A Covalent Molecular Weight ~92 000 Hybrid Plasminogen Activator Derived from Human Plasmin Fibrin-Binding and Tissue Plasminogen Activator Catalytic Domains†

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ABSTRACT: A covalent hybrid plasminogen activator was prepared from the sulfhydryl forms of the NH_2-terminal heavy (A) chain of human plasmin (Pln_A) containing the fibrin-binding domain and the COOH-terminal B chain of tissue plasminogen activator (t-PA_B) containing the catalytic domain. The sulfhydryl form of Pln_A [$Pln_A(SH)_2$] was isolated from reduced Lys-2-plasmin on an L-lysine-substituted Sepharose column, and the sulfhydryl form of t-PA_B [t-PA_B(SH)] was prepared from reduced two-chain tissue plasminogen activator (t-PA) by removing the tissue plasminogen activator NH_2-terminal A chain (t-PA_A) on an L-lysine-substituted Sepharose column from the chain mixture. The specific plasminogen activator activity, with soluble fibrin, of the isolated t-PA_B(SH) chain was determined to be 62 700 international units (IU)/mg of protein, about 13% of the specific plasminogen activator activity of the parent t-PA. The $Pln_A(SH)_2$ and the t-PA_B(SH) chains were mixed in a 1:1 molar ratio, and hybridization (reoxidation) was allowed to proceed by first dialyzing out the reducing agent at 4 °C and then concentrating the mixture. The time for maximum hybridization, or formation of the covalent hybrid activator, was 6 days, as determined by both specific plasminogen activator activity, with soluble fibrin, and specific amidolytic activity; sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed the continual formation of an M_r ~92 000 hybrid. The covalent Pln_A–t-PA_B hybrid activator was isolated from the 6-day hybridization mixture by a two-step affinity chromatography method. It was first adsorbed on an L-lysine-substituted Sepharose column and eluted with ε-aminocaproic acid and then adsorbed on a Zn-chelated agarose column with subsequent elution by imidazole. The protein yield of purified hybrid was 10%, with a major component (77%) of M_r ~92 000. The covalent Pln_A–t-PA_B hybrid activator contained 1 mol of each chain; after reduction, it gave the two parent chains, Pln_A and t-PA_B, also shown to be present by double immunodiffusion. The specific plasminogen activator activity, with soluble fibrin, and the specific amidolytic activity of the purified covalent hybrid activator were determined to be ~200 000 IU/mg of protein, about 40% of the specific activity of the parent t-PA. In a fibrin clot lysis assay, the covalent hybrid activator and t-PA have similar specific fibrinolytic activities, ~500 000 IU/mg of protein; however, the clot lysis time curves were not parallel. The binding of the covalent Pln_A–t-PA_B hybrid activator and t-PA to forming fibrin was found to be similar; at physiological fibrinogen concentrations, binding of both activators to forming fibrin was about 90%.

Plasminogen (Plg)[1] activators are a class of specific serine proteases which have a single function, the conversion of Plg, a plasma zymogen, to plasmin (Pln), a broad-spectrum serine proteinase, by the specific cleavage of the Arg_{560}–Val peptide bond in the zymogen (Robbins, 1982, 1987; Robbins et al., 1967, 1987; Summaria et al., 1967; Bachmann, 1987). Generally, as a unique class of proteins, Plg activators can exist as zymogens which are converted by specific proteolytic cleavage to the active enzyme. Prourokinase (pro-u-PA), a one-chain inactive molecule, is converted by Pln to a two-chain enzyme, urokinase (u-PA), of the same molecular weight (HMW-u-PA) (Nielsen et al., 1982). Also, tissue plasminogen activator (t-PA), a one-chain inactive molecule, is converted by Pln to a two-chain enzyme of the same molecular weight (Andreasen et al., 1984; Dano et al., 1985). However, completely active one-chain HMW-u-PA (Sumi et al., 1982) and one-chain t-PA (Rånby et al., 1982a; Rijken et al., 1982) have been reported. Streptokinase (SK), a bacterial protein, is a unique type of Plg activator since it itself is neither a zymogen nor an active enzyme but forms an active enzyme with human Plg (Pln) in a 1:1 equimolar complex (Plg·SK and Pln·SK) (Robbins, 1982, 1987). All Plg activators have catalytic and fibrin-binding domains; the COOH-terminal region, the B chain, contains the catalytic domain [u-PA_B (Sumi & Robbins, 1983), t-PA_B (Dodd et al., 1986; Holvoet et al., 1986; MacDonald et al., 1986; Rijken & Groeneveld, 1986; Van Zon-

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[1] Abbreviations: Plg, plasminogen; Pln, plasmin; Pln_A, plasmin NH_2-terminal heavy (A) chain; Pln_B, plasmin COOH-terminal light (B) chain; Glu_1, NH_2-terminal glutamic acid; Lys_{77}, NH_2-terminal lysine; Val_{561}, NH_2-terminal valine; Plg K1,2,3, plasminogen kringles 1, 2, and 3; HMW-u-PA, high molecular weight urokinase; u-PA_A, high molecular weight urokinase NH_2-terminal A chain; u-PA_B, high molecular weight urokinase COOH-terminal B chain; t-PA, tissue plasminogen activator; t-PA_A, tissue plasminogen activator NH_2-terminal A chain; t-PA_B, tissue plasminogen activator COOH-terminal B chain; SK, streptokinase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; (SH), one interchain sulfhydryl; $(SH)_2$, two interchain sulfhydryls; IU, international units; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTE, dithioerythritol; IgG, immunoglobulin G; EDTA, ethylenediaminetetraacetic acid.

