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**INTERACTIONS OF VAMPIRE BAT PLASMINOGEN ACTIVATOR, TISSUE-TYPE
PLASMINOGEN ACTIVATOR, AND TNK-VARIANTS
WITH FIBRIN, FIBRINOGEN, AND THE FIBRIN FRAGMENT (DD)E**

By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

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INTERACTIONS OF PG ACTIVATORS WITH THE FIBRIN FRAGMENT (DD)E

DOCTOR OF PHILOSOPHY (2000)
(Department of Medical Sciences;
Hemostasis, Thromboembolism, and
Atherosclerosis Programme)

McMaster University
Hamilton, Ontario

TITLE: **Interactions of Vampire Bat Plasminogen Activator,
Tissue-Type Plasminogen Activator, and TNK-Variants
with Fibrin, Fibrinogen, and the Fibrin Fragment (DD)E**

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NUMBER OF PAGES: **xvii, 248**

ABSTRACT

Fibrinolysis is the process by which blood clots are solubilized. For the purposes of intravascular fibrinolysis, tissue-type plasminogen activator (t-PA) catalyzes the rate-limiting step by converting the zymogen, plasminogen (Pg), to the active enzyme, plasmin. Plasmin then degrades the fibrin meshwork, generating soluble fibrin degradation products.

Because the catalytic efficiency of Pg activation by t-PA is higher in the presence of fibrin than that in the presence of fibrinogen(Fg), t-PA is designated a fibrin-specific Pg activator. Despite this designation, t-PA causes systemic plasminemia and fibrinogenolysis when given to patients. Recently, it was demonstrated that the fibrin-specificity of t-PA is compromised because (DD)E, a major soluble degradation product of crosslinked fibrin, stimulates Pg activation to the same extent as fibrin. This project was initiated to gain a better understanding of how (DD)E compromises the fibrin-specificity of t-PA by characterizing the interactions of t-PA and Pg with fibrin, (DD)E and Fg.

t-PA binds to fibrin through two classes of sites, one high affinity, finger-dependent site, and one low affinity, kringle-dependent site. In contrast, t-PA binds (DD)E and Fg solely through its second kringle domain, because these interactions are blocked by lysine or its analogues. Both t-PA and Pg bind (DD)E with affinities similar to those for fibrin, thereby explaining why (DD)E stimulates t-PA-mediated Pg activation to the same extent as fibrin. t-PA primarily binds to carboxy-terminal lysines on (DD)E because the affinity of t-PA for (DD)E is reduced 160-fold when (DD)E is exposed to carboxypeptidase B (CPB) or the active form of thrombin activatable fibrinolysis inhibitor (TAFIa), an endogenous

CPB-like enzyme. In contrast, the affinity of Pg for (DD)E is reduced only 2- to 4-fold when (DD)E is exposed to CPB or TAFIa, suggesting that Pg binds primarily internal lysine residues on (DD)E. t-PA and Pg have weak affinity for Fg, thereby explaining why Fg is a poor stimulator of Pg activation by t-PA. These studies demonstrate that the fibrin-specificity of t-PA is compromised by its kringle-dependent interactions with (DD)E and, to a lesser extent, Fg.

The limited fibrin-specificity of t-PA has prompted the development of Pg activators with greater selectivity for fibrin. Two such agents are the activator isolated from the saliva of the vampire bat (b-PA), and a bioengineered t-PA variant, TNK-t-PA. b-PA is structurally distinct from t-PA in that b-PA lacks a lysine-binding kringle. TNK-t-PA was designed to have a longer half-life than t-PA, resistance to inhibition by plasminogen activator inhibitor-1, and increased fibrin-specificity over t-PA.

When the fibrin-specificities of t-PA, b-PA, and TNK-t-PA were compared, the hierarchy of fibrin-specificity (b-PA>TNK-t-PA>t-PA) correlated with the ratio of their activity in the presence of fibrin relative to (DD)E. Whereas all activators have similar activities in the presence of fibrin, they are distinguished by their activity in the presence of (DD)E. b-PA, the most fibrin-specific, exhibits minimal stimulation by (DD)E, whereas t-PA, the least fibrin-specific, exhibits the greatest stimulation by (DD)E. Stimulation by (DD)E, in turn, reflects the affinity of the activator for (DD)E. b-PA does not bind (DD)E, presumably because it lacks a lysine-binding kringle. t-PA binds (DD)E with high affinity, whereas TNK-t-PA has ~9-fold lower affinity for (DD)E than t-PA.

These studies highlight the importance of (DD)E in compromising the fibrin-specificity of t-PA. Furthermore, they suggest that the fibrin-specificity of t-PA or t-PA variants could be improved by abolishing their lysine-binding properties.

ACKNOWLEDGEMENTS

No study is completed independently. Many people have helped me throughout my thesis work and I thank some of them here.

Firstly, I thank my supervisor Dr. Jeffrey Weitz. He has given me immense support with every aspect of my graduate work. His insight into both clinical and basic science is exceptional and I am fortunate to have had his guidance. I also thank Dr. James Fredenburgh for helping to shape my thesis. I appreciate his help in staying current with literature and have enjoyed our discussions. He is an excellent mentor. My thesis work has also benefited from the direction of Dr. Jack Hirsh and Dr. Laszlo Bajzar who have served as members of my supervisory committee.

I am indebted to the members of Dr. Weitz' laboratory. Beverly Leslie completed experiments which have given me further insight into my graduate work and some of these assays appear in this thesis. I have enjoyed working with Janice Rischke. She helped me with my first fibrin fragment isolation and has always provided me with (generous amounts of) fibrin(ogen) degradation products. Alan Stafford aided me in ways too numerous to do justice here. Alan instructed me in techniques and kept our computers running and up to date (despite considerable challenges). I also appreciate the interaction that I have had with other students. Debra Becker and I had many helpful discussions about course material and Patricia Liaw expanded my knowledge about thrombin and its inhibitors (the 'other side' of Haemostasis). I hope that all of the current and future students in this laboratory find the experience as enriching as I have. I thank the entire 'Weitz team' for making my time at the HCHRC enjoyable.

Finally, I thank my family. Jay (Dr. Stewart), Jen (Dr. Lailey), and Heidi have offered me love and inspiration. Norma (Dr. MacIntyre) has been my strength. More than anything, she is my most cherished friend. To Elaine and Jim, I offer my most heart-felt gratitude. Your love and support reaches every aspect of my life. I will never stop appreciating how fortunate I am to have you as parents.

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LIST OF ABBREVIATIONS

A	absorbance
α_2 -AP	α_2 -antiplasmin
AMI	acute myocardial infarction
b-PA	vampire bat plasminogen activator
CNBr	cyanogen bromide
CPB	carboxypeptidase B
CPI	carboxypeptidase inhibitor from potato tuber
DD	D-dimer
(DD)E	complex of D-dimer noncovalently associated with fragment E
dEGR-CMK	dansyl glutamyl-glycyl-arginine chloromethyl ketone
DSPA _{ri}	<i>Desmodus rotundus</i> salivary plasminogen activator
ϵ	extinction coefficient
EACA	ϵ -amino caproic acid
EC	endothelial cell
FDPs	fibrin degradation products
Fg	fibrinogen
<i>g</i>	gravitational force
Glu-Pg	native plasminogen with N-terminal Glutamic acid
HBS	HEPES-buffered saline (50 mM HEPES, 150 mM NaCl, pH 7.4)
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethansulfonic acid)

K2	second kringle domain of t-PA
LBS	lysine-binding site
Lys-Pg	plasmin-modified plasminogen with N-terminal Lysine
n-PA	nanoteplase
P	protease domain
PAGE	polyacrylamide gel electrophoresis
PAI-1	plasminogen activator inhibitor 1
Pg	plasminogen
PPACK	D-phenyl-prolyl-arginine chloromethyl ketone
r-PA	reteplase
SK	streptokinase
t-PA	tissue-type plasminogen activator
TAFI	thrombin activatable fibrinolysis inhibitor
TBS	Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl, pH 7.4)
TNK-t-PA	mutations in t-PA are abbreviated as follows:
	T, T103N (Threonine 103 replaced with Asparagine)
	N, N117Q (Asparagine 117 replaced with Glutamine)
	K, KHRR296-299AAAA (Lysine 296, Histidine 297, Arginine 298,
	and Arginine 299 each replaced with Alanine)
Tris	tris-(hydroxymethyl)-aminomethane
u-PA	urokinase-type plasminogen activator

VFKCK

D-valyl-phenyl-lysine chloromethyl ketone

ω -AA

ω -aminocarboxylic acid

CHAPTER 1 - INTRODUCTION

The hemostatic system serves to prevent catastrophic loss of blood while preserving vascular blood flow. Vascular integrity and blood fluidity are maintained through a dynamic balance between coagulation and fibrinolysis. The coagulation and fibrinolytic pathways involve proteolytic activation of circulating or cell surface zymogens, and both mechanisms are controlled by positive and negative feedback loops. Thrombosis occurs when the fibrinolytic capacity of the vasculature is overwhelmed, while excessive fibrinolysis causes hemorrhage.

Fibrinolysis is the process by which the fibrin meshwork of blood clots is solubilized. The rate limiting step in the fibrinolysis is the conversion of the zymogen, plasminogen (Pg), into the active enzyme, plasmin (for review, see Collen, 1999). For the purposes of intravascular fibrinolysis, Pg conversion to plasmin is primarily mediated by tissue-type plasminogen activator (t-PA) (Lijnen and Collen, 1995a; Collen, 1999). Plasmin then degrades the fibrin meshwork within a thrombus, resulting in the generation of soluble fibrin degradation products (FDPs).

t-PA is an exquisitely specific protease; its only known substrate *in vivo* is Pg (Madison et al., 1995). In contrast, plasmin is a relatively promiscuous enzyme that, in addition to degrading fibrin, also degrades plasma clotting factors, such as fibrinogen (Fg), factor V and factor VIII (Collen and Lijnen, 1995). Thus, to limit the systemic effects of Pg activation, the fibrinolytic system must ensure that plasmin activity is localized to the fibrin

surface. To accomplish this, t-PA is released from vascular endothelial cells at sites of fibrin deposition and fibrin acts as a template onto which both t-PA and Pg bind. As a functional consequence of this interaction, the catalytic efficiency of Pg activation by t-PA is 2 to 3 orders in magnitude higher in the presence of fibrin than in the absence of fibrin (Hoylaerts et al., 1982). In contrast to the potent stimulatory effect of fibrin, Fg, the precursor of fibrin, produces only modest enhancement in the catalytic efficiency of Pg activation by t-PA (Hoylaerts et al., 1982). Because it preferentially activates Pg in the presence of fibrin, rather than Fg, t-PA is considered a fibrin-specific Pg activator. Furthermore, plasmin and t-PA are protected from inhibition by α_2 -antiplasmin (α_2 -AP) and plasminogen activator inhibitor 1 (PAI-1), respectively (Collen and Lijnen, 1991; Krishnamurti and Barbara, 1992). In contrast, when these enzymes are free in the circulation, they are rapidly inactivated by their respective inhibitors. Thus, Pg activation and active plasmin are localized to the fibrin surface.

Because acute myocardial infarction usually results from coronary artery thrombosis superimposed on atherosclerotic plaque rupture, pharmacologic thrombus dissolution has become a mainstay of treatment (for review, see Pislaru and Van de Werf, 1999). Given its properties, t-PA is an excellent pharmacologic candidate for ensuring rapid clot lysis. Although t-PA produces rapid reperfusion of the occluded coronary artery (Forrester, 1995), it has two major limitations. First, despite its apparent fibrin-specificity, t-PA causes systemic activation of Pg (Stangl et al., 1998). The resulting plasmin then degrades circulating Fg and other clotting factors. This phenomenon, known as the systemic lytic state, may contribute to bleeding, the most serious complication of thrombolytic therapy

(Weitz, 1995). The second limitation of t-PA is its relatively short plasma half-life (3-5 min) which necessitates administration by infusion over 1.5 hours (COBALT Investigators, 1997). This complicates therapy and makes out-of-hospital administration difficult. In an attempt to improve on these limitations, variants of t-PA have been developed that have greater fibrin-specificities and a longer half-lives than t-PA (White and Van de Werf, 1998).

The following sections describe the current knowledge of physiologic fibrinolysis, describes the components of this system, and introduces the new t-PA variants used for pharmacologic fibrinolysis.

1.1 Physiologic Fibrinolysis

The three proteins critical for the initiation of fibrinolysis, namely Pg, t-PA, and fibrin, are described separately. Regulation of this system is detailed as it occurs at several levels, including secretion and clearance of t-PA, fibrin-mediated stimulation of Pg activation, and proteolytic inactivation of t-PA and plasmin.

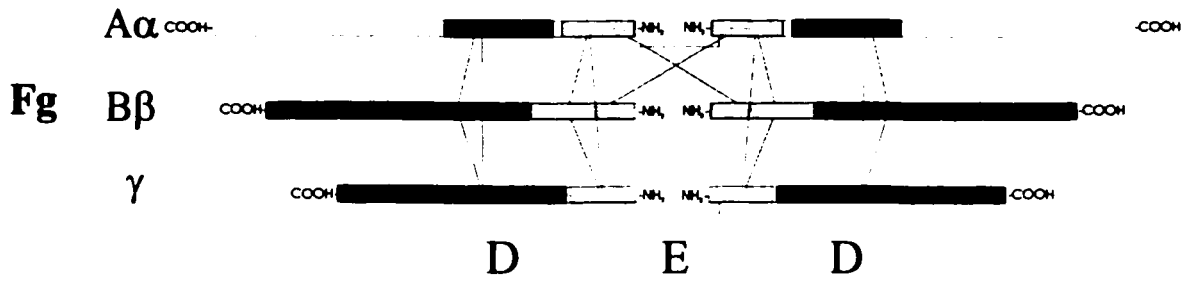
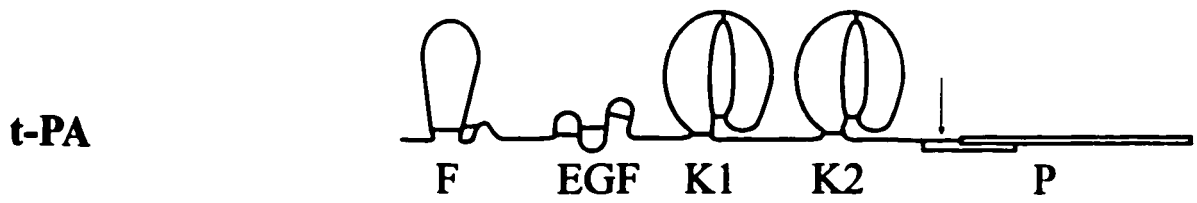
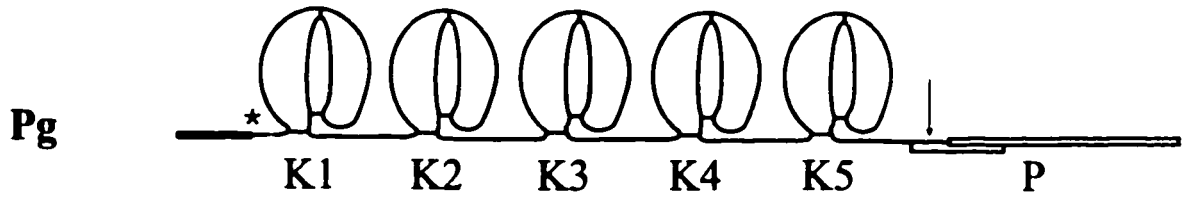
1.1.1 Plasminogen

Pg is a single-chain glycoprotein of 92 kDa that has a plasma concentration of 1.5 - 2 μ M. Its 791 amino acids are structured by 24 disulfide bonds into 7 distinct domains (Fig. 1.1) (Ponting et al., 1992). Residues 1-77, which comprise a "preactivation" peptide, are followed by 5 homologous kringle domains, each consisting of about 80 amino acids. Finally, residues 562 - 791 comprise the proteinase domain.

1.1.1i *Pg Glycoforms*

Pg is secreted as a mixture of two glycoforms (Castellino and Powell, 1981). Type I Pg contains an O-linked carbohydrate moiety at Thr 345 and an N-linked moiety at Asn

Figure 1.1 Secondary structures of Pg, t-PA, and Fg. Pg consists of five kringle domains (K1 to K5) and a protease domain (P). The position of activation cleavage of Pg to plasmin, mediated by Pg activators, is indicated by the arrow, and the position of plasmin-mediated removal of the 'preactivation peptide' is indicated by the asterisk. t-PA consists of five domains, including a finger domain (F), an epidermal growth factor domain (EGF), two kringle domains (K1 and K2, respectively) and a protease domain (P). Plasmin-mediated formation of two-chain t-PA is indicated by the arrow. Fg consists of two identical pairs of $A\alpha$, $B\beta$, and γ chains. The amino-termini of each chain is oriented towards the centre of the molecule. Inter-chain disulfide bonds are indicated by the thin lines. The portions of the chains which constitute the D domain are indicated in dark grey, and those that make up the E domain are indicated in light grey.



288, while type II Pg is glycosylated only at Thr 345. The glycosylation pattern may influence a variety of Pg functions. For example, more type II than type I Pg is located in the extravascular compartment, and type II Pg is catabolised 5-times faster than type I Pg (Hatton et al., 1994; Rudd et al., 1995). Furthermore, the efficiency of type II Pg activation by t-PA in the presence of Fg fragments is ~1.5-fold greater than that for type I Pg (Davidson and Castellino, 1993). This may reflect the fact that type II Pg has higher affinity for lysine and fibrin than type I Pg (Hayes and Castellino, 1979; Rudd et al., 1995).

1.1.1ii *Conversion of Glu-Pg to Lys-Pg*

Native Pg has an amino-terminal glutamic acid and is, therefore, referred to as Glu-Pg. Plasmin-mediated cleavage of Glu-Pg yields Lys-Pg, a truncated Pg molecule with amino-terminal Lys78 (Fig. 1.1). Proteolytic removal of the “preactivation peptide” (residues 1-77) is accompanied by a dramatic conformational change. Whereas Glu-Pg has a roughly circular shape with an average diameter 90 Å, Lys-Pg adopts a highly elongated shape, resembling a broad, shallow letter “u”, with average dimensions of 140 Å × 70 Å (Weisel et al., 1994). Thus, Glu-Pg exists in a closed (α) conformation whereas Lys-Pg is in an open, flexible (β) conformation.

Similar structural changes occur when Glu-Pg binds lysine or lysine analogues, such as ϵ -amino caproic acid (EACA) (Mangel et al., 1994). The open conformation of Pg can be induced by introducing mutations within kringles 1, 2 or 5 of Glu-Pg, that reduce their lysine-binding properties (McCance and Castellino, 1995). Furthermore, isolated kringles 1, 2 or 5 bind the “preactivation peptide” (An et al., 1998b). Taken together, these data suggest that intermolecular interactions between the “preactivation peptide” and one or more

kringles containing a lysine-binding site (LBS) maintain the closed conformation of Glu-Pg. Through site-directed mutagenesis, Lys50 and Lys62 have been identified as the amino acids within the preactivation peptide which may bind to LBS-containing kringles (Cockell et al., 1998; An et al., 1998a). An additional intramolecular interaction between kringles 3 and 4 has been described, which may contribute to the tightly closed conformation of Glu-Pg (Marshall et al., 1994).

The conformational change that occurs when Glu-Pg is converted to Lys-Pg gives rise to a change in functional properties. Lys-Pg binds fibrin with ~ 30-fold higher affinity than Glu-Pg (Horrevoets et al., 1997b). Furthermore, the efficiency of activation of Lys-Pg by t-PA is ~ 20-fold higher than that of Glu-Pg both in the absence or presence of fibrin (Hoylearts et al., 1982; Horrevoets et al., 1997a). Lys-Pg is a significant intermediate during clot lysis *in vitro* and its concentrations peak immediately prior to clot dissolution (Fredenburgh et al., 1992; Suenson and Thorsen, 1988). These data suggest that formation of Lys-Pg represents an important positive feedback mechanism in the fibrinolytic process.

1.1.1iii *Kringle domains of Pg*

The kringle domains of Pg mediate its interaction with fibrin. The structure of each kringle domain within Pg (like kringles in other proteins) is dictated by three disulfide bonds, leading to a predicted secondary structure that resembles the Danish pastry of the same name (Tulinski, 1991). Kringle 4 was the first kringle to be crystallized, giving valuable information about the structure of kringles and their interaction with lysine or lysine analogues (Mulichak et al., 1989). Each kringle within Pg binds lysine or ω -aminocarboxylic acids (ω -AA) to various degrees. The preferred ligand and affinity depends on the relative

positioning of the negatively charged side chains of Aspartic acids (Asp55 and 57 in K4) and positively charged side chains of arginines or lysines (Lys35 and Arg71 in K4) (Wu et al., 1991; Mulichak et al., 1991). Between these groups of charged residues is a hydrophobic groove, flanked by tryptophan and phenylalanine residues.

Using nuclear magnetic resonance and microcalorimetry, it was determined that kringles 1, 4, and 5 have the highest affinities for EACA ($K_d \approx 17, 30, \text{ and } 100 \mu\text{M}$, respectively) (Motta et al., 1987; Petros et al., 1989; Thewes et al., 1990), while kringles 2 and 3 bind EACA weakly ($K_d \approx 5 \text{ mM}$ for each) (Lerch et al., 1980; Marti et al., 1994). These data suggest that Pg interaction with fibrin is primarily mediated by kringles 1, 4, and 5. These kringles differ in their preference for internal *versus* carboxy-terminal lysine residues. K1 and K4 have higher affinities for ω -AAs containing carboxylate moieties, whereas K5 does not display this discrimination (Motta et al., 1987; Petros et al., 1989; Thewes et al., 1990). The presence of a carboxylate group distinguishes carboxy-terminal from internal lysine residues. These observations suggest that the lysine-binding properties of K1 or K4 may be of particular importance because carboxy-terminal lysine residues are exposed as fibrin undergoes plasmin-mediated degradation (discussed in section 1.1.4i).

1.1.1iv *Proteolytic activation of Pg*

The crystal structure of the proenzyme form of the catalytic domain of Pg has recently been solved (Peisach et al., 1999). Three mechanisms are in place to ensure inactivity of the zymogen. First, like most serine proteases, a salt bridge is formed between the aspartic acid residue flanking the active site serine (Asp740 in Pg) and a histidine residue (His586 in Pg). This interaction prevents the formation of the oxyanion hole that is necessary for catalytic

activity. Second, unlike most serine proteases of the chymotrypsin family, the catalytic residues in Pg (His603, Asp646, and Ser741) are not aligned. This precludes formation of the hydrogen bonding network necessary for increasing the nucleophilicity of Ser741. Third, unique to Pg is a 'foot in mouth' mechanism whereby Trp761 exists in the S1 specificity pocket, preventing substrate binding.

Pg is converted to plasmin by a single cleavage of the Arg560-Val561 peptide bond (Fig. 1.1). Pg can be activated by several Pg activators, including naturally occurring activators, such as t-PA and urokinase-type Pg activator (u-PA), or bacterially-derived activators, such as streptokinase (SK) and staphylokinase (for review, see Mayer, 1990). Activation cleavage capacitates the active site of plasmin by (1) forming a salt bridge between Asp740 and the newly formed amino-terminal Val561 which creates a functional oxyanion hole, (2) aligning the catalytic triad, and (3) removing Trp761 from the S1 pocket (Peisach et al., 1999). Like other serine proteases, plasmin cleaves carboxy-terminal to the basic amino acids lysine and arginine.

Structurally, plasmin is a two-chain molecule held together by two disulfide bridges (Cys558-Cys566 and Cys548-Cys666) (for review of plasmin structure, see Bachmann, 1994). The heavy chain, or A chain, is comprised of the preactivation peptide (in the case of Glu-plasmin) and the five kringle domains, whereas the catalytic domain constitutes the light chain, or B chain.

1.1.2 Tissue-type plasminogen activator

t-PA is a 65 kDa member of the trypsin family of serine proteases. The mature protein is organized by 16 disulfide bridges into 5 discrete structural domains that are

homologous to other plasma proteins (Fig. 1.1) (for review, see Madison, 1993). Residues 4-50 comprise a fibronectin finger-like domain, residues 50-87 constitute an epidermal growth factor (EGF) domain, two kringle domains are comprised by residues 87-176 and 176-262 (K1 and K2, respectively), and residues 276-527 constitute the catalytic domain. The amino-terminal portion of t-PA, consisting of the finger, EGF, K1 and K2 domains, is referred to as the A-chain while the protease domain is called the B-chain.

1.1.2i *t-PA Glycoforms*

t-PA is secreted as a glycoprotein containing complex oligosaccharides attached to Asn184 and 448 in the K2 and protease domain, respectively, a high-mannose carbohydrate attached to Asn117 in the K1 domain, and a single fucose residue attached to Thr61 within the EGF domain (Rijken, 1995). Asn117 is almost invariably occupied by oligomannose structures and mediates clearance of t-PA through liver endothelial cells (discussed in section 1.1.5iii). The O-linked fucose residue may also mediate clearance, although evidence for this mechanism is limited (Baenziger, 1994). The role of N-linked glycosylation at positions 184 and 448 is unclear. Removal or mutation of these sites causes little change in clearance of t-PA (Otter et al., 1992a). However, one or both may influence the functional properties of t-PA. Asn184 is variably glycosylated and species that contain an oligosaccharide at Asn184 are referred to as type I t-PA, whereas those that are not glycosylated at Asn184 are known as type II t-PA (Ranby et al., 1984). The two glycoforms differ in their affinities for lysine and are commonly separated using lysine-Sepharose affinity chromatography (Ranby et al., 1984; Parekh et al., 1989b). Although both t-PA glycoforms activate Pg to the same extent in the absence of a stimulator, type II t-PA is ~ 2-

fold more efficient at activating Pg in the presence of Fg fragments and ~ 1.3-fold more effective at promoting clot lysis than type I t-PA (Parekh et al., 1989a; Wittwer et al., 1989; Rudd et al., 1995). When N-linked glycosylation is blocked with tunicamycin, the resultant t-PA has ~ 2-fold greater fibrinolytic activity than type II t-PA (Wittwer et al., 1989). Together, these data suggest that one or more of the N-linked oligosaccharides within t-PA influence its ability to activate Pg.

1.1.2ii *Structure - function relationship of t-PA*

Domain deletion studies (Verheijen et al., 1986; van Zonneveld et al., 1986a; Lijnen et al., 1990a; Bakker et al., 1995a; Horrevoets et al., 1994), as well as the positioning of the exons coding the various domains (Banyai et al., 1983; Rogers, 1985; Pathy, 1985) suggest that each domain in t-PA is functionally autonomous. These studies also suggest that, like many enzymes in the hemostatic system, t-PA evolved from the trypsin gene through exon shuffling. The A-chain of t-PA mediates its binding to fibrin and plasma clearance, whereas the B-chain is responsible for its proteolytic activity. Because of the apparent functional autonomy of the domains of t-PA, many studies have characterized domain deletion and insertion variants of t-PA in terms of binding to fibrin, activation of Pg, and clearance (reviewed in Madison, 1993).

1.1.2iii *Fibrin binding of t-PA*

In a series of domain deletion mutants of t-PA, the largest reduction in fibrin binding was accomplished by removal of the finger domain (Verheijen, 1986; van Zonneveld, 1986b; de Munk, 1989; de Vries, 1989; Lijnen et al., 1990a). Indeed, a mutant activator consisting only of the finger and catalytic domains of t-PA binds fibrin with an affinity similar to that

of native t-PA (de Munk et al., 1989). In contrast, a mutant comprised only of the catalytic domain of t-PA does not bind fibrin (van Zonneveld et al., 1986a). Taken together, these studies suggest that binding of t-PA to fibrin is primarily mediated by its finger domain. However, deletion of the finger domain does not completely eliminate fibrin binding (de Vries, 1989), suggesting that t-PA binds fibrin through at least one additional domain.

Several groups have demonstrated that t-PA variants lacking a finger domain do not bind fibrin in the presence of lysine or lysine analogues (Nesheim et al., 1990; Kohnert et al., 1992; Bakker et al., 1995b). These studies indicate that finger-independent binding of t-PA to fibrin is mediated by a lysine-binding domain. Only the K2 domain in t-PA demonstrates significant lysine-binding properties (Lijnen et al., 1990a; Bakker et al., 1993; Byeon et al., 1995). Although two groups have suggested that K1 also exhibits lysine-binding properties and mediates stimulation of t-PA by fibrin(ogen) (Gething et al., 1988; Stern and Weidle, 1990), others have failed to confirm these results. In addition, t-PA mutants that lack a finger and K2 domain do not bind fibrin (van Zonneveld, 1986a). Thus, the most likely candidate for the second fibrin-binding domain in t-PA is K2.

Measurements of t-PA binding to fibrin are consistent with to a two-site model where t-PA binds through one high affinity site ($K_d = 0.1 - 0.3 \mu\text{M}$), mediated by its finger domain, and a low affinity site ($K_d = 0.6 - 1.5 \mu\text{M}$), mediated by its K2 domain (Husain et al., 1989; Nesheim et al., 1990). High affinity finger-dependent binding localizes t-PA to fibrin. The role of low affinity kringle-dependent binding of t-PA to fibrin is less clear.

1.1.2iv *Function of the K2 domain of t-PA*

Despite the fact that the second kringle of t-PA binds fibrin with low affinity,

elimination of this domain, or removal of its lysine-binding properties, results in ~3-fold reduction in Pg activation in the presence of fibrin and ~5-fold increase in clot lysis times (Bakker et al., 1995b; Urano et al., 1989). Although these data highlight the importance of the K2 domain in fibrinolysis, the mechanism by which K2 promotes Pg activation is debated. One possibility is that binding of the K2 domain to fibrin orients t-PA for optimal activation of Pg. Bakker et al. (1995b) have demonstrated that a t-PA variant consisting only of the K2 and protease domains (K2P) competitively inhibits the binding of a full-length t-PA mutant in which the lysine-binding properties of K2 are removed. These data suggest that the finger and K2 binding sites on fibrin are in close proximity. Furthermore, rearrangement of the position of the K2 domain within t-PA reduces its ability to stimulate Pg activation, even though the affinity of the activator for fibrin may increase (Bakker et al., 1995a). These data indicate that the relative positioning of the finger and K2 domains within t-PA is important for optimal stimulation. Together, these studies suggest that the K2 domain of t-PA is necessary for proper orientation of the activator on fibrin (Lijnen et al., 1990a; Horrevoets et al., 1994; Bakker et al., 1995b).

The K2 domain of t-PA may also mediate a positive feedback mechanism during fibrinolysis. Several investigators have shown that the rate of t-PA-mediated Pg activation increases as fibrinolysis progresses (Suenson et al., 1984; Norrman et al., 1985; Thorsen, 1992). When fibrin is degraded by plasmin (discussed in section 1.1.4i), carboxy-terminal lysine residues are exposed on the fibrin surface. The capacity of fibrin to bind t-PA increases 2- to 3-fold when fibrin is partially degraded by plasmin (Higgins and Vehar, 1987; de Vries et al., 1988). The increase in binding to partially degraded fibrin requires the K2

domain and is inhibited by lysine analogues (Higgins and Vehar, 1987; de Vries et al., 1988). Thus, by binding to newly exposed carboxy-terminal lysine residues on fibrin, the K2 domain of t-PA may upregulate Pg activation during fibrinolysis. This concept is further supported by two observations. First, using a noncatalytic mutant of Pg, Horrevoets et al. (1997a) demonstrated that the efficiency of fibrin-stimulated Pg activation by a t-PA variant lacking a K2 domain is similar to that of native t-PA. Second, several groups have demonstrated that the efficiency of fibrin-stimulated Pg activation by K2 deletion mutants is lower than that of native t-PA in the presence of plasmin (van Zonneveld, 1986b; Urano et al., 1986; Horrevoets et al., 1994). These data suggest that K2 only plays a significant role in the binding of t-PA to fibrin cleaved by plasmin.

Data presented in Chapter 3 demonstrate that, unlike the predominantly finger-mediated interaction with fibrin, t-PA binds to the fibrin degradation product (DD)E via its K2 domain (the structure of (DD)E is discussed in section 1.1.4ii). Furthermore, (DD)E is a potent stimulator of t-PA-mediated Pg activation (Weitz et al., 1991). These findings raise the possibility that the K2 domain of t-PA serves to promote plasmin formation on the surface of FDPs.

1.1.2v *Substrate specificity of t-PA*

Like plasmin, t-PA is a member of the trypsin family of serine proteases. The catalytic triad of t-PA, common to all serine proteases, consists of His322, Asp371, and Ser478 (Madison, 1993; Lamba et al., 1996). Unlike plasmin, however, t-PA is a remarkably specific enzyme. The only known substrate for t-PA *in vivo* is the Arg560-Val561 bond in Pg (Ding et al., 1995). The protease domain of t-PA displays the same substrate stringency

as full-length t-PA, indicating that the substrate specificity of t-PA is dictated by its protease domain (Madison, 1995). Whereas most serine proteases display substrate specificity based on the amino acids surrounding the target scissile bond, both t-PA and its protease domain exhibit little reactivity towards small peptides mimicking the t-PA cleavage site in Pg (Madison et al., 1995; Ding et al., 1995). However, Coombs et al. (1996) demonstrated that the K_M for t-PA-mediated cleavage of small peptides resembling the P2 to P4' site on Pg is reduced ~ 950-fold when these substrates are inserted into the amino-terminal portion of larger, nonspecific proteins. These data suggest that t-PA contains at least one secondary substrate recognition site distant from its S4 to S2' subsites. More recently, a hydrophobic exosite involving residues Leu420, Pro422, and Phe423 has been identified on the surface of the protease domain in t-PA (Ke et al., 1997). This site is required for efficient Pg activation in the absence of fibrin (Ke et al., 1997; Renatus et al., 1997a). The complimentary site on Pg to which this exosite binds has yet to be identified.

1.1.2vi *Single-chain versus two-chain t-PA*

t-PA is secreted as a single-chain polypeptide (sct-PA) that can be cleaved at the Arg275-Ile276 bond by plasmin to form two-chain t-PA (tct-PA) (Fig. 1.1). Although in the absence of a cofactor tct-PA is 2 to 3 times more efficient than sct-PA at activating Pg, the two forms of the activator have similar activities in the presence of fibrin (Loscalzo, 1988). Tachias and Madison (1995) produced an uncleavable sct-PA by introducing the mutation R275E. In the presence of fibrin, the efficiency of Pg activation by the R275E mutant was equivalent to that of native t-PA. In contrast, in the absence of a cofactor, the R275E t-PA was ~10-fold less efficient at activating Pg than native t-PA. These studies raise the

possibility that the differences between sct-PA and tct-PA could be exploited to produce an activator with greater fibrin-selectivity than native t-PA.

1.1.2vii *t-PA-mediated activation of Pg in the absence of a cofactor*

In addition to its substrate specificity, t-PA is distinguished from other serine proteases by the fact that the single-chain form is enzymatically active. Whereas most serine proteases, such as chymotrypsinogen, are secreted as zymogens (inactive proteins requiring proteolytic cleavage for catalytic activity), sct-PA is not a true zymogen (Stubbs et al., 1998). Although activation of Pg by sct-PA in the absence of a cofactor is inefficient, some activation occurs (Loscalzo, 1988). The activity of sct-PA has been attributed to both the absence of residues that normally stabilize the inactive zymogen and the presence of residues that stabilize the active form of t-PA.

Most serine proteases contain a 'zymogen triad', that prevents the formation of the functional oxyanion hole necessary for catalysis. For instance, in chymotrypsinogen, a salt bridge is established between Ser32, His40, and Asp194 (Wang et al., 1985). This 'zymogen triad' maintains Asp194 in a conformation such that Gly193 is oriented away from the active site Ser195. Activation cleavage of Arg15-Ile16 allows formation of a new salt bridge between the free α -amino group of Ile16 and Asp194, reorienting Asp194 and Gly193 to form a functional oxyanion hole with Ser195 (Wang et al., 1985). In t-PA, the analogous positions of Ser32 and His40 are occupied by Ala292 and Phe305, respectively, and no 'zymogen triad' is present (Madison et al., 1993). The two point mutations A292S and F305H introduce a 'zymogen triad' into t-PA and reduce the activity of sct-PA 140-fold in the absence of fibrin (Madison et al., 1993; Tachais and Madison, 1996).

The active conformation of chymotrypsin is stabilized by a salt bridge between the new amino-terminal Ile16 and Asp194, suggesting that the active conformation of sct-PA may involve an ionic interaction between Asp477 (analogous to Asp194 in chymotrypsin) and a free amine within its protease domain. Crystal structures of the protease domain of sct-PA indicate that the side-chain of Lys429 is inserted into the active site cleft of sct-PA (Renatus et al., 1997b). These data are consistent with the demonstration that mutation of Lys429 (K429Y) produces ~10-fold reduction in the activity of sct-PA in the absence of a cofactor (Tachias and Madison, 1997). However, mutation of Lys296 or Lys416, residues located on opposite sides of the active site cleft of t-PA (Renatus et al., 1997b), also reduces the activity of sct-PA in the absence of a cofactor ~10-fold (Bennett et al., 1991; Peterson et al., 1990). Together, these data suggest that one or more lysine residues within the protease domain of t-PA are involved in stabilizing the active conformation of sct-PA in the absence of a cofactor. In the presence of fibrin, mutant t-PA molecules that contain substitutions at Lys296, 416, or 429 have activity equivalent to that of wild-type t-PA, indicating that these lysine residues have no effect on the activity of t-PA in the presence of fibrin (Peterson et al., 1990; Bennett et al., 1991; Tachias and Madison, 1997).

1.1.2viii *t-PA-mediated activation of Pg in the presence of fibrin*

In the presence of fibrin, the efficiency of t-PA-mediated Pg activation is increased 2 to 3 orders in magnitude compared with that in its absence (Hoylearts et al., 1982). Kinetic analysis supports a model in which the activator or substrate binds to fibrin to form the respective binary species (Horrevoets et al., 1997a). Subsequently, the substrate or activator binds to form a ternary t-PA/Pg/fibrin complex. Although there is great variation in the

reported kinetic parameters for Pg activation by t-PA (reviewed in Bachmann, 1994), fibrin-mediated stimulation of Pg activation by t-PA is associated with a dramatic reduction in the K_M for Pg activation (from ~ 60 to ~ 0.2 μM) and a moderate increase in k_{cat} (from ~ 0.05 to 0.1 s^{-1}) (Hoylearts et al., 1982). Thus, the increase in catalytic efficiency (k_{cat}/K_M) for Pg activation in the presence of fibrin is primarily due to a decrease in K_M which is suggestive of an increase in the stability of the Michaelis complex (Hoylearts et al., 1982; Horrevoets et al., 1997a).

Several factors likely influence the stability of the ternary t-PA/Pg/fibrin complex. Using alanine scanning and screening 64 variants of t-PA, Bennett et al. (1991) demonstrated that reductions in the affinity of t-PA for fibrin parallel reductions in fibrin-stimulated Pg activation. In general, moderate reductions in affinity had little effect on Pg activation, whereas decreases in fibrin affinity of 50 to 80% were associated with reductions in Pg activation. However, several mutations that significantly affected binding did not alter stimulation of t-PA by fibrin, suggesting that fibrin affinity of t-PA is not the sole determinant of Pg activation by t-PA.

Both Pg and t-PA undergo conformational changes upon binding to fibrin that likely enhance Pg activation. Pg changes conformation, and its rate of activation increases, in the presence of lysine or lysine analogues (Green, 1986; Mangel et al., 1994). Binding of Pg to fibrin probably induces similar conformational changes that expose its activation site (Weisel et al., 1994). Conformational changes in t-PA that occur upon its binding to fibrin are not well understood. It is thought that two or more surface loops on the protease domain of t-PA may interact with fibrin, and change the conformation to shield the internal salt bridge that

orients Asp477 to form the functional oxyanion hole (Lamba et al., 1996; Bode and Renatus, 1997). Alternatively, binding of t-PA to fibrin via its finger and/or K2 domain may alter domain-domain interactions within t-PA and, in turn, induce conformational changes in the protease domain (Bakker et al., 1995a; Lamba et al., 1996; Renatus et al., 1997a). However, the activity of t-PA towards small substrates is enhanced only 2- to 4-fold by fibrin (Loscalzo, 1988; Bringmann et al., 1995), suggesting the majority of the fibrin stimulation of Pg activation results from optimal arrangement of t-PA and Pg on fibrin, orienting the active site of t-PA and the Pg activation site for a productive interaction.

1.1.2ix *Fibrin-specificity of t-PA*

The fibrin-specificity of a Pg activator is commonly determined by comparing the catalytic efficiency of Pg activation in the presence of fibrin with that in the presence of Fg (Fig. 1.2) (Hoylaerts et al., 1982; Weitz et al., 1999). This definition is clinically relevant (Holden, 1990; Collen, 1996). When used therapeutically, activators that are more fibrin-specific will target Pg activation to the thrombus and cause less systemic plasmin formation (the merits of this are discussed in section 1.2) (Collen, 1996). Although the reported values for the catalytic efficiency of t-PA-mediated Pg activation in the presence of fibrin and Fg vary considerably, it is generally agreed that the efficiency of Pg activation by t-PA is 10 to 100 times greater in the presence of fibrin than Fg (Hoylaerts et al., 1982; Paoni et al., 1993a; Bringmann et al., 1995). By these criteria, t-PA is designated a fibrin-specific Pg activator.

However, this definition of fibrin-specificity may be overly simplistic. Weitz et al. (1991) demonstrated that FDPs potentiate Pg activation by t-PA. Only those FDPs that bind both t-PA and Pg stimulate Pg activation. The species primarily responsible for stimulating

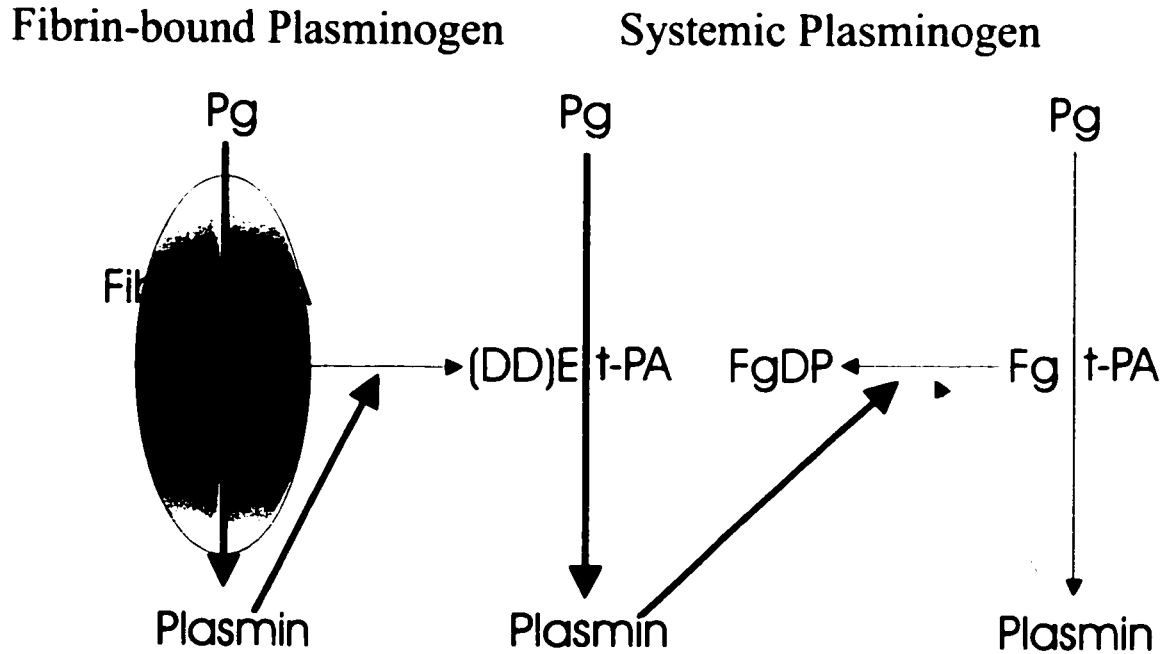


Figure 1.2 Activation of fibrin-bound Pg versus systemic Pg. Plasmin formed from fibrin-bound Pg degrades the fibrin clot into fibrin degradation products; a major degradation product of fibrin is (DD)E. In contrast to Pg activation on fibrin, plasmin formed systemically degrades Fg into Fg degradation products (FgDPs). The fibrin specificity of a Pg activator is commonly determined by comparing Pg activation in the presence of fibrin (left-most pathway) with that in the presence of Fg (right-most pathway). However, this determination fails to consider the effects of (DD)E (middle pathway). Because (DD)E is soluble and as potent as fibrin at stimulating Pg activation by t-PA, (DD)E compromises the fibrin-specificity of t-PA and promotes systemic plasmin formation.

t-PA was identified as (DD)E (the structure is discussed in section 1.1.4ii) (Weitz et al., 1991). (DD)E is as potent as fibrin at stimulating t-PA-mediated Pg activation (Weitz et al., 1991; Stewart et al., 2000). Although FDP levels are low under physiologic conditions, high levels of (DD)E may be generated during pharmacologic fibrinolysis (Gaffney, 1983; Francis et al., 1986; Soria et al., 1987; Lee et al., 1997); or in pathophysiologic conditions, such as disseminated intravascular coagulation (Whitaker et al., 1980; Gaffney, 1983), where there is increased fibrin formation and secondary fibrinolysis. Because (DD)E is soluble, it compromises the fibrin-specificity of t-PA by inducing systemic Pg activation. These findings suggest that the designation of the fibrin-specificity of Pg activators should consider the influence of (DD)E, as well as those of fibrin and Fg, on Pg activation (Fig. 1.2).

1.1.2x *Fibrin-specificity of vampire bat plasminogen activator*

To date, the most fibrin-specific Pg activator has been isolated from the saliva of the vampire bat (*Desmodus rotundus*) (Bergum and Gardell, 1992; Weitz et al., 1999). Full-length vampire bat salivary plasminogen activator (designated DSPA α_1 or b-PA) has over 72% amino acid identity to t-PA (Gardell et al., 1989; Schleuning et al., 1992). The structural difference between these molecules is that b-PA lacks a K2 domain. In the presence of fibrin, b-PA activates Pg with a catalytic efficiency similar to that of t-PA (Bringmann et al., 1995). Furthermore, b-PA and t-PA are equipotent thrombolytic agents (Witt et al., 1992; Gulba and Witt, 1995). Since both activators have finger domains and bind fibrin with similar affinities, it is not surprising that their activities are similar in the presence of fibrin. In contrast, the efficiency of Pg activation in the absence of a cofactor or presence of Fg is ~200-fold lower with b-PA than with t-PA (Bringmann et al., 1995). The

mechanism responsible for the reduced activity of b-PA relative to t-PA is not completely understood. Two differences between the protease domains of t-PA and b-PA may help to explain this phenomenon. First, b-PA contains a glutamine in place of lysine at position 296 (Renatus et al., 1997c). Because Lys296 may help stabilize the active conformation of t-PA in the absence of a cofactor, this difference may be responsible for at least some of the reduced basal activity of b-PA. Second, b-PA is a single-chain protein that can not be cleaved by plasmin because the Arg275 and Ile276 residues found in t-PA are replaced with His and Ser, respectively (Bringmann et al., 1995; Renatus et al., 1997c). The ability of b-PA to activate Pg in the absence of a cofactor increases as much as 20-fold when the activator is rendered cleavable by introduction of Arg275 and Ile276 (Bringmann et al., 1995). These observations do not exclude the possibility that there are as of yet undiscovered differences between the catalytic domains of b-PA and t-PA. Although the protease domain of b-PA does not mediate its binding to fibrin, the isolated protease domain of b-PA is 11-fold more fibrin-specific than isolated protease domain of t-PA (Toschi et al., 1998). These studies suggest that the catalytic domains of b-PA and t-PA may differ in their preference for fibrin-bound Pg *versus* free Pg.

Whereas these differences between b-PA and t-PA explain why b-PA has lower activity than t-PA in the absence of a cofactor, it is not known whether they contribute to the decreased activity b-PA in the presence of Fg or other soluble cofactors. Since fibrin-specificity refers to the relative abilities of fibrin and Fg to stimulate Pg activation, this difference between b-PA and t-PA is critical for defining the fibrin-specificity of b-PA. The data presented in Chapter 3 expand this definition to include the effects of (DD)E and offer

an explanation for the lower activity of b-PA in the presence of soluble cofactors (DD)E and Fg.

1.1.3 Fibrinogen

Fg, the precursor of fibrin, is a 340 kDa plasma protein consisting of three pairs of disulfide-bonded A α -, B β -, and γ -chains (Fig. 1.1) (reviewed by Shafer and Higgins, 1988). The three chains are arranged into a central globular E domain at their amino-termini, connected by coiled-coil regions to two identical globular D domains at their carboxy-termini (Weisel et al., 1985; Everse et al., 1995; Spraggon et al., 1997; Mosesson, 1998). Recent crystal structures (Brown et al., 2000) have demonstrated that the carboxy-terminal portion of the α -chains are free of the globular D domain and fold back towards the central E domain. This observation is consistent images of Fg taken using electron microscopy (Weisel et al., 1985).

1.1.3i *Fg degradation by plasmin*

The pattern of Fg degradation by plasmin has been extensively studied (Fig. 1.3)(for review, see Shafer and Higgins, 1988). The most labile chain of Fg is the A α chain. The carboxy-terminal portion of this chain, with a molecular weight of ~ 45,000, is the first to be liberated from Fg (Takagi and Doolittle, 1975b). Cleavage of one of the A α chains results in fragment X₁, whereas cleavage of both chains results in formation of fragment X₂ (Marder et al., 1969). An important property of fragment X is that it remains clottable, although fragment X clots are more susceptible to lysis than fibrin clots (discussed in section 1.2.2i). Cleavage of the A α chain is followed by liberation of 42 amino acids from the amino-terminal portion of the B β chain (Takagi and Doolittle, 1975a). Fragments with partially

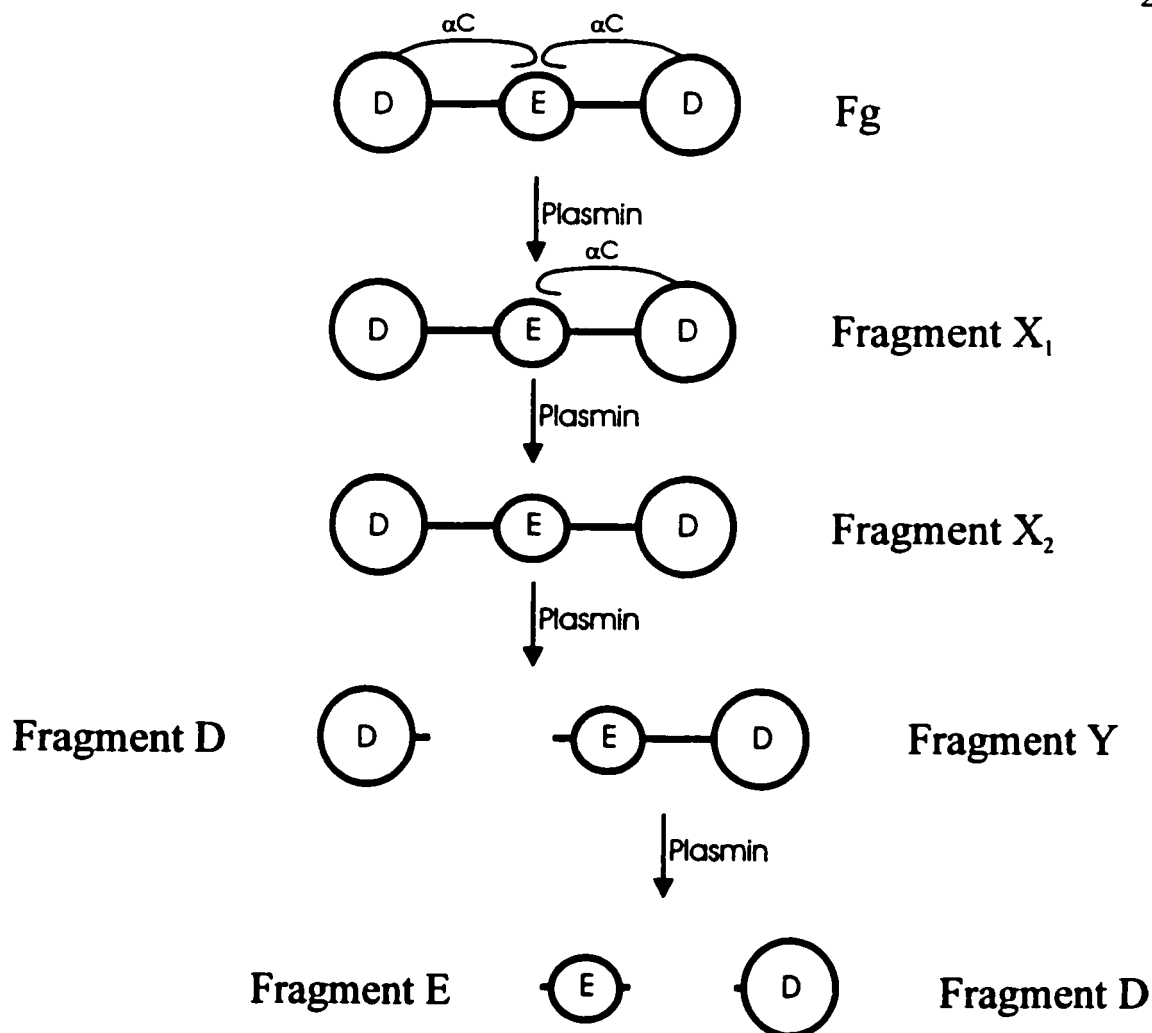


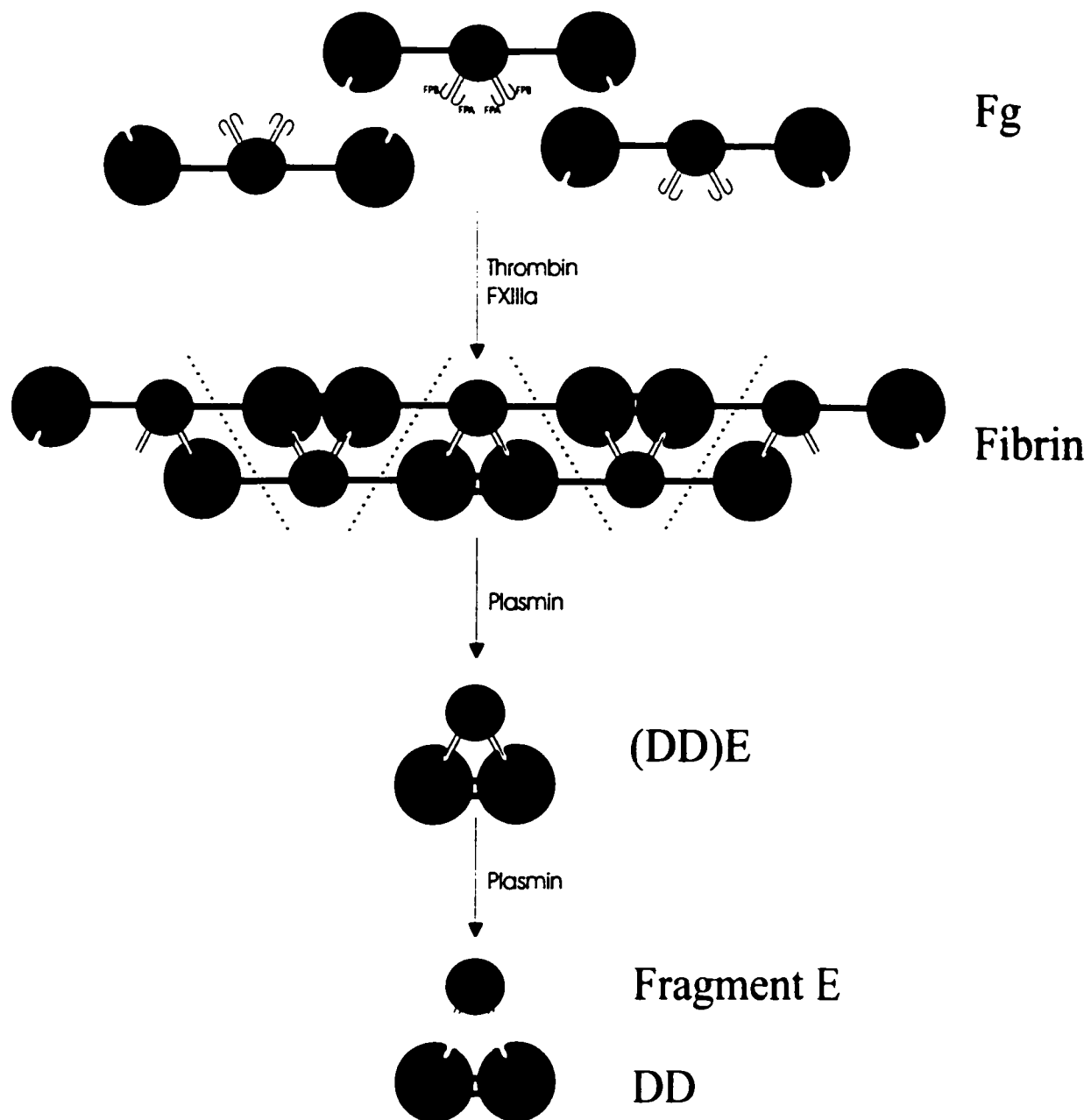
Figure 1.3 Plasmin-mediated degradation of Fg. Plasmin-mediated degradation of the carboxy-terminal portion of one A α chain (termed αC) gives rise to fragment X₁. Removal of the second αC region results in fragment X₂. Subsequent plasmin-mediated cleavage in the coiled-coil region between D and E domains generates fragments Y and D. Fragment Y consists of the E domain and remaining D domain which are separated by further plasmin-mediated cleavage.

degraded B β chains are still termed fragment X, although removal of B β 1-42 seriously compromises the ability of this fragment to form clots (Siebenlist et al., 1990). Further proteolysis of fragment X occurs in the region of the coiled coils which separate the D and E domains of Fg, and results in the formation of fragments D and Y (Budzynski et al., 1974). Fragment D varies in molecular weight from ~ 82,000 to 92,000 depending on the degree of plasmic degradation of the carboxy-termini of the γ chains (Shafer and Higgins, 1988). Fragment Y is comprised of one D domain and one E domain, and has a molecular weight of ~ 150,000 (Marder et al., 1969; Budzynski et al., 1974). Further degradation of fragment Y results in the separation of the D and E domains, the latter of which has a molecular weight of ~ 50,000 (Budzynski et al., 1974; Shafer and Higgins, 1988). Unlike fragment X, fragments Y, D, and E are not clottable (Marder et al., 1969).

