Single-Chain Urokinase-Type Plasminogen Activator Does Not Possess Measurable Intrinsic Amidolytic or Plasminogen Activator Activities†

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ABSTRACT: The question whether single-chain urokinase-type plasminogen activator (Sc-uPA) possesses an enzymatic activity has been a subject of intense investigation for a number of years but still remains unresolved. Recent studies from several laboratories suggest that Sc-uPA or its plasmin-resistant mutants obtained by site-directed mutagenesis possess significant, albeit low, amidolytic and plasminogen activator activities, ranging from 0.1% to 1% of that observed for two-chain urokinase (Tc-uPA). In an effort to characterize these putative intrinsic activities, Sc-uPA was repeatedly treated with dansyl-Glu-Gly-Arg chloromethyl ketone (dansyl-EGRck) or diisopropyl fluorophosphate (DFP) (0.1–0.25 mM added thrice over a period of 24 h at 0 °C). This treatment exhaustively inactivated the Tc-uPA contaminant but did not affect Sc-uPA, as evidenced by the lack of significant incorporation of radiolabeled inhibitor in Sc-uPA and full activation of the inhibitor-treated Sc-uPA by plasmin. Assayed in the presence of excess DFP or dansyl-EGRck to ensure trapping of any Tc-uPA generated in the assay mixture, Sc-uPA (84 μg/mL, 10500 latent units/mL) did not elicit any detectable cleavage of the chromogenic substrate S-2444 (detection limit 0.1 unit of Tc-uPA/mL). However, if the Tc-uPA inhibitors were removed prior to assay, a trace amount of amidolytic activity invariably reappeared in the Sc-uPA preparation. Incorporation experiments with [^{3}H]DFP suggested that the appearance of this amidolytic activity was due to formation of Tc-uPA. Plasminogen activator assay of DFP- and dansyl-EGRck-treated Sc-uPA (0.45–2.25 μM), performed in the presence of these inhibitors and Trasylol (10 μM) to ensure entrapment of any Tc-uPA or plasmin generated in the reaction mixture, showed no significant cleavage of ^{125}I-labeled plasminogen (detection limit 0.1 nM). However, if dansyl-EGRck and DFP were removed from the inhibitor-treated Sc-uPA and the assay was performed in the presence of Trasylol alone, there was significant cleavage of ^{125}I-plasminogen due to contamination by Tc-uPA. Fibrin, a positive effector of plasminogen activation by Tc-uPA or Sc-uPA preparations in the absence of DFP and dansyl-EGRck, did not promote cleavage of plasminogen or S-2444 by Sc-uPA in the presence of the Tc-uPA inhibitors. The present findings indicate that, under conditions stringently excluding Tc-uPA contamination, neither recombinant human Sc-uPA expressed in Chinese hamster ovary cells nor Sc-uPA secreted by fetal kidney cells or a transformed line of kidney cells shows measurable amidolytic activity above the detection limit of 0.001% or plasminogen activator activity above the detection limit of 0.01% of Tc-uPA activity. These studies suggest that the intrinsic activities ascribed to Sc-uPA or its plasmin-resistant mutants arise from small amounts of Tc-uPA, possibly generated from Sc-uPA by the action of traces of contaminating proteases that are not susceptible to inactivation by usual inhibitors of trypsin-like serine proteases.

