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Characterization of a Recombinant Fusion Protein of the Finger Domain of Tissue-type Plasminogen Activator with a Truncated Single Chain Urokinase-type Plasminogen Activator*

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Human recombinant single chain urokinase-type plasminogen activator (recombinant scu-PA) and a hybrid between human tissue-type plasminogen activator (t-PA) and scu-PA, obtained by ligation of cDNA fragments encoding the NH_2-terminal region (amino acids 1–67) of t-PA and the COOH-terminal region (amino acids 136–411) of scu-PA, were expressed in a mammalian cell system. The proteins were purified from conditioned culture media containing 2% fetal calf serum by chromatography on zinc chelate-Sepharose, immunoadsorption chromatography on an insolubilized murine monoclonal antibody directed against urokinase, benzamidine-Sepharose chromatography, and Ultrogel AcA 44 gel filtration. Between 180 and 230 μg of the purified proteins were obtained per liter of conditioned medium, with a yield of approximately 18% and a purification factor of 720–1900.

On sodium dodecyl sulfate gel electrophoresis under reducing conditions, the proteins migrated as single bands with approximate M_r 50,000 for recombinant scu-PA and M_r 43,000 for the t-PA/scu-PA hybrid. Following conversion to urokinase with plasmin, the proteins had a specific amidolytic activity comparable to that of natural scu-PA. Both proteins activated plasminogen directly with K_m = 0.53 and 1.4 μM and k_2 = 0.0034 and 0.0027 s^{-1}, respectively. Both proteins did not bind specifically to fibrin and had a comparable degree of fibrin selectivity as measured in a system composed of a whole human ^{125}I-fibrin-labeled plasma clot suspended in human plasma.

It is concluded that this chimeric protein, consisting of the NH_2-terminal "finger-like" domain of t-PA and the COOH-terminal region of scu-PA, has very similar enzymatic properties as compared to scu-PA, but has not acquired the fibrin affinity of t-PA.

Two physiological plasminogen activators with demonstrated fibrin specificity, namely tissue-type plasminogen activator (t-PA)[1] and single chain urokinase-type plasminogen activator (scu-PA) (1), are presently under clinical investigation as clot-specific thrombolytic agents (2–9). The molecular mechanisms regulating the fibrin specificity of t-PA and scu-PA are, however, different. t-PA is a poor activator of plasminogen in the absence of fibrin, characterized by a high Michaelis constant (65 μM). t-PA, however, binds specifically to fibrin (K_d = 0.14 μM), and fibrin-associated t-PA has a high affinity for plasminogen (K_m = 0.16 μM). The fibrin specificity of t-PA thus appears to be mediated via its specific binding to fibrin (10). t-PA contains an NH_2-terminal region, homologous to the "finger" domains of fibronectin, which has been claimed to be involved in its binding to fibrin (11, 12). We have reported that scu-PA has an intrinsic plasminogen-activating potential and that the fibrin specificity of scu-PA depends on its single chain structure (13, 14). Others (15), however, have reported that scu-PA is inactive toward plasminogen. Our results indicate that the activation of plasminogen by scu-PA obeys Michaelis-Menten kinetics characterized by a low K_m and a low k_2 (14). In plasma, however, scu-PA does not activate plasminogen, due to the presence of a reversible inhibitor, which is neutralized by fibrin (13).

On the basis of these mechanisms, we hypothesized that a hybrid molecule, consisting of the NH_2-terminal region of t-PA (the finger domain) and the COOH-terminal region of scu-PA (the B-chain and a carboxyl-terminal remnant of the A-chain), might combine the fibrin specificity of both molecules. The present study was undertaken to investigate this hypothesis.

EXPERIMENTAL PROCEDURES

Materials

Natural scu-PA (nscu-PA) was purified from the conditioned medium of a human lung adenocarcinoma cell line (CALU-3) as described (16). t-PA was purified from conditioned melanoma cell (Bowes) culture media as described (17). Thrombin (Topostasin®) was from Roche (Brussels, Belgium); bovine fibrinogen was from Organon (Oss, The Netherlands); aprotinin (Trasylol®) was from Bayer AG (Leverkusen-Bayerwerke, Federal Republic of Germany), and pyro-Glu-Gly-Arg-*p*-nitroanilide (S-2444) and D-Val-Leu-Lys-*p*-nitroanilide (S-2251) were from KabiVitrum (Brussels, Belgium). Human plasminogen and fibrinogen were purified as described (17). Plasmin was prepared by streptokinase activation of human plasminogen (18). The thrombin inhibitor D-Ile-Pro-Arg chloromethyl ketone was custom-synthetized at Union Chimique Belge (Brussels, Belgium) (19). The International Reference Preparation of Urokinase (66/46) was obtained from Dr. P. J. Gaffney (National Institute for Biological Standards and Control, London, Great Britain). Zinc chelate-Sepharose was prepared by the method of Porath *et al.* (20). Sephadex G-100 superfine was from Pharmacia P-L Biochemicals, and Ultrogel AcA 44 was from LKB (Bromma, Sweden). *p*-Aminobenzamidine (Aldrich) was coupled to CH-Sepharose 4B (Pharmacia P-L Biochem-