1.1.4 Fibrin

Fibrin is formed when thrombin (IIa) removes fibrinopeptides A and B (FPA and FPB, respectively) from the A α and B β chains, respectively, of Fg to produce fibrin monomers (Fig. 1.4) (Higgins et al., 1983; Shafer and Higgins, 1988). Fibrin monomers then spontaneously polymerize via a 'knob-in-hole' mechanism where the amino-terminal knobs formed by FPA and FPB removal fit into holes in D domains of two additional fibrin monomers (Hantgan et al., 1980; Weisel et al., 1993). Thus, the fibrin protofibril is arranged in a half-staggered fashion, where the E domain of one monomer is noncovalently bound to two D domains from the opposing strand. Factor XIIIa stabilizes the protofibril by forming glutamyl-lysyl crosslinks between the γ -chains of adjacent D domains (reviewed in Lorand, 2000). Fibers are formed as the protofibrils laterally aggregate and branching of the

Figure 1.4 Formation of fibrin and (DD)E. Each Fg molecule consists of two D domains flanking a central E domain. Fibrinopeptides A and B (FPA and FPB, respectively) constitute the amino-terminal portions of the A α and B β chains, respectively. Fibrin monomers are formed when thrombin removes FPA and FPB. Fibrin monomers spontaneously polymerize via a 'knob-in-hole' mechanism, forming a half-staggered fibrin protofibril. Active factor XIII (FXIIIa) catalyzes the crosslinking of adjacent D domains in the protofibril (indicated by a double line). Plasmin cleaves between adjacent D and E domains of the fibril, liberating FDPs, including (DD)E. (DD)E consists of two crosslinked D domains (DD), noncovalently associated with an E domain. Plasmin may further degrade the amino-terminal portion of the E domain until it no longer remains associated with DD, giving rise to fragments E and DD.



fibers forms a three-dimensional fibrin matrix. In addition to γ - γ crosslinking, factor XIIIa more slowly catalyzes the crosslinking of α -chains within and between protofibrils to further stabilize the fibrin meshwork (McDonagh et al., 1971; Francis and Marder, 1988).

Whereas Fg is a fairly inert molecule, fibrin participates in several physiologic processes, binds many plasma proteins, and interacts with cell receptors (Mosesson, 1999). These observations suggest that fibrin assembly produces conformational changes that expose multiple protein binding epitopes. Examples of these are t-PA and Pg binding sites (Mosesson, 1998). The affinity of t-PA for fibrin is ~15-fold higher than its affinity for Fg (Stewart et al., 1998b). Whereas Glu-Pg does not bind Fg, it binds fibrin with a K_d of 10 - 30 μ M (Horrevoets et al., 1997b; Stewart et al., 1998b). Attempts have been made to localize the t-PA and Pg binding sites on fibrin using monoclonal antibodies directed against epitopes on fibrin and various fibrin(ogen) fragments. Both t-PA and Pg bind the A α chain of fibrin between residues 148-160 (Nieuwenhuizen, 1994; Haddeland et al., 1996). Both of these interactions are inhibited by EACA, suggesting K2 of t-PA mediates its binding to this region (Nieuwenhuizen, 1994). t-PA also binds γ 312-324, an interaction not affected by EACA, suggesting that binding of t-PA to this epitope is finger-dependent (Nieuwenhuizen, 1994). Monoclonal antibodies directed against A α 148-160 and γ 312-324 bind fibrin, but not Fg, consistent with polymerization-mediated exposure of t-PA / Pg binding sites (Haddeland et al., 1996). Both, t-PA and Pg bind to fragment E from Fg or fibrin (Lucas et al., 1983; Varadi and Patthy, 1984; Weitz et al., 1991). Since A α 149-160 and γ 312-324 are located in the D domain, it is likely that t-PA and Pg bind to multiple sites on fibrin.

In addition to conformational changes that occur upon polymerization, exposure of epitopes in Fg also can occur with proteolytic cleavage or chemical modification of Fg. For example, proteolytic cleavage of Fg by plasmin exposes Pg binding sites in fragments D and E (Vardi and Patthy, 1983). Likewise, cyanogen bromide (CNBr)-mediated degradation of Fg results in a series of products that interact with t-PA or Pg and stimulate Pg activation to varying degrees (de Munk et al., 1989; Grailhe et al., 1994).

1.1.4i *Fibrin degradation by plasmin*

Fibrin plays a dual role in the Pg activation system, it is both a stimulator of Pg activation and a substrate for plasmin. Solubilization of fibrin by plasmin and the resulting FDPs have been extensively studied (reviewed in Shafer and Higgins, 1988). Plasmin cleavage sites on fibrin are well defined and occur in the coiled-coil region between E and D (Fig. 1.4) (Francis et al., 1980; Marder and Francis, 1983; Mosesson, 1995).

Cleavage of a protofibril requires cleavage of proximal α -, β -, and γ -chains in both strands of the protofibril. Recent statistical analysis of the integrity of the α -, β -, and γ -chains within FPDs has demonstrated that plasmin degradation of fibrin is not random (Walker and Nesheim, 1999). These data are supported by electron microscopy studies indicating that plasmin binds to end-to-end junctions between two fibrin molecules (Weisel et al., 1994) and cleaves laterally through an entire fiber, creating blunt ends in the fibrin meshwork (Veklich et al., 1998). Together, these data suggest that plasmin cleaves fibrin in a cooperative manner where cleavage of an adjacent strand in a protofibril is more likely than a second cleavage occurring elsewhere along the length of the protofibril. This mechanism also is supported by differences in spacing of plasmin binding sites on adjacent

strands of a protofibril *versus* those along a protofibril (i.e. lateral *versus* longitudinal directions). Plasmin binding sites on adjacent strands are 5 - 10 nm apart, whereas those along the length of a protofibril are 22.5 nm apart, making lateral cleavage of a fiber more likely (Veklich et al., 1998). Thus, plasmin may creep across the fibrin fiber by binding via two or more of its kringles, where one kringle binds an adjacent fibril as the other kringle releases the first fibril. This mechanism is consistent with multiple fibrin-binding kringles in plasmin and multiple Pg binding sites on fibrin(ogen). The concept that Pg moves across fibrin fibrils is also supported by studies using photoaffinity activated cross-linkers, in which a single Pg molecule was often found linked to the ends of two or more fibrin molecules (Weisel et al., 1994).

An important feature of fibrin degradation by plasmin is the positive feedback mechanism that occurs during the early stages of fibrinolysis. As fibrin is degraded by plasmin, the efficiency of Pg activation increases, caused by a decrease in K_M of ~16-fold (Norrman et al., 1985). This has been attributed to the plasmin-mediated exposure of new carboxy-terminal lysine residues on the fibrin surface. As a result, new binding sites are created for both Pg and t-PA (Higgins and Vehar, 1987; de Vries et al., 1989; Suenson et al., 1990; Fleury and Angles-Cano, 1991). Removal of the newly formed carboxy-terminal lysine residues by carboxypeptidase B (CPB), or endogenous CPB-like enzymes (discussed in section 1.1.5v), eliminates increased Pg and t-PA binding to fibrin and the upregulation of Pg activation that normally occurs (de Vries et al., 1989; Sakharov et al., 1997; Wang et al., 1998).

1.1.4ii Structure of (DD)E

(DD)E is a major degradation product of crosslinked fibrin (Olexa and Budzynski, 1979b; Gaffney and Joe, 1979). (DD)E is a complex of D-dimer (DD), noncovalently associated with fragment E (Fig. 1.4). The trinodular structure of (DD)E resembles the repeating trinodular unit in a fibrin fibril. Thus, (DD)E may represent a soluble form of fibrin. This concept is supported by the finding that (DD)E potentiates t-PA-mediated activation of Pg to the same extent as fibrin (Weitz et al., 1991; Stewart et al., 2000).

The structural integrity of (DD)E depends on the composition of the amino-terminal A α and B β chains. Plasmin can cleave the B β chain at Arg42 or Lys53 and the A α chain at Lys19 (Olexa et al., 1979). These cleavages eliminate the 'knob-in-hole' interactions and dissociate fragment E from DD (Olexa and Budzynski, 1979a; Olexa, 1979). It is important to note that the amino-terminal portions of the A α and B β chains are more resistant to plasminic degradation in the (DD)E complex than in Fg, probably because of the ionic interaction of these segments with their corresponding sites in DD (Moskowitz and Budzynski, 1994). This phenomenon may explain why (DD)E persists long after clot dissolution *in vitro* (Gaffney and Joe, 1979) and is found in patients with disseminated intravascular coagulation (Whitaker et al., 1980). Degradation of (DD)E to DD and fragment E may be of physiologic relevance because, unlike the potent stimulatory ability of (DD)E, the terminal degradation products DD and fragment E have little effect on Pg activation by t-PA (Stewart et al., 1998a). Therefore, (DD)E-stimulated Pg activation may be self-limiting and degradation of (DD)E by plasmin may be a mechanism by which systemic plasmin formation is minimized.

1.1.5 Regulation of Fibrinolysis

The high plasma concentration of Pg ($\sim 2 \mu\text{M}$) represents a considerable reservoir of potential proteolytic activity (Bachmann, 1994). Thus, regulation of fibrinolysis involves limiting Pg activation and plasmin activity to the site of a thrombus. Systemic Pg activation is limited by the low concentration and activity of t-PA in plasma, localization of Pg activation to fibrin by virtue of the stimulatory activity of fibrin, and inhibition of free plasmin by circulating inhibitors.

1.1.5i *Synthesis and secretion of t-PA*

The plasma level of t-PA is highly regulated by its rates of synthesis, secretion, and clearance (Lijnen and Collen, 1995; Redlitz and Plow, 1995). Although t-PA has been isolated from several different tissues, including heart and liver, the principal site of its synthesis is the vascular endothelial cell (EC) (Kooistra et al., 1994). Recently, it has been demonstrated that, like von Willebrand factor, t-PA is stored in Weibel-Palade bodies within ECs (Rosnoblet et al., 1999). t-PA is rapidly released into the circulation in response to various stimuli. Physical or mental exercise has been shown to augment circulating t-PA levels within minutes (Jern et al., 1989; Jern et al., 1994; Stein et al., 1998). This response has been attributed to both an increase in shear stress and a direct effect of adrenalin on ECs. Interestingly, pooling of blood, such as that which occurs with vascular occlusion of legs or forearms, also increases the local concentration of t-PA (Falkon et al., 1992; Kooistra et al., 1994). This phenomenon may be due to decreased clearance of the locally produced t-PA (clearance of t-PA is discussed in section 1.1.5iii) and/or stimulation of acute release of t-PA by various stimuli; for instance, hypoxia and acidosis both induce t-PA release from ECs

(Tappy et al., 1984). Although several chemical agonists stimulate t-PA release, most also increase synthesis and release of PAI-1 (Bachmann, 1994). One notable exception is thrombin, which binds specific cell receptors and activates membrane-bound protein kinase C, which, in turn, induces t-PA synthesis and release (Emeis, 1992).

The above mentioned phenomena are associated with vascular occlusion and inappropriate thrombus formation. When an atheromatous plaque with superimposed thrombus narrows a vessel lumen, shear stress increases. If the vessel becomes completely occluded, blood may pool at the site of the occlusion. Finally, thrombin concentrations are highest at the site of a thrombus. Consequently, the vessel responds appropriately to vascular occlusion and thrombus formation by releasing t-PA from ECs (Hoffmeister et al., 1998).

1.1.Sii *Inhibition of t-PA by PAI-1*

The activity of t-PA in plasma is dependent on the concentration of PAI-1, the primary inhibitor of t-PA (reviewed by Krishnamurti and Alving, 1992; Lijnen and Collen, 1995a). PAI-1 is a 52 kDa member of the serine protease inhibitor (serpin) family. Serving as a pseudo substrate for t-PA, PAI-1 is cleaved at the Arg346-Met347 peptide bond in the reactive center loop (Lindahl et al., 1990). This results in the formation of a covalent complex in which the active-site serine of t-PA forms an ester bond with the newly exposed carboxyl group of Arg346 within PAI-1. In addition to the active site of t-PA, a second site within the protease domain of t-PA is required for optimal inhibition by PAI-1. Deletion or mutation of a positively charged surface loop, comprised of residues 296-299 (Lys-His-Arg-Arg) in t-PA, renders the activator ~ 80-fold more resistant to inactivation by PAI-1 (Madison et al., 1990a). The proposed complimentary site within PAI-1 involves the

negatively charged residues Glu350, Glu351, and Asp355 (Madison et al., 1990b).

Inhibition of t-PA by PAI-1 is very rapid. The second order rate constant for PAI-1 inhibition of sct-PA or tct-PA is 5.5×10^6 and $1.8 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$, respectively (Thorsen et al., 1988). In healthy individuals, PAI-1 is in molar excess to t-PA in plasma (Chandler et al., 1990, Blood). Consequently, the majority of t-PA in plasma is complexed with PAI-1 and is inactive. However, a small amount (~ 5%, or 4 pM) of t-PA in plasma is active (Chandler, 1991). Since inactivation of t-PA by PAI-1 is so rapid, it is not clear why there is measurable t-PA activity in plasma. This observation may reflect the continual secretion of t-PA into circulation. Since inactivation of t-PA, especially sct-PA, is not immediate, there may always be a small amount of newly secreted, active t-PA (Chandler, 1991; Bachmann, 1994).

The rate of PAI-1-mediated inactivation of t-PA is 5- to 10-fold slower in the presence of fibrin than in the presence of Fg or absence of a cofactor (Masson and Angles-Cano, 1988; Carr et al., 1992; Lee et al., 2000). These data suggest fibrin-bound t-PA is partially protected from inactivation by PAI-1 (Fig. 1.5). PAI-1 has been shown to bind to the K2 domain of t-PA and inhibit binding of t-PA to fibrin in a competitive manner (Masson and Angles-Cano, 1988; Wilhelm, 1990). Binding of t-PA to fibrin via its K2 domain may inhibit the formation of the t-PA/PAI-1 complex by making the K2 domain of t-PA unavailable for PAI-1 interaction. However, this hypothesis is inconsistent with the observation that deletion of the K2 domain from t-PA enhances its inactivation by PAI-1 in the absence of a cofactor (de Serrano and Castellino, 1990). Others have suggested that, in addition to K2-dependent binding, PAI-1 binds t-PA via its finger domain (Kaneko et al., 1992; Stringer et al., 1994). Thus, the mechanism by which fibrin protects t-PA from

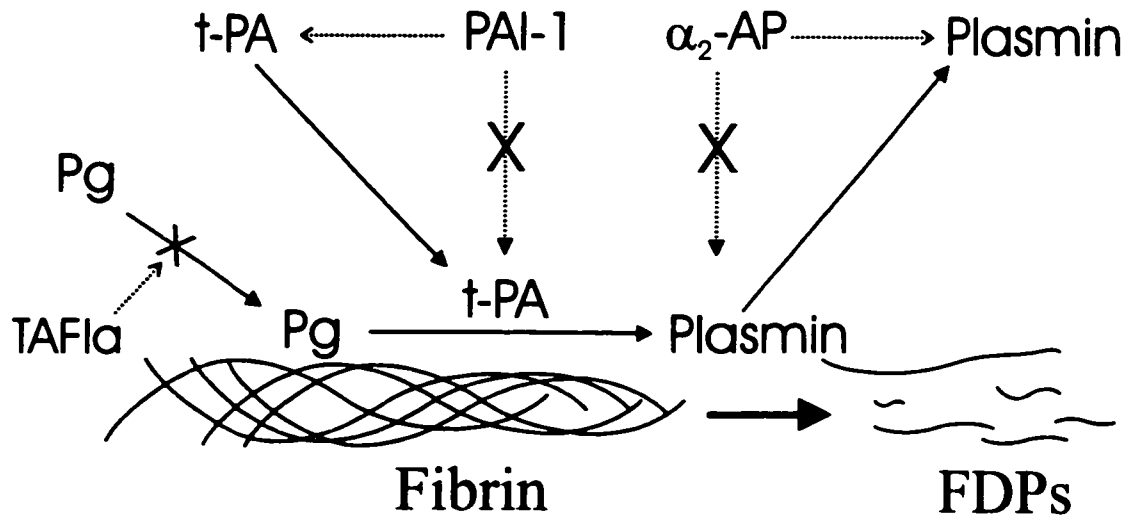


Figure 1.5 Regulation of the fibrinolytic system. Pg and t-PA bind fibrin to form a ternary Pg/t-PA/fibrin complex. As a result, Pg activation by t-PA is markedly enhanced in the presence of fibrin. Plasmin formed on the fibrin surface is protected from inhibition by α_2 -AP and degrades fibrin into soluble FDPs. Once plasmin is released from fibrin, it is rapidly inactivated by α_2 -AP. Like plasmin, t-PA is partially protected from its inhibitor, PAI-1, when bound to fibrin. Together, these mechanisms allow Pg activation on fibrin and limit systemic plasmin formation. TAFIa is a negative regulator of fibrinolysis. By limiting Pg binding to fibrin, TAFIa attenuates plasmin formation and subsequent fibrin degradation.

inactivation by PAI-1 remains controversial.

1.1.5iii Clearance of t-PA

The plasma concentration of t-PA is tightly regulated by the balance between t-PA release from ECs and t-PA clearance (Chandler, 1991; Bachmann, 1994). The plasma half-life of t-PA in humans is ~ 3.5 min and the organ mainly responsible for t-PA clearance is the liver (Rijken et al., 1990; Redlitz and Plow, 1995). At least two different t-PA receptors have been identified in the liver. First, parenchymal hepatocytes express a 39 kDa receptor for t-PA, identified as the low-density lipoprotein receptor-related protein or α_2 -macroglobulin receptor (Bu et al., 1992; Otter et al., 1992a; Warshawsky et al., 1993). This receptor recognizes both free t-PA and t-PA in complex with PAI-1, but has a preference for the complex (Orth et al., 1992). The second t-PA receptor is expressed on liver ECs and is a 175 kDa mannose receptor which recognizes the high mannose carbohydrate at Asn117 within K1 of t-PA (Otter et al., 1992b; Refino et al., 1993; Redlitz and Plow, 1995). Since the mannose receptor does not distinguish between free and complexed t-PA, this receptor is primarily responsible for clearance of pharmacological doses of t-PA, where plasma levels of t-PA are in excess over those of PAI-1 (Otter et al., 1992a). Indeed, the Asn117 glycosylation site on t-PA has been a target for prolonging the plasma half-life of t-PA (Larsen et al., 1991; Refino et al., 1993) (pharmacologic use of t-PA is discussed in section 1.1.2).

Other clearance receptors for t-PA have been postulated based on two experimental observations. First, several groups have demonstrated that point or domain deletion mutants of t-PA, with normal glycosylation and complex formation with PAI-1, have prolonged half-

lives in animals (Ahern et al., 1990; Yahara et al., 1992; Ikenada et al., 1992). Recently, however, Noorman et al. (1998) demonstrated that trypsin digestion products of t-PA, containing the high mannose oligosaccharide, bind to the high mannose receptor with an affinity as much as 7.5-fold lower than that of native t-PA. These data suggest that the conformation of the high mannose oligosaccharide, or other elements within t-PA, may be important for t-PA clearance and may explain the reductions in clearance seen with t-PA deletion and mutation variants. A second finding that suggests the presence of additional t-PA receptors in the liver comes from the work of Hajjar and Reynolds (1994) who demonstrated that binding of t-PA to human hepatoma cells (HepG2) requires the fucose residue attached to threonine 61 of t-PA. However, the importance of a fucose receptor in t-PA clearance is uncertain because other investigators have shown that the plasma clearance of non-fucosylated t-PA is similar to that of native t-PA (Narita et al., 1995; Camani and Kruithof, 1995; Camani et al., 1998).

1.1.5iv *Inhibition of plasmin by α_2 -AP*

To limit systemic plasmin activity, free plasmin is rapidly inactivated by circulating inhibitors. The principal *in vivo* inhibitor of plasmin is α_2 -AP, also called α_2 -plasmin inhibitor (for review, see Lijnen and Collen, 1986). Like PAI-1, α_2 -AP is a serpin. The mechanism of inhibition involves plasmin cleavage of the Arg364-Met365 peptide bond of α_2 -AP, followed by covalent bond formation between the active site serine of plasmin and Arg364 of α_2 -AP. What makes α_2 -AP unique among serpins is a carboxy-terminal extension of 51 amino acids. This region contains a secondary binding site for plasmin that binds to the LBS of kringles 1-3 in plasmin (Sugiyama et al., 1988). This endows α_2 -AP with a high

affinity for plasmin ($K_d = 0.2 \text{ pM}$) (Wiman et al., 1979). Lys464, the carboxy-terminal lysine of α_2 -AP, has been identified as the major contributor to its high affinity interaction with plasmin, although other lysine residues may also be involved (Hortin et al., 1988; Hortin et al., 1989).

The high affinity interaction between α_2 -AP and plasmin contributes to the extremely rapid inactivation of plasmin. The second order rate constant for this reaction is $\sim 3 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ (Wiman and Collen, 1978). The estimated half-life of active plasmin in circulation is 0.1 s (Lijnen and Collen, 1995b). This high rate of inhibition is dependent on the presence of both a free LBS and active site in plasmin. Plasmin molecules associated with fibrin have both their LBS and active site occupied. As a result, the rate of inactivation of plasmin by α_2 -AP in the presence of fibrin is 2 orders of magnitude slower than that in its absence (Rouy and Angles-Cano, 1990; Anonick and Gonias, 1991, Lijnen and Collen, 1995a).

Protection of fibrin-bound plasmin from inhibition by α_2 -AP ensures fibrin degradation and rapid inhibition of circulating plasmin prevents systemic plasmin activity once fibrin is degraded and plasmin is released from the clot (Fig. 1.5). However, the plasma concentration of α_2 -AP is $\sim 1 \text{ }\mu\text{M}$ (Lijnen and Collen, 1986), a concentration about half that of Pg. Thus, a high systemic level of plasmin, such as that which occurs during thrombolytic therapy, has the potential to overwhelm α_2 -AP.

1.1.5v *Role of Thrombin Activatable Fibrinolysis Inhibitor in fibrinolysis*

Recently, a latent CPB-like enzyme has been identified in plasma (Eaton et al., 1991; Wang et al., 1994; Bajzar et al., 1995). Because it is activated by the thrombin/thrombomodulin complex (Bajzar et al., 1996a; Bajzar et al., 1998), it is termed

thrombin activatable fibrinolysis inhibitor (TAFI). As fibrinolysis progresses, the activated form of TAFI (TAFIa) releases the carboxy-terminal lysine and arginine residues on fibrin that are exposed by plasmin (Wang et al., 1998). In so doing, TAFIa decreases Pg, and possibly t-PA, binding to partially degraded fibrin and eliminates the increased rate of Pg activation that normally occurs during the early stages of fibrinolysis (Sakharov et al., 1997; Wang et al., 1998). TAFIa also blocks the conversion of Glu-Pg to the more readily activated Lys-Pg (Nesheim et al., 1997; Wang et al., 1998). Thus, TAFIa attenuates fibrinolysis by eliminating the upregulation of Pg activation that occurs as fibrin is partially degraded by plasmin (Fig. 1.5).

TAFI is found in human plasma at a concentration of 75 nM (Bajzar et al., 1996b). The maximal prolongation of clot lysis time produced by TAFIa is 3 to 4-fold and occurs at 10 nM TAFIa, with half-maximal effect at 1 nM TAFIa (Bajzar et al., 1996a). Thus, it is likely that circulating TAFI concentrations are sufficient to attenuate fibrinolysis *in vivo*. Furthermore, TAFIa attenuates fibrinolysis over a range of t-PA concentrations that span physiologic and pharmacologic levels (Sakharov et al., 1997), suggesting that TAFIa may also influence thrombolysis effected by pharmacologic doses of Pg activator.

The activity of TAFIa appears to be limited by intrinsic instability. Recently, it was demonstrated that TAFIa undergoes a conformational change that leads to inactivation with a half life of ~ 8 - 9 min at body temperature July 14, 2000 (Boffa et al., 1998; Boffa et al., 2000; Marx et al., 2000).

Because TAFI is activated by thrombin and attenuates fibrin degradation, it defines a molecular connection between coagulation and fibrinolysis. By attenuating fibrinolysis,

TAFIa may ensure that fibrin persists long enough for wound repair to occur.

1.2 Pharmacologic Fibrinolysis

Acute myocardial infarction (AMI) is a leading cause of death in Western societies. AMI is usually caused by a thrombus superimposed on a ruptured atherosclerotic plaque. Thrombolytic therapy, a widely used method of AMI treatment, involves pharmacologic dissolution of the thrombus by intravenous administration of Pg activators (reviewed by Pislaru and Van de Werf, 1999).

1.2.1 Streptokinase

The first Pg activator to be tested in AMI was streptokinase (SK) (Fletcher et al., 1959). SK is a 48 kDa protein produced by β -hemolytic streptococci (McClintock and Bell, 1971). Unlike t-PA, SK is not an enzyme and does not directly convert Pg to plasmin. Instead, it forms a 1:1 stoichiometric complex with Pg, an interaction that induces a conformational change in Pg that capacitates its active site causing autocatalytic conversion to plasmin (Castellino, 1979; Wang et al., 1998). Recently, it was demonstrated that high affinity binding of SK to plasmin enhances the activity of the SK-plasmin complex by exposing a new Pg recognition exosite (Boxrud et al., 2000). The conformationally modified SK-plasmin complex then activates additional Pg molecules. SK has no affinity for fibrin and does not discriminate between free and fibrin-bound Pg (Collen, 1980). Furthermore, plasmin affinity for α_2 -AP is decreased when bound to SK (Lijnen et al., 1995). Thus, administration of SK results in a systemic lytic state, commonly characterized by depletion of plasma Fg (Rao et al., 1988).

1.2.1i *Streptokinase as a thrombolytic agent*

It was not until 1986 that it became clear that coronary thrombolysis reduces mortality in AMI patients. At that time, a clinical trial that included 11,712 patients was completed. In this study, AMI patients were randomized to receive SK or no thrombolytic agent (Gruppo Italiano per lo Studio Della Streptochinasi nell'Infarto Miocardico (GISSI), 1986). At 21 days, use of SK was associated with a 2.3% absolute reduction in mortality (18% relative risk reduction, $p = 0.0002$) compared with controls. Furthermore, the extent of the beneficial effect depended on the time from onset of symptoms to the administration of SK. In the subgroup of patients treated within 3 h of the onset of pain, the absolute reduction in mortality was 3.4%. Thus, the GISSI trial was the first to establish that a) the use of Pg activators to treat AMI reduces mortality, and b) early reperfusion of occluded coronary arteries is an important determinant of clinical outcome.

There are at least two limitations associated with the use of SK. First, SK causes bleeding. In the GISSI trial, use of SK was associated with a 0.5% absolute increase in major bleeding events (defined as need for transfusion of at least two units of blood) and a 3.2% increase in minor bleeding events. Second, because SK is bacterially derived, it is immunogenic. In the GISSI trial, 2.3 and 0.1% of patients given SK experienced allergic reactions and anaphylactic shock, respectively. Furthermore, most patients developed neutralizing antibodies to SK that persisted for several months and precluded readministration of the activator (Jennings, 1996). These limitations prompted the development of other activators for use in AMI patients.

1.2.2 t-PA as a thrombolytic agent

t-PA (or alteplase), a second generation thrombolytic agent, was developed to

overcome the limitations of SK. Because t-PA binds fibrin, it was hoped that t-PA would target occlusive thrombi and achieve reperfusion more rapidly than SK. Due to its fibrin-specificity, it was anticipated that t-PA would produce less systemic Pg activation and Fg depletion than SK, and this would translate into less bleeding. Finally, because t-PA is an endogenous activator, it was thought that t-PA would reduce the complications associated with the immunogenicity of SK.

In 1993, the Global Utilization of Streptokinase and Tissue Plasminogen Activator for Occluded Coronary Arteries (GUSTO) trial demonstrated that, when administered with aspirin and heparin, t-PA provides a survival benefit over SK (GUSTO Investigators, 1993). Use of t-PA was associated with a 1% reduction in mortality (14% relative risk reduction, $p = 0.001$) when compared with SK. In addition, significantly fewer allergic and anaphylactic reactions occurred with t-PA (absolute reductions of 4.2 and 0.4%, respectively, $p < 0.001$ for both) compared with SK. However, the requirement of red blood cell transfusion was only moderately lower with t-PA compared with SK (5.1 and 5.8 %, respectively, $p = 0.04$) and the rate of hemorrhagic stroke, although low in both groups, was higher with t-PA than with SK (0.72 vs. 0.54 %, respectively, $p = 0.03$). Thus, this study confirmed the beneficial effect of t-PA over SK, but called into question the relationship between fibrin-specificity of the activator, systemic Pg activation, and bleeding. In fact, results from the GUSTO trial raised the possibility that Pg activators with higher fibrin-specificity may increase the risk of intracranial hemorrhage.

1.2.2i *Mechanism of t-PA-induced bleeding*

Despite the increased fibrin-specificity of t-PA relative to SK, t-PA causes systemic

plasmin formation and Fg consumption when given to patients (Bell, 1997; Stangl et al., 1998). An explanation for this phenomenon is derived from the observation that (DD)E potentiates t-PA-mediated activation of Pg to the same extent as fibrin (Weitz et al., 1991). Thus, because it is soluble, (DD)E generated during lysis of thrombi has the potential to promote systemic plasmin generations. However, the systemic lytic state induced by t-PA in patients is not as severe as that caused by SK. Although Fg consumption is variable, it is consistently greater with SK than with t-PA (Bell, 1997; Pislaru and Van de Werf, 1999). Because t-PA causes the same rate of major bleeding and a higher rate of intracranial hemorrhage than SK, these observations seem inconsistent with a link between Fg degradation and bleeding.

When plasma samples from patients given t-PA for the treatment of AMI were subjected to polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis, Owen and colleagues (1987) found that Fg was largely degraded to fragment X, an early plasmin-mediated degradation product of Fg that remains clottable (Fig. 1.3). Furthermore, fragment X persisted in the circulation for at least 24 h. In contrast, in patients given SK, less fragment X was detected in plasma as Fg was rapidly degraded to smaller, nonclottable fragments Y, D and E. This finding is consistent with the concept that systemic plasmin concentrations are higher with SK than t-PA.

Weisel et al. (1994) have demonstrated that clots formed from fragment X polymerize more slowly than those formed from Fg. When compared at 0.5 mg/ml, fragment X increases the clotting time ~ 7-fold over Fg. The degree to which fragment X prolongs clotting time depends on the integrity of the β chain (Siebenlist et al., 1990). In addition to

polymerizing more slowly, fragment X clots are more susceptible to lysis than fibrin clots (Weitz et al., 1993). Fragment X decreases t-PA-induced clot lysis times in a concentration-dependent fashion. The mechanism for increased clot lysis likely involves both an increase in the rate of plasmin formation and a decrease in mechanical stability of a fragment X clot compared with that of a fibrin clot (Weisel et al., 1994; Private Communication from Amy Lazier: Dept. of Medical Sciences, McMaster University).

Plasma levels of Fg are commonly measured using a clotting method (so called Clauss assay), which determines total clottable protein (von Clauss, 1957). Because fragment X remains clottable, Fg levels measured in AMI patients likely includes fragment X. Thrombi are dynamic structures that undergo continuous formation and degradation (Nossel, 1981; Francis and Marder, 1994), raising the possibility that fragment X is incorporated into hemostatic plugs, thereby increasing their susceptibility to lysis. If this hypothesis is correct, bleeding should be diminished if t-PA-induced Fg degradation is attenuated. Two lines of evidence support this concept. The first involves inhibition of circulating plasmin with α_2 -AP and the second employs b-PA, an activator that is more fibrin-specific than t-PA.

Because fibrin-bound plasmin is partially protected from inhibition, supplemental α_2 -AP selectively blocks t-PA-mediated fibrinogenolysis without adversely affecting thrombolysis (Paramo et al., 1990; Weitz et al., 1993). In a rabbit jugular vein thrombolysis model, infusion of α_2 -AP prior to administration of t-PA produced only a modest reduction in thrombolysis (from 40% to 30%, $p = 0.12$), but a marked attenuation of Fg consumption (from 87% to 27%, $p < 0.0001$) and fragment X formation (Weitz et al., 1993). When

challenged with ear incisions, rabbits receiving supplemental α_2 -AP with t-PA experienced 8.5-fold less blood loss than those given t-PA alone ($p < 0.0001$) (Weitz et al., 1992).

In the same animal model, thrombolysis and bleeding induced by t-PA has been compared with that caused by b-PA (Klement et al., 1995; Weitz, 1995). When given doses that produce equivalent thrombolysis, b-PA caused significantly less fibrinogenolysis than t-PA (17% and 67%, respectively, $p < 0.0001$). Furthermore, less fragment X was detectable in b-PA-treated animals. When challenged, rabbits given b-PA experienced 10.2-fold less blood loss than those given t-PA. Several other studies using rabbits or rats with have demonstrated that b-PA causes less Fg degradation and bleeding than t-PA (Gardell et al., 1991; Witt et al., 1992; Muschick et al., 1993; Mellott et al., 1995). Taken together, these studies suggest that there is a link between Pg activator-induced fibrinogenolysis and bleeding.

Thus, t-PA has limitations as a thrombolytic agent. First, t-PA is not as fibrin-specific as anticipated, causes significant Fg degradation, and does not reduce the risk of bleeding. Second, because of its short half-life, t-PA must be given as an infusion over 1.5 h. Because time to treatment is the largest therapeutic determinant of AMI outcome, development of a thrombolytic agent with a sufficiently long half-life, enabling bolus out-of-hospital administration, is desirable. The limitations of t-PA have prompted the development of t-PA variants as third generation thrombolytic agents. One such agent is TNK-t-PA.

1.2.3 TNK-t-PA

TNK-t-PA (or tenecteplase) is a genetically engineered variant of t-PA (Fig. 1.6) that has a longer half-life, increased resistance to PAI-1, and increased fibrin-specificity

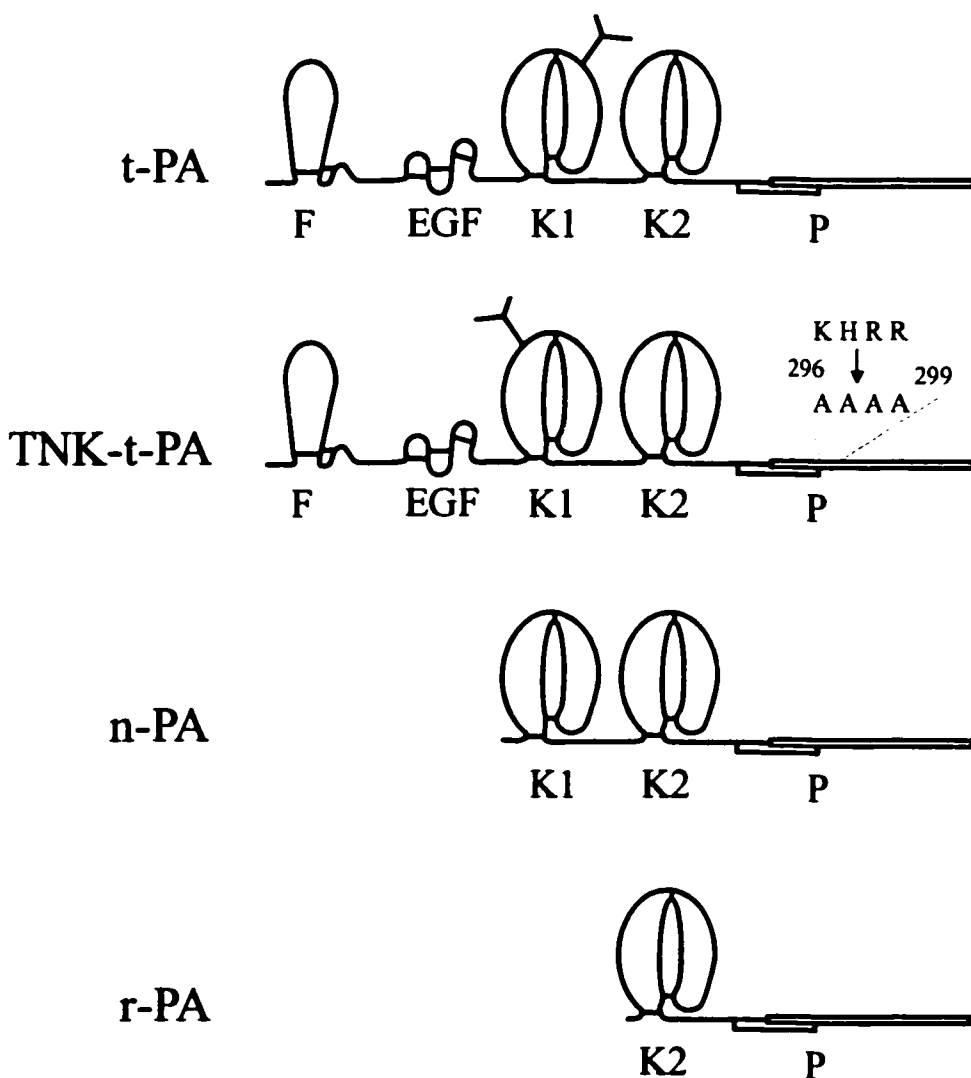


Figure 1.6 Comparison of the secondary structure of t-PA with t-PA variants. The domains of t-PA are defined in figure 1.1. TNK-t-PA differs from t-PA in that glycosylation within the first kringle is shifted 14 amino acids upstream and a tetra-alanine substitution (KHRR296-299AAA) is made in the protease domain. Lanoteplase (n-PA) is a truncated variant of t-PA missing the F and EGF domains. In addition, the glycosylation site within K1 is eliminated by the point mutation N117Q. Reteplase (r-PA) is a deletion variant of t-PA consisting of only the second kringle and protease domains.

agents were equally potent at lysing whole-blood clots, K-t-PA was 2.5-fold more potent at degrading platelet-rich clots, presumably because PAI-1, released from platelets, preferentially inhibited native t-PA. However, in this same model, T-t-PA and TK-t-PA were 2.4- and 4.2-fold more potent than t-PA, respectively, against platelet-rich clots, suggesting that mutations other than the protease change can increase the potency of the activator. Other studies have confirmed the observation that TNK-t-PA is more effective than t-PA at degrading platelet-rich clots (Paoni et al., 1993b; Graham et al., 1998), but no other study has demonstrated which mutation(s) contribute(s) to this phenomenon. Furthermore, TNK-t-PA is only moderately more effective at lysing whole-blood clots than t-PA. Thus, contribution of PAI-1 resistance to thrombolysis is questionable.

1.2.3i *TNK-t-PA as a thrombolytic agent*

TNK-t-PA has been compared with t-PA for treatment of AMI in a double-blind, randomised controlled trial that enrolled 16,949 patients (ASSENT-2 Investigators, 1999). The primary efficacy endpoint, 30-day mortality rate, was not different with TNK-t-PA from that with t-PA (6.18 and 6.15%, respectively, $p = 0.99$). These results are not surprising given that the activators are equipotent thrombolytic agents. Although the intracranial hemorrhage rate with TNK-t-PA was similar to that with t-PA (0.93 and 0.94 %, respectively, $p = 1.0$), patients given TNK-t-PA experienced fewer major bleeding episodes (requiring blood transfusion) than those given t-PA (4.66 and 5.94 %, respectively, $p = 0.0002$). The decreased incidence of bleeding was attributed to the increased fibrin-specificity of TNK-t-PA relative to t-PA.

As intended, TNK-t-PA has advantages over t-PA. Although the two agents are

equally effective, TNK-t-PA causes less bleeding. In addition, because of its long half-life, TNK-t-PA is easier to administer than t-PA. TNK-t-PA is given as a single bolus, instead of an infusion over 1.5 h (Cannon et al., 1997). This characteristic may facilitate prehospital treatment of AMI which, in turn, may improve outcome.

With a better understanding of its mechanism of action, it may be possible to improve on the fibrin-specificity of TNK-t-PA. Whether this would result in increased safety would have to be established in clinical trials.

1.2.4 Other derivatives of t-PA

1.2.4i *Retepase*

Retepase (r-PA) is a recombinant truncated t-PA variant comprised of the K2 and protease domains (Fig. 1.6) (Bode et al., 1995). Because r-PA is expressed in *Escherichia coli*, it is not glycosylated. The absence of carbohydrate side chains endows r-PA with a longer half-life than t-PA. With a half-life of about 15 min, r-PA is administered as two intravenous boluses, given 30 min apart (Noble and McTavish, 1996). Thus, r-PA is easier to administer than t-PA.

Because it lacks a finger domain, r-PA has 3- to 4-fold lower affinity for fibrin than t-PA, and its catalytic efficiency in the presence of fibrin is half that of t-PA (Kohnert et al., 1992; Kohnert et al., 1993). Furthermore, r-PA is 6.4-fold less potent than t-PA at lysing plasma clots (Martin et al., 1993). Although the affinity of r-PA for Fg or plasmin-derived degradation products of fibrin has yet to be determined, r-PA is stimulated by these soluble cofactors to the same extent as t-PA (Kohnert et al., 1993). With lower affinity for fibrin than t-PA, it is not surprising that r-PA is less fibrin-specific than t-PA and produces a

greater reduction in plasma levels of α_2 -AP and Fg (Smalling et al., 1998).

r-PA has been compared with t-PA for treatment of AMI in a randomised controlled trial that included 15,059 patients (GUSTO III Investigators, 1997). Although the 30 day mortality rate with r-PA was not significantly different from that with t-PA (7.47 and 7.24 %, respectively, $p = 0.54$), the trend was towards fewer deaths with t-PA. Rates of intracranial hemorrhage were similar with r-PA and t-PA (0.91 and 0.87 %, respectively, $p = 0.97$), and there was no difference in red blood cell transfusions (6.9 and 6.8 % for r-PA and t-PA, respectively, $p = 1.0$) With no greater effectiveness or safety, the advantage of r-PA over t-PA is restricted to ease of administration.

1.2.4ii *Lanoteplase*

Lanoteplase (n-PA) is a t-PA deletion mutant lacking the finger and epidermal growth factor domains (Fig. 1.6) (Larsen et al., 1991; den Heijer et al., 1998). Furthermore, the glycosylation site on K1 of n-PA is deleted by replacement of asparagine at position 117 with glutamine. Because it is expressed in Chinese hamster ovarian cells, n-PA retains the complex oligosaccharides present at position 184 and 448 on t-PA. The half-life of n-PA is ~30 min, likely because n-PA lacks the high mannose carbohydrate on K1, permitting administration via a single intravenous bolus (Smalling, 1996; den Heijer et al., 1998).

Because it lacks a finger domain, n-PA binds fibrin with ~5-fold lower affinity than t-PA (Nesheim et al., 1990). Consequently, ~3-fold higher concentrations of n-PA are required to produce clot lysis equivalent to that of t-PA (Larsen et al., 1988; Hansen et al., 1988). n-PA is stimulated by CNBr fragments of Fg to the same extent as t-PA (Hansen et al., 1988).

In a Phase II dose-finding study comparing n-PA with t-PA in patients with AMI (n = 602), the agents produced similar 90 min patency rates of the infarct-related coronary artery (den Heijer et al., 1998). However, data from a Phase III trial, conducted with 15,078 patients with AMI, has recently been presented that suggest n-PA is not as safe as t-PA (Neuhaus, 1999). The 30 day mortality rate with n-PA was similar to that with t-PA (6.77 and 6.60 %, respectively, $p = 1.0$). However, n-PA produced significantly more bleeding. Thus, patients given n-PA had higher rates of intracranial hemorrhage than those given t-PA (1.13 and 0.62 %, respectively, $p = 0.003$) and higher total bleeding rates (combined major and minor bleeding was 22 and 17 % for n-PA and t-PA, respectively, $p = 0.0001$). Given these data, it is unlikely that n-PA will be approved for clinical use.

Although no Pg activator has been proven to be more effective than t-PA for treatment of AMI, clinical trials with TNK-t-PA, as well as animal studies with b-PA, suggest that Pg activators that are more fibrin-specific than t-PA cause less bleeding. The fibrin-specificity of t-PA is compromised because (DD)E stimulates systemic activation of Pg. Understanding the mechanism by which (DD)E compromises the fibrin-specificity of t-PA may lead to the development of safer thrombolytic agents.

1.3 Overall Objective

The overall objective of this thesis was to investigate the mechanism by which (DD)E promotes t-PA-mediated Pg activation so as to (a) reveal new insights into the biochemistry of Pg activation and (b) identify directions for the development of more fibrin-specific thrombolytics.

1.3.1 Specific objectives

1: To quantify the binding of t-PA and Pg to Fg, fibrin, and (DD)E. Because b-PA lacks a K2 domain, b-PA also was investigated to distinguish the role of the K2 domain of t-PA in binding Fg, fibrin, and (DD)E.

2: To compare the ability of Fg, fibrin, and (DD)E to stimulate TNK-t-PA-mediated Pg activation with their ability to stimulate t-PA. In addition, the affinity of TNK-t-PA for Fg, fibrin or (DD)E was determined and compared with that of t-PA. These studies were done to gain insight into the mechanism responsible for the increased fibrin-specificity TNK-t-PA over t-PA. To help distinguish which mutation or combination of mutations within TNK-t-PA contributes to its increased fibrin-specificity over t-PA, the ability of Fg, fibrin, and (DD)E to stimulate and bind T-, K-, TK-, and NK-t-PA also was investigated.

3: To characterize the effects of TAFIa or CPB on the ability of (DD)E to stimulate Pg activation by t-PA and to bind t-PA or Pg. Because TAFIa and CPB remove carboxy-terminal lysine residues, and both t-PA and Pg bind (DD)E in a kringle-dependent fashion, TAFIa and CPB were used as tools to better define the interactions of t-PA and Pg with (DD)E.

CHAPTER 2 - EXPERIMENTAL PROCEDURES

Preface

This chapter provides a detailed compilation of the experimental procedures outlined in Chapters 3, 4, and 5 as well as a description of the procedures used to obtain the data presented in the Appendices.

2.1 Materials

2.1.1 Plasminogen activators

Wild-type recombinant t-PA (activase), TNK-t-PA, and its variants T-, K-, TK-, and NK-t-PA were provided by Dr. B. Keyt (Genentech Inc., S. San Francisco, CA). Recombinant b-PA (DSPA α_1) was a generous gift from Dr. W. Witt (Schering AG., Berlin, Germany). t-PA, TNK-t-PA and its variants were found to be $\geq 90\%$ single-chain species and b-PA was 100% single-chain, as determined by SDS-polyacrylamide gel electrophoresis (PAGE) analysis (Laemmli, 1970) on 4-15 % gels (Ready-Gel, Bio-Rad, Mississauga, ON). Protein band densities were analyzed by laser densitometry (Ultrosan XL, LKB-Pharmacia, Baie d'Urfe, PQ) or using an ImageMaster Video Documentation System (Amersham-Pharmacia Biotech Inc., San Francisco, CA). Active site-blocked, fluorescently labeled derivatives of the Pg activators were prepared by adding 1 ml of 0.05 M sodium pyrophosphate, 0.15 M NaCl, 0.5 M (NH₄)₂SO₄, pH 7.2 to 1 ml of a 2 mg/ml stock enzyme solution followed by incubation with a 5-fold molar excess of dansyl glutamyl-glycyl-arginine chloromethyl ketone (dEGR-CMK, Calbiochem, San Diego, CA) at 22 °C (Higgins

and Lamb, 1986). The residual activity of the active-site blocked Pg activators was evaluated by measuring their ability to hydrolyse the chromogenic substrate N-methylsulfonyl-D-phenyl-glycyl-arginine-*p*-nitroanilide-acetate (Chromozyme t-PA, Boehringer Mannheim, Laval, PQ). t-PA activity was abolished after 1 h incubation with dEGR-CMK, whereas 2 to 3 h incubations were needed to block the activity of activators containing the K mutation or b-PA. In all cases, activators were then dialysed against the pyrophosphate-containing buffer overnight at 4°C, and protein concentrations were determined by measuring absorbance at 280 and 335 nm. Absorbance at 335 nm was attributed to dansyl group absorbance and light scattering. Iteration was used to distinguish these phenomena (Nesheim et al., 1990). The absorbance at 280 nm was used for an initial estimate of protein concentration. This concentration was used to subtract the contribution of dansyl fluorescence from the reading at 335 nm, using a millimolar extinction coefficient of dansyl of 4.01 (Collen et al., 1988; Nesheim et al., 1990). The corrected 335 value was then multiplied by $(335/280)^4$ to correct the 280 value for scattering and to obtain a new estimate of protein concentration. This process was repeated until the protein estimate remained unchanged. Typically, three or four iterations yielded a stable result. Based on calculations of protein concentration, 80-95% of the Pg activators was recovered after dialysis against pyrophosphate buffer. Active-site blocked, unlabeled derivatives of the Pg activators were prepared by the same procedure except D-phenyl-prolyl-arginine chloromethyl ketone (PPACK, Calbiochem) was used in place of dEGR-CMK. Under these conditions, t-PA activity was abolished after 30 min incubation with PPACK, whereas a 1 to 2 h incubation was needed to block the activity of activators containing the K mutation or b-PA. The

protein concentrations of active site blocked, unlabeled derivatives were determined by measuring absorbance at 280 and 320 nm to distinguish tryptophan and phenylalanine absorbance from light scattering (Bloom et al., 1979). Immediately prior to use, a 1 ml volume of the Pg activator was dialysed against 2 L of 0.02 M Tris-HCl, 0.15 mM NaCl, 0.01% Tween-20, pH 7.4 (TBS) for 3 h with vigorous stirring, and then centrifuged at $12000 \times g$ for 7 min at 22°C in a microfuge to remove any aggregated material. Based on calculations of protein concentration, dialysis against TBS resulted in a 40-60% loss of t-PA, TNK, and TNK variants and a 30-40% loss of b-PA. The molecular weights and extinction coefficients used were 65,000 and $\epsilon_{1\%}^{280}=20.0$ for t-PA, TNK-t-PA, and its variants (Nesheim et al., 1990; Paoni et al., 1993b; Benedict et al., 1995), and 54,500 and $\epsilon_{1\%}^{280}=17.1$ for b-PA (Bringmann et al., 1995).

2.1.2 Plasminogen

Native Glu-Pg was isolated from freshly frozen plasma by lysine-Sepharose affinity chromatography (Castellino and Powell, 1981). Approximately 100 ml of lysine-Sepharose (Pharmacia Biotech Inc., Baie d'Urfe, PQ), packed into a 2.5 cm diameter chromatography column, was thoroughly washed with 0.3 M sodium phosphate, pH 8.0, followed by 40 mM EACA, pH 8.0 and equilibrated with TBS. Human plasma (~1 L) was loaded onto the resin at room temperature. The resin was then washed with 0.1 M sodium phosphate, pH 8.0, until the filtrate had an absorbance at 280 nm equivalent to that of the buffer, followed by 20 mM tris-Cl, pH 8.0. The bound material was then eluted directly onto a DEAE Fast-Flow column (1 × 20 cm) using 10 mM EACA, 20 mM Tris-Cl, pH 8.0. The DEAE column was washed with five column volumes of 20 mM Tris-Cl, pH 8.0, to remove the EACA, and Glu-Pg was

eluted with a 0-200 mM linear NaCl gradient in 20 mM Tris, pH 7.4. Glu-Pg was concentrated with 80% ammonium sulfate with subsequent solubilization and dialysis against TBS or by ultrafiltration using a Centriprep 30 concentrator fitted with a 30,000 MW cut-off membrane (Amicon Inc., Beverly, MA). As determined by urea/acetic acid PAGE analysis (Fredenburgh and Nesheim, 1992), isolated Glu-Pg was free of Lys-Pg and contained no plasmin activity as assessed using the plasmin-directed substrate D-valyl-leucyl-lysine *p*-nitroanilide dihydrochloride (S-2251; Chromogenix, Mississauga, ON). Glu-Pg concentrations were calculated by measuring absorbance at 280 and 320 nm and using a molecular weight of 90,000 and $\epsilon_{1\%}^{280} = 16.1$ (Castellino and Powell, 1981). Lys-Pg was purchased from Enzyme Research Laboratories Inc. (South Bend, IN). Lys-Pg was free of Glu-Pg and contained no plasmin, determined as described above.

2.1.3 Fibrinogen

Human Fg and Pg-depleted Fg were purchased from Enzyme Research Laboratories Inc. (South Bend, IN), and dissolved in TBS. In the case of Fg not previously depleted of Pg, residual Pg was removed by incubating the Fg (2 mg/ml) with 10 ml of lysine-Sepharose for 30 min at 22° C. After centrifugation at 3000 × *g* for 10 min at 22° C, the supernatant was incubated for 30 min at 22° C with 6 ml of gelatin-agarose (Sigma, Oakville, ON) to remove fibronectin, followed by centrifugation at 3000 × *g* for 10 min at 22° C. For factor XIII (FXIII)-free Fg, FXIII was removed by affinity chromatography using a sheep anti-FXIII IgG (Affinity Biologicals, Hamilton, ON) coupled to Affi-Gel (Bio-Rad), at a concentration of 7.4 mg IgG / ml gel. The Fg was then precipitated with 25 % ammonium sulfate. The pellet was then washed with 25 % ammonium sulfate, redissolved and dialyzed against TBS. For

clot lysis assays, Fg was trace labeled with ^{125}I using Iodo-beads (Pierce, Rockford, IL) (McFarlane, 1965) to a specific activity of $100 \pm 5 \mu\text{Ci/mg}$. Final Fg concentrations were calculated by measuring absorbance at 280 and 320 nm and using a molecular weight of 340,000 and $\epsilon_{1\%}^{280}=16.0$ (Dellenback and Chien, 1970).