Urokinase-type plasminogen activator (uPA)[1] is synthesized and secreted by cells as a 50-kDa single-chain glycoprotein that is variously referred to as single-chain urokinase (Sc-uPA) or prourokinase [review by Lijnen et al. (1987a) and references cited therein]. Sc-uPA is converted to two-chain urokinase (Tc-uPA) following cleavage of the Lys^{158}–Ile^{159} peptide bond by plasmin (Wun et al., 1982; Nielsen et al., 1982), plasma kallikrein, or some other serine proteases (Ichinose et al., 1986). Unlike Tc-uPA, Sc-uPA does not react with DFP (Wun et al., 1982; Eaton et al., 1984; Kasai et al., 1985; Pannell & Gurewich, 1987), peptide chloromethyl ketone derivatives (Lijnen et al., 1987b), and a variety of serine protease inhibitors (Eaton et al., 1984; Andreasen et al., 1986; Stump et al., 1986; Wun & Reich, 1987), suggesting that the single-chain form of urokinase represents the zymogen form of uPA. However, conflicting evidence has been presented to show that Sc-uPA possesses varying degrees of enzymatic activity. Initially believed to be catalytically as efficient a plasminogen activator as Tc-uPA (Collen et al., 1986), recent investigations have suggested that Sc-uPA possesses a low but significant intrinsic plasminogen activator activity between 0.4% and 6% (Ellis et al., 1987; Pannell & Gurewich, 1987; Lijnen et al., 1989, 1990) and amidolytic activity between 0.1% and 0.2% (Gurewich et al., 1984; Pannell & Gurewich, 1986; Booyse et al., 1988) of the corresponding activities of Tc-uPA. Other studies have, however, suggested that Sc-uPA may not possess a significant plasminogen activator activity (Kasai et al., 1985; Urano et al., 1988; Petersen et al., 1988).

The wide discrepancy in the plasminogen activator activity of Sc-uPA reported by various investigators is believed to be largely due to the technically difficult problem of avoiding facile conversion of Sc-uPA to Tc-uPA by the product of

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[1] Abbreviations: α_2-PI, α_2-plasmin inhibitor; BSA, bovine serum albumin; CHO cells, Chinese hamster ovary cells; dansyl, (dimethylamino)naphthalene-1-sulfonyl; DFP, diisopropyl fluorophosphate; EGRck, Glu-Gly-Arg chloromethyl ketone; KIU, kallikrein inactivator units; LMW-uPA, low-molecular-weight urokinase-type plasminogen activator; PAI-1, plasminogen activator inhibitor type 1; S-2444, pyro-Glu-Gly-Arg-*p*-nitroanilide; Sc-uPA, single-chain urokinase-type plasminogen activator (prourokinase); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tc-uPA, two-chain urokinase-type plasminogen activator; uPA, urokinase-type plasminogen activator.

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plasminogen activation, plasmin. To prevent this conversion, mutants of Sc-uPA in which the plasmin cleavage site Lys^{158} is replaced by various plasmin-resistant amino acids have been constructed by site-directed mutagenesis (Nelles et al., 1987; Gurewich et al., 1988; Lijnen et al., 1988a,b, 1990). The single-chain forms of these mutants have been shown to possess plasminogen activator activities ranging from 0.2% to 0.5% and amidolytic activites ranging from 0.1% to 0.2% of the corresponding activities of Tc-uPA. The expression of these activities by the plasmin-resistant mutants is believed to provide a confirmatory evidence for the view that Sc-uPA is an "active zymogen" that possesses a significant, albeit low, intrinsic enzymatic activity.

Sc-uPA and plasminogen are mutually stable in plasma milieu in the absence of fibrin clot. Formation of fibrin, however, provides a stimulus for activation of plasminogen by Sc-uPA. In view of the conflicting evidence about the activity of Sc-uPA, it is uncertain if plasminogen activation is initiated by the putative intrinsic activity of Sc-uPA or requires initial proteolytic conversion of an inactive Sc-uPA by another protease. There is general agreement, however, that the initial activation of plasminogen is followed by conversion of Sc-uPA to Tc-uPA by the product plasmin and that a feedback loop is established. Since the question of enzymatic activity of Sc-uPA is central to the elucidation of the mechanism of initiation of Sc-uPA-mediated clot lysis, I undertook to thoroughly characterize its putative intrinsic amidolytic and plasminogen activator activities and examine the effects of fibrin on these activities. I report that, after exhaustive inhibition of contaminating Tc-uPA, coupled with effective entrapment of any Tc-uPA or plasmin produced during assay, Sc-uPA does not cleave the chromogenic substrate pyroGlu-Gly-Arg-*p*-nitroanilide (S-2444) or the natural substrate plasminogen at a measurable rate.

Experimental Procedures

Materials

Human Sc-uPA expressed and purified from CHO cells was obtained from Collaborative Research, Inc., Bedford, MA. Sc-uPA purified from fetal kidney cell culture was provided by Abbot Chemical Co., Chicago, IL. Sc-uPA isolated from a transformed human kidney cell line TCL-598 was obtained from Sandoz, Vienna, Austria.