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neveld et al., 1986a), and Pln_B·SK (Summaria & Robbins, 1976)], and the NH_2-terminal region, the A chain, contains the fibrin-binding domain [u-PA_A and t-PA_A (Bachmann & Kruithof, 1984; Bachmann, 1987; Robbins et al., 1987)]. The NH_2-terminal fibrin-binding domain of human Plg (Pln_A) contains five triple-loop, three-disulfide bridge, regions of sequence homology called kringles (Sottrup-Jensen et al., 1978; Lucas et al., 1983; Vali & Patthy, 1984); the major fibrin-binding region is kringle 1 (Vali & Patthy, 1984). The NH_2-terminal u-PA_A chain has a single kringle region that shows extensive homology with the Plg kringle regions (Gunzler et al., 1982); however, pro-u-PA and two-chain u-PA (HMW-u-PA) do not readily bind to fibrin (Angles-Cano et al., 1986; Wijngaards et al., 1986), but one-chain u-PA does bind to fibrin (Sumi et al., 1982). The NH_2-terminal t-PA_A chain has two kringle regions which also shows homology with the Plg kringle regions (Pennica et al., 1983); the major fibrin-binding region of t-PA is kringle 2 (Van Zonneveld et al., 1986a,b). However, a "kringleless" recombinant t-PA was found to have fibrin specificity (Ehrlich et al., 1986). Fibrin greatly enhances the Plg activator activity of t-PA but not of u-PA (Holyaerts et al., 1982). The t-PA finger domain may also be involved in, but is not solely responsible for, fibrin binding (Banyai et al., 1983; Mattler et al., 1985; Van Zonneveld et al., 1986b); a "fingerless" recombinant t-PA had a high affinity for fibrin (Kagitani et al., 1985) probably due to kringle 2 only.

The NH_2-terminal Lys_{77}-Pln_A and the COOH-terminal Val_{561}-Pln_B chains of Pln have been isolated after reduction of the two interchain disulfide bonds of Pln, and a recombinant Lys_{77}-Pln was prepared from the sulfhydryl forms of the two chains by reoxidation (Summaria et al., 1979). A completely functionally active u-PA_B chain was isolated from two-chain HMW-u-PA after reduction of the single interchain disulfide bond (Sumi & Robbins, 1983). Recently, we developed methodology for the preparation of a new class of covalent hybrid Plg activators containing the fibrin-binding domains of human Plg (Pln_A) and the catalytic domain of human u-PA (u-PA_B) (Robbins & Tanaka, 1986; also see Figure 9). A covalent Pln_A–u-PA_B hybrid activator was prepared of M_r ~92 000, containing 1 mol of each chain. It had a specific activity of 71 300 IU/mg of protein in an amidolytic assay (tripeptide chromogenic substrate) compared to a specific activity of 128 000 IU/mg of protein for HMW-u-PA; however, in a fibrin clot lysis assay, the specific Plg activator activity was determined to be 279 000 IU/mg of protein, a 2-fold higher specific activity than HMW-u-PA. With soluble fibrin, the specific Plg activator activity was significantly stimulated, about 5-fold over HMW-u-PA, and was more strongly bound (adsorbed) to a fibrin clot, above 8-fold over HMW-u-PA, from 3% to 25%.

In this paper, we will report our studies on a second type of covalent hybrid Plg activator, Pln_A–t-PA_B, prepared from the sulfhydryl forms of the Pln_A and t-PA_B chains. Its isolation and characterization will be described. This new covalent hybrid activator contains the Plg fibrin-binding domain and the t-PA catalytic domain. Studies on fibrin binding and fibrin clot lysis will be reported.

Experimental Procedures

Materials. The plasmin chromogenic substrate H-D-Val-Leu-Lys-*p*-nitroanilide (S-2251) and human fibrinogen grade L (KabiVitrum AB) were purchased from Helena Laboratories. Glu_1-Plg used in the assays was a gift from KabiVitrum AB. The t-PA chromogenic substrate CH_3SO_2-D-cyclohexyl-Tyr-Gly-L-Arg-*p*-nitroanilide (Spectrozyme t-PA), t-PA (two chain), and goat anti-human melanoma t-PA IgG were gifts from American Diagnostica, Inc. Leupeptin, Ac-L-Leu-L-Leu-L-arginal·$^1/_2H_2SO_4$·H_2O (M_r 476) (Peptide Institute, Inc.), Trasylol (M_r 6512, 6600 kallikrein inhibitor units/mg of protein) (Bayer, West Germany), human serum albumin (fraction V) (Sigma), bovine thrombin Plg free (Pentex), DTE (Aldrich), agarose (Litex, type HSA), glycerol (Baker), and Tween 80 (Baker) were purchased. Ancrod, 150 units/ampule (Abbott Laboratories), was a gift from G. H. Barlow. The human melanoma t-PA reference preparation, 83/517, 1000 IU/ampule (established by the International Committee on Thrombosis and Haemostasis), was obtained from P. J. Gaffney, National Institute for Biological Standards and Control, London, England. The molecular weight markers (Pharmacia) for SDS–PAGE were purchased. All other reagents and chemicals were of the highest grade available.

