Krause, K. L., Voltz, K. W., & Lipscomb, W. N. (1987) *J. Mol. Biol. 193*, 527-553.
Ladjimi, M. M., & Kantrowitz, E. R. (1987) *J. Biol. Chem. 262*, 312-318.
Ladjimi, M. M., Ghellis, C., Feller, A., Cunin, R., Glansdorff, N., Pierard, A., & Hervê, G. (1985) *J. Mol. Biol. 186*, 715-724.
London, R. E., & Schmidt, P. G. (1972) *Biochemistry 11*, 3136-3142.
London, R. E., & Schmidt, P. G. (1974) *Biochemistry 13*, 1170-1179.
Matsumoto, S., & Hammes, G. G. (1973) *Biochemistry 12*, 1388-1394.
Pastra-Landis, S. C., Foote, J., & Kantrowitz, E. R. (1981) *Anal. Biochem. 118*, 358-363.
Porter, R. W., Modebe, M. O., & Stark, G. R. (1969) *J. Biol. Chem. 244*, 1846-1859.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660-672.
Suter, P., & Rosenbusch, J. P. (1976) *J. Biol. Chem. 251*, 5986-5991.
Suter, P., & Rosenbusch, J. P. (1977) *J. Biol. Chem. 252*, 8136-8141.
Thiry, L., & Hervê, G. (1978) *J. Mol. Biol. 125*, 515-539.
Tondre, C., & Hammes, G. G. (1974) *Biochemistry 13*, 3131-3136.
Voltz, K. W., Krause, K. L., & Lipscomb, W. N. (1986) *Biochem. Biophys. Res. Commun. 136*, 822-826.
Wild, J. R., Loughrey-Chen, S. J., & Corder, T. S. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 46-50.
Winlund, C. C., & Chamberlin, M. J. (1970) *Biochem. Biophys. Res. Commun. 40*, 43-49.
Wu, C. W., & Hammes, G. G. (1973) *Biochemistry 12*, 1400-1408.
Yates, R. A., & Pardee, A. B. (1956) *J. Biol. Chem. 221*, 757-770.
Zhang, Y., Ladjimi, M. M., & Kantrowitz, E. R. (1988) *J. Biol. Chem. 263*, 1320-1324.
Zoller, M. J., & Smith, M. (1982) *Nucleic Acids Res. 10*, 6487-6500.

Binding of Tissue-Type Plasminogen Activator to Lysine, Lysine Analogues, and Fibrin Fragments

G. A. W. de Munk,[‡] M. P. M. Caspers,[§] G. T. G. Chang,[‡] P. H. Pouwels,[§] B. E. Enger-Valk,[§] and J. H. Verheijen*[,‡]
Gaubius Institute TNO and Medical Biological Laboratory TNO, P.O. Box 612, 2300 AP Leiden, The Netherlands
Received January 5, 1989; Revised Manuscript Received May 12, 1989

ABSTRACT: Human tissue-type plasminogen activator (t-PA) consists of five domains designated (starting from the N-terminus) finger, growth factor, kringle 1, kringle 2, and protease. The binding of t-PA to lysine–Sepharose and aminohexyl-Sepharose was found to require kringle 2. The affinity for binding the lysine derivatives 6-aminohexanoic acid and *N*-acetyllysine methyl ester was about equal, suggesting that t-PA does not prefer C-terminal lysine residues for binding. Intact t-PA and a variant consisting only of kringle 2 and protease domains were found to bind to fibrin fragment FCB-2, the very fragment that also binds plasminogen and acts as a stimulator of t-PA-catalyzed plasminogen activation. In both cases, binding could completely be inhibited by 6-aminohexanoic acid, pointing to the involvement of a lysine binding site in this interaction. Furthermore, the second site in t-PA involved in interaction with fibrin, presumably the finger, appears to interact with a part of fibrin, different from FCB-2.

Polymerized fibrin forms the network that keeps a blood clot together. After fibrin has fulfilled its function, it is degraded to soluble products by the relatively nonspecific serine protease plasmin. Plasmin is formed from its inactive zymogen plasminogen by plasminogen activators among which tissue-type plasminogen activator (t-PA)[1] has a prominent role. Plasminogen activation by t-PA is virtually confined to the fibrin surface by specific binding of t-PA to fibrin and the very low activity of t-PA that is not bound to fibrin (Rånby & Wallén, 1985; Thorsen et al., 1972; Hoylaerts et al., 1982). Upon binding to fibrin, plasminogen activation by t-PA is considerably accelerated. The formation of a ternary complex between fibrin, plasminogen, and t-PA appears to constitute the basis of the mechanism of acceleration of t-PA-catalyzed plasminogen activation by fibrin (Hoylaerts et al., 1982; Rånby, 1982). The involvement of lysine residues in fibrin in this stimulation was suggested by Radcliffe (1983). During the last few years, it has become clear that several enzymatically or chemically derived fragments of fibrin(ogen), such as D and FCB-2, retain a large part of the ability to accelerate t-PA-catalyzed plasminogen activation whereas other fragments, such as E, have no such effect (Verheijen et al., 1982a,b; Nieuwenhuizen et al., 1983a,b).

Fibrin fragment FCB-2, the most effective fragment for acceleration of t-PA, was shown to be able to bind t-PA and plasminogen and seems to be a good candidate for the fibrin site involved in formation of the ternary complex (Bosma et al., 1988). The involvement of lysine residue Aα 157 in this acceleration was shown in a series of elegant experiments using synthetic peptides (Voskuilen et al., 1987).

In the last few years, much knowledge has been gained concerning the structure and properties of t-PA. The cDNA

* Address correspondence to this author.
‡ Gaubius Institute TNO.
§ Medical Biology Laboratory TNO.

[1] Abbreviations: t-PA, tissue-type plasminogen activator; mt-PA, melanoma cell derived t-PA; rt-PA, recombinant t-PA; ϵACA, 6-aminohexanoic acid; CHO, Chinese hamster ovary.

0006-2960/89/0428-7318$01.50/0

Table I: Plasmids and Expression Products[a]

plasmid	product	residues deleted in mature protein compared to melanoma	no. of amino acids in mature protein	calcd mol wt of mature protein	sp act. (IU/pmol)
pEV1-t-PA pEV2-t-PA	FGK_1K_2P or rt-PA		527	59039	23.5
pEV1-t-PA2	k_1K_2P	8–145	389	43445	23.5
pEV1-t-PA3	K_2P	7–168	365	40579	32.7
pEV1-t-PA4	GK_1K_2P	5–44	487	54133	20.6
pEV1-t-PA5	P	8–254	280	31391	0.41
pEV2-t-PA6	FGK_1k_2P	207, 210–253	482	53948	3.38
pEV2-t-PA11	FP	47–261	312	35343	1.23

[a] For details of plasmid construction and sequences, see Materials and Methods and Figure 1. The products are schematically indicated according to the domains they have retained. Domain names F, G, K_1, K_2, and P are according to Patthy (1985). k_1 and k_2 respectively indicate K_1 and K_2 with parts of their sequence deleted. Amino acid numbering is according to Ny et al. (1984). It has been assumed that normal processing occurs and that mature proteins start at residue 1. Molecular weights are calculated excluding the carbohydrate part of the molecule. Variants K_2P, GK_1K_2P, and P as well as rt-PA have been described before (Verheijen et al., 1986). Specific activities were determined as described under Materials and Methods; for melanoma t-PA, a specific activity of 30 IU/pmol was found.