2.1.4 (DD)E

The soluble fibrin fragment, (DD)E, was prepared by plasmin-mediated lysis of a crosslinked fibrin clot. Fg (100 mg) in TBS, was clotted with 64 nM thrombin (Enzyme Research Laboratories) and 10 mM CaCl_2 in the presence of 93 nM activated recombinant factor XIII (a generous gift from Dr. P. Bishop, Zymogenetics, Inc., Seattle, WA), 0.4 μM Glu-Pg, and 2 pM or 4.3 nM t-PA. Clotting occurred within 30 s. Using 2 pM t-PA, fibrin was completely degraded after 55 h, whereas with 4.3 nM t-PA, the fibrin clot was degraded after 20 min. In both cases, the reaction was terminated by the addition of 5 μM D-valyl-phenyl-lysine chloromethyl ketone (VFKCK, Calbiochem) to block plasmin activity, and 1 μM PPACK to block both t-PA and thrombin activity. The clot lysate was then concentrated to a 2 ml volume by ultrafiltration using a Centriprep 10 or Centriprep 30 concentrator fitted with a 10,000 or 30,000 MW cut-off membrane, respectively (Amicon). After removing aggregates by centrifugation at $12,000 \times g$ for 5 min, the fibrin degradation products were isolated by passing the material over a Biosep-Sec-S3000 size exclusion column (Phenomenex, Torrance, CA) fitted to a liquid chromatograph (System Gold; Beckman Instruments Inc., Palo Alto, CA) equipped with two model 126 solvent delivery systems, and a model 506 automatic injector. Presence of protein was determined with a model 167 variable wavelength absorbance detector set at 280 nm. Peak protein-containing fractions

were pooled and subjected to PAGE on 4-15% non-denaturing gels. (DD)E-containing fractions were identified based on their apparent molecular weight and by immunoblot analysis using antibodies against DD and E (Weitz et al., 1991). (DD)E concentrations were calculated by measuring absorbance at 280 and 320 nm using a molecular weight of 250,000 and $\epsilon_{1\%}^{280}=16.0$ (Olexa and Budzynski, 1979b). When (DD)E was incubated with 10 mM H-Gly-Pro-Arg-Pro-OH (Calbiochem) prior to non-denaturing PAGE analysis, two lower molecular weight bands were apparent, corresponding to DD and E, respectively.

2.1.5 CPB and TAFIa

CPB, potato tuber-derived CPB inhibitor (CPI), and the CPB-directed synthetic substrate hippuryl-L-arginine were purchased from Sigma. CPB activity was assessed by incubating 20 nM CPB with 0.4 mM hippuryl-L-arginine dissolved in TBS in a quartz cuvette. Increases in absorbance at 254 nm were monitored for 20 min at 22 °C using a DU 7400 Spectrophotometer from Beckman (Mississauga, Canada). Under these conditions, CPB has a specific activity of 41 units / mg, where one unit hydrolyzes 1 μ mol hippuryl-L-arginine / min. When the experiment was repeated in the presence of 1 μ M CPI, the lowest concentration used to inhibit CPB prior to Pg activation assays, no increase in A_{254} was observed, indicating complete CPB inhibition. TAFIa was kindly provided by Dr. L Bajzar (Hamilton Civic Hospitals Research Centre, Hamilton, ON). The specific activity of TAFIa against hippuryl-L-arginine was similar to that of CPB. Because TAFIa activity is unstable at room temperature (Boffa, et al., 2000; Marx, et al. 2000), TAFIa was used immediately, or kept on ice and used within 2 h of activation. Like CPB, the activity of 20 nM TAFIa was completely inhibited by 1 μ M CPI. CPB was used for the majority of experiments because

its activity is more stable than that of TAFIa and it does not require preactivation. To demonstrate that TAFIa has effects similar to CPB, however, confirmatory experiments were done using TAFIa.

2.2 Methods

2.2.1 Quantification of Pg activator-induced fibrin(ogen)olysis in the absence or presence of plasma clots

To assess the fibrinolytic potential of t-PA, TNK-t-PA, variants of TNK-t-PA, and b-PA, these agents were incubated with ^{125}I -labeled plasma clots for 1 h and the residual radioactivity in the clot was used as a measure of clot lysis. To assess their fibrinogenolytic potential, these activators were incubated in plasma for 1 h in the absence or presence of a plasma clot and B β 1-42 levels were determined as a sensitive index of plasmin-mediated Fg degradation. Comparisons of the fibrinolytic and fibrinogenolytic potentials of the activators were used as a measure of their fibrin-specificities.

2.2.1i Preparation of platelet-poor plasma

Blood was collected from the antecubital veins of 10 to 15 healthy volunteers into 1/10 volume of 3.8 % sodium citrate. After sedimenting the red cells by centrifugation at $2000 \times g$ for 20 min at 4 °C, harvested platelet-poor plasma was collected, pooled and stored in aliquots at -70 °C until needed.

2.2.1ii Preparation of ^{125}I -labeled plasma clots

Thawed plasma, supplemented with ^{125}I -labeled fibrinogen (approximately 60,000 cpm/ml), was clotted with Thromborel S (DADE International Inc, Miami, FL) to 0.1 % v/v and calcium chloride to 15 mM and rapidly dispensed in 500 μl aliquots into 1.5 ml

polypropylenemicrocentrifuge tubes each containing a plastic inoculation loop (Weitz et al., 1991). Clots formed around these loops were incubated for 60 min at 37 °C and subjected to 3 washes, each with 2 ml of TBS. Washed clots were then counted for radioactivity for 1 min using a Clinigamma counter (LKB Instruments, Inc., Gaithersburg, MD). Based on SDS-PAGE analysis, clots formed in this fashion are cross-linked because no free α - or γ -chains are detected (Francis et al., 1980; Weitz et al., 1991).

2.2.1iii *Quantification of fibrinolysis and BB1-42 generation in the absence or presence of plasma clots*

t-PA, TNK-t-PA, a TNK-t-PA variant or b-PA in concentrations ranging from 0 to 60 nM was incubated for 60 min at 37 °C in 500 μ l aliquots of plasma in the absence or presence of a washed 125 I-labeled plasma clot and clot lysis and fibrinogenolysis were measured as follows. (a) *Clot lysis*. At the end of the incubation period, clots were removed, washed once with 1 ml TBS and counted for residual radioactivity for 1 min. The extent of clot lysis was calculated by subtracting the residual radioactivity from the initial amount and expressing this value as a percentage of initial radioactivity. (b) *Fibrinogenolysis*. The extent of fibrinogenolysis in the absence or presence of a clot was determined by measuring the levels of BB1-42 and Fg in the plasma after the 60 min incubation with the plasminogen activator. For BB1-42 analysis, 100 μ l of plasma was removed and unreacted Fg was precipitated by addition of 300 μ l ethanol. After a 30 min incubation on ice and centrifugation at 15,000 \times g for 4 min, the ethanol supernatants were evaporated to dryness in a Speedvac concentrator (Savant Instruments Inc, Farmingdale, NY) and reconstituted to original volume with distilled water. BB1-42 levels were determined by

radioimmunoassay using an antibody that does not cross-react with fibrinopeptide B or BB15-42 (Weitz et al., 1986; Weitz et al., 1991). For Fg determination, an additional 100 μ l aliquot of plasma was removed and 100 KIU/ml aprotinin (American Diagnostica Inc, Greenwich, CT) was added to inhibit plasmin activity. These samples were frozen at -70°C and later assayed for Fg using the Clauss method (von Clauss, 1957).

2.2.2 Characterization of the binding of Pg activators or Pg to fibrin, (DD)E, Fg, lysine, or EACA

2.2.2i Fluorescent and light scattering measurements

For the purposes of measuring binding of Pg activators or Pg to various cofactors, all fluorescence and light scattering intensities were measured in a LS50B luminescence spectrometer (Perkin-Elmer, Etobicoke, ON) using a cuvette thermostated at 22°C . Fluorescence measurements were performed in a 1 ml quartz microcuvette and right angle light scattering measurements were made in a 3 ml quartz cuvette with stirring. To measure the fluorescence of individual samples, three fluorescence intensity readings, each recorded over a 3 s integration time, were averaged. Scattering intensities were continuously monitored in time drive with the interval time set at 1 or 2 s, and the response time at 2 or 3 s. Intensity values were determined by averaging scattering intensities observed over a period of at least 100 s. Thus, each scattering intensity value represents the mean of 50 to 100 individual readings.

2.2.2ii Binding of Pg activators or Pg to fibrin

The binding of dEGR-labeled t-PA, TNK-t-PA, TNK-t-PA variant or b-PA to fibrin was determined by adding increasing concentrations of Pg activator to a series of microfuge

tubes (Sarstedt 72.702) containing fixed amounts of Fg in TBS containing 2 mM CaCl_2 . A 10 μl aliquot of thrombin (final concentration, 10 nM) was then added to induce clotting. The final reaction volume was 200 μl . After incubation at 22 °C for 1 h, the clots were vortexed and centrifuged at $12000 \times g$ for 2.5 min to compact the fibrin into the 10 μl tip of the microtube. The fluorescence intensity of 150 μl of clot supernatant in 350 μl of Tris buffer was measured with $\lambda_{\text{ex}} = 280 \text{ nm}$, $\lambda_{\text{em}} = 530 \text{ nm}$, a 515 nm cut-off filter, and 15 nm slit widths. A parallel titration was done in the absence of thrombin to establish a standard curve for each ligand. The binding of Lys-Pg or Glu-Pg to fibrin was determined using the same procedure except unbound Pg was quantified by measuring its intrinsic fluorescence and the standard curve of Pg concentrations was established in the absence of Fg. Clots were also formed in the absence of Pg and the intrinsic fluorescence of unclotted material in the supernatants were determined and subtracted from values obtained in the presence of Pg. The conditions for measuring intrinsic fluorescence include $\lambda_{\text{ex}} = 280 \text{ nm}$, $\lambda_{\text{em}} = 340 \text{ nm}$, a 290 nm cut-off filter, and slit widths set to 2.5 nm.

For analysis of fibrin binding, the fluorescence intensities of the supernatants were used to calculate the concentrations of unbound proteins by comparison with fluorescence intensities of known concentrations of protein. The concentrations of bound proteins were determined by calculating the difference between the total and unbound protein concentrations. These values were divided by the Fg concentration to determine the number of moles of dEGR-labeled Pg activator, Lys-Pg, or Glu-Pg bound per mole of fibrin (B). For each point in the titration, these values were then plotted against the concentration of unbound protein. Scatchard plots also were constructed, and if these appeared linear,

reflecting a single class of binding sites, the binding isotherm was analysed by nonlinear regression analysis (Table Curve, Jandel Scientific, San Rafael, CA) of the relationship,

$$B = \frac{n \cdot L}{K_d + L} \quad (\text{equation 1})$$

where [L] is the concentration of unbound protein, n is the stoichiometry, and K_d is the dissociation constant. Scatchard plots that were not linear were curved downward. For analysis of these data, the binding isotherms were best fit to a two-site model by nonlinear regression analysis (Table Curve, Jandel Scientific) according to the expression,

$$B = \frac{n_1 \cdot L}{K_{d1} + L} + \frac{n_2 \cdot L}{K_{d2} + L} \quad (\text{equation 2})$$

The effect of EACA on the binding of dEGR-t-PA, dEGR-b-PA, Glu-Pg or Lys-Pg to fibrin was determined by repeating the fibrin titrations in the presence of 20 mM EACA. In addition, clots formed by incubating 2 μ M Fg with 10 nM thrombin in the presence of 0.8 μ M dEGR-t-PA, dEGR-b-PA, Glu-Pg or Lys-Pg were titrated with EACA (in concentrations ranging from 0 to 20 mM) and the amount of ligand displaced was determined by measuring the concentration of unbound protein in the clot supernatant as described above.

To determine whether t-PA and b-PA compete for the same fibrin binding sites, various concentrations of unlabeled, active-site blocked b-PA or t-PA, with or without 20

mM EACA, were added to a series of microfuge tubes charged with 2 μ M Fg and 0.8 μ M dEGR-t-PA or dEGR-b-PA. Thrombin (10 nM) was added, and after incubation for 60 min at 22°C, fibrin was pelleted by centrifugation. The amount of unbound fluorescently-labeled enzyme in the supernatant was then compared with that found in control samples prepared in the absence of thrombin.

2.2.2iii *Binding of Pg activators or Pg to (DD)E or Fg*

The binding of t-PA, TNK-t-PA, TNK-t-PA variants, b-PA, Glu-Pg, or Lys-Pg to Fg or (DD)E was studied using solution-phase titrations. Interactions were monitored using right angle light scattering spectroscopy where the solution was excited at a fixed wavelength ($\lambda=400$ or 440 nm) and emission intensities were measured at the same wavelength with both excitation and emission slit widths set to either 8 or 12 nm. In the case of Fg, aliquots (5 or 10 μ l) of 15 μ M Fg were added to 2 ml of 0.1 μ M active site blocked Pg activator, or 0.3 μ M Glu-Pg or Lys-Pg. Control titrations were done to determine the intensity of light scattering of Fg alone. In the case of (DD)E, aliquots (5 or 10 μ l) of 5 μ M (DD)E were added to 2 ml of 0.1 μ M active site blocked Pg activator. Interactions of Glu-Pg and Lys-Pg with (DD)E were monitored in a similar fashion except 0.1 μ M (DD)E was titrated with 80 or 100 μ M Glu-Pg or 5 μ M Lys-Pg. To ensure that none of the target proteins was undergoing self-association, the light scattering intensity of active site blocked Pg activator, (DD)E, Glu- or Lys-Pg (in concentrations ranging from 0.05 μ M to 0.5 μ M) was monitored over a 30 min period under the conditions outlined for the binding experiments. In each case, there was no change in scattering intensity over time, indicating that the target proteins were not aggregating. For analysis of the binding data, the emission intensity after each addition of

ligand was corrected for changes because of ligand scattering and dilution (I) and compared to the emission intensity of the target alone (I_0) and these data were fit by nonlinear regression analysis (Table Curve, Jandel Scientific) to the equation,

$$\frac{I}{I_0} = 1 + \frac{\alpha}{2} \left(1 + \frac{K_d + L_0}{n \cdot P_0} - \sqrt{\left(1 + \frac{K_d + L_0}{n \cdot P_0} \right)^2 - 4 \cdot \frac{L_0}{n \cdot P_0}} \right) \quad (\text{equation 3})$$

where L_0 is the total concentration of ligand added, P_0 is the concentration of target protein, K_d is the dissociation constant, n is the stoichiometry, and α is the maximum change in emission intensity (Boskovic et al., 1990; Stewart et al., 1998b). Reverse titrations were used to confirm binding parameters. Thus, 0.1 μM (DD)E or Fg was titrated with 5 or 6 μM active site blocked activator and 0.1 μM Glu- or Lys-Pg was titrated with 5 μM (DD)E or 15 μM Fg. Data from these titrations were analyzed as described above.

2.2.2iv Lysine affinity of Pg activators

To compare their lysine binding properties, fluorescently-labeled t-PA or b-PA was subjected to affinity chromatography on a lysine-Sepharose column. The fluorescence intensity of a 500 μl sample of 4.9 μM dEGR-t-PA or 3.7 μM dEGR-b-PA was quantified with excitation (λ_{ex}) and emission (λ_{em}) wavelengths set to 280 and 530 nm, respectively, a 515 nm cut-off filter, and excitation and emission slit widths both set to 5 nm. The Pg activator was then passed over a lysine-Sepharose column (1 \times 5 cm) and, after washing with five column volumes of TBS, bound material was eluted with 40 mM EACA, and 500 μl fractions were collected. Fractions containing dansyl fluorescence were pooled and total I_{530}

determined. The amount of Pg activator that bound was then calculated by expressing the I_{530} of the eluted material as a percent of the total I_{530} loaded onto the column.

To compare the affinity of t-PA, TNK-t-PA, TNK-t-PA variants and b-PA for lysine or EACA, changes in intrinsic fluorescence were monitored as each plasminogen activator was titrated with L-lysine or EACA. Additions of 10 to 20 μ l of 20 mM L-lysine or EACA were made to a 2 ml solution containing 0.1 μ M active-site blocked activator. Intrinsic fluorescence was monitored with $\lambda_{ex} = 280$ nm, $\lambda_{em} = 340$ nm, a 290 nm cut-off filter and slit widths set to 5 nm. The emission intensity after each addition of L-lysine or EACA was corrected for dilution and compared to the emission intensity of the activator alone, and these data, together with the total concentration of L-lysine or EACA were fit by nonlinear regression analysis to equation 3.

2.2.3 Fibrin, (DD)E or Fg stimulation of Pg activators

2.2.3i Fibrin, (DD)E or Fg stimulation of Pg activation

The effect of fibrin, (DD)E or Fg on t-PA-, TNK-t-PA-, TNK-t-PA variant-, or b-PA-mediated Pg activation was determined by comparing plasmin generation in the absence of these cofactors with that in their presence. 20- μ l aliquots containing 2 mM S-2251 and Pg activator in concentrations ranging from 0.5 - 5 nM were added to wells of a 96-well microtitre plate containing Pg (0 - 80 μ M) and (DD)E (0 - 1 μ M) or Fg (0 - 10 μ M) suspended in 80 μ l of TBS. Plasmin generation was monitored by measuring absorbance at 405 nm at 30 s intervals for 20 to 30 min using a Spectramax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). To measure Pg activation in the presence of fibrin, 5 μ l aliquots of Pg activator and thrombin (final concentrations, 0.1 and 10 nM, respectively)

were separately placed in wells prior to the addition of 90 μ l of a solution of Pg (0 - 20 μ M), fibrinogen (0 - 1 μ M), 2 mM CaCl₂ and 0.44 mM S-2251. The wells were monitored at 490 and 405 nm and A₄₉₀ values were then subtracted from those for A₄₀₅. In all cases, rates of plasmin formation were calculated by dividing the slope determined from the plot of A₄₀₅ *versus* time squared by the specific activity of plasmin (0.017 O.D./s/ μ M), which was determined in a separate experiment. Rates of Pg activation were then divided by the concentration of activator and subjected to nonlinear regression analysis (TableCurve; Jandel Scientific) according to the Michaelis-Menten equation ,

$$v' = \frac{[S] \cdot k_{cat}}{K_M + [S]} \quad (\text{equation 4})$$

where v' is the rate of Pg activation corrected for activator concentration and $[S]$ is the equilibrium concentration of free substrate, calculated for each input concentration of Pg and cofactor (Horrevoets et al., 1997a), using 13 and 5.4 μ M for the K_d values of Glu-Pg for fibrin and (DD)E, respectively, and 0.13, 0.03, and 0.23 μ M for the K_d values of Lys-Pg for fibrin, (DD)E, and Fg, respectively (Stewart et al., 1998b). Because binding of Glu-Pg to Fg was not detected and has not been previously reported, all of the Glu-Pg was assumed free in the presence of Fg. Analysis using equation 4 yielded the rate constant, k_{cat} , and Michaelis constant, K_M . The relative effects of fibrin, (DD)E and Fg on Pg activation were determined by comparing the catalytic efficiencies (k_{cat}/K_M) calculated in the presence of saturating concentrations of each cofactor. Cofactor concentrations were considered saturating when

the catalytic efficiency reached a maximum.

2.2.3ii *Fibrin or (DD)E stimulation of the amidolytic activity of Pg activators*

To determine the activity of t-PA, TNK-t-PA or b-PA against small substrates, 5 nM Pg activator was incubated with 2 mM Chromozyme t-PA or 4 mM H-D-isoleucyl-prolyl-arginine-p-nitroanilide-dihydrochloride (S-2288), dissolved in 100 μ l TBS. Absorbance at 405 nm was monitored for 20 min at 22 °C in a Spectramax microplate spectrophotometer. The rate of change in A_{405} was used as a measure of the activity of the Pg activator against the substrate. To determine if the cofactor fibrin or (DD)E affects the activity of t-PA, TNK-t-PA or b-PA, the experiments were repeated in the presence of 1 μ M cofactor. In the case of fibrin, 5 nM thrombin and 2 mM CaCl_2 were used to initiate clotting and changes in A_{405} were corrected for the presence of thrombin. The effect of the cofactor on the cleavage of chromogenic substrate was determined by comparing the rate of change in A_{405} in the presence of cofactor with that in its absence.

2.2.4 Characterization of the effects of CPB or TAFIa on (DD)E

2.2.4i *Effect of (DD)E exposure to CPB or TAFIa on the ability of (DD)E to stimulate Glu- or Lys-Pg activation by t-PA*

The effect of (DD)E exposure to CPB or TAFIa on the ability of (DD)E to stimulate Glu-Pg activation was assessed by monitoring rates of Glu-Pg activation in the presence of (DD)E exposed to CPB or TAFIa for various amounts of time. The effect of (DD)E exposure to CPB or TAFIa on the kinetic parameters for (DD)E-stimulated Glu- or Lys-Pg activation also was determined. (a) *Rates of Glu-Pg activation*: 1.0 ml of a 8 μ M (DD)E solution was incubated with 20 nM CPB or TAFIa for 40 min at 22°C. At intervals, 20 μ l

aliquots were removed and made to 3.6 μM with CPI to inhibit the CPB or TAFIa. Complete CPB or TAFIa inhibition was achieved because no residual activity was detected using the CPB-directed synthetic substrate hippuryl-L-arginine. The ability of TAFIa- or CPB-treated (DD)E to stimulate Pg activation was then compared with that of the starting material by adding 20 μl of 2 mM S-2251 and 0.5 nM t-PA to wells of a 96-well microtitre plate containing 0.5 μM Glu-Pg and 0.5 μM (DD)E, dissolved in 80 μl of TBS. Plasmin generation was assessed by measuring absorbance at 405 nm at 30-s intervals for 60 min using a Spectramax microplate spectrophotometer thermostated at 37°C. Initial rates of plasmin formation were calculated as described above. *(b) Kinetic parameters for Glu- or Lys-Pg activation:* After exposure of 8 μM (DD)E to 20 nM CPB or TAFIa for 40 min at 22 °C, the reaction was terminated with CPI. Different concentrations of CPI were used to maintain a concentration of CPI in the Pg activation assay between 0.2 and 0.8 μM . This range of CPI concentration was maintained to ensure CPI levels were well above the K_i for CPB inhibition (Ryan et al., 1974), but low enough so as not to inhibit plasmin (5 μM CPI causes a 7 % reduction in the activity of 0.01 μM plasmin). 0.8 μM CPI has no effect on the rate of plasmin formation. Glu- or Lys-Pg, in concentrations ranging from 0 to 16 μM , was incubated with 0.1 nM t-PA in the absence or presence of native (DD)E or TAFIa- or CPB-exposed (DD)E at concentrations up to 6 μM . Plasmin formation was then monitored using the plasmin-directed substrate S-2251. Rates of plasmin formation were determined and subjected to nonlinear regression analysis according to equation 4 as described above, with one exception. Since CPB or TAFIa causes a moderate reduction in the affinity of (DD)E for Glu- or Lys-Pg; free Glu- and Lys-Pg concentrations in the presence of CPB- or TAFIa-

exposed (DD)E were calculated based on K_d values of 11 and 0.35 μM for Glu- and Lys-Pg, respectively.

2.2.4ii *Effect of CPB or TAFIa on (DD)E degradation during t-PA-mediated Pg activation*

Plasmin degrades (DD)E to DD and fragment E (Olexa and Budzynski, 1979a; Gaffney and Joe, 1979). Because exposure of (DD)E to CPB or TAFIa reduces its ability to potentiate Pg activation, we examined whether CPB or TAFIa attenuates (DD)E degradation during t-PA-mediated Pg activation. 4 μM (DD)E that had been exposed to 20 nM CPB for various intervals, or to 20 nM TAFIa for 40 min, was compared with 4 μM native (DD)E in terms of its ability to stimulate 0.4 μM Glu-Pg activation by 1 nM t-PA. Under these conditions, (DD)E concentrations were sufficient to monitor its degradation by PAGE analysis. After 1 h incubation, the reaction was made to 1 μM VFKCK and 10 nM PPACK to inhibit plasmin and t-PA, respectively, and a 4 μl aliquot of the reaction solution was then subjected to PAGE analysis under nondenaturing conditions on 4-15% gradient gels, on 10% gels in the presence of SDS, and on 15% gels in the presence of SDS and β -mercaptoethanol. Gels were stained with Fast Stain (Zoion Research, Shrewsbury, MA) and bands were quantified using an ImageMaster Video Documentation System. For amino acid sequencing, protein bands were transferred onto polyvinylidene difluoride membranes and stained with Ponceau S. Appropriate bands were cut from the membrane and subjected to amino-terminal sequence analysis which was performed by Biotechnology Service Centre (University of Toronto, Toronto, Canada).

2.2.4iii *Effect of (DD)E exposure to CPB or TAFIa on its affinity for t-PA, Glu- or Lys-Pg*

(DD)E (1 μM) was incubated with 20 nM CPB or TAFIa for 40 min at 22 $^{\circ}\text{C}$ and the

reaction was terminated with 1 μM CPI. The affinity of CPB- or TAFIa-exposed (DD)E for t-PA, Glu-Pg or Lys-Pg was then determined using right angle light scattering spectroscopy, as described above, except 15- or 20- μl aliquots of active site blocked t-PA, Glu-Pg or Lys-Pg (at concentrations of 6, 100, and 10 μM , respectively) were added to 2 ml of a 0.1 μM solution of CPB- or TAFIa-exposed (DD)E. These results were compared to those obtained with native (DD)E.

2.2.4iv Determination of the release of free lysine and arginine from (DD)E by CPB or TAFIa

To determine whether CPB or TAFIa releases lysine or arginine residues from (DD)E, (DD)E was incubated with CPB or TAFIa for intervals up to 60 min and free lysine and arginine were measured using methods similar to those described by Wang et al. (1998). After dialysis of (DD)E into 50 mM HEPES, 150 mM NaCl, pH 7.4, an 8 μM (DD)E solution was incubated with 20 nM CPB or TAFIa for 60 min at 22 °C. At various times, 100 μl aliquots were removed and the reaction was made to 3.6 μM CPI to inactivate the CPB or TAFIa. The samples were deprotonated by bringing the solution to 0.2 M with perchloric acid followed by centrifuging at $12000 \times g$ for 5 min. After neutralizing the supernatants with potassium hydroxide, the samples were placed on ice and insoluble potassium perchlorate was removed by centrifugation at $12000 \times g$ for 5 min. The concentration of lysine and arginine in the supernatants was determined enzymatically by methods established by Nakatani et al. (1972) and Gaede et al. (1974), respectively. For lysine determination, 50 μl of supernatant was added to 40 μl of 0.5 mM NADH (Roche Diagnostics, Laval, Canada) and 2.5 mM α -ketoglutaric acid (Roche Diagnostics) in 50 mM

HEPES, 150 mM NaCl, pH 7.0 (HBS). Dehydration of NADH was initiated by the addition of 0.33 units of saccharopine dehydrogenase (Sigma) suspended in 10 μ l HBS. Decreases in fluorescence intensity were monitored over 25 min in a Spectra Max, Gemini XS fluorescent plate reader (Molecular Devices, Sunnyvale, CA), with excitation and emission wavelengths set to 340 and 450 nm, respectively, and fitted with a 435 nm emission cutoff filter. The concentration of lysine was calculated based on a standard curve generated by plotting changes in fluorescence intensity produced by known concentrations of L-lysine (Sigma). Determination of free arginine was accomplished in the same manner using pyruvate (Roche Diagnostics) in place of α -ketoglutaric acid, and 0.5 units of octopine dehydrogenase (Sigma) in place of saccharopine dehydrogenase. Standard curves for free arginine determination were generated by plotting changes in fluorescence intensity produced by known concentrations of L-arginine (Sigma).

CHAPTER 3 - CHARACTERIZATION OF THE INTERACTIONS OF
PLASMINOGEN AND TISSUE AND VAMPIRE BAT PLASMINOGEN
ACTIVATORS WITH FIBRINOGEN, FIBRIN, AND THE COMPLEX OF D-DIMER
NONCOVALENTLY LINKED WITH FRAGMENT E

Preface

This manuscript has been published by the Journal of Biological Chemistry (Vol. 273, No. 29, Issue of July 17, pp. 18292-18299, 1998). The authors are Ronald J. Stewart, James C. Fredenburgh, and Jeffrey I. Weitz. The corresponding author is Dr. Weitz. Permission to include copyright material in this thesis has been obtained from the Journal of Biological Chemistry (p.75).

All of the experiments presented in this manuscript were performed by me, with guidance from Drs. Fredenburgh and Weitz.

This work was supported by grants from the Heart and Stroke Foundation of Ontario and the Medical Research Council of Canada. R.J. Stewart is the recipient of a Research Traineeship from the Heart and Stroke Foundation of Canada. Dr. J.I. Weitz is a Career Investigator of the Heart and Stroke Foundation of Ontario.

The abbreviations used are: t-PA, tissue-type plasminogen activator; b-PA, vampire bat plasminogen activator; DSPA_{α1}, *Desmodus rotundus* salivary plasminogen activator; DD, D-dimer; (DD)E, complex of D-dimer noncovalently associated with fragment E; EACA, ε-

amino caproic acid; Pg, plasminogen; Glu-Pg, native plasminogen with N-terminal Glu; Lys-Pg, plasmin-modified plasminogen with N-terminal Lys; Fg, fibrinogen; K2, second kringle domain of t-PA; PAGE, polyacrylamide gel electrophoresis; PPACK, D-phenyl-prolyl-arginine chloromethyl ketone; dEGR-CMK, dansyl glutamyl-glycyl-arginine chloromethyl ketone; VFKCK, D-valyl-phenyl-lysine chloromethyl ketone.

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3.1 Summary

Vampire bat plasminogen activator (b-PA) causes less fibrinogen (Fg) consumption than tissue-type plasminogen activator (t-PA). Herein, we demonstrate that this occurs because (DD)E, the most abundant degradation product of crosslinked fibrin, as well as Fg, stimulate Pg activation by t-PA more than b-PA. To explain these findings, we characterized the interactions of t-PA, b-PA, Lys-Pg and Glu-Pg with Fg and (DD)E using right angle light scattering spectroscopy. In addition, interactions with fibrin were determined by clotting Fg in the presence of various amounts of t-PA, b-PA, Lys-Pg or Glu-Pg and quantifying unbound material in the supernatant after centrifugation. Glu-Pg and Lys-Pg bind fibrin with K_d values of 13 and 0.13 μM , respectively. t-PA binds fibrin through two classes of sites with K_d values of 0.05 and 2.6 μM , respectively. The second kringle (K2) of t-PA mediates the low affinity binding that is eliminated with ϵ -amino caproic acid (EACA). In contrast, b-PA binds fibrin through a single kringle-independent site with a K_d of 0.15 μM . t-PA competes with b-PA for fibrin binding, indicating that both activators share the same finger-dependent site on fibrin. Glu-Pg binds (DD)E with a K_d of 5.4 μM . Lys-Pg binds to (DD)E and Fg with K_d values of 0.03 and 0.23 μM , respectively. t-PA binds to (DD)E and Fg with K_d values of 0.02 and 0.76 μM , respectively; interactions eliminated with EACA, consistent with K2-dependent binding. Because it lacks a K2-domain, b-PA does not bind to either (DD)E or Fg, thereby explaining why b-PA is more fibrin-specific than t-PA.

3.2 Introduction

Tissue-type plasminogen activator (t-PA) is a naturally occurring serine protease that initiates fibrinolysis by converting plasminogen (Pg) to plasmin (Lijnen and Collen, 1995b). Not only is fibrin the target for plasmin attack, but fibrin also stimulates t-PA-mediated Pg activation (Madison and Sambrook, 1982; Hoylaerts et al., 1982). To accomplish this, fibrin acts as a template to which both t-PA and Pg bind (Horrevoets et al., 1997a). The fibrin-binding properties of t-PA have been ascribed to its finger and second kringle (K2) domains (Verheijen et al., 1986; van Zonneveld et al., 1986), although recent studies suggest that the protease domain may also influence the interaction of t-PA with fibrin (Bennet et al., 1991; Paoni et al., 1993a; Horrevoets et al., 1997a). The binding of both Glu- and Lys-plasminogen (Glu-Pg and Lys-Pg, respectively) to fibrin is entirely kringle-mediated with Lys-Pg having higher affinity for fibrin than Glu-Pg (Lucas et al., 1983).

As a functional consequence of t-PA interaction with fibrin, the catalytic efficiency of t-PA-mediated Pg activation is two to three orders in magnitude higher in the presence of fibrin than in its absence (Hoylaerts et al., 1982; Bringmann et al., 1995). In contrast to fibrin, fibrinogen (Fg) stimulates Pg activation by t-PA only 25-fold (Hoylaerts et al., 1982; Bringmann et al., 1995). Based on these considerations, t-PA is designated a fibrin-specific Pg activator (Collen and Lijnen, 1992). Despite this designation, t-PA causes systemic plasminemia and fibrinogenolysis when given to patients (Vaughan et al., 1987). In recent studies, we have demonstrated that t-PA causes systemic plasminemia because, like intact fibrin, soluble fibrin degradation products also stimulate t-PA-mediated Pg activation (Weitz et al., 1993). Furthermore, we have identified the (DD)E complex as the fibrin derivative

primarily responsible for this effect (Weitz et al., 1991), and have shown that the stimulatory activity of (DD)E is similar to that of fibrin (Stewart et al., 1998a). (DD)E, a complex of D-dimer noncovalently bound to fragment E, is the major degradation product of crosslinked fibrin (Olexa and Budzynski, 1979b). As a potent stimulator of t-PA-mediated activation of Pg, (DD)E generated during thrombus dissolution has the potential to induce systemic plasminemia (Olexa and Budzynski, 1979b; Vaughan et al., 1987).

The limited fibrin-specificity of t-PA has prompted the development of Pg activators with greater selectivity for fibrin (Bachmann, 1995). One such agent is the Pg activator isolated from the saliva of vampire bats (*Desmodus rotundus*) (Gulba et al., 1995). Full length vampire bat salivary Pg activator (designated DSPA α_1) has over 72% amino acid sequence identity to t-PA (Krätzschar et al., 1991). The major structural difference is that vampire bat Pg activator (b-PA) contains only one kringle domain, whereas t-PA has two. The single kringle domain of b-PA more closely resembles the first kringle domain of t-PA in that it lacks a lysine-binding site (Gardell et al., 1989; Krätzschar et al., 1991).

Although fibrin stimulates Pg activation by b-PA to the same extent as t-PA (Bringmann et al., 1995), b-PA causes less α_2 -antiplasmin and Fg consumption than t-PA in experimental animals when the two agents are used in concentrations that produce equivalent thrombolysis (Gardell et al., 1991; Witt et al., 1992; Klement et al., 1995; Mellott et al., 1995). This has been attributed to the fact that Fg potentiates Pg activation by t-PA more than b-PA (Gardell et al., 1990; Bergum and Gardell, 1992; Schleuning et al., 1992; Bringmann et al., 1995). Because our studies demonstrated that (DD)E compromises the fibrin specificity of t-PA, we examined the possibility that the greater fibrin-specificity of b-

PA over t-PA reflects less (DD)E-mediated stimulation of Pg activation by b-PA relative to t-PA. Herein we demonstrate that (DD)E and Fg stimulate plasmin formation by t-PA to a greater extent than b-PA. To explore the possibility that differences in potentiation reflect differences in binding parameters, we measured the affinities of t-PA, b-PA, Glu-Pg, and Lys-Pg for (DD)E, as well as for fibrin and Fg. Binding was quantified in the absence and presence of the lysine analogue ϵ -amino caproic acid (EACA) to identify kringle-dependent interactions.

3.3 Experimental Procedures

3.3.1 Materials

3.3.1i *Plasminogen activators*: Wild-type recombinant t-PA was kindly provided by Dr. B. Keyt (Genentech Inc., South San Francisco, CA.), and recombinant b-PA (DSPA α_1) was a generous gift from Dr. W. Witt (Schering AG., Berlin, Germany). t-PA and b-PA were found to be 93% and 100% single-chain, respectively, when analysed by SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) on 4-15% gels (Ready-Gel, Bio-Rad, Mississauga, ON), as determined by laser densitometry (Ultrosan XL, LKB-Pharmacia, Baie d'Urfe, PQ). The chromogenic substrate used in Pg activation studies was the plasmin-directed substrate S-2251 (D-valyl-leucyl-lysine-*p*-nitroanilide dihydrochloride) from Chromogenix (Helena, Mississauga, ON). Active site-blocked, fluorescently labeled derivatives of t-PA or b-PA were prepared by adding 1 ml of 0.05 M sodium pyrophosphate, 0.15 M NaCl, 0.5 M (NH₄)₂SO₄, pH 7.2 to 1 ml of a 2 mg/ml stock enzyme solution followed by incubation with a 5-fold molar excess of dansyl glutamyl-glycyl-arginine chloromethyl ketone (dEGR-CMK, Calbiochem, San Diego, CA) at 22 °C (Higgins and Lamb, 1986). The residual activity of the active site blocked Pg activators was evaluated by measuring their ability to hydrolyse the chromogenic substrate N-methylsulfonyl-D-Phe-Ala-Gly-Arg-4-nitroanilide-acetate (Chromozyme t-PA, Boehringer Mannheim, Laval, PQ). t-PA activity was abolished after 1 h incubation with dEGR-CMK, whereas a 3 h incubation was needed to block b-PA activity. Both enzymes were then dialysed against the pyrophosphate-containing buffer overnight at 4°C. The protein concentrations were determined by measuring absorbance at 280 and 320 nm. Absorbance at 335 nm was used to distinguish

dansyl group absorbance from light scattering, as described previously (Nesheim et al., 1990). Based on calculations of protein concentration, 90-95% of the Pg activators were recovered after dialysis against pyrophosphate buffer. Active site blocked, unlabeled derivatives of t-PA or b-PA were prepared by the same procedure except D-phenyl-prolyl-arginine chloromethyl ketone (PPACK, Calbiochem) was used in place of dEGR-CMK. Under these conditions, t-PA activity was abolished after 30 min incubation with PPACK, whereas a 2 h incubation was needed to block b-PA activity. Immediately prior to use, a 1 ml volume of the Pg activator was dialysed against 2 L of 0.02 M Tris-HCl, 0.15 mM NaCl, 0.01% Tween-20, pH 7.4 (TBS) for 3 h with vigorous stirring, and then centrifuged at $12000 \times g$ for 7 min at 22°C in a microfuge to remove any aggregated material. Based on calculations of protein concentration, dialysis against TBS resulted in a 40-60% loss of t-PA and a 30-40% loss of b-PA. The molecular weights and extinction coefficients used were 65,000 and $\epsilon_{1\%}^{280}=20.0$ for t-PA (Nesheim et al., 1990), and 54,500 and $\epsilon_{1\%}^{280}=17.1$ for b-PA (Bringmann et al., 1995).

3.3.1ii *Plasminogen*: Native Glu-Pg was isolated from fresh-frozen plasma by lysine-Sepharose affinity chromatography as described previously (Castellino and Powell, 1981), but in the absence of aprotinin. Subsequently, the column was washed extensively with 0.1 M sodium phosphate, pH 8.0, followed by 20 mM Tris-Cl, pH 8.0. Adsorbed Pg was eluted with 10 mM EACA, 20 mM Tris-Cl, pH 8.0, directly onto a DEAE-Fast Flow column (1 × 20 cm). The DEAE column was washed with 20mM Tris-Cl, pH 8.0, to remove the EACA, and Glu-Pg was then eluted with a 0-200 mM linear NaCl gradient in TBS. Glu-Pg was concentrated by ammonium sulfate precipitation with subsequent solubilization and dialysis

against TBS. As determined by urea/acetic acid polyacrylamide gel electrophoresis (Fredenburgh and Nesheim, 1992), isolated Glu-Pg was free of Lys-Pg and contained no plasmin chromogenic activity using S-2251. Glu-Pg concentrations were calculated by measuring absorbance at 280 and 320 nm, and using a molecular weight of 90,000 and $\epsilon_{1\%}^{280}=16.1$ (Castellino and Powell, 1981). Lys-Pg was purchased from Enzyme Research Laboratories.

3.3.1iii *Fibrinogen*: Human Fg, purchased from Enzyme Research Laboratories Inc. (South Bend, IN), was dissolved in a 0.02 M Tris-HCl, 0.15 M NaCl, pH 7.4. Prior to use, Fg (2 mg/ml) was incubated for 30 min at 22° C with 10 ml of lysine-Sepharose (Pharmacia Biotech Inc., Baie d'Urfe, PQ) to remove residual Pg. After centrifugation at 3000 × g for 10 min at 22° C, the supernatant was incubated for 30 min at 22° C with 6 ml of gelatin-Sepharose (Sigma Chemical Co., St. Louis, MO) to remove fibronectin. After centrifugation at 3000 × g for 10 min at 22° C, the final Fg concentration in the supernatant was calculated by measuring absorbance at 280 and 320 nm and using a molecular weight of 340,000 and $\epsilon_{1\%}^{280}=16.0$ (Nesheim et al., 1979).

3.3.1iv *Isolation of (DD)E*: The soluble fibrin fragment, (DD)E, was prepared by plasmin-mediated lysis of a crosslinked fibrin clot. Briefly, a 12 ml solution of Fg (8.3 mg/ml) in 0.02 M Tris-HCl, 0.15 M NaCl, pH 7.4, was clotted with 64 nM thrombin (Enzyme Research Laboratories) and 10 mM CaCl₂ in the presence of 93 nM activated recombinant factor XIII (a generous gift from Dr. P. Bishop, Zymogenetics, Inc., Seattle, WA), 0.4 μM Glu-Pg, and 2 pM t-PA. Clotting occurred within 30 s and the resultant fibrin was completely degraded after 55 h. The reaction was terminated by the addition of 5 μM D-valyl-phenyl-lysine

chloromethyl ketone (VFKCK, Calbiochem) to block plasmin activity, and 1 μ M PPACK to block both t-PA and thrombin activity. The clot lysate was then concentrated to a 2 ml volume by ultrafiltration using a Centriprep 10 concentrator fitted with a 10,000 MW cut-off membrane (Amicon Inc., Beverly, MA). After removing aggregates by centrifugation at $12,000 \times g$ for 5 min, the fibrin degradation products were isolated by passing the material over a Biosep-Sec-S3000 size exclusion column (Phenomenex, Torrance, CA) fitted to a liquid chromatograph (System Gold; Beckman Instruments Inc., Palo Alto, CA). Presence of protein was determined with a model 167 variable wavelength absorbance detector set at 280 nm. Peak protein-containing fractions were pooled and subjected to PAGE analysis on 4-15% non-denaturing gels. (DD)E-containing fractions were identified based on their apparent molecular weight and by immunoblot analysis using antibodies against DD and E (Weitz et al., 1991). (DD)E concentrations were calculated by measuring absorbance at 280 and 320 nm and using a molecular weight of 250,000 and $\epsilon_{1\%}^{280}=16.0$ (Olexa and Budzynski, 1979b). When (DD)E was incubated with 10 mM H-Gly-Pro-Arg-Pro-OH (Calbiochem) prior to non-denaturing PAGE analysis, or when subjected to SDS-PAGE, two lower molecular weight bands appeared, corresponding to DD and E, respectively.

3.3.2 Methods

3.3.2i *(DD)E or Fg stimulation of Pg activation*: The effect of (DD)E or Fg on t-PA- and b-PA-mediated Pg activation was determined by comparing plasmin generation in the absence of these cofactors with that in their presence. 20- μ l aliquots containing 2 mM S-2251 and 1 nM t-PA or 5 nM b-PA were added to wells of a 96-well microtitre plate containing 0.4 μ M Giu-Pg in the absence or presence of either (DD)E or Fg. Plasmin generation was

monitored by measuring absorbance at 405 nm at 30 s intervals for 20 to 30 min using a Spectramax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Point-to-point slopes were determined and converted to plasmin concentration based on the specific activity of plasmin with S-2251 ($0.017 \text{ O.D.}\cdot\text{s}^{-1}\mu\text{M}^{-1}$), which was determined in a separate experiment. Plots of plasmin concentration *versus* time were used to calculate the rate of plasmin formation.

3.3.2ii Fluorescence and light scattering measurements: All fluorescence and light scattering intensities were measured in a LS50B luminescence spectrometer (Perkin-Elmer, Etobicoke, ON) using a cuvette thermostated at 22 °C. Fluorescence measurements were performed in a 1 ml quartz microcuvette and right angle light scattering measurements were made in a 3 ml quartz cuvette with stirring. To measure the fluorescence of individual samples, three fluorescence intensity readings, each recorded over a 3 s integration time, were averaged. Scattering intensities were continuously monitored in time drive with the interval time set at 1 or 2 s, and the response time at 2 or 3 s. Intensity values were determined by averaging scattering intensities observed over a period of at least 100 s. Thus, each scattering intensity value represents the mean of 50 to 100 individual readings.

3.3.2iii Lysine affinity of t-PA and b-PA: To compare their affinities for lysine, fluorescently-labeled t-PA or b-PA was subjected to affinity chromatography on a lysine-Sepharose column. The fluorescence intensity of a 500 μl sample of dEGR-t-PA or dEGR-b-PA was quantified with excitation (λ_{ex}) and emission (λ_{em}) wavelengths set to 280 and 530 nm, respectively, a 515 nm cut-off filter, and excitation and emission slit widths both set to 5 nm. The Pg activator was then passed over a lysine-Sepharose column (1 \times 5 cm) and,

after washing, bound material was eluted with 40 mM EACA, and 500 μ l fractions were collected. Fractions containing dansyl fluorescence were pooled and total I_{530} determined. The amount of Pg activator that bound was then calculated by expressing the I_{530} of the eluted material as a percent of the total I_{530} loaded onto the column.

As another method of comparing the relative affinities of t-PA and b-PA for lysine, changes in intrinsic fluorescence were monitored as each Pg activator was titrated with the lysine analogue, EACA. Additions of 20-40 μ l of 20 mM EACA were made to a 2 ml solution containing 0.3 μ M PPACK-t-PA or PPACK-b-PA. Intrinsic fluorescence was monitored with $\lambda_{ex}=280$ nm, $\lambda_{em}=340$ nm, a 290 nm cut-off filter, and slit widths set to 5 nm.

3.3.2iv *Binding to fibrin:* The binding of dEGR-t-PA or dEGR-b-PA to fibrin was determined by adding increasing concentrations of Pg activator to a series of microfuge tubes (Sarstedt 72.702) containing fixed amounts of Fg and 2 mM CaCl_2 in TBS (29). A 10 μ l aliquot of thrombin (final concentration, 10 nM) was then added to induce clotting. The final reaction volume was 200 μ l. After incubation at 22 °C for 1 h, the clots were vortexed and centrifuged at $12000 \times g$ for 2.5 min to compact the fibrin into the 10 μ l tip of the microtube. The fluorescence intensity of 150 μ l of clot supernatant in 350 μ l of Tris buffer was measured with $\lambda_{ex}=280$ nm, $\lambda_{em}=530$ nm, a 515 nm cut-off filter, and 15 nm slit widths. A parallel titration was done in the absence of thrombin to establish a standard curve for each ligand. The binding of Lys-Pg and Glu-Pg to fibrin was determined using the same procedure except unbound Pg was quantified by measuring intrinsic fluorescence and the standard curve of Pg concentrations was established in the absence of Fg. Because the affinity of Pg for fibrin is lower than that of the Pg activators, higher Pg concentrations were

used in these experiments, thereby obviating the need to use fluorescently labeled Pg. The conditions for measuring intrinsic fluorescence include $\lambda_{\text{ex}}=280$ nm, $\lambda_{\text{em}}=340$ nm, a 290 nm cut-off filter, and slit widths set to 2.5 nm.

The effect of EACA on the binding of dEGR-t-PA, dEGR-b-PA, Glu-Pg or Lys-Pg to fibrin was determined by repeating the same titrations in the presence of 20 mM EACA. In addition, clots formed by incubating 2 μM Fg with 10 nM thrombin in the presence of 0.8 μM dEGR-t-PA, dEGR-b-PA, Glu-Pg or Lys-Pg were titrated with EACA (in concentrations ranging from 0 to 20 mM) and the amount of ligand displaced was determined by measuring the concentration of unbound protein in the clot supernatant as described above.

To determine whether t-PA and b-PA compete for the same fibrin binding sites, various concentrations of unlabeled, active site blocked b-PA or t-PA, with or without 20 mM EACA, were added to a series of microfuge tubes charged with 2 μM Fg and 0.8 μM dEGR-t-PA or dEGR-b-PA. Thrombin (10 nM) was added, and after incubation for 60 min at 22 °C, fibrin was pelleted by centrifugation. The amount of unbound fluorescently-labeled enzyme in the supernatant was then compared with that found in control samples prepared in the absence of thrombin.

3.3.2v Binding of t-PA, b-PA, and Pg to Fg or (DD)E: The binding of t-PA, b-PA, Glu-Pg, and Lys-Pg to Fg or (DD)E was studied using solution-phase titrations. Interactions were monitored using right angle light scattering spectroscopy where the solution was excited at a fixed wavelength ($\lambda=400$ or 440 nm) and emission intensities were measured at the same wavelength with both excitation and emission slit widths set to either 8 or 12 nm. In the case of Fg, aliquots (5 or 10 μl) of 15 μM Fg were added to 2 ml of 0.1 μM active site

blocked t-PA or b-PA, or 0.3 μM Glu-Pg or Lys-Pg. Control titrations were done to determine the intensity of light scattering of Fg alone. In the case of (DD)E, aliquots (5 or 10 μl) of 5 μM (DD)E were added to 2 ml of 0.1 μM PPACK-t-PA or PPACK-b-PA. Interactions of Glu-Pg and Lys-Pg with (DD)E were monitored in a similar fashion except 0.1 μM (DD)E was titrated with 80 μM Glu-Pg or 5 μM Lys-Pg. To ensure that none of the target proteins was undergoing self-association, the light scattering intensity of PPACK-t-PA, PPACK-b-PA, or (DD)E (in concentrations ranging from 0.05 μM to 0.25 μM) was monitored over a 30 min period under the conditions outlined for the binding experiments. In each case, there was no change in scattering intensity over time, indicating that the target proteins were not aggregating.

3.3.2vi *Data analyses:* For analysis of fibrin binding, the fluorescence intensities of the supernatants were used to calculate the concentrations of unbound proteins by comparison with fluorescence intensities of known concentrations of protein. The concentrations of bound proteins were determined by calculating the difference between the total and unbound protein concentrations. These values were divided by the Fg concentration to determine the number of moles of dEGR-t-PA, dEGR-b-PA, Lys-Pg, or Glu-Pg bound per mole of fibrin (B). For each point in the titration, these values were then plotted against the concentration of unbound protein (L). Scatchard plots also were constructed, and if these appeared linear, reflecting a single class of binding sites, the binding isotherm was analysed by nonlinear regression analysis (Table Curve, Jandel Scientific, San Rafael, CA) of the relationship:

$$B = \frac{n \cdot L}{K_d + L} \quad (\text{equation 1})$$

where n is the stoichiometry, and K_d is the dissociation constant. All binding isotherms were linear, except for that corresponding to the binding of dEGR-t-PA to fibrin in the absence of EACA, which curved downward. These data were best fit to a two-site model by nonlinear regression analysis (Table Curve, Jandel Scientific) according to the expression:

$$B = \frac{n_1 \cdot L}{K_{d1} + L} + \frac{n_2 \cdot L}{K_{d2} + L} \quad (\text{equation 2})$$

where L represents the concentration of unbound protein, n_1 and n_2 are the stoichiometries for each of the two sites, and K_{d1} and K_{d2} are their respective dissociation constants.

For analysis of solution-phase binding of PPACK-t-PA, PPACK-b-PA, Lys-Pg, or Glu-Pg to Fg or (DD)E, the emission intensity (I) of the incident beam after each addition of ligand was corrected for changes due to dilution and ligand scattering. Corrected values were compared with the emission intensity before the addition of ligand (I_o), and these data, together with the total ligand concentration (L_o), were fit by nonlinear regression analysis (Table Curve, Jandel Scientific) to the following equation:

$$\frac{I}{I_o} = 1 + \frac{\alpha}{2} \left(1 + \frac{K_d + L_o}{n \cdot P_o} - \sqrt{\left(1 + \frac{K_d + L_o}{n \cdot P_o} \right)^2 - 4 \cdot \frac{L_o}{n \cdot P_o}} \right) \quad (\text{equation 3})$$

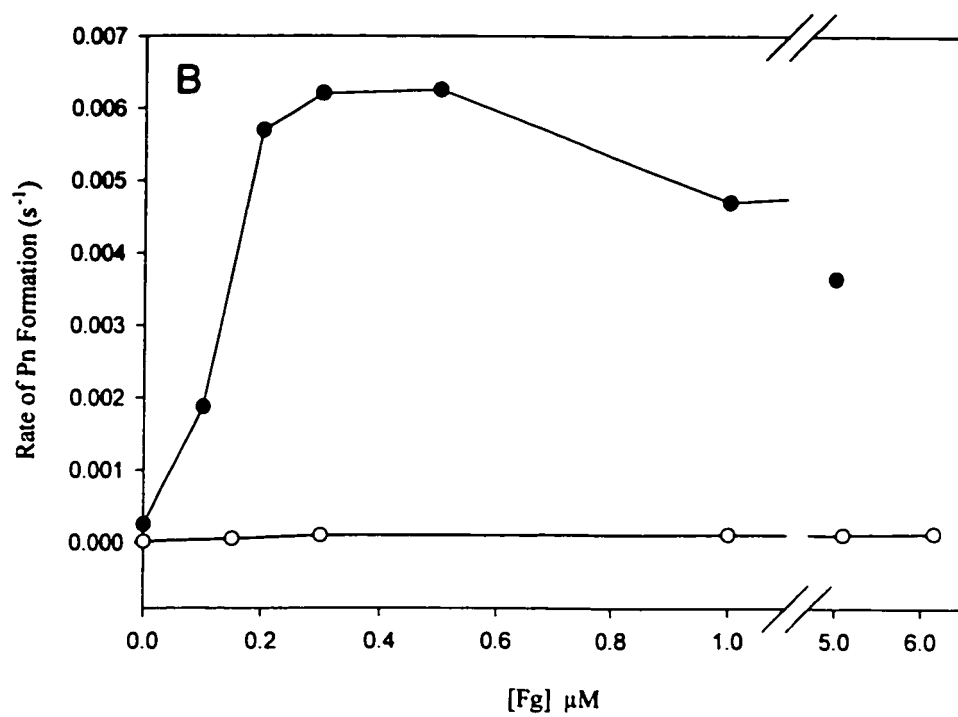
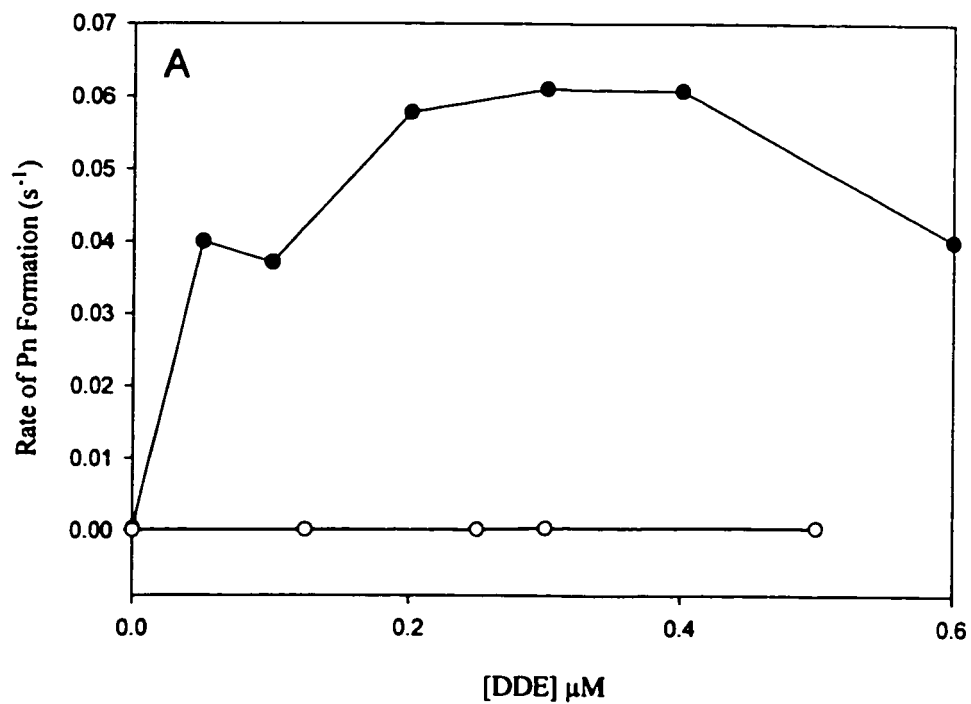
where L_0 is the concentration of ligand added, P_0 is the concentration of target protein, and α is the maximum change in emission intensity. Using α as a measure of 100% ligand bound, the amount of unbound ligand was determined after each addition of ligand, and Scatchard analysis was used to confirm the binding parameters derived from equation 3.