Preparation of $Pln_A(SH)_2$ (Lys_{77}–Arg_{560}) Chain. Human Glu_1-2-Plg, Lys_{77}-2-Pln, and $Pln_A(SH)_2$ (Lys_{77}–Arg_{560}) were prepared by methods previously described (Robbins & Tanaka, 1986). In the final step, the $Pln_A(SH_2)_2$ chain was eluted from the L-lysine-substituted Sepharose column at 4 °C with a linear 0–20 mM ε-aminocaproic acid gradient, in 100 mM sodium phosphate buffer, pH 7.4, containing 3 mM DTE, 5 mM EDTA, and 0.22 mM leupeptin. The eluted fractions were collected in 100% glycerol, to give a final concentration of 25% glycerol, pooled and concentrated by using the Micro-ConFilt system, with a 10000 molecular weight cutoff membrane, and washed with 4 volumes of 50 mM Tris-HCl buffer, pH 9.0, containing 20 mM lysine, 5 mM EDTA, 3 mM DTE, and 0.22 mM leupeptin. The final concentration of the $Pln_A(SH)_2$ chain was 4.2 mg/mL; the fraction was stored at −70 °C. This chain has a molecular weight of ~60,000 with an $E^{1\%}_{1cm}$(280 nm) of 17.0.

Preparation of t-PA(SH) (Ile_{276}–Pro_{527}) Chains. Two-chain t-PA (1.39 mg in 1.26 mL, 694 000 IU) in 1000 mM NH_4HCO_3, pH 7.7, was adjusted to pH 9.0 by adding $^1/_{20}$th volume of deaerated 1000 mM Tris-HCl, buffer, pH 9.0, containing 100 mM EDTA, 2000 mM NaCl, 4.4 mM leupeptin, and 0.2% Tween 80, and then 100% glycerol to a final concentration of 25% glycerol. The two-chain t-PA was reduced with 3 mM DTE at 25 °C for 90 min under a nitrogen atmosphere. The preparation was applied to a column of L-lysine-substituted Sepharose (0.9 × 3.5 cm, column volume of 2 mL) previously equilibrated with 100 mM sodium phosphate buffer, pH 7.4, containing 5 mM EDTA, 3 mM DTE, 0.22 mM leupeptin, 0.22 nM Trasylol, and 0.01% Tween 80, at 4 °C, in order to adsorb the t-PA_A(SH) chain. The COOH-terminal t-PA_B(SH) chain (Ile_{276}–Pro_{527}) passed through the column unadsorbed and was collected (3 mL) in 100% glycerol (1 mL) to give a final concentration of 25% glycerol. The t-PA_B(SH) chain fraction, in a volume of 4.9 mL, contained 42 000 IU of Plg activator activity, 495 000 IU of amidolytic activity, and 0.68 mg of protein calculated from an $E^{1\%}_{1cm}$(280 nm) of 16.0 (assumed to be similar to the Pln_B chain). This chain has a molecular weight of ~32 000.

Hybridization of $Pln_A(SH)_2$ with t-PA(SH). The $Pln_A(SH)_2$ chain (1.3 mg in 0.32 mL) was mixed with the t-PA_B(SH) chain (0.66 mg in 4.8 mL) in an equimolar ratio, and the solution was dialyzed (10 000 molecular weight cutoff membrane) against 190 volumes of oxygenated 100 mM NH_4HCO_3/25% glycerol, pH 7.8, containing 5 mM EDTA, 2.2 mM leupeptin, 0.22 nM Trasylol, and 0.01% Tween 80, at 4 °C, with four hourly buffer changes and continuous stirring. Trasylol was omitted from the final two buffer changes. The mixture, 7.1 mL, was then concentrated over-

night at 4 °C, to 1.2 mL. The hybridization mixture was placed in a plastic tube (1.2 × 7.5 cm) and stirred continuously at 4 °C for 5 additional days, with samples removed at selected daily intervals. On day 6, the mixture became opalescent.

Purification of Covalent Pln_A–t-PA_B Hybrid Activator. The Pln_A–t-PA_B hybrid was purified by a two-step affinity chromatography method at 4 °C. The 6-day hybridization mixture was clarified by centrifugation at 2000 rpm for 20 min at 4 °C, and a slight insoluble residue was discarded. The supernatant was passed through a L-lysine-substituted Sepharose column (0.9 × 1.8 cm, column volume of 1 mL) equilibrated with 100 mM sodium phosphate buffer, pH 7.4, containing 5 mM EDTA, 0.22 mM leupeptin, and 0.01% Tween 80. After the column was washed with several column volumes of the equilibration buffer, the Pln_A–t-PA_B hybrid and unhybridized Pln_A chain, which was also adsorbed to the column, were eluted with 200 mM ε-aminocaproic acid; the fractions were collected and pooled (2 mL). The unhybridized t-PA_B chain which passed through the column was collected and pooled (3.7 mL), concentrated to 1.8 mL, clarified, and stored at −70 °C. The Pln_A–t-PA_B hybrid and unhybridized Pln_A chain mixture were then passed through a Zn-chelated agarose 6B column (0.9 × 1.8 cm, column volume of 1 mL) equilibrated with a 50 mM Tris-HCl buffer, pH 7.5, containing 1000 mM NaCl, 0.22 mM leupeptin, and 0.01% Tween 80. The contaminating unhybridized Pln_A chain passed through the column. The Pln_A–t-PA_B hybrid was eluted with the equilibration buffer containing 50 mM imidazole, and the fractions were collected in 100% glycerol to give a final concentration of 25% glycerol and pooled (2.5 mL). The purified covalent Pln_A–t-PA_B hybrid fraction was concentrated to 0.65 mL. It contained 0.356 mg of protein, calculated from an $E^{1\%}_{1cm}$(280 nm) of 16.7, and 71 000 IU of Plg activator activity. One mole of each parent chain was found in the hybrid: on a weight basis, two thirds Pln_A chain, M_r ~60 000, with an $E^{1\%}_{1cm}$(280 nm) of 17.0 and one-third t-PA_B chain, M_r ~32 000, with an $E^{1\%}_{1cm}$(280 nm) of 16.0.

