 2, and corresponding protein SEQ ID 111) fused in frame with encoding tpA (SEQ ID 9, and corresponding protein SEQ ID 120) and another segment of DNA fused in frame encoding EGF4,5,6 (SEQ ID 8, and corresponding protein SEQ ID 111) was chemically synthesized purify and cloned in pUC19. It was sequenced to establish its validity (SEQ ID 8, and corresponding protein SEQ ID 111). This construct refer to as EGF-tpA-EGF (SEQ ID 10, and corresponding protein SEQ ID 121). In addition to this gene construct wherein EGF4,5,6 domains gene encoding segments were fused either at the N-terminal site of tPA (SEQ ID 11, and corresponding protein SEQ ID122) or fused at the C-terminal of tpA (SEQ ID 12, and corresponding protein SEQ ID 123). The tPA encoding sequences were first amplified by primer tPAFp 1 and tPA Rp2 (refer to table 2 for primer sequence) primers. The PCR conditions use for this reaction are as follows: Hot start, complete denaturation at 95°C for 5 minutes, denaturation at 95°C for 45 seconds, annealing at 55°C for 45 seconds, extension at 72°C for 4 minute, a total no of 28 cycles and a final extension of 72°C for 10 minutes for the complete amplification of incomplete PCR products. Thus primers used for this reaction tPAFp 1 contains Xho I restriction site at its 5' end and tPARp 2 contains Not I restriction site. Obtained PCR product was digested with the Xho I and Not I restriction enzymes and ligated in to pET 23-d. Final construct formed by this process was named as pET 23-d tPA (refer to Fig.2F), and sequenced to validate the tPA open reading frame. In order to make N-terminal (N_EGF_tPA) and C-terminal (tPA_EGF) fusions, a simple restriction digestion and ligation scheme was followed. To make both the constructs in a single step, pET23-d tPA and pUC-19_N_EGF _tPA_EGF were cleaved with Xho I and BSrG I, both being unique cutters; this gave the smaller and larger fragments. Smaller fragment of pUC-19_N_EGF_tPA_EGF digestion contains N_EGF_tPA sequence, which was ligated with the lager fragment of pET23-d_tPA, step that gave the N_EGF_tPA (DNA SEQ ID 11, and corresponding protein SEQ ID 122) construct. tPA_EGF (DNA SEQ ID 12, and corresponding protein SEQ ID 123) construct was made by ligation of larger fragment of pUC-19_N_EGF_tPA_EGF with smaller fragment of pET 23-d_tPA, which gave tPA_EGF (refer to Fig.1 J) for this construction. N_EGF_tPA (refer

to Fig.1 K) and tPA_EGF (refer to Fig. 2 G) were digested with the Xho I and Not I restriction enzymes and the isolated insert fragment was ligated into pPIC-9K, where construct was placed in-frame to the α-secretory signal sequence. All these constructs were expressed in *Pichiapastrois* (*GS115*) under the influence of methanol inducing promoter located in the vector after integration to the host genome.

Table 2. Primers used for the various EGF 4,5,6 and tissue plasminogen activator gene fusion construct preparations.

S. No.	SEQ ID Nos.	Primer Name	Primers Sequence
1	SEQ ID 71	tPA_Fp 1	5' GGATAACTCGAGAAAAGAGAGGCTCAAGAGATTCATGCTAGATTCAGA 3'
2	SEQ ID 72	tPA Rp 2	5'TCATATCGCGGCCGCTGGTCTCATGTTATCTCTGATCCAGTCCA 3'
3	SEQ ID 73	Fin Fp 1	5' GGATAACTCGAGAAAAGAGAGGCTCAAGAGATTCATGCTAGATTCAGA 3'
4	SEQ ID 74	Fin Rp2	5' GGCTCTAAAGCAAGGGTCAACTGATTTAACTGGAACACTATG3'
5	SEQ ID 75	EGF Fp3	5' CATAGTGTTCCAGTTAAATCAGTTGACCCTTGCTTTAGAGCC3'
6	SEQ ID 76	EGF RP 4	5 AGCTCTGGTTCCGGAATCACAGTCAGTACCTATATGTCTTGCCAA3
7	SEQ ID 77	K1 Fp 5	5' TTGGCAAGACATATAGGTACTGACTGTGATTCCGGAACCAGAGCT 3'
8	SEQ ID 78	CD Rp 2	5'TCATATCGCGGCCGCTGGTCTCATGTTATCTCTGATCCAGTCCA 3'
9	SEQ ID 79	Fg Fp 1	5' GGATAACTCGAGAAAAGAGAGGCTCAAGAGATTCATGCTAGATTCAGA 3'
10	SEQ ID 80	Fg Rp2	5' TCTAAAGCAAGGGTCAACTGATTTAACTGGAACACTATGACA3'
11	SEQ ID 81	EFI Fp3	5' TGTCATAGTGTTCCAGTTAAATCAGTTGACCCTTGCTTTAGA3'

12	SEQ ID 82	EFI RP 4	5'ATCACTGTTTCCGGAATCACAATCAGTACCAATATGTCTTGCC AA 3'
13	SEQ ID 83	K2 Fp 5	5'TTGGCAAGACATATTGGTACTGATTGTGATTCCGGAAACAGTG AT 3'
14	SEQ ID 84	K2CD Rp 6	5'TCATATCGCGGCCGCTGGTCTCATGTTATCTCTGATCCAGTCCA 3'
15	SEQ ID 85	N_EtPA Fp1	5'GGATATCTCGAGAAAAGAGTTGACCCTTGCTTCAGAGCCAAC 3'
16	SEQ ID 86	N_EtPA Rp 2	5' AAAGTAACAATCACTATTTGACTTGACTGGAACACTATGACA 3'
17	SEQ ID 87	EK2 CD Fp 3	5' TGTCATAGTGTTCCAGTCAAGTCAAATAGTGATTGTTACTTT 3'
18	SEQ ID 88	K2 CD Rp 4	5'TCATATCGCGGCCGCTGGTCTCATGTTATCTCTGATCCAGTCCA 3'
19	SEQ ID 89	Finger Fp1	5' GGATAACTCGAGAAAAGAGAGGCTCAAGAGATTCATGCTA-GATTCAGA 3'
20	SEQ ID 90	Finger Rp 2	5' AAAGTAACAATCACTATTTGACTTGACTGGAACACTATGACA 3'
21	SEQ ID 91	K2 CD Fp 3	5' TGTCATAGTGTTCCAGTCAAGTCAAATAGTGATTGTTACTTT 3'
22	SEQ ID 92	EGF Rp 4	5'CCATATCGCGGCCGCTTAACCTGAGTCACAATCTGTTCCAATA 3'
23	SEQ ID 93	EGF CD Fp1	5' GTTGACCCTTGCTTTAGAGCCAACTGTGAATACCAATGCCAG 3'
24	SEQ ID 94	EF CD Rp 2	5' CTGGCATTGGTATTCACAGTTGGCTCTAAAGCAAGGGTCAAC 3'
25	SEQ ID 95	K2 CD Fp 3	5'GTTGACCCTTGCTTTAGAGCCAACTGTGAATACCAATGCCAG 3'
26	SEQ ID 96	K2 CD Rp 4	5' TTTATACGCGGCCGCTGGTCTCATGTTATCTCTGATCCAGT 3'
27	SEQ ID 97	Met rep Val Fp	5'CATGAACCACATAGATGTCAAGTATTCTGCAACCAGACTG 3'
28	SEQ ID 98	Met rep Val Rp	5' CAGTCTGGTTGCAGAATACTTGACATCTATGTGGTTCATG3'
29	SEQ ID 99	Met rep Ala Fp	5' CATGAACCACATAGATGTCAAGCATTCTGCAACCAGACTG 3'
30	SEQ	Met rep Ala Rp	5' CAGTCTGGTTGCAGAATGCTTGACATCTATGTGGTTCATG 3'

	ID 100		
31	SEQ ID 101	Met rep Glu Fp	5' CATGAACCACATAGATGTCAACAATTCTGCAACCAGACTG 3'
32	SEQ ID 102	Met rep Glu Rp	5' CAGTCTGGTTGCAGAATTGTTGACATCTATGTGGTTCATG 3'
33	SEQ ID 103	T 115 N Fp	5'CAGGGAATCTCATATAGAGGTAATTGGTCTACAGCTGAGT 3'
34	SEQ ID 104	T 115 N Rp	5'ACTCAGCTGTAGACCAATTACCTCTATATGAGATTCCCTG 3'
35	SEQ ID 105	N129 Q Fp	5' AATGTACTAACTGGCAATCTTCCGCTTTGGC 3'
36	SEQ ID 106	N129 Q Rp	5' GCCAAAGCGGAAGATTGCCAGTTAGTACATT 3'
37	SEQ ID 107	KH Fp 1	5' GGATAACTCGAGAAAAGAGAGGCTCAAGAGATTCATGCTA-GATTCAGA 3'
38	SEQ ID 108	KH Rp 2	5'AGCAGCGGCAGCAGCAAAAATGGCAGCCTGCCA3'
39	SEQ ID 109	KH Fp 3	5'GCTGCTGCCGCTGCTTCCCCTGGAGAGAGATTCCTTT 3''
40	SEQ ID 110	KH Rp4	5'TCATATCGCGGCCGCTGGTCTCATGTTATCTCTGATCCAGTCCA 3'

Example 4

CONSTRUCTION OF VARIOUS HYBRID GENES BETWEEN TISSUE PLASMINOGEN ACTIVATOR AND EGF 4,5,6 DOMAINS OF HUMAN THROMBOMODULIN:

(i)Construction of inter-domain EGF 4,5,6-tPA construct: Tissue plasminogen activator contains different domains, in the following order: N-terminal peptide-finger domain-EGF like domain-kringle 1 –kringle 2-catalytic domain. A new non-natural, hybrid design was constructed at gene level, where the intrinsic EGF domain/s of tPA were replaced by the EGF 4,5,6 domains of thrombomodulin. These constructs were made by the overlap extension PCR strategy used earlier for the generation of other hybrid genes.

(a) Construction of a hybrid gene block, where the intrinsic egf domain of tPA was replaced with EGF4,5,6 domains of human thrombomodulin: In order to make this construct, pET 23-d_tPA (SEQ ID 9, and corresponding protein SEQ ID 120) was taken as reference where intrinsic egf coding region started from 187 bp to 297 bp. In the first step, the finger domain encoding DNA was isolated by the use of following primer Fin Fp 1 and Fin Rp2 (refer table 2 for the sequence of these primers), where upstream primer contains a Xho I restriction site at its 5' terminus, while on the other hand, the second primer contains downstream sequence encoding for finger domain with overlapping sequence for the 4^{th} domain of EGF4,5,6 domains. The PCR conditions were as follows: Complete denaturation at 95°C for 5 minutes, followed next by 28 cycles : 95°C for 45 seconds, annealing at 48°C for 45 seconds, extension at 72°C for 1 minute, and a final extension done at 72°C for 10 minutes. In a second PCR, EGF 4,5,6 domain segment was amplified where EGF Fp 3 and EGF Rp 4 refer table 2 for the sequence of these primers) primer set was used. Primer EGF Fp3 contains downstream sequence of finger domain and 4^{th} domain of EGF; on the other hand, the downstream primer EGF Rp 4 contains overlapping sequence of kringle 1 at its 5'terminal end. The PCR conditions were as follows: Complete denaturetion at 95°C for 5 minutes, followed next by 28 cycles : 95°C for 45 seconds, annealing at 48°C for 45 seconds, extension at 72°C for 1 minute, and finally ending with an extension segment at 72°C for 10 minutes. Gene block obtained by this set of primers contains finger domain sequence at 5' terminus and overlapping sequence of kringle 1 at its 3' end. In a third PCR reaction, the sequence encoding for kringle 1 to the end of the catalytic domain was selectively amplified by the

use of K1 Fp 5 and CD Rp 6 (refer table 2 for the sequence of these primers) set of primers. Primer K1 Fp1 contains overlapping sequence for the 6th domain of EGF4,5,6 at its 5' terminus, and primer CD Rp 6 contains a termination codon and a Not I restriction site at the 5'end. PCR condition were as follows: Complete denaturation at 95°C for 5 minutes, followed for the next 28 cycles which were: 95°C for 45 seconds, annealing at 48°C for 45 seconds, extension at 72°C for 3 minutes, and final extension done at 72°C for 10 minutes for all partial-length fragments' synthesis completion. Gene block obtained in this reaction contains the EGF 6th domain overlapping sequence at 5'terminus and also the catalytic domain encoding sequences, a termination codon and a Not I site at its 3' end. All the three PCR products were gel-purified and subjected in to a single-pot SOE for the amplification of the complete hybrid gene construct, where the order of assembly was to be as follows: finger domain-EGF4,5,6 domains –kringle 1-kringle 2 and tPA (refer to Fig.1 Q and Fig.2J). catalytic domain. This was carried out with the help of Fin Fp 1 and CD Rp 6(refer table 2 for the sequence of these primers) set of primers. Final PCR conditions were as follows: Complete denaturation at 95°C for 5 minutes, with the next 28 cycles being: 95°C for 45 seconds, annealing at 48°C for 45 seconds, extension at 72°C for 4 minutes, and final extension (single segment) at 72°C for 10 minutes. This gene block was purified by gel extraction and digested with Xho I and Not I enzymes, and ligated with similarly digested and purified pET 23-d vector, and cloned into E. coli XL Blue as described before. A few random clones were subjected to Sanger's sequencing for the complete insert/gene block, and validated to contain the correct in-frame incorporation of EGF 4,5,6 domains into that of tPA (DNA SEQ ID 13, and corresponding protein SEQ ID 124). This gene block was then transferred to pPIC-9K vector, cloned into Pichia pastoris, as before, and screened for high levels of plasminogen activator activity as described under 'general methods used in Examples'.

(b) Construction of inter-domain tPA and EGF hybrid gene construct where intrinsic egf and kringle 1 domains of tPA were replaced with EGF 4,5,6 domains of human thrombomodulin: In order to make this construct, three different PCRs were done separately, where resultant gene blocks contained overlapping sequences at the terminals of each gene block, and they were then amplified in a single-pot SOE reaction in order to make the following hybrid construct: tPA finger domain-EGF4,5,6 –tPAkringle 2 – tPA catalytic (serine protease) domain (refer to Fig. 1R and Fig. 2H). In order to make this construct, pET 23-d_tPA (SEQ ID 9, and corresponding pro-

tein SEQ ID 120) was taken as the template where intrinsic egf- and kringle 1-encoding nucleotide region starts from 187 bp to 564 bp. In the first step, finger domain encoding segment was isolated by the use of the following primers: FgFp 1 and Fg Rp2 (refer table 2 for the sequence of these primers), where the upstream primer contain as Xho I restriction site at 5' terminus, while on the other hand, the second primer contains downstream sequences of finger domain and overlapping sequence of the 4th domain of EGF4,5,6 domains. The PCR condition were as follows: Complete denaturation at 95°C for 5 minutes, with the next 28 cycles being: 95°C for 45 seconds, annealing at 48°C for 45 seconds, extension at 72°C for 1 minute, and final extension being done at 72°C for 10 minutes. In a second PCR, EGF 4,5,6 domains were amplified with primers EFI Fp 3 and EFI Rp 4 (refer table 2 for the sequence of these primers) . Primer EFI Fp 3 contained downstream sequence of finger domain and 4th domain of EGF while on the other hand the downstream primer contained overlapping sequence of kringle 2 at its 5'terminal end. PCR conditions were as follows: Complete denaturation at 95°C for 5 minutes,and next 28 cycles were: 95°C for 45 seconds, annealing at 48°C for 45 seconds, extension at 72°C for 1 minute and final extension done at 72°C for 10 minutes. The Gene block obtained by this set of primers contained finger domain sequence at the 5' terminus, and overlapping sequence of kringle 2 at its 3' end. In a third PCR reaction, sequences starting from kringle 2 to the tPA catalytic domain were amplified by the use of K2 Fp 5 and K2 CD Rp 6 (refer to table 2 for the sequence of these primers), a set of two primers. Primer K2 Fp5 contains overlapping sequence of 6th domain of EGF 4,5,6 at its 5' terminus and primer K2 CD Rp6 contains termination codon and Not I restriction site at the 5'end. PCR condition were as follows: A 'hot start' with complete denaturation at 95°C for 5 minutes, with the next 28 cycles being- 95°C for 45 seconds, annealing at 48°C for 45 seconds, extension at 72°C for 3 minute- and a final extension being done at 72°C for 10 minutes. The gene block obtained in this reaction contained the 6th domain overlapping sequence at its 5'terminus and the catalytic domain encoding sequence, followed by a termination codon, and a Not I site at its 3' end. All the three PCR products were gel-purified and subjected to single- pot SOE reaction with the Fg Fp 1 and K2CD Rp 6 set of primer (refer table 2 for the sequence of these primers) for the amplification for obtaining the complete hybrid gene construct, where the order of assembly of the various protein encoding segments was as follows (finally confirmed by DNA sequencing after cloning): finger domain-EGF4,5,6 domains -kringle 2 and catalytic domain(DNA SEQ ID 14, and corresponding protein SEQ ID 126) . Final PCR

conditions were as follows: Complete denaturation at 95°C for 5 minutes, next 28 cycles being-95°C for 45 seconds, annealing at 48°C for 45 seconds, extension at 72°C for 4 minutes, and a final extension done at 72°C for 10 minutes. This gene block was purified by gel extraction and digested with Xho I and Not I enzymes and ligated with pET 23-d vector, and cloned in E. coli XL Blue, wherein the complete gene block was then sequenced and validated for the correct in-frame incorporation of EGF 4,5,6 domains into the partially truncated tPA gene. This final, hybrid gene-block was transferred to pPIC-9K vector and checked for its ORF expression in pichiapastorisusing standardized procedures as described before.