3.4 Results

3.4.1 Influence of (DD)E or Fg on t-PA- and b-PA-mediated activation of Pg: To compare the effect of (DD)E and Fg on t-PA- and b-PA-mediated Pg activation, 0.4 μM Glu-Pg was incubated with 1 nM t-PA or 5 nM b-PA in the absence or presence of various concentrations of (DD)E or Fg for 10 min at 37 °C and the rate of plasmin formation was monitored (Fig. 3.1). In the presence of (DD)E the rate of t-PA-mediated plasmin formation is increased a maximum of 244-fold (from $2.5 \times 10^{-4} \text{ s}^{-1}$ to $6.1 \times 10^{-2} \text{ s}^{-1}$). Fg increases the rate of t-PA-mediated plasmin formation 25-fold (from $2.5 \times 10^{-4} \text{ s}^{-1}$ to $6.2 \times 10^{-3} \text{ s}^{-1}$). In contrast, b-PA-mediated plasmin formation is increased only 20-fold with (DD)E (from $1.3 \times 10^{-5} \text{ s}^{-1}$ to $2.6 \times 10^{-4} \text{ s}^{-1}$) and 8-fold with Fg (from $1.3 \times 10^{-5} \text{ s}^{-1}$ to $1.0 \times 10^{-4} \text{ s}^{-1}$). Thus, (DD)E, and to a lesser extent Fg, are more potent stimulators of Pg activation by t-PA than b-PA.

3.4.2 Affinities of t-PA and b-PA for EACA: To begin to explore why (DD)E and Fg are less potent stimulators of Pg activation by b-PA than t-PA, we first compared the lysine-binding properties of the Pg activators because the affinity of t-PA for lysine determines, at least in part, its affinity for fibrin (Bakker et al., 1995b). To compare their relative affinities for lysine, aliquots containing 0.32 mg/ml dEGR-t-PA or 0.2 mg/ml dEGR-b-PA were subjected to affinity chromatography on a lysine-Sepharose column. Pg activator that bound to the lysine-Sepharose was eluted with 40 mM EACA. Whereas 90% of the t-PA bound to lysine-Sepharose, only 3% of the b-PA bound. The affinities of t-PA and b-PA for the lysine analogue, EACA, were compared by quantifying changes in intrinsic fluorescence when each agent was titrated with EACA. Titration of active site-blocked t-PA with EACA results in a concentration-dependent and saturable increase in its intrinsic fluorescence (Fig. 3.2).

Figure 3.1 Influence of (DD)E or Fg on t-PA- and b-PA-mediated activation of Glu-Pg. Glu-Pg (0.4 μM) was incubated with 1 nM t-PA (●) or 5 nM b-PA (○) for 10 min at 37 °C in the absence or presence of (DD)E (panel A) or Fg (panel B) in the concentrations indicated, and plasmin activity was monitored by measuring the hydrolysis of 0.4 mM S-2251. Plasmin concentrations were calculated based on the specific activity of plasmin for S-2251 (0.017 O.D. $\text{s}^{-1}\mu\text{M}^{-1}$), and rates of plasmin formation were determined by plotting plasmin concentration as a function of time.



Based on analysis of these data, EACA binds to t-PA with a $K_d = 214 \mu\text{M}$ and $n = 0.91$ EACA/t-PA. In contrast, there is no change in intrinsic fluorescence when active site blocked b-PA is titrated with EACA (Fig. 3.2). This finding is consistent with our observation that unlike t-PA, b-PA does not bind lysine-Sepharose.

3.4.3 Interactions of t-PA, b-PA, Glu-Pg and Lys-Pg with fibrin: Since fibrin has been reported to stimulate Pg activation by t-PA and b-PA to a similar extent (Bringmann et al., 1995), we quantified the binding of the Pg activators and Pg to fibrin. The Scatchard plot for the binding of dEGR-t-PA is nonlinear (Fig. 3.3A), indicating heterogeneous binding sites or negative cooperativity (Boeynaems and Dumont, 1975). A plot of the double reciprocal ($1/B$ vs. $1/F$) yields a straight line, whereas a plot of B^2/F versus B yields a sigmoidal curve, where B and F represent the amount of bound and free t-PA, respectively (data not shown). These findings are indicative of binding site heterogeneity (Boeynaems and Dumont, 1975). Accordingly, the data were fit to a two-site model (equation 2) by nonlinear regression analysis, and the resulting binding parameters are $K_{d1} = 0.053 \mu\text{M}$; $n_1 = 0.25$ t-PA/fibrin, and $K_{d2} = 2.6 \mu\text{M}$; $n_2 = 1.4$ t-PA/fibrin. When fibrin is titrated with dEGR-t-PA in the presence of 20 mM EACA (Fig. 3.3B), Scatchard analysis yields a straight line, indicating a single class of binding sites ($K_d = 0.47 \mu\text{M}$, $n = 0.25$ t-PA/fibrin) that more closely resembles the high affinity interaction of t-PA with fibrin seen in the absence of EACA. Like other investigators (Nesheim et al., 1990), we interpret this as indicating that EACA blocks the interaction of the K2 domain of t-PA with fibrin, while finger-dependent binding is maintained.

In contrast to the results obtained with t-PA, the Scatchard plot of b-PA binding to

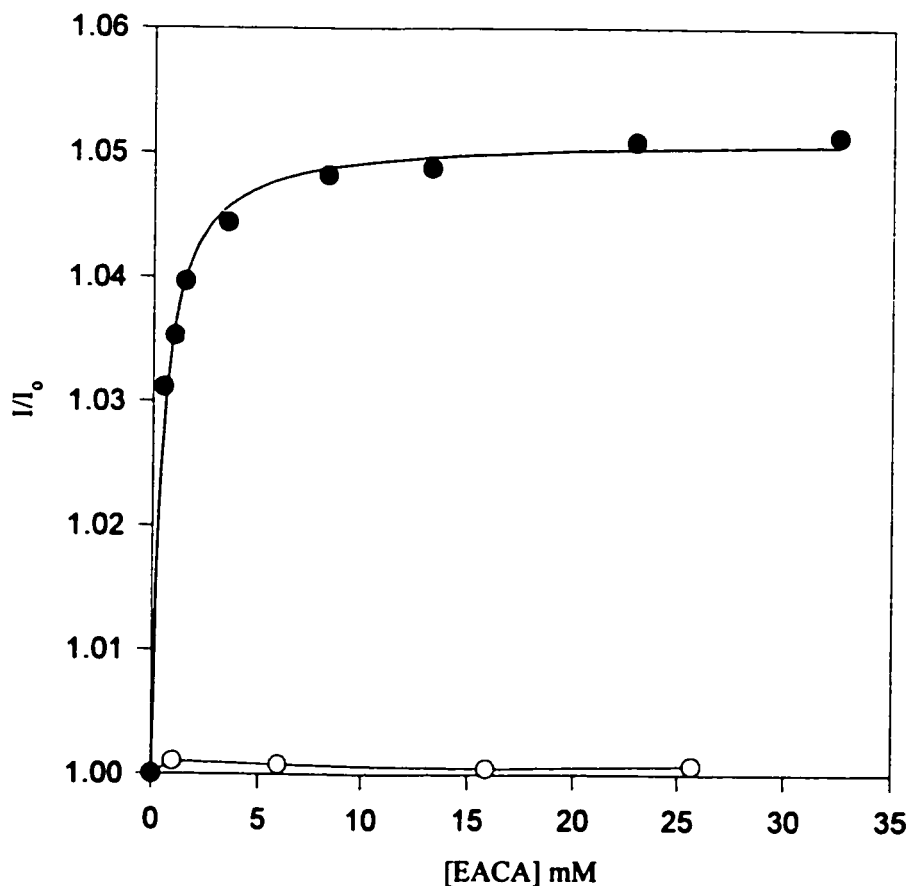
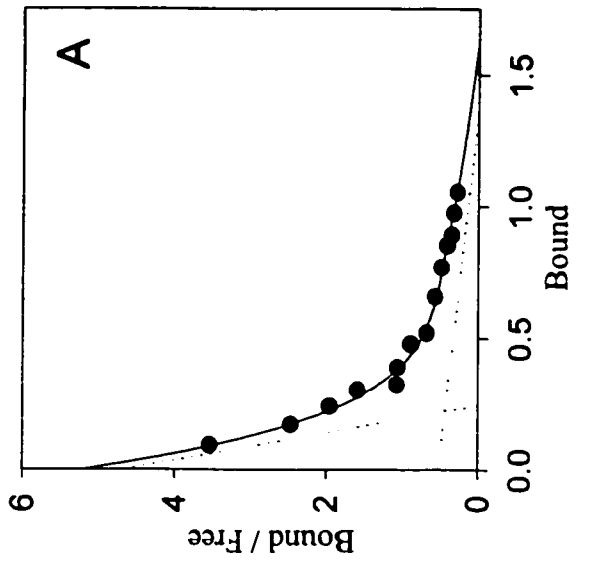
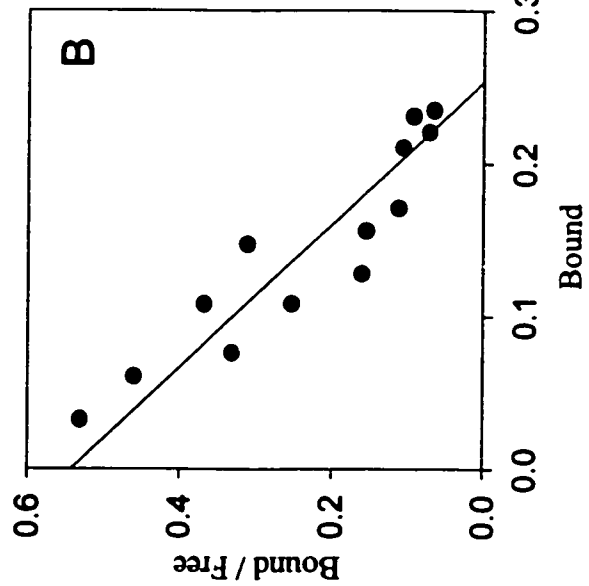
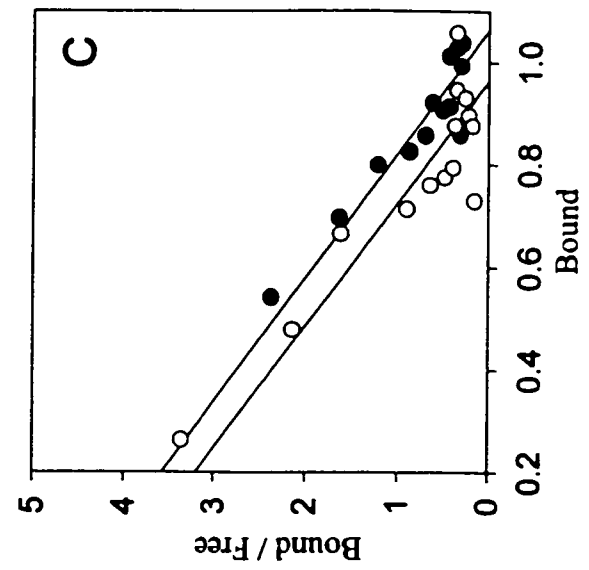


Figure 3.2 Relative intrinsic fluorescence intensity plots for the interactions of EACA with PPACK-t-PA and PPACK-b-PA. 0.3 μM PPACK-t-PA (●) or PPACK-b-PA (○) was titrated with EACA. The intrinsic fluorescence intensities throughout the titrations are shown relative to the intensity of the Pg activator alone. Analysis of these results indicates saturable binding of EACA to PPACK-t-PA with a $K_d = 214 \mu\text{M}$ and $n = 0.91$ EACA/t-PA. The lack of an increase in the intrinsic fluorescence intensity of PPACK-b-PA when titrated with EACA indicates that EACA does not bind to PPACK-b-PA.

Figure 3.3 Scatchard plots of the binding of dEGR-t-PA or dEGR-b-PA to fibrin in the absence and presence of EACA. Fg (1 μM) was clotted with 10 nM thrombin in the presence of various concentrations of dEGR-t-PA or dEGR-b-PA. The concentration of Pg activator that bound to fibrin was calculated by comparing the dansyl fluorescence of the clot supernatant with that in control titrations containing all reagents except thrombin. The term *Bound* (horizontal axis) refers to the moles of Pg activator bound to fibrin per mole of input Fg, whereas *Free* refers to the moles of unbound Pg activator. Panel A illustrates the binding of dEGR-t-PA to fibrin in the absence of EACA. The solid line represents nonlinear regression analysis of the indicated data which best fit a two-site model with $K_{d1} = 0.053 \mu\text{M}$, $K_{d2} = 2.6 \mu\text{M}$, and $n_1 = 0.25$ t-PA/fibrin, $n_2 = 1.4$ t-PA/fibrin, respectively. The dashed lines represent the theoretical Scatchard lines for the binding of t-PA to these two classes of sites. Panel B shows the binding of dEGR-t-PA to fibrin in the presence of 20 mM EACA. The solid line represents linear regression of these data and indicates that, under these conditions, dEGR-t-PA binds to fibrin through a single class of sites with a $K_d = 0.47 \mu\text{M}$ and $n = 0.25$ t-PA/fibrin. Panel C represents the binding of dEGR-b-PA to fibrin in the absence (\bullet) or presence (\circ) of 20 mM EACA. Linear regression analyses of these data indicate that dEGR-b-PA binds to fibrin through a single class of sites with a $K_d = 0.15 \mu\text{M}$ and $n = 1.0$ b-PA/fibrin in the absence of EACA, and a $K_d = 0.14 \mu\text{M}$ and $n = 0.9$ b-PA/fibrin in the presence of EACA.



fibrin is linear (Fig. 3.3C), indicating a single class of binding sites. Based on analysis of these data, b-PA binds fibrin with a $K_d = 0.15 \mu\text{M}$, and $n = 1.0$ b-PA/fibrin. Virtually identical results are obtained in the presence of 20 mM EACA ($K_d = 0.14 \mu\text{M}$, $n = 0.9$ b-PA/fibrin), consistent with the concept that the interaction of b-PA with fibrin is lysine-independent, and reflects the binding of its finger domain to fibrin.

When fibrin clots charged with a fixed concentration of either dEGR-t-PA or dEGR-b-PA were titrated with increasing concentrations of EACA, the EACA competed for approximately 50% of the t-PA binding to fibrin, but had no effect on b-PA binding to fibrin (not shown). These findings were taken as further evidence that t-PA binds to fibrin through two classes of sites; a high affinity, finger-independent site and a low affinity, kringle-dependent site. In contrast, b-PA binds to fibrin through a single class of high affinity, kringle-independent sites.

The ability of t-PA and b-PA to compete for the same fibrin binding sites was assessed by titrating fibrin clots containing fixed amounts of either dEGR-t-PA or dEGR-b-PA with increasing concentrations of PPACK-b-PA or PPACK-t-PA, respectively. As illustrated in Fig. 3.4, t-PA competes for virtually all of the b-PA binding sites on fibrin. In contrast, b-PA is only able to compete for about 50% of the t-PA binding to fibrin. However, the combination of excess b-PA and EACA competes for almost all of the t-PA binding sites on fibrin (Fig. 3.4). These data support the concept that t-PA and b-PA share a high affinity, lysine-independent class of binding sites on fibrin, and that t-PA binds fibrin through a second class of low affinity sites that are lysine-dependent.

The Scatchard plots for the binding of Glu-Pg and Lys-Pg to fibrin are linear (data

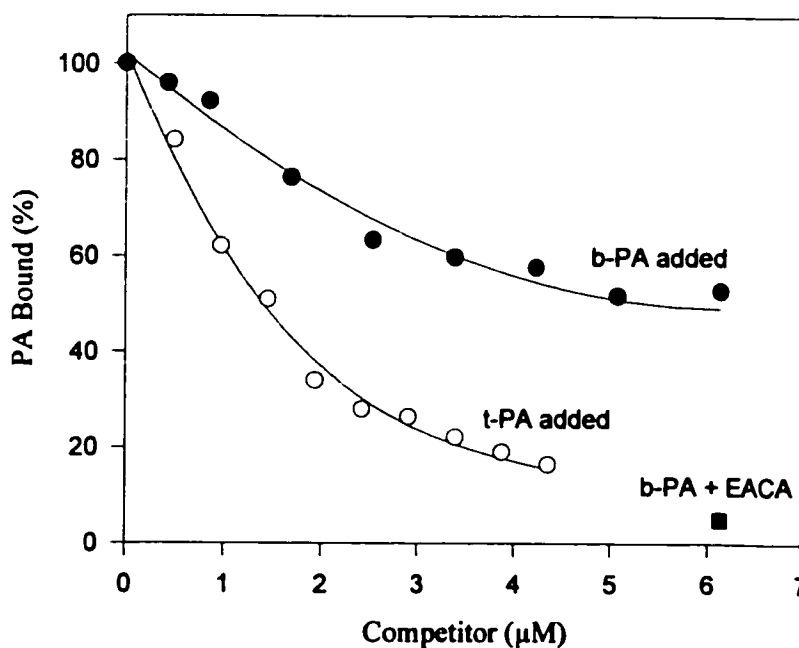


Figure 3.4 Pg activator (PA) competition for fibrin binding sites. Clots made with 2 μM Fg, 10 nM thrombin, and either 0.8 μM dEGR-t-PA or 0.8 μM dEGR-b-PA were titrated with PPACK-b-PA or PPACK-t-PA, respectively. The amount of fluorescently labeled Pg activator that remained bound to fibrin was calculated by comparing the dansyl fluorescence of the supernatant with that in control titrations containing all reagents except thrombin. These values were then expressed as a percentage of the amount of fluorescently labeled Pg activator that bound in the absence of competing unlabeled, active-site-blocked Pg activator. t-PA competes for almost all of the b-PA binding sites on fibrin (○), whereas b-PA only competes for 50% of the t-PA binding sites on fibrin (●). The combination of saturating concentrations of b-PA and 20 mM EACA competes for all of the t-PA binding sites on fibrin (■).

not shown), indicating that both Glu-Pg and Lys-Pg interact with fibrin through a single class of binding sites. Glu-Pg binds to fibrin with a $K_d = 13 \mu\text{M}$ and $n = 0.72$ Glu-Pg/fibrin, whereas Lys-Pg binds to fibrin with a $K_d = 0.13 \mu\text{M}$ and $n = 0.71$ Lys-Pg/fibrin. No binding of either Glu-Pg or Lys-Pg to fibrin was detected when the experiments were repeated in the presence of 20 mM EACA, indicating that their interaction with fibrin is entirely kringle-dependent.

3.4.4 Interactions of t-PA, b-PA Glu-Pg and Lys-Pg with Fg The relative scatter plots for the interactions of t-PA and b-PA with Fg are shown in Fig. 3.5. Under the conditions outlined in methods ($\lambda_{\text{ex}}, \lambda_{\text{em}} = 400\text{nm}$, slit widths = 12nm), the scattering intensity of 0.1 μM PPACK-t-PA is 1.0 (I_0). At saturating levels of Fg, the maximum relative scattering intensity (I/I_0) is 42; a value in good agreement with a calculated maximum relative scattering intensity of 39, if the stoichiometry is 1:1 (Blanchoin et al., 1996). The solid line represents the fit of the data to equation 3 by nonlinear regression analysis. Based on this analysis, t-PA binds to Fg with a $K_d = 0.76 \mu\text{M}$ and $n = 0.59$ t-PA/Fg. When t-PA is titrated with Fg in the presence of 20 mM EACA, there is no increase in the scattering intensity relative to Fg alone. Thus, the binding of t-PA to Fg is entirely kringle-dependent. The scattering intensity of 0.1 μM PPACK-b-PA is 0.8, and the relative scattering intensity does not change when Fg is added. Therefore, in contrast to the findings with t-PA, b-PA does not interact with Fg.

The interactions of Glu-Pg and Lys-Pg with Fg are shown in Fig. 3.6. Relative scattering intensity increases when Lys-Pg is titrated with Fg. I_0 for 0.3 μM Lys-Pg ($\lambda_{\text{ex}}, \lambda_{\text{em}} = 440$, slit widths = 12 nm) is 2.7. If one Lys-Pg molecule binds to each Fg molecule, the theoretical I/I_0 at saturating Fg concentrations is 24. Titrations of Lys-Pg with Fg reach a

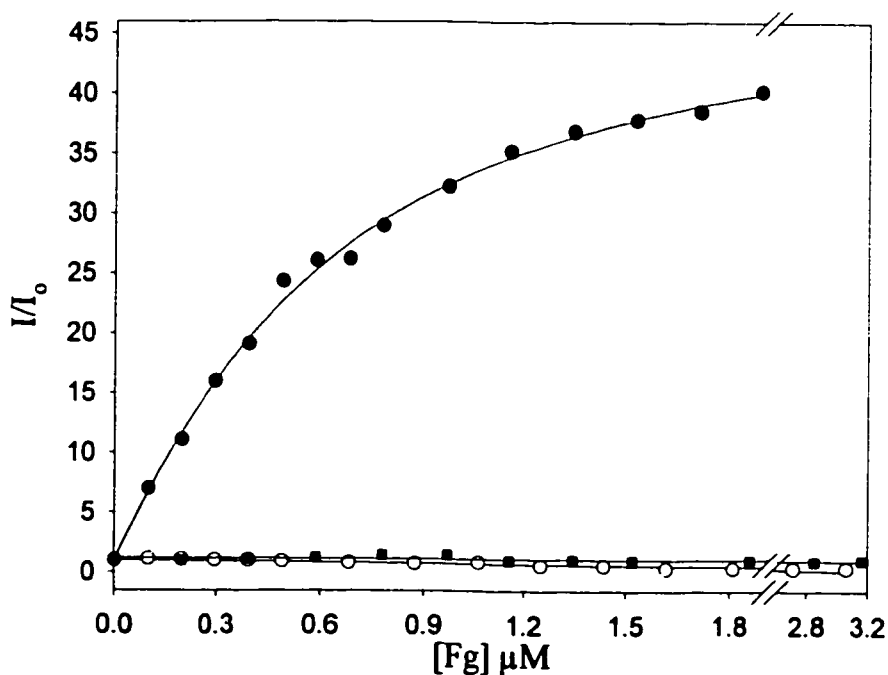


Figure 3.5 The binding of PPACK-t-PA or PPACK-b-PA to Fg. 0.1 μM PPACK-t-PA (●) or PPACK-b-PA (■) was titrated with Fg at the concentrations indicated. Using λ_{ex} and $\lambda_{\text{em}} = 400\text{nm}$, the relative increases in scattering intensities when the Pg activator was titrated with Fg (I) were compared with the scattering intensity of the Pg activator alone (I_0). I_0 values for PPACK-t-PA and PPACK-b-PA were 1.0 and 0.8, respectively. In the case of t-PA, the titration was repeated in the presence of 20 mM EACA (○). t-PA binds saturably to Fg with a $K_d = 0.76 \mu\text{M}$ and $n = 0.6$ t-PA/Fg, where the curved solid line represents the best fit to equation 3 by nonlinear regression analysis. No increases in the relative scattering intensities were detected when Fg was titrated with t-PA in the presence of EACA, indicating that EACA completely abolishes t-PA binding to Fg. Similarly, no interaction was detected between b-PA and Fg.

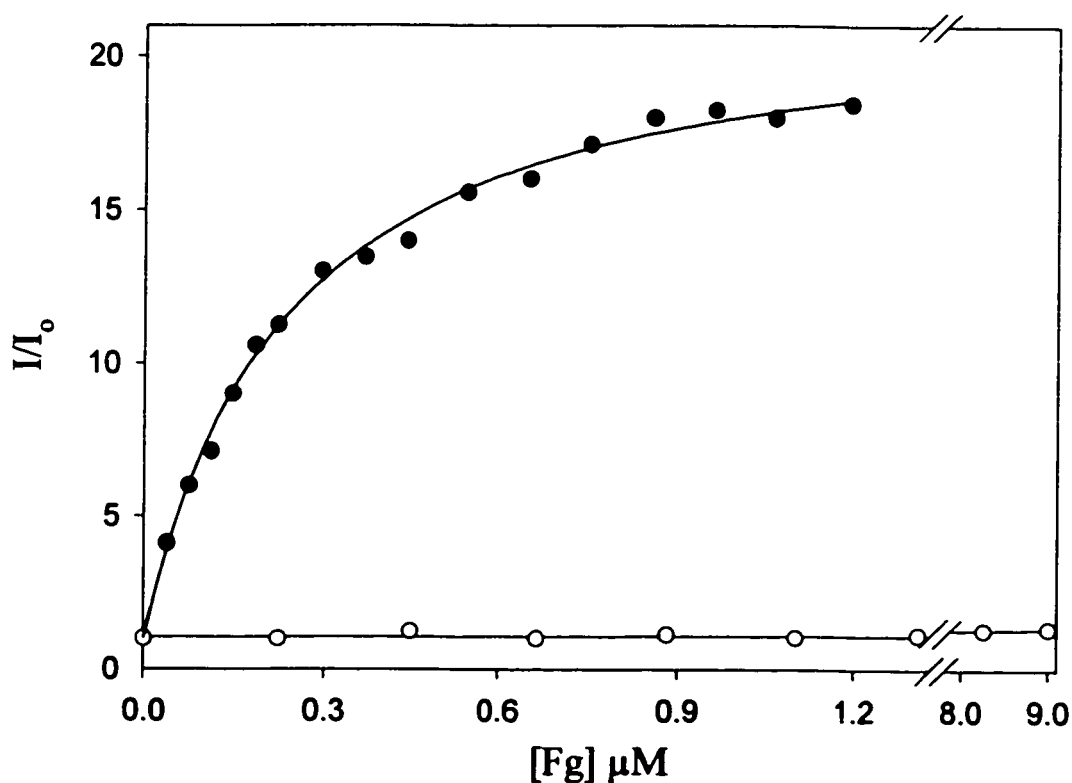


Figure 3.6 The binding of Glu-Pg or Lys-Pg to Fg. 0.3 μM Lys-Pg (●) or Glu-Pg (○) was titrated with Fg at the concentrations indicated and light scattering was monitored at 440 nm (I). Since Glu-Pg was titrated with high concentrations of Fg, excitation and emission slit widths were both narrowed to 8 nm; in contrast, interactions with Lys-Pg were monitored with slit widths of 12 nm. Under these conditions, I_0 values for Glu- and Lys-Pg were 1.6 and 2.7, respectively. Increases in the scattering intensities when Lys-Pg is titrated with Fg indicate saturable binding of Lys-Pg to Fg with a $K_d = 0.23$ μM and $n = 0.64$ Lys-Pg/Fg. The solid line represents the best fit to equation 3. In contrast, when compared with the scattering caused by Glu-Pg alone, Fg does not increase the relative scattering intensity, indicating that binding of Glu-Pg to Fg is not detected.

maximum I/I_0 value of 19; a value compatible with 1:1 stoichiometry. Analysis of the binding data by nonlinear regression analysis indicates that Lys-Pg interacts with Fg with a $K_d = 0.23 \mu\text{M}$ and $n = 0.64$ Lys-Pg /Fg. The interaction of Lys-Pg with Fg is kringle-dependent because it is completely abrogated by EACA (data not shown). In contrast to Lys-Pg, there is almost no increase in the scattering intensity over baseline when Glu-Pg is titrated with Fg.

3.4.5 Interaction of t-PA, b-PA, Glu-Pg and Lys-Pg with (DD)E: The interactions of t-PA and b-PA with (DD)E are illustrated in Fig. 3.7. Titrations with (DD)E were performed under the same conditions as titrations with Fg titrations, and I_0 values for the Pg activators were identical to those previously determined (1.0 for t-PA and 0.8 for b-PA). When t-PA is titrated with (DD)E, the maximum I/I_0 observed is 22; a value identical to theoretical I/I_0 for a one to one t-PA:(DD)E interaction. Based on analysis of the binding data, t-PA binds to (DD)E with a $K_d = 0.023 \mu\text{M}$ and $n = 0.8$ t-PA/(DD)E. No increase in scattering intensity was detected when t-PA was titrated with (DD)E in the presence of 20 mM EACA, indicating that the interaction of t-PA with (DD)E is entirely kringle-dependent. In contrast to the findings with t-PA, no increase in scattering occurred when b-PA was titrated with (DD)E, indicating that b-PA does not interact with (DD)E.

The interactions of Glu-Pg and Lys-Pg with (DD)E are illustrated in Fig. 3.8A and 3.8B, respectively. With λ_{ex} and $\lambda_{\text{em}} = 440\text{nm}$ and slits widths set to 12nm, 0.1 μM (DD)E has a scattering intensity of 7.4. Titration of (DD)E with Glu-Pg results in a maximum I/I_0 of 2.0; a value similar to a predicted I/I_0 of 1.9 for a one to one Glu-Pg to (DD)E interaction. Analysis of the binding curve indicates that Glu-Pg binds to (DD)E with a $K_d =$

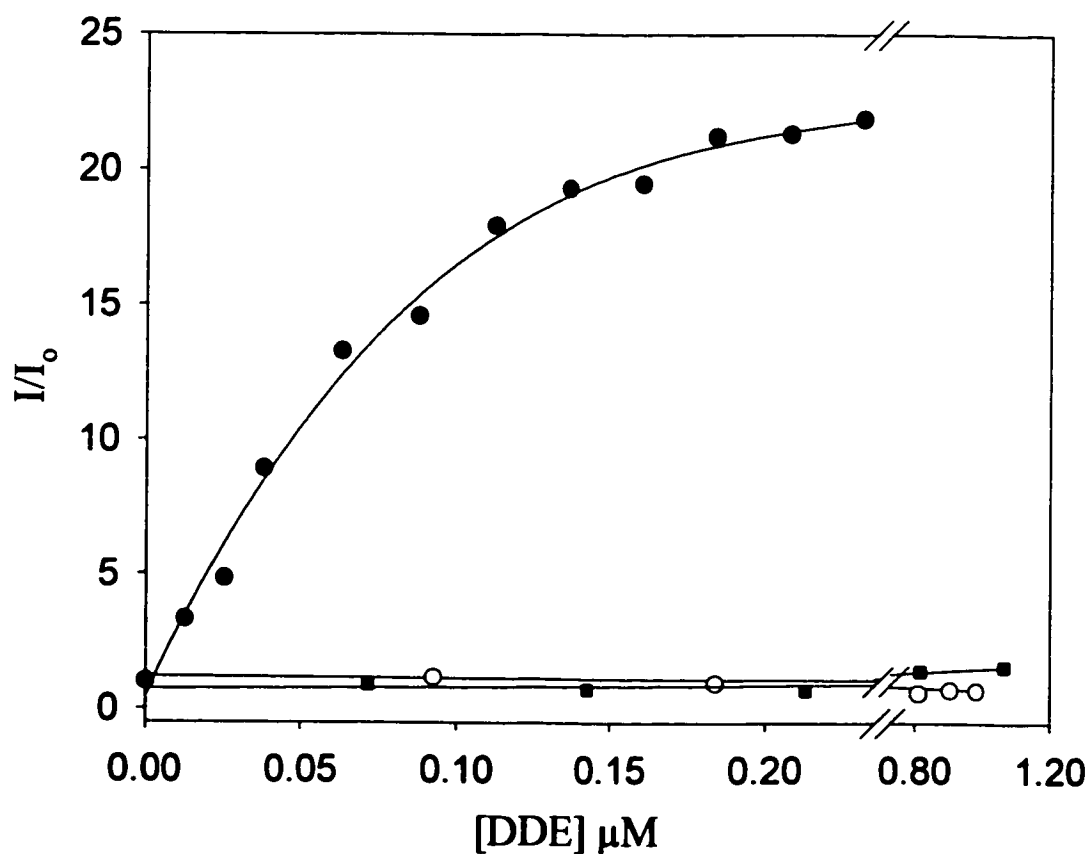
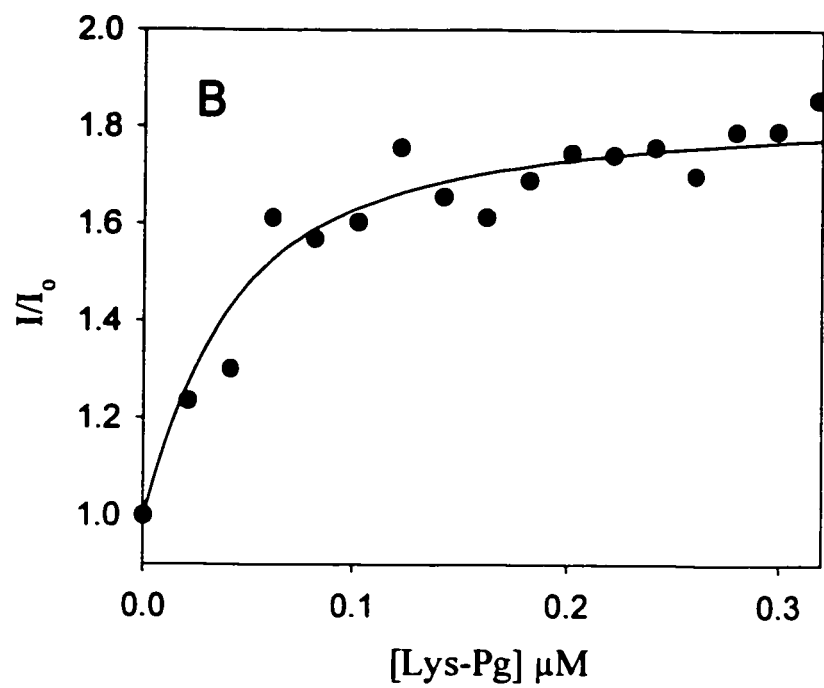
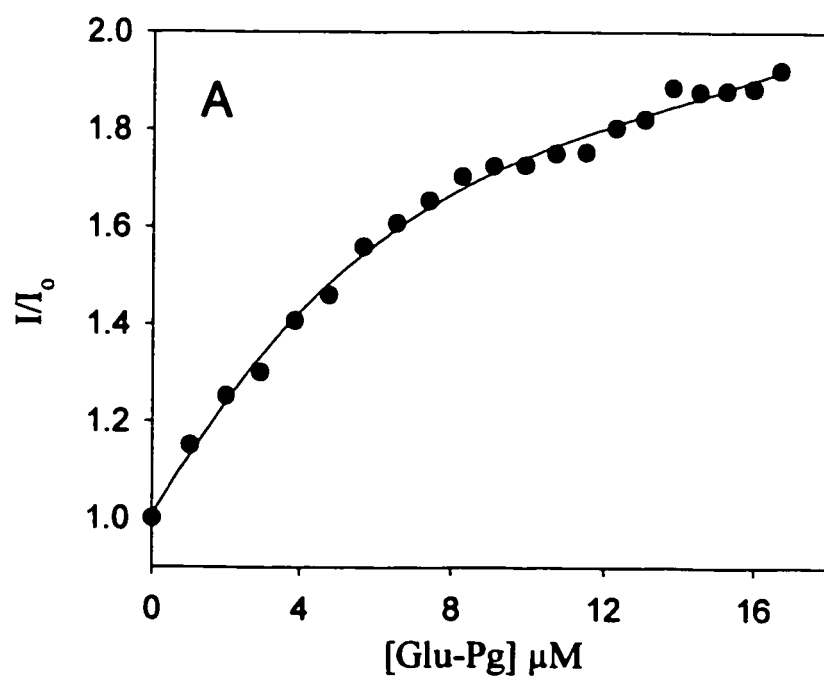


Figure 3.7 The binding of PPACK-t-PA or PPACK-b-PA to (DD)E. 0.1 μM PPACK-t-PA (●) or PPACK-b-PA (■) was titrated with (DD)E at the concentrations indicated. Using λ_{ex} and $\lambda_{\text{em}} = 400$ nm, scattering intensities obtained in the presence of (DD)E (I) were compared with those obtained with Pg activator alone (I_0). PPACK-t-PA binds saturably to (DD)E with a $K_d = 0.023$ μM and $n = 0.8$ t-PA/(DD)E. When the titration is repeated in the presence of EACA (○) there is no increase in the relative scattering intensity, indicating that EACA completely blocks the interaction of PPACK-t-PA with (DD)E. In contrast to t-PA, b-PA does not interact with (DD)E.

Figure 3.8 The binding of Glu-Pg or Lys-Pg to (DD)E. Glu- and Lys-Pg interactions with (DD)E were analyzed by titrating 0.1 μM (DD)E with Glu-Pg (Panel A) or Lys-Pg (Panel B) at the concentrations indicated, and monitoring the light scattering intensity at 440 nm. Under these conditions, I_0 for (DD)E was 7.4. Analysis of these data indicates Glu-Pg binds to (DD)E with a $K_d = 5.4 \mu\text{M}$ and $n = 1.2$ Glu-Pg/(DD)E, whereas Lys-Pg binds to (DD)E with a $K_d = 0.03 \mu\text{M}$ and $n = 1.1$ Lys-Pg/(DD)E.



5.4 μM and $n = 1.2$ Glu-Pg/(DD)E. Lys-Pg titration of (DD)E results in a maximum I/I_0 of 1.8, a value identical to that predicted by 1:1 stoichiometry. Nonlinear regression analysis of the data indicates saturable binding of Lys-Pg to (DD)E with a $K_d = 0.03 \mu\text{M}$ and $n = 1.1$ Lys-Pg/(DD)E. The interactions of both Glu-Pg and Lys-Pg with (DD)E are completely inhibited by 20 mM EACA, indicating that their binding is kringle-dependent (data not shown).

3.5 Discussion

Previously, we demonstrated that t-PA causes systemic plasminemia and subsequent fibrinogenolysis because (DD)E generated during the thrombolytic process stimulates t-PA-mediated Pg activation (Weitz et al., 1991; Weitz et al., 1993). We, and others, have shown that t-PA produces more Fg consumption than b-PA in experimental animals (Gardell et al., 1991; Witt et al., 1992; Klement et al., 1995; Mellott et al., 1995). Figure 3.1 provides a plausible explanation for the greater fibrin-specificity of b-PA over t-PA. Thus, (DD)E and Fg are less potent stimulators of Pg activation by b-PA than t-PA. To explore the possibility that this reflects differences in the affinities of the Pg activators for (DD)E and Fg, we compared the binding interactions of t-PA and b-PA with (DD)E and Fg. Since efficient Pg activation requires the formation of a ternary enzyme-cofactor-substrate complex (Horrevoets et al., 1997a), the affinity of both native Glu-Pg and plasmin-derived Lys-Pg for (DD)E and Fg also were quantified. For comparative purposes, we also measured the affinities of the activators and substrates for fibrin.

The binding parameters for the interactions of the Pg activators (t-PA and b-PA) and substrates (Glu-Pg and Lys-Pg) with the cofactors (Fg, fibrin, and (DD)E) are listed in Table 3.1, and the structural domains responsible for these interactions are summarized in Table 3.2. Interactions of t-PA and b-PA with (DD)E and Fg elucidate the principal differences between the two activators. t-PA binds to both Fg and (DD)E via its K2 domain. In contrast, b-PA does not bind Fg or (DD)E because it lacks a functional lysine-binding site. Thus, the presence of a lysine-binding kringle, in addition to its finger domain, gives t-PA a wider binding repertoire than b-PA.

TABLE 3.1

Summary of Binding Parameters

All values are presented as mean \pm S.E.

Ligand	Fibrin		(DD)E		Fg	
	K_d	n	K_d	n	K_d	n
	μM	mol / mol Fn	μM	mol / mol (DD)E	μM	mol / mol Fg
t-PA	0.053 \pm 0.019 2.6 \pm 0.4	0.25 \pm 0.08 1.4 \pm 0.1	0.023 \pm 0.008	0.80 \pm 0.16	0.76 \pm 0.04	0.59 \pm 0.28
b-PA	0.15 \pm 0.04	1.0 \pm 0.2	N.B. ^a	N.B.	N.B.	N.B.
Glu-Pg	13 \pm 4	0.72 \pm 0.21	5.4 \pm 1.1	1.2 \pm 0.2	N.B.	N.B.
Lys-Pg	0.13 \pm 0.03	0.71 \pm 0.21	0.030 \pm 0.005	1.1 \pm 0.2	0.23 \pm 0.05	0.64 \pm 0.16

^aN.B. denotes no binding detected

TABLE 3.2

Domains Responsible for the Binding of t-PA, b-PA, and Pg to Fg, Fibrin, and (DD)E

Ligand	t-PA	b-PA	Pg
Fg	Kringle	N.B. ^a	Kringle
Fibrin	Finger + Kringle	Finger	Kringle
(DD)E	Kringle	N.B.	Kringle

^aN.B. denotes no binding detected

Both the finger and domains of t-PA independently contribute to its interaction with fibrin (Fig. 3.3A). Binding is reduced by EACA (Fig. 3.3B), and we interpret these results as indicating that EACA blocks the low affinity, K₂-dependent interaction of t-PA with fibrin. Although the stoichiometry of the high affinity site is unchanged in the presence of EACA, its affinity decreases from a K_d of 0.053 μM to 0.47 μM . Nesheim et al. (1990) also

reported that EACA increases the K_d of the high affinity interaction of t-PA with fibrin. The reduced affinity attributed to finger-mediated binding may reflect the conformational changes in t-PA that occur when its K2-domain is occupied by EACA, a concept supported by our observation that EACA induces changes in the intrinsic fluorescence of t-PA (Fig. 3.2), and the report that the fluorescence of eosin-t-PA changes when it is titrated with poly-L-lysine (Loscalzo, 1988).

In contrast to t-PA, b-PA binds to fibrin through a single class of high affinity sites (Fig. 3.3C). Similar results were obtained by Bringmann et al. (1995). Since EACA has no effect on binding (Fig. 3.3C), the interaction is kringle-independent. The finger domain of both b-PA and t-PA recognize the same high affinity binding site on fibrin because t-PA inhibits b-PA binding to fibrin in a concentration-dependent fashion. In contrast, b-PA partially inhibits t-PA binding by competing only with those t-PA molecules that are bound via their finger domains (Fig. 3.4). This concept is supported by the observation that complete inhibition of t-PA binding to fibrin occurs with a combination of b-PA and EACA (Fig. 3.4). Thus, t-PA and b-PA demonstrate comparable high affinity, finger-mediated binding to intact fibrin, whereas t-PA binds additionally to fibrin through a distinct low affinity, kringle-dependent binding site. The observation that the finger domain of t-PA binds fibrin with a stoichiometry of 0.25 mol t-PA per mol fibrin both in the absence and presence of EACA, whereas the finger domain of b-PA binds fibrin with 1:1 stoichiometry (Table 3.1), suggests that the kringle domain of t-PA sterically limits the access of its finger domain to fibrin binding sites.

It is evident from Table 3.1 that kringle-dependent affinities of t-PA and Pg vary

depending on the fibrin(ogen) derivative. Kringle-dependent interactions with Fg and fibrin are weak, whereas (DD)E binding is much stronger. The affinity of the site on (DD)E that binds the K2 domain of t-PA is 112-fold higher than its counterpart on fibrin. Consequently, t-PA binds to (DD)E via its K2 domain with an affinity similar to that of its finger-domain for fibrin. These findings indicate that when fibrin is solubilized by plasmin to form (DD)E, the binding site for the finger domain is lost, whereas the binding site for the K2 domain is modified such that its affinity increases. These findings are consistent with previous studies reporting increased binding of t-PA to fibrin that was partially degraded by plasmin or to fibrin formed from Fg that was plasmin cleaved (Higgins and Vehar, 1987; de Vries et al., 1989). The observation that (DD)E retains high affinity for t-PA may explain its fibrin-like ability to stimulate t-PA-mediated activation of Pg.

Both Glu- and Lys-Pg bind to intact fibrin, although the affinity of Lys-Pg is much higher than that of Glu-Pg (Table 3.1); a finding consistent with previous reports (Lucas et al., 1983). Both forms of Pg bind via their kringle domains, and share the same binding site on fibrin, as evidenced by competition studies (not shown). Plasmin-mediated exposure of new carboxy-terminal lysine residues may explain why the affinities of Glu-Pg and Lys-Pg for (DD)E are higher than those for fibrin. In support of this concept, fibrin exposed to limited plasmin digestion has been reported to exhibit higher affinity for both forms of Pg (Higgins and Vehar, 1987).

Three lines of evidence indicate that (DD)E and Fg serve as templates onto which the enzyme and substrate assemble. First, near unity stoichiometries for the interactions of t-PA, Glu-, and Lys-Pg with (DD)E and Fg were obtained by nonlinear regression analysis of the

binding data. Second, as an independent assessment of stoichiometry, increases in right angle light scattering intensities were compared with those predicted by 1:1 interactions, based on the observation that right angle scattering intensity is related to the square of the molecular mass (Blanchoin et al., 1996). In all cases, the observed increase was similar to that predicted for simple binary interactions. Third, t-PA and Lys-Pg bind to distinct sites on (DD)E and Fg because high concentrations of Lys-Pg have no effect on t-PA binding to these derivatives (not shown); a finding similar to that observed with intact fibrin (Nesheim et al., 1990). Taken together, these data suggest that the cofactor serves as a template onto which one enzyme and one substrate molecule assemble. This hypothesis is supported by the recent observation that t-PA-mediated stimulation of Pg activation by fibrin requires binding of both t-PA and Pg to fibrin (Horrevoets et al., 1997a).

Our results suggest that the affinity of the Pg activator for fibrin(ogen) derivatives determines the stimulatory activity of the cofactor. Thus, we have shown that high affinity Pg activator-cofactor interactions (b-PA/fibrin, t-PA/fibrin, and t-PA/(DD)E) result in marked stimulation of Pg activation, whereas weaker interactions (t-PA/Fg, b-PA/Fg, and b-PA/(DD)E) elicit modest to poor stimulation. A correlation between a cofactor's affinity for t-PA and its ability to stimulate Pg activation is supported by the observation that the affinity of t-PA mutants for fibrin corresponds with their ability to degrade plasma clots (Lijnen et al., 1990a). Furthermore, our findings suggest that, as a determinant of stimulatory activity, the affinity of the cofactor for the activator is more important than the mode of binding. Thus, high affinity, kringle-dependent interactions (t-PA/(DD)E) stimulate Pg activation to the same extent as high affinity, finger-dependent interactions (b-PA/fibrin

and t-PA/fibrin), thereby challenging the concept that the domain of t-PA serves only a docking function that facilitates finger-dependent stimulation (Horrevoets et al., 1994; Stringer et al., 1994).

Our studies give considerable insight into the biochemical differences between t-PA and b-PA and provide direction for further study. Although t-PA-mediated Pg activation is stimulated in the presence of fibrin, t-PA has only modest fibrin-specificity because it binds to (DD)E and fibrin with equally high affinity, and displays moderate affinity for Fg. These data explain why (DD)E is as potent as fibrin at stimulating t-PA-mediated Pg activation (Stewart et al., 1998a), and why Fg is a weaker stimulator (Hoylaerts et al., 1982). In contrast, b-PA is more fibrin-specific than t-PA (Klement et al., 1995; Gardell et al., 1991; Witt et al., 1992; Mellott et al., 1995) because it only has affinity for fibrin. Since it is the K2 domain of t-PA that limits its fibrin-specificity by mediating t-PA binding to (DD)E and Fg, our studies also suggest that targeted removal of the lysine-binding properties within this domain would render t-PA as fibrin-specific as b-PA.

**CHAPTER 4 - IDENTIFICATION OF THE MECHANISM RESPONSIBLE FOR THE
INCREASED FIBRIN-SPECIFICITY OF TNK-TISSUE PLASMINOGEN
ACTIVATOR RELATIVE TO TISSUE PLASMINOGEN ACTIVATOR**

Preface

This manuscript has been published in the Journal of Biological Chemistry (Vol. 275, No. 14, Issue of April 7, pp. 10112-10120, 2000). The authors are: Ronald J. Stewart, James C. Fredenburgh, Beverly A. Leslie, Bruce A. Keyt, Janice A. Rischke, and Jeffrey I. Weitz. The corresponding author is Dr. Weitz. Written permission to include copyright material in this thesis has been obtained from the Journal of Biological Chemistry (p.75).

All of the binding and kinetic analyses were performed by me. Beverly Leslie completed the clot lysis and B β 1-42 measurements (Fig. 4.2, 4.3, and 4.5). Dr. Keyt provided the TNK variants. Janice Rischke assisted in the isolation of (DD)E and Drs. Fredenburgh and Weitz provided guidance throughout the project.

This work was supported by operating grants from the Heart and Stroke Foundation of Ontario and the Medical Research Council of Canada. R.J. Stewart is the recipient of a Research Traineeship from the Heart and Stroke Foundation of Canada. Dr. J.I. Weitz is a Career Investigator of the Heart and Stroke Foundation of Ontario.

The abbreviations used are¹: PAI-1, plasminogen activator inhibitor 1; Mutations in t-PA are abbreviated as follows: T, T103N; N, N117Q; K, K296A/H297A/R298A/R299A.

¹To avoid repetition, the abbreviations listed here are only those that are not defined in the preface of the previous manuscript.

4.1 Summary

TNK-tissue plasminogen activator (TNK-t-PA), a bioengineered variant of tissue-type plasminogen activator (t-PA), has a longer half-life than t-PA because the glycosylation site at amino acid 117 (N117Q, abbreviated N) has been shifted to amino acid 103 (T103N, abbreviated T) and is resistant to inactivation by plasminogen activator inhibitor 1 because of a tetra-alanine substitution in the protease domain (KHRR296-299AAAA, abbreviated K). TNK-t-PA is more fibrin-specific than t-PA for reasons that are poorly understood. Previously, we demonstrated that the fibrin-specificity of t-PA is compromised because t-PA binds to (DD)E, the major degradation product of crosslinked fibrin, with an affinity similar to that for fibrin. To investigate the enhanced fibrin-specificity of TNK-t-PA, we compared the kinetics of plasminogen activation for t-PA, TNK-, T-, K-, TK- and NK-t-PA in the presence of fibrin, (DD)E or fibrinogen. Although the activators have similar catalytic efficiencies in the presence of fibrin, the catalytic efficiency of TNK-t-PA is 15-fold lower than that for t-PA in the presence of (DD)E or fibrinogen. The T and K mutations combine to produce this reduction via distinct mechanisms because T-containing variants have a higher K_M , whereas K-containing variants have a lower k_{cat} than t-PA. These results are supported by data indicating that T-containing variants bind (DD)E and fibrinogen with lower affinities than t-PA, whereas the K and N mutations have no effect on binding. Reduced efficiency of plasminogen activation in the presence of (DD)E and fibrinogen, but equivalent efficiency in the presence of fibrin, explain why TNK-t-PA is more fibrin-specific than t-PA.

4.2 Introduction

Tissue-type plasminogen activator (t-PA) is a trypsin-like serine protease that initiates fibrinolysis by converting plasminogen (Pg) to plasmin (Collen and Lijnen, 1986). Plasmin then solubilizes fibrin, yielding fibrin degradation products. Through a positive feedback mechanism, fibrin enhances its own degradation by stimulating t-PA-mediated Pg activation (Hoylaerts et al., 1982). To enhance this reaction, fibrin binds t-PA and Pg, thereby increasing the catalytic efficiency of t-PA-mediated Pg activation 1000-fold over that in the absence of fibrin (Hoylaerts et al., 1982; de Vries et al., 1990; Lijnen et al., 1990b; Horrevoets et al., 1997a; Mosesson et al., 1998). In contrast to the potent stimulatory effect of fibrin, fibrinogen (Fg) produces only modest enhancement in the catalytic efficiency of Pg activation by t-PA (Hoylaerts et al., 1982; Verheijen et al., 1982; Nieuwenhuizen et al., 1983). Because it preferentially activates Pg in the presence of fibrin rather than Fg, t-PA is designated a fibrin-specific Pg activator.

Despite its apparent fibrin-specificity, t-PA causes systemic fibrinogenolysis and α_2 -antiplasmin consumption when given to patients (Rao et al., 1988; Collen, 1997). Recently, we demonstrated that the fibrin-specificity of t-PA is compromised because (DD)E, the major degradation product of crosslinked fibrin (Olexa and Budzynski, 1979b), stimulates Pg activation by t-PA to a similar extent as fibrin (Weitz et al., 1991; Stewart et al., 1998a). This occurs because, like fibrin, (DD)E also binds t-PA and Pg with high affinity, thereby enhancing their interaction (Stewart et al., 1998b). As a potent stimulator of t-PA-mediated activation of Pg, (DD)E generated during thrombus dissolution has the potential to induce systemic plasminemia (Olexa and Budzynski, 1979b; Vaughan et al., 1987). These data

suggest that the fibrin-specificity of an activator not only depends on its relative stimulation by fibrin and Fg, but also on the degree to which it is stimulated by (DD)E. Furthermore, activators that have reduced stimulation by (DD)E, but retain activity in the presence of fibrin, should be more fibrin-specific than t-PA.

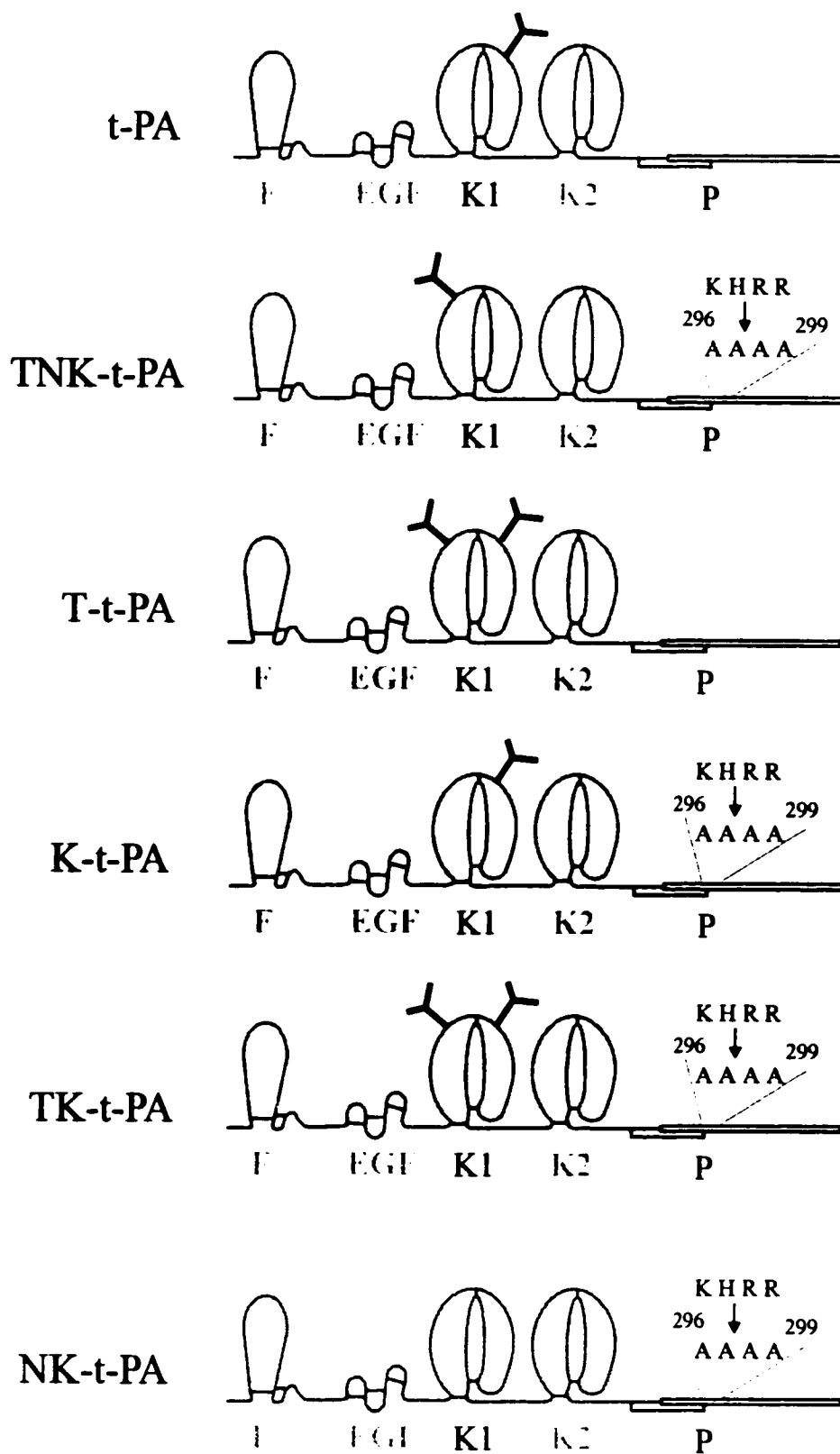
Whereas binding of t-PA to fibrin is predominantly mediated by its finger domain, high affinity t-PA binding to (DD)E is mediated solely by its second kringle domain (Nesheim et al., 1990; Stewart et al., 1998b). Supporting the concept that interaction with (DD)E limits the fibrin-specificity of t-PA, we demonstrated that vampire bat plasminogen activator (b-PA), an agent homologous to t-PA but lacking a second kringle domain, is more fibrin-specific than t-PA because it fails to bind to (DD)E, yet retains its finger-mediated interaction with fibrin (Klement et al., 1995; Stewart et al., 1998b).