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[1] The abbreviations used are: t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; scu-PA, single chain u-PA; nscu-PA, natural scu-PA obtained from a lung adenocarcinoma cell line (CALU-3); rscu-PA, recombinant scu-PA obtained by expression of the cDNA encoding scu-PA (with $Cys^{131} \rightarrow Trp^{131}$ and $Gly^{366} \rightarrow Cys^{366}$ mutations) in a mammalian cell system; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay; DMEM, Dulbecco's modified Eagle's medium.

icals) as described by Holmberg *et al.* (21). All other chemicals and reagents were of standard laboratory grade.

The monoclonal antibody MA-4D1E8, which reacts with an epitope in the B-chain of urokinase, was obtained as described elsewhere (22). It was coupled to CNBr-activated Sepharose 4B (Pharmacia P-L Biochemicals) following the instructions of the manufacturer, with a substitution level of 6 mg of antibody/g of gel. Polyclonal rabbit antisera against u-PA and against t-PA were obtained as described elsewhere (17, 22).

Methods

Construction of Expression Vectors for rscu-PA and for t-PA/scu-PA Hybrid

The urokinase cDNA clone pULB1000 (23), obtained from a cDNA library of Detroit 562 carcinoma cells (ATCC CRL 138), was used. This clone encodes a protein which differs in three positions from the published urokinase sequence (24, 25): Cys^{131} is replaced by tryptophan, Gly^{366} by cysteine, and Ala^{410} by valine. To reconstruct the cDNA coding for pre-scu-PA, the cDNA of pULB1000 was fused with a synthetic adaptor corresponding to the urokinase signal peptide (25) up to amino acid −16 (26). In addition, the codon specifying Val^{410} was replaced by a codon specifying alanine by means of a synthetic linker (26) to yield construct ppUK.410 (26).

The t-PA/scu-PA hybrid cDNA was constructed by fusion of a 392-bp *Hin*dIII-*Rsa*I cDNA fragment, derived from the t-PA clone DSP1.1TPA25.BGH (26) which encodes the NH_2-terminal region (up to amino acid 67) of t-PA via a synthetic 43-bp adaptor molecule, to a *Bal*I-*Sac*I fragment of cDNA, encoding amino acids 149–411 of u-PA (26). In this way, the NH_2-terminal region of t-PA, encompassing amino acids 1–67, is fused via a tyrosine codon to Lys^{136} in u-PA, whereby the plasmin cleavage site Lys^{135}-Lys^{136} in urokinase is destroyed, yielding construct Fg.t-PA/UK.410 (26).

Both the recombinant pre-scu-PA and the pre-t-PA/scu-PA hybrid were further subcloned as *Hin*dIII-*Sac*I cassettes into a eukaryotic transient expression vector DSP1.1BGH (27). This expression system is under SV40 early promoter and bovine growth hormone polyadenylation control regions, which are flanked by polylinkers with unique *Sal*I sites. Thus, the generated "*Sal*I expression cassettes" can be easily obtained from the transient expression vector and inserted into the unique *Sal*I site of a stable plasmid expression vector (28). This latter vector contains bovine polyoma virus sequences and either the neomycin resistance gene (t-PA/scu-PA hybrid containing plasmid) or the mouse dihydrofolate reductase cDNA (29) (scu-PA containing plasmid), which confer resistance to G418 and methotrexate, respectively.[2]