and genomic DNA were cloned (Pennica et al., 1983; Ny et al., 1984; Browne et al., 1985), and the predicted amino acid sequence of the protein was confirmed by protein chemistry (Pohl et al., 1984). Several regions with similarity to regions of other proteins were identified within the t-PA molecule: a protease part (P) similar to trypsin; two kringles (K_1, K_2) similar to structures found in plasminogen, prothrombin, factor XII, urokinase, and apolipoprotein (a); a region similar to epidermal growth factor (G); and a region with similarity to the finger structures in fibronectin (F) (Pennica et al., 1983; Ny et al., 1984; Bányai et al., 1983; Patthy, 1985; McLean et al., 1987). Fibrin binding and acceleration of activity by fibrin require the presence of the N-terminal heavy or A chain of t-PA, comprising the F, G, K_1, and K_2 regions (Rijken & Groeneveld, 1986; Dodd et al., 1986). Using deletion mutant proteins prepared by expression of plasmids containing portions of the coding sequence for t-PA, it was shown that both the F and K_2 domains are involved in binding to fibrin and that a variant enzyme consisting only of K_2 and P was still stimulated in activity by fibrinogen fragments (Van Zonneveld et al., 1986a; Verheijen et al., 1986). A lysine binding site was found in K_2 of t-PA, and its involvement in stimulation of t-PA activity by fibrin has been suggested (Ichinose et al., 1986; Van Zonneveld et al., 1986b).

In this paper, we have further studied the lysine binding of t-PA using immobilized and soluble lysine analogues and compared these properties with binding to fibrin fragments.

Materials and Methods

Construction of Plasmids. The construction of plasmids pEV1-t-PA, pEV1-t-PA3, pEV1-t-PA4, and pEV1-t-PA5 coding for respectively rt-PA (FGK_1K_2P), K_2P, GK_1K_2P, and P (see Table I for explanation of nomenclature and structure of proteins) has been described previously (Verheijen et al., 1986).

Plasmid pEV1-t-PA2 coding for variant k_1K_2P was constructed as follows. Partial *Pst*I digestion of p-t-PA 8FL (Van Zonneveld et al., 1986c) and religation of the partials resulted in p-t-PA2 lacking a 414 bp *Pst*I fragment within the coding region of the mature t-PA. A 1416 bp *Bgl*II fragment of p-t-PA2 was inserted into the *Bgl*II site of pEV1-t-PA (Verheijen et al., 1986), resulting in a plasmid pEV1-t-PA2 coding for amino acid residues −35 through 7 and 146–527 (Table I).

The following strategy was used for the construction of plasmid pEV1-t-PA6 coding for variant FGK_1k_2P. Plasmid pEV1-t-PA was digested with *Sca*I, ligated to phosphorylated *Eco*RI–*Sca*I adapters (5′AATTCCATTCTAGAGT3′, 5′ACTCTAGAATGG3′) to convert the *Sca*I site to an *Eco*RI site, and digested with *Eco*RI. From this mixture, a 321 bp *Eco*RI fragment, coding for t-PA amino acid residues 205, 206, 208, 209, and 254–362, was isolated. A 4668 bp fragment, containing the coding sequence for t-PA amino acid residues 363–527, the non-t-PA coding pEV1 vector sequences, and the coding sequences for t-PA amino acid residues −35 through 204, was isolated from partially *Eco*RI-digested pEV1-t-PA and dephosphorylated with bovine alkaline phosphatase. Ligation of the 321 bp fragment with the 4668 bp fragment results in pEV1-t-PA6 coding for the residues −35 through 206, 208, 209, and 254–527 of t-PA (Table I).

Plasmid pEV2-t-PA, having a rabbit β-globin poly(A) signal instead of the SV40 splice and poly(A) signal present in pEV1-derived plasmids, was constructed as follows. Plasmid pEV1-t-PA was digested with *Bam*HI followed by partial digestion with *Bgl*II. A 4292 bp *Bam*HI–*Bgl*II fragment was isolated and ligated to a 443 bp *Xho*II fragment of pSVMT328A+ containing the rabbit β-globin poly(A) signal (a gift of Dr. E. Swarthof, Erasmus University, Rotterdam). The resulting plasmid pEV2-t-PA (4735 bp) contains the SV40 early promoter, the t-PA coding sequence, part of the 3′-noncoding sequence, the rabbit β-globin poly(A) signal, and the pBR sequences for selection and replication in *Escherichia coli* (Figure 1).

From this plasmid, pEV2-t-PA11, coding for variant FP (Table I), was constructed as follows. Plasmid pEV2-t-PA was digested with *Dra*III and treated with bovine alkaline phosphatase. A 3684 bp fragment, containing the coding sequence for t-PA amino acid residues 394–527, the non-t-PA-coding pEV2 vector sequences, and the sequence coding for t-PA amino acid residues −35 through 45, was isolated. Plasmid pEV1-t-PA was digested with *Rsa*I, ligated with phosphorylated *Rsa*I–*Dra*III adaptors (5′GTGTCGACC-TGCGGCCTGAGGCAGT3′, 5′ACTGCCTCAGGCCG-CAGGTCGACACTGA3′) to convert the *Rsa*I site to a *Dra*III site, and digested with *Dra*III. A 400 bp *Dra*III fragment, coding for t-PA amino acid residues 46 and 262–393, was isolated. This fragment ligated to the 3684 bp fragment of pEV2-t-PA results in pEV2-t-PA11 (4090 bp) coding for residues −35 through 46 and 262–527 of t-PA (Table I).

Isolation of Stably Transformed Cells. Since the amount of variant protein that can be isolated from the media of transiently expressing Chinese hamster ovary (CHO) cells is very limited, we have isolated stably transformed cells expressing t-PA or variant proteins. CHO cells (ca. 3×10^6 cells in a 56 cm^2 dish) were cotransfected with 8 μg of one of the t-PA expression plasmids (pEV2-t-PA, pEV1-t-PA2, pEV1-t-PA3, pEV1-t-PA4, pEV1-t-PA5, pEV1-t-PA6, or pEV2-t-

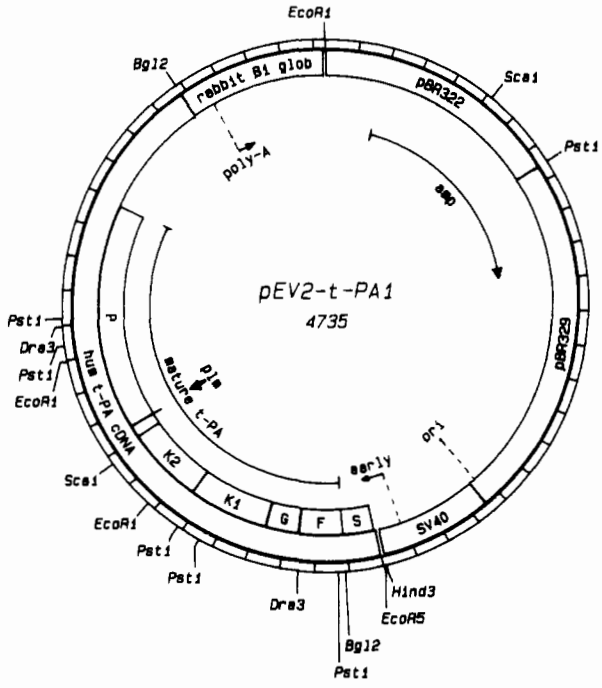

FIGURE 1: Structure of plasmid pEV2-t-PA. The construction of this plasmid is described under Materials and Methods. "Early" stands for the SV40 early promoter; S, F, G, K_1, K_2, and P are the nucleotide sequences coding for signal propeptide, finger, growth factor, two kringles, and the protease domain of t-PA, respectively; poly(A) is the rabbit β-globin polyadenylation signal, and amp and ori are the *E. coli* ampicillin resistance gene and replication origin, respectively. Some restriction sites used for construction of variant plasmids are indicated.