Recently, TNK-t-PA, a genetically engineered variant of t-PA, has been compared with t-PA for treatment of patients with acute myocardial infarction (Cannon et al. 1997; Cannon et al., 1998; ASSENT-2 Investigators, 1999). Although the two agents produce similar patency rates, TNK-t-PA is easier to administer because it has a half-life ~5-fold longer than that of t-PA (Modi et al., 1998). Consequently, TNK-t-PA can be given as a single intravenous bolus, whereas t-PA must be given by bolus followed by an infusion (Stassen et al., 1991; Holden, 1990; Smalling, 1997; Werz and Werner, 1998). The longer half-life of TNK-t-PA, relative to t-PA, is the result of two mutations introduced into the first kringle of t-PA; removal of the high mannose carbohydrate at position 117 by substitution of asparagine with glutamine (N117Q, abbreviated N), and creation of a new glycosylation site by replacement of the threonine residue at position 103 with an asparagine (T103N,

abbreviated T) (Keyt et al., 1994). A third modification is introduced into the protease domain of TNK-t-PA. By replacing the amino acids at positions 296 to 299 with alanine residues (K296A/H297A/R298A/R299A, abbreviated K), TNK-t-PA is rendered relatively resistant to inactivation by type-1 plasminogen activator inhibitor (PAI-1) (Collen et al., 1994; Keyt et al., 1994).

In vivo studies indicate that despite producing equivalent thrombolysis, TNK-t-PA causes less Fg and α_2 -antiplasmin consumption than t-PA (Cannon et al., 1997; Cannon et al., 1998). The current study was undertaken to explore the mechanism responsible for the greater fibrin-specificity of TNK-t-PA relative to t-PA. The fibrin-specificity of TNK-t-PA and variants with only one or two modifications [T-, K-, TK- and NK-t-PA (Figure 4.1)] were compared by incubating them in plasma in the absence or presence of a plasma clot, and measuring BB1-42 generation as a sensitive index of fibrinogenolysis. Based on our previous work demonstrating that (DD)E-mediated stimulation of systemic Pg activation compromises the fibrin-specificity of t-PA (Weitz et al., 1991; Weitz et al., 1993; Stewart et al., 1998b), we examined the possibility that differences in fibrin-specificity of the activators reflect the extent to which they are stimulated by (DD)E or Fg. To accomplish this, we compared the kinetics of Pg activation by t-PA to that of the TNK variants in the presence of fibrin, (DD)E or Fg, and determined the affinities of the activators for these fibrin(ogen) derivatives.

Figure 4.1 Secondary structure of t-PA, TNK-t-PA and its variants. TNK-t-PA differs from t-PA in the K1 and protease domains. T refers to the replacement of threonine 103 with asparagine (T103N) and N refers to the replacement of asparagine 117 with glutamine (N117Q). When T and N are combined, the glycosylation site within K1 is shifted 14 amino acids upstream. K refers to the tetra-alanine substitution in the protease domain (KHRR296-299AAAA).



4.3 Experimental Procedures

To reduce repetition, procedures are discussed in detail only when they are different from those presented in the previous manuscript.

4.3.1 Materials

4.3.1i *Plasminogen activators*: Wild-type recombinant t-PA (activase), TNK-t-PA and its variants were provided by Dr. B. Keyt (Genentech Inc., S. San Francisco, CA). All were greater than or equal to 90 % single-chain when analysed by SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) on 4-15 % gels (Ready-Gel, Bio-Rad, Mississauga, ON), as determined by laser densitometry (Ultrosan XL, LKB-Pharmacia, Baie d'Urfe, PQ). Active site blocked fluorescent and nonfluorescent derivatives of Pg activators were prepared as described in section 3.3.1i. The molecular weight and extinction coefficient used for TNK-t-PA and its variants were 65,000 and $\epsilon_{1\%}^{280}=20.0$, respectively (Paoni et al., 1993b; Benedict et al., 1995).

4.3.1ii *Plasminogen*: Native Glu-Pg was isolated from fresh-frozen plasma by lysine-Sepharose affinity chromatography as reported in section 3.3.1ii.

4.3.1iii *Fibrinogen*: Human Pg-depleted Fg, purchased from Enzyme Research Laboratories Inc. (South Bend, IN), was rendered fibronectin-free as detailed in section 3.3.1iii. Factor XIII (FXIII) was removed by affinity chromatography using a sheep anti-FXIII antibody (Affinity Biologicals, Hamilton, ON) coupled to Affi-Gel (Bio-Rad) and the Fg was then precipitated with 25 % ammonium sulfate. The pellet was then washed with 25 % ammonium sulfate, redissolved and dialyzed against TBS. Fg concentration was calculated by measuring the absorbances at 280 and 320 nm and using $\epsilon_{1\%}^{280}=16.0$ (Dellenback and

Chien, 1970; Nesheim et al., 1979). For clot lysis assays, Fg was trace labeled with ^{125}I using Iodo-beads (Pierce, Rockford, IL) to a specific activity of $100 \pm 5 \mu\text{Ci}/\text{mg}$ as described in detail elsewhere (McFarlane, 1965; Ikeno et al., 1981; Weitz et al., 1991).

4.3.1iv *(DD)E*: (DD)E was prepared by plasmin-mediated lysis of crosslinked fibrin clots as outlined in section 3.3.1iv, except 4.3 nM t-PA (in place of 2 pM t-PA) was used to induce fibrin degradation. This concentration of t-PA allowed for more rapid fibrin clot lysis and higher yields of (DD)E.

4.3.2 Methods:

4.3.2i *Preparation of platelet-poor plasma*: Blood was collected from the antecubital veins of 10 to 15 healthy volunteers into 1/10 volume of 3.8 % sodium citrate. After sedimenting the red cells by centrifugation at $2000 \times g$ for 20 min at 4°C , harvested platelet-poor plasma was collected, pooled and stored in aliquots at -70°C until needed.

4.3.2ii *Preparation of ^{125}I -labeled plasma clots*: Thawed plasma, supplemented with ^{125}I -labeled Fg (approximately 60,000 cpm/ml), was clotted with Thromborel S (DADE International Inc, Miami, FL) to 0.1 % v/v and calcium chloride to 15 mM and rapidly dispensed in 500 μl aliquots into 1.5 ml polypropylene microcentrifuge tubes each containing a plastic inoculation loop (Weitz et al., 1991). Clots formed around these loops were incubated for 60 min at 37°C and subjected to 3 washes, each with 2 ml of TBS. Washed clots were then counted for radioactivity for 1 min using a Clinigamma counter (LKB Instruments, Inc., Gaithersburg, MD). Based on SDS-PAGE analysis, clots formed in this fashion are crosslinked because no free α - or γ -chains are detected (Francis et al., 1980; Weitz et al., 1991).

4.3.2iii *Quantification of Pg activator-induced fibrin(ogen)olysis in the absence or presence of ¹²⁵I-labeled plasma clots:* t-PA, TNK-t-PA or a TNK-t-PA variant in concentrations ranging from 0 to 60 nM was incubated for 60 min at 37 °C in 500 µl aliquots of plasma in the absence or presence of a washed ¹²⁵I-labeled plasma clot and clot lysis and fibrinogenolysis were measured as follows. (a) *Clot lysis.* At the end of the incubation period, clots were removed, washed once with 1 ml TBS and counted for residual radioactivity for 1 min. The extent of clot lysis was calculated by subtracting the residual radioactivity from the initial amount and expressing this value as a percentage of initial radioactivity. (b) *Fibrinogenolysis.* The extent of fibrinogenolysis in the absence or presence of a clot was determined by measuring the levels of BB1-42 and Fg in the plasma after the 60 min incubation with the Pg activator. For BB1-42 analysis, 100 µl of plasma was removed and unreacted Fg was precipitated by addition of 300 µl ethanol. After a 30 min incubation on ice and centrifugation at 15,000 × g for 4 min, the ethanol supernatants were evaporated to dryness in a Speedvac concentrator (Savant Instruments Inc, Farmingdale, NY) and reconstituted to original volume with distilled water. BB1-42 levels were determined by radioimmunoassay using an antibody that does not cross-react with fibrinopeptide B or BB15-42 (Weitz et al., 1986; Weitz et al., 1991). For Fg determination, an additional 100 µl aliquot of plasma was removed and 100 KIU/ml aprotinin (American Diagnostica Inc, Greenwich, CT) was added to inhibit plasmin activity. These samples were frozen at -70°C and later assayed for Fg using the Clauss method (von Claus, 1957).

4.3.2iv *Fibrin, (DD)E or Fg stimulation of Pg activation:* The effect of fibrin, (DD)E or Fg on t-PA, TNK-, T-, K-, TK- and NK-t-PA-mediated Pg activation was determined by

comparing plasmin generation in the absence of these cofactors with that in their presence. 20- μ l aliquots containing 2 mM S-2251 and Pg activator in concentrations ranging from 0.5 - 5 nM were added to wells of a 96-well microtitre plate containing Pg (0 - 50 μ M) and (DD)E (0 - 0.75 μ M) or Fg (0 - 7.5 μ M) suspended in 80 μ l of TBS. Plasmin generation was monitored by measuring absorbance at 405 nm at 30 s intervals for 20 to 30 min using a Spectramax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). To measure Pg activation in the presence of fibrin, 5 μ l aliquots of Pg activator and thrombin (final concentrations, 0.1 and 10 nM, respectively) were separately placed in wells prior to the addition of 90 μ l of a solution of Pg (0 - 6.7 μ M), fibrinogen (0 - 1 μ M), 2 mM CaCl₂ and 0.44 mM S-2251. The wells were monitored at 490 and 405 nm and A₄₉₀ values were then subtracted from those for A₄₀₅. In all cases, rates of plasmin formation were calculated by dividing the slope determined from the plot of A₄₀₅ versus time squared by the specific activity of plasmin (0.017 O.D./s/ μ M), which was determined in a separate experiment. Rates of Pg activation (v) were then divided by the concentration of activator and subjected to nonlinear regression analysis (TableCurve; Jandel Scientific, San Rafael, CA) according to the Michaelis Menten equation $v = ([S] \times k_{cat}) / (K_M + [S])$, where v ' is the rate of Pg activation corrected for activator concentration and [S] is the equilibrium concentration of free substrate, calculated for each input concentration of Pg and cofactor as described (Horrevoets et al., 1997a), using 13 and 5.4 μ M for the K_d values of Glu-Pg for fibrin and (DD)E, respectively (Stewart et al., 1998b). This analysis yielded the rate constant, k_{cat}, and Michaelis constant, K_M. The relative effects of fibrin, (DD)E and Fg on Pg activation were determined by comparing the catalytic efficiencies (k_{cat}/K_M) calculated in the presence of

saturating concentrations of each cofactor. Cofactor concentrations were considered saturating when the catalytic efficiency reached a maximum.

4.3.2v *Fluorescence and Light Scattering Measurements:* All fluorescence and light scattering intensities were measured as described in section 3.3.2ii.

4.3.2vi *Binding of t-PA, TNK-t-PA or its Variants to Fibrin:* The binding of dEGR-labeled t-PA, TNK-t-PA or its variants to fibrin was determined as indicated in section 3.3.2iv. Scatchard plots for the binding of t-PA, TNK-t-PA and its variants to fibrin were curved downward and these data were best fit to the two-site model by nonlinear regression analysis (Table Curve, Jandel Scientific, San Rafael, CA) according equation 2 presented in section 3.3.2vi.

4.3.2vii *Binding of t-PA, TNK-t-PA or its variants to (DD)E or Fg:* Interactions of t-PA, TNK-t-PA or its variants with (DD)E or Fg were monitored using right angle light scattering spectroscopy as described in section 3.3.2v. These solution-phase interactions were analyzed according to equation 3, presented in section 3.3.2vi.

4.3.2viii *Lysine Affinity of t-PA, TNK-t-PA or its Variants:* To compare the affinity of the activators for lysine, changes in intrinsic fluorescence were monitored as each Pg activator was titrated with L-lysine or EACA (Stewart et al., 1998b). Additions of 10 to 20 μ l of 20 mM L-lysine or EACA were made to a 2 ml solution containing 0.1 μ M active site blocked activator. Intrinsic fluorescence was monitored as described in section 3.3.2iii and these data were fit to equation 3, as outlined in section 3.3.2vi, to determine the affinities of the activators for lysine or EACA.

4.4 Results

4.4.1 t-PA- or TNK-t-PA-induced clot lysis: To compare the thrombolytic activities of t-PA and TNK-t-PA, plasma clots prepared with trace amounts of ^{125}I -labeled Fg were incubated for 1 h at 37 °C with either t-PA or TNK-t-PA in concentrations ranging from 0 to 60 nM. Using residual thrombus radioactivity as a measure of clot lysis, both activators produce a concentration dependent and saturable increase in clot lysis (Fig. 4.2). When added in equimolar concentrations, there is no difference in the extent of clot lysis produced by each activator. These data indicate that t-PA and TNK-t-PA are equipotent fibrinolytic agents.

4.4.2 t-PA- or TNK-t-PA-induced fibrinogenolysis in the absence or presence of plasma clots: Aliquots of plasma containing increasing concentrations of t-PA or TNK-t-PA were incubated in the absence or presence of ^{125}I -labeled plasma clots for 1 h at 37 °C. Small quantities of BB1-42, a sensitive index of fibrinogenolysis, are generated when t-PA or TNK-t-PA is incubated in plasma in the absence of a plasma clot (Fig. 4.3A). Only with 60 nM t-PA, the highest concentration tested, does the BB1-42 concentration exceed 1 % of total releasable BB1-42 (based on a plasma Fg concentration of 9 μM (Shafer and Higgins, 1988). Presumably sufficient plasmin is generated at this t-PA concentration to overcome inhibition by α_2 -antiplasmin.

Both t-PA and TNK-t-PA generate more BB1-42 in the presence of a clot than in its absence (Fig. 4.3B). The higher BB1-42 levels in the presence of a clot are not due to BB1-42 trapped within the clot because, in previous studies, we demonstrated that less than 10 nM BB1-42 is recovered when clots suspended in buffer are completely lysed by t-PA (Weitz et

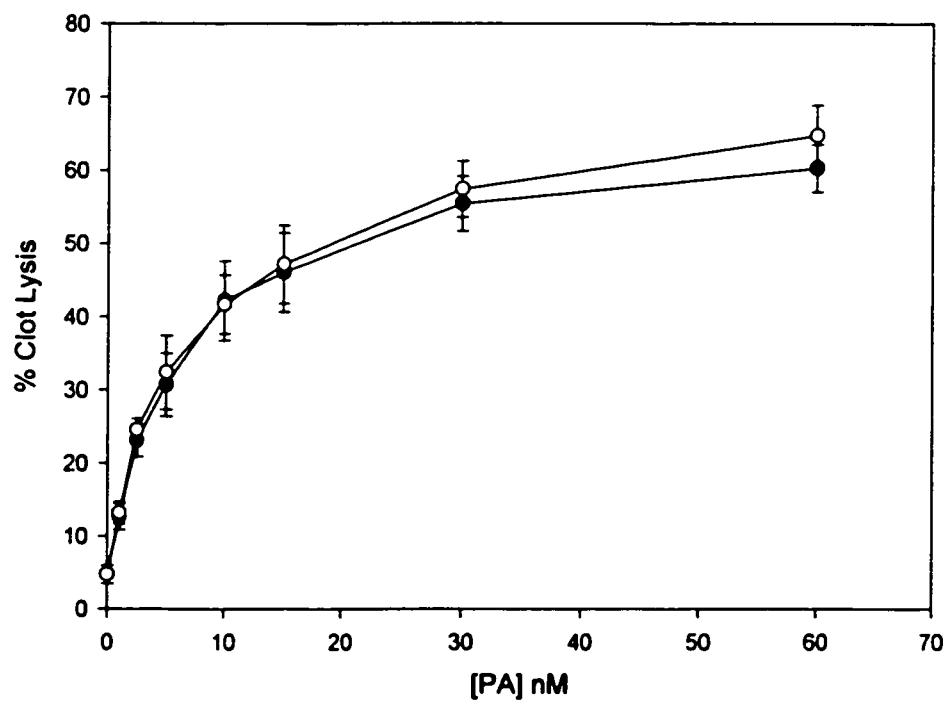


Figure 4.2 Comparison of t-PA- or TNK-t-PA-induced fibrinolysis. To compare the fibrinolytic activities of t-PA and TNK-t-PA, ^{125}I -labeled plasma clots were incubated for 1 h at 37 °C with either TNK-t-PA (○) or t-PA (●) at the indicated concentrations and residual thrombus radioactivity was used as a measure of clot lysis. Points represent the means \pm S.E. of six experiments, each done in duplicate. At each concentration of activator, t-PA and TNK-t-PA produce equivalent fibrinolysis.

Figure 4.3 Comparison of t-PA- or TNK-t-PA-induced fibrinogenolysis in the absence or presence of plasma clots. TNK-t-PA (○) or t-PA (●), at the concentrations indicated, was incubated in plasma for 60 min at 37 °C in the absence (panel A) or presence (panel B) of a plasma clot and BB1-42 levels were measured as an index of fibrinogenolysis. Points represent the means \pm S.E. of six experiments, each done in duplicate. Where error bars are not visible, the size of the error is less than the symbol size. t-PA causes more fibrinogenolysis than TNK-t-PA in the presence of a plasma clot and at high concentrations in the absence of a clot.

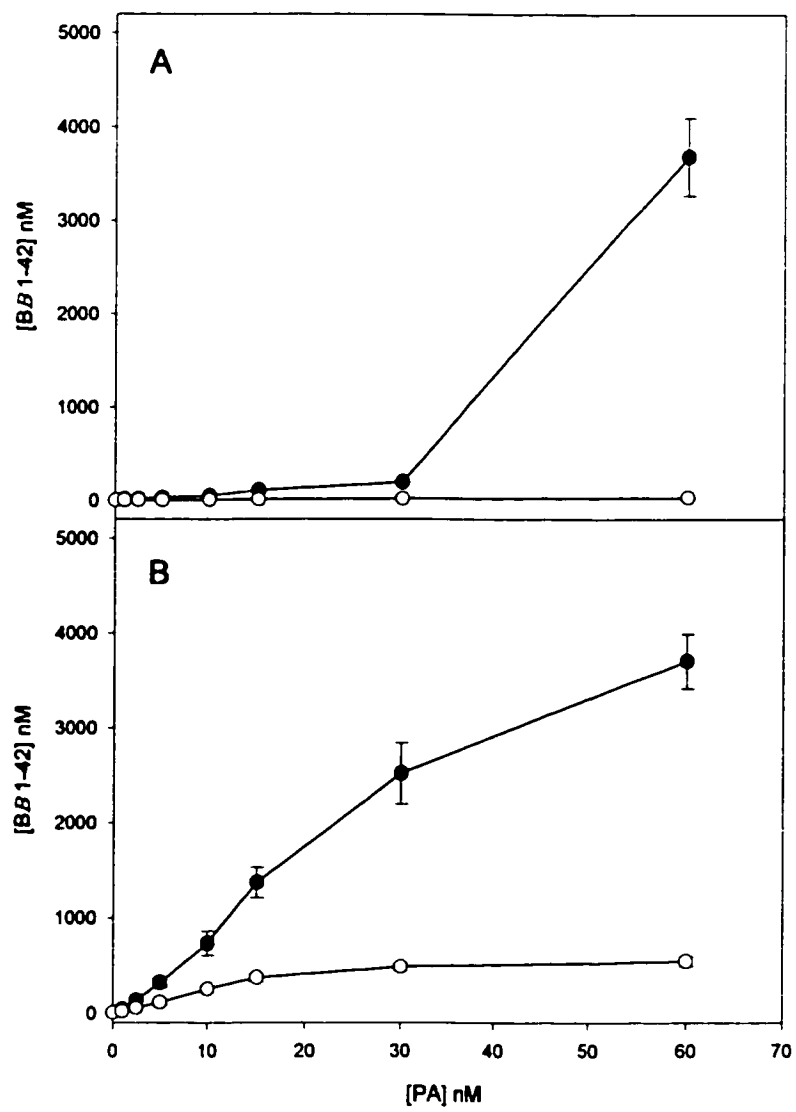
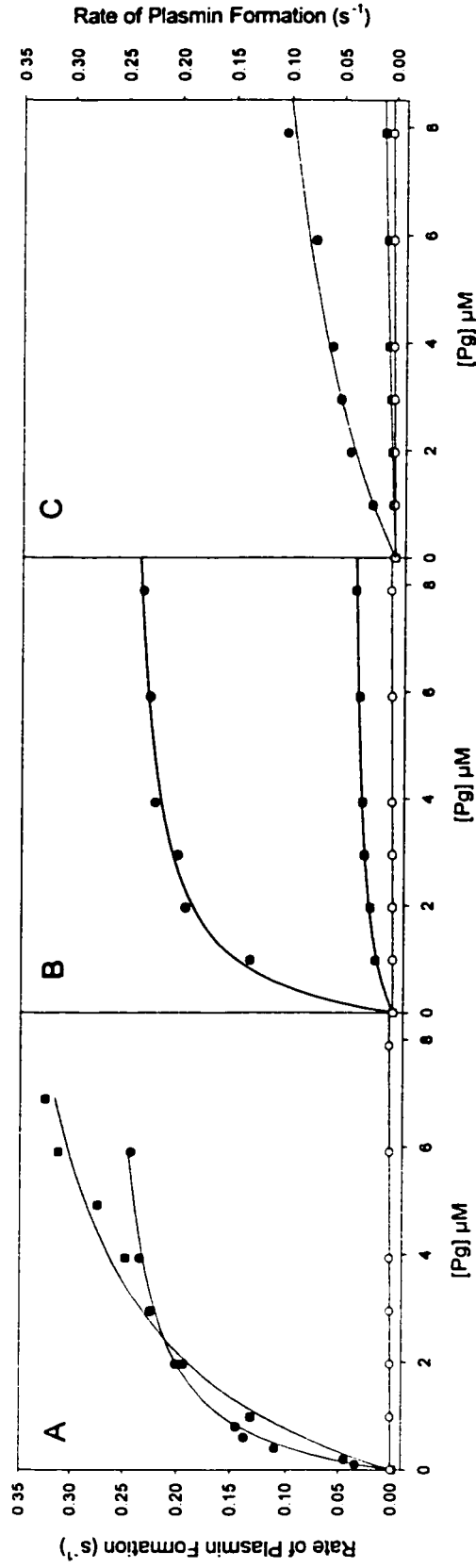


Figure 4.4 Potentiation of t-PA- or TNK-t-PA-mediated Glu-Pg activation by fibrin, (DD)E or Fg. 0.5 nM TNK-t-PA (■) or t-PA (●) was incubated with Glu-Pg, at the concentrations indicated, in the absence (○) or presence of 0.3 μM fibrin (panel A), 0.3 μM (DD)E (panel B) or 2 μM Fg (panel C). Plasmin formation was monitored using the plasmin-directed substrate S-2251, and rates of plasmin formation were calculated. Fibrin increases the rates of t-PA- and TNK-t-PA-mediated plasmin formation to a similar extent. In contrast, (DD)E and Fg have less effect on the rate of plasmin formation by TNK-t-PA than by t-PA.



4.4.4 Fibrinogenolysis induced by TNK variants: To begin to determine which mutation (or combination of mutations) in TNK-t-PA contributes to its increased fibrin-specificity over t-PA, additional t-PA variants containing single (T- and K-t-PA) or double (TK- and NK-t-PA) mutations were examined (Fig. 4.1). All of the activators produce equivalent plasma clot lysis (data not shown). To compare the fibrinogenolysis induced by the TNK variants, 15 nM t-PA, TNK-, T-, K-, TK- or NK-t-PA was incubated in plasma in the presence of a plasma clot for 1 h at 37 °C and BB1-42 generation was used to compare the extent of Fg degradation. As illustrated in Figure 4.5, t-PA causes the most BB1-42 generation in the presence of a clot. When compared with t-PA, T-t-PA produces 50 % less BB1-42, K- and NK-t-PA produce 65 % less BB1-42, and TK- and TNK-t-PA produce 80 % less BB1-42. These data suggest that both the T- and K-mutations contribute to the enhanced fibrin-specificity of TNK-t-PA, and that their effects are additive.

4.4.5 Effect of Fibrin, (DD)E or Fg on the kinetics of Glu-Pg activation by TNK variants:

To determine whether the increased fibrin-specificity of the TNK-variants reflects the degree to which the activators are stimulated by various cofactors, we compared the effect of fibrin, (DD)E or Fg on the kinetics of Glu-Pg activation by t-PA, TNK-, T-, K-, TK- or NK-t-PA. The kinetic parameters for Glu-Pg activation in the presence of saturating levels of these cofactors were determined with a constant amount of activator, and systematic variations in both the Pg and cofactor concentration. Data were fit to the Michaelis-Menten equation by nonlinear regression to determine the values of k_{cat} and K_M . Cofactor concentrations were considered saturating when the catalytic efficiency (k_{cat}/K_M) reached a maximum. A maximum catalytic efficiency was achieved with both fibrin and (DD)E at concentrations

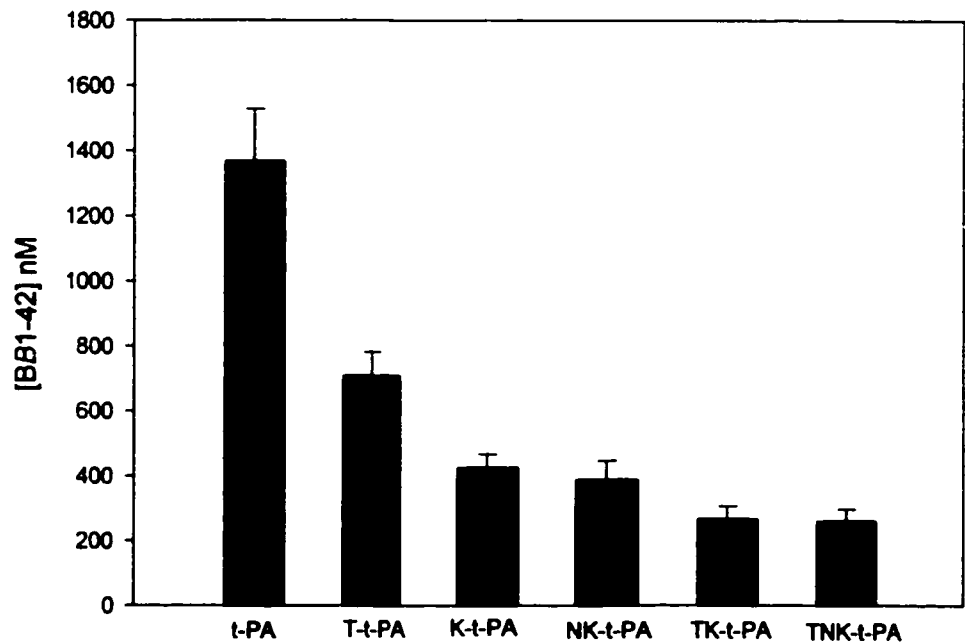


Figure 4.5 Comparison of the fibrinolytic activity of TNK variants. An equimolar concentration (15 nM) of t-PA, TNK-, T-, K-, TK- or NK-t-PA was incubated in plasma for 1 h at 37 °C in the presence of a plasma clot, and the extent of fibrinogenolysis was determined by measuring plasma levels of BB1-42. Each bar represents the mean \pm S.E. of six experiments, each done in duplicate for t-PA and TNK-t-PA, and four experiments, each done in duplicate, for T-, K-, TK- and NK-t-PA. T-t-PA generates 50 % less BB1-42 than t-PA, and K-t-PA and NK-t-PA both produce 65 % less BB1-42 than t-PA. Like TK-t-PA, BB1-42 production by TNK-t-PA is 80 % less than that generated by t-PA.

TABLE 4.1
Kinetic Parameters for Glu-Pg Activation by t-PA, TNK-t-PA or its Variants
in the Absence or Presence of Fibrin, (DD)E or Fg

All values are presented as the means \pm S.E. of at least three experiments

Activator	No cofactor		Fibrin		(DD)E		Fg	
	$k_{cat}/K_M \times 10^{-4}$	K_M	k_{cat}	K_M	k_{cat}	K_M	k_{cat}	K_M
	$\mu M^{-1}s^{-1}$	μM	s^{-1}	μM	s^{-1}	μM	s^{-1}	μM
t-PA	9.0 ± 0.4	0.87 ± 0.25	0.27 ± 0.05	0.87 ± 0.25	0.26 ± 0.05	0.85 ± 0.23	0.26 ± 0.05	9.0 ± 0.8
TNK	0.87 ± 0.11	2.8 ± 0.5	0.54 ± 0.09	2.8 ± 0.5	0.050 ± 0.004	2.5 ± 0.2	0.040 ± 0.009	20 ± 1.8
T	4.2 ± 0.8	1.1 ± 0.5	0.28 ± 0.04	1.1 ± 0.5	0.27 ± 0.04	2.7 ± 0.6	0.28 ± 0.04	11 ± 4
TK	1.1 ± 0.1	2.8 ± 0.3	0.50 ± 0.03	2.8 ± 0.3	0.044 ± 0.005	2.0 ± 0.2	0.039 ± 0.006	20 ± 4
K	1.0 ± 0.3	2.6 ± 0.3	0.52 ± 0.05	2.6 ± 0.3	0.042 ± 0.005	1.1 ± 0.2	0.032 ± 0.006	10 ± 1.5
NK	0.89 ± 0.15	2.2 ± 0.2	0.48 ± 0.08	2.2 ± 0.2	0.040 ± 0.015	1.0 ± 0.2	0.025 ± 0.004	7.8 ± 0.9

TABLE 4.2

**Relative Catalytic Efficiencies of Glu-Pg Activation by t-PA, TNK-t-PA
or its Variants in the Presence of Fibrin, (DD)E or Fg**

Activator	Ratio of Catalytic Efficiencies ^a		
	(DD)E / Fg	Fibrin / (DD)E	Fibrin / Fg
t-PA	11.1	1	11.1
TNK	10	9.5	95
T	9.5	3.2	30
TK	11.6	8.2	94.7
K	12.3	5.4	66.7
NK	12.5	5.5	68.8

^a Cofactor activities are compared by dividing the catalytic efficiencies (derived from Table 4.1) of Pg activation in the presence of the various cofactors fibrin, (DD)E or Fg.

4.4.6 Binding of t-PA, TNK-t-PA or its variants to fibrin: To characterize the binding of t-PA, TNK-t-PA and its variants to fibrin, Scatchard plots were generated. The plots for all activators are nonlinear and indicate binding site heterogeneity (Fig. 4.6) (Boeynaems and Dumont, 1975; Nesheim et al., 1990; Stewart et al., 1998b). Each activator binds to fibrin via a high and low affinity site. High affinity binding is mediated by the finger domain, whereas low affinity binding is mediated by the second kringle domain of the activator (Nesheim et al., 1990; Stewart et al., 1998b). The dissociation constant for high affinity binding of t-PA to fibrin is 0.05 μM and that for low affinity binding is 2.6 μM (Table 4.3). K- and NK-t-PA bind to fibrin with affinities similar to that of t-PA. K_d values for the high and low affinity sites are 0.1 and 2.5 μM , respectively, for K-t-PA and 0.087 and 2.2 μM ,

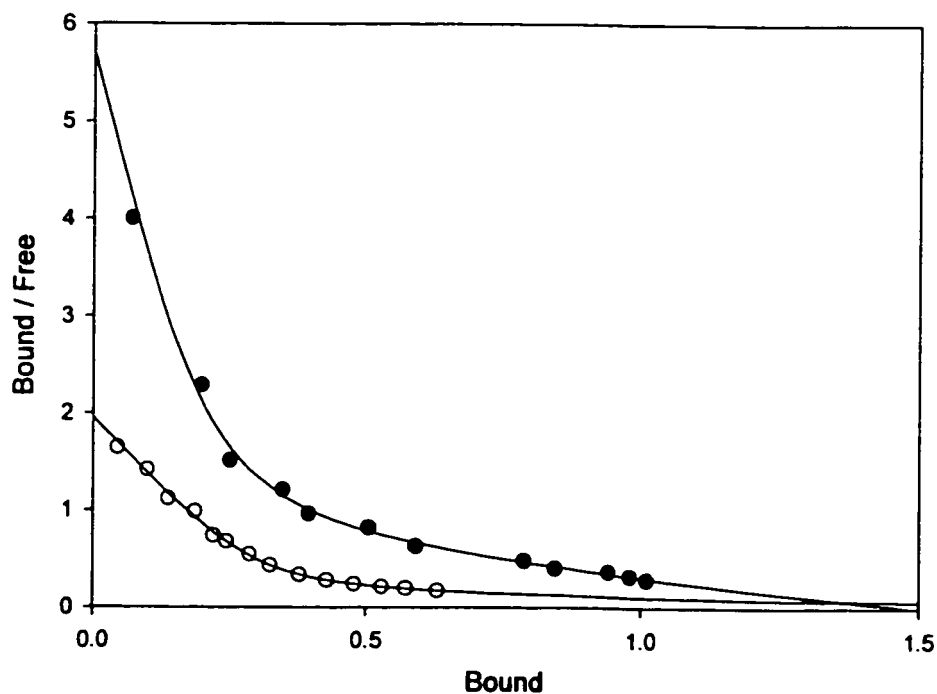


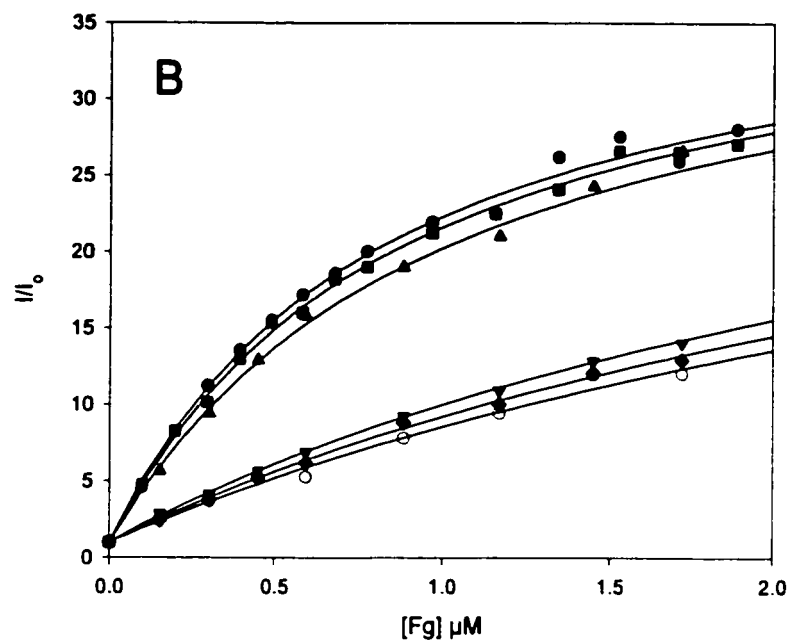
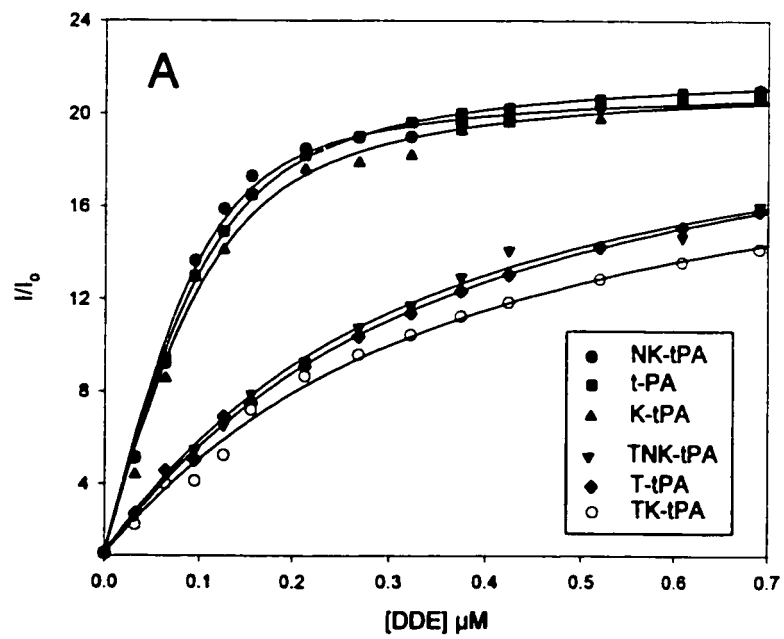
Figure 4.6 Scatchard plots for the binding of t-PA or TNK-t-PA to fibrin. After clotting $1 \mu\text{M}$ Fg with 10 nM thrombin in the presence of various concentrations of dEGR-TNK-t-PA (○) or dEGR-t-PA (●), the concentration of Pg activator bound to fibrin was calculated by comparing dansyl fluorescence of the clot supernatant with that of control titrations containing all reagents except thrombin. Solid lines represent nonlinear regression analysis of the data. Both activators bind to fibrin via a high affinity and low affinity site with K_d values of $0.05 \mu\text{M}$ and $2.6 \mu\text{M}$, respectively, for t-PA and $0.15 \mu\text{M}$ and $15 \mu\text{M}$, respectively, for TNK-t-PA.

respectively, for NK-t-PA, whereas T-containing mutants (TNK-, T- and TK-t-PA) exhibit a 3-fold reduction in affinity for finger-dependent binding and a 5-fold lower affinity for kringle-dependent binding (Table 4.3).

4.4.7 Interactions of t-PA, TNK-t-PA or its variants with (DD)E: To determine whether differences in the catalytic efficiencies of Pg activation in the presence of (DD)E reflect changes in the affinity of the activators for (DD)E, the binding of each activator to (DD)E was measured by light scattering (Stewart et al., 1998b). Figure 4.7A illustrates the relative scatter plots for the interactions of t-PA, TNK-, T-, K-, TK- and NK-t-PA with (DD)E. Under the conditions outlined in “Methods”, the scattering intensity of 0.1 μ M PPACK-t-PA is 1.0 (I_0). At saturating levels of (DD)E, the maximum relative scattering intensity (I/I_0) is 21; a value in good agreement with a calculated maximum relative scattering intensity of 22 if the stoichiometry is 1:1 (Blanchoin et al., 1996; Stewart et al., 1998b). The *solid lines* represent the fit of the data to Equation 2 by nonlinear regression analysis. Based on this analysis, t-PA binds to (DD)E with high affinity ($K_d = 0.029 \mu$ M). The affinities of K- and NK-t-PA ($K_d = 0.03$ and 0.02μ M, respectively) for (DD)E are similar to that of t-PA. In contrast, the T-containing variants (TNK-, T- and TK-t-PA) bind to (DD)E with lower affinity than t-PA (K_d values of 0.25, 0.3 and 0.3μ M, respectively).

4.4.8 Interactions of t-PA, TNK-t-PA or its variants with Fg: To determine whether the T, N or K mutations affect the affinity of the activator for Fg, we measured the binding of t-PA, TNK-, T-, K-, TK- and NK-t-PA to Fg. The scattering intensity for t-PA was identical to that previously determined ($I_0 = 1.0$). Figure 4.7B illustrates the relative scatter plots for the interactions of the various activators with Fg. The *solid lines* represent the fit of the data to

Figure 4.7 Binding of t-PA, TNK-t-PA or its variants to (DD)E or Fg. To measure binding to (DD)E (panel A), (DD)E was titrated into 0.1 μ M active-site blocked activator and scattering intensities obtained in the presence of (DD)E (I) were compared with those obtained in its absence (I_0). Solid lines represent nonlinear regression analysis of the data. T-containing activators (T-, TK- and TNK-t-PA) bind to (DD)E with lower affinity than activators without this mutation (K-, NK- and t-PA), indicating that the T mutation compromises activator binding to (DD)E. Binding to Fg, which was quantified using the same technique, yields similar results with T-containing activators (T-, TK- and TNK-t-PA) exhibiting lower affinity for Fg than K-, NK- and t-PA.



equation 3 (section 3.3.2vi) by nonlinear regression analysis. The maximum I/I_0 observed when each activator was titrated with Fg is approximately 42, a value similar to a predicted I/I_0 of 39 for a 1:1 activator interaction with Fg (Blanchoin et al., 1996; Stewart et al., 1998b). Analysis of these binding data indicates that t-PA binds to Fg with a K_d of 0.8 μM ; K- and NK-t-PA bind to Fg with similar affinities ($K_d = 0.9$ and $0.75 \mu\text{M}$, respectively). In contrast, TNK-, T- and TK-t-PA bind to Fg with lower affinities than t-PA ($K_d = 3.2, 4.0$ and $3.8 \mu\text{M}$, respectively).

4.4.9 Affinities of t-PA, TNK-t-PA or its variants for lysine: Previously, we demonstrated that t-PA binds to (DD)E via its lysine-binding second kringle domain (Stewart et al., 1998b). To determine whether the different affinities of the TNK variants for (DD)E and Fg reflect alterations in their lysine affinities, we quantified changes in intrinsic fluorescence when each agent was titrated with L -lysine. Titration of active-site blocked activator with L -lysine results in a concentration-dependent and saturable increase in intrinsic fluorescence (Stewart et al., 1998b). Based on analysis of these data, L -lysine binds to t-PA with a K_d of 230 μM (Table 4.3). Activators containing the T-mutation (TNK-, T- and TK-t-PA) have a lower affinity for lysine (K_d of 340 μM , 460 μM and 510 μM , respectively). The lysine affinity of K-t-PA is similar to that of t-PA ($K_d = 180 \mu\text{M}$), and NK-t-PA has a moderately higher lysine affinity ($K_d = 150 \mu\text{M}$). Titration of the activators with the lysine analogue EACA results in similar increases in intrinsic fluorescence (data not shown). EACA binds to t-PA with an affinity similar to that of L -lysine (K_d of 210 μM and 230 μM , respectively). Like L -lysine, the affinity of EACA for T-containing mutants is lower than its affinity for t-PA.

4.5 Discussion

The fibrin-specificity of Pg activators is traditionally defined as the ratio of stimulation of Pg activation that occurs in the presence of fibrin relative to that in the presence of Fg. However, this definition overlooks the fact that (DD)E compromises the fibrin-specificity of t-PA by binding and stimulating t-PA to the same extent as fibrin (Weitz et al., 1991; Stewart et al., 1998a; Stewart et al., 1998b; Weitz et al., 1999). Systemic plasmin generation triggered by (DD)E may explain why t-PA causes Fg and α_2 -antiplasmin consumption when given to patients.

TNK-t-PA, a genetically modified variant of t-PA, is as effective as t-PA for treatment of patients with acute myocardial infarction (Cannon et al., 1997; Cannon et al., 1998; Modi et al., 1998; ASSENT-2 Investigators, 1999). However, TNK-t-PA is more convenient to use because its longer half-life permits single bolus administration, whereas t-PA must be given as a bolus followed by an infusion. TNK-t-PA is more fibrin-specific than t-PA and causes less Fg and α_2 -antiplasmin consumption (Cannon et al., 1997; Cannon et al., 1998). This phenomenon could explain why the need for red blood cell transfusion was lower in patients treated with TNK-t-PA than it was in those given t-PA (ASSENT-2 Investigators, 1999).

The mechanism responsible for the enhanced fibrin-specificity of TNK-t-PA, relative to t-PA, is unclear. Keyt et al. (1994) demonstrated that TNK-t-PA has less enzymatic activity in the presence of Fg than t-PA, although both agents have similar activities in the presence of fibrin. Presumably differences in the presence of Fg reflect less stimulation of TNK-t-PA than t-PA. However, these studies fail to consider the potential influence of

(DD)E. The current study was undertaken to explore the mechanism for the greater fibrin-specificity of TNK-t-PA relative to t-PA, and to identify the responsible mutations so that structure-function relationships could be better defined.

Over a range of activator concentrations that approximates those achieved after intravenous bolus injection (Cannon et al., 1997; Modi et al., 1998), t-PA and TNK-t-PA produce equivalent clot lysis (Fig. 4.2); results that are in agreement with clinical trial data indicating that TNK-t-PA and t-PA are equally effective (Cannon et al., 1997; Cannon et al., 1998; ASSENT-2 Investigators, 1999). In contrast, in the presence of a plasma clot, TNK-t-PA induces less BB1-42 generation than t-PA (Fig. 4.3B). Since our previous studies revealed that clot-derived (DD)E is responsible for the increase in Fg consumption (Weitz et al., 1991), we hypothesized that TNK-t-PA would be less responsive to (DD)E than t-PA.

To test this hypothesis, we determined the kinetics of Pg activation by t-PA or TNK-t-PA in the presence of fibrin, (DD)E or Fg (Fig. 4.4). Whereas fibrin stimulates plasmin formation by t-PA and TNK-t-PA to a similar extent, (DD)E markedly increases the rate of Pg activation by t-PA, but not TNK-t-PA. Likewise, Fg has little effect on Pg activation by TNK-t-PA. Since the soluble cofactors, (DD)E and Fg, stimulate Pg activation by TNK-t-PA to a lesser extent than t-PA, whereas fibrin potentiates both activators to a similar extent, these data explain why TNK-t-PA is more fibrin-specific than t-PA. The importance of (DD)E in defining the fibrin-specificity of plasminogen activators is highlighted by the observation that (DD)E is as potent as fibrin as a stimulator of t-PA, but not TNK-t-PA.

As illustrated in Figure 4.4A, the relative rate of t-PA- and TNK-t-PA-mediated plasmin formation in the presence of fibrin depends on the Pg concentration. The rate of t-

PA-mediated Pg activation is higher at low Pg concentrations (0-2 μM), whereas TNK-t-PA appears more active at higher Pg concentrations. This observation may explain some of the discrepancies in the literature. Although the rate of TNK-t-PA-mediated Pg activation in the presence of fibrin has not been reported, Paoni et al. (1993b) noted that the turnover number for TK-t-PA-mediated activation of Pg in the presence of fibrin is 3-fold higher than that for t-PA. In contrast, Key and colleagues (1994) reported that TNK-t-PA has 1.2-fold lower activity in the presence of a plasma clot than t-PA. It is unlikely that this difference reflects the absence or presence of the N-mutation, since we and others have found that removal of this glycosylation site has little effect on the kinetics of Pg activation (Tables 4.1 and 4.2, Benedict et al., 1995). The higher relative rate of plasmin formation determined by Paoni et al. (1993b) for the TK-t-PA variant may reflect the fact that these investigators used Pg concentrations ranging from 0.9 to 19 μM to measure the maximum rate of Pg activation. In contrast, Key and colleagues (1994) used 1 μM Pg to compare Pg activation by t-PA and TNK-t-PA.

Although the k_{cat} for Pg activation is higher for TNK-t-PA than for t-PA in the presence of fibrin (Table 4.1), so is the K_{M} , suggesting that the stability of the ternary activator-substrate-cofactor complex is decreased. However, it is important to note that at 2 μM Pg, the concentration of Pg found in plasma, the rate of plasmin formation in the presence of fibrin is equivalent for TNK-t-PA and t-PA (Fig. 4.4A). These data may explain why the two activators produce equivalent thrombolysis *in vitro* (Fig. 4.2) and in patients (Cannon et al., 1998; ASSENT-2 Investigators, 1999).

To identify the mutations that influence the fibrin-specificity of TNK-t-PA, we

compared TNK-t-PA-induced B β 1-42 generation in plasma in the absence and presence of a plasma clot with that obtained with T-, K-, TK- or NK-t-PA (Figs. 4.1 and 4.5). Although all of these activators produce equivalent plasma clot lysis, the variants induce less fibrinogenolysis than t-PA (Fig. 4.5). TK- and TNK-t-PA are the most fibrin-specific, generating the least B β 1-42. T-t-PA and K-t-PA produce B β 1-42 levels intermediate to those produced by t-PA and TNK-t-PA, suggesting that the T- and K- mutations contribute to the fibrin-specificity of TNK-t-PA in an additive fashion.

Detailed kinetic analysis reveals that, in the presence of fibrin, the k_{cat} and K_M for K-containing variants are 2- and 3-fold higher, respectively, than those for t-PA, resulting in a catalytic efficiency approximately 1.5-fold lower than that of t-PA (Table 4.1). Our results are consistent with those of others who demonstrated that both the k_{cat} and K_M are higher for K-t-PA than for t-PA in the presence of fibrin (Paoni et al., 1993b; Eastman et al., 1992). These data support the concept that although the K-mutation increases the rate of TNK-t-PA-mediated Pg activation in the presence of fibrin, it decreases the stability of the ternary cofactor-enzyme-substrate complex.

The soluble cofactor, (DD)E, distinguishes the TNK-variants from t-PA. In the presence of (DD)E, the catalytic efficiencies of all the mutants are lower than that of t-PA. Furthermore, the catalytic efficiencies in the presence of (DD)E parallel the extent of B β 1-42 generation produced by the various activators in the presence of clots. These data suggest that the extent of stimulation by (DD)E has a major impact on Fg degradation.

In the presence of (DD)E, the k_{cat} for Glu-Pg activation by activators containing the K mutation is approximately 6-fold lower than that for t-PA (Table 4.1). In support of this

concept, Eastman et al.(1992) reported that K-t-PA has a 5-fold lower catalytic efficiency than t-PA in the presence of Fg that was partially degraded by plasmin, reflecting a reduction in k_{cat} . In contrast, we demonstrate that T-containing mutants have a 2.5- to 3-fold increase in K_M compared with t-PA in the presence of (DD)E (Table 4.1). These data suggest that although both the K and T mutations decrease the catalytic efficiency of Pg activation in the presence of (DD)E, they do so via different kinetic mechanisms. Thus, it is the combination of a lower k_{cat} (reflecting the K mutation) and higher K_M (reflecting the T mutation) that results in the marked reduction in catalytic efficiencies of TK- and TNK-t-PA relative to t-PA in the presence of (DD)E.

Examination of the ratios of catalytic efficiency in the presence of fibrin, (DD)E or Fg for t-PA, TNK-t-PA or its variants (Table 4.2) reveals that TK- and TNK-t-PA are the most fibrin-specific since their fibrin / (DD)E and fibrin / Fg ratios are the highest, whereas t-PA is the least fibrin-specific because it has the lowest ratios. The N mutation has no effect on fibrin-specificity since K- and NK-t-PA have similar kinetic ratios. In contrast, both T- and K-t-PA have higher catalytic efficiencies in the presence of fibrin than in the presence of (DD)E or Fg. Again, these data demonstrate that the T and K mutations combine to increase the fibrin-specificity of TNK-t-PA. These results are consistent with the hierarchy of fibrin-specificity determined by BB1-42 generation, and suggest that the fibrin-specificity of the activator is determined by the catalytic efficiency of the activator in the presence of fibrin relative to that in the presence of the soluble cofactors, (DD)E and Fg.

To determine whether differences in the catalytic efficiencies of Pg activation reflect changes in the affinity of the activators for the cofactor, we measured the binding of t-PA,

TNK-t-PA or its variants to fibrin, (DD)E or Fg. All activators bind to fibrin via two distinct classes of sites (Fig. 4.6, Table 4.3). Previously, we and others demonstrated that high affinity binding of t-PA to fibrin is mediated by its finger domain, whereas low affinity binding is mediated by its second kringle domain (Nesheim et al., 1990; Stewart et al., 1998b). The results reported here indicate that the K and N mutations do not alter the affinity of the activator for fibrin because K- and NK-t-PA bind to fibrin with affinities similar to that of t-PA. In contrast, the T mutation produces a 3-fold reduction in high affinity binding and a 5-fold decrease in low affinity binding. Although Keyt et al. (1994) demonstrated similar reductions in binding to fibrin for T-containing mutants, these investigators reported apparent K_d values based on half-maximal binding of activator to various amounts of fibrin, values which do not differentiate between high and low affinity binding. Our findings indicate that the T mutation influences the low affinity, kringle-dependent binding of the activator to fibrin to a greater extent than the high affinity, finger-dependent interaction.

Despite the fact that T-t-PA has reduced affinity for fibrin relative to t-PA, both activators have similar catalytic efficiencies in the presence of fibrin. TK- and K-t-PA also have similar efficiencies in the presence of fibrin, despite differences in low affinity binding of the activators to fibrin. These data suggest that moderate reductions in affinity of these activators for fibrin, caused by additional glycosylation at amino acid 103 (T), do not significantly affect the kinetics of Pg activation in the presence of fibrin.

Like the results with fibrin, T-containing mutants exhibit reduced affinity for (DD)E (Fig. 4.7A). However, the T mutation produces a greater reduction in the affinity for (DD)E

than for fibrin (Table 4.3). In contrast, K- and NK-t-PA bind to (DD)E with affinities similar to that of t-PA. Reduced affinity for (DD)E explains why T-t-PA has a lower catalytic efficiency than t-PA and why both TK- and TNK-t-PA have lower catalytic efficiencies than K-t-PA in the presence of (DD)E. Thus, reduced affinity of the activator for (DD)E and, in turn, reduced catalytic efficiency, contributes to the increased fibrin-specificity of TNK-t-PA.

Affinities of the activators for Fg parallel those for (DD)E (Fig. 4.7B, Table 4.3). However, the catalytic efficiency of Pg activation in the presence of (DD)E is an order of magnitude higher than in the presence of Fg. Furthermore, the fold reduction in affinity of the T-containing activators for (DD)E is higher than that for Fg. Taken together, these findings suggest that alterations in Fg affinity have little influence on TNK-t-PA-mediated Pg activation in the presence of a clot.

Based on our previous observation that the binding of t-PA to (DD)E is mediated by its second kringle domain (Stewart et al., 1998b), we determined whether differences in the affinities of the activators for (DD)E reflect their affinities for ϵ -lysine. Consistent with binding of the activators to (DD)E and Fg, ϵ -lysine binds to T-containing variants with affinities lower than that for t-PA (Table 4.3). These data confirm that the lysine-binding properties of the activator are reduced by the addition of glycosylation at amino acid 103 and are consistent with a reduced affinity of T-containing activators for (DD)E and Fg.

Our studies give insight into the biochemical differences between t-PA and TNK-t-PA, and help to explain why TNK-t-PA is more fibrin-specific than t-PA. The two agents have equivalent fibrinolytic properties because fibrin stimulates TNK-t-PA and t-PA to a similar extent. In contrast, TNK-t-PA produces less fibrinogenolysis than t-PA because

(DD)E produces less stimulation of TNK-t-PA than t-PA. These observations highlight the importance of (DD)E as a determinant of the fibrin-specificity of Pg activators. We have demonstrated that both the T and K mutations contribute to the decreased stimulation of TNK-t-PA by (DD)E via two distinct mechanisms. Glycosylation addition within the first kringle (T) decreases the affinity of TNK-t-PA for (DD)E by reducing its lysine-binding properties, whereas the tetra-alanine substitution in the protease domain (K) decreases the rate of plasmin formation in the presence of (DD)E. Thus, the T and K mutations combine to decrease the catalytic efficiency of Pg activation by TNK-t-PA in the presence of (DD)E and this, in turn, increases the fibrin-specificity of TNK-t-PA relative to t-PA. Our studies also suggest that the fibrin-specificity of TNK-t-PA could be enhanced further by eliminating its lysine-binding properties.