Transfection and Stable Expression in Mammalian Cell Systems

Hamster kidney cells (27) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (Gibco/Bethesda Research Laboratories, Gent, Belgium or Flow, Irvine, Scotland) and 2 mM L-glutamine. Transfection was performed in 80-cm^2 tissue culture flasks with approximately 3×10^6 cells in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 50 μg/ml gentamycin (Sigma) for expression of the t-PA/scu-PA hybrid or in a mixture (1:1 volume) of DMEM/F12 special (Gibco/Bethesda Research Laboratories) lacking thymidine and hypoxanthine (for expression of scu-PA). Twenty μg of recombinant plasmid DNA, twice purified by CsCl gradient centrifugation, was added to the cells which were transfected using the calcium phosphate coprecipitation procedure (30). After 4 h of incubation, the cells were treated with 10% glycerol in DMEM for 3–4 min. After 2 days of incubation in DMEM, the cells were subcultured with a split ratio of 1:10 and subjected to selective pressure by addition of either 400 μg/ml G418 (Geneticin, Gibco/Bethesda Research Laboratories) in DMEM containing 2% fetal calf serum (t-PA/scu-PA hybrid selection) or 200–400 nM methotrexate in DMEM/F12 special supplemented with 2% dialyzed fetal calf serum (scu-PA selection). The selective medium was changed after 4–5 days, and 1–3-mm colonies became visible after 11–14 days. These colonies were isolated with a cloning cylinder and further propagated, and the culture supernatants were screened for u-PA-related antigen by ELISA and for amidolytic activity with the chromogenic urokinase substrate S-2444. Cell clones, selected on the basis of their expression level of both urokinase-like activity and u-PA-related antigen, were further expanded to 175-cm^2 cell culture flasks (Gibco/Bethesda Research Laboratories) and maintained and passaged under standard culturing conditions with split ratios of 1:10 to 1:15 at weekly intervals. Finally, two clones (clone 31 for rscu-PA and clone 138 for the t-PA/scu-PA hybrid) secreting, respectively, between 2 and 5 μg/ml or between 0.6 and 2 μg/ml urokinase equivalent (S-2444 amidolytic) activity per 48 h when cultured in 175-cm^2 flasks containing 50 ml of medium were selected for larger scale production of conditioned medium. Seven 175-cm^2 flasks of each clone were used to inoculate a 6000-cm^2 Nunc cell factory (Gibco/Bethesda Research Laboratories) containing 1.8 liters of DMEM supplemented with 2% fetal calf serum and 50 μg/ml of gentamycin. At 2- or 3-day intervals, the conditioned medium was harvested, and aprotinin (Sigma) was added to a final concentration of 20 μg/ml. After three harvests, the cell monolayers began to detach; and therefore, the cell factories were discarded.

Assay Techniques

ELISA utilizing rabbit anti-urokinase IgG-coated polystyrene plates and the murine monoclonal antibody MA-4D1E8, directed against an epitope in the B-chain of urokinase, was performed as described elsewhere (22). Clot lysis was assayed as described (22). Binding of u-PA to fibrin was performed in thrombin-clotted citrated human plasma or buffer containing plasminogen-free human fibrinogen (13). SDS-polyacrylamide gel electrophoresis was performed on 12% slab gels according to Laemmli (31), and immunoblotting on nitrocellulose sheets was performed according to Towbin *et al.* (32). Amino acid analysis was performed on a Beckman 119CL amino acid analyzer after 20 h of hydrolysis in 6 M HCl at 110 °C *in vacuo*. Specific activities were determined on fibrin plates (33) by comparison with the International Reference Preparation of Urokinase.

Characterization of Purified rscu-PA and t-PA/scu-PA Hybrid

Treatment with Plasmin—nscu-PA, rscu-PA, or the t-PA/scu-PA hybrid at a final concentration of 1 μM in 0.038 M NaCl, 0.05 M Tris·HCl buffer, pH 7.4, containing 0.01% Tween 80 was incubated at 37 °C with plasmin (final concentration, 6.8 nM). At timed intervals (10–40 min), 10 μl was removed and added to 800 μl of the same buffer containing S-2444 at a final concentration of 0.3 mM. Urokinase activity was determined from measurements of the absorbance at 405 nm and expressed in international units by comparison with the International Reference Preparation (66/46).

Activation of Plasminogen in Presence of Excess Plasmin Substrate—rscu-PA at a concentration of 7.5 nM or the t-PA/scu-PA hybrid at a concentration of 10.5 nM was incubated with varying concentrations of plasminogen (final concentration, 0.08–1 μM) in 0.038 M NaCl, 0.05 M Tris·HCl buffer, pH 7.4, containing 0.01% Tween 80 and 1 mM S-2251 (14). The generation of plasmin was determined from the absorbance at 405 nm, monitored over 3 min, and expressed in nanomolar by comparison with a plasmin calibration curve.