PA11) and 2 μg of pSV3gpt--H+ (a gift of Dr. J. Hoeijmakers, Erasmus University, Rotterdam). Plasmid pSV3gpt--H+ is a derivative of pSV2gpt (Mulligan & Berg, 1981). This plasmid contains the *E. coli gpt* gene coding for the enzyme xanthine–guanine phosphoribosyltransferase conferring resistance to mycophenolic acid. The *gpt* gene is used as a selectable marker for *gpt*-transformed cells which will frequently also contain t-PA expression sequences.

For transfection of CHO cells, the calcium phosphate DNA precipitation method with glycerol shock was used as described before (Verheijen et al., 1986). After 17-h recovery in growth medium (DMEM + 10% FCS), XHATM selection medium (DMEM + 10% FCS, 10 μg/mL xanthine, 15 μg/mL hypoxanthine, 0.2 μg/mL aminopterin, 5 μg/mL thymidine, and 25 μg/mL mycophenolic acid) was applied on the cells for 2–4 days. Confluent cell monolayers were treated with trypsin–EDTA, and 10^4 cells were dispersed on two to four 56 cm^2 dishes in 10 mL of XHATM medium. Medium was refreshed every 3 days. Mycophenolic acid resistant colonies became visible after 7–10 days.

Colonies producing t-PA or variant protein were identified with the fibrin/agarose overlay method (Jones & Benedict, 1975) as follows. Selection medium was removed from the dishes with resistant colonies, and dishes were washed with serum-free medium (DMEM). The cells were overlaid with a sterile fibrin/agarose mixture of 42 °C [final composition 1% (w/v) low-melting agarose, 0.14% (w/v) plasminogen containing bovine fibrinogen, and 0.1 NIH unit/mL thrombin]. Dishes were incubated for 3–4 h at 37 °C in a 5% CO_2 atmosphere. Colonies producing a clear lysis zone in an opaque fibrin/agarose layer were marked. After longer incubation times (4–10 h), almost all colonies are surrounded by lysis zones due to low-level expression of human t-PA or endogenic CHO t-PA. The size of the lysis zones is a measure of the t-PA production level. A control plate with non-t-PA-transformed CHO cells shows the level of background endogenous t-PA production.

The colonies producing high t-PA levels were marked; the fibrin/agarose layer was carefully removed and replaced by XHATM medium for 2–3 days. These colonies were removed from the dish and transferred to a 7 cm^2 dish with 2 mL of XHATM. After 10–14 days of culturing, these colonies yield confluent monolayers. After being washed with phosphate-buffered saline, 1 mL of serum-free DMEM per dish was added and incubated for 17 h at 37 °C. The t-PA or variant production in this period in these media was determined with the spectrophotometric activity assay. The clones with the highest production of t-PA or the different variant proteins were grown on a larger scale (56 cm^2 dish, 15×10^6 cells, 5 mL of serum-free DMEM). In general, media were harvested after 17 h.

Isolation of t-PA Variants. Media were centrifuged for 5 min at 625*g* to remove cells and debris and stored at −20 °C. Media were applied to small columns (0.2 mL) of monoclonal antibody ESP2 (Bioscot, Edinburgh, Scotland) coupled to CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden). This monoclonal binds the P domain (Van Zonneveld et al., 1986b) and can be used for purification of all variant t-PAs having this domain. The column was washed with 10–20 bed volumes of 0.02 mol/L Tris·HCl, pH 7.5, and 0.01% (v/v) Tween 80, 10–20 volumes of the same buffer containing 1 mol/L NaCl, and again with the first buffer, and then the plasminogen activator or variant was eluted with 0.02 mol/L Tris·HCl, pH 7.5, 0.01% (v/v) Tween 80, and 3 mol/L KSCN. Activator-containing fractions were dialyzed against 0.1 mol/L Tris·HCl, pH 7.5, and 0.1% (v/v) Tween 80 and stored at −70 °C until further use. All preparations were in the two-chain form (not shown).

Determination of Specific Activity. The activity of t-PA variants was determined in a coupled photometric plasminogen activation assay in the presence of CNBr-digested fibrinogen (Verheijen, 1982b). Activities were expressed in IU of t-PA using t-PA preparation 83-517 (Gaffney & Curtis, 1985) as a standard. The molar concentration of the variants was determined from their amidolytic activity on the substrate S-2288 (H-D-Ile-Pro-Arg-pNA) (Kabi, Mölndal, Sweden). It has been shown before that the amidolytic activity of different t-PA variants on a molar basis is very similar if they are all in the two-chain form (Rehberg et al., 1989; Bergum & Erickson, 1988). Purified human two-chain melanoma t-PA (specific activity 5×10^5 IU/mg of protein corresponding with 30 IU/pmol) was used as a reference. Amidolytic activity was measured in 0.1 mol/L Tris·HCl, 0.1% v/v Tween 80, and 0.4 mmol/L S-2288 (final volume 0.25 mL) at 37 °C.

Radiolabeling of t-PA and Variants. For labeling of t-PA or variants, an iodinated inhibitor was used. The inhibitor 4-aminobenzoyl-Gly-Arg chloromethyl ketone (a kind gift of Dr. E. Shaw) was iodinated with ^{125}I and purified as described (Rauber et al., 1988). Proteins in 0.1 mol/L Tris·HCl, pH 7.5, and 0.1% v/v Tween 80 were incubated with 2 μmol/L iodinated inhibitor for 4 h at room temperature and overnight at 4 °C. The material was brought on a 1-mL column of zinc chelate–Sepharose and extensively washed with 0.02 mol/L Tris·HCl, pH 7.6, 1 mol/L NaCl, and 0.01% v/v Tween 80, and proteins were eluted with 100 mmol/L imidazole in the same buffer.

Binding of t-PA to Lysine–Sepharose and Aminohexyl-Sepharose: Localization of the Binding Domain. Binding studies were performed as follows: 50 IU of t-PA in 0.1 mol/L

Tris·HCl, pH 7.5, 0.4 mol/L NaCl, and a variable concentration of ϵACA (final volume 0.25 mL) was brought on 75-μL columns of lysine–Sepharose, aminohexyl-Sepharose, or control normal Sepharose. The t-PA present in the flow-through was determined by using the substrate S-2288. Bound t-PA was calculated from the t-PA missing in the flow-through compared with control Sepharose and expressed as percentage of control.