Tcp-uPA was prepared from Sc-uPA by treatment with plasmin (2:100 molar ratio) for 3 h at 37 °C. Plasmin was then removed by adsorption on aprotinin–Sepharose. SDS–PAGE of the product showed complete conversion of Sc-uPA to Tc-uPA.

Glu-plasminogen was prepared from fresh human citrated plasma by chromatography on Lys–Sepharose (Deutsch & Mertz, 1970) in the presence of Trasylol (50 KIU/mL). The eluant ϵ-aminocaproic acid was removed from the plasminogen preparation by gel filtration. Any Lys-plasminogen contaminant present in the preparation was removed by chromatography on DEAE-Sephadex (Wallen & Wiman, 1972).

Human fibrinogen (grade L, Kabi) was obtained from Helena Laboratories, Beaumont, TX. Plasminogen was removed from the product by two consecutive passages through columns of lysine–Sepharose.

Fibrin monomer was prepared as described by Laudano and Doolittle (1980). A sonicated suspension of fibrin was prepared by the method of Lucas et al. (1983) as modified by Husain et al. (1989).

Other chemicals and supplies were obtained from the following sources: pyroGlu-Gly-Arg-*p*-nitroanilide (S-2444), Kabi Vitrum, Stockholm, Sweden; dansyl-Glu-Gly-Arg chloromethyl ketone and protein-grade Tween 80, Calbiochem, San Diego, CA; DFP, Aldrich, Milwaukee, WI; [^{3}H]DFP, New England Nuclear, Boston, MA; fibrinogen fragment FCB-2, American Diagnostica, Greenwich, CT; Trasylol, Mobay Corp., New York, NY; NCS tissue solubilizer, Amersham, Arlington Heights, IL; Econo-Pac 10DG prepacked gel filtration columns, Bio-Rad, Richmond, CA; Centricon 10 and 30 microconcentrators, Amicon, Danvers, MA.

Buffers. Buffer 1 was 0.05 M Tris-HCl, 0.15 M NaCl, 0.02% Tween 80, 50 KIU/mL Trasylol, pH 7.4. Buffer 2 was the same as buffer 1, but omitting Trasylol. Buffer 3 was 0.01 M sodium acetate, 0.15 M NaCl, 0.02% Tween 80, and 50 KIU/mL Trasylol, pH 4.8.

Methods

Radioiodination of Plasminogen. Plasminogen was radiolabeled with ^{125}I by using solid-phase lactoperoxidase. Glu-plasminogen (10 μg) was incubated with ^{125}I (1 mCi), β-D-glucose (0.25 mg), and immobilized lactoperoxidase in 100 mM sodium phosphate buffer, pH 7.2 (final volume of 150 μL), for 8 min at room temperature. Excess ^{125}I was removed by gel filtration on an Econo-Pac DG10 column, equilibrated with 0.01 M sodium phosphate, 0.15 M NaCl, and 0.02% Tween 80, pH 7.4. Specific activity of the radiolabeled plasminogen was (4–6) $\times$ 10^6 cpm/μg.

Assay for Amidolytic Activity. Amidolytic assays were performed with 0.25 mM S-2444 in buffer 1 at 37 °C. The change in absorbance at 406 nm was continuously monitored by using a Hewlett Packard diode array spectrophotometer.

Determination of Latent Amidolytic Activity of Sc-uPA. Sc-uPA was incubated with plasmin at a molar ratio of 2% in buffer 2 at 37 °C. Aliquots were withdrawn at various time intervals, diluted with the appropriate amount of buffer 1, and assayed as above.

Effects of DFP or Dansyl-EGRck on Amidolytic Activities of Sc-uPA and Tc-uPA. Rates of inhibition of amidolytic activities of Sc-uPA preparation and Tc-uPA by DFP were compared following treatment of Sc-uPA (225 μg/mL, 28 125 latent units/mL) and Tc-uPA (600 units/mL) with 100 μM DFP in buffer 1. Further additions of similar amounts of DFP were made at 4 and 20 h, and the mixture was incubated for a total of 24 h at 0 °C. Aliquots were withdrawn at various time intervals and assayed with S-2444 following 3–6-fold dilution in the assay solution.