Binding to Purified Fibrin—Human fibrinogen (final concentration, 0–3.3 mg/ml) in 0.038 M NaCl, 0.05 M Tris·HCl buffer, pH 7.4, containing 0.01% Tween 80 and 1 mg/ml bovine serum albumin was mixed with rscu-PA, nscu-PA, the t-PA/scu-PA hybrid, or t-PA (final concentration, 50–100 ng/ml). The mixture was clotted by addition of thrombin to a final concentration of 20 NIH units/ml. Following a 1-min incubation at 37 °C, thrombin was inactivated by the addition of D-Ile-Pro-Arg chloromethyl ketone (final concentration, 10 μM) to prevent inactivation of urokinase (34), and the samples were centrifuged for 1 min at 10,000 × *g*. The concentrations of u-PA-related antigen in the supernatants were determined by ELISA as described above; t-PA was assayed with ELISA described elsewhere (35).

^{125}I-Fibrin-labeled Plasma Clot Lysis in Plasma Milieu—The relative fibrin specificity of nscu-PA, rscu-PA, the t-PA/scu-PA hybrid, and t-PA was measured in a system composed of a ^{125}I-fibrin-labeled human plasma clot suspended in human citrated plasma as described elsewhere (18). Fibrinogen levels were measured with a coagulation rate assay (36).

RESULTS

Purification and Physicochemical Characterization of rscu-PA and t-PA/scu-PA Hybrid

The results of the purification of rscu-PA and the t-PA/scu-PA hybrid from conditioned culture media are summarized in Table I. The first purification step involved the

[2] D. Gheysen, unpublished data.

TABLE I

Purification of rscu-PA and the t-PA/scu-PA hybrid

The data represent mean values of two independent preparations (total volume of starting material 3.8 and 12 liters). The first three columns are expressed per 1000 ml of starting material.

	Volume	Protein	u-PA antigen	Yield	Purification
	ml	*mg*	*mg*	*%*	*-fold*
scu-PA					
Starting material	1000	1450	1.40	100	1
Zinc chelate-Sepharose	37	51	0.60	42	12
Immunoadsorption	4.3	1.2	0.70	49	600
Benzamidine-Sepharose	7.2	0.75	0.50	35	690
Ultrogel AcA 44	4.0	0.33	0.23	17	720
t-PA/scu-PA					
Starting material	1000	1450	0.97	100	1
Zinc chelate-Sepharose	42	52	0.60	62	16
Immunoadsorption	4.4	0.75	0.34	35	650
Benzamidine-Sepharose	6.5	0.45	0.18	18	560
Ultrogel AcA 44	3.8	0.13	0.18	18	1900

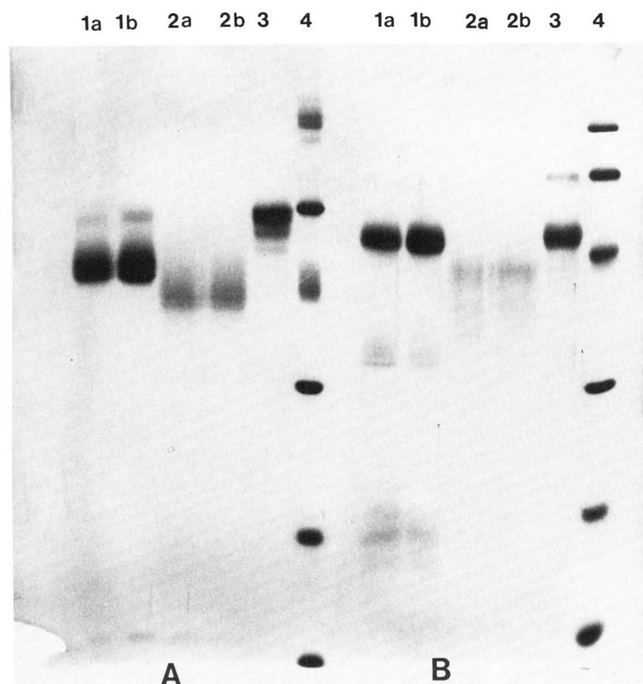

FIG. 1. **Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** *A*, without reduction; *B*, after reduction with dithioerythritol. *Lane 1*, rscu-PA (preparations *a* and *b*); *lane 2*, t-PA/scu-PA hybrid (preparations *a* and *b*); *lane 3*, nscu-PA from CALU-3; *lane 4*, calibration mixture containing phosphorylase *b* (M_r 94,000), albumin (M_r 67,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), soybean trypsin inhibitor (M_r 20,000), and α-lactalbumin (M_r 14,400).