(c) Construction of hybrid gene blocks encoding for truncated versions of tPA where EGF 4,5,6 encoding segments were fused in-frame at the N-terminal and C-terminal encoding ends of the former: These constructs were prepared by the help of previously made pET-23-d N_EGF_tPA (SEQ ID 11, and corresponding protein SEQ ID 121) and pET 23-d_tPA_EGF (SEQ ID 12, and corresponding protein SEQ ID 123) as templates. In bothconstructs, the intrinsic egf and kringle 1 domains were removed with the help of the overlapping PCR scheme. In order to remove intrinsic egf and kringel 1 domains of tPA from N_EGF_tPA (refer to Fig 1.N) construct, two sets of primers were designed. The first set of primers, N_EtPAFp 1 and N_EtPA Rp2 (refer table 2 for the sequence of these primers) were used for the amplification of contiguous nucleotide sequences of EGF4,5,6 domains and tPA finger domain. In this case, upstream primer contained 4^{th} domain sequence of EGF and Xho I restriction site at its 5' end, and primer N_EtPA Rp2 contained overlapping sequence of tPAkringle 2. A second set of primers, namely EK2 CD Fp3 and K2CD Rp 4 (refer table 2 for the sequence of these primers) was used for the amplification of kringle 2 and catalytic domain of tPA. Primer EK2 CD Fp 3 contained downstream overlapping sequence of finger domain at 5'end and other primer contains downstream sequence of tPA catalytic domain and a Not I restriction site (PCR conditions: Hot start, 95°C for 5 minutes, 28 cycles of 95°C for 45 seconds, 52°C for 45 seconds, 72°C for 2 minutes, and a final extension at 72°C for 10 minutes). Resultant gene block obtained in this reaction contained tPA finger domain overlapping sequence at its 5' end and a Not I restriction site at its 3' end. Both PCR products were gel- purified and subjected for the construct of a complete gene block where intrinsic egf and kringle 1 were deleted with the help of primers N_EtPA Fp1 and K2 CD Rp4 (refer table 2 for the sequence of these primers), primer set. The resultant gene block was purified and di-

gested with the Xho I and Not I restriction enzymes and ligated into pET 23-d vector , sub cloned in E. coli, and DNA- sequenced for the correct in-frame joining after removal of egf and kringle 1 from the tPA (DNA SEQ ID 15, and corresponding protein SEQ ID 126; Fig. 2.I) encoding gene-block. After validation by sequencing for the correct open reading frame of gene block, this cassette was ligated into pPIC-9K, transformed into Pichia as before, screened for activity, and checked for extracellular expression. In a similar way, intrinsic egf and kringel 1 were deleted from the tPA encoding gene block in the construct, tPA_EGF(see Fig.1P and Fig 2 H). Here, pET 23-d –tPA_EGF plasmid DNA was used as template for the amplification of truncated tPA nucleotide sequence. Using the primer set, Finger Fp1 and Finger Rp2 (refer table 2 for the sequence of these primers) for the amplification of finger domain, where Finger Fp1 primer contained a Xho I restriction site at its 5'end and the other primer, Finger Rp2 contained downstream sequence of finger domain and overlapping sequence of kringle 2 of tPA. PCR conditions were as follows: complete denaturation at 95°C for 5 minutes, followed by 30 cycles of : denaturation at 95°C for 45 seconds, annealing at 44°C for 45 seconds and extension 72°C for 1 minute, ending with a final extension at 72°C for 10 minutes. This gave the gene block containing tPAfinger domain over-lapping sequences, followed by anXho I restriction site at its 5' end, and at its 3'end containedkringle 2 overlapping sequences. A second primer set was used for the amplification of kringle2-catalytic encoding domain and EGF 4,5,6 domains. The first primer, K2 CD Fp 3 (refer table 2 for the sequence of these primer), contained overlapping sequences to downstream end of tPA finger domain and a small part of kringle 2; on the other hand, the second primer EGF Rp 4 (refer table 2 for the sequence of these primer) contained downstream sequence of EGF and a Not I restriction site. PCR conditions were as follows: complete denaturation at 95°C for 5 minutes, and 30 cycles of: denaturation at 95°C for 45 seconds, annealing at 44°C for 45 seconds and extension 72°C for 1 minute, and final extension at 72°C for 10 minutes. Both gene blocks were purified from gel and subjected to the overlap extension reaction in presence of primers Finger Fp1 and EGF Rp 4 (seeTable 2 for the sequence of these primers), and the PCR product obtained from this was gel purified and ligated into pet 23-d in (DNA SEQ ID 16, and corresponding protein SEQ ID 127) E. coli as before, and sequencedto validate the complete gene block. Finally this gene block wasligated into pPIC-9K and transformed into Pichia pastoris for screening of expression and functional properties as described before.

(d) Incorporation of different mutations in various EGF containing tissue plasminogen activator gene constructs: In different fusion constructs of EGF 4,5,6 described above, and tPA, following amino acid changes were made : Threonine 115th amino acid changed by aspargine (T 115 N), Aspargine 129th amino acid changed by glutamine (N 129 Q) and KHRR encoding region of tPA 308 -311 is replaced by a tetra-Alanine stretch of mutations (KHRR (308-311)AAAA) (DNA SEQ ID 20, and corresponding protein SEQ ID 128). These mutations are known to confer additional fibrin specificity to the resultant molecule, as well as increase the *in vivo* half life of the tPA (Keytt et al., 1994). For introduction of theses mutations, we used the site directed mutagenesis approach (refer to the 'methods used in examples') and incorporated these changes in the oxidation resistant (where the 129th methionine of EGF4,5,6 domain was replaced valine/alanine/glutamine) gene templates also. The following primers were used for the site directed mutagenesis: 1. T 115 N Fp and T115 N Rp (refer table 2 for sequence of these primers) 2. N129 Q Fp an N129 Q Rp. (refer table 2 for sequence of these primers)

In case of tPA mutations KHRR (where residues 308-311 of tPA were replaced by the tetra Alanine amino acid residues) overlapping extension PCR (refer to the methods used in examples) using KHFp 1 and KHRp 2 as end-primers (refer to table 2 for sequence of these primers i.e. at Serial Nos.37 and 38) were used for the amplification of the 1- 933 bp long stretch of polynucleotide, where KHFp 1 primer contains a Xho I restriction site, and upstream sequence of tPA; on the other hand, the primer KHRp2 contains a four- alanine mutation at its 5' end. The following PCR conditions were used: Hot start, complete denaturation at 95°C for 5 minutes, followed by denaturation at 95°C for 45 seconds, annealing at 45°C for 45 seconds, extension at 72°C for 1.5 minute, with a total of 28 cycles, and a final extension of 72°C for 10 minutes for the complete amplification of any incomplete PCR products. Finally, the gene block obtained by this method contained a Xho I restriction site at the 5' end, and its 3' end contained a tetra-alamine mutation. In another set of PCR reaction, KHFp3 and KHRp 4 (refer table 2 for sequence of these primers) were used for the amplification of the DNA between 918th bp to 1617th bp of tPA. Primer KHFp 3 contains a tetra alanine mutation at it 5' end, while the second primer, KHRp 4 contains a Xho I restriction site. Following PCR condition were used for the reaction: Hot start, complete denaturation at 95°C for 5 minutes, followed by denaturation at 95°C for 45 seconds, annealing at 45°C for 45 seconds, extension at 72°C for 1.5 minute, with a

total of 28 cycles, and a final extension of 72°C for 10 minutes for the complete amplification of any incomplete PCR products. The resultant gene block contains tetra-alanine mutation at its 5'end, and its 3'end contains a Not I restriction site. Both PCR products were gel purified and mixed into a common 'Overlap' PCR reaction single pot, where KHFp1 and KHRp 4 (refer to table 2 for the sequence of these primers) were used for the amplification of the whole gene block. Finally, the obtained gene block was transferred to pET 23-d and after validation of DNA sequence and correct open reading frame, transferred into pPIC9K where the hybrid polypeptide was expressed checked by standard conditions in *Pichia* (please refer to 'methods used in examples').

Example 5

CONSTRUCTION OF HYBRID GENES BETWEEN STAPHYLOKINASE AND THROMBOMODULIN DOMAINS EGF 4,5,6 :

*Construction of SAK_EGF and EGF_SAK encoding hybrid gene blocks:*For the construction of EGF-SAK fusions, EGF 4,5,6 PCR block was isolated with the help of N_EGF_SAK Fp1 and N_EGF_SAK Rp 2 set (refer table 3 for the sequence of these primers)of primers, where pET 23-d _EGF 4,5,6 (Seq ID 2, and corresponding protein SEQ ID 111) was used as template. Here, N_EGF_SAK Fp 1 contained Xho I restriction site at its 5' end, while N_EGF_SAK Rp 2 contained overlapping sequence of SAK nucleotides at the 5' end. PCR conditions were as follows: complete denaturation at 95°C for five minutes in the 1st(hot start) cycle, with the next 28 cycles being: denaturation at 95°C for 45 seconds, annealing at 50°C for 45 seconds, extension at 72°C for 1 minute, and final step was at 72°C for 10 minutes for completion of any uncompleted PCR products. The PCR product contained aXho I restriction site at its 5'end, and 3' end contained overlapping sequence of SAK. In a second step, SAK PCR block was amplified with the help of N_EGF_SAK Fp3 and N_EGF_SAK Rp 4 set of primers and pGMEX_SAK (SEQ ID 17, and corresponding protein SEQ ID 130) construct was used as template. In this reaction, primer N_EGF_SAK Fp 3 contained downstream overlapping sequence of the 6th domain of EGF 4,5,6; N_EGF_SAK Rp 4 contained a termination codon followed by a Not I restriction site. PCR condtions were as follows: complete denaturation at 95°C for five minutes in the 1st cycle, followed next by 28 cycles of denaturation at 95°C for 45 seconds, annealing at 50°C for 45 seconds, ex-

tension at 72°C for 1 minute, and final step at 72°C for 10 minute for completion of uncompleted PCR products. This gave the gene block which had 6thEGF domain overlapping sequences at the 5' end, and 3' end contained a termination codon followed by a Not I restriction site. Both the PCR products were gel-extracted and purified with the help of gel purification kit and mixed into a single SOE PCR reaction in 1:1 molar ratio with N_EGF_SAK upstream Fp 1 and N_EGF_SAK Rp 4 downstream primers being used for the hybrid gene intermediate (obtained by overlap extension) amplification. As a result of this, we got a contiguous gene fragment which contained the N_ EGF_SAK (DNA SEQ ID 18, and corresponding protein SEQ ID 118)sequence. This gene block was then digested with Xho I and Not I, and transferred into pPIC-9K vector. DNA sequencing validated in-frame fusion expected in the EGF_SAK (refer to Fig. 1T) construct and absence of any other mutations. This construct was transformed into the GS 115 strain of *Pichia pastoris* and checked for expression under the influence of the alcohol oxidase promoter after integration into host genome, as before.

The construction of SAK_EGF (refer to Fig.1. S) construct was carried out in a similar way where EGF4,5,6 domain segment was fused at the C-terminal end of SAK. In the first step, SAK gene block was isolated with the help of SAK_EGF Fp 1 and SAK_EGF Rp 2 set of primer. Primer SAK_EGF Fp 1 contains aXho I restriction site at the 5' end; on the other hand SAK_EGF Rp 2 contains overlapping sequence of 4th domain of EGF 4,5,6. The resultant gene block contains Xho I site among its upstream sequences and overlapping 4th domain EGF sequence at its downstream ones. In a second step, SAK gene block was isolated by the use of SAK_EGF Fp 3 and SAK_EGF Rp 4 primers, where the SAK_EGF Fp3 contains overlapping sequence of SAK, and SAK_EGF Rp 4 contains termination codon and a Not I restriction site. The gene block isolated from PCR using these set of primers contained SAK overlapping sequence upstream, and termination codon and Not I site at its downstream end. Both gene blocks were purified from agarosegel and mixed into a single SOE reaction with the SAK_EGF Fp 1and SAK_EGF Rp 4 in order to obtain the complete SAK_EGF (DNA SEQ ID 19, and corresponding protein SEQ ID 119) gene block. Thus, the final PCR product gave a hybrid gene block containing the SAK_EGF sequence which was purified from gel and digested with Xho I and Not I restriction enzymes and transferred into pPIC-9K, after sequencing correct in-frame validation done. This construct transform into *GS 115* strain of *Pichia pastoris* where, after genome integra-

tion by standardized procedures, its expression (which was checked by SDS-PAGE as well as casein –overlay plasminogen activator assays) under the influence of the alcohol oxidase promoter situated in the expression plasmid pPIC-9K. Whole SAK_EGF construct was incorporated in-frame upstream of the α-secretory signal sequence which helps in transport of hybrid gene product across the membrane into the medium.

Bacterial expression of N_EGF_SAK and SAK _EGF: The above-described N_EGF_SAK and SAK _EGF DNA constructs were also expressed under the influence of IPTG inducible lac promoter. In order to make these constructs, the pPIC-9K_N_EGF_SAK and pPIC-9K_SAK_EGF plasmids were used as template.

(i) Construction of bacterial expression cassette of N_EGF_SAK gene block: N_EGF_SAK gene block was first amplified with the help of BacFp 1 and BacRp 2 primers by using pPIC-9K _N_EGF_SAK as template. Bac Fp1 primer contained aNco I site at its 5' end, to help in providing a required AUG codon in the beginning of the gene block, and and provide the initiator Met residueue encoding codon in the mRNA. Primer BacRp 2 contained aXho I restriction site to facilitate in-frame introduction of 6 histidine amino acid-encoding nucleotides at the end of the transcribed mRNA before the *ter* codon that helps in detection of gene product and its purification. This gene block was isolated from the gel and digested with Nco I and Xho I restriction enzymes, finally ligated with T7 promoter based pET 23-d vector and transformed into *E. coli*. These two sites, which are unique, help the hybrid gene construct's insertion under the influence of T7 RNA polymerase promoter (Studier and Moffatt, 1986). This construct pET 23-d_N_EGF_SAK was transformed into XL 1B cells (rec A^- and end A^-) where plasmid propagates and sequenced. Now this plasmid transfer to BL21 (DE3) cells (expression host), where induction of T7 RNA polymerase was done by IPTG and expression in intra-cellular form as inclusion bodies (please refer to ' methods used in examples' for expression conditions, isolation of inclusion bodies and refolding protocols). Finally, the refolded protein was obtained in highly purified form through chromatography and subjected to activity assays.