The minimum concentration of DFP or dansyl-EGRck required for complete inactivation of the amidolytic activity of Tc-uPA was determined by titration of Tc-uPA with these inhibitors. For this purpose, Tc-uPA (600 units/mL) was incubated with a series of solutions containing increasing concentrations of DFP (0, 10, 20, 40, 80, 160, 320, 640, and 1280 μM) or dansyl-EGRck (0, 5, 10, 20, 40, 80, and 160 μM) in buffer 1 for 20 h at 0 °C. The amidolytic activities were determined with 0.25 mM S-2444 following 4-fold dilution in the assay mixture.

Effect of the lowest concentration of DFP (1280 μM) or dansyl-EGRck (160 μM) that completely blocked the amidolytic activity of Tc-uPA was examined on the activity of Sc-uPA (225 μg/mL, 28 125 latent units/mL) as described above. In other experiments, amidolytic activity of Sc-uPA was determined following repeated treatment with DFP (3 $\times$ 250 μM) or dansyl-EGRck (3 $\times$ 100 μM) over a period of 20 h at 0 °C.

Dialysis of Sc-uPA after treatment with DFP (3 $\times$ 250 μM) or dansyl-EGRck (3 $\times$ 100 μM) was performed by microconcentration in Centricon 30. The reaction mixture (200 μL)

was diluted to 2 mL with the dialysis buffer and concentrated to 50–100 μL. After repeating the cycles of dilution and concentration three more times, the concentrate was collected and used for amidolytic assay. The whole dialysis procedure took 3–4 h.

Amidolytic Activity of DFP- or Dansyl-EGRck-Treated Sc-uPA after Activation with Plasmin. Samples of the inhibitor-treated Sc-uPA (10 μL containing 2.25 μg of protein) were diluted with 2 mL of buffer 2 containing 0.1 mg/mL BSA and then dialyzed by microconcentration in a Centricon 30 using buffer 2 as described above. In a parallel control experiment, a similar quantity of Sc-uPA, containing a determined amount of latent activity, was dialyzed. Following activation of the dialyzed samples with plasmin for 2 h at 37 °C, amidolytic activities were determined with S-2444 as described above.

Incorporation of [^{3}H]DFP in Tc-uPA and Sc-uPA. Sc-uPA (112.5 μg/mL, 14060 units/mL) was incubated with 0.1, 0.2, 0.5, and 1 mM [^{3}H]DFP (specific activity 97 μCi/μmol) in buffer 1 for 15 h in ice. Excess [^{3}H]DFP was then removed by extensive dialysis with buffer 3 in Centricon 30. Control experiments containing [^{3}H]DFP but no Sc-uPA were performed in parallel. Incorporation of [^{3}H]DFP in Sc-uPA was determined by scintillation counting.

The reaction of Tc-uPA (29 μg/mL) with [^{3}H]DFP (0.005, 0.01, 0.02, and 0.05 mM) was carried out as above.

Determination of Traces of Tc-uPA in Sc-uPA Preparations from [^{3}H]DFP Incorporation. Sc-uPA (10 μL containing 22.5 μg of protein), buffer 1 (70 μL), and [^{3}H]DFP (20 μL of 330 μM solution containing 20 μCi) were mixed together and incubated for 2 h at room temperature. Excess DFP was then dialyzed with buffer 1 in Centricon 30. The dialyzed, concentrated solution was mixed with unlabeled Sc-uPA and LMW-uPA (2 μg each) as markers, treated with reducing sample buffer for 3 min at 100 °C, and analyzed by SDS–PAGE. After Coomassie Blue staining, bands corresponding to Sc-uPA and LMW-uPA (catalytic chain of Tc-uPA) as well as adjacent bands were cut out and extracted by incubation with NCS tissue solubilizer (120 μL of 50% solution) for 24 h at 60 °C. Radioactivity in the suspension was determined by liquid scintillation counting.