For the determination of the localization of the binding site of t-PA or variants to lysine–Sepharose or aminohexyl-Sepharose, radiolabeled t-PA or variants were used (see above). Columns of 1 mL of lysine–Sepharose (containing 4.5 μmol of lysine/mL swollen gel) or aminohexyl-Sepharose (containing 8 μmol of aminohexane/mL swollen gel) (Pharmacia) were used. Labeled variants of control t-PA (0.06–1 pmol in 1 mL of 0.1 mol/L Tris·HCl, pH 7.5, 0.4 mol/L NaCl, and 0.1% v/v Tween 80) was loaded on the columns, and the flow-through was collected. The columns were washed with 2.5 mL of the same buffer, and finally the columns were eluted with 2.5 mL of 50 mmol/L ϵACA in the same buffer. Radioactivity present in flow-through, wash buffer, elution buffer, and still present on the column was determined in a γ counter. The percentage of radioactivity present in the elution fraction was defined as bound to the column. After elution, columns did not retain more than a few percent of radioactivity.

Determination of the Binding Constant of t-PA for ϵACA and N-Acetyllysine Methyl Ester. Aminohexyl-Sepharose and Sepharose were mixed in variable ratios, giving ligand concentrations ranging from 0 to 4.5 mmol/L total gel, and washed with Tris/Tween buffer [0.1 mol/L Tris·HCl, pH 7.5, and 0.1% (v/v) Tween 80]. Aliquots of 0.10 mL of packed, swollen gel were taken from these mixtures and mixed with 0.03 mL of 4 mol/L NaCl in Tris/Tween, 0, 0.015, or 0.030 mL of either 3 mmol/L ϵACA or 3 mmol/L *N*-acetyllysine methyl ester (Aldrich), 0.10 mL of 50 IU/mL melanoma t-PA (mt-PA), and Tris/Tween buffer, giving a total volume of 0.30 mL. The tubes were shaken for 3 h at 4 °C and centrifuged. Unbound mt-PA present in the supernatant was determined by incubating 0.05-mL aliquots of the supernatant fluid with 0.4 mmol/mL chromogenic t-PA substrate S-2288 (H-D-Ile-Pro-Arg-pNA; Kabi) in 0.25 mL of Tris/Tween buffer at 37 °C. The t-PA bound to the gel for the different mixtures of aminohexyl-Sepharose and Sepharose, and different concentrations of competing ligands (ϵACA or N-acetyllysine methyl ester), was expressed as the fraction of the total amount of t-PA. Total t-PA was defined as the t-PA activity found in the supernatant when only Sepharose and no aminohexyl-Sepharose was added.

Binding of t-PA and Its Variant K_2P to Fibrinogen Fragment FCB-2. PVC microtiter plates (Flow Laboratories, Irvine, Scotland) were coated with rabbit IgGs against fibrin degradation products by incubation of 0.125 mL of a 10 μg/mL solution of the IgGs in coating buffer (50 mmol/L $NaHCO_3$, pH 9.6) overnight at room temperature. The wells were emptied and filled with 0.125 mL of bovine serum albumin (Sigma A7030) in coating buffer (10 mg/mL). After 2-h incubation at room temperature, the wells were washed twice with PET buffer [0.01 mol/L NaH_2PO_4, 0.15 mol/L NaCl, 5 mmol/L EDTA, and 0.1% (v/v) Tween 80, pH 7.4]. In each well, 0.125 mL of a solution of 400 nmol/L fragment FCB-2 in PET buffer with 1% (w/v) bovine serum albumin, or only PET buffer with albumin (in the controls), was incubated for 5 h at room temperature. The wells were washed twice with PET buffer and filled with 0–0.06 mL of mt-PA or variant K_2P (60 IU/mL). To investigate the effect of ϵACA on the binding of t-PA or K_2P, 0.025 mL of 50 mmol/L this compound was added to some wells. The volume was brought to 0.125 mL with PET buffer containing 1% albumin, and the plates were incubated overnight at room temperature. After the plates were washed 5 times with PET buffer, 0.10 mL of 0.7 mmol/L S-2251 (H-D-Val-Leu-Lys-pNA, Kabi) and 0.025 mL of plasminogen (1.5 μmol/L), both in 0.1 mol/L Tris·HCl, pH 7.5, and 0.1% (v/v) Tween 80, were added. The plates were incubated at 25 °C, and the A_{405} was determined after 6 h.

The amount of mt-PA or K_2P bound to FCB-2 was expressed as A_{405} with FCB-2 during coating minus A_{405} with only buffer with albumin during coating.

Fibrin Zymography. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate using nonreducing conditions was performed on 10% gels with 5% stacking gels (Laemmli, 1970). After electrophoresis, the gels were washed sequentially in respectively 2.5% (v/v) Triton X-100 and 0.05 mol/L Tris·HCl/0.1 mol/L NaCl, pH 7.7, to remove the dodecyl sulfate and placed on plasminogen-containing fibrin–agarose layers (Granelli-Piperno & Reich, 1978). Active plasminogen activators result in clear lysis zones on an opaque background.

Fibrin Binding of t-PA and Variant K_2P. Fibrin binding was essentially performed as described before (Verheijen et al., 1986) with some changes: fibrinogen was passed over a lysine–Sepharose column to remove contaminating plasminogen; t-PA or variant K_2P was labeled with ^{125}I-4-aminobenzoyl-Gly-Arg chloromethyl ketone as described above. About 0.06 pmol of labeled t-PA or K_2P was used in a final volume of 0.80 mL containing 0–1.2 mg/mL fibrinogen. Furthermore, Trasylol was added to a final concentration of 500 KIE/mL to prevent any action of traces of plasmin. The experiment was also performed in the presence of 5 mmol/L ϵACA. Clotting was done by addition of thrombin (2 NIH units/mL) and 45-min incubation at 37 °C. After centrifugation, radioactivity was counted in the supernatant.

Miscellaneous Materials. Human melanoma t-PA (mt-PA) in its two-chain form was purified as described previously (Kluft et al., 1983). The specific activity was about 500 000 IU/mg or 30 IU/pmol, using t-PA preparation 83-517 (Gaffney & Curtis, 1985) as a standard. Fibrinogen fragment FCB-2 and rabbit IgG against fibrin degradation products were kindly provided by Dr. Nieuwenhuizen of this institute.

Results

Purification of Variant t-PAs. The P domain specific monoclonal antibody ESP2 was found to bind human t-PA or its variants and not endogenous CHO cell derived t-PA. The bound human material was eluted in KSCN-containing buffers. The purified variant preparations were analyzed by fibrin zymography, no CHO t-PA was detectable, and the variants had approximately the size expected from the sequence of their cDNA (Figure 2, Table I).

Specific Activity of Variant t-PAs. The molar specific activities of variant t-PAs containing an intact K_2 ranged from 20.6 to 32.7 IU/pmol, comparable to the 30 IU/pmol found for mt-PA. The variants lacking a K_2 or having a partially deleted K_2 show a much lower specific activity, although those for FGK_1k_2P and FP are significantly higher than for P (Table I).