Determination of Plg Activator Activity. Plg activator activity was determined by an end-point method with H-D-Val-Leu-Lys-*p*-nitroanilide (Wohl et al., 1979, 1980) with soluble fibrin (Rånby et al., 1982b). Soluble fibrin was prepared by dissolving 1 g of human fibrinogen in 50 mL of H_2O and adding 20 units of Ancrod; the mixture was incubated at 25 °C for 4 h, and at 4 °C overnight. The resulting clot was dissolved in 7 M urea and stored at −70 °C. The assay was carried out in 100 mM Tris-HCl buffer, pH 7.5, containing 0.05% human serum albumin and 0.01% Tween 80, to which was added 200 μg of Glu_1-Plg, 150 μg of soluble fibrin, and varying amounts of activator, in a final volume of 975 μL. The activation mixture was incubated at 37 °C for 5 min, and 25 μL of 10 mM chromogenic substrate was added and incubated at 37 °C for 5 min; 100 μL of 50% acetic acid was added, and the absorbance was read at 405 nm. A t-PA standard curve was used for the calculation of international units.

Determination of Amidolytic Activity. Amidolytic activity was determined by an end-point method with CH_3SO_2-D-cyclohexyl-Tyr-Gly-L-Arg-*p*-nitroanilide. The assay was carried out in 300 mM Tris-HCl/300 mM imidazole buffer, pH 8.4, to which was added varying amounts of the activator, in a final volume of 900 μL. The mixture was incubated at 37 °C for 2 min, and 100 μL of the 3 mM chromogenic substrate was added and incubated at 37 °C for 5 min; 100 μL of 50% acetic acid was added, and the absorbance was read at 405 nm. A t-PA standard curve was used for the calculation of international units.

Determination of Fibrinolytic Activity in a Fibrin Clot Lysis Assay. Fibrinolytic activity was measured in a clot lysis assay, carried out in 1.0 × 7.5 cm glass tubes, in a system containing 250 μL of 2 mg/mL human fibrinogen in 10 mM sodium phosphate buffer, pH 7.4, containing 140 mM NaCl, 0.003% Tween 80, 150 μL of 0.5 mg/mL Glu-Plg in the same buffer, and 500 μL of varying amounts of activator from 0.2 to 8 IU in the same buffer. Thrombin, 2 IU in 50 μL, was added to form the clot. Clot lysis was carried out at 37 °C, with ~300 mg of glass bead placed over the surface of the clot 1 min after the addition of thrombin. The end point, the clot lysis time, was noted when the glass bead dropped to the bottom of the tube.

Binding (Adsorption) of Covalent Hybrid Activator to Forming Fibrin Clots. The binding (adsorption) of activators to forming fibrin clots was carried out by a modification of a previously described method (Rijken et al., 1982). The experiments were carried out in 50 mM Tris-HCl buffer, pH 7.2, containing 0.22 nM Trasylol, 0.01% Tween 80, and 1 mg/mL human serum albumin, to which was added 250 ng of activator and varying concentrations of human fibrinogen from 0.03 to 1.0 mg, in a final volume of 995 μL. One unit of thrombin in 5 μL was added and the mixture incubated at 37 °C for 60 min. The clot was removed by centrifugation at 12 000 rpm for 30 min. The supernatant solution was assayed for plasminogen activator activity in the presence of soluble fibrin; the percent binding was determined from the difference in plasminogen activator activity between the amount added and the amount recovered.

SDS–PAGE. SDS–PAGE was carried by using a previously described method (Laemmli, 1970) in the Hoefer apparatus (14 × 16 cm, 1.5 mm thick gel). Samples were treated in 100 mM sodium phosphate buffer, pH 7.0, containing 2 mM EDTA and 0.1% SDS (gel and running buffer), with or without 3 mM DTE; reduced samples were treated for 90 min at 25 °C under a N_2 atmosphere. The samples were placed on a 5% stacking gel over a 10% running gel and run at a constant voltage of 130 V (150 mA) at 20 °C for 3 h. Molecular weight markers were run in each gel. After electrophoresis, the gel was stained with 0.25% Coomassie Brilliant Blue R-250 dissolved in 45% methanol/10% acetic acid and destained with 25% methanol/7% acetic acid. The gel was scanned after drying.

Antibodies to Pln_A and t-PA_B Domains: Double Immunodiffusion Analysis. Antibodies specific for the human Pln_A domain were prepared from a goat anti-Plg IgG preparation by adsorption of antibodies to a Plg K1,2,3–L-lysine-substituted Sepharose column and elution of specific anti-Plg K1,2,3 IgG antibodies from the column. Plg K1,2,3 was prepared from an elastase digest of human Plg (Sottrup-Jensen et al., 1978).

Double immunodiffusion analysis was carried out by the Ouchterlony method in 1% agarose in a 80 mM Tris-HCl/0.15 M NaCl buffer, pH 7.2, containing 0.02% NaN_3, at 25 °C, for 48–72 h. Goat anti-t-PA IgG (5 mg/mL) and goat anti-Plg K1,2,3 IgG (5 mg/mL) were used at a final concentration of 100 and 20 μg, respectively, per well. The hybrid antigen was used at a final concentration of 10 μg per well. The gel was washed with the buffer for several days at 4 °C, stained with Coomassie Brilliant Blue R-250 in 25% methanol/10% acetic acid, destained with 90% methanol/10% acetic acid, and dried.