CHAPTER 5 - THROMBIN ACTIVATABLE FIBRINOLYSIS INHIBITOR
ATTENUATES (DD)E-MEDIATED STIMULATION OF PLASMINOGEN
ACTIVATION BY REDUCING THE AFFINITY OF (DD)E FOR TISSUE-TYPE
PLASMINOGEN ACTIVATOR

Preface

This manuscript has been accepted for publication by the Journal of Biological Chemistry. The article is scheduled for a November/December 2000 hard copy release and is currently published as a 'JBC Papers in Press' (article M0:05483) at www.jbc.org. Permission to include copyright material is presented on page 153. The authors are Ronald J. Stewart, James C. Fredenburgh, Janice A. Rischke, Laszlo Bajzar, and Jeffrey I. Weitz. The corresponding author is Dr. Weitz.

All of the experiments presented in this manuscript were performed by me. Janice Rischke assisted in the isolation of (DD)E. Dr. Bajzar provided the TAFIa and Drs. Fredenburgh and Weitz provided guidance throughout the project.

This work was supported by operating grants from the Heart and Stroke Foundation of Ontario and the Medical Research Council of Canada. R.J. Stewart is the recipient of a Research Traineeship from the Heart and Stroke Foundation of Canada. Dr. L. Bajzar is a Research Scholar of the Heart and Stroke Foundation of Canada. Dr. J.I. Weitz is a career investigator of the Heart and Stroke Foundation of Ontario and recipient of the J. Fraser

Mustard Endowed Chair in Cardiovascular Research.

The abbreviations used are²: CPB, carboxypeptidase B; TAFI, thrombin activatable fibrinolysis inhibitor; CPI, carboxypeptidase inhibitor from potato tuber

To Whom It May Concern

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I am completing a Ph.D. at McMaster University in Hamilton, Ontario, Canada. I would like your permission to reprint an article which is currently in press for the Journal of Biological Chemistry. The manuscript number is M0:05483 (article no. 9020) and is entitled "Thrombin Activatable Fibrinolysis Inhibitor Attenuates (DD)E-Mediated Stimulation of Plasminogen Activation by Reducing the Affinity of (DD)E for Tissue Plasminogen Activator: A Potential Mechanism for Enhancing the Fibrin-Specificity of Tissue Plasminogen Activator".

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² To avoid repetition, the abbreviations listed here are only those that are not defined in the prefaces of the previous manuscripts.

5.1 Summary

(DD)E, a degradation product of crosslinked fibrin that binds tissue plasminogen activator (t-PA) and plasminogen (Pg) with affinities similar to those of fibrin, compromises the fibrin-specificity of t-PA by stimulating systemic Pg activation. In this study, we examined the effect of thrombin activatable fibrinolysis inhibitor (TAFI), a latent carboxypeptidase B (CPB)-like enzyme, on the stimulatory activity of (DD)E. Incubation of (DD)E with activated TAFI (TAFIa) or CPB, (a) produces a 96% reduction in its capacity to stimulate t-PA-mediated activation of Glu- or Lys-Pg by reducing k_{cat} and increasing K_M for the reaction, (b) induces the release of 8 mol lysine / mol of (DD)E, although most of the stimulatory activity is lost after release of only 4 mol lysine / mol (DD)E, and (c) reduces the affinity of (DD)E for Glu-Pg, Lys-Pg, and t-PA by 2-, 4-, and 160-fold, respectively. Because TAFIa- or CPB-exposed (DD)E produces little stimulation of Glu-Pg activation by t-PA, (DD)E is not degraded into fragment E and D-dimer (DD), the latter of which has been reported to impair fibrin polymerization. These data suggest a novel role for TAFIa. By attenuating systemic Pg activation by (DD)E, TAFIa renders t-PA more fibrin-specific.

5.2 Introduction

Intravascular fibrinolysis is initiated when plasminogen (Pg) is converted to plasmin by tissue-type plasminogen activator (t-PA) (Hoylaerts et al., 1982; Collen and Lijnen, 1986). Plasmin then degrades fibrin, yielding soluble fibrin degradation products. Through a positive feedback mechanism, fibrin enhances its own degradation by stimulating t-PA-mediated Pg activation. To potentiate this reaction, fibrin acts as a template on to which both t-PA and Pg bind (Horrevoets, et al., 1997a). The activator and its substrate bind to independent sites on intact fibrin because the t-PA interaction is primarily mediated by its fibronectin finger-like domain, whereas Pg binding is kringle-dependent (Castellino and Powell, 1981; Nesheim et al., 1990; Stewart et al., 1998b). As a functional consequence of t-PA and Pg interaction with fibrin, the catalytic efficiency of t-PA-mediated Pg activation is 2 to 3 orders of magnitude greater in the presence of fibrin than in its absence (Hoylaerts et al., 1982; Horrevoets et al., 1997a; Mosesson et al., 1998). In contrast to the potent stimulatory effect of fibrin, fibrinogen (Fg) produces only a 25-fold enhancement in the catalytic efficiency of Pg activation by t-PA (Hoylaerts et al., 1982; Nieuwenhuizen et al., 1988). Because t-PA preferentially activates Pg in the presence of fibrin rather than Fg, it is designated a fibrin-specific Pg activator.

When crosslinked fibrin is solubilized by plasmin, a major degradation product is (DD)E, a complex of D-dimer (DD) noncovalently associated with fragment E (Gaffney and Joe, 1979; Olexa and Budzynski, 1979b). Recently, we demonstrated that (DD)E compromises the fibrin-specificity of t-PA because this soluble fragment is as potent as fibrin at stimulating Pg activation by t-PA (Stewart et al., 1998a; Stewart et al., 2000). Like fibrin,

(DD)E binds t-PA and Pg with high affinity (Weitz et al., 1991; Stewart et al., 1998a; Stewart et al., 1998b). In contrast to its predominantly finger-dependent interaction with fibrin, t-PA binds to (DD)E via its second kringle domain (Nesheim et al., 1990; Stewart et al., 1998b; Stewart et al., 2000). Although Pg also binds to (DD)E in a kringle-dependent fashion, the activator and substrate do not compete for (DD)E binding, indicating that they have distinct binding sites (Stewart et al., 1998b).

A latent carboxypeptidase B (CPB)-like enzyme, termed thrombin activatable fibrinolysis inhibitor (TAFI), has recently been identified in plasma (Eaton et al., 1991; Wang et al., 1994; Bajzar et al., 1995). When activated by the thrombin/thrombomodulin complex, activated TAFI (TAFIa) attenuates fibrinolysis, presumably by removing carboxy-terminal lysine residues on fibrin, thereby removing the newly exposed binding sites for Pg that are generated as fibrin is degraded by plasmin (Sakharov et al., 1997; Nesheim, 1999). TAFIa prevents the 2.5-fold rate enhancement of Pg activation that occurs during the early stages of fibrinolysis and blocks the conversion of Glu-Pg to the more readily activated Lys-Pg, a process also dependent on exposure of new carboxy-terminal lysine residues (Nesheim et al., 1997; Wang et al., 1998). Thus, TAFIa eliminates the enhancement of Pg activation associated with partial degradation of fibrin by plasmin.

Given its mechanism of action, we speculated that TAFIa or CPB would compromise the cofactor activity of (DD)E by abrogating Pg and/or t-PA binding. To explore this possibility, the ability of (DD)E to potentiate Pg activation by t-PA was examined before and after (DD)E exposure to TAFIa or CPB. Herein we demonstrate that the stimulatory activity of (DD)E is nearly abolished upon exposure to TAFIa or CPB. Because plasmin generation

in the presence of TAFIa- or CPB-exposed (DD)E is less than that with native (DD)E, TAFIa or CPB attenuates degradation of (DD)E into its constitutive fragments, E and DD, the latter of which can impair fibrin polymerization. To explore the mechanism responsible for this phenomenon, light scattering spectroscopy was used to compare the affinities of TAFIa- or CPB-exposed (DD)E for Glu-Pg, Lys-Pg or t-PA with those of native (DD)E. TAFIa or CPB exposure reduces the affinity of (DD)E for Glu- or Lys-Pg 2- and 4-fold, respectively. In contrast, the affinity of (DD)E for t-PA is reduced 160-fold. These data raise the possibility that by attenuating the systemic plasmin generation induced by (DD)E, TAFIa renders t-PA more fibrin-specific.

5.3 Experimental Procedures

To avoid repetition, the procedures are described in detail only when they are not reported in the previous manuscripts.

5.3.1 Materials

5.3.1i *Plasminogen activator*: Wild-type recombinant t-PA was the same as that used in section 3.3.1i. Procedures used to analyze t-PA and generate active site blocked t-PA were similar to those reported in section 3.3.1i, except an ImageMaster Video Documentation System (Amersham-Pharmacia Biotech Inc., San Francisco, CA) was used to measure protein band densities following SDS-polyacrylamide gel electrophoresis (PAGE).

5.3.1ii *Plasminogen*: Glu-Pg was isolated from freshly frozen plasma as described in section 3.3.1ii and Lys-Pg was purchased from Enzyme Research Laboratories Inc. (South Bend, IN). The purity of both species were confirmed by the methods outlined in section 3.3.1ii.

5.3.1iii *Fibrinogen*: Human Pg-depleted Fg was purchased from Enzyme Research Laboratories and rendered fibronectin- and factor XIII-free as indicated in sections 3.3.1iii and 4.3.1iii, respectively.

5.3.1iv *(DD)E*: The fibrin degradation product, (DD)E, was prepared by plasmin-mediated lysis of crosslinked fibrin clots as described in section 4.3.1iv.

5.3.1v *Carboxypeptidase B and TAF1a*: CPB, potato tuber-derived CPB inhibitor (CPI), and the CPB-directed synthetic substrate hippuryl-L-arginine were purchased from Sigma. CPB activity was assessed by incubating 20 nM CPB with 0.4 mM hippuryl-L-arginine dissolved in 0.02 M Tris-HCl, 0.15 M NaCl, 0.01 % Tween 20, pH 7.4 (TBS) in a quartz cuvette. Increases in absorbance at 254 nm were monitored for 20 min at 22 °C using a DU 7400

Spectrophotometer from Beckman (Mississauga, Canada). Under these conditions, CPB has a specific activity of 41 units / mg, where one unit hydrolyzes 1 μ mol hippuryl-L-arginine / min. When the experiment was repeated in the presence of 1 μ M CPI, the lowest concentration used to inhibit CPB prior to Pg activation assays, no increase in A_{254} was observed, indicating complete CPB inhibition. TAFI was isolated from fresh frozen plasma by Pg-Sepharose affinity chromatography and activated by thrombin and soluble thrombomodulin as described elsewhere (Bajzar et al., 1996a). The specific activity of the resultant TAFIa against hippuryl-L-arginine was similar to that of CPB. Because TAFIa activity is unstable at room temperature (Boffa et al., 2000; Marx et al., 2000), TAFIa was used immediately or kept on ice until used. Like CPB, the activity of 20 nM TAFIa was completely inhibited by 1 μ M CPI. We used CPB for the majority of experiments because its activity is more stable than that of TAFIa and it does not require preactivation. To demonstrate that TAFIa has effects similar to CPB, however, confirmatory experiments were done using TAFIa.

5.3.2 Methods

5.3.2i Effect of TAFIa or CPB on the rate of (DD)E-stimulated Glu- and Lys-Pg Activation by t-PA: To examine the effect of TAFIa or CPB on the ability of (DD)E to stimulate Pg activation by t-PA, 1.0 ml of a 8 μ M (DD)E solution was incubated with 20 nM TAFIa or CPB for 40 min at 22°C. At intervals, 20 μ l aliquots were removed and 2 μ l of 40 μ M CPI was added to inhibit the TAFIa or CPB. Complete TAFIa or CPB inhibition was achieved because no residual activity was detected using the CPB-directed synthetic substrate hippuryl-L-arginine. The ability of TAFIa- or CPB-treated (DD)E to stimulate Pg activation

was then compared with that of the starting material by adding 20 μl of 2 mM S-2251 and 0.5 nM t-PA to wells of a 96-well microtitre plate containing 0.5 μM Glu-Pg or 0.12 μM Lys-Pg and 0.5 μM (DD)E, dissolved in 80 μl of TBS. Plasmin generation was assessed by measuring absorbance at 405 nm at 30-s intervals for 60 min using a Spectramax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) thermostated at 37 °C. Initial rates of plasmin formation were calculated by dividing the slope determined from the linear portion of the plot of A_{405} versus time squared by the specific activity of plasmin (0.017 O.D.s⁻¹ μM^{-1}), which was determined in a separate experiment (Stewart et al. 1998b).

5.3.2ii Effect of CPB or TAFIa on (DD)E Degradation During t-PA-mediated Pg Activation:

Plasmin degrades (DD)E to DD and fragment E (Gaffney and Joe, 1979; Olexa and Budzynski, 1979b). Because exposure of (DD)E to CPB or TAFIa reduces its ability to potentiate Pg activation, we examined whether CPB or TAFIa attenuates (DD)E degradation during t-PA-mediated Pg activation. 4 μM (DD)E that had been exposed to 20 nM CPB for various intervals, or to 20 nM TAFIa for 40 min, was compared with 4 μM native (DD)E in terms of its ability to stimulate 0.4 μM Glu-Pg activation by 1 nM t-PA. Under these conditions, (DD)E concentrations were sufficient to monitor its degradation by PAGE analysis. After 1 h incubation, the reaction was made to 1 μM VFKCK and 10 nM PPACK to inhibit plasmin and t-PA, respectively, and a 4 μl aliquot of the reaction solution was then subjected to PAGE analysis under nonreducing conditions on 4-15% gradient gels (Ready-Gels; BioRad), on 10 % gels in the presence of SDS, and on 15 % gels in the presence of SDS and β -mercaptoethanol. Gels were stained with Fast Stain (Zoion Research, Shrewsbury, MA) and bands were quantified using an ImageMaster Video Documentation

System. For amino acid sequencing, protein bands were transferred onto polyvinylidene difluoride membranes and stained with Ponceau S. Appropriate bands were cut from the membrane and subjected to amino-terminal sequence analysis which was performed by Biotechnology Service Centre (University of Toronto, Toronto, Canada).

5.3.2iii Determination of the Release of Free Lysine and Arginine From (DD)E by CPB or TAFIa: To determine whether CPB or TAFIa releases lysine or arginine residues from (DD)E, (DD)E was incubated with CPB or TAFIa for intervals up to 60 min and free lysine and arginine were measured using methods similar to those described by Wang et al. (1998). After dialysis of (DD)E into 50 mM HEPES, 150 mM NaCl, pH 7.4, an 8 μ M (DD)E solution was incubated with 20 nM CPB or TAFIa for 60 min at 22 °C. At various times, 100 μ l aliquots were removed and 10 μ l of 40 μ M CPI was added to inactivate the CPB. The samples were deprotonated by bringing the solution to 0.2 M with perchloric acid followed by centrifuging at 12000 \times g for 5 min. After neutralizing the supernatants with potassium hydroxide, the samples were placed on ice and insoluble potassium perchlorate was removed by centrifugation at 12000 \times g for 5 min. The concentration of lysine and arginine in the supernatants was determined enzymatically by methods established by Nakatani et al. (1972) and Gaede and Grieshaber. (1974), respectively. For lysine determination, 50 μ l of supernatant was added to 40 μ l of 0.5 mM NADH (Roche Diagnostics, Laval, Canada) and 2.5 mM α -ketoglutaric acid (Roche Diagnostics) in 50 mM HEPES, 150 mM NaCl, pH 7.0 (HBS). Dehydration of NADH was initiated by the addition of 0.33 units of saccharopine dehydrogenase (Sigma) suspended in 10 μ l HBS. Decreases in fluorescence intensity were monitored over 25 min in a Spectra Max, Gemini XS fluorescent plate reader (Molecular

Devices, Sunnyvale, CA), with excitation and emission wavelengths set to 340 and 450 nm, respectively, and fitted with a 435 nm emission cutoff filter. The concentration of lysine was calculated based on a standard curve generated by plotting changes in fluorescence intensity produced by known concentrations of L-lysine (Sigma). Determination of free arginine was accomplished in the same manner using pyruvate (Roche Diagnostics) in place of α -ketoglutaric acid, and 0.5 units of octopine dehydrogenase (Sigma) in place of saccharopine dehydrogenase. Standard curves for free arginine determination were generated by plotting changes in fluorescence intensity produced by known concentrations of L-arginine (Sigma). To determine whether TAFIa has the same effect on (DD)E as CPB, (DD)E was exposed to 20 nM TAFIa for 60 min and release of free lysine and arginine residues was quantified as described above.

5.3.2iv Effect of (DD)E Exposure to TAFIa or CPB on the Kinetics of (DD)E-Stimulated Glu- and Lys-Pg Activation by t-PA: To determine the effects of exposure of (DD)E to TAFIa or CPB on the kinetics of (DD)E-stimulated Pg activation, kinetic parameters for Glu- and Lys-Pg activation by t-PA, measured in the presence of TAFIa- or CPB-exposed (DD)E, were compared with those obtained with native (DD)E, or in the absence of a cofactor. After exposure of 8 μ M (DD)E to 20 nM TAFIa or CPB for 40 min at 22 $^{\circ}$ C, the reaction was terminated with 8 or 1 μ M CPI. Different concentrations of CPI were used to maintain a concentration of CPI in the Pg activation assay between 0.2 and 0.8 μ M. This range of CPI concentration was maintained to ensure CPI levels were well above the K_i for inhibiting CPB (Ryan et al., 1974), but low enough so as not to inhibit plasmin (5 μ M CPI causes a 7 % reduction in the activity of 0.01 μ M plasmin). 0.8 μ M CPI has no effect on the rate of

plasmin formation (data not shown). Glu- or Lys-Pg, in concentrations ranging from 0 to 16 μM , was incubated with 0.1 nM t-PA in the absence or presence of native (DD)E or TAFIa- or CPB-exposed (DD)E at concentrations up to 6 μM . Values of k_{cat} and K_{M} for Pg activation were determined according to the Michealis Menton equation, as detailed in section 4.3.2iv. For these analyses, free Glu- and Lys-Pg concentrations were calculated based on their affinities for native (DD)E (K_{d} values of 5.5 and 0.09, respectively) or CPB-exposed (DD)E (K_{d} values of 11 and 0.35 μM , respectively) which were determined as described below.

5.3.2v Effect of (DD)E exposure to TAFIa or CPB on its affinity for t-PA, Glu- or Lys-Pg:

The affinities of native or TAFIa- or CPB-exposed (DD)E for t-PA, Glu-Pg or Lys-Pg were quantified using right angle light scattering spectroscopy in a LS50B luminescence spectrometer (Perkin-Elmer, Etobicoke, Canada) (Stewart et al., 1998b; Stewart et al., 2000). 15 - or 20 - μl aliquots of active-site blocked t-PA, Glu- or Lys-Pg (at concentrations of 6, 100, and 10 μM , respectively) were added to 2 ml of a 0.1 μM solution of native or TAFIa- or CPB-exposed (DD)E in a 3 ml quartz cuvette thermostated at 22 °C with stirring. Light scattering was measured as detailed in section 3.3.2v and analyzed according to equation 3, defined in section 3.3.2vi. To confirm the binding parameters obtained in this fashion, reverse titrations also were performed wherein 0.1 μM active-site blocked t-PA, Glu or Lys-Pg was titrated with native or TAFIa- or CPB-exposed (DD)E.

5.4 Results

5.4.1 Effect of CPB or TAFIa on the Ability of (DD)E to Stimulate t-PA-mediated Pg

Activation: To determine whether CPB or TAFIa treatment of (DD)E modulates its ability to stimulate Pg activation by t-PA, (DD)E was incubated with CPB or TAFIa for intervals up to 40 min. After inhibiting CPB or TAFIa with CPI, the stimulatory activity of CPB- or TAFIa-treated (DD)E was then compared with that of the starting material. When t-PA-mediated activation of Glu-Pg is monitored (Fig. 5.1 inset), plasmin formation decreases as (DD)E is progressively exposed to CPB prior to Pg activation. From these data, rates of plasmin formation were calculated (Fig. 5.1). After 40 min exposure of (DD)E to CPB, the rate of Glu-Pg activation in the presence of CPB-treated (DD)E is similar to that in the absence of a cofactor. Analogous results are obtained when Lys-Pg is substituted for Glu-Pg. Although rates of plasmin formation are higher with Lys-Pg than with Glu-Pg, 40 min exposure of (DD)E to CPB almost completely abolishes its stimulatory activity (Fig. 5.2). Exposure of (DD)E to TAFIa reduces the rate of Glu- and Lys-Pg activation to the same extent as CPB-exposure (Fig. 5.2).

Plasmin can degrade (DD)E into DD and fragment E (Gaffney and Joe, 1979; Olexa and Budzynski, 1979b). Because DD and fragment E are less potent stimulators of Pg activation than intact (DD)E (Weitz et al., 1991), this phenomenon could influence the kinetics of (DD)E-stimulated Pg activation. However, when analyzed by PAGE, no detectable (DD)E degradation was observed during the first 10 min of Pg activation (data not shown), the time over which the initial rates of plasmin formation were calculated. These observations suggest that degradation of (DD)E did not influence the kinetics of Pg

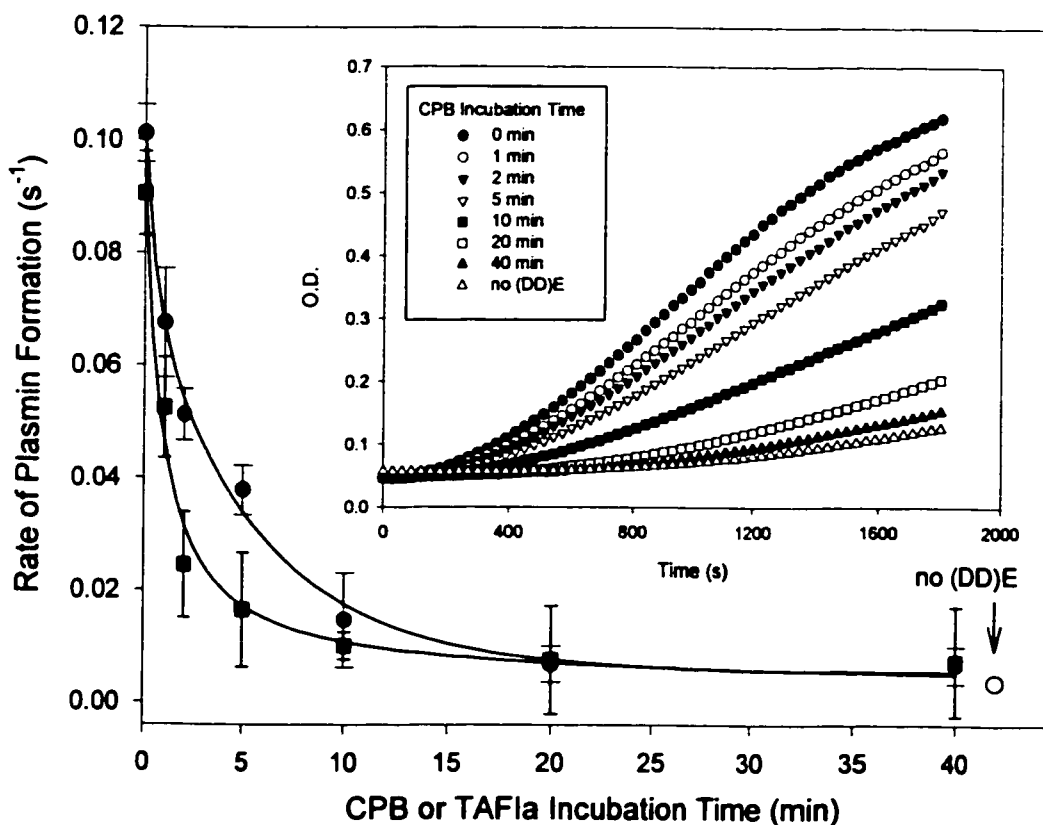


Figure 5.1 Effect of CPB or TAFIa on the rate of (DD)E-stimulated activation of Glu-Pg by t-PA. 8 μM (DD)E was incubated with 20 nM CPB or TAFIa for 40 min at 22 °C. At the indicated intervals, aliquots were removed and CPB or TAFIa was inactivated with CPI. CPB- or TAFIa-exposed (DD)E (0.4 μM) was incubated with 0.4 μM Glu-Pg and 0.1 nM t-PA for 60 min at 37 °C and plasmin formation was monitored at 405 nm using the plasmin-directed substrate S-2251 (inset). From these data, rates of plasmin formation were calculated. Each point represents the mean \pm S.E. of at least three experiments. The rate of Pg activation in the presence of (DD)E incubated for 40 min with CPB (●) or TAFIa (■) is similar to that measured in the absence of (DD)E (○).

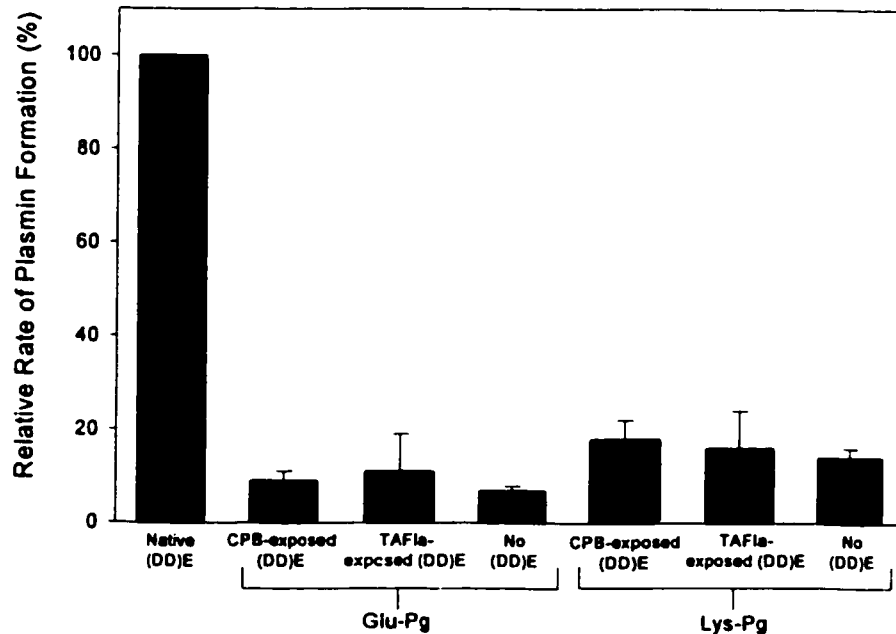


Figure 5.2 The effect of CPB or TAFIa on the rate of (DD)E-stimulated activation of Glu- or Lys-Pg by t-PA. To determine whether exposure of (DD)E to CPB decreases its ability to stimulate the activation of Lys-Pg to the same extent as Glu-Pg, and whether TAFIa has the same effects as CPB, 8 μ M (DD)E was incubated with 20 nM CPB or TAFIa for 40 min at 22 °C. After addition of CPI, 0.4 μ M CPB- or TAFIa-exposed (DD)E was incubated with 0.4 μ M Glu-Pg or 0.1 μ M Lys-Pg and 0.1 nM t-PA, and rates of plasmin formation were determined and compared with those obtained in the presence of 0.4 μ M native (DD)E. Each bar represents the mean \pm S.E. of at least two experiments. Exposure of (DD)E to CPB or TAFIa reduces its ability to stimulate Glu- or Lys-Pg activation by t-PA to almost background levels.

activation in the presence of (DD)E.

5.4.2 Effect of CPB or TAFIa on the Degree to Which (DD)E is Degraded During t-PA-mediated Pg Activation: Figure 5.3, which illustrates the formation of (DD)E from fibrin and its subsequent degradation by plasmin, helps to describe the gels presented in Figure 5.4. (DD)E, which is formed when plasmin degrades two-stranded fibrin protofibrils between adjacent D and E domains, consists of two crosslinked D domains noncovalently associated with an E domain (Fig. 5.3). The E domain remains bound to DD provided that the amino-terminal portion of at least one α - and β -chain within the E domain (α_E and β_E , respectively) remains intact (Olexa et al., 1981; Moskowitz and Budzynski, 1994). Plasmin first cleaves β_E at Lys53, and then α_E at Arg19. The E moiety is characterized by changes in molecular weight that occur upon β_E cleavage (Olexa and Budzynski, 1979b; Olexa et al., 1981). Fragment E₁, which has a molecular weight of ~ 60,000, has the Lys53-Lys54 peptide bonds of both β_E chains intact, whereas fragment E₂, with a molecular weight of 55,000, has one β_E chain cleaved at Lys53. Both fragments E₁ and E₂ remain associated with DD. In contrast, fragment E₃, which has a molecular weight of ~ 50,000, no longer binds DD because both β_E chains are cleaved at Lys53.

When (DD)E is exposed to plasmin, it is degraded to DD and fragment E (Gaffney and Joe, 1979; Olexa and Budzynski, 1979b). Since TAFIa attenuates fibrin degradation by limiting plasmin formation (Bajzar et al., 1995; Sakharov et al., 1997), we explored the possibility that CPB or TAFIa modulates (DD)E degradation. (DD)E was incubated with CPB for 40 min. At intervals, aliquots were removed and CPB was inhibited with CPI. Using PAGE analysis, the extent to which the various (DD)E samples were degraded after

Figure 5.3 Model of (DD)E formation and degradation. Thrombin releases fibrinopeptide A ($A\alpha_{1-16}$) and fibrinopeptide B ($B\beta_{1-14}$) from Fg, thereby generating fibrin monomers. These aggregate to form two-stranded protofibrils that are arranged in a half-staggered fashion, whereby the E domain of a fibrin monomer of one strand is noncovalently associated with D domains of two adjacent fibrin monomers on the opposite strand. Factor XIIIa catalyzes the crosslinking of γ -chains of adjacent D domains. Plasmin cleaves the α , β , and γ chains between E and D domains to yield (DD) E_1 , wherein fragment E_1 remains noncovalently associated with DD via two 'knob and hole' interactions on each D domain. These interactions only occur when the amino-terminals of the α and β chains of fragment E are intact. Exposure of (DD) E_1 to plasmin results in progressive cleavage of the β and α chains of fragment E. The E moiety is characterized by cleavage of the Lys53-Lys54 peptide bonds on the β -chains. Fragment E_2 , which has one β chain cleaved, remains noncovalently associated with DD via one 'knob and hole' interaction. In contrast, fragment E_3 , which has both β chains cleaved at position 53, no longer interacts with DD. Cleavage of the α chains at position 19 within fragment E occurs subsequent to cleavage of at least one β chain.

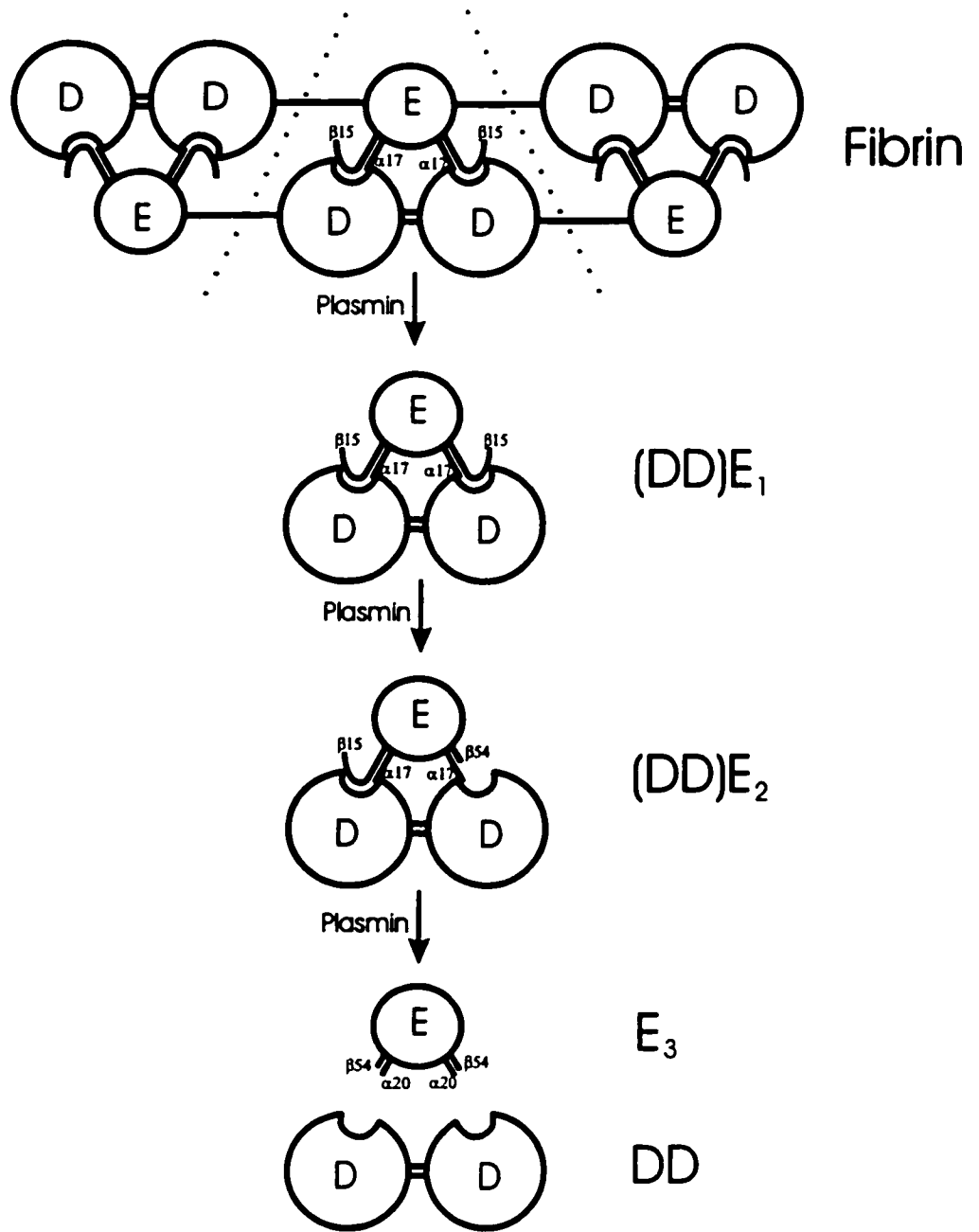
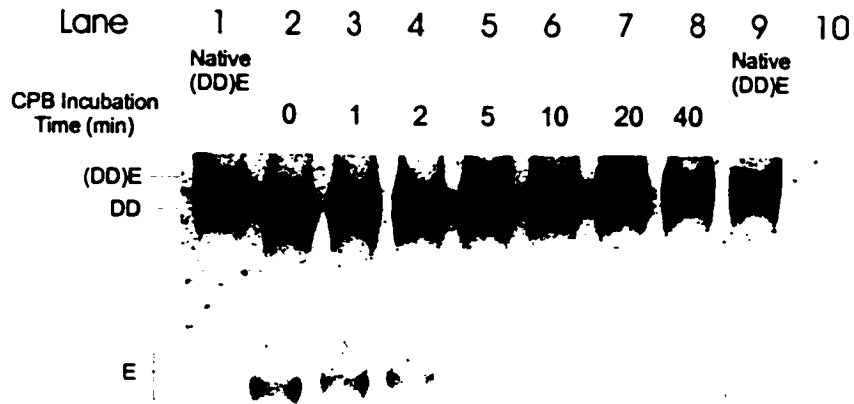
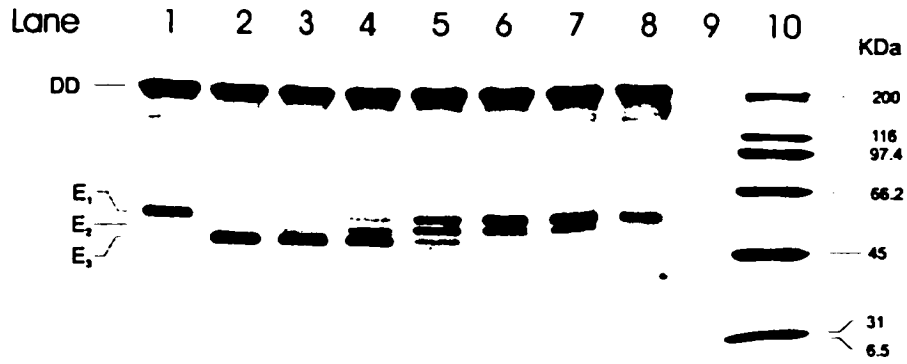


Figure 5.4 Effect of CPB on (DD)E degradation during t-PA-mediated Pg activation.

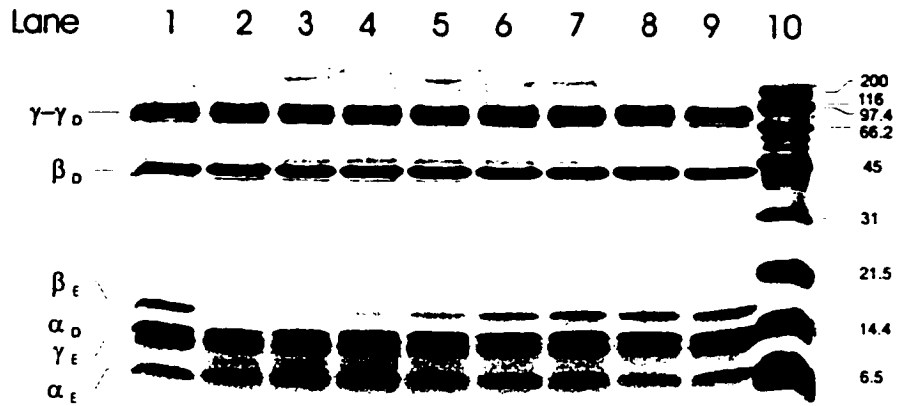
8 μM (DD)E was incubated with 20 nM CPB for 40 min at 22 °C. At intervals, aliquots were removed and CPB was inhibited with CPI. 4 μM of CPB-treated (DD)E or native (DD)E was used to stimulate the activation of 0.4 μM Glu-Pg by 1 nM t-PA for 1 h, at which time plasmin and t-PA were inhibited with VFKCK and PPACK, respectively. The reaction mixture was then subjected to PAGE analysis under nondenaturing conditions on a 4-15 % gradient gel (A), denaturing conditions on 10 % gels (B), or denaturing and reducing conditions on 15 % gels (C). Lane numbers are indicated above each gel and the time (DD)E was exposed to CPB prior to Pg activation is indicated above gel A. Native (DD)E is in lanes 1 and 9 and Molecular weight markers, when present, are in lane 10. Native (DD)E consists only of DD and fragment E_1 . When native (DD)E is used to stimulate Pg activation, the β_E chain is completely degraded, fragment E_1 is degraded to E_3 , and the E moiety no longer remains associated with DD. In contrast, when CPB-exposed (DD)E is used, the β_E chain is not degraded and fragment E_1 remains intact and associated with DD. Thus, by limiting plasmin formation, CPB preserves the structural integrity of (DD)E.



A



B



C

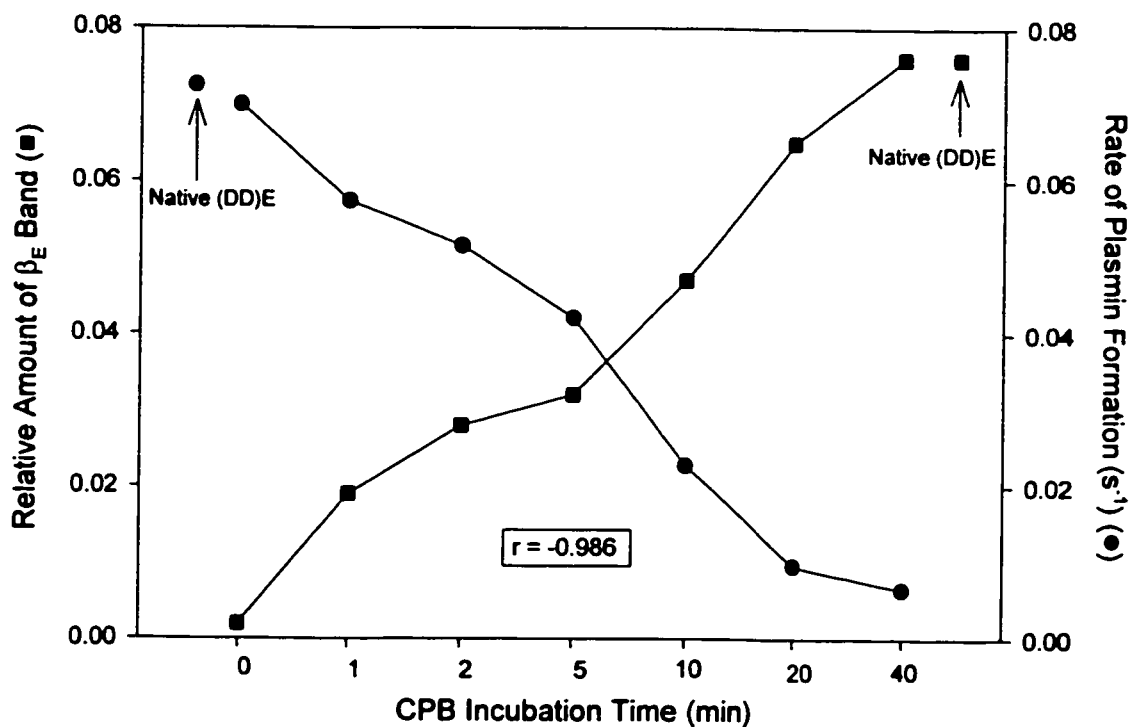


Figure 5.5 Correlation between β_E chain degradation and the ability of (DD)E to stimulate Pg activation. The relative amounts of β_E in each lane of figure 5.4C (■), determined by densitometry, are plotted against the time that (DD)E was exposed to CPB prior to Pg activation. The rate of Pg activation by CPB-exposed (DD)E (●) is plotted against time of CPB-exposure on the secondary y-axis. Both of these values are compared with those obtained with native (DD)E. These data demonstrate that the integrity of the β_E chain is inversely correlated ($r = -0.986$) with the extent to which (DD)E stimulates Pg activation.

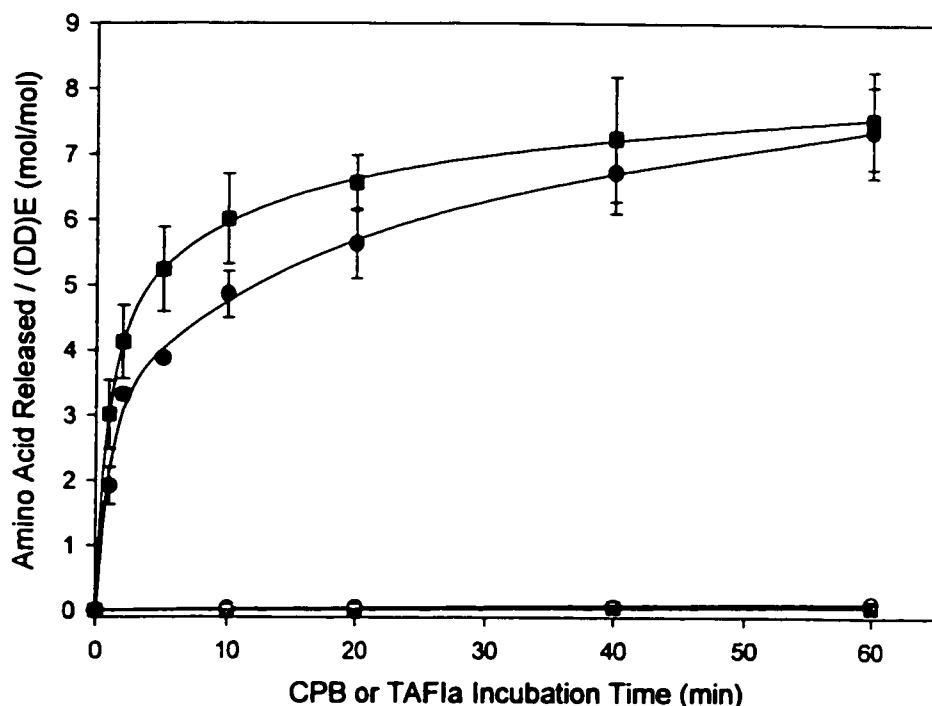


Figure 5.6 CPB- or TAFIa-mediated lysine and arginine release from (DD)E. 8 μ M (DD)E was incubated with 20 nM CPB or TAFIa for various times at 22 °C. After addition of CPI, the amount of free lysine or arginine release was determined by monitoring dehydration of NADH using saccharopine or octopine dehydrogenase, respectively. The total concentration of lysine or arginine was divided by the concentration of (DD)E to calculate moles of free amino acid released / mol of (DD)E. CPB (●) or TAFIa (■) causes the release of 8 mol lysine / mol (DD)E (●). Under the conditions employed here, ~ 4 mol lysine are released within the first 10 min or 2 min of CPB or TAFIa incubation, respectively. The remaining 4 mol lysine are released within 60 min incubation with either CPB or TAFIa. Neither CPB (○) nor TAFIa (□) releases arginine residues from (DD)E.

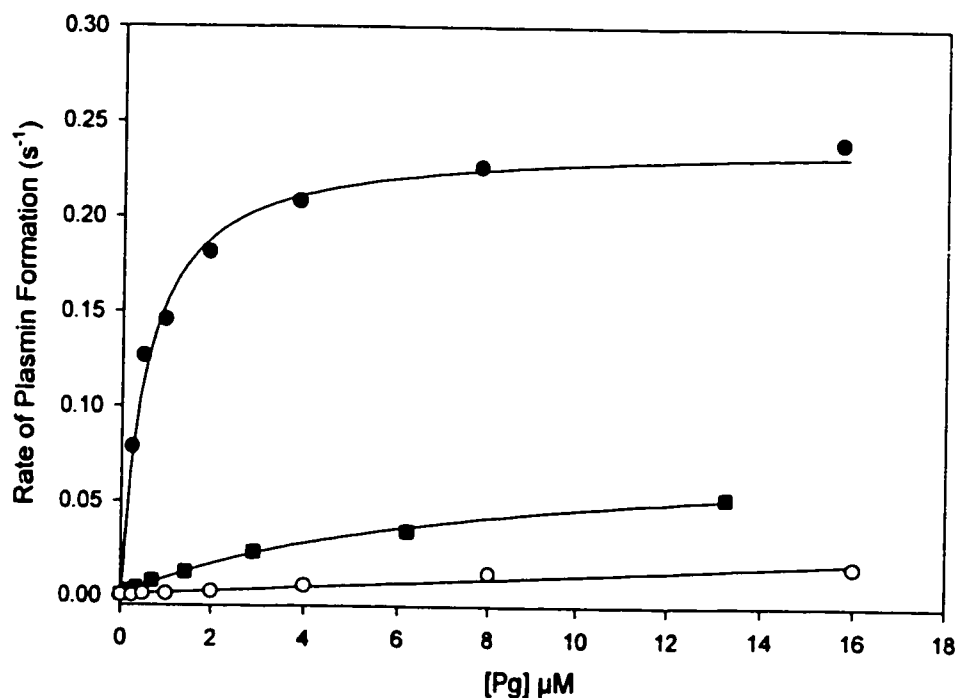


Figure 5.7 Effect of CPB on the ability of (DD)E to potentiate t-PA-mediated activation of Glu-Pg. 0.1 nM t-PA was incubated with various concentrations of Glu-Pg in the presence of 0.4 μM native (DD)E (●), 5 μM CPB-treated (DD)E (■), or in the absence of (DD)E (○). Plasmin formation was monitored using the plasmin-directed substrate S2251, and rates of plasmin formation were calculated. CPB markedly reduces the rate of (DD)E-stimulated Pg activation at all Pg concentrations. These data were fit to the Michaelis-Menten equation by nonlinear regression analysis to determine k_{cat} and K_{M} (Table 5.1).

TABLE 5.1

**Kinetic Parameters for Glu- and Lys-Pg Activation in the Absence of a Cofactor,
in the Presence of (DD)E Before and After its Exposure to CPB or TAFIa,
or in the Presence of Fibrin or Fg**

All kinetic parameters are presented as the mean \pm S.E. of at least three experiments, except those for TAFIa-exposed (DD)E, which are in parentheses and reported as the mean of two experiments.

Cofactor		k_{cat}	K_M	k_{cat}/K_M	Stimulation
		s^{-1}	μM	$\mu M^{-1}s^{-1}$	fold ^a
Glu-Pg	no Cofactor	—	—	0.00088 ± 0.00019	1
	Native (DD)E	0.25 ± 0.10	0.78 ± 0.21	0.32 ± 0.04	360
	CPB- or (TAFIa-) exposed (DD)E	0.080 ± 0.021 (0.12)	7.5 ± 2.3 (8.2)	0.011 ± 0.003 (0.014)	12
	Fibrin	0.27 ± 0.13	0.87 ± 0.36	0.31 ± 0.12	350
	Fg	0.21 ± 0.07	9.5 ± 1.4	0.022 ± 0.007	25
Lys-Pg	no Cofactor	—	—	0.010 ± 0.001	1
	Native (DD)E	0.10 ± 0.02	0.022 ± 0.004	4.5 ± 0.5	450
	CPB- or (TAFIa-) exposed (DD)E	0.049 ± 0.010 (0.081)	0.27 ± 0.06 (0.31)	0.18 ± 0.05 (0.26)	18
	Fibrin	0.16 ± 0.09	0.033 ± 0.005	4.8 ± 1.3	480
	Fg	0.060 ± 0.010	0.31 ± 0.03	0.19 ± 0.04	19

^aFold stimulation is calculated by dividing the catalytic efficiency (k_{cat}/K_M) determined in the presence of the indicated cofactor by that determined in the absence of a cofactor.

determine whether the reduced stimulatory activity of TAFIa- or CPB-treated (DD)E reflects changes in its affinity for t-PA and/or Pg, light scattering spectroscopy was used to compare the affinities of t-PA or Pg for native (DD)E with those for TAFIa- or CPB-exposed (DD)E. The relative scatter plots for the interactions of t-PA or Glu-Pg with (DD)E before and after CPB treatment are illustrated in Figures 5.8A and 5.8B, respectively. Under the conditions outlined in section 5.3.2v, the scattering intensity of 0.1 μM (DD)E is 15 (I_0). At saturating levels of t-PA, the maximum relative scattering intensity (I/I_0) in the presence of untreated (DD)E is 1.5 (Fig. 5.8A); a value in good agreement with a calculated maximum relative scattering intensity of 1.6 if the stoichiometry is 1:1 (Blanchoin et al. 1996; Stewart et al. 1998b). The maximum I/I_0 when (DD)E was titrated with Glu-Pg was 2.1 (Fig. 5.8B), a value similar to a predicted I/I_0 of 1.9 for a 1:1 substrate interaction with (DD)E (Blanchoin et al, 1996; Stewart et al., 1998b). The solid lines represent the fit of the data to equation 3 (section 3.3.2vi) by nonlinear regression analysis and these results are summarized in Table 5.2.

t-PA binds to native (DD)E with a K_d of 0.04 μM (Table 5.2). In contrast, t-PA binds to CPB-exposed (DD)E with a K_d of 6.5 μM , an affinity 160-fold lower than that of the activator for native (DD)E. Glu-Pg binds to native and CPB-exposed (DD)E with K_d values of 5.5 μM and 11 μM , respectively. Thus, CPB treatment produces only a 2-fold decrease in the affinity of (DD)E for Glu-Pg. Similar results are obtained with Lys-Pg which binds to CPB-exposed (DD)E with a 4-fold lower affinity than that for native (DD)E ($K_d = 0.35$ and 0.09 μM , respectively). The affinities of TAFIa-exposed (DD)E for t-PA, Glu- or Lys-Pg are similar to those of CPB-exposed (DD)E (Table 5.2).

Figure 5.8 Effect of CPB on the Affinity of (DD)E for t-PA or Glu-Pg. 0.1 μ M native (DD)E (\bullet) or CPB-exposed (DD)E (\circ) was titrated with active site blocked t-PA (A) or Glu-Pg (B) and scattering intensities obtained in the presence of titrant (I) were compared with those obtained in its absence (I_0). Solid lines represent nonlinear regression analysis of the data to determine K_d values, which are summarized in Table 5.2. Whereas exposure of (DD)E to CPB causes a modest reduction in its affinity for Glu-Pg, CPB it markedly reduces the affinity of (DD)E for t-PA.

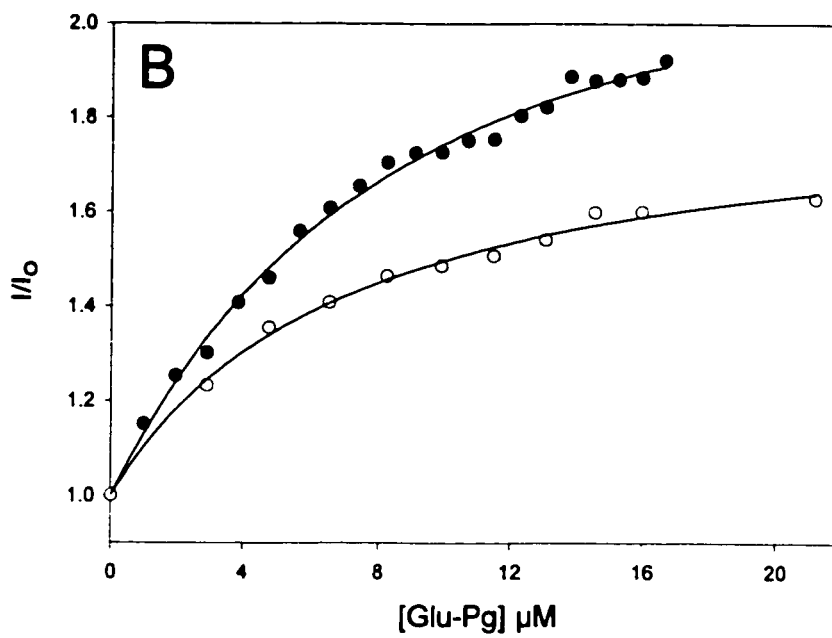
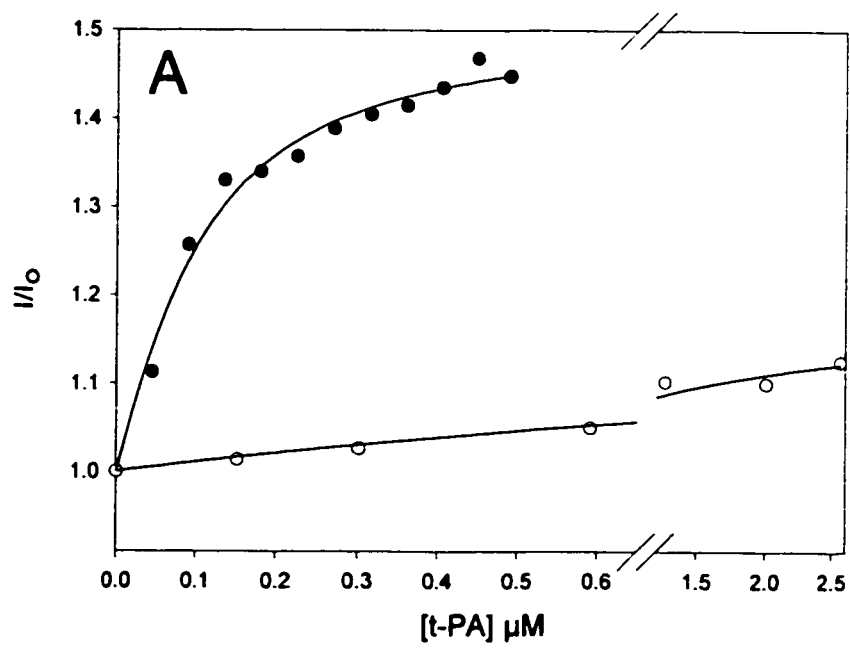


TABLE 5.2

Effect of CPB or TAFIa on the Affinity of (DD)E for t-PA, Glu- or Lys-Pg

All dissociation constants are presented as the means \pm S.E. of at least three titrations, except those using TAFIa-exposed (DD)E, which are in parentheses and reported as the means of two titrations.