application of between 3.8 or 12 liters of conditioned media to a 5 × 10-cm column of zinc chelate-Sepharose, previously equilibrated with 0.3 M NaCl, 0.2 M Tris·HCl buffer, pH 7.5, containing 0.01% Tween 80 and 10 kallikrein inhibitor units/ml aprotinin (Bayer), at a flow rate of 100 ml/h at 4 °C. Only between 50 and 70% of the u-PA-related antigen was adsorbed during the first passage, and the material was therefore recycled on a second column. After the conditioned media were applied, the column was washed with equilibration buffer and eluted with buffer containing 50 mM imidazole, collecting 20-ml fractions. Fibrinolytic activity, as measured by clot lysis, and u-PA-related antigen, as measured by ELISA, eluted together in a peak partially separated from the major protein peak. A recovery of u-PA-related antigen of 40–60% with 12–16-fold purification and 20–30-fold reduction in volume was achieved. Aliquots of about 100 ml of these pooled fractions were then chromatographed on a 0.8 × 5-cm immunoadsorp-

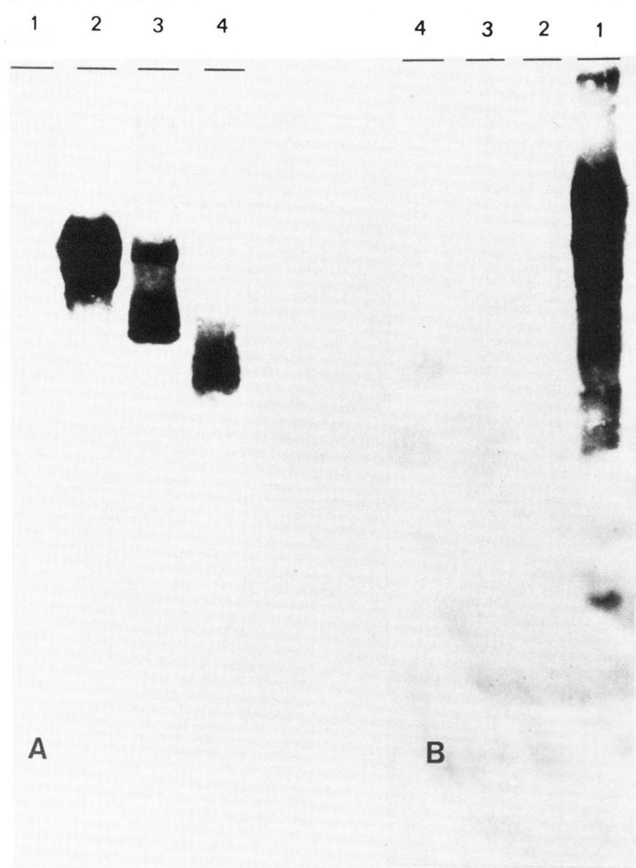

FIG. 2. **Immunoblotting of nonreduced SDS gels.** *A*, with anti-u-PA antiserum; *B*, with anti-t-PA antiserum. *Lane 1*, t-PA; *lane 2*, nscu-PA; *lane 3*, rscu-PA; *lane 4*, t-PA/scu-PA hybrid.

TABLE II

Amino acid composition of scu-PA and the t-PA/scu-PA hybrid

The numbers of amino acids were normalized to a total of 378 for nscu-PA, 377 for rscu-PA ($Gly^{366} \rightarrow Cys^{366}$ substitution), and 315 for the t-PA/scu-PA hybrid, which is the number of amino acids (excluding cysteine and tryptophan, which were not determined) expected from the primary sequences (23–26, 39). The data represent the means of three analyses.

Amino acid	nscu-PA	rscu-PA	Sequence	t-PA/scu-PA hybrid	Sequence
Asx	39	38	37	24	22
Thr	23	26	26	23	22
Ser	32	32	30	29	29
Glx	43	43	40	43	40
Pro	31	29	23	20	19
Gly	38	34	35 (34)	28	25
Ala	18	15	16	13	13
Val	16	14	19	17	17
Met	6	7	7	6	6
Ile	16	12	19	13	19
Leu	28	30	31	26	25
Tyr	15	17	18	15	16
Phe	13	12	12	11	10
His	13	16	17	11	11
Lys	24	27	26	20	20
Arg	23	22	22	19	21

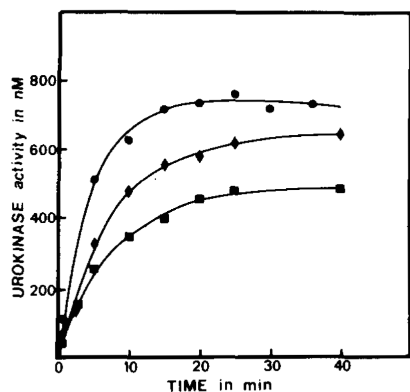

FIG. 3. **Generation of amidolytic activity by treatment with plasmin.** ●, nscu-PA; ♦, rscu-PA; ■, t-PA/scu-PA hybrid.