RESULTS

Reduction of Two-Chain t-PA and Preparation of t-PA_B-(SH) Chain. Reduction of the two-chain t-PA gave a mixture of t-PA_A(SH) and t-PA_B(SH) chains. There was a 94% loss

Table I: Preparation and Purification of Covalent Pln_A–t-PA_B Hybrid Activator

step	mg of protein	total Plg activator act. (IU)	total amidolytic act. (IU)	sp Plg activator act. (IU/mg of protein)	Plg activator act. yield (%)	amidolytic act.:Plg activator act. ratio
(1) parent t-PA	1.39	694 000	765 000	500 000	100	1.1
(2) reduced t-PA mixture	1.39	46 600	466 000	33 500	7	10.0
(3) isolated t-PA_B(SH) chain	0.68	42 000	495 000	62 700	6	11.8
(4) 6-day hybridization mixture	1.97	186 000	888 000	83 300	24	4.8
(5) two-step affinity chromatography method[a]	0.36	70 500	71 600	199 000	10	1.0

[a] L-Lysine-substituted Sepharose + Zn-chelated agarose.

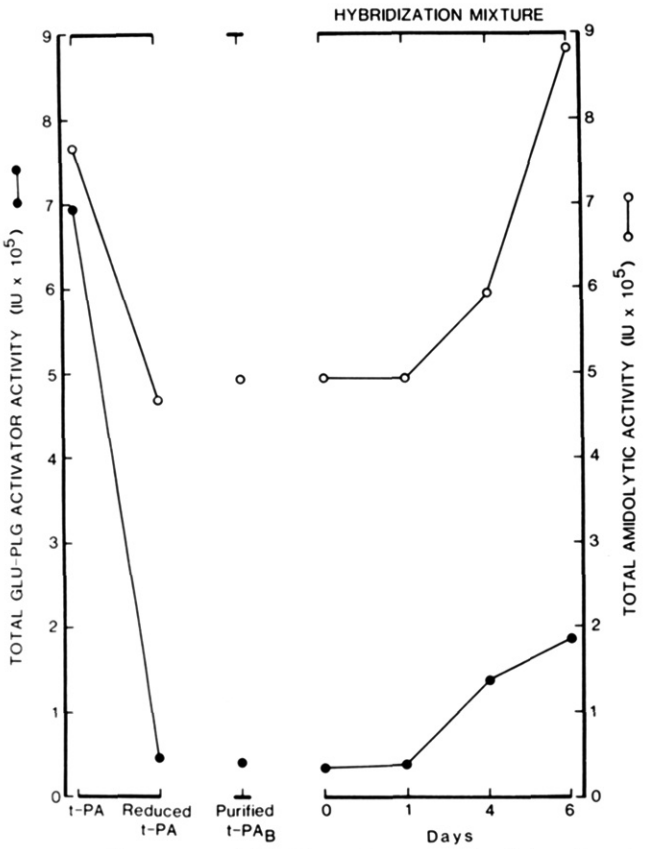

FIGURE 1: Data on total Glu-Plg activator activity (●) and total amidolytic activity (○) of t-PA, reduced t-PA, the isolated t-PA_B(SH) chain, and the hybridization reaction (days 0, 1, 4, and 6).

in Plg activator activity with a concomitant 39% loss in amidolytic activity (Table I and Figure 1). The total specific Plg activator activity decreased from 694 000 to 46 600 IU, whereas the total amidolytic activity decreased from 765 000 to 466 000 IU. It is interesting to note that the ratio of amidolytic activity to Plg activator activity was ~1 for the parent t-PA and rose to ~10 for the reduced mixture. The measurement of amidolytic activity of parent t-PA gives the same value as the measurement of Plg activator activity, using t-PA standard curves.

The isolated t-PA_B(SH) chain, of M_r ~32 000, had a low specific Plg activator activity, 62 700 IU/mg of protein, about 13% of the specific Plg activator activity of the parent t-PA. The Plg activator yield was 6%, and the ratio of amidolytic activity to Plg activator activity was ~12.

Hybridization of $Pln_A(SH)_2$ Chain with t-PA_B(SH) Chain. The $Pln_A(SH)_2$ chain, 1.30 mg in 0.32 mL, was hybridized with the t-PA_B(SH) chain, 0.67 mg in 4.8 mL, at a 1:1 molar ratio of the two chains. The hybridization mixture (day 0) was dialyzed extensively to remove the reducing agents. It was concentrated (day 1), and the hybridization (reoxidation) continued for 12 days. Maximum hybrid formation occurred

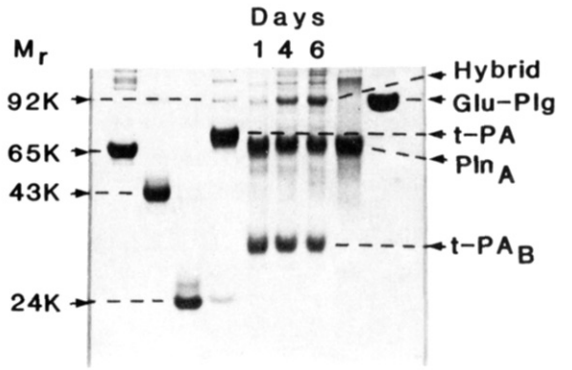

FIGURE 2: SDS–PAGE analysis of the hybridization mixtures on days 1 (lane 5), 4 (lane 6), and 6 (lane 7). The marker proteins are as follows: lane 1, bovine serum albumin (65K); lane 2, ovalbumin (43K); lane 3, trypsinogen (24K); lane 4, t-PA (70K); lane 8, Pln_A (60K); and lane 9, Glu-Plg (88K). The forming hybrid (92K), and t-PA_B (32K), is identified in the hybridization mixtures (lanes 5–7).