(ii) Construction of SAK_EGF gene block for bacterial expression: SAK_EGF construct was prepared by the help of SAK_EGF Fp1 and SAK_EGF Rp 2 primers, where pPIC-9K _SAK_EGF was taken as the template for amplification. The primer SAK_bac Fp1 contained a

Nco I restriction site at the 5' end to help in the introduction of a AUG codon; on the other hand, SAK_bac Rp2 contained a Xho I restriction site to facilitate the introduction of 6 histidine amino acid-coding nucleotides prior to the termination codon at the downstream end of the intended SAK_EGF gene-construct. The PCR conditions were as follows: complete denaturation at 95°C for five minutes in 1st cycle, next 28 cycles were: denaturation at 95°C for 45 seconds, annealing at 50°C for 45 seconds, extension at 72°C for 1.5 minute, and a final step 72°C for 10 minute for completion of partial-length PCR products. Resultant gene block contained a Nco I site at its upstream end, and Xho I site present at its downstream end, and was purified by gel extraction and ligated into pET 23-d vector. This ligation product was transformed into *E. coli* XL1B (rec A^- and end A^-) cells by electroporation and the resultant plasmids sequenced for the validation of correct open reading frame. Propagation of plasmid was done in XL1B cells but for expression of hybrid gene product plasmid was transformed into *E. coli* BL 21 (DE 3) cell (Novagen Inc.). Finally, protein was expressed in presence of IPTG, which forms inclusion bodies which were isolated and refolded in presence of oxidized and reduced glutathione as detailed under 'methods used in examples', and subjected to plasminogen activator and thrombin inhibition activity assays as for the EGF_SAK construct, above.

*(iii) Introduction of thrombin cleavable sequences at the junction of EGF and SAK:*Thrombin cleavable sequences were introduced at the junction of EGF and SAK in the *N*-terminal fusion construct. For this construct, EGF_SAK DNA in the plasmid pET-23-d was taken as template for EGF and SAK amplification. Thrombin cleavable primers were designed. For EGF amplification, named E 4 Fp 1 and E6 Rp 2 (refer to table 3 for sequence of these primers at Serial No's. 13 and 14) primers were used. E4 Fp 1 primer contains Xho I restriction site and upstream sequence of EGF4,5,6 domains at its 5'end; on the other hand E6 Rp2 contains downstream sequence of EGF 4,5,6 and thrombin cleavable nucleotide sequence at its 5'end. Following PCR conditions were used for the amplification: complete denaturation at 95°C, followed by next 28 cycles: 95° C for 45 seconds, annealing at 45°C for 45 second, extension at 72°C for 1 minutes, and finally, an extension for a further 10 min at 72 °C. Gene block obtained by this PCR reaction contains sequence encoding in-frame for the 4th domain of EGF at its 5'end, and its 3'end contains downstream sequence of EGF4,5,6 and a thrombin cleavable sequence. In a second PCR reaction, the SAK gene block was amplified with the help of TCS

SAK Fp 3 and TCS SAK Rp4 (refer to table 3 for sequence of these primers i.e. S.No. 15,16). TCS SAK Fp3 contains a thrombin cleavable sequence and upstream sequence of SAK nucleotide; similarly the other primer TCS SAK Rp4 contains downstream sequence of SAK and termination codon, followed by a Not I restriction site. The resulatnat, amplified gene block contains thrombin cleavable nucleotide sequence at its 5'end and termination codon and Not I site situated at its 3' end. The following PCR conditions were used for the amplification of the SAK gene block: complete denaturation at 95°C, followed by next 28 cycles as follows: denaturation at 95° C for 45 seconds, annealing at 45°C for 45 seconds, extension at 72°C for 1 minutes, and final extension for a further 10 min at 72 °C. Both the PCR products were gel-eluted and subjected in a common Overlap Extension PCR reaction for the amplification of EGF_TCS_SAK gene block with the help of E4 Fp 1 and TCS_SAK Rp 4 (refer to table 3 for sequence of these primers i.e. at S.No. 13 and 16 of the table) under the following conditions: complete denaturation at 95°C, followed by denaturation at 95° C for 45 seconds, annealing at 45°C for 45 seconds, extension at 72°C for 2 minutes for 28 cycles and a final extension for a further 10 min at 72 °C. The obtained gene block contained a Xho I restriction site at its 5'end and a Not I site at its 3' end. This PCR product was digested with Xho I and Not I enzymes, and ligated into pPIC-9K after being sequenced for correct in-frame insertion of thrombin cleavable sequence subsequent to cloning in pET 23-d.

Table 3. Primers used for the various EGF 4,5,6 and SAK fusion construct preparations.

S No.	SEQ ID Nos.	Primer Name	Primer Sequence
1	SEQ	N_EGF_SAK Fp 1	5'ATGGATCTCGAGAAAAGAGTGGACCCGTGCTTCAGA 3'

	ID 55		
2	SEQ ID 56	N_EGF_SAK Rp 2	5'ATATTTTCTTTGTCGAATGAACTTGACATGCCGGAGTCACAGTC 3'
3	SEQ ID 57	N_EGF_SAK Fp3	5'GACTGTGACTCCGGCATGTCAAGTTCATTCGACAAAGGAAAATAT 3'
4	SEQ ID 58	N_EGF_SAK Rp 4	5'TTATATCGCGGCCGCTTATTTCTTTTCTATAACAACCTTT 3'
5	SEQ ID 59	SAK_EGF Fp 1	5'ATCCCTCTCGAGAAAAGATCAAGTTCATTCGACAAAGGAA 3'
6	SEQ ID 60	SAK_EGF Rp 2	5'AGTTGGCTCTGAAGCACGGGTCCACGGGCTCTTTCTTTTCTATAACAACCTT3'
7	SE	SAK_EGF	5'AAGGTTGTTATAGAAAAGAAAGAGCCCGTGGACCCGTGCTT

	Q ID 61	Fp 3	CAGAGCCAACT 3'
8	SE Q ID 62	SAK_EGF Rp 4	5'TTTTACGCGGCCGCTCCTGAGTCACAGTCTGTGCCAATGT 3'
9	SE Q ID 63	BacFp 1	5'ATATAGGCCATGGGTGGACCCGTGCTTCAGAGCCAACT 3'
10	SE Q ID 64	BacRp 2	5'CCTATATCTCGAGTTTCTTTTCTATAACAACCTTT 3'
11	SE Q ID 65	SAK_bac Fp1	5'ATGGATCCATGGTCAAGTTCATTCGACAAAGGAAAATATA 3'
12	SE Q ID 66	SAK_bac Rp2	5'TATATTCTCGAGTCCTGAGTCACAGTCTGTGCCAATGT 3'

13	SEQ ID 67	E 4 Fp 1	5'ATGGATCTCGAGAAAAGAGTGGACCCGTGCTTCAGA 3'
14	SEQ ID 68	E6 Rp 2	5' CCGACAATTCTAGGTTTAATGCCGGAGTCACAGT-CAGTGCCAA3'
15	SEQ ID 69	TCS SAK Fp 3	5'GGCATTAAACCTAGAATTGTCGGATCAAGTTCATTCGATAA AGGAAAAT 3
16	SEQ ID 70	TCS SAK Rp4	5'TTATATCGCGGCCGCTTATTTCTTTTCTATAACAACCTTT 3'

Example 6

BIOLOGICAL ACTIVITIES OF TPA-EGF AND SAK-EGF FUSION CONSTRUCTS:

The different hybrid gene constructs of EGF and tPA, where chemically synthesized (N_EGF_tPA_EGF), hybrid tPA fusion constructs (where EGF 4,5,6 was inserted to replace the intrinsic domains of tPA i.e. egf and Kringle 1), internally deleted forms of tPA and EGF fusions (where intrinsic egf and kringle 1 were deleted and EGF 4,5,6 fused at either N and C terminii), and oxidation resistant variants of above-mentioned constructs were cloned in-frame upstream of

α-secretory signal sequence of pPIC-9K vector essentially as described before. Each construct was separately checked by the restriction endonucleases and validated by DNA sequencing. All these constructs were individually transferred to *Pichia pastoris* (*GS 115*) by electroporation (please refer to 'general methods used in examples' section). Individual clones were grown on BMGY and BMMY media (BMGY=1% yeast extract, 2% peptone, 1X glycerol, 1X yeast nitrogen base without amino acids and 100 mM of potassium phosphate buffer pH 5.5 and BMMY =1% yeast extract, 2% peptone, 1X Methanol, 1X yeast nitrogen base without amino acids and 100 mM of potassium phosphate buffer pH 5.5) for 5 days alongwith by inducing with methanol and supernatants were tested by casein overlay method for plasminogen activation ability (refer to 'methods used in examples' section); apart from this, the supernatant of each positive clone tested for plasminogen activation in one-stage assay. Then each hyper-active clone was grown at one-liter level, where the presence of tPA polypeptide in each protein was validated by Western blotting, and followed by lysine affinity and ion-exchange chromatography (for detailed protocol refer to 'methods used in examples'). Different fusion constructs obtained by chromatography were 92-95% pure by SDS-PAGE. These purified proteins were subjected for the plasminogen activation assays. The specific activity of each purified protein was closely similar to *pichia* derived native tissue plasminogen activator. However, constructs where the finger, egf, and kringle 1 and kringle 2 domains were deleted and replaced by EGF 4,5,6 domains showed comparatively weaker zones of hydrolysis by the Casein Overlay method, and also generally exhibited relatively lowered plasminogen activator activity by the quantitative micro-titer plate-based assay using plasminogen and chromogenic substrate. Moreover, the extent of stimulation in activity seen in presence of soluble fibrin in the internally deleted constructs were much lower as compared to tPA/tPA mutants where the fusions of EGF were at the former's terminii. In case of the tPA mutant (containing the Threonine 115th amino acid changed into aspargine (T 115 N), Aspargine 129th amino acid changed to glutamine (N 129 Q) and KHRR encoding region of tPA i.e. residues 308 -311 being replaced by a tetra-Alanine stretch of

mutations (KHRR (308-311→AAAA; see Example, above), the plasminogen activator activity was native-like in terms of both 'basal' activity as well as its stimulation in presence of soluble fibrin. At the same time, this contruct exhibited the highest thrombin inhibiting property using well defined assays (see 'Methods used in Examples' section) of thrombin inhibition and protein C activation. In contrast, thrombin inhibition, as checked by the clotting time assay, wherein fibrin mesh formation in presence or absence of native tPA upto high micro molar concentrations was essentially unaltered compared to buffer controls, the addition of nanomolar concentrations of different purified chimeric constructs including the 'tetra-ala' mutant above, or where EGF 4,5,6 was incorporated in different places internally or at the ends in tPA, gave significantly increased clotting time in a dose-dependent manner. The observed increase in clotting was more prominent in N_EGF_tPA _EGF construct compared to EGF domains fused at either end. This type of effect was also observed in thrombin mediated protein C activation assays, where amount of protein C generated by N_EGF_tPA_EG was 2-4 fold higher than the single EGF 4,5,6 domain containing tPA fusion constructs and the internally fused EGF-tPA constructs in general. In another set of results, where oxidation resistant forms with methionine substitutions in the EGF portion of the EGF-tPA fusions were compared for their thrombin mediated protein C activation capability, the oxidation resistant forms where methionine was replaced with Valine invariably showed 15-20% higher protein C activation.

In a similar way staphylokinase and EGF fusion constructs were prepared and expressed in *Pichia pastoris* as well as in Bl21 DE3 cells. Streptokinase expression in *Pichia pastoris* and glycosylation of its 26th amino acid have been reported in literature, where this glycosylation seems to hamper the plasminogena activation function, but if cultures were grown in presence of tunicamycin containing media, the same plasminogen activation profile like native bacterial purified SAK is obtained. Keeping this fact in consideration, the same gene blocks were prepared and designed for both bacterial and *pichia* expression. Using SAK and EGF fusion

gene blocks for SAK- EGF fusions at the N-terminal and C-terminal ends, the initial expression was carried out in *E. coli* BL 21 DE cells where they the polypeptide synthesis was induced by 1 mM concentration of IPTG (please refer to 'methods used in examples'). Finally, EGF_SAK and SAK_EGF were obtained in form of inclusion bodies where refolding conditions for these polypeptides were optimized with different ratio of oxidized and reduced glutathione as well as other solution conditions (please refer to 'methods used in examples'); side by side, both the constructs were expressed in *Pichia pastoris* in presence of tunicamycin. Finally, fusion polypeptides obtained through both methods were purified and subjected to the plasminogen activation, thrombin inhibition and protein C activation assays. Plasminogen activation of *pichia*-derived, on the one hand, and refolded fusion constructs obtained from *E. coli* IBs, on the other, were essentially the same, but in case of thrombin inhibition and protein C activation the *pichia* derived EGF_SAK and SAK_EGF polypeptides were only approx. 2-fold more active compared to the *E. coli* derived protein. The underlying reason viz. methionine oxidation of EGF in presence of oxidizing conditions particularly during the refolding was validated by quantitative amino acid analysis. The oxidation and activity dimunition problem was solved by putting valine in place of methionine by site directed mutagenesis in the EGF section of the hybrids, as described in the earlier Examples, above.

ADVANTAGES OF THE PRESENT INVENTION

The present invention has advantages over anti-thrombin thrombolytics used in the art. In the past, attempts were made to make anti-thrombin thrombolytics where Kringle of tissue plasminogen activator, peptidic sequences containing arginine (R), glycine (G) and aspartic (D), and the anti-thrombin part of hirudin were introduced at the C-terminal of staphylokinase, the premise being that this form of staphylokinase would help in platelet inhibition by the 'RGD peptide', increased fibrin affinity by the tPA derived kringle, and the thrombin inactivating hirudin

part conferring the anti-thrombin property through inhibition of the transiently formed thrombin at the early stages(Szemraj, Walkowiak et al. 2005). Efforts were also made to combat re-occlusion problem after thrombolysis through recombinant lipoprotein associated cogulation inhibitors (LACI) to effect inhibition of tissue factor and factor VII (Haskel, Torr et al. 1991). This helps in making inactive complex with tissue factor but cannot thwart transient and clot-bound thrombin.

In this invention, however, strategically designed new-generation thrombolytics which contain plasminogen activation as well as thrombin inhibition properties of both kinds (early-stage transient thrombin, as well as the procoagulant generated Protein C pathway) have been designed and validated.

Presently available medication for myocardial infarction /circulatory diseases require co-administration of heparin, hirudin and other thrombin inhibitors but available/marketed thrombin inhibitors target transiently generated thrombin, and do not inhibit the feed-back generation of thrombin.

In contrast to presently available medication, we have strategically designed new class of improved thrombolytics that perform both the thrombin inhibitory functions simultaneously apart from their plasminogen activation property specificity and thereby help to prevent unwanted side effects during clot lysis without not only not compromising their original properties such as fibrin enhancement (as in case of tPA) but also additional properties (such as plasmin- and thrombin dependent activation in cases where none existed in the 'parent' molecules). In cases where there is non-specific plasminogen activation in the original molecule (as is the case of streptokinase) we have designed hybrid constructs that exhibit clot specificity apart from the antithrombin properties.

In the present invention, we have disclosed non-natural chimeric versions of thrombolytics like streptokinase, staphylokinase and tissue plasminogen activator which contain both fibrinolytic and anti-thrombin potential. The already known

'parent' molecules of these constructs, including the second- and third- generation engineered ones, can dissolve clots but cannot suppress thrombin's activity which is primarily responsible for re-thrombosis. The present constructs not only dissolve the fibrin clots but also directly suppress thrombin and also activate protein C when thrombin shifts the equilibrium through the intrinsic thrombomodulin-catalyzed anticoagulant pathway.

In accordance with the present invention, in one embodiment, EGF 4,5,6 domains were introduced at the different domain junctions within the streptokinase molecule,, after placement of suitable linkers, or in place of a natural inter-domain linker. Different active constructs were then selected on the basis of plasminogen activation profile as well as thrombin inhibition properties. Similarly, various muteins of SK fused with the EGF domains in various combinations/motifs were created, expressed and then screened using a simple assay system where functionally surviving chimeras were selected out having the desired properties.