In order to determine if Sc-uPA preparations, treated with DFP and dansyl-EGRck to exhaustively inactivate contaminating Tc-uPA, regenerate traces of Tc-uPA after removal of the inhibitors, Sc-uPA (22.5 μg) in 170 μL of buffer 1 was treated with 10 μL of 5 mM unlabeled DFP solution at 0 °C. After 4 h, an additional 10 μL of DFP was added and incubation continued for a total of 20 h in ice. Finally, 10 μL of 1 mM DFP and 2 mM dansyl-EGRck was added and the solution incubated at room temperature for 2 h. Excess unlabeled inhibitors were then dialyzed off by microconcentration in Centricon 30. Any Tc-uPA regenerated after removal of the unlabeled inhibitors from Sc-uPA was determined by treatment of the dialyzed preparation with [^{3}H]DFP, followed by SDS–PAGE as described above.

Effects of Fibrin Monomer, Polymeric Fibrin, or Fibrin Fragment FCB-2 on the Amidolytic Activity of DFP-Treated Sc-uPA. The effect of fibrin monomer (100 μg/mL) on the amidolytic activity of DFP-treated Sc-uPA (84 μg/mL) was investigated in the presence of 2 mM Gly-Pro-Arg-Pro as a polymerization inhibitor (Laudano & Doolittle, 1980) with S-2444 (0.25 mM) in buffer 1. The change in absorbance at 406 nm was monitored.

The effect of fibrin fragment FCB-2 (final concentration 50 μg/mL) on the amidolytic activity of DFP-treated Sc-uPA was investigated as above, omitting the polymerization inhibitor.

The effect of polymeric fibrin on the amidolytic activity of DFP-treated Sc-uPA was investigated by incubating the Sc-uPA preparation with fibrin suspension (1 mg/mL final concentration) and S-2444 (0.25 mM) in buffer 1 at 37 °C. Aliquots were removed at various time intervals, fibrin was separated by brief centrifugation in a microcentrifuge, and the absorbance of the supernatant was measured at 406 nm.

Assay for Activation of Plasminogen by Sc-uPA or Tc-uPA. Prior to the assay, plasminogen and Sc-uPA were treated with DFP and dansyl-EGRck to exhaustively inactivate traces of active proteases as follows. A mixture of plasminogen (0.1 mg of protein) and ^{125}I-plasminogen (0.25 μg of protein containing 1.5×10^6 cpm) in 200 μL of buffer 2 was treated with 0.1 mM DFP at 0 °C. Two additional increments of similar amounts of DFP were added at 4 and 20 h, and the incubation was continued for a total of 24 h in ice. Sc-uPA (22.5 μg) in 200 μL of buffer 2 was treated with DFP in a similar fashion. Before use, Sc-uPA was mixed with an equal volume of a solution containing 1 mM DFP, 0.2 mM dansyl-EGRck, and 2000 KIU/mL Trasylol in buffer 2. The concentration of DFP in this mixture was 0.65 mM, which was slightly lower than the minimum concentration of 1.28 mM found from the titration experiment for complete inhibition of Tc-uPA, but 0.25 mM dansyl-EGRck in the mixture, compared to the minimum of 0.16 mM required, assured a Tc-uPA-free Sc-uPA before mixing with plasminogen. The combination of the two inhibitors plus Trasylol assured effective trapping of Tc-uPA as well as plasmin generated during the assay.

The reaction was started by combining the treated Sc-uPA (200 μL) and plasminogen (200 μL) with buffer 2 containing 2000 KIU/mL Trasylol, 1 mM DFP, and 0.2 mM dansyl-EGRck (100 μL) to give a solution having a final composition of 0.45 μM Sc-uPA (a higher concentration of 2.25 μM was used in some studies), 2.2 μM plasminogen, 600 KIU/mL Trasylol, 580 μM DFP and hydrolyzed products, and 80 μM dansyl-EGRck and hydrolyzed products. The mixture was incubated at 37 °C, and aliquots (50 μL) were withdrawn at 0-, 10-, 20-, 30-, 40-, 50-, and 60-min intervals, mixed with equal volume of SDS–PAGE sample buffer, containing 2% SDS and 40 mM dithiothreitol, and heated for 3 min at 100 °C. Ten microliters from each prepared sample was analyzed by SDS–PAGE. After staining the gel with Coomassie Blue, autoradiography was performed on Kodak X-Omat AR or Ortho G films using Quanta III intensifying screens. The later film was slower than the former but gave a much clearer background.