Binding of t-PA to Lysine–Sepharose and Aminohexyl-Sepharose. The binding of t-PA to small columns of lysine–Sepharose and aminohexyl-Sepharose was determined in the presence of increasing concentrations of ϵACA. As shown in Figure 3, t-PA was bound to both of these column materials,

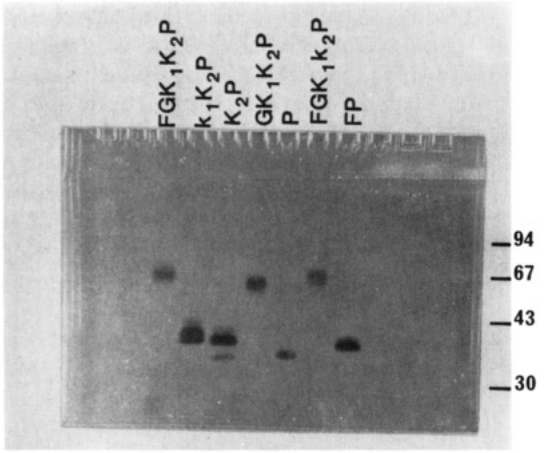

FIGURE 2: Fibrin zymography of rt-PA and variant t-PA's. rt-PA (FGK_1K_2P) and variants (k_1K_2P, K_2P, GK_1K_2P, P, FGK_1k_2P, and FP as indicated) were isolated from media by affinity chromatography on ESP2–Sepharose. Gel electrophoresis and zymography were performed as described under Materials and Methods. Molecular weight marker proteins were run on a parallel gel and stained with Coomassie brilliant blue. Their positions are indicated.

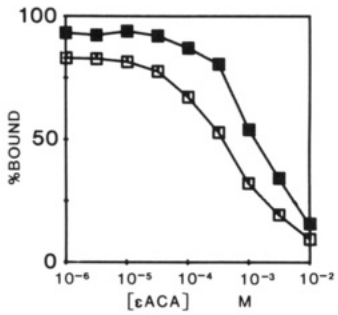

FIGURE 3: Binding of t-PA to lysine–Sepharose and aminohexyl-Sepharose. t-PA was brought on 75-μL columns of lysine–Sepharose (■) or aminohexyl-Sepharose (□) in the presence of increasing concentrations of ϵACA as indicated. The t-PA present in the flow-through was determined, and t-PA bound to the column was calculated from this quantity and expressed as a percentage of the total t-PA determined in control experiments with normal Sepharose (for details, see Materials and Methods).

even in the presence of 0.4 mol/L NaCl to prevent ion-exchange behavior. In both cases, relatively low concentrations of ϵACA interfere with this binding.

Localization of the Binding Site in t-PA for Lysine–Sepharose and Aminohexyl-Sepharose. After it was found that t-PA also binds to aminohexyl-Sepharose and not only to lysine–Sepharose, an experiment was performed to find out whether one or two different domains of t-PA are involved in the interaction with these two materials. In this experiment, a set of deletion mutants of t-PA was used consisting of molecules missing certain domains. It was found (Figure 4) that melanoma t-PA (mt-PA), rt-PA (FGK_1K_2P), and variants GK_1K_2P, k_1K_2P, and K_2P bind considerably better to both materials than variants P, FGK_1k_2P, and FP. These experiments suggest that t-PA binds to both lysine–Sepharose and aminohexyl-Sepharose with the K_2 domain.

Binding of t-PA to Lysine Analogues. The interaction of t-PA with the lysine analogues ϵACA and *N*-acetyllysine methyl ester in solution was studied quantitatively. This was done by determining the binding of t-PA to aminohexyl-Sepharose in the presence of various concentrations of ϵACA or *N*-acetyllysine methyl ester. The interactions can be described by two equilibria:

$$L + T \leftrightarrow TL \quad (1a)$$

$$I + T \leftrightarrow TI \quad (1b)$$

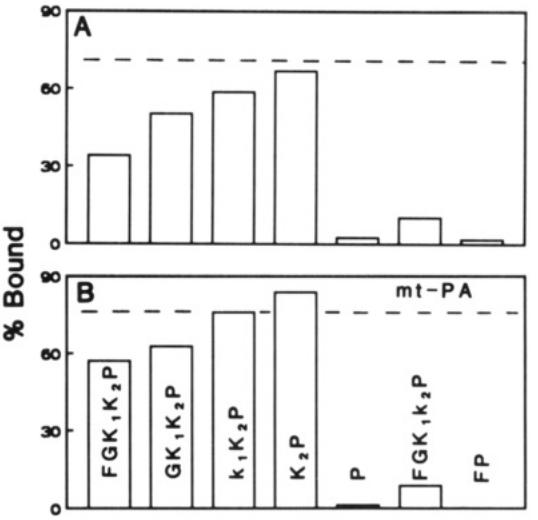

FIGURE 4: Binding of variant t-PA's to lysine–Sepharose and aminohexyl-Sepharose. Radiolabeled variants were brought on 1-mL columns of lysine–Sepharose (panel B) or aminohexyl-Sepharose (panel A). Flow-through was collected; the columns were washed with 2.5 mL of buffer and eluted with 2.5 mL of buffer with 50 mmol/L ϵACA. The radioactivity present in the flow-through, washing buffer, and elution buffer and remaining on the columns was determined. The radioactivity present in the elution fraction expressed as a percentage of the total radioactivity was plotted. For details, see Materials and Methods.

where L is the concentration of free aminohexyl groups on aminohexyl-Sepharose, T is the concentration of t-PA free in solution, TL is the concentration of t-PA bound to aminohexyl-Sepharose, I is the concentration of free competing ligand, i.e., ϵACA or *N*-acetyllysine methyl ester in solution, and TI is the concentration of t-PA bound to ligand in solution. When both the immobilized ligand concentration and the soluble ligand concentration are much higher than the t-PA concentration, the binding can be described by the equations

$$1/b = (1/L_t)K_{app} + 1 \quad (2a)$$

$$K_{app} = K_d(1 + I_t/K_i) \quad (2b)$$

where b is the concentration of t-PA bound to aminohexyl-Sepharose expressed as the fraction of total t-PA, L_t is the concentration of total aminohexyl groups on aminohexyl-Sepharose, K_{app} is the apparent dissociation constant of the t-PA/aminohexyl-Sepharose complex, K_d is the real dissociation constant of the t-PA/aminohexyl-Sepharose complex, I_t is the total concentration of competing ϵACA or *N*-acetyllysine methyl ester, and K_i is the dissociation constant of the t-PA/ϵACA or t-PA/*N*-acetyllysine methyl ester complex. A graph of $1/b$ against $1/L_t$ yields straight lines with slopes equal to K_{app} (Figure 5). When K_{app} at each concentration of soluble ligand is plotted against the soluble ligand concentration, a straight line is found, with the y-axis intercept equal to the binding constant of t-PA to the immobilized ligand, whereas the slope is the ratio of binding constants with immobilized and soluble ligands (Figure 6). From Figure 6, binding constants for ϵACA and *N*-acetyllysine methyl ester with t-PA of 0.07 and 0.08 mmol/L, respectively, are calculated. The binding constant for the interaction of t-PA with the immobilized ligand is, however, 0.8 mmol/L as calculated from the mean y-axis intercepts of the lines in Figure 5.