in 6 days, under the conditions of this experiment; there was a continual loss in Plg activator activity after 6 days. SDS–PAGE analysis (Figure 2) shows the continual formation of a M_r ~92 000 hybrid from day 1, to day 4, to day 6. The gel scan showed 4% hybrid on day 1, 17% on day 4, and 26% on day 6. The Pln_A chain decreased from 56% (day 1), to 49% (day 4), to 46% (day 6), whereas the t-PA_B chain decreased from 40% (day 1), to 34% (day 4), to 28% (day 6). Some of the data on the Plg activator activity and the amidolytic activity of the hybridization mixture, days 0, 1, 4, and 6, are shown in Figure 1 and Table 1. The Plg activator activity of the hybridization mixture rose from 35 000 IU (day 0), to 37 000 IU (day 1), to 137 000 (day 4), to 186 000 (day 6). The amidolytic activity rose from 495 000 IU (days 1 and 2), to 590 000 IU (day 4), to 888 000 IU (day 6). In 6 days, there was a ~5-fold increase in Plg activator activity and a ~2-fold increase in amidolytic activity.

Purification and Properties of Covalent Pln_A–t-PA_B Hybrid Activator. The 6-day hybridization mixture was passed through a L-lysine-substituted Sepharose column which adsorbed the covalent Pln_A–t-PA_B hybrid and the unhybridized Pln_A chain. The unhybridized, excess, t-PA_B chain passed through the column. After elution of the hybrid and Pln_A chain with ε-aminocaproic acid, the eluate was immediately passed through the Zn-chelated agarose column to adsorb the hybrid. The unhybridized, excess, Pln_A chain passed through the column. The hybrid was eluted with imidazole added to the column buffer, and the eluate was concentrated. The original mixture of 1.3 mg of protein of the $Pln_A(SH)_2$ chain and 0.67 mg of protein of the t-PA_B(SH) chain yielded 0.36 mg of covalent hybrid protein with a specific Plg activator activity of 199 000 IU/mg of protein, which is ~40% of the specific Plg activator activity of the parent t-PA preparation. The ratio of amidolytic activity to Plg activator activity was ~1, the same as for the parent t-PA. Analysis of the purified

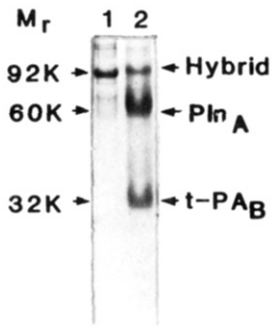

FIGURE 3: SDS–PAGE analysis of the purified covalent Pln_A–t-PA_B hybrid activator. Lane 1, purified covalent hybrid (6 μg) (92K); lane 2, reduced purified hybrid activator (12 μg) (Pln_A, 60K; t-PA_B, 32K).

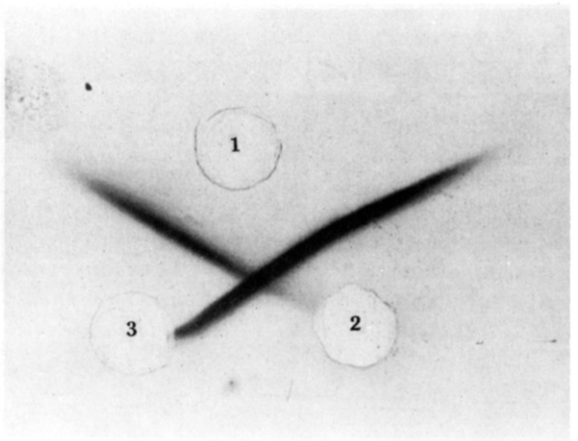

FIGURE 4: Double immunodiffusion analysis of the covalent Pln_A–t-PA_B hybrid activator. Well 1 contained 10 μg of covalent hybrid activator, well 2 contained 20 μg of anti-Plg K1,2,3 IgG, and well 3 contained 100 μg of anti-t-PA IgG.

covalent Pln_A–t-PA_B hybrid by SDS–PAGE showed a major component of M_r ~92 000 (77%) with both some higher molecular weight (14%) and some lower (9%) molecular weight components (Figure 3); reduction of the purified hybrid showed two major chains of M_r ~60 000 (Pln_A) and M_r ~ 32 000 (t-PA_B), with ~10% unreduced hybrid. The theoretical molecular weight of the covalent hybrid is ~92 000 [Pln_A chain (M_r ~60 000) plus t-PA_B chain (M_r ~32 000)], showing that it contains 1 mol of each chain. Immunodiffusion analysis (Figure 4) showed that the hybrid contains both the Pln_A and t-PA_B domains.

Fibrinolytic Activity of the Covalent Pln_A–t-PA_B Hybrid Activator: In Vitro Fibrin Clot Lysis. Standard fibrin clots containing Glu-Plg were used for clot lysis experiments with different activators with from 0.2 to 8 IU to measure fibrinolytic activity (Figure 5). Parallel curves were obtained with the covalent Pln_A–t-PA_B hybrid, t-PA_B chain, u-PA, and Pln_B·SK. There was no parallelism between t-PA and the other activators, the covalent hybrid/t-PA_B chain/u-PA/Pln_B·SK. It is interesting to note that the covalent hybrid behaves more like u-PA and Pln_B·SK than t-PA. The most active plasminogen activator, in these fibrin clot lysis experiments, was found to be Pln_B·SK, followed by u-PA, t-PA_B chain, t-PA, and the covalent hybrid; the covalent hybrid was found to be very similar to t-PA in fibrinolytic activity. At 1 IU of activator, the clot lysis times varied from 5.5 min for Pln_B·SK, to 8.8 min for u-PA, to 20 min for t-PA_B chain, to 25 min for t-PA, and to 30 min for the covalent hybrid. In this clot lysis assay, the covalent hybrid and t-PA have similar specific fibrinolytic activities, at 3–4 IU (where the curves intersect); however, at 1 IU, there is a 2-fold difference, with the covalent