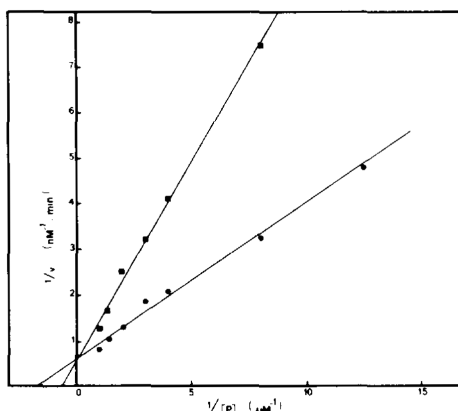

FIG. 4. **Lineweaver-Burk plot of activation of plasminogen.** ●, rscu-PA (7.5 nM); ■, t-PA/scu-PA hybrid (10.5 nM).

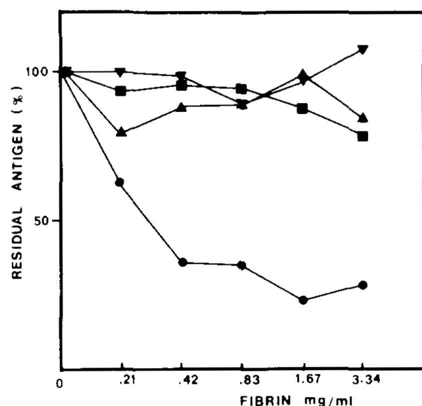

FIG. 5. **Binding to fibrin clots.** ▲, nscu-PA; ■, rscu-PA; ▼, t-PA/scu-PA hybrid; ●, t-PA. Concentrations of u-PA- or t-PA-related antigen were measured in the supernatant after thrombin-induced clotting of purified human fibrinogen.

tion column at a flow rate of 3 ml/h. The column was washed with 0.3 M NaCl, 0.02 M Tris·HCl buffer, pH 7.5, containing 0.01% Tween 80 and 10 kallikrein inhibitor units/ml aprotinin and then eluted with 2 M KSCN in the same buffer. The fractions containing u-PA-related antigen were pooled, dialyzed against buffer, and passed over a 0.6 × 3-cm column of benzamidine-Sepharose at a flow rate of 2 ml/h. This procedure efficiently eliminated small amounts of contaminating urokinase (reduction from between 1.5 and 2% to less than 0.3%). The eluates were concentrated to 10 ml by dialysis against solid polyethylene glycol 20,000 (Merck), and final purification was obtained by gel filtration on a 1.6 × 90-cm column of Ultrogel AcA 44 equilibrated with 0.3 M NaCl, 0.02 M Tris·HCl buffer, pH 7.5, containing 0.01% Tween 80 and 10 kallikrein inhibitor units/ml aprotinin. Table I summarizes the yields and purification factors in the consecutive purification steps obtained with two independent preparations of rscu-PA and the t-PA/scu-PA hybrid. The specific activities of the final products measured on fibrin plates were 63,000 IU/mg for rscu-PA and 33,000 IU/mg for the t-PA/scu-PA hybrid.

The purified proteins were analyzed by SDS-polyacrylamide gel electrophoresis as shown in Fig. 1. Purified rscu-PA migrated as a triplet band under nonreducing conditions but as a single main band with M_r ~50,000 under reducing conditions. All three bands seen on the unreduced gels represented rscu-PA as evidenced by their reactivity with urokinase antiserum on immunoblotting (Fig. 2*A*). The purified t-PA/scu-PA hybrid also migrated as a triplet band under nonreducing conditions and as one broad band with M_r ~43,000 under reducing conditions (Fig. 1). All bands reacted with antibodies against u-PA (Fig. 2*A*) and also, but very weakly, with antibodies against t-PA (Fig. 2*B*).

The amino acid compositions of rscu-PA and the t-PA/scu-PA hybrid are represented in Table II. The observed amino acid compositions are in agreement with those derived from the known sequences of rscu-PA and the t-PA/scu-PA hybrid.