An interesting and useful phenomenon observed in streptokinase (the drug molecule with highest thrombolytic potential) when EGF 4,5,6 was fused at its N-terminal end was that the chimeric construct showed delayed plasminogen activation kinetics and this delayed activation was significantly reduced if small amounts of plasmin were present in the reaction mixture. The serendipitous observation that these chimeric constructs exhibited plasmin-dependent activation (as opposed to 'spontaneous' zymogenic activation of plasminogen by unmodified native streptokinase) also makes the resultant molecules clot specific in that these willbe circulating initially in an inactive state (since there is usually no free plasmin in blood, as it is rapidly inactivated by the anti-plasmin serpins) but upon encountering the clot-bound plasmin, will be activated due to its plasmin dependent mechanism of action. This would greatly minimize the hemorrhage risk of SK and confer additional advantages on a widely used drug, besides the one conferred by the functional fusion of the EGF domains.

Other interesting constructs in accordance with the present invention were obtained by the fusion of EGF 4,5,6 domains at the inter-domain junction of alpha (α) and beta (β) domain, on the one hand, and beta(β) and gamma(γ) domains of streptokinase, on the other. Amongst these constructs we found plasminogen activation as well as anti-thrombin activity only where we fused EGF between the alpha and beta domains. Interestingly, the fusion construct where EGF domains were introduced between the beta and gamma domains failed to show any plasmin activity. These results suggest that it is not predictable *a priori* whether a given domain fusion design will allow the 'parent' properties to co-exist unless actually tested by experimentation.

An advantage that was observed in the N-terminal EGF-SK fusion construct, and N-terminal fusion of EGF domains with truncated SK(residues 5-383) constructs was their clear plasmin dependent activation property apart from thrombin inactivation, which is potentially of great advantage for clot specific thrombolysis and thrombin inactivation.

When EGF 4,5,6 domains were fused with N-terminal and C-terminal regions of staphylokinase, itwas observed that the N-terminal part was removed during the 1:1 plasmin complex formation, which is likely to be helpful in the independent function of EGF domain during the lysis as also in generating a high level of clot specificity. In the case of C-terminal EGF fusion, it was found to remain attached to SAK. In both cases, thus, plasminogen activation and anti-thrombotic properties were found to be integrated successfully in the chimeric polypeptides.

Compositions including compounds of the invention can have prodrug forms. Prodrugs of the compounds of the invention are useful in the methods of this invention. Any compound that will be converted in vivo to provide a biologically, pharmaceutically or therapeutically active form of a compound of the invention is a prodrug. Various examples and forms of prodrugs are well known in the art. A biomolecule such as a precursor protein or precursor nucleic acid can be a prodrug.

Examples of prodrugs are found, inter alia, in Design of Prodrugs, edited by H. Bundgaard, (Elsevier, 1985), Methods in Enzymology, Vol. 42, at pp. 309-396, edited by K. Widder, et. al. (Academic Press, 1985); A Textbook of Drug Design and Development, edited by Krosgaard-Larsen and H. Bundgaard, Chapter 5, "Design and Application of Prodrugs," by H. Bundgaard, at pp. 113-191, 1991); H. Bundgaard, Advanced Drug Delivery Reviews, Vol. 8, p. 1-38 (1992); H. Bundgaard, et al., Journal of Pharmaceutical Sciences, Vol. 77, p. 285 (1988); and Nogrady (1985) Medicinal Chemistry A Biochemical Approach, Oxford University Press, New York, pages 388-392).

The invention has been described with reference to various specific and preferred embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention. It will be apparent to one of ordinary skill in the art that compositions, methods, devices, device elements, materials, procedures and techniques other than those specifically described herein can be applied to the practice of the invention as broadly disclosed herein without resort to undue experimentation. All art-known functional equivalents of compositions, methods, devices, device elements, materials, procedures and techniques described herein are intended to be encompassed by this invention. Whenever a range is disclosed, all subranges and individual values are intended to be encompassed. This invention is not to be limited by the embodiments disclosed, including any shown in the drawings or exemplified in the specification, which are given by way of example or illustration and not of limitation. The scope of the invention shall be limited only by the claims.

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5

Seq listing

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Seq Id 3= interdoamin SK_EGF

Between alpha andbeta

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We CLAIM:

1. A chimeric protein construct comprising the 4, 5, and 6 epidermal growth factor-like domains (EGF 4,5,6) of thrombomodulin fused to a thrombolytic protein selected from the group consisting of a streptokinase, a tissue plasminogen activator, a staphylokinase, a urokinase, and derivatives and analogs thereof.

2. A chimeric protein construct according to claim 1, where said thrombomodulin EGF 4,5,6 domains are fused to said thrombolytic protein, or derivative or analog thereof at the N-terminus, C-terminus, or both N- and C- termini of said thrombolytic protein, or derivative or analog thereof.

3. A chimeric protein construct according to claim 1, wherein said thrombolytic protein comprises a streptokinase with one or more amino acid substitutions, insertions, deletions, or truncations, and wherein the construct possesses plasminogen activation, thrombin inhibition, and anticoagulant protein C pathway activation activity.

4. A chimeric protein construct according to claim 1, where the thrombomodulin EGF 4,5,6 domains are fused between the alpha and beta or beta and gamma domains of said streptokinase or between the alpha and beta or beta and gamma domains ofa streptokinase derivative or analog, wherein the streptokinase derivative or analog comprises one or more mutations, additions, insertions, or truncations, and wherein said construct activates plasminogen, inhibits thrombin, and activates the anticoagulant protein C pathway.

5. A chimeric protein construct according to claim 2, where the EGF 4,5,6 domains are fused in-frame to streptokinase, or a streptokinase derivative or analog, at one or more positions selected from the streptokinase N-terminus, streptokinase C-terminus, both N- and C- streptokinase termini, or at an inter-domain position of streptokinase.

6. A chimeric protein construct according to claim 5, wherein the streptokinase derivative spans residues 5-383 or 5-414.

7. A chimeric protein construct according to claim 6, wherein the streptokinase derivativespans residues 16-383.

8. A chimeric protein construct according to claim 2, where the EGF 4,5,6 domains are fused in-frame at one or more positions selected from the streptokinase N-terminus, streptokinase C-terminus, or both N- and C- streptokinase termini, wherein the Met 41 of the EGF 4,5,6 domains is replaced by either valine, alanine, or glutamine; or the C-terminal Met 435 is replaced by valine, alanine, or glutamine; or in the simultaneous N- and C-terminal fusion constructs, Met 41 and Met 435 are independently replaced by either valine, alanine, or glutamine.

9. A chimeric protein construct according to claim 1 further comprising transglutaminase recognition sequences.

10. A chimeric protein construct according to claim 1 further comprising one or more thrombin cleavable sequences at the junction of the EGF 4,5,6 domain and the thrombolytic protein, or derivative or analog thereof.

11. A method of treating thrombosis in a mammal, comprising administering to said mammal in need of treatment a therapeutically effective amount of a chimeric protein construct according to any of claims 1 to 10.

12. A chimeric protein construct comprising the EGF 4,5,6 domain of thrombomodulin fused to a tissue plasminogen activator (tPA) derivative, analog, or fragment at the N-terminus of the tPA derivative, analog, or fragment, at the C-terminus of the tPA derivative, analog, or fragment, at both termini of the tPA derivative, analog, or fragment, or internally within the tPA derivative, analog, or fragment.

13. A chimeric protein construct according to claim 12, wherein the fusion between the EGF 4,5,6 domain and the tPA derivative, analog, or fragment further comprises one or more linker fragments.

14. A chimeric protein construct according to claim 13, wherein the one or more linker fragments comprise one or more amino acids that promote flexibility of said construct.

15. A chimeric protein construct according to claim12, wherein the tPA derivative, analog or fragment comprisesone or more mutations, additions, insertions, or truncations, and wherein said tPA derivative, analog or fragment activates plasminogen, inhibits thrombin, and activates anticoagulant protein C.

16. A chimeric protein construct accoriding to claim 12 comprising a fragment of tissue plasminogen activator (tPA), or a truncated or modified form thereof, fused to one or morethrombomodulin EGF 4,5,6 domains, such that the EGF domains of tPA are replaced by the one or morethrombomodulin EGF 4,5,6 domains, and wherein the construct has both antithrombin and plasminogen activation activity.

17. A chimeric protein construct accoriding to claim 12 comprising a fragment of tissue plasminogen activator (tPA), or a truncated or modified form thereof, fused to one or morethrombomodulin EGF 4,5,6 domains, such that the kringle 1 and EGF domains of tPA are replaced by the one or morethrombomodulin EGF 4,5,6 domains, and wherein the construct has both antithrombin and plasminogen activation activity.

18. A chimeric protein construct according to claim 17, wherein the tPA EGF domains and kringle 1 domain are replaced by one or more thrombomodulin EGF 4,5,6 domains at the N-terminus or C-terminus of the tPA fragment.

19. A chimeric protein construct of any one of claims 17 or18, further comprising one or more linker fragments between the tPA fragment or truncated or modified form thereof and the thrombomodulin EGF 4,5,6domains,the linker fragments comprisingamino acid residues that promote flexibility of the constructso thatthe construct has thrombin inhibition, protein C activation,and plasminogen activation capabilities.

20. A chimeric protein construct of any one of claims 17 to 19, wherein the methionine 41 of the EGF 4,5,6 component is replaced by either alanine, valine or glutamine.

21. A chimeric protein construct comprising an EGF 4,5,6 domain of thrombomodulin fused in-frame to the N-terminal or C-terminal end of staphylokinase (SAK).

22. A chimeric protein construct according to claim 21, wherein methionine 41 of the thrombomodulin EGF 4,5,6 domain is replaced by either alanine, valine or glutamine.

23. A chimeric protein construct according to either of claims 21 or 22, further comprising thrombin cleavable sequencesbetween the SAK and EGF 4,5,6 domain regions.

24. A chimeric protein construct according to any of claims 22 to 23 further comprising transglutaminase cross-linking sequences.

25. A chimeric protein construct according to any one of claims 1-10 or 12-24, wherein said constructs are soluble in aqueous or saline solution.

26. A method of inhibiting thrombin comprising the use of a chimeric protein construct according to any one of claims 1-10 or 12-24.

27. A method of activating protein C comprising the use of a chimeric protein construct according to any one of claims 1-10 or 12-24.

28. A method of providing both antithrombin and plasminogen activation comprising the use of a chimeric protein construct according to any one of claims 1-10 or12-24.

29. A pharmaceutical formulation comprising a pharmaceutically effective amount of a chimeric protein construct according to any one of claims 1-10 or12-24.

30. A method of thrombolysis in a mammal comprising administering to said mammal in need thereof a therapeutically effective amount of a chimeric protein construct according to any one of claims 1-10 or 12-24to a subject in need thereof.

31. A chimeric construct according to any one of claims 1-10 or 12-24that is prepared by expression in a bacterial system.

32. A chimeric construct according to any one of claims 1-10 or 12-24that is prepared by expression in a eukaryotic system.

33. The chimeric construct according to claim 32, wherein the eukaryotic expression system is selected from the group consisting of an animal cell, Pichia pastoris, and a fungi.

34. The chimeric protein construct according to claim 33 is, secreted into an extracellular medium.

35. A chimeric protein construct according to claim 13, wherein one or more amino acids is chosen fromthe group consisting of Gly, Asn, Pro, Ser, Gln, Arg and Lys.

36. A nucleic acid sequence encoding a chimeric protein construct according to any of claims 1-10 ,12-25 or 31-35.

37. A vector comprising a nucleic acid sequence of claim 36.

38. A host cell comprising a vector of claim 37.

39. A chimeric protein construct according to any of claims 1 to 10 12-25 or 31-35that is prepared by expression of a nucleic acid sequence encoding said protein in a host cell expression system.

40. A method of preparing a chimeric protein construct according to any of claims 1 to 10 and 12-25 or 31-35 by expression of a nucleic acid sequence encoding said protein in a host cell expression system.

41. The method according to claim 40, in which the host cell expression system is a eukaryotic expression system.

42. The method according to claim 41, in which the host cell expression system is a bacterial expression system.

43. The method according to claim 42, in which the bacteria is E. coli.

44. The method according to claim 43, in which the eukaryotic expression system is an animal cell, or a yeast cell.

45. The method according to claim 44, in which the yeast is Pischia pastoris.

46. The method according to any of claims 40 to 45, wherein the chimeric protein construct is secreted from the host cell into an extracellular medium.

Figure 1.

Schematic Representation of Various Constructs

(A)

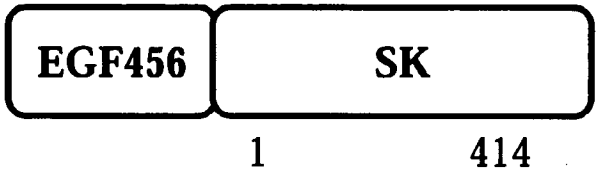


Fig. 1(A)

(B)

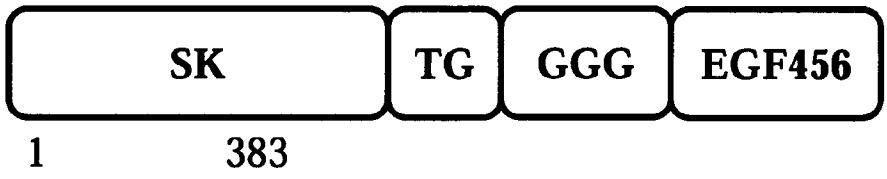


Fig. 1(B)

(C)

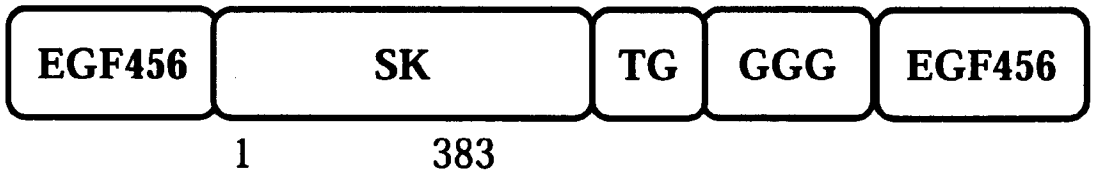


Fig. 1(C)

(D)

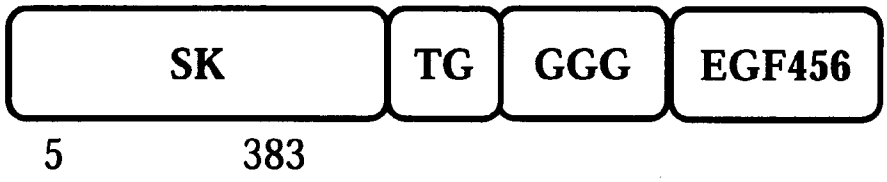


Fig. 1(D)

Schematic Representation of Various Constructs (cont,d)

(E)

Fig. 1 (E)

(F)

Fig. 1 (F)

(G)

Fig. 1 (G)

(H)

Fig. 1 (H)

Schematic Representation of Various Constructs

(I)

Fig. 1(I)

(J)

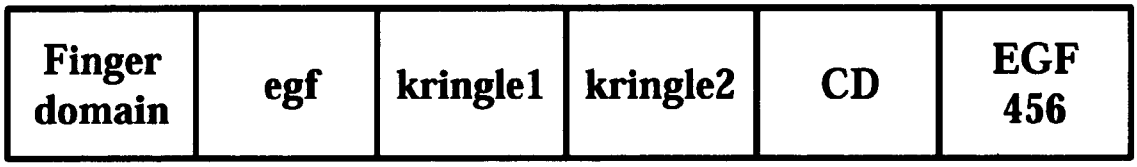


Fig. 1(J)

(K)

Fig. 1(K)

Schematic Representation of Various Constructs (Fig. 1, cont,d)

(L)

Fig. 1 (L)

(M)

Fig. 1 (M)

(N)