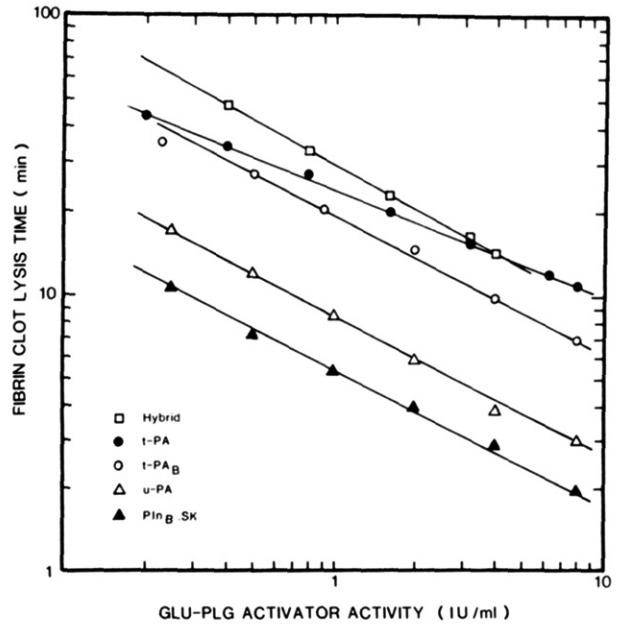

FIGURE 5: Fibrinolytic activity of different plasminogen activators, including the covalent Pln_A–t-PA_B hybrid activator, in a standard fibrin clot lysis assay using between 0.2 and 8 IU of activator. log–log plot of fibrin clot lysis time vs. Glu-Plg activator activity.

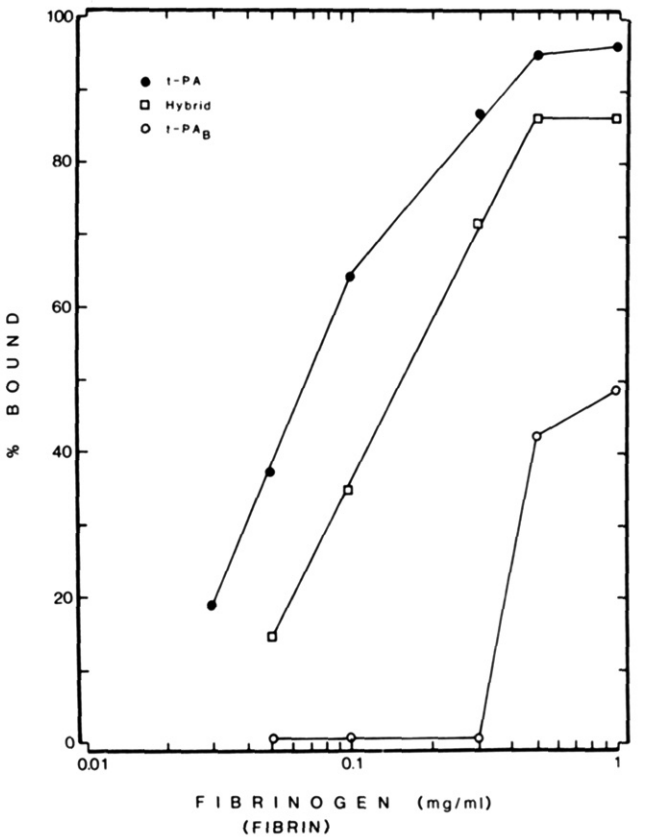

FIGURE 6: Binding (adsorption) of the covalent Pln_A–t-PA_B hybrid activator, t-PA, and t-PA_B chain to forming fibrin clots using 250 ng of each activator protein at each fibrinogen (fibrin) concentration from 0.03 to 1 mg/mL; percent binding determined by measuring the plasminogen activator activity in the supernatant, after removal of the fibrin clot.

hybrid having the lower specific fibrinolytic activity. At 3–4 IU of covalent hybrid, u-PA has a 10-fold higher specific fibrinolytic activity.

Binding (Adsorption) of Covalent Pln_A–t-PA_B Hybrid Activator to Forming Fibrin Clots. The results of the binding

Table II: Comparative Data on t-PA and u-PA Hybrids

	M_r	IU/mg of protein: sp Plg activator act. (+fibrin)	IU/mg of protein: sp amidolytic act.	IU/mg of protein: sp fibrinolytic act.	fibrin binding (%)
(1) t-PA	~70 000	500 000	500 000	500 000	95
t-PA hybrid	~92 000	200 000	200 000	500 000	87
(2) u-PA (HMW)	~54 000	192 000	128 000	128 000	3
u-PA hybrid	~92 000	360 000	71 300	279 000	25

(adsorption) of the covalent Pln_A–t-PA_B hybrid activator to forming fibrin clots are shown in Figure 6. The data compare native t-PA and t-PA_B chain binding with covalent hybrid activator binding. The binding was compared at fibrin (fibrinogen) concentrations from about 0.03 to 1.0 mg/mL, using 250 ng of activator protein at each fibrinogen concentration. Fifty percent binding of t-PA was found at a fibrin (fibrinogen) concentration of ~0.07 mg/mL whereas 50% binding of the covalent hybrid was found at a fibrin (fibrinogen) concentration of ~0.16 mg/mL, a ~2-fold higher fibrin concentration. There was no binding of the t-PA_B chain at a fibrin (fibrinogen) concentration of 0.30 mg/mL. At a fibrin (fibrinogen) concentration of 0.5 mg/mL, ~95% t-PA, ~87% covalent hybrid, and ~42% t-PA_B chain were found to be bound. At physiological fibrinogen concentrations, when fibrin is forming, the binding of t-PA and the covalent hybrid activator are both nearly complete. Neither u-PA nor Pln_B·SK binds to fibrin at concentrations of 250 ng of activator protein at fibrin (fibrinogen) concentrations from 0.05 to 1.00 mg/mL.