To investigate activation of plasminogen by Sc-uPA in the absence of the Tc-uPA inhibitors, plasminogen and Sc-uPA were treated with DFP and dansyl-EGRck as described above. The proteins were then extensively dialyzed with buffer 2 or 3 in Centricon 30. Activation of plasminogen was examined in the presence of 600 KIU/mL Trasylol as described above.

Activation of plasminogen by Tc-uPA (0.002 μM) was investigated without pretreatment of the proteins with DFP and dansyl-EGRck. The assay was performed in the presence of 600 KIU/mL Trasylol.

The effect of hydrolyzed DFP and dansyl-EGRck on plasminogen activation by Sc-uPA (treated with DFP and dansyl-EGRck and then dialyzed) or Tc-uPA was investigated by including hydrolyzed DFP (2.5 mM) and hydrolyzed dansyl-EGRck (350 μM) in the assay mixture containing 600 KIU/mL Trasylol but no DFP or dansyl-EGRck. The hydrolyzed inhibitors were obtained by incubating DFP (100

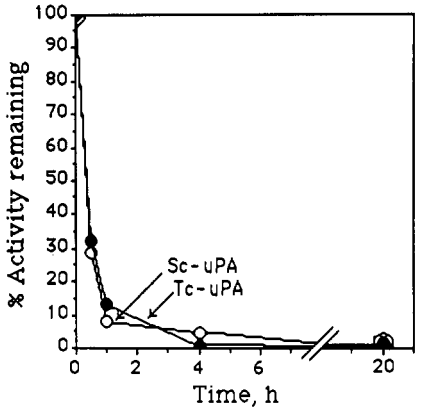

FIGURE 1: Inhibition of amidolytic activities of Tc-uPA and Sc-uPA by DFP. Sc-uPA (4.5 μM) or Tc-uPA (0.1 μM) was incubated with DFP (0.1 mM) at 0 °C. Additional amounts of 0.1 mM DFP were added at 4 and 20 h. At various time intervals aliquots were removed and assayed with S-2444 as described under Experimental Procedures.

mM) and dansyl-EGRck (14 mM) at pH 10.

Effect of Fibrin on Plasminogen Activation. Activation of plasminogen by Sc-uPA was investigated in the presence of a sonicated suspension of fibrin (final concentration 3 mg/mL) with or without DFP and dansyl-EGRck. Aliquots of the reaction suspension were withdrawn and heated at 100 °C with the sample preparation buffer as described above. Fibrin was solubilized during this treatment, allowing application of the samples to polyacrylamide gel without any other manipulation.

RESULTS

Amidolytic Activities of Sc-uPA Preparations. The chromogenic substrate S-2444 was cleaved by the preparations of recombinant Sc-uPA, fetal kidney Sc-uPA, and transformed kidney cell Sc-uPA at rates of 0.6%, 0.4%, and 5% compared to the rates obtained after activation of these preparations by plasmin.

Effects of DFP and Dansyl-EGRck on Amidolytic Activities of Sc-uPA Preparations. To distinguish the contribution of the putative intrinsic amidolytic activity of Sc-uPA from that of Tc-uPA contaminant usually present in Sc-uPA preparations, effects of DFP and dansyl-EGRck on the amidolytic activity of Sc-uPA were investigated.

Treatment of Sc-uPA with DFP (0.1 mM) with repeat additions of similar amounts of the inhibitor at 4- and 15-h intervals inhibited the amidolytic activity in a time-dependent manner. After 20-h incubation on ice, essentially all the amidolytic activity of Sc-uPA was abolished. Reaction of Tc-uPA with DFP under identical conditions gave an inactivation profile that was similar to the loss of the Sc-uPA amidolytic activity (Figure 1).

Activation of the DFP-treated Sc-uPA with plasmin resulted in an active preparation which had an amidolytic activity essentially indistinguishable from that of the untreated preparation, suggesting that DFP does not impair the latent activity of Sc-uPA.

Incorporation experiments with [^{3}H]DFP showed that while Tc-uPA was maximally labeled (0.6 mol of [^{3}H]DFP/mol of Tc-uPA) at 50 μM inhibitor concentration, Sc-uPA did not incorporate significant amounts (<0.05 mol/mol of Sc-uPA) of the labeled inhibitor even at 1 mM DFP.