Functional Characterization of rscu-PA and t-PA/scu-PA Hybrid

Generation of Amidolytic Activity following Treatment with Plasmin—Plasmin (0.7% on a molar basis) causes a time-dependent conversion of nscu-PA, rscu-PA, and the t-PA/scu-PA hybrid to amidolytically active urokinase (Fig. 3). Maximal conversion results in an amidolytic activity measured with S-2444, corresponding to a specific activity of about 70,000 IU/mg for nscu-PA, 67,000 IU/mg for rscu-PA, and 49,000 IU/mg for the t-PA/scu-PA hybrid.

Kinetics of Activation of Plasminogen—The activation of plasminogen by both rscu-PA and the t-PA/scu-PA hybrid obeys Michaelis-Menten kinetics, as evidenced by linear double reciprocal plots of the initial activation rate (v) *versus* the plasminogen concentration ([P]) (Fig. 4). From these Lineweaver-Burk plots, $K_m = 0.53$ μM and $k_2 = 0.0034$ s^{-1} for rscu-PA and $K_m = 1.4$ μM and $k_2 = 0.0027$ s^{-1} for the t-PA/scu-PA hybrid were obtained, which are similar to the constants previously reported for nscu-PA (16, 37).

Binding to Fibrin—Fig. 5 illustrates that nscu-PA, rscu-PA, and the t-PA/scu-PA hybrid, in contrast to t-PA, do not bind specifically to fibrin.

Thrombolytic Properties—rscu-PA, the t-PA/scu-PA hybrid, and nscu-PA caused a very similar time- and concentration-dependent lysis of a ^{125}I-fibrin-labeled clot immersed in citrated human plasma (Fig. 6). Clot lysis was associated with comparable degrees of fibrinogen degradation, which at equipotent doses, was more pronounced than that observed with t-PA.

DISCUSSION

The molecular structures involved in the fibrin specificity of t-PA and scu-PA are different. The clot selectivity of t-PA is mediated via its binding to fibrin (10), and the NH_2-terminal finger-like domain has been claimed to be involved in this interaction (11, 12). We have hypothesized that the fibrin specificity of scu-PA depends on its single chain structure (13, 14); and recently, it was demonstrated that the amino-terminal region is not involved in this interaction (22). Based on the hypothesis that the structures in t-PA and scu-