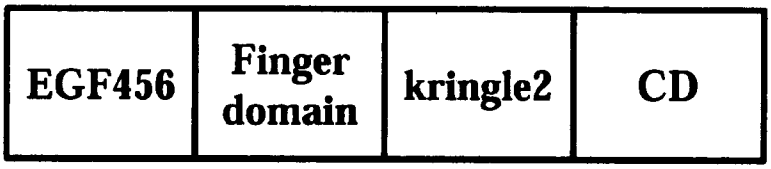


Fig. 1 (N)

(O)

Fig. 1 (O)

(P)

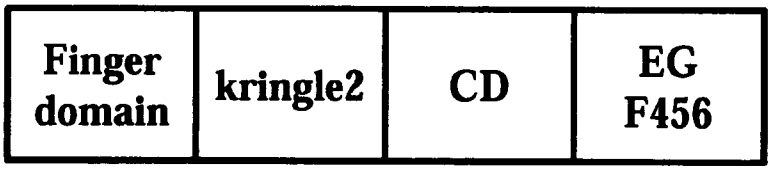


Fig. 1 (P)

Schematic representation of various fusion gene Constructs (cont,d)

(Q)

Fig.1.(Q)

(R)

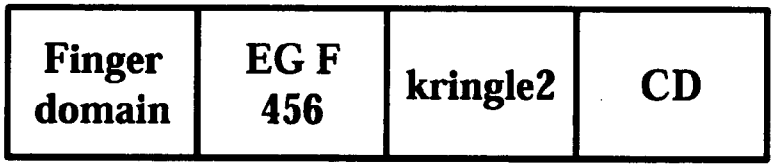


Fig. 1(R)

(S)

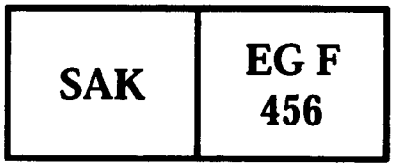


Fig. 1(S)

(T)

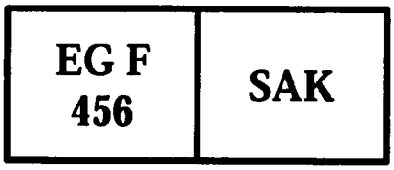
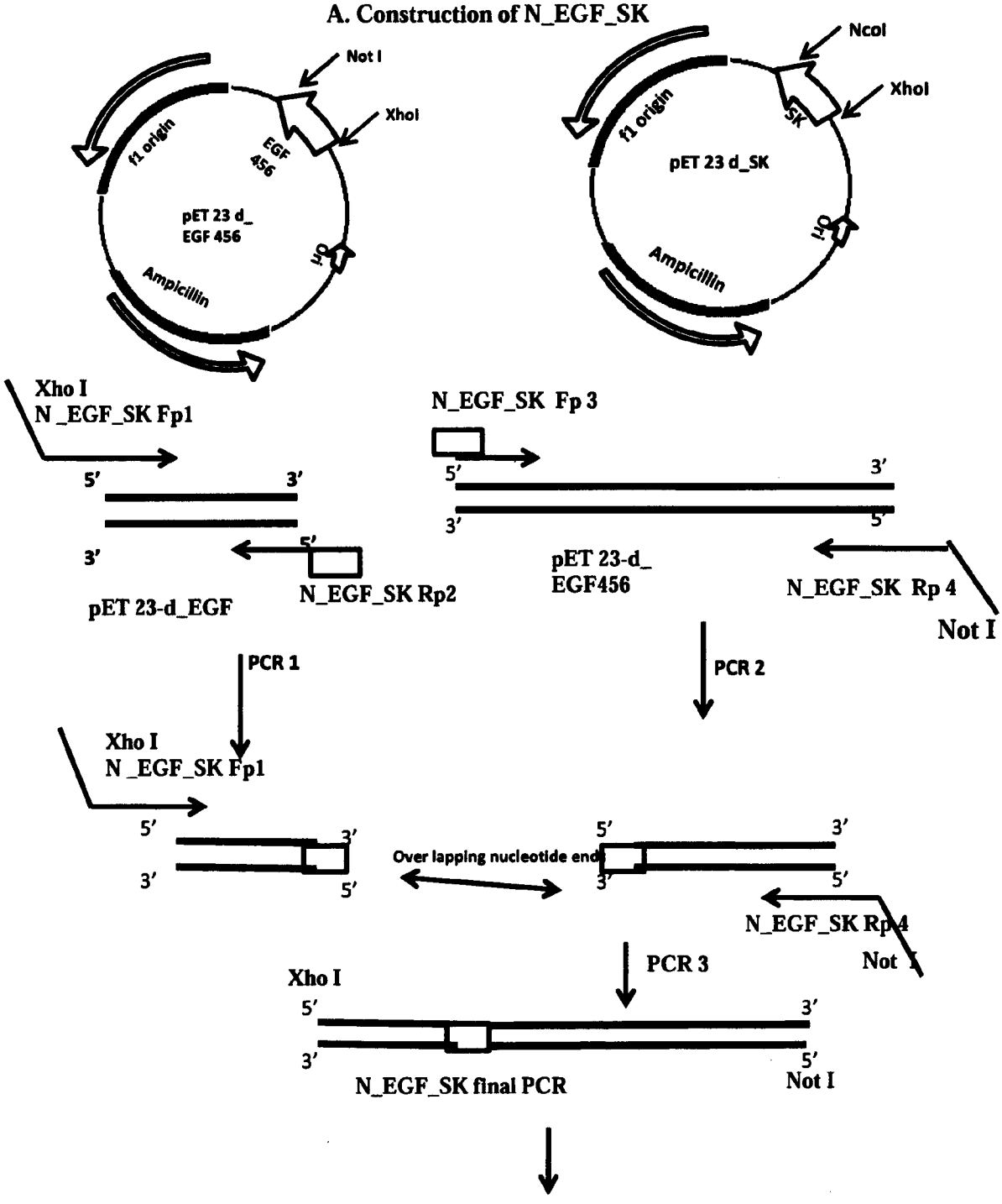


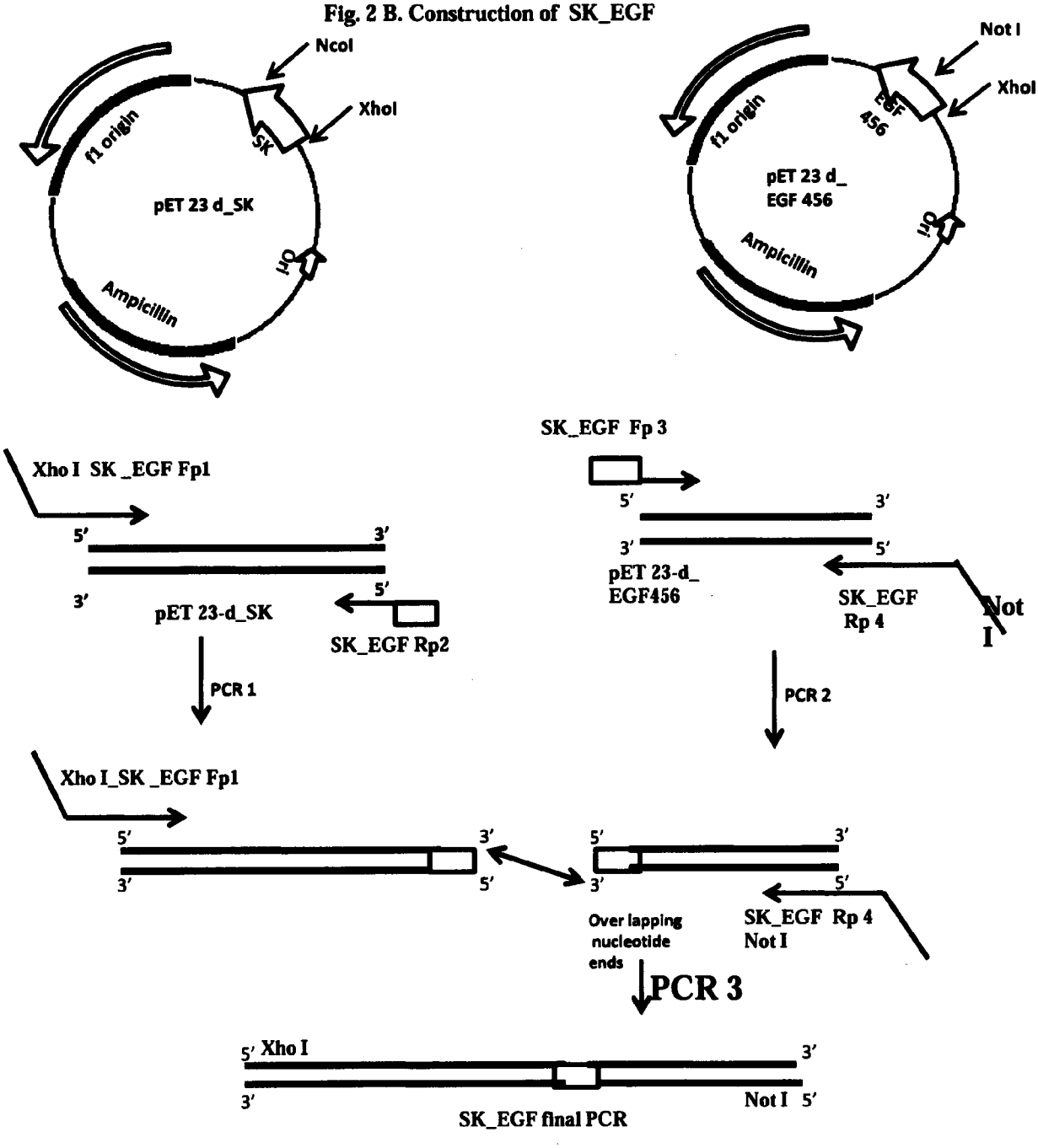
Fig. 1(T)

Fig. 2 Cloning sechemes of various fusion constructs
A. Construction of N_EGF_SK



Digest with Xho I and Not I and clone into pET 23-d, and pPIC-9K for expression

Fig. 2 B. Construction of SK_EGF



Digest with Xho I and Not I and cloned into pET 23-d and pPIC-9K for expression

Fig. 2 C. Construction of N_EGF_SK_EGF

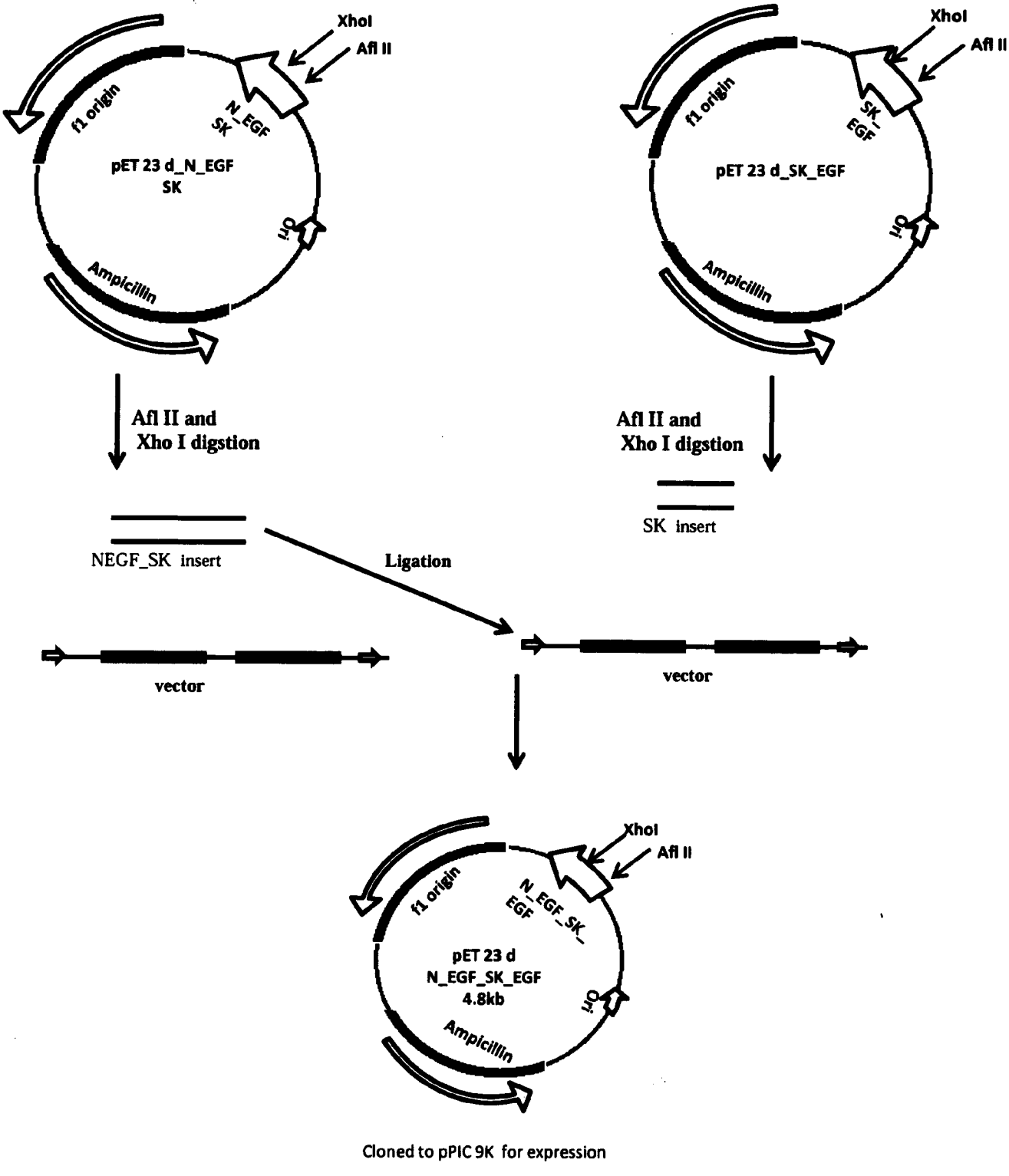
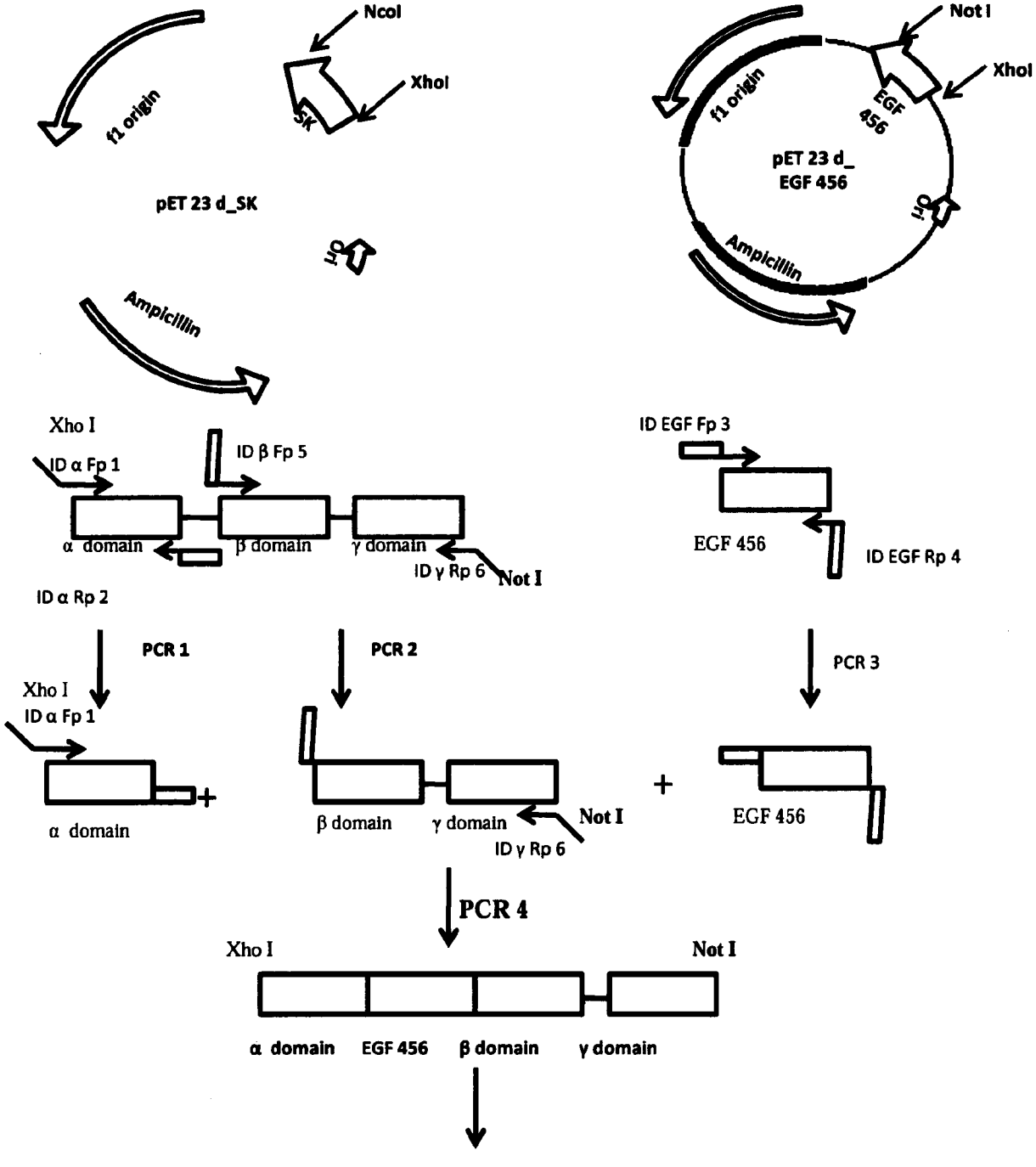
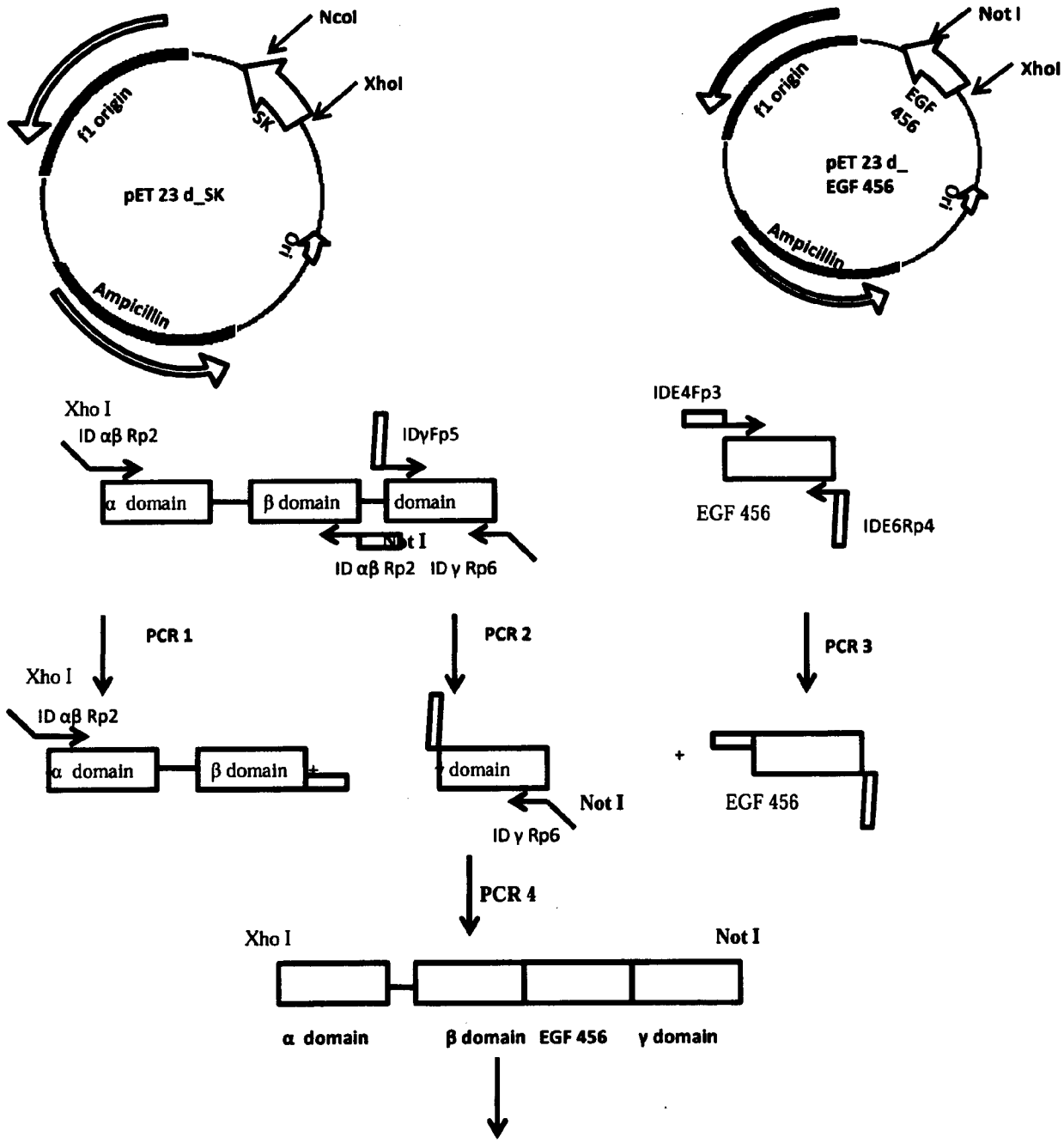