Ligand	Native (DD)E	CPB- or (TAFIa-)exposed (DD)E	Decrease in Affinity
	K_d (μ M)	K_d (μ M)	fold ^a
t-PA	0.04 \pm 0.01	6.5 \pm 1.5 (5.8)	160
Glu-Pg	5.5 \pm 1.8	10.8 \pm 3.1 (9.8)	2
Lys-Pg	0.09 \pm 0.01	0.35 \pm 0.10 (0.46)	4

^a Fold decrease in affinity is calculated by dividing the dissociation constant for CPB-exposed (DD)E and the indicated ligand by the dissociation constant for native (DD)E and the same ligand.

5.5 Discussion

(DD)E, a soluble degradation product of crosslinked fibrin, compromises the fibrin-specificity of t-PA by promoting systemic activation of Pg (Olexa and Budzynski, 1979b; Weitz et al., 1991; Stewart et al., 1998b). The stimulatory activity of (DD)E, like that of fibrin, reflects its capacity to bind t-PA and Pg (Weitz et al., 1991; Stewart et al., 1998a; Stewart et al., 1998b). In contrast to its predominantly finger-mediated interaction with fibrin, t-PA binds to (DD)E via its second kringle domain (Stewart et al., 1998b; Stewart et al., 2000). Although Pg binding also is kringle-dependent, Pg does not compete with t-PA for (DD)E binding, indicating that Pg and t-PA bind to distinct lysine residues on (DD)E (Stewart et al., 1998b). By releasing carboxy-terminal lysine residues, TAFIa, a CPB-like enzyme, attenuates fibrin degradation (Bajzar et al., 1995; Nesheim, 1999). In this study, we explored the possibility that TAFIa modulates the stimulatory activity of (DD)E via a similar mechanism.

(DD)E is as potent as fibrin at stimulating t-PA-mediated Pg activation. When (DD)E is incubated with TAFIa or CPB, its ability to stimulate the activation of either Glu- or Lys-Pg by t-PA is reduced by 96% and becomes comparable to that of Fg. Reduced stimulation by (DD)E reflects both a decrease in k_{cat} and an increase in K_M for both Glu- and Lys-Pg. The larger effect is on K_M , however, suggesting that exposure of (DD)E to TAFIa or CPB serves to destabilize the ternary t-PA/plasminogen/(DD)E complex.

Lysine residues are released in parallel with the loss of stimulatory activity that occurs as (DD)E is incubated with TAFIa or CPB. A total of 8 mol of lysine/mol (DD)E is generated. Based on the molecular weight and amino-terminal sequence of the 6 individual

chains within (DD)E, as well as the known plasmin cleavage sites on fibrin, the 8 releasable lysine residues correspond to the 6 carboxy-terminal lysine residues on the α , β , and γ -chains of fragment E at positions 78, 133 and 62, respectively, as well as the 2 carboxy-terminal lysine residues on the α -chains of each D-domain at position 206 (Olexa and Budzynski, 1979b; Olexa et al., 1981; Walker and Nesheim, 1999). Under the conditions employed in this study, most of the stimulatory activity of (DD)E is lost after 2 or 10 min incubation with TAFIa or CPB, respectively, times at which at which ~ 4 mol of lysine/mol of (DD)E are released. These findings suggest that some lysine residues on (DD)E are more susceptible to TAFIa- or CPB-mediated release than others, and that those most susceptible to release are the lysine residues important for formation of the ternary t-PA/Pg/(DD)E complex.

Because plasmin generation in the presence of TAFIa- or CPB-exposed (DD)E is less than that with native (DD)E, TAFIa or CPB serves to indirectly preserve the structural integrity of (DD)E. This may be of physiologic relevance because DD binds to fibrin with high affinity and inhibits its polymerization, whereas intact (DD)E has no effect on fibrin polymerization (Olexa and Budzynski, 1980; Budzynski et al., 1979; Husain et al., 1989). Limiting DD formation may promote clot stability because fibrin clots are dynamic structures that undergo continuous formation and degradation. Thus, the anti-fibrinolytic properties of TAFIa may include clot stabilization by the prevention of (DD)E degradation to DD and fragment E.

To explore the mechanism by which TAFIa or CPB eliminates the cofactor activity of (DD)E, we compared the affinities of t-PA and Pg for TAFIa- or CPB-treated (DD)E with those for native (DD)E. Exposure of (DD)E to TAFIa or CPB reduces its affinity for Glu-

and Lys-Pg 2- and 4-fold, respectively. In contrast, the affinity of (DD)E for t-PA is reduced 160-fold after TAFIa or CPB exposure. The fact that TAFIa or CPB exposure lowers the affinity of (DD)E for t-PA more than it reduces its affinity for Glu- or Lys-Pg, suggests that TAFIa or CPB modulates the stimulatory activity of (DD)E primarily by decreasing its affinity for the activator rather than for Pg.

Because TAFIa- or CPB-exposed (DD)E has little affinity for t-PA, it is a poor stimulator of Glu- or Lys-Pg activation by t-PA. This observation is consistent with our previous work with the Pg activator from the saliva of the vampire bat (b-PA). Lacking a lysine-binding kringle, b-PA does not bind (DD)E (Stewart et al., 1998b). Consequently, (DD)E is a poor stimulator of Pg activation by b-PA (Stewart et al., 1998b; Weitz et al., 1999). These data support the concept that only Pg activators that bind to (DD)E are significantly potentiated by this fragment.

Although it does not bind to (DD)E, b-PA binds fibrin with high affinity via its finger domain and is stimulated by fibrin to the same extent as t-PA (Bringmann et al., 1995; Stewart et al., 1998b). Because (DD)E and fibrin stimulate t-PA to a similar extent, whereas b-PA is potentiated only by fibrin, t-PA is less fibrin-specific than b-PA. Thus, (DD)E compromises the fibrin-specificity of t-PA. Our current findings raise the possibility that by decreasing the capacity of (DD)E to bind t-PA, TAFIa enhances the fibrin-specificity of t-PA.

Upon exposure to TAFIa or CPB, the affinity of (DD)E for Glu- or Lys-Pg is only modestly reduced suggesting that Pg binds to internal lysines, as well as carboxy-terminal lysine residues. In contrast, our data suggest that t-PA predominantly binds to carboxy-terminal lysine residues on (DD)E because the affinity of t-PA for (DD)E is markedly

reduced when (DD)E is exposed to TAFIa or CPB. These findings are consistent with the previously reported noncompetitive binding of t-PA and Pg to (DD)E (Stewart et al., 1998b).

Like their effect on (DD)E, TAFIa and CPB also release lysine residues from fibrin partially degraded by plasmin (de Vries et al., 1989; Wang et al., 1998). This results in a reduction in the affinity of plasmin-exposed fibrin for Glu- or Lys-Pg and blocks the accumulation of fluorescently-labeled Pg on fibrin (Fleury and Angles-Cano, 1991; Sakharov et al., 1997). TAFIa also prevents the conversion of Glu-Pg to Lys-Pg; an early event in the course of t-PA-mediated clot lysis that serves as a positive feedback mechanism because Lys-Pg, which has higher affinity for fibrin than Glu-Pg, is more readily activated by t-PA (Nesheim et al., 1997; Wang et al., 1998). Although the effects of TAFIa on the affinity of the activator for fibrin has yet to be investigated, CPB blocks the 2- to 3-fold increase in t-PA binding that occurs when fibrin is exposed to plasmin, suggesting that this increase reflects kringle-dependent binding of t-PA to newly exposed carboxy-terminal lysine residues (de Vries et al., 1989). The inhibitory effect of TAFIa or CPB on these positive feed-back events results in a 3- to 4-fold prolongation of the rate of t-PA-mediated fibrinolysis (Bajzar et al., 1995; Bajzar et al., 1996a).

Our results with (DD)E have similarities to those with fibrin. TAFIa or CPB causes the release of free lysine residues from (DD)E, reduces the affinity of the substrate and activator for the cofactor, and attenuates cofactor degradation. However, other features distinguish the effects of TAFIa or CPB on (DD)E from those on fibrin. Although fibrin degradation is attenuated by TAFIa or CPB, it is not inhibited, suggesting that even in the presence of these enzymes, fibrin remains a competent stimulator of Pg activation by t-PA

(Bajzar et al., 1996a). Furthermore, the inhibitory effects of TAFIa on fibrinolysis can be overcome by substituting Lys-Pg for Glu-Pg (Nesheim et al., 1997; Wang et al., 1998). In contrast, exposure of (DD)E to TAFIa or CPB produces a 96% reduction in its ability to stimulate the activation of either Glu- or Lys-Pg by t-PA. These differences between fibrin and (DD)E are explained by the observation that TAFIa or CPB reduces the affinity of t-PA for (DD)E more than its affinity for fibrin. Whereas t-PA binding to plasmin-degraded fibrin is only partially compromised by CPB (Higgins and Vehar, 1987; de Vries et al., 1989), TAFIa or CPB almost abolishes t-PA binding to (DD)E.

(DD)E compromises the fibrin-specificity of t-PA because it binds t-PA with high affinity and stimulates Pg activation to the same extent as fibrin. By reducing the affinity of (DD)E for t-PA, TAFIa converts (DD)E from a fibrin-like stimulator to one that has minimal stimulatory activity, much like Fg. Thus, our data suggest a novel role for TAFIa. By reducing the stimulatory activity of (DD)E and attenuating systemic Pg activation, TAFIa may enhance the fibrin-specificity of t-PA. Furthermore, since DD can impair fibrin polymerization, limiting (DD)E degradation may augment the anti-fibrinolytic properties of TAFIa.

CHAPTER 6 - SUMMARY AND PERSPECTIVES

Coronary thrombolysis with t-PA is a mainstay of pharmacologic reperfusion therapy for AMI. t-PA, a second generation thrombolytic agent, is defined as a fibrin-specific Pg activator because it is a more efficient activator of Pg in the presence of fibrin than in the presence of Fg (Hoylaerts et al., 1982; Collen, 1996; Weitz et al., 1999). However, this definition of fibrin-specificity overlooks the fact that (DD)E, which is as potent as fibrin as a stimulator of Pg activation by t-PA, potentiates systemic plasmin generation (Weitz et al., 1991; Weitz et al., 1999). Once plasmin overwhelms α_2 -AP, Fg degradation results. Thus, (DD)E limits the fibrin-specificity of t-PA. The limited fibrin-specificity of t-PA and its short half-life prompted the development of third generation activators such as TNK-t-PA.

This thesis work was undertaken to characterize the interaction of t-PA, b-PA, and TNK-t-PA with Fg, fibrin, and (DD)E so as to gain a better understanding of the factors that govern the fibrin-specificity of these activators. The thesis has three main goals: (a) characterization the interactions of t-PA and b-PA with Fg, fibrin, and (DD)E, (b) elucidation of the mechanism responsible for the increased fibrin-specificity of TNK-t-PA relative to t-PA, and (c) characterization of the effects of TAFIa or CPB on the stimulatory activity of (DD)E. This chapter focuses on the biochemical and physiological relevance of these studies and identifies directions for additional work in this area.

6.1 Interactions of t-PA and b-PA with Fg, fibrin, and (DD)E

The fibrinolytic system has several mechanisms in place to ensure that plasmin is

localized to the fibrin surface so that fibrin is degraded and systemic plasmin is limited (Lijnen and Collen, 1995a; Collen, 1999). One of these is the ability of fibrin to stimulate Pg activation by t-PA. This stimulatory activity fibrin depends on its capacity to bind t-PA. The experiments presented in Chapter 3 indicate that t-PA binds fibrin through two classes of sites, one high affinity, finger-dependent site, and one low affinity, K2-dependent site. These findings confirm the work of other investigators (Verheijen et al., 1986; van Zonneveld et al., 1986b; Nesheim et al., 1990).

The novel aspect of the work presented in Chapter 3 relates to the interaction of t-PA with soluble cofactors. t-PA binds Fg and (DD)E solely through its K2 domain. The affinity of t-PA for (DD)E is ~110-fold higher than its affinity for the corresponding K2-dependent binding site on fibrin. These observations suggest that finger binding sites on fibrin are lost and K2 binding sites become more accessible and/or new K2-dependent binding sites are generated as fibrin is degraded into soluble fragments by plasmin. These findings are consistent with previous observations that K2-dependent binding of t-PA to fibrin increases when fibrin is partially degraded by plasmin (Higgins and Vehar, 1987; de Vries et al., 1989).

It would be of interest to identify the t-PA and Pg binding sites on (DD)E. To this end, photoaffinity crosslinkers could be used to irreversibly bind t-PA or Pg to (DD)E, thereby creating an electrophoretically stable complex (Weisel et al., 1994). After degrading the complex with various enzymes or chemical agents, fragments can be subjected to PAGE analysis to (a) identify whether t-PA and Pg bind to DD, fragment E or both, and (b) identify the chains within these fragments responsible for t-PA and Pg binding. These studies would provide further insight into the assembly of the t-PA/Pg/(DD)E complex. Although

photoaffinity crosslinkers, in combination with electron microscopy, have been used to identify the region on fibrin to which Pg binds (Weisel et al., 1994), these techniques have not been used to identify the chain(s) to which Pg binds. The studies proposed here could be used to determine if t-PA and Pg bind to similar sites on (DD)E and fibrin.

The studies presented in Chapter 3 give insight into the mechanism of cofactor stimulation of Pg activation by t-PA. The affinity of t-PA for the various cofactors correlates with the extent to which t-PA is stimulated by the cofactor. Weak interaction (t-PA / Fg) elicits little stimulation, whereas high-affinity interactions (t-PA / fibrin; t-PA / (DD)E) result in greater stimulation. The degree to which t-PA is stimulated by the cofactor is independent of the domain responsible for the interaction because the high affinity K2 binding of t-PA to (DD)E results in stimulation similar to the high affinity finger dependent binding of t-PA to fibrin. Thus, K2-dependent binding of t-PA to (DD)E, a cofactor that also binds Pg, is sufficient to arrange t-PA and Pg on (DD)E in a productive manner. This finding challenges previous reports that K2 functions merely to orient t-PA on fibrin (Lijnen et al., 1990a; Bakker et al., 1995b), and raises the possibility that at least one of the functions of K2 is to stimulate Pg activation on fibrin degradation products, thereby ensuring their breakdown into the smallest possible components.

These studies also have important implications on mechanisms governing the fibrin-specificity of t-PA. t-PA binding to fibrin is primarily mediated by its finger domain, whereas binding to soluble cofactors, (DD)E and Fg, is K2-dependent. This suggests that activators with competent finger domains, but no lysine-binding properties, should be more fibrin-specific than t-PA. This hypothesis was tested using the activator isolated from the

saliva of the vampire bat.

Although b-PA shares over 72% amino acid identity to t-PA, b-PA lacks a lysine-binding kringle (Gardell et al., 1989; Schleuning et al., 1992), making it an excellent tool for assessing the function of the K2 domain. The binding of b-PA to fibrin presented in Chapter 3 is almost identical to that previously reported by Bringmann and colleagues (1995). The data in Chapter 3 extend previous studies by demonstrating that b-PA inhibits t-PA binding to fibrin by competing with t-PA for finger-dependent binding. Because b-PA binds to fibrin with an affinity similar to that of t-PA, the efficiency of Pg activation in the presence of fibrin is similar for b-PA and t-PA. In contrast to t-PA, b-PA does not bind (DD)E or Fg because b-PA lacks a K2 domain. Consequently, these soluble cofactors produce little stimulation of Pg activation by b-PA. These studies help to explain why b-PA is more fibrin-specific than t-PA.

It is conceivable that the Pg activator in the saliva of the vampire bat evolved to be exquisitely fibrin-specific so as to maintain the fluidity of its victim's blood for easy consumption. Furthermore, because plasmin can generate inflammatory mediators, localization of Pg activation to the fibrin surface may prevent a host inflammatory response when bitten (Renatus, et al., 1997c). This phenomenon may explain why, as nocturnal feeders, vampire bats can feed for long periods of time without awakening their victims.

Because b-PA is more fibrin-specific than t-PA, it causes less Fg degradation and fragment X formation *in vitro* (Bergum and Gardell, 1992; Schleuning et al., 1992) and *in vivo* (Gardell et al., 1991; Mellot et al., 1995; Weitz, 1995) and, when challenged, animals given b-PA bleed less than those given t-PA (Mellott et al., 1995; Weitz, 1995). These

findings raise the possibility that b-PA would cause less bleeding than t-PA in AMI patients. Notwithstanding the homology between b-PA and t-PA, however, b-PA is a non-human protein which may be immunogenic. In fact, antibodies against b-PA have been detected in rats and monkeys given this agent (Gulba and Witt, 1995). Although subtherapeutic doses of b-PA did not induce antibody formation in humans (Verstraete et al., 1995), it is possible that antibody formation may occur with pharmacologically relevant doses. Therefore, for therapeutic purposes, it may be better to develop a t-PA mutant with reduced lysine-binding properties (discussed in detail in section 6.3).

Using established rabbit models, it may be possible to further define the link between fragment X formation and bleeding. Studies are underway to isolate and characterize rabbit fragment X for administration to rabbits. Rabbits treated with low-dose t-PA will be infused with various amounts of fragment X or Fg and challenged with ear incisions to assess bleeding. If incorporation of fragment X into hemostatic plugs renders them more susceptible to lysis, rabbits given fragment X will bleed more than those given Fg. These studies will help to establish a causal relationship between fragment X formation and bleeding.

6.2 Mechanism of the fibrin-specificity of TNK-t-PA

The limitations of t-PA prompted the development of Pg activators with longer half-lives and increased fibrin-specificity. One such agent is TNK-t-PA. The increased half-life of TNK-t-PA is the result of a shift in the glycosylation site within K1 from amino acid 117 to 103 (Keyt et al., 1994). Not only does this combination of mutations displace the carbohydrate responsible for clearance at pharmacologic doses, but it also alters the

carbohydrate moiety from a high mannose to a complex-type. These changes, which increase the plasma half-life of the activator ~ 5-fold, permit TNK-t-PA administration as a single bolus (Cannon et al., 1997).

The data presented in Chapter 4 give insight into why TNK-t-PA is more fibrin-specific than t-PA. Activators containing the T-mutation (glycosylation addition to amino acid 103), including TNK-, T-, and TK-t-PA, bind to (DD)E, Fg, and ϵ -lysine with ~ 9-, 4-, and 2-fold lower affinity, respectively, than t-PA. These data suggest that glycosylation at position 103 interferes with the lysine-binding properties of the activator, and that this, in turn, decreases its affinity for soluble cofactors. TNK-, T-, and TK-t-PA also have higher K_M values for Pg activation in the presence of (DD)E or Fg, suggesting the glycosylation addition at amino acid 103 destabilizes the Michaelis complex, a finding consistent with reduced binding of the activator to the cofactor.

Carbohydrates normally present in t-PA alter its lysine-binding properties. Type II t-PA binds lysine with higher affinity than type I t-PA (discussed in section 1.1.2vi). These findings indicate that glycosylation at position 184 within the K2 domain of t-PA influences its lysine-binding properties. Fg and CNBr fragments of Fg stimulate type I t-PA to a lesser degree than type II t-PA (Parekh et al., 1989a; Wittwer et al., 1989). These findings are consistent with the observation that binding of t-PA to Fg and Fg fragments is mediated by its K2 domain (de Munk et al., 1989; Stewart et al., 1998b), and raise the possibility that type I t-PA might be more fibrin-specific than type II t-PA because binding of type I t-PA to soluble cofactors is compromised. Against this concept is the observation that type I t-PA has less fibrinolytic activity and binds fibrin with lower affinity than type II t-PA (Wittwer

et al., 1989; Rudd et al., 1995). It would be of interest, therefore, to compare the fibrin-specificities of type I and type II t-PA. This would entail separating the two species using lysine-sepharose affinity chromatography, and comparing the ability of Fg, fibrin, or (DD)E to bind and stimulate the two different glycoforms. The results obtained with type I and type II t-PA could then be compared with those for the TNK variants to determine the relative contributions of K1 and K2 glycosylation to Pg activation. Interestingly, the efficiency of Pg activation by type I t-PA in the presence of Fg fragments is ~ 1.5-fold lower than that of type II t-PA, primarily because of a lower k_{cat} (Rudd et al., 1995). Results presented in Chapter 4 with TNK-t-PA indicate that glycosylation addition within K1 decreases the efficiency of Pg activation in the presence of Fg or (DD)E by increasing K_M for the reaction. Taken together, these findings suggest that, although glycosylation of both K1 and K2 affects Pg activation, the mechanism by which they exert their effects may be different.

In addition to the T-mutation, the K-mutation contributes to the fibrin-specificity of TNK-t-PA. The likely mechanism for increased fibrin-specificity of K-containing variants involves the mutation of lysine at position 296 (K296A) which normally stabilizes the active conformation of t-PA by forming a salt bridge with Asp477 (discussed in section 1.1.2vii). The ability of K-t-PA to activate Pg in the absence of a cofactor is ~ 10-fold less than that of t-PA. Binding to fibrin overcomes the K296A mutation. In contrast, binding to (DD)E or Fg does not overcome the diminished activity introduced by the K-mutation. Since binding of the activator to fibrin is mediated by its finger domain, whereas binding to (DD)E is K2-dependent, these observations raise the possibility that conformational changes that occur in the protease domain of the activator upon cofactor binding are dependent on the

domain that mediates binding. Alternatively, epitopes within the protease domain of the activator that interact with fibrin to stabilize the active site (Lamba et al., 1996; Bode and Renatus, 1997b) may not interact to the same extent with (DD)E. Although the currently available data make it difficult to distinguish between these two possibilities, two lines of evidence suggest that the active site conformation of Pg activators containing the K mutation is different in the presence of (DD)E compared with fibrin. First, K-t-PA binds (DD)E and fibrin with similar affinities, but its efficiency of Pg activation is 5.2-fold lower in the presence of (DD)E compared with fibrin because k_{cat} for the reaction is lower (sections 4.4.7 and 4.4.5, respectively). Second, the amidolytic activity of TNK-t-PA is increased 5-fold by fibrin, but only 2-fold by (DD)E (Appendix 1). These observations suggest that the activity of K-t-PA in the presence of fibrin or (DD)E is distinguished by differences in the conformation of the protease domain that alter its activity. The catalytic domain of a K-containing activator has yet to be crystalized. Comparison of this structure with the recently crystalized catalytic domain of t-PA (Lamba et al., 1996) may help to determine how the K mutation contributes to fibrin-specificity of the activator.

The data presented in Chapter 4 indicate that both the T and K mutations contribute to the increased fibrin-specificity of TNK-t-PA, albeit by different mechanisms. The T mutation reduces the affinity of TNK-t-PA for (DD)E, and Fg, and reduces the K_M for Pg activation in the presence of these soluble cofactors, whereas the K mutation reduces k_{cat} for Pg activation in the absence of a cofactor and presence of (DD)E or Fg.

6.3 Comparison of the fibrin-specificity t-PA, b-PA, and TNK-t-PA

t-PA, TNK-t-PA, and b-PA are compared in Appendices 2 and 3 in terms of their

fibrin-specificities and stimulation by fibrin and (DD)E, respectively. When incubated with plasma clots, the three activators produce the same degree of clot lysis at all concentrations tested (0 - 60 nM, Appendix 2). In contrast, when B β 1-42 in the bathing plasma is used as an index of fibrinogenolysis, TNK-t-PA induces less Fg degradation than t-PA, but produces more than b-PA. At the highest concentration tested, TNK-t-PA or b-PA produces 7- and 34-fold less B β 1-42 generation than t-PA, respectively. Thus, TNK-t-PA is more fibrin-specific than t-PA, and b-PA is more fibrin-specific than TNK-t-PA.

The hierarchy of fibrin-specificity (b-PA>TNK>t-PA) correlates with the relative catalytic efficiencies of the activators in the presence of fibrin compared with (DD)E (fibrin/(DD)E ratio, Appendix 3). Because the efficiency of Pg activation by t-PA is equivalent in the presence of fibrin or (DD)E, t-PA has a fibrin/(DD)E ratio of 1. TNK-t-PA and b-PA have fibrin/(DD)E ratios of 9.5 and 700, respectively. Although the efficiency of Pg activation by TNK-t-PA or b-PA is ~2-fold lower than that for t-PA in the presence of fibrin, TNK-t-PA and b-PA are 16- and 1400-fold less active than t-PA in the presence of (DD)E. The degree to which (DD)E stimulates Pg activation by each activator correlates with the increase in amidolytic activity of the activator in the presence of (DD)E (Appendices 3 and 1, respectively). Thus, (DD)E increases the catalytic efficiency of Pg activation and the amidolytic activity of t-PA the most, whereas (DD)E has the least effect on Pg activation and small substrate cleavage by b-PA. However, the effect of (DD)E on Pg activation is ~100-fold greater than its effect on amidolytic activity for each Pg activator, consistent with the hypothesis that the majority of cofactor-mediated stimulation of Pg activation results from optimal orientation of t-PA and Pg on the cofactor and the stability of the ternary

complex, rather than conformational changes in the protease domain of t-PA that capacitate its active site (Loscalzo, 1988; Bringmann et al., 1995; Horrevoets et al., 1997a).

Because both T-t-PA and K-t-PA have higher fibrin/(DD)E ratios than t-PA (3.2 and 5.4, respectively, section 4.4.5), it is clear that both the T mutation, which reduces the affinity of the activator for (DD)E, and the K mutation, which reduces the activity of the activator in the absence of a cofactor, contribute to the higher fibrin/(DD)E ratio for TNK-t-PA relative to t-PA. The mechanism(s) responsible for the increased fibrin-specificity of b-PA relative to t-PA is less clear. The catalytic efficiency of Pg activation in the absence of a cofactor is ~ 100-fold lower for b-PA compared with t-PA (Appendix 3). Because b-PA binds fibrin with an affinity similar to that of t-PA, the catalytic efficiency b-PA-mediated Pg activation is similar to t-PA in the presence of fibrin. In contrast, because b-PA does not bind (DD)E, b-PA-mediated Pg activation is only moderately enhanced by (DD)E (Appendix 3). However, as detailed in section 1.1.2x, differences between the protease domains of b-PA and t-PA also may contribute to the reduced activity of b-PA in the presence of soluble cofactors. It would be of interest to characterize the relative contributions of the various differences between b-PA and t-PA to the increased fibrin-specificity of b-PA. To this end, the catalytic efficiency of Pg activation by mutant t-PA molecules containing various combinations of three mutations D236N (to remove lysine binding properties of t-PA), R275E (to make sct-PA uncleavable to tct-PA), and K296A (to destabilize the active conformation of t-PA in the absence of a cofactor) could be assessed in the presence of (DD)E or fibrin. Mutants with greater fibrin-specificity than t-PA could be candidates for pharmacologic thrombolysis. However, given the numbers of patients required to investigate

thrombolytic agents, development of new Pg activators has become extremely expensive.

6.4 Effect of TAFIa or CPB on (DD)E stimulation of Pg activation

The data presented in Chapter 5 give further insight into the interactions of t-PA and Pg with (DD)E. Whereas t-PA binds primarily to carboxy-terminal lysine residues on (DD)E, Pg binds to both carboxy-terminal and internal lysines. These data are consistent with the lack of competition between t-PA and Pg for binding to (DD)E.

Future experiments could be designed to elucidate which lysine residues on (DD)E are critical for stimulating Pg activation. To accomplish this, (DD)E could be incubated with CPB for various amounts of time. Conditions were established in Chapter 5 to isolate species of (DD)E with various numbers of lysines removed. Each (DD)E species could be characterized in terms of its ability to stimulate and bind t-PA. A separate aliquot could be subjected to PAGE analysis under reducing and denaturing conditions to separate the individual chains within (DD)E. The individual chains could then be subjected to further CPB-mediated degradation to determine which chains have CPB-releasable lysine residues. These results could be correlated with those obtained using native (DD)E to determine which lysines were removed by CPB prior to PAGE analysis. Furthermore, by correlating the CPB-released lysine residues with the stimulatory activity of (DD)E, the specific lysines required for stimulating Pg activation could be identified. These data could be compared with the photoaffinity crosslinker studies, proposed earlier, to determine if the lysine residues required for stimulation are the same residues that mediate binding of (DD)E to t-PA.

The studies presented in Chapter 5 also highlight some critical differences between the effects of TAFIa or CPB on fibrin and their effect on (DD)E. TAFIa or CPB attenuates

fibrin degradation by eliminating the upregulation of Pg activation that occurs during the early stages of fibrinolysis (Bajzar et al., 1995; Sakharov et al., 1997; Wang et al., 1998). However, even in the presence of these enzymes, fibrin remains a competent stimulator of Pg activation. In contrast, TAFIa or CPB causes a 96% reduction in the ability of (DD)E to stimulate activation of Pg. Furthermore, the effects of TAFIa on fibrinolysis are overcome by substituting Lys-Pg for Glu-Pg (Nesheim et al., 1997; Wang et al., 1998), whereas this change does not diminish the effect of TAFIa on (DD)E. These differences are explained by the observation that TAFIa or CPB causes a dramatic reduction in the affinity of (DD)E for t-PA. These data raise the possibility that TAFIa enhances the fibrin-specificity of t-PA by reducing its stimulation by (DD)E.

This hypothesis could be tested in t-PA-treated animals. Rabbits could be given native (DD)E or TAFIa- or CPB-exposed (DD)E, and Fg degradation could be measured. If TAFIa- or CPB-exposure of (DD)E reduces its ability to potentiate systemic plasmin formation, rabbits given TAFIa- or CPB-exposed (DD)E would have less Fg degradation than those given native (DD)E. Alternatively, t-PA-treated rabbits could be given (DD)E and various doses of CPI or saline as a control. If TAFIa attenuates systemic plasmin formation by reducing the stimulatory activity of (DD)E, rabbits given CPI should have more Fg degradation than controls. CPI is currently being investigated for its potential to enhance clot lysis when used as an adjunct to t-PA (Stefansson et al., 1998; Klement et al., 1999). If CPI concurrently promotes systemic Pg activation, this phenomenon could complicate the use of CPI as a thrombolytic adjunct by enhancing bleeding. However, as detailed in section 1.2, the relationship between systemic plasmin formation and bleeding is complex, and needs

further clarification.

6.5 Conclusions

None of the new fibrinolytic agents has been shown to be more effective than t-PA in AMI patients. Therefore, recent attention has shifted to improving the effectiveness of adjunctive therapy. The recent discovery of TAFI, raises the possibility that TAFIa inhibitors may enhance fibrinolysis. Other agents under investigation include potent antiplatelet drugs, such as GPIIb/IIIa antagonists, and direct thrombin inhibitors (Antman et al., 1999; Kennedy and Stadius, 1999; Neuhaus et al., 1999; Hirsh and Weitz, 1999).

Although not more effective than t-PA, new thrombolytic agents include Pg activators with longer half-lives, thereby facilitating drug delivery, and greater fibrin-specificity than t-PA. Clinical results with TNK-t-PA suggest that activators that are more fibrin-specific than t-PA may cause less bleeding, the most feared side-effect of thrombolytic therapy. The studies presented in this thesis give insight into the mechanisms that govern the fibrin-specificity of t-PA. Kringle dependent interactions of t-PA with (DD)E and, to a lesser extent, Fg compromise the fibrin-specificity of t-PA. Pg activators with competent finger domains, but compromised lysine-binding properties, are more fibrin-specific than t-PA. In addition, mutation of Arg275 and Lys296 in the protease domain, reducing the basal activity t-PA, may also increase its fibrin-specificity. Therefore, these studies provide direction for development of new thrombolytic agents. Removal of the lysine-binding properties of t-PA, in combination with point mutations in the protease domain, may result in a fibrinolytic agent that is more fibrin-specific than those that are currently available. Whether greater fibrin-specificity will increase safety requires investigation in clinical trials.

REFERENCES

- Ahern, T. J., Morris, G. E., Barone, K. M., Horgan, P. G., Timony, G. A., Angus, L. B., Henson, K. S., Stoudemire, J. B., Langer-Safer, P. R., and Larsen, G. R. (1990). Site-directed mutagenesis in human tissue-plasminogen activator. Distinguishing sites in the amino-terminal region required for full fibrinolytic activity and rapid clearance from the circulation. *Journal of Biological Chemistry* **265**, 5540-5545.
- An, S. S., Carreno, C., Marti, D. N., Schaller, J., Albericio, F., and Llinas, M. (1998a). Lysine-50 is a likely site for anchoring the plasminogen N-terminal peptide to lysine-binding kringles. *Protein Science* **7**, 1960-1969.
- An, S. S., Marti, D. N., Carreno, C., Albericio, F., Schaller, J., and Llinas, M. (1998b). Structural/functional properties of the Glu1-Ser57 N-terminal fragment of human plasminogen: conformational characterization and interaction with kringle domains. *Protein Science* **7**, 1947-1959.
- Anonick, P. K. and Gonias, S. L. (1991). Soluble fibrin preparations inhibit the reaction of plasmin with alpha 2-macroglobulin. *Biochemical Journal* **275**, 53-59.
- Antman, E. M., Giugliano, R. P., Gibson, C. M., McCabe, C. H., Coussement, P., Kleiman, NS, Vahanian, A., Adgey, A. A., Menown, I., Rupprecht, H. J., Van der Wieken, R., Ducas, J., Scherer, J., Anderson, K., Van de Werf, F., and Braunwald, E. (1999). Abciximab facilitates the rate and extent of thrombolysis: results of the thrombolysis in myocardial infarction (TIMI) 14 trial. The TIMI 14 Investigators. *Circulation* **99**, 2720-2732.

- ASSENT-2 Investigators (1999). Single-bolus tenecteplase compared with front-loaded alteplase in acute myocardial infarction: the ASSENT-2 double-blind randomised trial. Assessment of the safety and efficacy of a new thrombolytic (ASSENT-2) investigators. *Lancet* **354**, 716-722.
- Bachmann, F. (1994). The plasminogen-plasmin enzyme system. In "Hemostasis and Thrombosis: Basic Principles and Clinical Practice" (R. W. Colman, J. Hirsh, V. J. Marder, and E. W. Salzman, Eds.), pp. 1592-1622. J.B. Lipincott Company, Philadelphia.
- Bachmann, F. (1995). Fibrinolytic agents. *Fibrinolysis* **9 Suppl 1**, 9-15.
- Baenziger, J. U. (1994). Tissue-type plasminogen activator: A role for O-linked fucose. *Journal of Clinical Investigation* **93**, 459-459.
- Bajzar, L., Manuel, R., and Nesheim, M. E. (1995). Purification and characterization of TAFI, a thrombin-activatable fibrinolysis inhibitor. *Journal of Biological Chemistry* **270**, 14477-14484.
- Bajzar, L., Morser, J., and Nesheim, M. (1996a). TAFI, or plasma procarboxypeptidase B, couples the coagulation and fibrinolytic cascades through the thrombin-thrombomodulin complex. *Journal of Biological Chemistry* **271**, 16603-16608.
- Bajzar, L., Nesheim, M. E., and Tracy, P. B. (1996b). The profibrinolytic effect of activated protein C in clots formed from plasma is TAFI-dependent. *Blood* **88**, 2093-2100.
- Bajzar, L., Nesheim, M., Morser, J., and Tracy, P. B. (1998). Both cellular and soluble forms of thrombomodulin inhibit fibrinolysis by potentiating the activation of thrombin-activatable fibrinolysis inhibitor. *Journal of Biological Chemistry* **273**, 2792-2798.

- Bakker, A. H. F., Van der Greef, W., Rehberg, E. F., Marotti, K. R., and Verheijen, J. H. (1993). Introduction of lysine and clot binding properties in the kringle one domain of tissue-type plasminogen activator. *Journal of Biological Chemistry* **268**, 18496-18501.
- Bakker, A. H. F., Rehberg, E. F., Marotti, K. R., and Verheijen, J. H. (1995a). The position of the structurally autonomous kringle 2 domain influences the functional features of tissue-type plasminogen activator. *Protein Engineering* **8**, 293-300.
- Bakker, A. H. F., Weening-Verhoeff, E. J. D., and Verheijen, J. H. (1995b). The role of the lysyl binding site of tissue-type plasminogen activator in the interaction with a forming fibrin clot. *Journal of Biological Chemistry* **270**, 12355-12360.
- Banyai, L., Varadi, A., and Patthy, L. (1983). Common evolutionary origin of the fibrin-binding structures of fibronectin and tissue-type plasminogen activator. *FEBS Letters* **163**, 37-41.
- Bell, W. R. (1997). Evaluation of thrombolytic agents. *Drugs* **3**, 11-17.
- Benedict, C. R., Refino, C. J., Keyt, B. A., Pakala, R., Paoni, N. F., Thomas, G. R., and Bennett, W. F. (1995). New variant of human tissue plasminogen activator (TPA) with enhanced efficacy and lower incidence of bleeding compared with recombinant human TPA. *Circulation* **92**, 3032-3040.
- Bennett, W. F., Paoni, N. F., Keyt, B. A., Botstein, D., Jones, A. J. S., Presta, L., Wurm, F. M., and Zoller, M. J. (1991). High resolution analysis of functional determinants on human tissue-type plasminogen activator. *Journal of Biological Chemistry* **266**, 5191-5201.

- Bergum, P. W. and Gardell, S. J. (1992). Vampire bat salivary plasminogen activator exhibits a strict and fastidious requirement for polymeric fibrin as its cofactor, unlike human tissue-type plasminogen activator: a kinetic analysis. *Journal of Biological Chemistry* **267**, 17726-17731.
- Blanchoin, L., Didry, D., Carlier, M. F., and Pantaloni, D. (1996). Kinetics of association of myosin subfragment-1 to unlabeled and pyrenyl-labeled actin. *Journal of Biological Chemistry* **271**, 12380-12386.
- Bode, C., Kohler, B., Smalling, R. W., and Runge, M. S. (1995). Reteplase (r-PA): A novel recombinant plasminogen activator. *Fibrinolysis* **9 Suppl 1**, 97-99.
- Bode, W. and Renatus, M. (1997). Tissue-type plasminogen activator: variants and crystal/solution structures demarcate structural determinants of function. *Current Opinion in Structural Biology* **7**, 865-872.
- Boeynaems, J. M. and Dumont, J. E. (1975). Quantitative analysis of the binding of ligands to their receptors. *J. Cyclic Nucleotide Res.* **1**, 123-142.
- Boffa, M. B., Wang, W., Bajzar, L., and Nesheim, M. E. (1998). Plasma and recombinant thrombin-activable fibrinolysis inhibitor (TAFI) and activated TAFI compared with respect to glycosylation, thrombin/thrombomodulin-dependent activation, thermal stability, and enzymatic properties. *Journal of Biological Chemistry* **273**, 2127-2135.
- Boffa, M. B., Bell, R., Stevens, W. K., and Nesheim, M. E. (2000). Roles of thermal instability and proteolytic cleavage in regulation of activated thrombin-activatable fibrinolysis inhibitor. *Journal of Biological Chemistry* **275**, 12868-12878.
- Bok, R. A. and Mangel, W. F. (1985). Quantitative characterization of the binding of

- plasminogen to intact fibrin clots, lysine-sepharose, and fibrin cleaved by plasmin. *Biochemistry* **24**, 3279-3286.
- Boxrud, P. D., Fay, W. P., and Bock, P. E. (2000) Streptokinase binds to human plasmin with high affinity, perturbs the plasmin active site, and induces expression of a substrate recognition exosite for plasminogen. *Journal of Biological Chemistry* **275**, 14579-14589.
- Bringmann, P., Gruber, D., Liese, A., Toschi, L., Krätzschmar, J., Schleuning, W. D., and Donner, P. (1995). Structural features mediating fibrin selectivity of vampire bat plasminogen activators. *Journal of Biological Chemistry* **270**, 25596-25603.
- Brown, J. H., Volkmann, N., Jun, G., Henschen-Edman, A. H., and Cohen, C. (2000). The crystal structure of modified bovine fibrinogen. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 85-90.
- Bu, G., Williams, S., Strickland, D. K., and Schwartz, A. L. (1992). Low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor is an hepatic receptor for tissue-type plasminogen activator. *Proceedings of the National Academy of Sciences:USA* **89**, 7427-7431.
- Budzynski, A. Z., Marder, V. J., and Shainoff, J. R. (1974). Structure of plasmic degradation products of human fibrinogen. *Journal of Biological Chemistry* **249**, 2294-2302.
- Budzynski, A. Z., Olexa, S. A., and Brizuela, B. S. (1979). The interference of plasmic degradation products of human crosslinked fibrin with clot formation. *Biochimica et Biophysica Acta* **584**, 284-287.
- Byeon, I.-J. L., Kelley, R. F., Mulkerrin, M. G., An, S. S. A., and Llinás, M. (1995). Ligand

binding to the tissue-type plasminogen activator kringle 2 domain: Structural characterization by ¹H-NMR. *Biochemistry* **34**, 2739-2750.

Camani, C. and Kruithof, E. K. O. (1995). The role of the finger and growth factor domains in the clearance of tissue-type plasminogen activator by hepatocytes. *Journal of Biological Chemistry* **270**, 26053-26056.

Camani, C., Gavin, O., Bertossa, C., Samatani, E., and Kruithof, E. K. (1998). Studies on the effect of fucosylated and non-fucosylated finger/growth-factor constructs on the clearance of tissue-type plasminogen activator mediated by the low-density-lipoprotein-receptor-related protein. *European Journal of Biochemistry* **251**, 804-811.

Cannon, C. P., McCabe, C. H., Gibson, C. M., Ghali, M., Sequeira, R. F., McKendall, G. R., Breed, J., Modi, N. B., Fox, N. L., Tracy, R. P., Love, T. W., and Braunwald, E. (1997). TNK-tissue plasminogen activator in acute myocardial infarction. Results of the Thrombolysis in Myocardial Infarction (TIMI) 10A dose-ranging trial. *Circulation* **95**, 351-356.

Cannon, C. P., Gibson, M., McCabe, C. H., Adgey, A. A., Schweiger, M. J., Sequeira, R. F., Grollier, G., Giugliano, R. P., Frey, M., Mueller, H. S., Steingart, R. M., Weaver, W. D., Van de Werf, F., and Braunwald, E. (1998). TNK-tissue plasminogen activator compared with front loaded alteplase in acute myocardial infarction. Results of the TIMI 10B trial. *Circulation* **98**, 2805-2814.

Cappiello, M., Vilaro, P. G., Del Corso, A., and Mura, U. (1998). Hirunorms, novel hirudin-like direct thrombin inhibitors. *General Pharmacology* **30**, 565-568.

Carr, M. E., Krishnamurti, C., and Alving, B. M. (1992). Effect of plasminogen activator inhibitor-1 on tissue-type plasminogen activator-induced fibrinolysis. *Thrombosis*

and Haemostasis **67**, 106-110.

Castellino, F. J. (1979). A unique enzyme-protein substrate modifier reaction: plasmin/streptokinase interaction. *Trends in Biochemical Sciences* 1-5.

Castellino, F. J. and Powell, J. R. (1981). Human plasminogen. *Methods in Enzymology* **80**, 365-379.

Chandler, W. L. (1991). A kinetic model of the circulatory regulation of tissue plasminogen activator. *Thrombosis and Haemostasis* **66**, 321-328.

Chandler, W. L., Trimble, S. L., Loo, S. C., and Mornin, D. (1990). Effect of PAI-1 levels on the molar concentrations of active tissue plasminogen activator (t-PA) and t-PA/PAI-1 complex in plasma. *Blood* **76**, 930-937.

COBALT Investigators (1997). A comparison of continuous infusion of alteplase with double-bolus administration for acute myocardial infarction. *The New England Journal of Medicine* **337**, 1124-1130.

Cockell, C. S., Marshall, J. M., Dawson, K. M., Cederholm-Williams, S. A., and Ponting, C. P. (1998). Evidence that the conformation of unliganded human plasminogen is maintained via an intramolecular interaction between the lysine binding site of kringle 5 and the N Terminal peptide. *Biochemical Journal* **333**, 99-105.

Collen, D. (1980). On the regulation and control of fibrinolysis. *Thrombosis and Haemostasis* **43**, 77-89.

Collen, D. (1996). Fibrin-selective thrombolytic therapy for acute myocardial infarction. *Circulation* **93**, 857-865.

- Collen, D. (1997). Thrombolytic therapy. *Thrombosis and Haemostasis* **78**, 742-746.
- Collen, D. (1999). The plasminogen (fibrinolytic) system. *Thrombosis and Haemostasis* **82**, 259-270.
- Collen, D. and Lijnen, H. R. (1986). Tissue-type plasminogen activator. Mechanisms of action and thrombolytic properties. *Haemostasis* **16 Suppl 3:25-32**, 25-32.
- Collen, D. and Lijnen, H. R. (1991). Basic and clinical aspects of fibrinolysis and thrombolysis. *Blood* **78**, 3114-3124.
- Collen, D. and Lijnen, H. R. (1992). Fibrin-specific fibrinolysis. *Annals of the New York Academy of Sciences* **667**, 259-271.
- Collen, D., Stassen, J. M., Yasuda, T., Refino, C., Paoni, N., Keyt, B., Roskams, T., Guerrero, J. L., Lijnen, H. R., Gold, H. K., and Bennett, W. F. (1994). Comparative thrombolytic properties of tissue-type plasminogen activator and of a plasminogen activator inhibitor-1-resistant glycosylation variant, in a combined arterial and venous thrombosis model in the dog. *Thrombosis and Haemostasis* **72**, 98-104.
- Coombs, G. S., Dang, A. T., Madison, E. L., and Corey, D. R. (1996). Distinct mechanisms contribute to stringent substrate specificity of tissue-type plasminogen activator. *Journal of Biological Chemistry* **271**, 4461-4467.
- Davidson, D. J. and Castellino, F. J. (1993). The influence of the nature of the asparagine 289-linked oligosaccharide on the activation by urokinase and lysine binding properties of natural and recombinant human plasminogens. *Journal of Clinical Investigation* **92**, 249-254.
- de Munk, G. A. W., Caspers, M. P. M., Chang, G. T. G., Pouwels, P. H., Enger-Valk, B. E.,

- and Verheijen, J. H. (1989). Binding of tissue-type plasminogen activator to lysine, lysine analogues, and fibrin fragments. *Biochemistry* **28**, 7318-7325.
- de Vries, C., Veerman, H., and Pannekoek, H. (1989). Identification of the domains of tissue-type plasminogen activator involved in the augmented binding to fibrin after limited digestion with plasmin. *Journal of Biological Chemistry* **264**, 12604-12610.
- de Vries, C., Veerman, H., Koornneef, E., and Pannekoek, H. (1990). Tissue-type plasminogen activator and its substrate Glu-plasminogen share common binding sites in limited plasmin-digested fibrin. *Journal of Biological Chemistry* **265**, 13547-13552.
- Dellenback, R. J. and Chien, S. (1970). The extinction coefficient of fibrinogen from man, dog, elephant, sheep, and goat at 280 m μ . *Proceedings of the Society for Experimental Biology and Medicine* **134**, 353-355.
- den Heijer, P., Vermeer, F., Ambrosioni, E., Sadowski, Z., Lopez-Sendon, J. L., von Essen, R., Beaufils, P., Thadani, U., Adgey, J., Pierard, L., Brinker, J., Davies, R. F., Smalling, R. W., Wallentin, L., Caspi, A., Pangerl, A., Trickett, L., Hauck, C., Henry, D., and Chew, P. (1998). Evaluation of a weight-adjusted single-bolus plasminogen activator in patients with myocardial infarction: a double-blind, randomized angiographic trial of lanoteplase versus alteplase. *Circulation* **98**, 2117-2125.
- DeSerrano, V. S. and Castellino, F. J. (1990). Structural determinants of the noncatalytic chain of tissue-type plasminogen activator that modulate its association rate with plasminogen activator inhibitor-1. *Journal of Biological Chemistry* **265**, 10473-10478.
- Ding, L., Coombs, G. S., Strandberg, L., Navre, M., Corey, D. R., and Madison, E. L.

- (1995). Origins of the specificity of tissue-type plasminogen activator. *Proceedings of the National Academy of Sciences:USA* **92**, 7627-7631.
- Eastman, D., Wurm, F. M., van Reis, R., and Higgins, D. L. (1992). A region of tissue plasminogen activator that affects plasminogen activation differentially with various fibrin(ogen)-related stimulators. *Biochemistry* **31**, 419-422.
- Eaton, D. L., Malloy, B. E., Tsai, S. P., Henzel, W., and Drayna, D. (1991). Isolation, Molecular cloning, and partial characterization of a novel carboxypeptidase B from human plasma. *Journal of Biological Chemistry* **266**, 21833-21838.
- Emeis, J. J. (1992). Regulation of the acute release of tissue-type plasminogen activator from the endothelium by coagulation activation products. *Annals of the New York Academy of Sciences* **249-258**.
- Everse, S. J., Pelletier, H., and Doolittle, R. F. (1995). Crystallization of fragment D from human fibrinogen. *Protein Science* **4**, 1013-1016 --.
- Falkon, L., Gari, I. M., Borrel, M., and Fontcuberta, J. (1992). The release of plasminogen activators (t-PA and u-PA) and plasminogen activator inhibitor (PAI-1) after venous stasis. *Blood Coagulation and Fibrinolysis* **3**, 33-38.
- Fletcher, A. P., Alkjaersig, N., Smyrniotis, F. E., and Sherry, S. (1959). The maintenance of a sustained thrombolytic state in man. I. Induction and effects. *Journal of Clinical Investigation* **38**, 1110-1119.
- Fleury, V. and Angles-Cano, E. (1991). Characterization of the binding of plasminogen to fibrin surfaces: the role of carboxy-terminal lysines. *Biochemistry* **30**, 7630-7638.
- Forrester, J. S. (1995). New standard for success of thrombolytic therapy - An earnest

proposal. *Circulation* **92**, 2026-2028.

Francis, C. W., Marder, V. J., and Martin, S. E. (1980). Plasmic degradation of crosslinked fibrin. I. Structural analysis of the particulate clot and identification of new macromolecular-soluble complexes. *Blood* **56**, 456-464.

Francis, C. W., Connaghan, D. G., and Marder, V. J. (1986). Assessment of fibrin degradation products during fibrinolytic therapy for acute myocardial infarction. *Circulation* **74**, 1027-1036.

Francis, C. W. and Marder, V. J. (1988). Increased resistance to plasmic degradation of fibrin with highly crosslinked alpha-Polymer chains formed at high factor XIII concentrations. *Blood* **71**, 1361-1365.

Fredenburgh, J. C. and Nesheim, M. E. (1992). Lys-plasminogen is a significant intermediate in the activation of Glu-plasminogen during fibrinolysis in vitro. *Journal of Biological Chemistry* **267**, 26150-26156.

Gaede, G. and Grieshaber, M. (1974). A rapid and specific enzymatic method for the estimation of L-arginine. *Analytical Biochemistry* **66**, 393-399.

Gaffney, P. J. (1983). The occurrence and clinical relevance of fibrin fragments in blood. *Annals of the New York Academy of Sciences* **408:407-23**, 407-423.

Gaffney, P. J. and Joe, F. (1979). The lysis of crosslinked human fibrin by plasmin yields initially a single molecular complex, D dimer-E. *Thrombosis Research* **15**, 673-687.

Gardell, S. J., Duong, L. T., Diehl, R. E., York, J. D., Hare, T. R., Register, R. B., Jacobs, J. W., Dixon, R. A. F., and Friedman, P. A. (1989). Isolation, characterization, and cDNA cloning of a vampire bat salivary plasminogen activator. *Journal of Biological*

Chemistry **264**, 17947-17952.

- Gardell, S. J., Hare, T. R., Bergum, P. W., Cuca, G. C., O'Neill-Palladino, L., and Zavodny, S. M. (1990). Vampire bat salivary plasminogen activator is quiescent in human plasma in the absence of fibrin unlike human tissue plasminogen activator. *Blood* **76**, 2560-2564.
- Gardell, S. J., Ramjit, D. R., Stabilito, I. I., Fujita, T., Lynch, J. J., Cuca, G. C., Jain, D., Wang, S. P., Tung, J. S., and Mark, G. E. (1991). Effective thrombolysis without marked plasminemia after bolus intravenous administration of vampire bat salivary plasminogen activator in rabbits. *Circulation* **84**, 244-253.
- Gorkun, O. V., Veklich, Y. I., Medved, L. V., Henschen, A. H., and Weisel, J. W. (1994). Role of the α C domains of fibrin in clot formation. *Biochemistry* **33**, 6986-6997.
- Graham, D. A., Huang, T. C., Keyt, B. A., and Alevriadou, B. R. (1998). Real-time measurement of lysis of mural platelet deposits by fibrinolytic agents under arterial flow. *Ann.Biomed.Eng.* **26**, 712-724.
- Grailhe, P., Nieuwenhuizen, W., and Anglés-Cano, E. (1994). Study of tissue-type plasminogen activator binding sites on fibrin using distinct fragments of fibrinogen. *European Journal of Biochemistry* **219**, 961-967.
- Green, G. D. J. (1986). Poly-lysines as modifiers of one- and two-chain tissue-type plasminogen activator activity. *Thrombosis Research* **44**, 849-857.
- Gruppo Italiano per lo Studio della Streptokinasi nell'Infarto Miocardico (GISSI) (1986). Effectiveness of intravenous thrombolytic treatment in acute myocardial infarction. *Lancet* **1**, 397-402.

- Gulba, D. C., Praus, M., and Witt, W. (1995). DSPA alpha--Properties of the plasminogen activators of the vampire bat *Desmodus rotundus*. *Fibrinolysis* **9 Suppl 1**, 91-96.
- GUSTO Investigators (1993). An international randomized trial comparing four thrombolytic strategies for acute myocardial infarction. The GUSTO investigators. *New England Journal of Medicine* **329**, 673-682.
- GUSTO III Investigators (1997). A comparison of reteplase with alteplase for acute myocardial infarction. The Global Use of Strategies to Open Occluded Coronary Arteries (GUSTO III) Investigators. *New England Journal of Medicine* **337**, 1118-1123.
- Haddeland, U., Sletten, K., Bennick, A., Nieuwenhuizen, W., and Brosstad, F. (1996). Aggregated, conformationally changed fibrinogen exposes the stimulating sites for t-PA-catalysed plasminogen activation. *Thrombosis and Haemostasis* **75**, 326-331.
- Hajjar, K. A. and Reynolds, C. M. (1994). α -Fucose-mediated binding and degradation of tissue-type plasminogen activator by HepG2 cells. *Journal of Clinical Investigation* **93**, 703-710.
- Hansen, L., Blue, Y., Barone, K., Collen, D., and Larsen, G. R. (1988). Functional effects of asparagine-linked oligosaccharide on natural and variant human tissue-type plasminogen activator. *Journal of Biological Chemistry* **263**, 15713-15719.
- Hantgan, R., Fowler, W., Erickson, H., and Hermans, J. (1980). Fibrin assembly: a comparison of electron microscopic and light scattering results. *Thrombosis and Haemostasis* **44**, 119-124.
- Hatton, M. W. C., Southward, S., and Ross-Ouellet, B. (1994). Catabolism of plasminogen

- glycoforms I and II in rabbits: Relationship to plasminogen synthesis by the rabbit liver in vitro. *Metabolism: Clinical and Experimental* **43**, 1430-1437.
- Hayes, M. L. and Castellino, F. J. (1979). Carbohydrate of the human plasminogen variants II. Structure of the asparagine-linked oligosaccharide. *Journal of Biological Chemistry* **254**, 8772-8776.
- Higgins, D. L. and Lamb, M. C. (1986). The incorporation of a fluorescent probe into the active sites of one- and two-chain tissue-type plasminogen activator. *Archives of Biochemistry and Biophysics* **249**, 418-426.
- Higgins, D. L., Lewis, S. D., and Shafer, J. A. (1983). Steady state kinetic parameters for the thrombin-catalyzed conversion of human fibrinogen to fibrin. *Journal of Biological Chemistry* **258**, 9276-9282.
- Higgins, D. L. and Vehar, G. A. (1987). Interaction of one-chain and two-chain tissue plasminogen activator with intact and plasmin-degraded fibrin. *Biochemistry* **26**, 7786-7791.
- Hirsh, J. and Weitz, J. I. (1999). New antithrombotic agents. *Lancet* **353**, 1431-1436.
- Hoffmeister, H. M., Jur, M., Ruf-Lehmann, M., Helber, U., Heller, W., and Seipel, L. (2000). Endothelial tissue-type plasminogen activator release in coronary heart disease. *Journal of the American College of Cardiology* **31**, 547-551.
- Holden, R. W. (1990). Plasminogen activators: pharmacology and therapy. *Radiology* **174**, 993-1001.
- Horrevoets, A. J. G., Smilde, A., de Vries, C., and Pannekoek, H. (1994). The specific roles of finger and kringle 2 domains of tissue-type plasminogen activator during in vitro

fibrinolysis. *Journal of Biological Chemistry* **269**, 12639-12644.