Binding of t-PA to Fibrin. Binding of t-PA to fibrin involves at least two domains, K_2 and probably F (Van Zonneveld et al., 1986a,b; Verheijen et al., 1986). The binding of t-PA and variant K_2P to fibrin was determined quantitatively both in

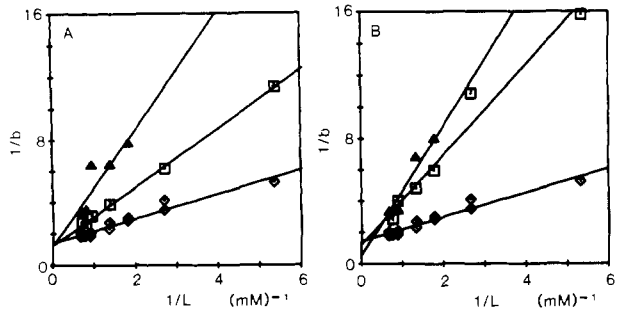

FIGURE 5: Effect of ϵACA and *N*-acetyllysine methyl ester on the binding of t-PA to aminohexyl-Sepharose. Binding of mt-PA to aminohexyl-Sepharose in the presence of 0 (◇), 0.15 (⊡), or 0.30 mmol/L (▲) ϵACA (panel A) or *N*-acetyllysine methyl ester (panel B) was determined. The t-PA bound (*b*) to aminohexyl-Sepharose was expressed as the fraction of total t-PA, and $1/b$ was plotted along the *y* axis. The concentration of Sepharose-bound aminohexyl groups is *L*, and $1/b$ was plotted along the *x* axis. For details, see Materials and Methods and equations under Results.

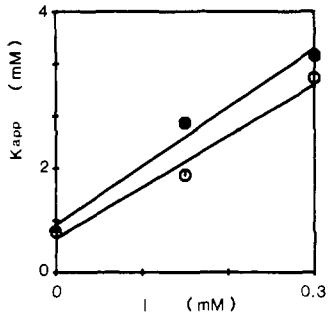

FIGURE 6: Determination of the affinity of t-PA for ϵACA and *N*-acetyllysine methyl ester. From the slopes of the lines in Figure 5, the apparent dissociation constant of t-PA for immobilized aminohexyl groups can be obtained (see eq 2a under Results). Plotting these constants against the concentration of competing soluble ligand I, ϵACA (open symbols) or *N*-acetyllysine methyl ester (closed symbols), gives straight lines from which the dissociation constant for the binding of t-PA to the Sepharose-bound aminohexyl group and to the soluble competing ligands ϵACA and *N*-acetyllysine methyl ester can be obtained (see eq 2b under Results).

the absence and in the presence of ϵACA. It was found (Figure 7) that in the absence of ϵACA both t-PA and K_2P bind to fibrin. In the presence of ϵACA, binding of t-PA is reduced, and binding of K_2P is completely blocked. This result is in accordance with previous findings.

Binding of t-PA and Variant K_2P to Fragment FCB-2. For kinetic studies on plasminogen activation, soluble fragments of fibrin have frequently been used as fibrin mimicks (Cassels et al., 1987; Geiger & Binder, 1984, 1985; Nieuwenhuizen et al., 1988; Zamarron et al., 1984). It has, however, never been shown whether t-PA binds to such fragments in the same way as to intact fibrin. Therefore, we have studied the interaction between t-PA and fibrin fragment FCB-2, known to stimulate t-PA-catalyzed plasminogen activation and to bind both t-PA and plasminogen. A system was used consisting of FCB-2 bound to specific antibodies coated to a plastic multiwell plate. Such an indirect immobilization was preferred to a direct one in which FCB-2 is coated to the plastic to prevent conformational changes caused by binding to plastic as observed for fibrinogen. It was found (Figure 8) that both t-PA and variant K_2P bind to immobilized fragment FCB-2. This binding could completely be prevented by including ϵACA during incubation of the coated plates with the plasminogen activator (Figure 8). This was the case for variant K_2P and remarkably also for intact t-PA. This result is different for binding to a fibrin clot, in which case binding of intact t-PA could not be prevented by ϵACA [Figure 7 and Van Zonneveld et al. (1986b)].

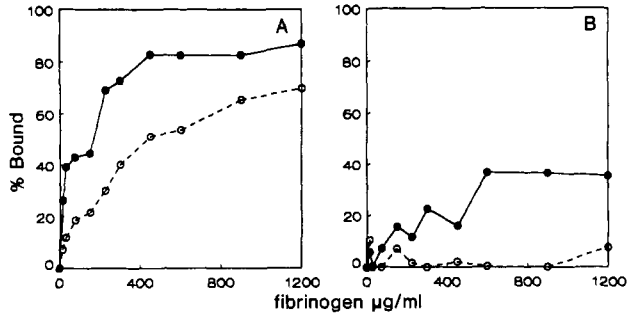

FIGURE 7: Binding of t-PA and variant K_2P to forming fibrin clots in the absence and presence of ϵACA. Mixtures of labeled t-PA (A) or K_2P (B) and variable concentrations of fibrinogen were clotted with thrombin in the absence (●) or presence (○) of 5 mmol/L ϵACA. Clots were centrifuged, and labeled t-PA in the supernatant was determined by using a γ counter. Bound t-PA was calculated from total t-PA, determined in control experiments with fibrinogen or thrombin, and t-PA in the supernatant. For details, see Materials and Methods.

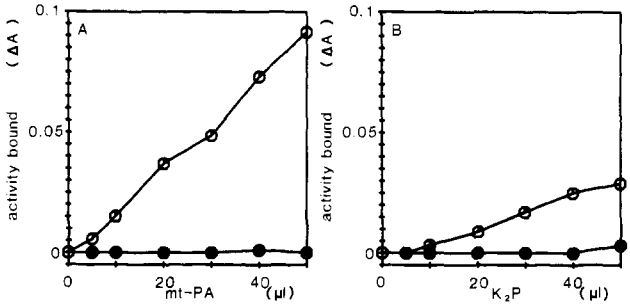

FIGURE 8: Binding of t-PA and variant K_2P to fibrinogen fragment FCB-2. Binding of mt-PA (A) or variant K_2P (B) to immobilized fibrinogen fragments FCB-2 (open symbols) was determined as described under Materials and Methods. Closed symbols represent the binding to FCB-2 in the presence of 5 mmol/L ϵACA.

Discussion

The specific activities of all variants having an intact K_2 are similar to that of t-PA itself whereas those of variants without K_2 or a partially deleted K_2 are much lower (Table I). It is striking that the specific activity of FGK_1k_2P, although low compared with t-PA, is considerably higher than that of P. A comparable phenomenon is seen in the binding experiments to lysine–Sepharose and aminohexyl-Sepharose (Figure 4). Also, in this case, some binding of FGK_1k_2P is observed. This might indicate either that the remaining part of K_2 still has still some contribution to these properties or that another domain, F of K_1, contributes also.

Binding of t-PA and plasminogen to fibrin was demonstrated some time ago (Thorsen et al., 1972; Cederholm-Williams, 1977). The fibrin binding of plasminogen has been thoroughly studied, and the involvement of lysine binding sites in the kringles of plasminogen is established (Castellino, 1988; Nieuwenhuizen, 1988). Different types of lysine binding sites have been discerned, so-called high-affinity lysine binding sites, low-affinity lysine binding sites, and an aminohexyl or AH site requiring only a positive charge (Christensen, 1984).