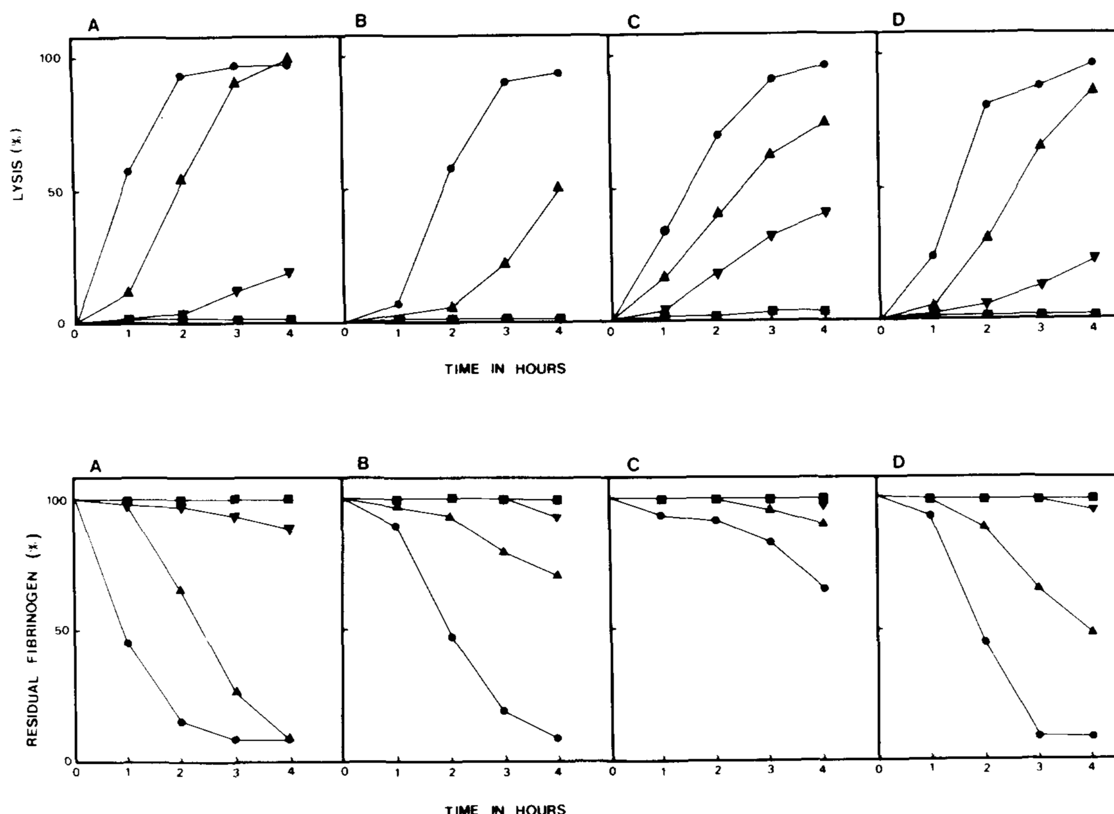

FIG. 6. **Lysis of ^{125}I-fibrin-labeled human plasma clots immersed in human plasma.** *A*, rscu-PA at 0.5 (■), 1 (▼), 2 (▲), and 4 (●) μg/ml; *B*, t-PA/scu-PA hybrid at 0.5 (■), 1 (▼), 2 (▲), and 4 (●) μg/ml; *C*, t-PA at 35 (■), 70 (▼), 150 (▲), and 300 (●) ng/ml; *D*, nscu-PA at 0.5 (■) 1 (▼), 2 (▲), and 4 (●) μg/ml.

PA responsible for fibrin binding are localized in independent, autonomously folding domains (12), we have purified and functionally characterized a hybrid protein, obtained by fusion of the cDNA encoding the finger region of t-PA (67 amino-terminal amino acids) to that encoding the carboxyl-terminal region of u-PA (amino acids 136–411), and compared it with rscu-PA, obtained by expression of the cDNA encoding full-length scu-PA in eukaryotic cells.

rscu-PA was obtained in the single chain form by preventing conversion to two-chain u-PA during purification by addition of aprotinin to all buffers. The resulting protein was indistinguishable from nscu-PA on the basis of its M_r (reduced SDS gels), amino acid composition, specific activity, and reactivity with anti-u-PA antiserum. However, on nonreduced SDS gels, its mobility was significantly faster than that of scu-PA. This may be due to abnormal folding of the rscu-PA protein, which differs in two amino acids from the natural scu-PA. Indeed, the rscu-PA expressed by the pULB1000 cDNA has $Cys^{131} \rightarrow Trp^{131}$ and $Gly^{366} \rightarrow Cys^{366}$ substitutions, which may give rise to the formation of a Cys^{50}-Cys^{366} disulfide bond, which is not present in the natural protein. In this case, the incorrect folding of the protein does, however, no longer appear to alter its enzymatic properties.

The t-PA/scu-PA hybrid migrated on both reduced and unreduced SDS gels in a position compatible with its anticipated M_r of 43,000, although the band on reduced gels was unexpectedly broad and stained only weakly. Thus, the t-PA/scu-PA hybrid was also obtained with an intact Lys^{158}-Ile^{159} peptide bond. The hybrid protein, with a very low intrinsic amidolytic activity, was converted to an amidolytically active two-chain structure by plasmin. It activated plasminogen obeying Michaelis-Menten kinetics, with kinetic constants very similar to those of rscu-PA. It did, however, not bind specifically to fibrin. In a plasma milieu, the hybrid protein had a relative fibrin specificity comparable, but not superior, to that of rscu-PA. Thus, surprisingly, fusion of the finger domain of t-PA to the truncated scu-PA did not confer affinity for fibrin to the hybrid molecule. This may be due to the fact that, contrary to our working hypothesis, the finger region of t-PA is not the main domain responsible for the fibrin binding of t-PA, as indeed has been recently demonstrated (12, 38). Alternatively, by fusion of the amino-terminal 67 amino acids in t-PA to the truncated scu-PA, Cys^{56} of t-PA, located in the epidermal growth factor-like domain, can no longer form a disulfide bond with Cys^{73}, but instead, might react with the mutagenized Cys^{366} of rscu-PA. This would, however, not explain an incorrect folding of the finger region, if this region indeed constitutes an autonomous structural and functional domain (12).

In conclusion, the present study shows that the truncated rscu-PA may serve as an acceptor for fusion of t-PA domains to its amino-terminal end, without loss of its enzymatic properties. Whether fusion proteins of other domains of the t-PA molecule with this truncated rscu-PA, such as constructed and described elsewhere (26), may constitute plasminogen activators with improved fibrin selectivity remains to be investigated.

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