Fig. 2 D. Interdomain SK _EGF (where EGF456 introduced between α and β domain)



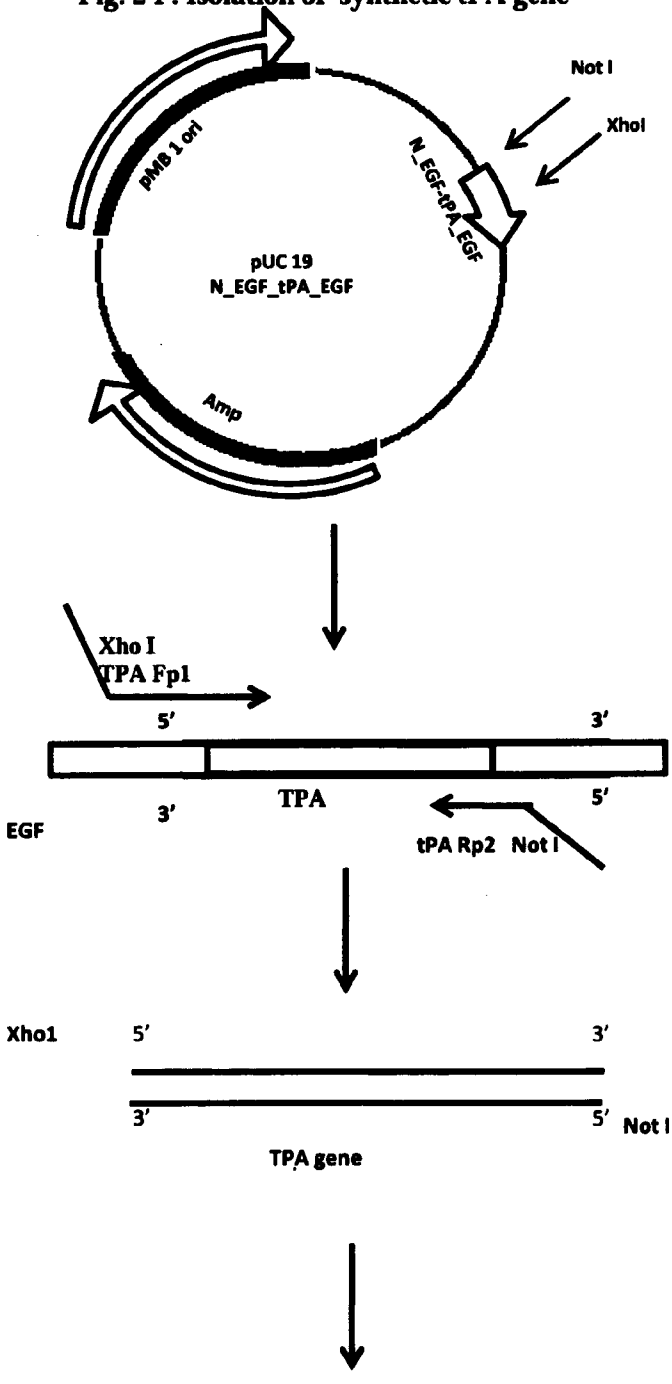
Digest with Xho I and Not I and cloned into pET-23-d, and pPIC-9K for expression

Fig. 2 E. Interdomain SK _ EGF (where EGF456 introduced between the junction of β and γ domain)



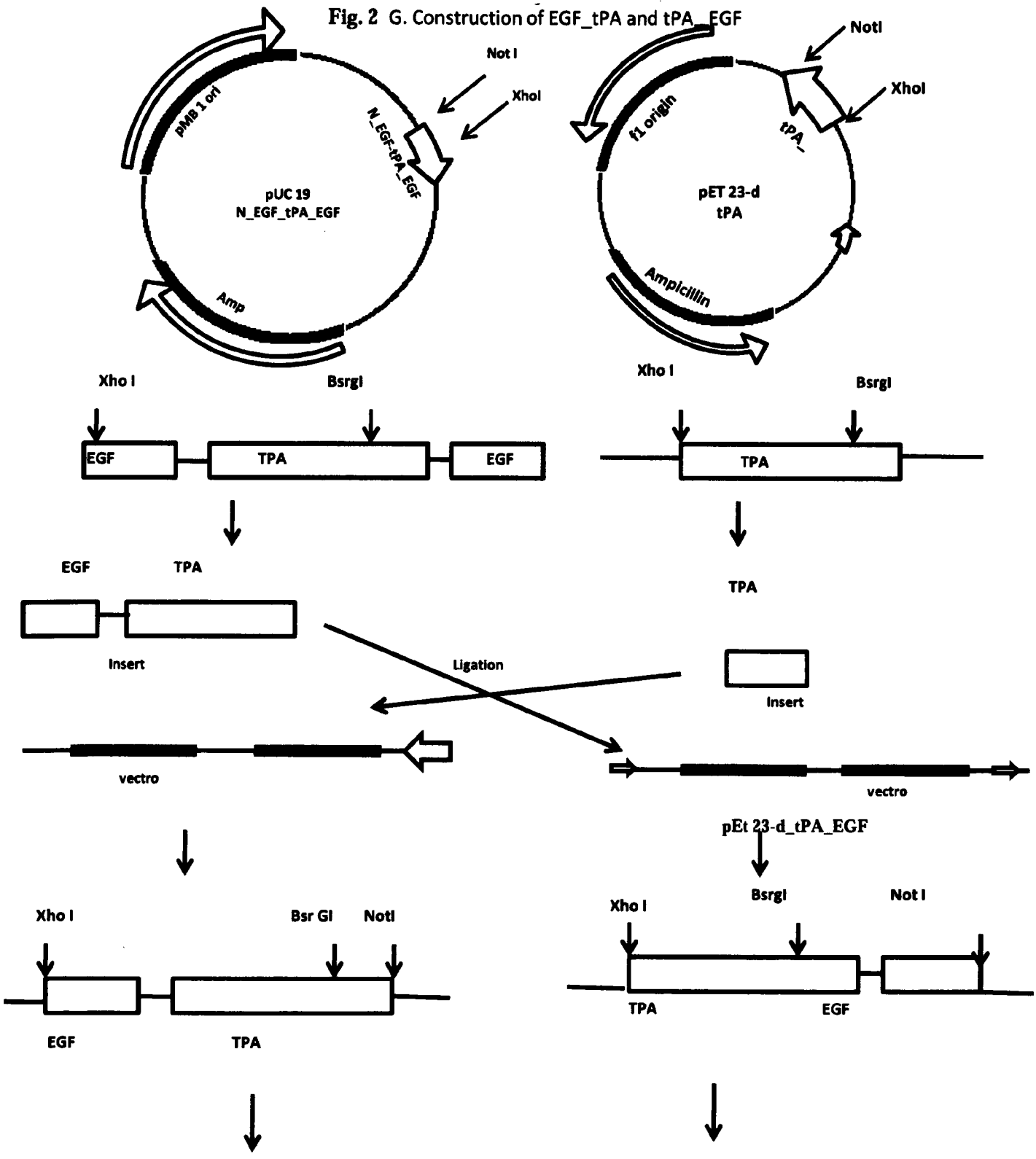
Digest with Xho I and Not I and ligated wnto pET-23-d and pPIC-9K

Fig. 2 F. Isolation of synthetic tPA gene



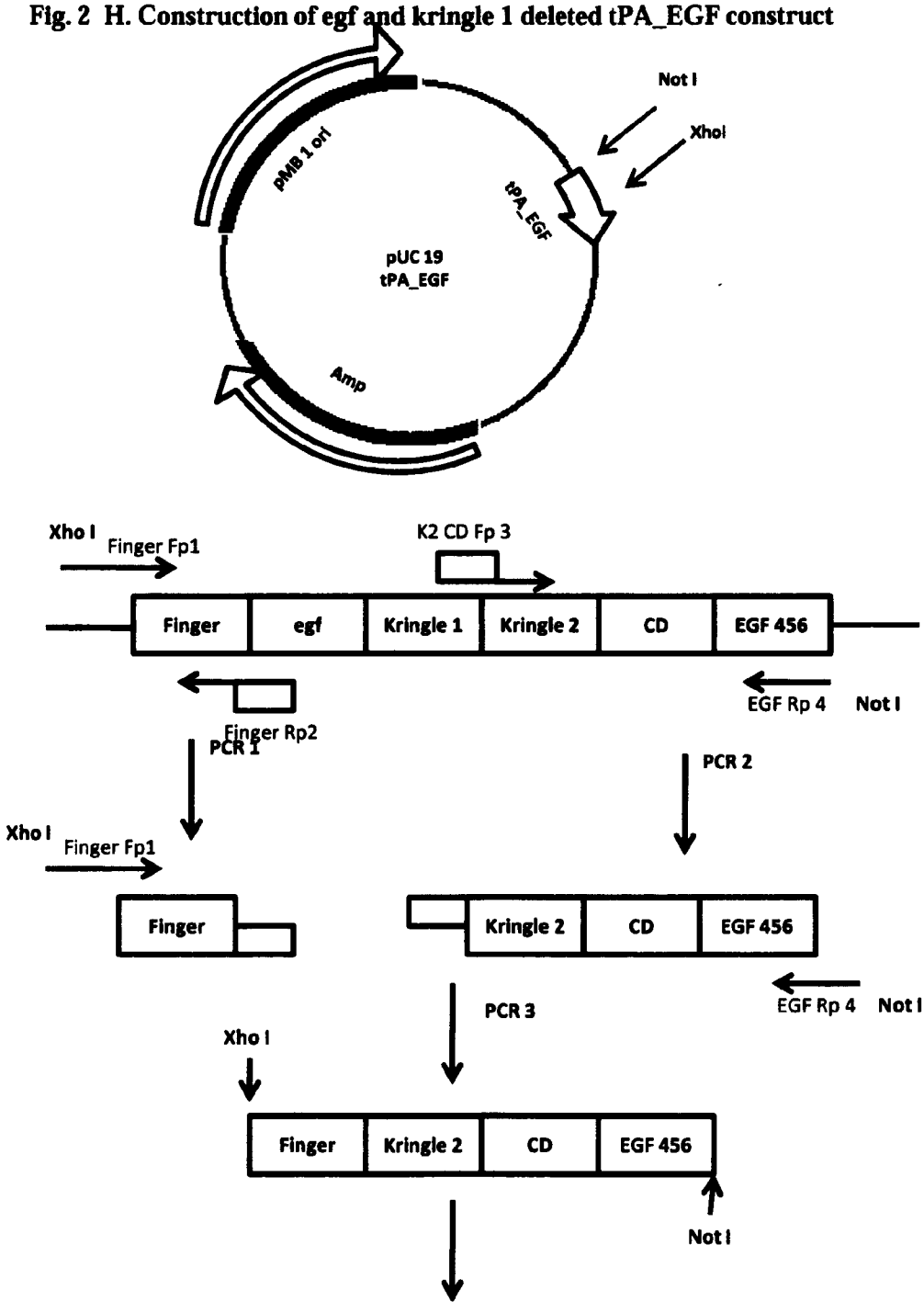
tPA gene (synthetically prepared and cloned in pUC 19 vector) was digested with Xho I and Not I and cloned into pET23-d, and pPIC-9K for expression