Affinity for lysine has been described for t-PA also (Radcliffe, 1978), and the presence of a lysine binding site in K_2 has been shown (Van Zonneveld et al., 1986b; Ichinose et al., 1986). We have further analyzed the binding of t-PA to lysine analogues.

It was found that t-PA not only binds to lysine–Sepharose but also binds to aminohexyl-Sepharose. In both cases, ϵACA

interferes with binding (Figure 3). Experiments using deletion variants of t-PA show that for binding to either of these two materials the K_2 domain of t-PA is required (Figure 4), suggesting that the actual binding site or sites might be located in this domain.

The binding of t-PA to the lysine analogues ϵACA and *N*-acetyllysine methyl ester, model compounds for C-terminal and intrachain lysine residues, respectively, was studied quantitatively. These soluble analogues were preferred to Sepharose-bound analogues to exclude effects of the matrix and availability of ligands for proteins. It was found that ϵACA and *N*-acetyllysine methyl ester bind to t-PA with affinities of 0.07 and 0.08 mmol/L, respectively (Figures 5 and 6). These affinities lay between those described for the high- and low-affinity lysine binding sites in plasminogen. A difference with plasminogen is that in the case of t-PA lysine binding and aminohexyl binding are mediated by the same kringle, whereas in plasminogen different kringles appear to be responsible (Castellino, 1988; Christensen, 1984; Nieuwenhuizen, 1988).

Soluble fragments of fibrin(ogen) such as FCB-2 have frequently been used as fibrin mimics in the study of the kinetics and mechanism of t-PA-catalyzed plasminogen activation. It has been shown that fragment FCB-2 stimulates t-PA-catalyzed plasminogen activation (Nieuwenhuizen et al., 1983b) and binds t-PA as well as plasminogen (Bosma et al., 1988). It has not been shown, however, which domains in t-PA are involved in this interaction, either F, K_2, or both. Therefore, the binding of t-PA and variant K_2P to FCB-2 was studied. It was found that both t-PA and K_2P, which has retained only one of the two domains thought to be involved in interaction with fibrin, bind to FCB-2. In this case, binding of both t-PA and K_2P could be completely blocked by ϵACA (Figure 8). Binding of K_2P to FCB-2 appears less efficient than that of t-PA; this might be due to steric effects in this amputated molecule, since the ϵACA effect makes the involvement of another domain in t-PA not likely. Binding of t-PA to fibrin, however, cannot be blocked completely by ϵACA in contrast to that of K_2P (Figure 7). This indicates that K_2 alone is involved in the interaction of t-PA with the FCB-2 part of fibrin, whereas in fibrin two domains, F and K_2 are involved. The F domain likely binds to a part of fibrin different from FCB-2. The kinetics of t-PA-catalyzed plasminogen in the presence of fibrin or derivatives are similar, suggesting that for stimulation of t-PA by fibrin, interaction with the K_2 domain is responsible for the dominant effect. It is dangerous, however, to extrapolate findings in simple model systems using purified components to the lysis of real clots in vivo. A real clot contains many more components than fibrin, and the way fibrin exposes its binding sites for t-PA and/or plasminogen could very well depend on clot structure. A further complication is that this structure changes during clot lysis, leading to changes in kinetics during lysis (Norrman et al., 1985; Suenson et al., 1984; Suenson & Petersen, 1986). The data presented in this paper suggest that t-PA not necessarily binds only to C-terminal lysine residues in fibrin, formed upon limited degradation, but could also bind to certain specific intrachain lysine residues in intact t-PA. This is in agreement with the findings of Bosma et al. (1988) that t-PA does bind to fragment FCB-2 and does not bind to fragment E, despite the fact that the first fragment contains zero and the latter contains six C-terminal lysine residues. Furthermore, it has been shown that upon deliberate generation of C-terminal lysine residues in fibrin by limited plasmic digestion of fibrinogen before clotting, indeed new high-affinity binding sites for t-PA are formed. The number of these sites is, however, much smaller than that of the sites available in undegraded fibrin, and binding to these new sites is not inhibited by ϵACA and so seems not to involve a lysine binding site in t-PA (Higgins & Vehar, 1987). Since a clot made of degraded fibrinogen is different from a fibrin clot which is degraded after clotting, also in this case, extrapolation to a real clot is risky.

During the preparation of this paper, a very interesting paper, describing a new set of t-PA variants, appeared (Gething et al., 1988). In this paper, it is concluded that both K_1 and K_2 contain a lysine binding site and that each one alone is able to mediate stimulation of activity by fibrin. These very interesting results are partly in contrast with our present data and those published before (Verheijen et al., 1986; Van Zonneveld et al., 1986). A major difference between this new set of variants and sets described before and used in this paper is that, indeed, in the latter cases unpaired cysteines could occur, possibly leading to misfolding of the protein as suggested. This is very difficult to verify, however, because of the small quantities of variant proteins available and because of the fact that the theoretical disulfide pattern of t-PA, based on homology with other proteins, has not been confirmed by experimental data yet.

Recently, model building experiments of plasminogen and t-PA kringles, based on the known structure of a prothrombin kringle, have been performed (Tulinsky et al., 1988). These structures indicate that in a kringle only one binding site for a lysine-like compound can be accommodated, leading to the likely conclusion that ϵACA, lysine–Sepharose, aminohexyl-Sepharose, and FCB-2 interact with the same site in K_2 of t-PA. In both t-PA kringles, the two aspartic acid residues thought to form the binding site for the positive charge on lysine are present. This would be consistent with the presence of a lysine binding site in each of these kringles (Gething et al., 1988). However, some structural differences between K_1 and K_2 of t-PA could also be consistent with the lack of a lysine binding site in K_1 (Tulinsky et al., 1988). Interestingly, both t-PA kringles lack the arginine residue thought to bind the negative charge on lysine in plasminogen K_4. This could explain our finding that the affinity of t-PA K_2 for lysine analogues with and without a negative charge is almost equal. The t-PA kringle structure is based on computer modeling using the coordinates of other kringles; therefore, this structure is probably not completely correct, and very detailed conclusions should not be drawn. Further mutagenesis experiments, preferably small changes instead of extensive deletions, could be useful to solve these interesting questions.

References

Bányai, L., Váradim, L., & Patthy, L. (1983) *FEBS Lett. 163*, 37–41.

Bergum, P. W., & Erickson, L. A. (1988) *Enzyme 40*, 122–129.

Bosma, P., Rijken, D. C., & Nieuwenhuizen, W. (1988) *Eur. J. Biochem. 172*, 399–404.

Browne, M. J., Tyrrel, A. W. R., Chapman, C. G., Cavey, J. E., Glover, D. M., Grosveld, F. G., Dodd, I., & Robinson, J. H. (1985) *Thromb. Haemostasis 54*, 422–424.

Cassels, R., Fears, R., & Smith, A. G. (1987) *Biochem. J. 247*, 395–400.

Castellino, F. J. (1988) in *Tissue-type Plasminogen Activator (t-PA). Physiological and Clinical Aspects* (Kluft, C., Ed.) pp 145–170, CRC Press, Boca Raton, FL.

Cederholm-Williams, S. A. (1977) *Thromb. Res. 11*, 421–423.

Christensen, U. (1984) *Biochem. J. 223*, 413–421.