To investigate if Sc-uPA possesses a trace amount (0.1–0.2%) of intrinsic amidolytic activity as suggested by some investigators, high concentrations of recombinant Sc-uPA, fetal kidney cell Sc-uPA, and transformed kidney cell Sc-uPA were repeatedly treated with DFP and dansyl-EGRck to exhaustively inactivate Tc-uPA contaminant. Amidolytic assay was performed after dilution but without complete removal of the inhibitors (the concentration of the inhibitor plus decomposition products was 100–250 μM in the assay mixture). The presence of excess inhibitors assured that any Tc-uPA generated in the reaction mixture was effectively trapped. Under these conditions, none of the Sc-uPA preparations cleaved S-2444 at a measurable rate, suggesting that Sc-uPA does not possesses an amidolytic activity above the detection limit of 0.001% of the Tc-uPA activity. Amidolytic activities of various Sc-uPA preparations before and after treatment with DFP are summarized in Table I.

Table I: Cleavage of S-2444 by Various Preparations of Sc-uPA[a]

type of sample	A_{406}, increase in 10 min	% latent act.
(1) Sc-uPA Preparation without Plasmin Activation		
recombinant	1.15	0.6
fetal kidney cell	0.534	0.375
transformed kidney cell[b]	1.0 (in 2.5 min)	5.4
(2) Sc-uPA Preparation after Plasmin Activation		
recombinant	1.24 × 150[c]	100
fetal kidney cell	0.65 × 220[c]	100
transformed kidney cell	0.645 × 125[c]	100
(3) Sc-uPA Preparation after Treatment with DFP or Dansyl-EGRck		
recombinant	<0.001	<0.001
fetal kidney cell	<0.002	<0.002
transformed kidney cell	<0.001	<0.001
(4) Inhibitor-Treated Sc-uPA after Plasmin Activation		
recombinant	1.36 × 150[c]	110
fetal kidney cell	ND[d]	
transformed kidney cell	ND[d]	

[a] The amidolytic activities in groups 1 and 3 are compared to the plasmin-activatable latent activities of the respective samples in group 2. The activity of the sample in group 4 is compared to the untreated, dialyzed control sample as described under Methods. [b] A preparation possessing a relatively high starting amidolytic activity was used to show the effectiveness of the inhibitor treatment in abolishing activity. Other preparations had less than 0.5% activity. [c] Dilution factor compared to the samples in group 1. [d] ND, not determined.

Table II: Titration of Tc-uPA with DFP or Dansyl-EGRck[a]

μM DFP	0	40	80	160	320	620	1280
% active	100	1.0	0.8	0.6	0.5	0.2	<u>0.1</u>[b]
μM EGRck	0	5	10	20	40	80	160
% active	100	0.7	0.6	0.4	0.4	0.2	<u>0.1</u>[b]

[a] Titration was performed to determine the lowest concentration of DFP or dansyl-EGRck that destroys all detectable traces of Tc-uPA activity. Tc-uPA (600 units/mL) was incubated at the given concentrations of the inhibitors in buffer 1 for 20 h at 0 °C. The amidolytic activity was determined with 0.25 mM S-2444 following 4-fold dilution with the incubation buffer. [b] The underlined numbers indicate the limit of detection.

To examine the possibility that DFP or dansyl-EGRck block the putative intrinsic amidolytic activity of Sc-uPA by a reversible mechanism and not by blockage of Tc-uPA contaminant in the Sc-uPA preparation, Tc-uPA was titrated with DFP and dansyl-EGRck, and the lowest concentration of these inhibitors found to completely block the amidolytic activity of Tc-uPA was used to treat Sc-uPA. Table II shows that although 99% of Tc-uPA is inactivated at 40 μM DFP, for 99.9% inhibition, the detection limit under the condition of the assay, treatment with 1280 μM DFP is required. A minimum concentration of 160 μM dansyl-EGRck is required for inhibition of Tc-uPA to the detection limit. Sc-uPA treated with 1280 μM DFP or 160 μM dansyl-EGRck and subsequently assayed under identical conditions as for Tc-uPA showed no amidolytic activity. Because of the instability of