Discussion

The activation of Plg by Plg activators at the surface of a fibrin–thrombus depends upon different factors: the binding of the Plg activator both to Plg and to fibrin, and the binding of Plg to fibrin (Robbins, 1983, 1987). The generation of Pln results in the proteolytic cleavage of fibrin, which exposes new Plg and Plg activator binding regions, and the proteolytic cleavage of native Glu_1-Plg generates Lys_{77}-Plg, a more avid-binding Plg. Also, zymogen Plg activators, like pro-u-PA, will be activated by Pln to u-PA at the surface of the thrombus. The activation mechanisms at the surface of the fibrin–thrombus are complex and probably require the presence of both the catalytic and fibrin-binding domains of the Plg activator.

The concept of improving Plg activators by covalently linking fibrin-binding domains from Plg to the catalytic domains of the activator may permit us to develop new types of activators with unusual properties, which may result in more effective and efficient fibrinolytic/thrombolytic agents. The catalytic domain of u-PA, both in HMW-u-PA and in LMW-u-PA, is very efficient as an activator, and the removal of the NH_2-terminal domain/chain with a fibrin-binding region (kringle) does not decrease the catalytic efficiency of the activator (Sumi & Robbins, 1983). The catalytic domain of t-PA, in the native molecule, is very efficient as an activator in the presence of fibrin but not in the absence of fibrin (Hoylaerts et al., 1982; Bachmann, 1987). However, the isolated catalytic domain, t-PA_B, after removal of the NH_2-terminal chain, is not an efficient activator in the presence of fibrin (Dodd et al., 1986; Rijken & Groeneveld, 1986; this study). Therefore, the NH_2-terminal domain of t-PA, but not of u-PA, is required for fibrin binding and fibrin specificity. The NH_2-terminal chain of Pln (Pln_A) when covalently linked, to the catalytic domain either of u-PA or of t-PA, could give enhanced fibrin binding and fibrin specificity to these catalytic domains. This concept was proven to be experimentally possible with the preparation of a covalent Pln_A–u-PA_B hybrid (Robbins & Tanaka, 1986) and a covalent Pln_A–t-PA_B hybrid (this study). The covalent Pln_A–u-PA_B hybrid (M_r ~92 000), when compared to HMW-u-PA (M_r ~54 000), had a 2-fold lower specific activity in an amidolytic assay and a 2-fold higher specific Plg activator activity in a clot lysis assay. Its Plg activator activity was stimulated 5-fold by soluble fibrin compared to 1.5-fold for HMW-u-PA. Also, it was more strongly adsorbed to a fibrin clot than was HMW-u-PA, from 3% to 25%. In this study, the purified covalent Pln_A–t-PA_B hybrid (M_r ~92 000), when compared to t-PA (M_r ~70 000), had a 2.5-fold lower specific Plg activity, with soluble fibrin, and a 2.5-fold lower specific amidolytic activity, but with similar specific fibrinolytic activities (nonparallel clot lysis time curves). Comparative data on the two hybrids are shown in Table II. It is difficult to explain why the specific activity of the covalent hybrid is only 40% that of the parent t-PA; on a total weight basis or on a catalytic chain basis, the activity of the hybrid is still lower than t-PA. Since the t-PA_B chain also has a very low specific plasminogen activator activity, with soluble fibrin, ~8-fold lower than t-PA, the structure and configuration of the t-PA_A chain must contribute significantly to the catalytic efficiency of the native activator in the presence of fibrin. The catalytic efficiency of the activator could not be entirely duplicated, or enhanced, by replacing the t-PA_A chain with the Pln_A chain, even though the Pln_A chain has strong fibrin-binding regions. With the covalent Pln_A–u-PA_B hybrid, the Pln_A fibrin-binding domains significantly increased the catalytic efficiency of the native u-PA in clot lysis assays, which was not so with the covalent Pln_A–t-PA_B hybrid. The u-PA_A chain has weak, or little, fibrin-binding capacity or fibrin specificity.

An important aspect of determining specific plasminogen activator activities of activators is the use of the clot lysis time assay (fibrinolytic activity) which is a reflection of the ability of the activator to activate plasminogen in a forming (formed) fibrin clot. log–log plots of clot lysis times vs. standard units of activator permit one to compare different preparations of a single activator (e.g., t-PA), and perhaps different activators, if the curves are parallel (Gaffney & Curtis, 1985). However, an international unit of t-PA is not the same as an international unit of either u-PA or SK, since the three activators have standards/reference preparations with different definitions of an international unit. Of the activators compared, t-PA does not give a curve parallel with the curves obtained for the covalent Pln_A–t-PA_B hybrid, t-PA_B chain, u-PA, and Pln_B·SK which are all parallel to each other (see Figure 5); therefore, it is difficult to compare the fibrinolytic activity of the t-PA hybrid with t-PA. Also, it is difficult to compare t-PA and the t-PA hybrid with u-PA and Pln_B·SK since the catalytic efficiencies of the latter two activators (in the presence of soluble fibrin) are much higher, by more than 10-fold, than the former two activators (Robbins et al., 1987). On an international unit basis (standard units), Pln_B·SK (assayed against an SK standard) is the most active activator. Plg·SK complexes, including Pln_B·SK, have the highest catalytic efficiencies of all the activators studied, in both the absence and presence of fibrin (Robbins, 1987; Robbins et al., 1987).

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