Dodd, I., Fears, R., & Robinson, J. H. (1986) *Thromb. Haemostasis 54*, 94–97.
Erickson, L. A., Bergum, P. W., Hubert, E. V., Theriault, N. Y., Rehberg, E. F., Palermo, D. P., De Munk, G. A. W., Verheijen, J. H., & Marotti, K. R. (1987) *Thromb. Haemostasis 58*, 287.
Gaffney, P. J., & Curtis, A. D. (1985) *Thromb. Haemostasis 53*, 134–136.
Geiger, M., & Binder, B. R. (1984) *J. Biol. Chem. 259*, 2976–2981.
Geiger, M., & Binder, B. R. (1985) *Thromb. Haemostasis 54*, 413–414.
Gething, M. J., Adler, B., Boose, J. A., Gerard, R. D., Madison, E. L., McGookey, D., Meidell, R. S., Roman, L. M., & Sambrook, J. (1988) *EMBO J. 7*, 2731–2740.
Gheysen, D., Lijnen, H. R., Piérard, L., De Foresta, F., Demarsin, E., Jacobs, P., De Wilde, M., Bollen, A., & Collen, D. (1987) *J. Biol. Chem. 262*, 11779–11784.
Granelli-Piperno, A., & Reich, E. (1978) *J. Exp. Med. 148*, 223–234.
Higgins, D. L., & Vehar, G. A. (1987) *Biochemistry 26*, 7786–7791.
Hoylaerts, M., Rijken, D. C., Lijnen, H. R., & Collen, D. (1982) *J. Biol. Chem. 257*, 2912–2919.
Ichinose, A., Takio, K., & Fujikawa, K. (1986) *J. Clin. Invest. 78*, 163–169.
Jones, P., & Benedict, W. (1975) *Cell 5*, 323–329.
Kluft, C., Van Wezel, A. L., Van der Velden, C. A. M., Emeis, J. J., Verheijen, J. H., & Wijngaards, G. (1983) in *Advances in Biotechnological Processes* (Mizrahi, A., & Van Wezel, A. L., Eds.) Vol. 2, pp 97–110, Alan R. Liss, New York.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Larsen, G. R., Henson, K., & Blue, Y. (1988) *J. Biol. Chem. 263*, 1023–1029.
Lijnen, H. R., Van Hoe, B., & Collen, D. (1984) *Eur. J. Biochem. 144*, 541–544.
McLean, J. W., Tomlinson, J. E., Kuang, W. J., Eaton, D. L., Chen, E. Y., Fless, G. M., Scanu, A. M., & Lawn, R. M. (1987) *Nature 330*, 132–137.
Mulligan, R. C., & Berg, P. (1981) *Mol. Cell Biol. 1*, 449–459.
Nieuwenhuizen, W. (1988) in *Tissue-type Plasminogen Activator (t-PA). Physiological and Clinical Aspects* (Kluft, C., Ed.) pp 171–188, CRC Press, Boca Raton, FL.
Nieuwenhuizen, W., Vermond, A., Voskuilen, M., Traas, D. W., & Verheijen, J. H. (1983a) *Biochim. Biophys. Acta 748*, 86–92.
Nieuwenhuizen, W., Verheijen, J. H., Vermond, A., & Chang, G. T. G. (1983b) *Biochim. Biophys. Acta 755*, 531–533.
Nieuwenhuizen, W., Voskuilen, M., Vermond, A., Hoegee-de Nobel, B., & Traas, D. W. (1988) *Eur. J. Biochem. 174*, 163–169.
Norrman, B., Wallén, P., & Ranby, M. (1985) *Eur. J. Biochem. 149*, 193–200.
Ny, T., Elgh, F., & Lund, B. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5355–5359.
Patthy, L. (1985) *Cell 41*, 657–663.
Pennica, D., Holmes, W. E., Kohr, W. J., Harkins, R. N., Vehar, G. A., Ward, C. A., Bennett, W. F., Yelverton, E., Seeburg, P. H., Heyneker, H. L., Goeddel, D. V., & Collen, D. (1983) *Nature 301*, 214–221.
Pohl, G., Kälström, M., Bergsdorf, N., Wallén, P., & Jörnvall, H. (1984) *Biochemistry 23*, 3701–3707.
Radcliffe, R. (1983) *Biochim. Biophys. Acta 743*, 422–430.
Radcliffe, R., & Heinze, T. (1978) *Arch. Biochem. Biophys. 189*, 185–194.
Ranby, M. (1982) *Biochim. Biophys. Acta 704*, 461–469.
Ranby, M., & Wallén, P. (1985) in *Thrombolysis: Biological and Therapeutic Properties of New Thrombolytic Agents* (Collen, D., Lijnen, H. R., & Verstraete, M., Eds.) p 31, Churchill Livingstone, Edinburgh.
Rauber, P., Wikstrom, P., & Shaw, E. (1988) *Anal. Biochem. 168*, 259–264.
Rehberg, E. F., Theriault, N. Y., Carter, J. B., Palermo, D. P., Hubert, E. V., Bergum, P. W., Wunderlich, C. J., Erickson, L. A., & Marotti, K. R. (1989) *Protein Eng. 2*, 371–377.
Rijken, D. C., & Groeneveld, E. (1986) *J. Biol. Chem. 261*, 3098–3102.
Suenson, E., & Petersen, L. C. (1986) *Biochim. Biophys. Acta 870*, 510–519.
Suenson, E., Lützen, O., & Thorsen, S. (1984) *Eur. J. Biochem. 140*, 513–522.
Thorsen, S., Glas-Greenwalt, P., & Strup, T. (1972) *Thromb. Diath. Haemorrh. 28*, 65–74.
Tulinsky, A., Park, C. H., Mao, B., & Llinás, M. (1988) *Proteins 3*, 85–96.
Van Zonneveld, A. J., Veerman, H., & Pannekoek, H. (1986a) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4670–4674.
Van Zonneveld, A. J., Veerman, H., & Pannekoek, H. (1986b) *J. Biol. Chem. 261*, 14214–14218.
Van Zonneveld, A. J., Chang, G. T. G., Van den Berg, J., Kooistra, T., Verheijen, J. H., Pannekoek, H., & Kluft, C. (1986c) *Biochem. J. 235*, 385–390.
Verheijen, J. H., Nieuwenhuizen, W., & Wijngaards, G. (1982a) *Thromb. Res. 27*, 377–385.
Verheijen, J. H., Mullaart, E., Chang, G. T. G., Kluft, C., & Wijngaards, G. (1982b) *Thromb. Haemostasis 48*, 266–269.
Verheijen, J. H., Nieuwenhuizen, W., Traas, D. W., Chang, G. T. G., & Hoegee, E. (1983) *Thromb. Res. 32*, 87–92.
Verheijen, J. H., Caspers, M. P. M., Chang, G. T. G., De Munk, G. A. W., Pouwels, P. W., & Enger-Valk, B. E. (1986) *EMBO J. 5*, 3525–3530.
Voskuilen, M., Vermond, A., Veeneman, G. H., Van Boom, J. H., Klasen, E. A., Zegers, N. D., & Nieuwenhuizen, W. (1987) *J. Biol. Chem. 262*, 5944–5946.
Zamarron, C., Lijnen, H. R., & Collen, D. (1984) *J. Biol. Chem. 259*, 2080–2083.