Fig. 2 G. Construction of EGF_tPA and tPA_EGF



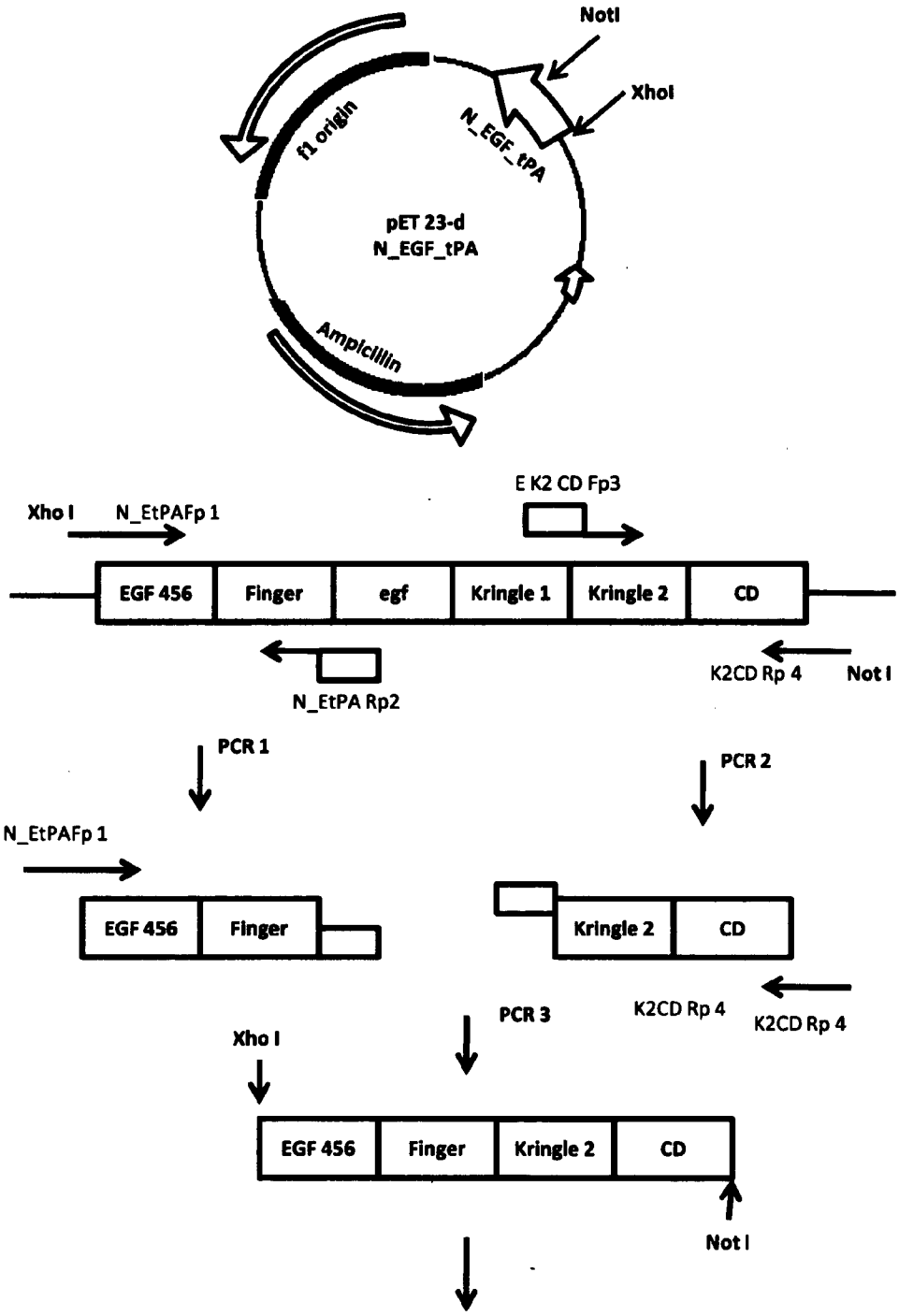
Digested vector cassettes of N_EGF_tPA and tPA_EGF cloned in to pET 23-d, and pPIC-9K for expression

Fig. 2 H. Construction of egf and kringle 1 deleted tPA_EGF construct



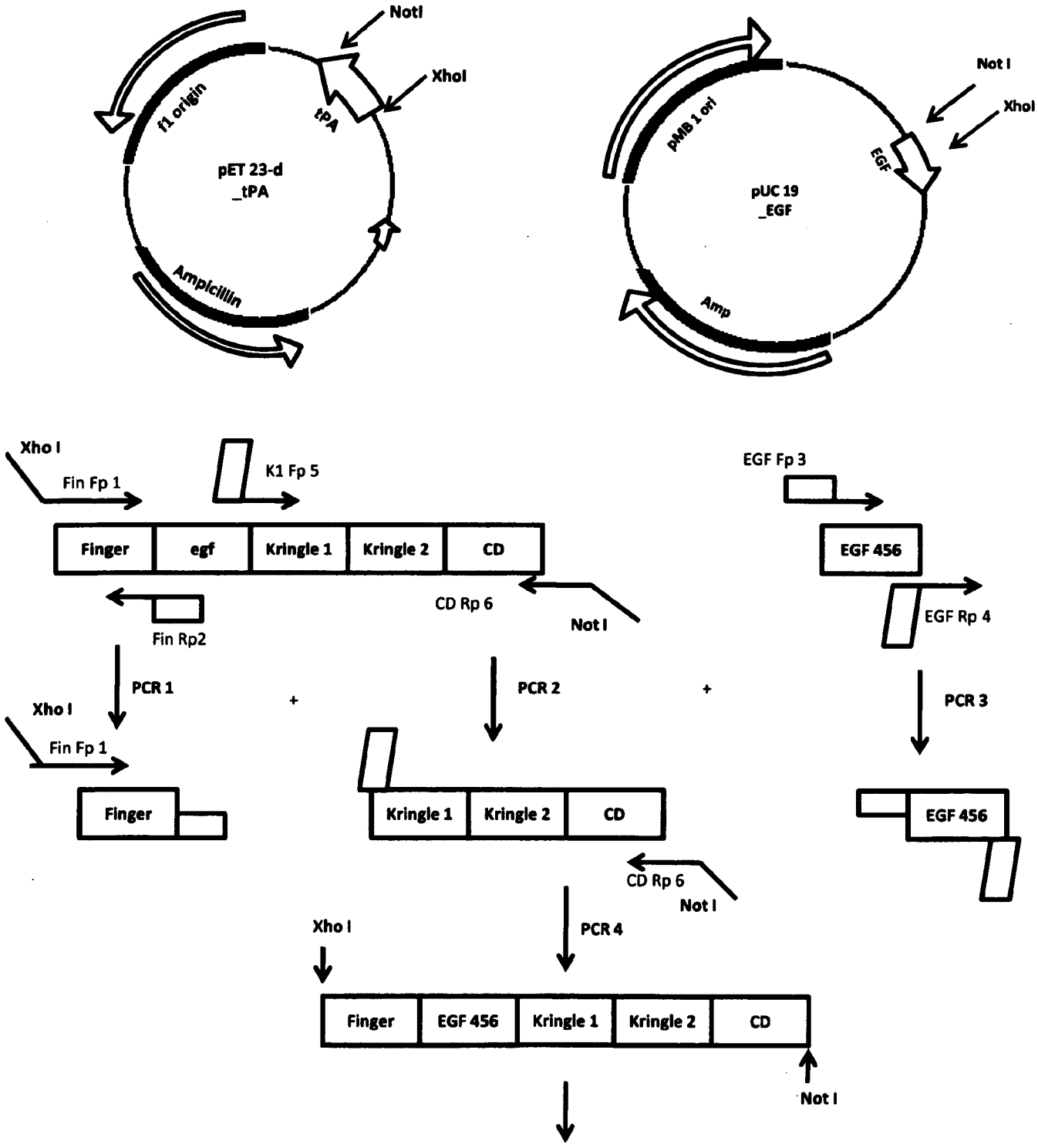
Digested with Xho I and Not I and cloned into pET 23- d, and pPIC 9K for expression

Fig. 2 I. Construction of egf and kringle 1 deleted N_EGF_tPA construct



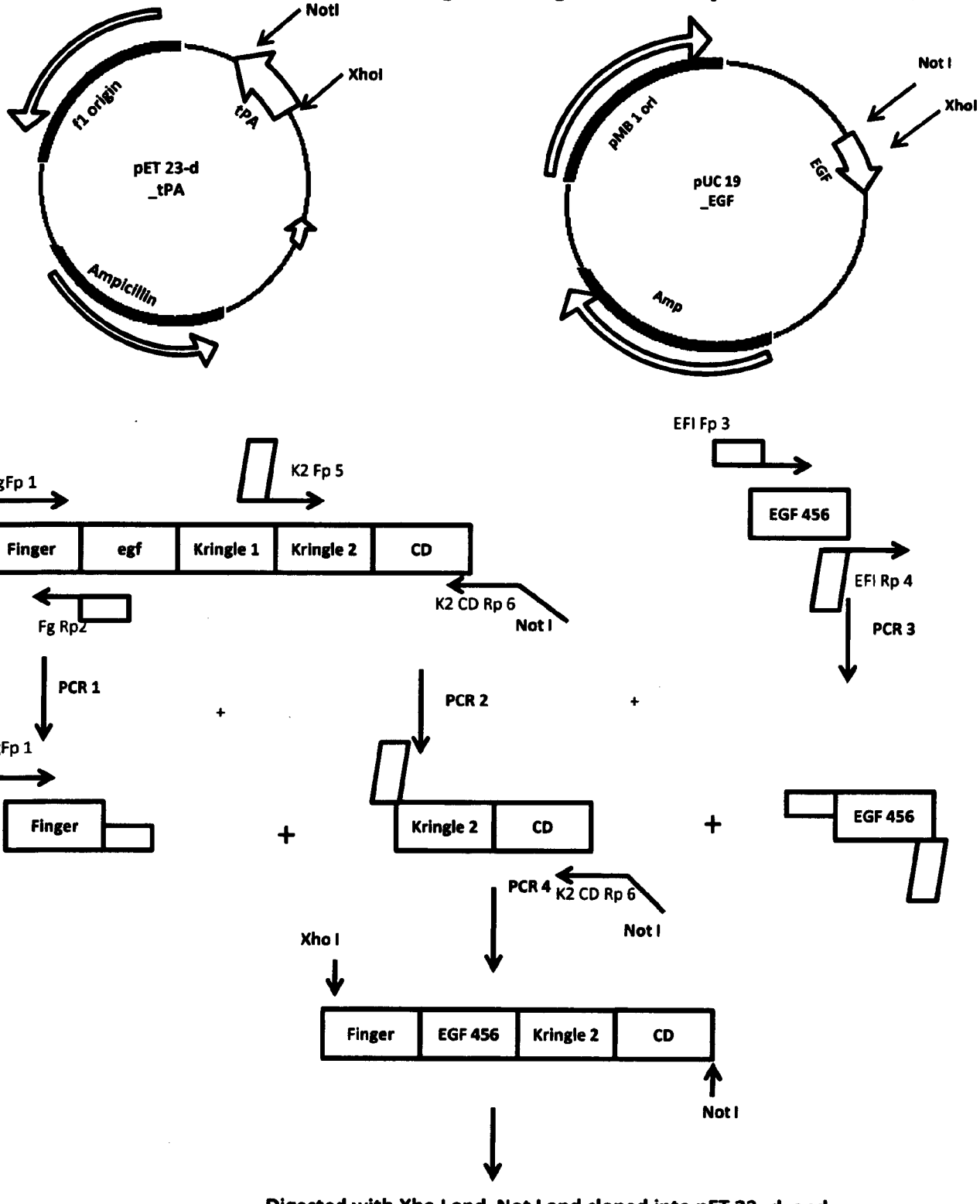
Digested with Xho I and Not I and cloned into pET 23- d, and pPIC 9K for expression

Fig. 2 J. Interdomain EGF _tPA (where egf of tPA is repalced with EGF 456)



Digested with Xho I and Not I and cloned into pET 23- d, and pPIC 9K for expression

Fig. 2 K Interdomain EGF _tPA (where egf and kringle 1 of tPA is replaced with EGF 456)



Digested with Xho I and Not I and cloned into pET 23- d, and pPIC 9K for expression

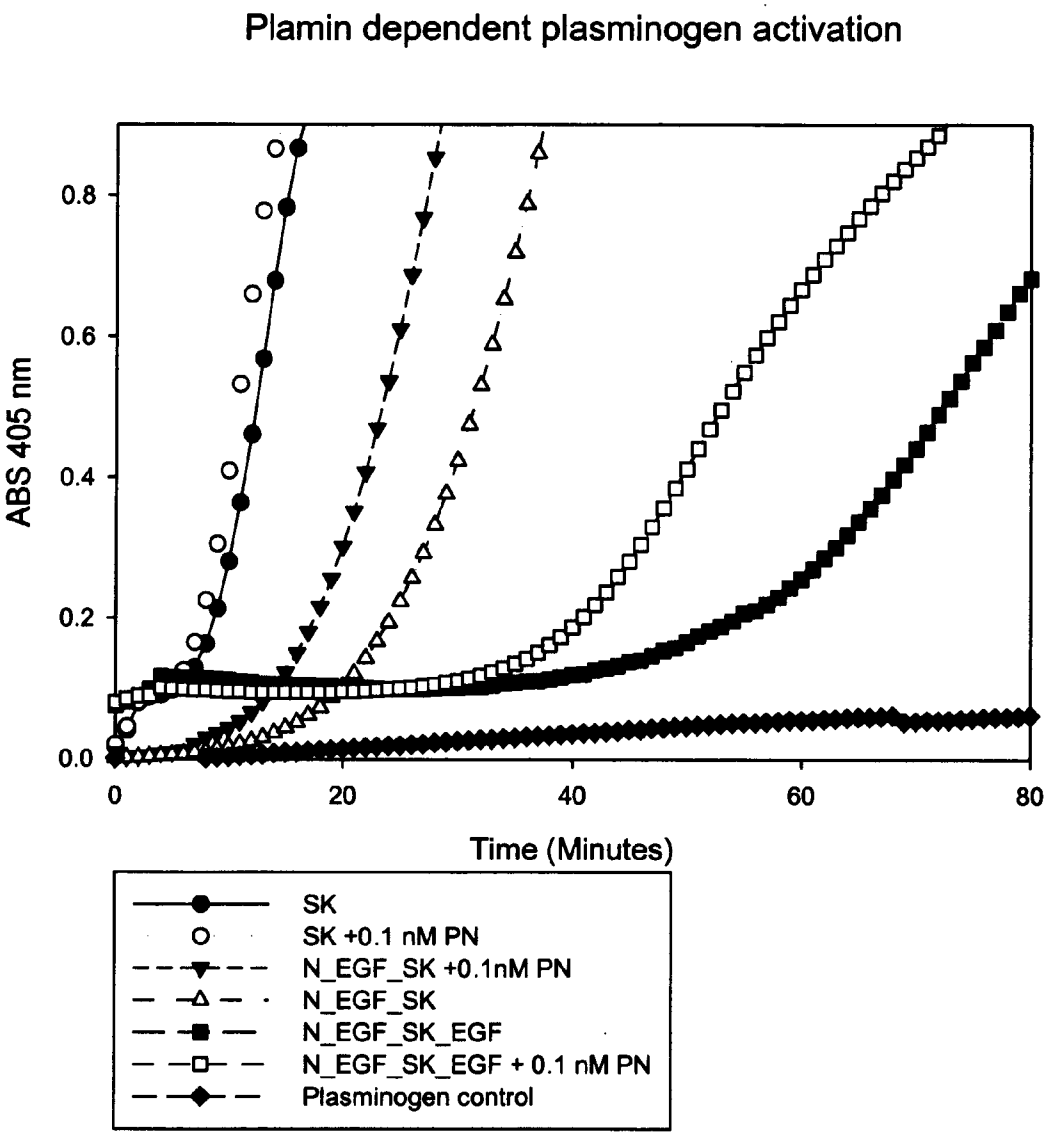

Plamin dependent plasminogen activation
ABS 405 nm
0.0
0.2
0.4
0.6
0.8
0
20
40
60
80
Time (Minutes)
SK
SK +0.1 nM PN
N_EGF_SK +0.1nM PN
N_EGF_SK
N_EGF_SK_EGF
N_EGF_SK_EGF + 0.1 nM PN
Plasminogen control