Horrevoets, A. J. G., Pannekoek, H., and Nesheim, M. E. (1997a). A steady-state template model that describes the kinetics of fibrin-stimulated [Glu1]- and [Lys78]plasminogen activation by native tissue-type plasminogen activator and variants that lack either the finger or kringle-2 domain. *Journal of Biological Chemistry* **272**, 2183-2191.

Horrevoets, A. J. G., Pannekoek, H., and Nesheim, M. E. (1997b). Production and characterization of recombinant human plasminogen (S741C-fluorescein). A novel approach to study zymogen activation without generation of active protease. *Journal of Biological Chemistry* **272**, 2176-2182.

Hortin, G. L., Gibson, B. L., and Fok, K. F. (1988). Alpha 2-antiplasmin's carboxy-terminal lysine residue is a major site of interaction with plasmin. *Biochemical and Biophysical Research Communications* **155**, 591-596.

Hortin, G. L., Trimpe, B. L., and Fok, K. F. (1989). Plasmin's peptide-binding specificity: characterization of ligand sites in alpha2-antiplasmin. *Thrombosis Research* **54**, 621-632.

Hoylaerts, M., Rijken, D. C., Lijnen, H. R., and Collen, D. (1982). Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin. *Journal of Biological Chemistry* **257**, 2912-2919.

Husain, S. S., Weisel, J. W., and Budzynski, A. Z. (1989). Interaction of Fibrinogen and its Derivatives with Fibrin. *Journal of Biological Chemistry* **264**, 11414-11420.

Ikenada, Y., Yajima, K., and Yahara, H. (1992). Characterization of human tissue-type

- plasminogen activator variants with amino acid mutations in kringle 1 domain. *Blood Coagulation and Fibrinolysis* **3**, 381-387.
- Ikeno, L. C., Bowmen, B. M., and Der, M. (1981). Commercial production of ¹²⁵I-fibrinogen injection. *J.Radioanal.Chem.* **65**, 179-188.
- Jennings, K. (1996). Antibodies to streptokinase - Once is enough. *BMJ* **312**, 393-394.
- Jern, C., Eriksson, E., Tengborn, L., Risberg, B., Wadenvik, H., and Jern, S. (1989). Changes of plasma coagulation and fibrinolysis in response to mental stress. *Thrombosis and Haemostasis* **62**, 767-771.
- Jern, C., Selin, L., and Jern, S. (1994). In vivo release of tissue-type plasminogen activator across the human forearm during mental stress. *Thrombosis and Haemostasis* **72**, 285-291.
- Kaneko, M., Sakata, Y., Matsuda, M., and Mimuro, J. (1992). Interactions between the finger and kringle-2 domains of tissue-type plasminogen activator and plasminogen activator inhibitor-1. *Journal of Biochemistry* **111**, 244-248.
- Ke, S. H., Tachias, K., Lamba, D., Bode, W., and Madison, E. L. (1997). Identification of a hydrophobic exosite on tissue type plasminogen activator that modulates specificity for plasminogen. *Journal of Biological Chemistry* **272**, 1811-1816.
- Kennedy, J. W. and Stadius, M. L. (1999). Combined thrombolytic and platelet glycoprotein IIb/IIIa inhibitor therapy for acute myocardial infarction. *Circulation* **99**, 2714-2716.
- Keyt, B. A., Paoni, N. F., Refino, C. J., Berleau, L., Nguyen, H., Chow, A., Lai, J., Pena, L., Pater, C., and Ogez, J. (1994). A faster-acting and more potent form of tissue plasminogen activator. *Proceedings of the National Academy of Sciences:USA* **91**,

3670-3674.

- Klement, G., Klement, P., Smith, S., and Weitz, J. (1995). Bat plasminogen activator causes less fibrinogenolysis and bleeding than tissue plasminogen activator in rabbits. *Thromb Haemost* **73**, 1339 (abstr.).
- Klement, P., Liao, P., and Bajzar, L. (1999). A novel approach to arterial thrombolysis. *Blood* **94**, 2735-2743.
- Kohnert, U., Rudolph, R., Verheijen, J. H., Weening-Verhoeff, E. J. D., Stern, A., Optiz, U., Martin, U., Lill, H., Prinz, H., Lechner, M., Kresse, G. B., Buckel, P., and Fischer, S. (1992). Biochemical properties of the kringle 2 and protease domains are maintained in the refolded t-PA deletion variant BM 06.022. *Protein Engineering* **5**, 93-100.
- Kohnert, U., Horsch, B., and Fischer, S. (1993). A variant of tissue plasminogen activator (t-PA) comprised of the kringle 2 and the protease domain shows a significant difference in the in vitro rate of plasmin formation as compared to the recombinant human t-PA from transformed Chinese hamster ovary cells. *Fibrinolysis* **7**, 365-372.
- Kooistra, T., Schrauwen, Y., Arts, J., and Emeis, J. J. (1995). Regulation of endothelial cell t-PA synthesis and release. *International Journal of Hematology* **59**, 233-255.
- Kratzschmar, J., Haendler, B., Langer, G., Boidol, W., Bringmann, P., Alagon, A., Donner, P., and Schleuning, W. D. (1991). The plasminogen activator from the salivary gland of the vampire bat *desmodus rotundus*: cloning and expression. *Gene* **105**, 229-237.
- Krishnamurti, C. and Alving, B. M. (1992). Plasminogen activator inhibitor type 1: biochemistry and evidence for modulation of fibrinolysis in vivo. *Seminars in*

Thrombosis and Hemostasis **18**, 67-79.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lamba, D., Bauer, M., Huber, R., Fischer, S., Rudolph, R., Kohnert, U., and Bode, W. (1996). The 2.3 Å crystal structure of the catalytic domain of recombinant two-chain human tissue-type plasminogen activator. *Journal of Molecular Biology* **258**, 117-135.

Larsen, G. R., Henson, K., and Blue, Y. (1988). Variants of human tissue-type plasminogen activator fibrin binding, fibrinolytic, and fibrinogenolytic characterization of genetic variants lacking the fibronectin finger-like and/or the epidermal growth factor domains. *Journal of Biological Chemistry* **263**, 1023-1029.

Larsen, G. R., Timony, G. A., Horgan, P. G., Barone, K. M., Henson, K. S., Angus, L. B., and Stoudemire, J. B. (1991). Protein engineering of novel plasminogen activators with increased thrombolytic potency in rabbits relative to activase. *Journal of Biological Chemistry* **266**, 8156-8161.

Lee, A. Y. Y., Fredenburgh, J. C., Stewart, R. J., Rischke, J. A., and Weitz, J. I. (2000). The profibrinolytic soluble fibrin degradation product (DD)E protects plasmin from inhibition from alpha-2 antiplasmin. *manuscript in preparation*.

Lee, L. V., Ewald, G. A., McKenzie, C. R., and Eisenberg, P. R. (1997). The relationship of soluble fibrin and cross-linked fibrin degradation products to the clinical course of myocardial infarction. *Arteriosclerosis, Thrombosis, and Vascular Biology* **17**, 628-633.

- Lerch, P. G., Rickli, E. E., Lergier, W., and Gillessen, D. (1980). Localization of individual lysine-binding regions in human plasminogen and investigations on their complex-forming properties. *European Journal of Biochemistry* **107**, 7-13.
- Lijnen, H. R. and Collen, D. (1986). Alpha 2-Antiplasmin. In "Proteinase Inhibitors" (A. J. Barrett and G. Salvesen, Eds.), pp. 457-476. Elsevier Science Publishers BV, New York.
- Lijnen, H. R., Nelles, L., Van Hoef, B., De Cock, F., and Collen, D. (1990a). Biochemical and functional characterization of human tissue-type plasminogen activator variants obtained by deletion and/or duplication of structural/functional domains. *Journal of Biological Chemistry* **265**, 5677-5683.
- Lijnen, H. R., Van Hoef, B., and Collen, D. (1990b). On the molecular interactions between fibrin, tissue-type plasminogen activator and plasminogen. *Thrombosis Research* **57**(Suppl.X), 45-54.
- Lijnen, H. R. and Collen, D. (1995a). Mechanisms of physiological fibrinolysis. *Balliere's Clinical Haematology* **8**, 277-290.
- Lijnen, H. R. and Collen, D. (1995b). Molecular and cellular basis of fibrinolysis. In "Hematology. Basic Principles and Practice" (R. Hoffman, E. J. Benz, S. J. Shattil, B. Furie, H. J. Cohen, and L. E. Silberstein, Eds.), pp. 1588-1596. Churchill Livingstone, New York.
- Lijnen, H. R., Stassen, J.-M., and Collen, D. (1995). Differential inhibition with antifibrinolytic agents of staphylokinase and streptokinase induced clot lysis. *Thrombosis and Haemostasis* **73**, 845-849.

- Lindahl, T. L., Ohlsson, P. I., and Wiman, B. (1990). The mechanism of the reaction between human plasminogen-activator inhibitor 1 and tissue plasminogen activator. *Biochemical Journal* **265**, 109-113.
- Lorand, L. (2000). Sol Sherry lecture in thrombosis: Research on clot stabilization provides clues for improving thrombolytic therapies. *Arteriosclerosis, Thrombosis, and Vascular Biology* **20**, 2-9.
- Loscalzo, J. (1988). Structural and kinetic comparison of recombinant human single and two-chain tissue plasminogen activator. *Journal of Clinical Investigation* **82**, 1391-1397.
- Lucas, M. A., Fretto, L. J., and McKee, P. A. (1983). The binding of human plasminogen to fibrin and fibrinogen. *Journal of Biological Chemistry* **258**, 4249-4256.
- Madison, E. L., Goldsmith, E. J., Gerard, R. D., Gething, M. J. H., Sambrook, J. F., and Bassel-Duby, R. S. (1990a). Amino acid residue that affect interaction of tissue-type plasminogen activator with plasminogen activator inhibitor 1. *Proceedings of the National Academy of Sciences:USA* **87**, 3530-3533.
- Madison, E. L., Goldsmith, E. J., Gething, M. J. H., Sambrook, J. F., and Gerard, R. D. (1990b). Restoration of Serine Protease-Inhibitor Interaction by Protein Engineering. *Journal of Biological Chemistry* **265**, 21423-21426.
- Madison, E. L., Kobe, A., Gething, M. J., Sambrook, J. F., and Goldsmith, E. J. (1993). Converting tissue plasminogen activator to a zymogen: A regulatory triad of Asp-His-Ser. *Science* **262**, 419-421.
- Madison, E. L. and Sambrook, J. F. (1993). Probing structure-function relationships of

- tissue-type plasminogen activator by oligonucleotide-mediated site-specific mutagenesis. *Methods in Enzymology* **223**, 249-271.
- Madison, E. L., Coombs, G. S., and Corey, D. R. (1995). Substrate specificity of tissue type plasminogen activator. Characterization of the fibrin independent specificity of t-PA for plasminogen. *Journal of Biological Chemistry* **270**, 7558-7562.
- Mangel, W. F., Lin, B., and Ramakrishnan, V. (1990). Characterization of an extremely large, ligand-induced conformational change in plasminogen. *Science* **248**, 69-73.
- Marder, V. J., Shulman, N. R., and Carroll, W. R. (1969). High molecular weight derivatives of human fibrinogen produced by plasmin. *Journal of Biological Chemistry* **211**.
- Marder, V. J. and Francis, C. W. (1983). Plasmin degradation of cross-linked fibrin. *Annals of the New York Academy of Sciences* **408**, 397-406.
- Marshall, J. M., Brown, A. J., and Ponting, C. P. (1994). Conformational studies of human plasminogen and plasminogen fragments: Evidence for a novel third conformation of plasminogen. *Biochemistry* **33**, 3599-3606.
- Marti, D., Schaller, J., Ochensberger, B., and Rickli, E. E. (1994). Expression, purification and characterization of the recombinant kringle 2 and kringle 3 domains of human plasminogen and analysis of their binding affinity for omega-aminocarboxylic acids. *European Journal of Biochemistry* **219**, 455-462.
- Martin, U., Sponer, G., and Strein, K. (1993). Differential fibrinolytic properties of the recombinant plasminogen activator BM 06.022 in human plasma and blood clot systems in vitro. *Blood Coagulation and Fibrinolysis* **4**, 235-242.
- Marx, P. F., Hackeng, T. M., Dawson, P. E., Griffin, J. H., Meijers, J. C. M., and Bouma, B.

- N. (2000). Inactivation of active thrombin-activatable fibrinolysis inhibitor takes place by a process that involves conformational instability rather than proteolytic cleavage. *Journal of Biological Chemistry* **275**, 12410-12415.
- Masson, C. and Angles-Cano, E. (1988). Kinetic analysis of the interaction between plasminogen activator inhibitor-1 and tissue-type plasminogen activator. *Biochemical Journal* **256**, 237-244.
- Mayer, M. (1990). Biochemical and biological aspects of the plasminogen activation system. *Clinical Biochemistry* **23**, 197-211.
- McCance, S. G. and Castellino, F. J. (1995). Contributions of individual kringle domains toward maintenance of the chloride-induced tight conformation of human glutamic acid-1 plasminogen. *Biochemistry* **34**, 9581-9586.
- McClintock, D. K. and Bell, P. H. (1971). The mechanism of activation of human plasminogen by streptokinase. *Biochemical and Biophysical Research Communications* **43**, 694-702.
- McDonagh, R. P., McDonagh, J., Blomback, M., and Blomback, B. (1971). Crosslinking of Human Fibrin: Evidence for Intermolecular Crosslinking Involving Alpha-chains. *FEBS Letters* **14**, 33-36.
- McFarlane, A. S. (1965). Labelling of plasma proteins with radioactive iodine. *Biochemical Journal* **62**, 135-143.
- Mellott, M. J., Ramjit, D. R., Stabilito, I. I., Hare, T. R., Senderak, E. T., Lynch, J. J., Jr., and Gardell, S. J. (1995). Vampire bat salivary plasminogen activator evokes minimal bleeding relative to tissue-type plasminogen activator as assessed by a rabbit cuticle

- bleeding time model. *Thrombosis and Haemostasis* **73**, 478-483.
- Modi, N. B., Eppler, S., Breed, J., Cannon, C. P., Braunwald, E., and Love, T. W. (1998). Pharmacokinetics of a slower clearing tissue plasminogen activator variant, TNK-tPA, in patients with acute myocardial infarction. *Thrombosis and Haemostasis* **79**, 134-139.
- Mosesson, M. W. (1995). Terminology for macromolecular derivatives of crosslinked fibrin. *Thrombosis and Haemostasis* **73**, 725-726.
- Mosesson, M. W. (1998). Fibrinogen structure and fibrin clot assembly. *Seminars in Thrombosis and Hemostasis* **24**, 169-174.
- Mosesson, M. W. (1999). Fibrinogen and fibrin polymerization and functions. *Blood Coagulation and Fibrinolysis* **10 Suppl 1**, S45-S48.
- Mosesson, M. W., Siebenlist, K. R., Voskuilen, M., and Nieuwenhuizen, W. (1998). Evaluation of the factors contributing to fibrin-dependent plasminogen activation. *Thrombosis and Haemostasis* **79**, 796-801.
- Moskowitz, K. A. and Budzynski, A. Z. (1994). The (DD)E complex is maintained by a composite fibrin polymerization site. *Biochemistry* **33**, 12937-12944.
- Motta, A., Laursen, R. A., Llinas, M., Tulinsky, A., and Park, C. H. (1987). Complete Assignment of the aromatic proton magnetic resonance spectrum of the kringle 1 domain from human plasminogen: structure of the ligand-binding site. *Biochemistry* **26**, 3827-3836.
- Mulichak, A. M., Park, C. H., Tulinsky, A., Petros, A. M., and Llinas, M. (1989). Human plasminogen kringle 4. Crystallization and preliminary diffraction data of two

- different crystal forms. *Journal of Biological Chemistry* **264**, 1922-1923.
- Mulichak, A. M., Tulinsky, A., and Ravichandran, K. G. (1991). Crystal and molecular structure of human plasminogen kringle 4 Refined at 1.9 Å resolution. *Biochemistry* **30**, 10576-10588.
- Muschick, P., Zeggert, D., Donner, P., and Witt, W. (1993). Thrombolytic properties of desmodus (vampire bat) salivary plasminogen activator DSPAalpha1, alteplase and streptokinase following intravenous bolus injection in a rabbit model of carotid artery thrombosis. *Fibrinolysis* **7**, 284-290.
- Nakatani, Y., Fujioka, M., and Higashino, K. (1972). Enzymic determination of L-lysine in biological materials. *Analytical Biochemistry* **49**, 225-231.
- Narita, M., Bu, G., Herz, J., and Schwartz, A. L. (1995). Two receptor systems are involved in the plasma clearance of tissue-type plasminogen activator (t-PA) in vivo. *Journal of Clinical Investigation* **96**, 1164-1168.
- Nesheim, M. E. (1999). TAFI. *Fibrinolysis and Proteolysis* **13**, 72-77.
- Nesheim, M. E., Prendergast, F. G., and Mann, K. G. (1979). Interactions of a fluorescent active-site-directed inhibitor of thrombin: dansylarginine N-(3-Ethyl-1,5-pentanediy) amide. *Biochemistry* **18**, 996-1003.
- Nesheim, M. E., Fredenburgh, J. C., and Larsen, G. R. (1990). The dissociation constants and stoichiometries of the interactions of Lys-plasminogen and chloromethyl ketone derivatives of tissue plasminogen activator and the variant dFEIX with intact fibrin. *Journal of Biological Chemistry* **265**, 21541-21548.
- Nesheim, M., Wang, W., Boffa, M., Nagashima, M., Morser, J., and Bajzar, L. (1997).

Thrombin, thrombomodulin and TAFI in the molecular link between coagulation and fibrinolysis. *Thrombosis and Haemostasis* **78**, 386-391.

Neuhaus, K. L. (1999). In TIME 2 trial. *Presented at the American College of Cardiology Scientific Sessions, New Orleans (March)*.

Neuhaus, K. L., Molhoek, G. P., Zeymer, U., Tebbe, U., Wegscheider, K., Schroder, R., Camez, A., Laarman, G. J., Grollier, G. M., Lok, D. J., Kuckuck, H., and Lazarus, P. (1999). Recombinant hirudin (lepirudin) for the improvement of thrombolysis with streptokinase in patients with acute myocardial infarction: results of the HIT-4 trial. *Journal of the American College of Cardiology* **34**, 966-973.

Nieuwenhuizen, W. (1994). Sites in fibrin involved in the acceleration of plasminogen activation by t-PA. Possible role of fibrin polymerization. *Thrombosis Research* **75**, 343-347.

Nieuwenhuizen, W., Verheijen, J. H., Vermond, A., and Chang, G. T. G. (1983). Plasminogen activation by tissue plasminogen activator is accelerated in the presence of fibrin(ogen) cyanogen bromide fragment FCB-2. *Biochimica et Biophysica Acta* **755**, 531-533.

Nieuwenhuizen, W., Voskuilen, M., Vermond, A., Hoegge-de Nobel, B., and Traas, D. W. (1988). The Influence of fibrin(ogen) fragments on the kinetic parameters of the tissue-type plasminogen-activator-mediated activation of different forms of plasminogen. *Journal of Biochemistry* **174**, 163-169.

Noble, S. and McTavish, D. (1996). Reteplase. A review of its pharmacological properties and clinical efficacy in the management of acute myocardial infarction. [Review] [55 refs]. *Drugs* **52**, 589-605.

- Norman, B., Wallen, P., and Ranby, M. (1985). Fibrinolysis mediated by tissue plasminogen activator. Disclosure of kinetic transition. *European Journal of Biochemistry* **149**, 193-200.
- Noorman, F., Barrett-Bergshoeff, M. M., and Rijken, D. C. (1998). Role of carbohydrate and protein in the binding of tissue-type plasminogen activator to the human mannose receptor. *European Journal of Biochemistry* **251**, 107-113.
- Olexa, S. A. and Budzynski, A. Z. (1979a). Binding phenomena of isolated unique plasmonic degradation products of human cross-linked fibrin. *Journal of Biological Chemistry* **254**, 4925-4932.
- Olexa, S. A. and Budzynski, A. Z. (1979b). Primary soluble plasmonic degradation product of human cross-linked fibrin. Isolation and stoichiometry of the (DD)E complex. *Biochemistry* **18**, 991-995.
- Olexa, S. A. and Budzynski, A. Z. (1980). Evidence for four different polymerization sites involved in human fibrin formation. *Proceedings of the National Academy of Sciences:USA* **77**, 1374-1378.
- Olexa, S. A., Budzynski, A. Z., Doolittle, R. F., Cottrell, B. A., and Greene, T. C. (1981). Structure of fragment E species from human cross-linked fibrin. *Biochemistry* **20**, 6139-6145.
- Orth, K., Madison, E. L., Gething, M. J., Sambrook, J. F., and Herz, J. (1992). Complexes of tissue-type plasminogen activator and its serpin inhibitor plasminogen-activator inhibitor type 1 are internalized by means of the low density lipoprotein receptor-related protein/alpha2-macroglobulin receptor. *Proceedings of the National Academy of Sciences:USA* **89**, 7422-7426.

- Otter, M., Kuiper, J., van Berkel, T. J., and Rijken, D. C. (1992a). Mechanisms of tissue-type plasminogen activator (tPA) clearance by the liver. *Annals of the New York Academy of Sciences* **667**:431-42, 431-442.
- Otter, M., Zockova, P., Kuiper, J., van Berkel, T. J., Barrett-Bergshoeff, M. M., and Rijken, D. C. (1992b). Isolation and characterization of the mannose receptor from human liver potentially involved in the plasma clearance of tissue-type plasminogen activator. *Hepatology* **16**, 54-59.
- Owen, J., Friedman, K. D., Grossman, B. A., Wilkins, C., Berke, A. D., and Powers, E. R. (1987). Quantitation of fragment X formation during thrombolytic therapy with streptokinase and tissue plasminogen activator. *Journal of Clinical Investigation* **79**, 1642-1647.
- Paoni, N. F., Chow, A. M., Peña, L. C., Keyt, B. A., Zoller, M. J., and Bennett, W. F. (1993a). Making tissue-type plasminogen activator more fibrin specific. *Protein Engineering* **6**, 529-534.
- Paoni, N. F., Keyt, B. A., Refino, C. J., Chow, A. M., Nguyen, H. V., Berleau, L. T., Badillo, J., Peña, L. C., Brady, K., Wurm, F. M., Ogez, J., and Bennett, W. F. (1993b). A slow clearing, fibrin-specific, PAI-1 resistant variant of t- PA (T103N, KHRR 296-299 AAAA). *Thrombosis and Haemostasis* **70**, 307-312.
- Paramo, J. A., Gascoine, P. S., Pring, J. B., and Gaffney, P. J. (1990). The relative inhibition by alpha2-antiplasmin and plasminogen activator inhibitor-1 of clot lysis in vitro. *Fibrinolysis* **4**, 169-175.
- Parekh, R. B., Dwek, R. A., Rudd, P. M., Thomas, J. R., Rademacher, T. W., Warren, T., Wun, T. C., Hebert, B., Reitz, B., Palmier, M., Ramabhadran, T., and Tiemeier, D.

- C. (1989a). N-Glycosylation and in vitro enzymatic activity of human recombinant tissue plasminogen activator expressed in chinese hamster ovary cells and a murine cell line. *Biochemistry* **28**, 7670-7679.
- Parekh, R. B., Dwek, R. A., Thomas, J. R., Opdenakker, G., Rademacher, T. W., Wittwer, A. J., Howard, S. C., Nelson, R., Siegel, N. R., Jennings, M. G., Harakas, N. K., and Feder, J. (1989b). Cell-type specific and site-specific N-glycosylation of type I and type II human tissue plasminogen activator. *Biochemistry* **28**, 7644-7662.
- Pathy, L. (1985). Evolution of the proteases of blood coagulation and fibrinolysis by assembly from modules. *Cell* **41**, 657-663.
- Peisach, E., Wang, J., Santos, T., Reich, E., and Ringe, D. (1999). Crystal structure of the proenzyme domain of plasminogen. *Biochemistry* **38**, 11180-11188.
- Petersen, L. C., Boel, E., Johannessen, M., and Foster, D. (1990). Quenching of the amidolytic activity of one-chain tissue-type plasminogen activator by mutation of lysine-416. *Biochemistry* **29**, 3451-3457.
- Petros, A. M., Ramesh, V., and Llinas, M. (1989). ¹H-NMR studies of aliphatic ligand binding to human plasminogen kringle 4. *Biochemistry* **28**, 1368-1376.
- Pislaru, S. V. and Van de Werf, F. J. (1999). The current role of thrombolytic therapy in the treatment of acute myocardial infarction. *Fibrinolysis and Proteolysis* **13**, 78-90.
- Ponting, C. P., Marshall, J. M., and Cederholm-Williams, S. A. (1992). Plasminogen: a structural review. *Blood Coagulation and Fibrinolysis* **3**, 605-614.
- Ranby, M., Bergsdorf, N., Pohl, G., and Wallen, P. (1982). Isolation of two variants of native one-chain tissue plasminogen activator. *FEBS Letters* **146**, 289-292.

- Rao, A. K., Pratt, C., Berke, A., Jaffe, A., Ockene, I., Schreiber, T. L., Bell, W. R., Knatterud, G., Robertson, T. L., and Terrin, M. L. (1988). Thrombolysis in Myocardial Infarction (TIMI) Trial--phase I: hemorrhagic manifestations and changes in plasma fibrinogen and the fibrinolytic system in patients treated with recombinant tissue plasminogen activator and streptokinase. *Journal of the American College of Cardiology* **11**, 1-11.
- Redlitz, A. and Plow, E. F. (1995). Receptors for plasminogen and t-PA: an update. *Balliere's Clinical Haematology* **8**, 313-327.
- Refino, C. J., Paoni, N. F., Keyt, B. A., Pater, C. S., Badillo, J. M., Wurm, F. M., Ogez, J., and Bennett, W. F. (1993). A variant of t-PA (T103N, KHRR 296-299 AAAA) that, by bolus, has increased potency and decreased systemic activation of plasminogen. *Thrombosis and Haemostasis* **70**, 313-319.
- Renatus, M., Bode, W., Huber, R., Sturzebecher, J., Prasa, D., Fischer, S., Kohnert, U., and Stubbs, M. T. (1997a). Structural mapping of the active site specificity determinants of human tissue-type plasminogen activator. Implications for the design of low molecular weight substrates and inhibitors. *Journal of Biological Chemistry* **272**, 21713-21719.
- Renatus, M., Engh, R. A., Stubbs, M. T., Huber, R., Fischer, S., Kohnert, U., and Bode, W. (1997b). Lysine 156 promotes the anomalous proenzyme activity of tPA: X-ray crystal structure of single-chain human tPA. *EMBO Journal* **16**, 4797-4805.
- Renatus, M., Stubbs, M. T., Huber, R., Bringmann, P., Donner, P., Schleuning, W. D., and Bode, W. (1997c). Catalytic domain structure of vampire bat plasminogen activator: a molecular paradigm for proteolysis without activation cleavage. *Biochemistry* **36**,

13483-13493.

- Rijken, D. C. (1995). Plasminogen activators and plasminogen activator inhibitors: biochemical aspects. *Balliere's Clinical Haematology* **8**, 291-312.
- Rijken, D. C., Otter, M., Kuiper, J., and van Berkel, T. J. (1990). Receptor-mediated endocytosis of tissue-type plasminogen activator (t-PA) by liver cells. *Thrombosis Research Supplement X*, 63-71.
- Rogers, J. (1985). Exon Shuffling and Intron Insertion in Serine Protease Genes. *Nature* **315**, 458-459.
- Rosnoblet, C., Vischer, U. M., Gerard, R. D., Irminger, J. C., Halban, P. A., and Kruithof, E. K. O. (1999). Storage of tissue-type plasminogen activator in weibel-palade bodies of human endothelial cells. *Arteriosclerosis, Thrombosis, and Vascular Biology* **19**, 1796-1803.
- Rouy, D. and Angles-Cano, E. (1990). The mechanism of activation of plasminogen at the fibrin surface by tissue-type plasminogen activator in a plasma milieu in vitro. Role of antiplasmin. *Biochemical Journal* **271**, 51-57.
- Rudd, P. M., Woods, R. J., Wormald, M. R., Opdenakker, G., Downing, A. K., Campbell, I. D., and Dwek, R. A. (1995). The effects of variable glycosylation on the functional activities of ribonuclease, plasminogen and tissue plasminogen activator. *Biochimica et Biophysica Acta* **1248**, 1-10.
- Ryan, C. A., Hass, G. M., and Kuhn, R. W. (1974). Purification and properties of a carboxypeptidase inhibitor from potatoes. *Journal of Biological Chemistry* **249**, 5495-5499.

- Sakharov, D. V., Plow, E. F., and Rijken, D. C. (1997). On the mechanism of the antifibrinolytic activity of plasma carboxypeptidase B. *Journal of Biological Chemistry* **272**, 14477-14482.
- Schleuning, W. D., Alagon, A., Boidol, W., Bringmann, P., Petri, T., Kratzschmar, J., Haendler, B., Langer, G., Baldus, B., Witt, W., and Donner, P. (1992). Plasminogen activators from the saliva of *desmodus-rotundus* (common vampire bat) - unique fibrin specificity. *Annals of the New York Academy of Sciences* **395**-403.
- Shafer, J. A. and Higgins, D. L. (1988). Human fibrinogen. *CRC Critical Reviews in Clinical Laboratory Sciences* **26**, 1-41.
- Siebenlist, K. R., Di Orto, J. P., Budzynski, A. Z., and Mosesson, M. W. (1990). The polymerization and thrombin-binding properties of des-(B β 1-42)-Fibrin. *Journal of Biological Chemistry* **265**, 18650-18655.
- Smalling, R. W. (1996). Molecular biology of plasminogen activators: what are the clinical implications of drug design? *American Journal of Cardiology* **78**, 2-7.
- Smalling, R. W. (1997). A fresh look at the molecular pharmacology of plasminogen activators: from theory to test tube to clinical outcomes. *Am.J.Health Syst.Pharm.* **54** (Suppl 1), S17-S22.
- Smalling, R. W., Bode, C., Kalbfleisch, J., Sen, S., Limbourg, P., Forycki, F., Habib, G., Feldman, R., Hohnloser, S., and Seals, A. (1995). More rapid, complete, and stable coronary thrombolysis with bolus administration of reteplase compared with alteplase infusion in acute myocardial infarction. RAPID Investigators. *Circulation* **91**, 2725-2732.

- Soria, J., Soria, C., Mirshahi, M., Mirshahi, M., Samama, M. M., and Caen, J. P. (1987). Specific determination of cross-linked fibrin degradation products in patients under thrombolytic therapy for myocardial infarction. *Seminars in Thrombosis and Hemostasis* **13**, 223-227.
- Spraggon, G., Everse, S. J., and Doolittle, R. F. (1997). Crystal structures of fragment D from human fibrinogen and its crosslinked counterpart from fibrin. *Nature* **389**, 455-462.
- Stangl, K., Laule, M., Tenckhoff, B., Stangl, V., Glicch, V., Dubel, P., Grohmann, A., Melzer, C., Langel, J., Wernecke, K. D., Baumann, G., and Ziemer, S. (1998). Fibrinogen breakdown, long-lasting systemic fibrinolysis, and procoagulant activation during alteplase double-bolus regimen in acute myocardial infarction. *American Journal of Cardiology* **81**, 841-847.
- Stassen, J. M., Lijnen, H. R., Vanlinthout, I., and Collen, D. (1991). Thrombolysis with bolus injections and infusions of tissue-type plasminogen activator in rabbits with experimental jugular vein thrombosis. *Fibrinolysis* **5**, 165-170.
- Stefánsson, S., Lawrence, D. A., Herren, T., and Plow, E. F. (1998). Novel approaches to thrombolysis based on modulation of endogenous fibrinolysis. *Coronary Artery Disease* **9**, 99-104.
- Stewart, R. J., Fredenburgh, J. C., Leslie, B. A., Rischke, J. A., and Weitz, J. I. (1996). Importance of (DD)E in limiting the fibrin-specificity of plasminogen activators. *Blood* **88**, 521A.
- Stewart, R. J., Fredenburgh, J. C., Lee, A. Y., Rischke, J. A., and Weitz, J. I. (1998a). Functional comparison of (DD)E with fibrin. *Blood Coagulation and Fibrinolysis* **9**,

691-692.

- Stewart, R. J., Fredenburgh, J. C., and Weitz, J. I. (1998b). Characterization of the interactions of plasminogen and tissue and vampire bat plasminogen activators with fibrinogen, fibrin, and the complex of D-dimer noncovalently linked to fragment E. *Journal of Biological Chemistry* **273**, 18292-18299.
- Stewart, R. J., Fredenburgh, J. C., Leslie, B. A., Keyt, B. A., Rischke, J. A., and Weitz, J. I. (2000). Identification of the mechanism responsible for the increased fibrin-specificity of TNK-tissue plasminogen activator relative to tissue plasminogen activator. *Journal of Biological Chemistry* **275**, 10112-10120.
- Stien, C. M., Brown, N., Vaughan, D. E., Lang, C. C., and Wood, A. J. J. (1998). Regulation of local tissue-type plasminogen activator release by endothelium-dependent and endothelium-independent agonists in human vasculature. *Journal of the American College of Cardiology* **32**, 117-122.
- Stringer, H. A. R., Van Swieten, P., Horrevoets, A. J. G., Smilde, A., and Pannekoek, H. (1994). The role of the finger domain of tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor 1 (PAI-1) in initiation and progression of thrombolysis. *Thrombosis and Haemostasis* **72**, 900-905.
- Stubbs, M. T., Renatus, M., and Bode, W. (1998). An active zymogen: unravelling the mystery of tissue-type plasminogen activator. *Biological Chemistry* **379**, 95-103.
- Suenson, E., Lutzen, O., and Thorsen, S. (1984). Initial plasmin-degradation of fibrin as the basis of a positive feed-back mechanism in fibrinolysis. *European Journal of Biochemistry* **140**, 513-522.

- Suenson, E. and Thorsen, S. (1988). The course and prerequisites of Lys-plasminogen formation during fibrinolysis. *Biochemistry* **27**, 2435-2443.
- Suenson, E., Bjerrum, P., Holm, A., Lind, B., Meldal, M., Selmer, J., and Petersen, L. C. (1990). The role of fragment X polymers in the fibrin enhancement of tissue plasminogen activator-catalyzed plasmin formation. *Journal of Biological Chemistry* **265**, 22228-22237.
- Sugiyama, N., Sasaki, T., Iwamoto, M., and Abiko, Y. (1988). Binding site of alpha 2-plasmin inhibitor to plasminogen. *Biochimica et Biophysica Acta* **952**, 1-7.
- Tachias, K. and Madison, E. L. (1995). Variants of tissue-type plasminogen activator which display substantially enhanced stimulation by fibrin. *Journal of Biological Chemistry* **270**, 18319-18322.
- Tachias, K. and Madison, E. L. (1996). Converting tissue-type plasminogen activator into a zymogen. *Journal of Biological Chemistry* **271**, 28749-28752.
- Tachias, K. and Madison, E. L. (1997). Converting tissue type plasminogen activator into a zymogen. Important role of Lys156. *Journal of Biological Chemistry* **272**, 28-31.
- Takagi, T. and Doolittle, R. F. (1975a). Amino acid sequence studies on plasmin-derived fragments of human fibrinogen. Amino-terminal sequences of intermediate and terminal fragments. *Biochemistry* **14**, 940-946.
- Takagi, T. and Doolittle, R. F. (1975b). Amino acid sequence studies on the alpha chain of human fibrinogen. Location of four plasmin attack points and a covalent cross-linking site. *Biochemistry* **14**, 5149-5156.
- Tappy, L., Hauert, J., and Bachmann, F. (1984). Effects of hypoxia and acidosis on vascular

- plasminogen activator release in the pig ear perfusion system. *Thrombosis Research* **33**, 117-122.
- Thewes, T., Constantine, K., Byeon, I. J. L., and Llinas, M. (1990). Ligand interactions with the kringle 5 domain of plasminogen: a study by H NMR spectroscopy. *Journal of Biological Chemistry* **265**, 3906-3915.
- Thorsen, S. (1992). The mechanism of plasminogen activation and the variability of the fibrin effector during tissue-type plasminogen activator-mediated fibrinolysis. *Annals of the New York Academy of Sciences* 52-63.
- Thorsen, S., Philips, M., Selmer, J., Lecander, I., and Astedt, B. (1988). Kinetics of inhibition of tissue-type and urokinase-type plasminogen activator by plasminogen-activator inhibitor type 1 and type 2. *European Journal of Biochemistry* **175**, 33-39.
- Toschi, L., Bringmann, P., Petri, T., Donner, P., and Schleuning, W. D. (1998). Fibrin selectivity of the isolated protease domains of tissue-type and vampire bat salivary gland plasminogen activators. *European Journal of Biochemistry* **252**, 108-112.
- Tulinsky, A. (1991). The structures of domains of blood proteins. *Thrombosis and Haemostasis* **66**, 16-31.
- Urano, S., Metzger, A. R., and Castellino, F. J. (1989). Plasmin-mediated fibrinolysis by variant recombinant tissue plasminogen activators. *Proceedings of the National Academy of Sciences:USA* **86**, 2568-2571.
- van Zonneveld, A. J., Veerman, H., and Pannekoek, H. (1986a). Autonomous functions of structural domains on human tissue-type plasminogen activator. *Proceedings of the National Academy of Sciences:USA* **83**, 4670-4674.

- van Zonneveld, A. J., Veerman, H., and Pannekoek, H. (1986b). On the interaction of the finger and the kringle-2 domain of tissue-type plasminogen activator with fibrin. Inhibition of kringle-2 binding to fibrin by epsilon-amino caproic acid. *Journal of Biological Chemistry* **261**, 14214-14218.
- Varadi, A. and Patthy, L. (1983). Location of plasminogen-binding sites in human fibrin(ogen). *Biochemistry* **22**, 2440-2446.
- Varadi, A. and Patthy, L. (1984). Beta(Leu121-Lys122) segment of fibrinogen is in a region essential for plasminogen binding by fibrin fragment E. *Biochemistry* **23**, 2108-2112.
- Vaughan, D. E., Goldhaber, S. Z., Kim, J., and Loscalzo, J. (1987). Recombinant tissue plasminogen activator in patients with pulmonary embolism: correlation of fibrinolytic specificity and efficacy. *Circulation* **75**, 1200-1203.
- Veklich, Y., Francis, C. W., White, J., and Weisel, J. W. (1998). Structural studies of fibrinolysis by electron microscopy. *Blood* **92**, 4721-4729.
- Verheijen, J. H., Nieuwenhuizen, W., and Wijngaards, G. (1982). Activation of plasminogen by tissue activator is increased specifically in the presence of certain soluble fibrin(ogen) fragments. *Thrombosis Research* **27**, 377-385.
- Verheijen, J. H., Caspers, M. P. M., Chang, G. T. G., de Munk, G. A. W., Pouwels, P. H., and Enger-Valk, B. E. (1986). Involvement of finger domain and kringle 2 domain of tissue-type plasminogen activator in fibrin binding and stimulation of activity by fibrin. *EMBO Journal* **5**, 3525-3530.
- Verstraete, M., Lijnen, H. R., and Collen, D. (1995). Thrombolytic agents in development. *Drugs* **50**, 29-42.

- von Clauss, A. (1957). Gerinnungsphysiologische Schnellmethode zur Bestimmung des Fibrinogens. *Acta Haematol.* **17**, 237.
- Walker, J. B. and Nesheim, M. E. (1999). The molecular weights, mass distribution, chain composition, and structure of soluble fibrin degradation products released from a fibrin clot perfused with plasmin. *Journal of Biological Chemistry* **274**, 5201-5212.
- Wang, D., Bode, W., and Huber, R. (1985). Bovine chymotrypsinogen: X-ray crystal structure analysis and refinement of a new crystal form at 1.8 Å resolution. *Journal of Molecular Biology* **185**, 595-624.
- Wang, W., Hendriks, D. F., and Scharpé, S. S. (1994). Carboxypeptidase U, a plasma carboxypeptidase with high affinity for plasminogen. *Journal of Biological Chemistry* **269**, 15937-15944.
- Wang, W., Boffa, M. B., Bajzar, L., Walker, J. B., and Nesheim, M. E. (1998). A study of the mechanism of inhibition of fibrinolysis by activated thrombin-activable fibrinolysis inhibitor. *Journal of Biological Chemistry* **273**, 27176-27181.
- Wang, X., Lin, X., Loy, J. A., Tang, J., and Zhang, X. C. (1998). Crystal structure of the catalytic domain of human plasmin complexed with streptokinase. *Science* **281**, 1662-1665.
- Warshawsky, I., Bu, G., and Schwartz, A. L. (1993). 39-kD protein inhibits tissue-type plasminogen activator clearance in vivo. *Journal of Clinical Investigation* **92**, 937-942.
- Weisel, J. W., Stauffacher, C. V., Bullitt, E., and Cohen, C. (1985). A model for fibrinogen: domains and sequence. *Science* **230**, 1388-1391.

- Weisel, J. W. and Papsun, D. M. (1987). Involvement of the COOH-terminal portion of the alpha-chain of fibrin in the branching of fibers to form a clot. *Thrombosis Research* **47**, 155-163.
- Weisel, J. W., Veklich, Y., and Gorkun, O. (1993). The sequence of cleavage of fibrinopeptides from fibrinogen is important for protofibril formation and enhancement of lateral aggregation in fibrin clots. *Journal of Molecular Biology* **232**, 285-297.
- Weisel, J. W., Nagaswami, C., Korsholm, B., Petersen, L. C., and Suenson, E. (1994). Interactions of plasminogen with polymerizing fibrin and its derivatives, monitored with a photoaffinity cross-linker and electron microscopy. *Journal of Molecular Biology* **235**, 1117-1135.
- Weitz, J. I. (1995). Limited fibrin specificity of tissue-type plasminogen activator and its potential link to bleeding. *J.Vasc.Interv.Radiol.* **6**, 19S-23S.
- Weitz, J. I., Koehn, J. A., Canfield, R. E., Landman, S. L., and Friedman, R. (1986). Development of a radioimmunoassay for the fibrinogen-derived peptide B β 1-42. *Blood* **67**, 1014-1022.
- Weitz, J. I., Leslie, B., and Ginsberg, J. (1991). Soluble fibrin degradation products potentiate tissue plasminogen activator-induced fibrinogen proteolysis. *Journal of Clinical Investigation* **87**, 1082-1090.
- Weitz, J. I., Leslie, B., Hirsh, J., and Klement, P. (1993). Alpha 2-antiplasmin supplementation inhibits tissue plasminogen activator-induced fibrinogenolysis and bleeding with little effect on thrombolysis. *Journal of Clinical Investigation* **91**, 1343-1350.

- Weitz, J. I., Stewart, R. J., and Fredenburgh, J. C. (1999). Mechanism of action of plasminogen activators. *Thrombosis and Haemostasis* **82**, 974-982.
- Werz, W. and Werner, R. G. (1998). Recombinant tissue plasminogen activator (rt-PA) in fibrinolytic therapy. *Journal of Biotechnology* **61**, 157-158.
- Whitaker, A. N., Rowe, E. A., Masci, P. P., and Gaffney, P. J. (1980). Identification of D dimer-E complex in disseminated intravascular coagulation. *Thrombosis Research* **18**, 453-459.
- White, H. D. and Van de Werf, F. J. (1998). Thrombolysis for acute myocardial infarction. *Circulation* **97**, 1632-1646.
- Wilhelm, O. G., Jaskunas, S. R., Vlahos, C. J., and Bang, N. U. (1990). Functional properties of the recombinant kringle-2 domain of tissue plasminogen activator produced in *escherichia coli*. *Journal of Biological Chemistry* **265**, 14606-14611.
- Wiman, B. and Collen, D. (1978). On the kinetics of the reaction between human antiplasmin and plasmin. *European Journal of Biochemistry* **84**, 573-578.
- Wiman, B., Lijnen, H. R., and Collen, D. (1979). On the specific interaction between the lysine-binding sites in plasmin and complementary sites in alpha2-antiplasmin and in fibrinogen. *Biochimica et Biophysica Acta* **579**, 142-154.
- Witt, W., Baldus, B., Bringmann, P., Cashion, L., Donner, P., and Schleuning, W. D. (1992). Thrombolytic properties of *desmodus rotundus* (vampire bat) salivary plasminogen activator in experimental pulmonary embolism in rats. *Blood* **79**, 1213-1217.
- Wittwer, A. J., Howard, S. C., Carr, L. S., Harakas, N. K., Feder, J., Parekh, R. B., Rudd, P. M., Dwek, R. A., and Rademacher, T. W. (1989). Effects of N-glycosylation on in

vitro activity of bowes melanoma and human colon fibroblast derived tissue plasminogen activator. *Biochemistry* **28**, 7662-7669.

Wu, T. P., Padmanabhan, K., Tulinsky, A., and Mulichak, A. M. (1991). The refined structure of the epsilon aminocaproic acid complex of human plasminogen kringle 4. *Biochemistry* **30**, 10589-10594.

Yahara, H., Matsumoto, K., Maruyama, H., Nagaoka, T., Ikenaka, Y., Yajima, K., Fukao, H., Ueshima, S., and Matsuo, O. (1992). Recombinant variants of tissue-type plasminogen activator containing amino acid substitutions in the finger domain. *Thrombosis and Haemostasis* **68**, 672-677.

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Peer-reviewed Publications

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Stewart RJ, Fredenburgh JC, Leslie BA, Keyt, BA, Rischke JA, Weitz JI. Identification of the mechanism responsible for the increased fibrin specificity of TNK-tissue plasminogen activator relative to tissue plasminogen activator. J Biol Chem, 2000; 275:10112-10120.

Weitz JI, Stewart RJ, Fredenburgh JC. Fundamental aspects of how thrombolytics work. Thromb Haem, 1999; 82:974-982.

Stewart RJ, Fredenburgh JC, Weitz JI. Characterization of the interactions of plasminogen and tissue and vampire bat plasminogen activators with fibrinogen, fibrin, and (DD)E. J Biol Chem, 1998; 273:18292-18299.

Submitted Manuscripts

Lee AYY, Fredenburgh JC, Stewart RJ, Rischke JA, Weitz JI. The profibrinolytic soluble fibrin degradation product (DD)E protects plasmin from inhibition by α_2 -antiplasmin. Thromb Haem, 2000; submitted.

Abstracts

Stewart RJ, Leslie BA, Fredenburgh JC, Weitz JI. Identification of the mechanism responsible for the decreased fibrin-specificity of reteplase relative to tissue-type plasminogen activator. Blood, 2000; in press.

Stewart RJ, Fredenburgh JC, Leslie BA, Rischke JA, Keyt BA, Weitz JI. The glycosylation site within kringle 1 modulates the fibrin-specificity of TNK-t-PA. Blood, 1999; 94(Suppl. 1, part 1 of 2):232A.

Leslie B, Vasan H, Kremer S, Stewart RJ, Weitz JI. Impact of size-restricted diffusion of thrombin inhibitors into thrombi on the susceptibility of fibrin-bound thrombin to inactivation. Blood, 1999; 94(Suppl. 1, part 1 of 2): 22A.

Stewart RJ, Fredenburgh JC, Leslie BA, Rischke JA, Keyt BA, Weitz JI. Evidence that change in the kringle-1 glycosylation site contributes to the increased fibrin-specificity of TNK-t-PA relative to t-PA. Thromb Haem, 1999; Supplement: pp.708.

Stewart RJ, Fredenburgh, JC, Rischke JA, Weitz JI. Carboxypeptidase B reduces the affinity of (DD)E for t-PA: a potential role for TAFI in limiting systemic plasmin generation. Blood, 1998; 92(10 Suppl. 1, part 1 of 2): 39A.

Stewart RJ, Fredenburgh JC, Lee AYY, Rischke JA, Weitz JI. Functional comparison of (DD)E with fibrin. Blood Coagulation and Fibrinolysis, 1998; 9(7):691-692.

Stewart RJ, Fredenburgh JC, Keyt BA, Weitz JI. The fibrin-specificities of tissue-type plasminogen activator and, to a lesser extent, the TNK variant are compromised by kringle-dependent interactions with (DD)E. Blood, 1997; 90(10 Suppl. 1, part 1 of 2): 144A.

Lee AYY, Fredenburgh JC, Rischke J, Stewart RJ, Weitz JI. Plasmin generated on the (DD)E surface is protected from inactivation by antiplasmin. Thromb Haem 1997; Supplement: pp. 358(PS-1465).

Stewart RJ, Fredenburgh JC, Leslie BA, Keyt BA, Weitz JI. The fibrin-specificity of plasminogen activators is compromised by their interactions with (DD)E. Thromb Haem 1997; Supplement: pp. 193(OC-779).

Stewart RJ, Fredenburgh JC, Leslie BA, Rischke JA, Weitz JI. Importance of (DD)E in limiting the fibrin-specificity of plasminogen activators. Blood 1996; 88(10 Suppl. 1, part 1 of 2): 521A.

Stewart RJ, Fredenburgh JC, Weitz JI. Characterization of the interactions of lys-plasminogen and tissue and vampire bat plasminogen activators with fibrin and (DD)E. Blood 1995; 86(10 Suppl. 1): 287A.

Stewart RJ, Fredenburgh JC, Weitz, JI. Characterization of tissue and vampire bat plasminogen activator and lys-plasminogen binding to fibrin(ogen). Can J Cardiol 1995; 11(Suppl. E):108E.

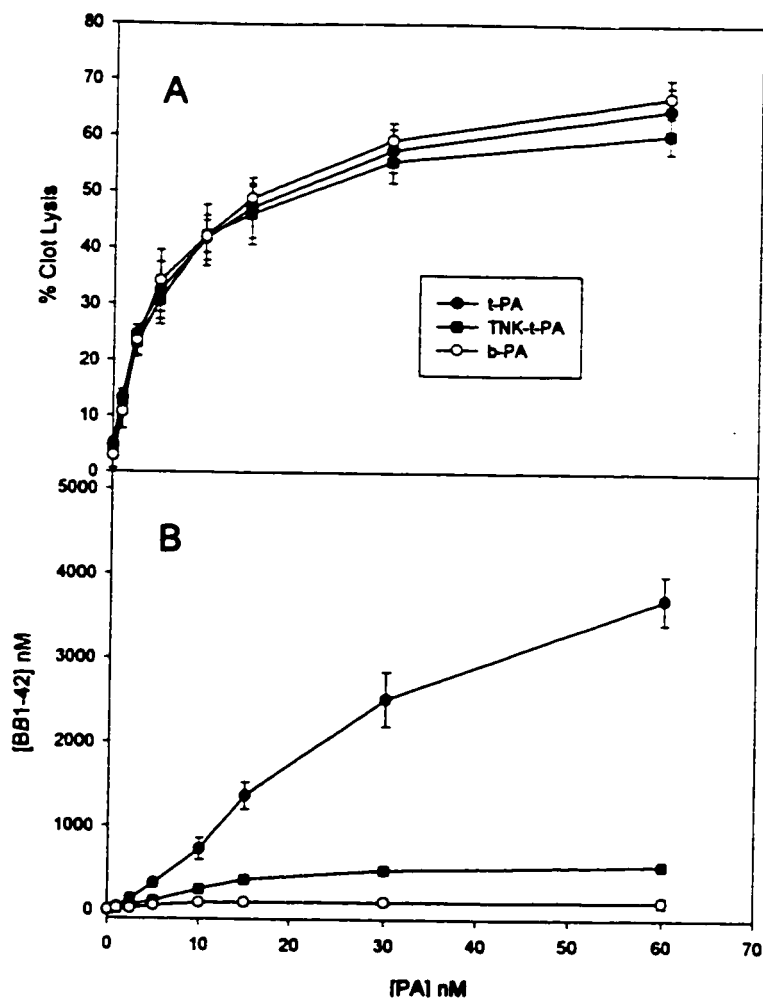
Bajzar L, Stewart RJ, Nesheim ME. The effect of fibrin on the activation of thrombin by antithrombin III in the presence and absence of heparin. FASEB 1994; 8(7):A1376.

Effector	t-PA		TNK-t-PA		b-PA	
	Rate ^a	Stimulation ^b	Rate	Stimulation	Rate	Stimulation
	A_{405}/min	fold	A_{405}/min	fold	A_{405}/min	fold
No Cofactor	0.025	—	0.013	—	0.0065	—
Fibrin	0.095	3.8	0.065	5	0.046	7
(DD)E	0.1	4	0.027	2.1	0.0078	1.2

^aRate of change of absorbance measured at 405 nm.

^bStimulation (fold) was calculated by dividing the rate of absorbance change in the presence of fibrin or (DD)E by that in the absence of a cofactor.

Appendix 1. Effect of fibrin or (DD)E on the amidolytic activity of t-PA, TNK-t-PA or b-PA. To determine if fibrin or (DD)E affect the activity of t-PA, TNK-t-PA or b-PA against small substrates, 5 nM Pg activator and 2 mM Chromozyme t-PA was incubated in the absence or presence of 1 μM Fg, clotted with 5 nM thrombin, or 1 μM (DD)E. Absorbance at 405 nm was monitored for 20 min. In the case of fibrin, absorbance readings were corrected for cleavage of the substrate by thrombin. Rates of substrate hydrolysis were calculated by dividing the change in absorbance by time for the linear portions of the plots of A_{405} versus time. Fibrin and (DD)E increase the amidolytic activity of t-PA to a similar extent. In contrast, fibrin increases the rate of chromogenic substrate hydrolysis by TNK-t-PA or b-PA more than (DD)E. Similar results were obtained using 4 mM S-2288 in place of Chromozyme t-PA (data not shown).



Appendix 2. Comparison of t-PA-, TNK-t-PA- or b-PA-induced fibrin(ogen)olysis. t-PA, TNK-t-PA or b-PA, at the concentrations indicated, was incubated in plasma for 60 min at 37 °C in the presence of 125 -I-labeled plasma clots. Residual thrombus radioactivity was used as a measure of clot lysis (A) and B β 1-42 levels in the bathing plasma were used as an index of fibrinogenolysis (B). Each point represents the mean \pm S.E. of at least six measurements. At each concentration, t-PA, TNK-t-PA, and b-PA produce equivalent clot lysis. In contrast, t-PA generates the most B β 1-42 and b-PA generates the least. By these criteria, b-PA is the most fibrin-specific and t-PA is the least fibrin-specific of the three Pg activators.

Effector	k_{cat}/K_M		
	t-PA	TNK-t-PA	b-PA
		$\mu\text{M}^{-1}\text{s}^{-1}$	
No Cofactor	9.0×10^{-4}	8.7×10^{-5}	1.0×10^{-5}
Fibrin	0.31	0.19	0.16
(DD)E	0.31	0.02	0.00022
Fibrin / (DD)E ^a	1	9.5	730

^aRelative catalytic efficiency of Pg activation was calculated by dividing the catalytic efficiency in the presence of fibrin by that in the presence of (DD)E.

Appendix 3. Effect of fibrin or (DD)E on Glu-Pg activation by t-PA, TNK-t-PA or b-PA. The effect of fibrin or (DD)E on the catalytic efficiency (k_{cat}/K_M) of Glu-Pg activation by t-PA, TNK-t-PA or b-PA was determined by monitoring plasmin formation, using the plasmin-directed substrate S-2251, in the presence of a fixed concentration of Pg activator and various amounts of Glu-Pg and fibrin or (DD)E as described in section 2.2.3i. (DD)E stimulates Glu-Pg activation by t-PA to the same extent as fibrin. In contrast, the catalytic efficiency of Glu-Pg activation by TNK-t-PA or b-PA is lower in the presence of (DD)E compared with fibrin. These differences explain why TNK-t-PA or b-PA is more fibrin-specific than t-PA.