Fig. 3

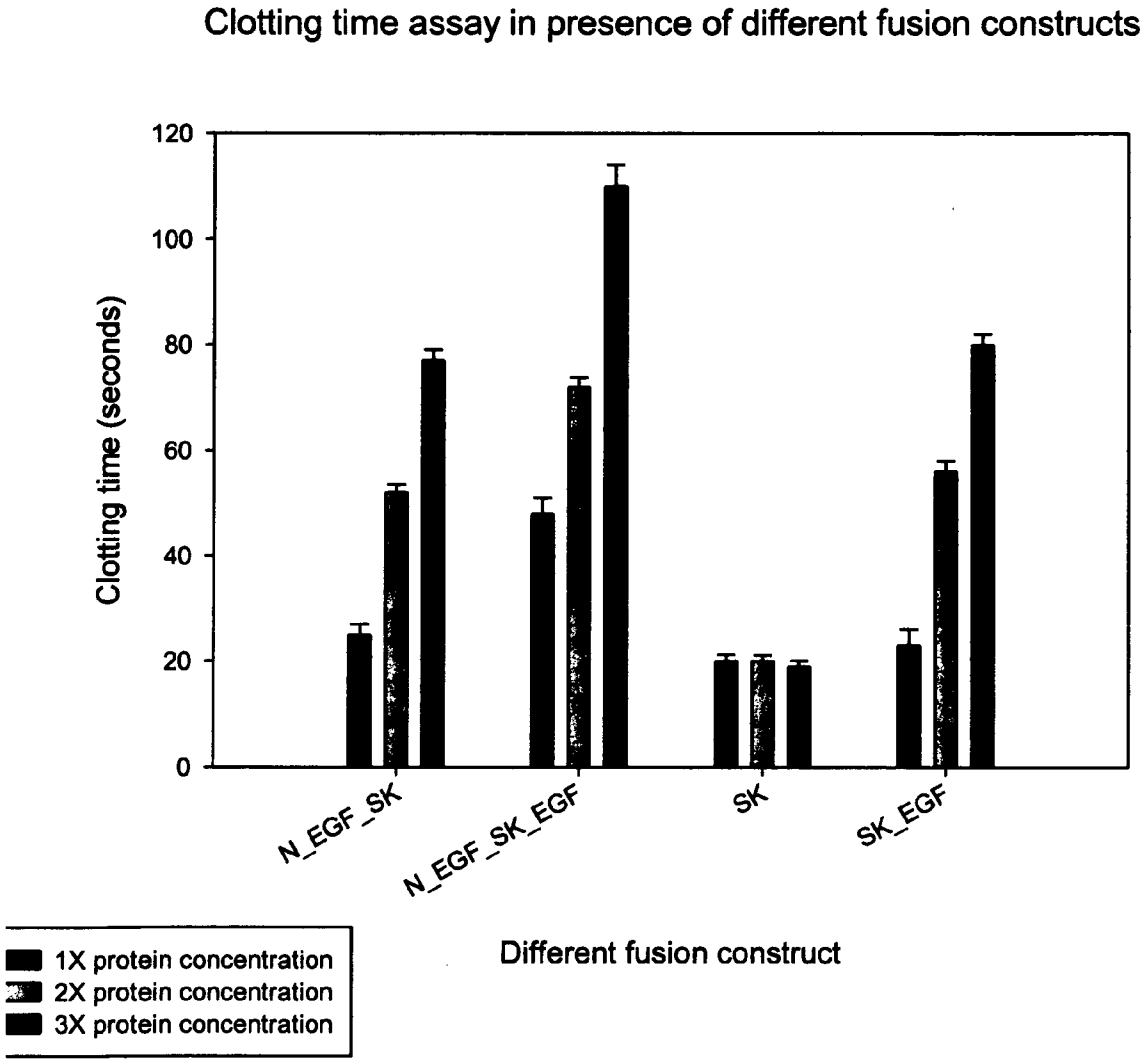

Clotting time assay in presence of different fusion constructs
Clotting time (seconds)
120
100
80
60
40
20
0
N_EGF_SK
N_EGF_SK_EGF
SK
SK_EGF
Different fusion construct
1X protein concentration
2X protein concentration
3X protein concentration

Fig. 4

Protein C activation in presence of different SK-EGF fusion constructs

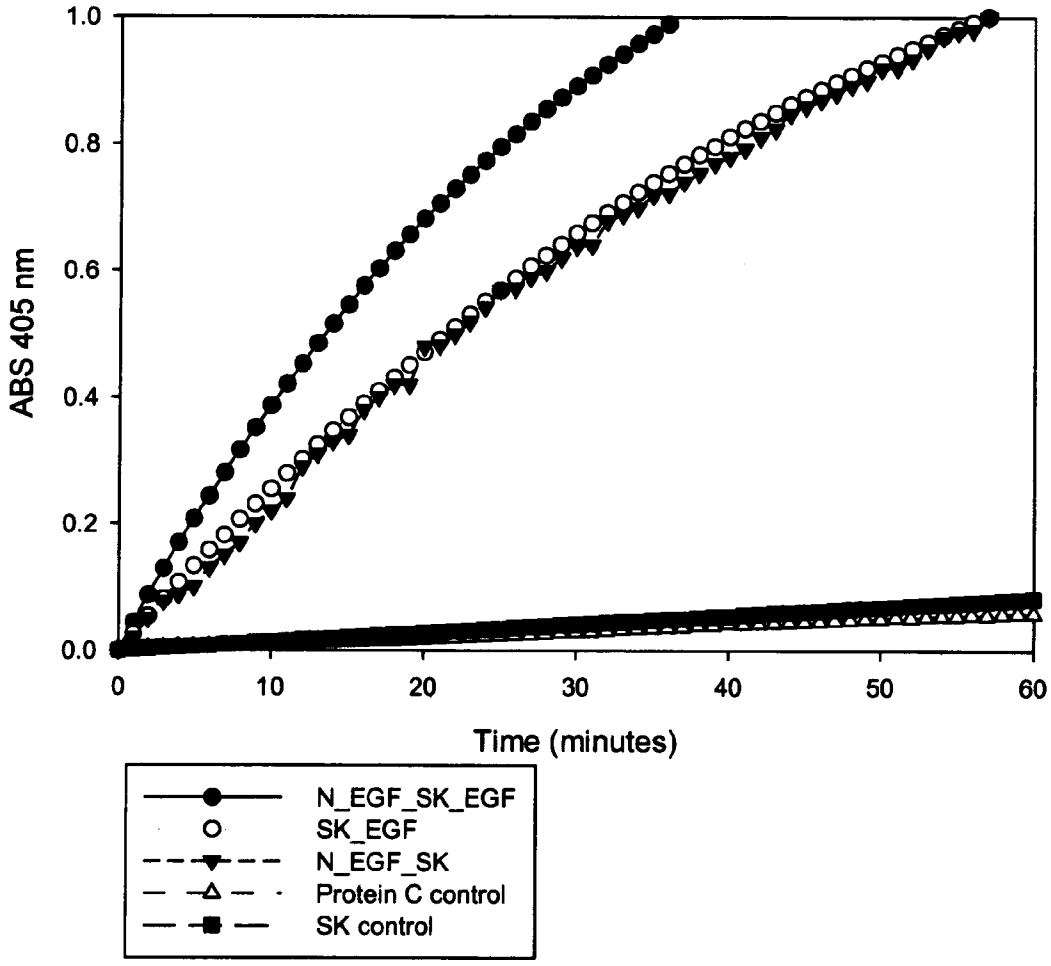


Fig. 5

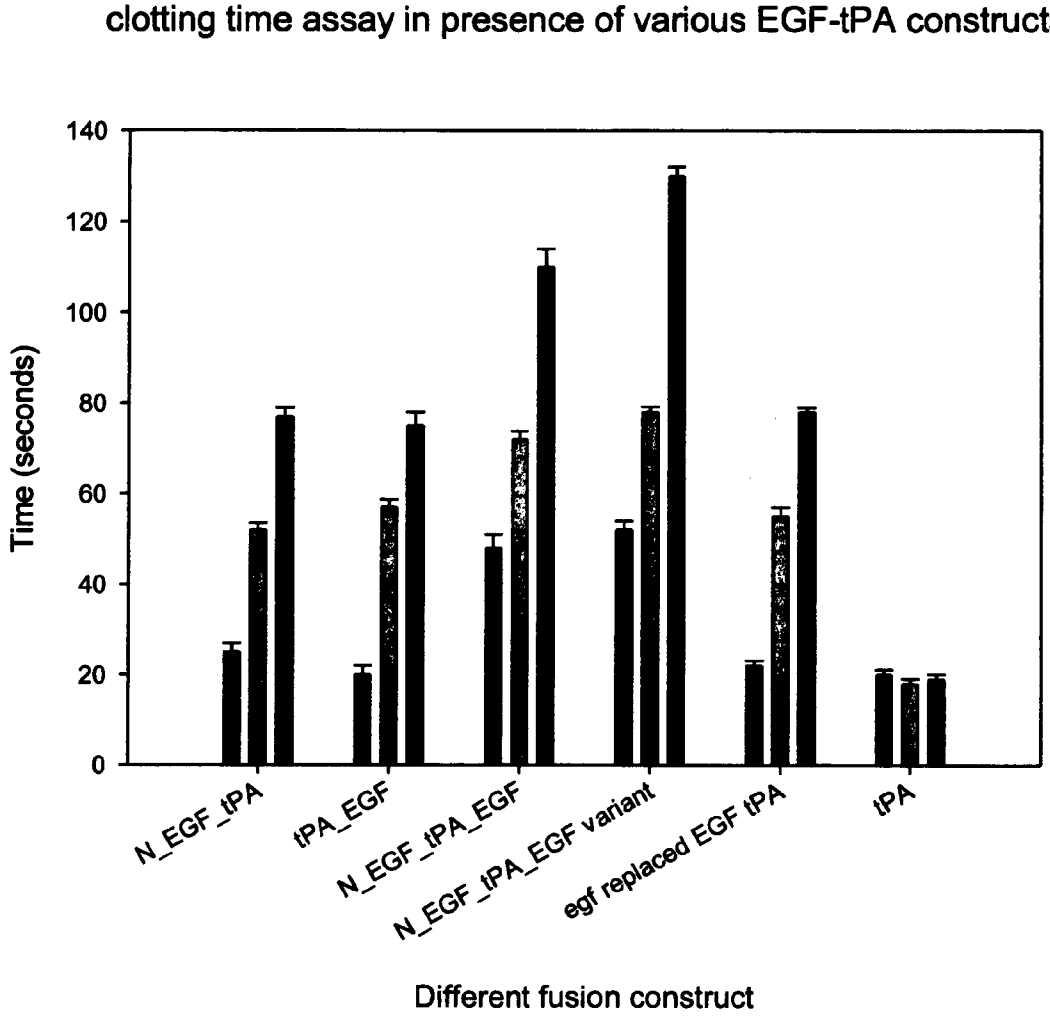

clotting time assay in presence of various EGF-tPA constructs
Time (seconds)
140
120
100
80
60
40
20
0
N_EGF_tPA
tPA_EGF
N_EGF_tPA_EGF
N_EGF_tPA_EGF variant
egf replaced EGF tPA
tPA
Different fusion construct
1X protein concentration
2X protein concentration
3X protein concentration

Fig.6

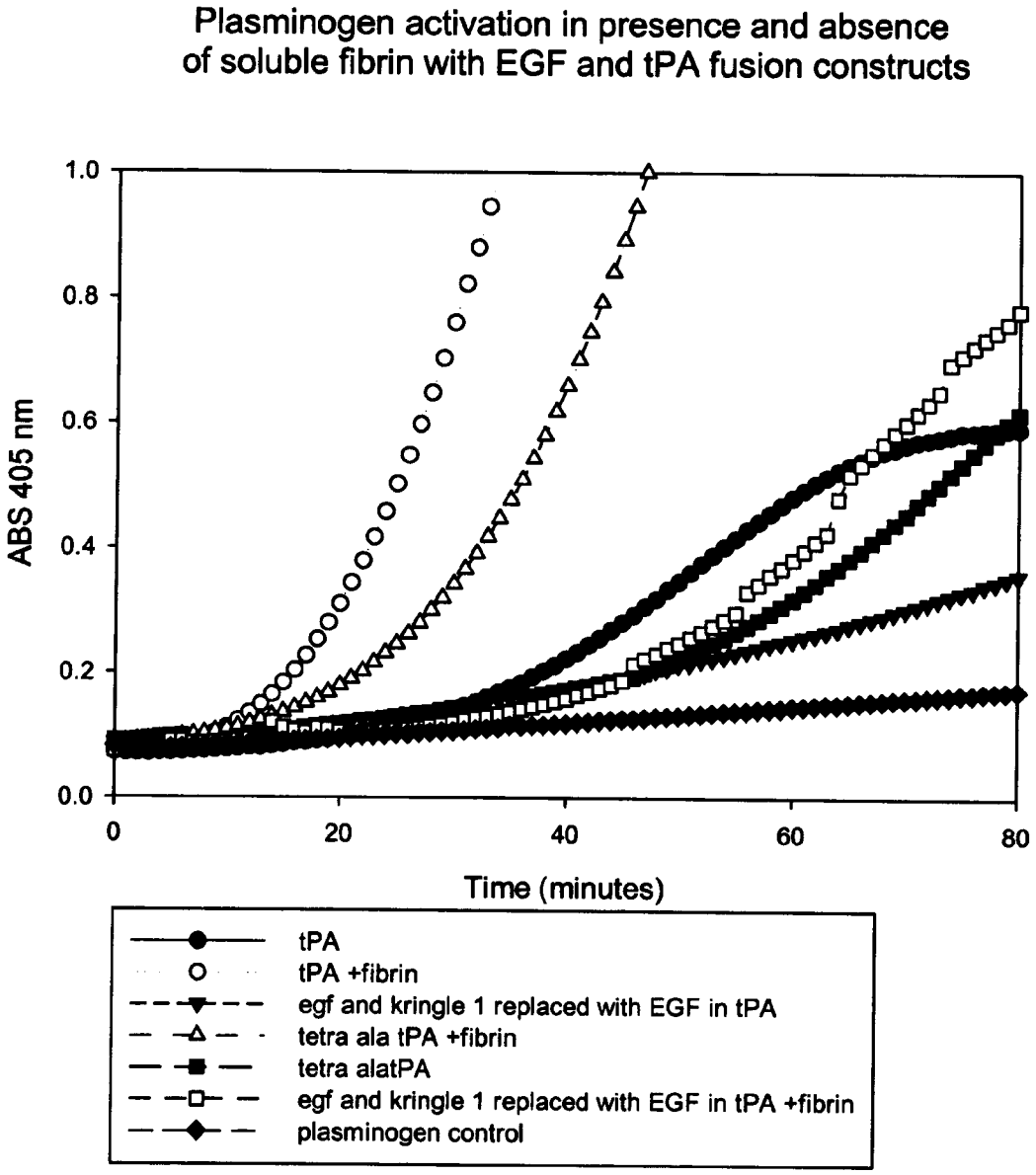

Plasminogen activation in presence and absence of soluble fibrin with EGF and tPA fusion constructs
ABS 405 nm
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
Time (minutes)
tPA
tPA +fibrin
egf and kringle 1 replaced with EGF in tPA
tetra ala tPA +fibrin
tetra alatPA
egf and kringle 1 replaced with EGF in tPA +fibrin
plasminogen control

Fig. 7