Table III: Amidolytic Activity of DFP- and Dansyl-EGRck-Treated Sc-uPA after Removal of the Tc-uPA Inhibitors

sample	% latent act.
(1) starting Sc-uPA	0.6
(2) inhibitor-treated Sc-uPA, dialyzed with buffer 2	0.33
(3) inhibitor-treated Sc-uPA, dialyzed with buffer 1	0.05
(4) inhibitor-treated Sc-uPA, dialyzed with buffer 3	0.02
(5) inhibitor-treated Sc-uPA, undialyzed	<0.001

[a] Recombinant Sc-uPA was treated with DFP and dansyl-EGRck to exhaustively inactivate Tc-uPA contaminant. Amidolytic assay was performed without removal of the inhibitors or after dialysis by microconcentration with buffer 1 (0.05 M Tris-HCl, 0.15 M NaCl, 0.02% Tween 80, 50 KIU/mL Trasylol, pH 7.4), buffer 2 (same as buffer 1, but omitting Trasylol), or buffer 3 (0.01 M sodium acetate, 0.15 M NaCl, 0.02% Tween 80, 50 KIU/mL Trasylol, pH 4.8). The amidolytic activities in rows 1 and 5 are compared to the plasmin-activatable latent activity of the undialyzed preparation while those in rows 2–4 are compared to the latent activity of control Sc-uPA samples dialyzed with buffer 2.

DFP and dansyl-EGRck in the incubation buffer, complete inactivation of the Tc-uPA contaminant is Sc-uPA preparations is achieved at lower effective concentrations of DFP or dansyl-EGRck by using multiple additions over a period of time than with a single dose used in the titration experiment. Treatment of Sc-uPA with 3 × 250 μM DFP over a period of 20 h at 0 °C, for example, is as effective as a single-dose treatment with 1280 μM DFP for complete destruction of Tc-uPA activity.

In order to investigate whether fibrin promotes an expression of activity in Sc-uPA without proteolytic cleavage, actions of monomeric fibrin, fibrin fragment FCB-2, and polymeric fibrin on the amidolytic activity were examined. None of these effectors promoted any significant cleavage of S-2444 by Sc-uPA in the presence of the Tc-uPA inhibitors.

Activity and Proteolytic Stability of DFP and Dansyl-EGRck-Treated Sc-uPA after Removal of the Tc-uPA Inactivators. Preparations of Sc-uPA that had been treated with DFP and dansyl-EGRck to exhaustively inactivate the Tc-UPA contaminant, thereby destroying all traces of amidolytic activity, invariably regenerated traces of activity when the Tc-uPA inhibitors were removed from the treated Sc-uPA. This regeneration of activity, seen to varying extents in every preparation of Sc-uPA studied, was more pronounced when an alkaline buffer without Trasylol (e.g., buffer 2) was used for dialysis. Inclusion of Trasylol in the buffer (buffer 1) or, more effectively, use of an acidic buffer (buffer 3) reduced, but did not completely prevent, the regeneration of activity. Amidolytic activities of recombinant Sc-uPA that had been treated with DFP and dansyl-EGRck to exhaustively inactivate any Tc-uPA contaminant and then dialyzed by microconcentration against various buffers are shown in Table III. Assays were performed within 6 h of the commencement of dialysis. A longer delay resulted in higher activities.

Continuous monitoring of the amidolytic activity of DFP- and dansyl-EGRck-treated Sc-uPA that had been dialyzed free of the inhibitors with buffer 3 and then incubated in buffer 1 containing 0.25 mM S-2444 at 37 °C showed a slow but persistent increase in the activity (Figure 2). The increase in the amidolytic activity was more pronounced with the recombinant Sc-uPA preparation from CHO cells than with the fetal kidney or transformed kidney cell preparations. Addition of pepstatin (100 μM) did not slow the increase im the amidolytic activity. Addition of EDTA (2.5 mM), however, produced a retarding effect on the generation of activity.

Treatment of recombinant Sc-uPA (a preparation with an amidolytic activity of 0.6% of that of Tc-uPA) with [^{3}H]DFP followed by SDS–PAGE analysis showed a major labeled band

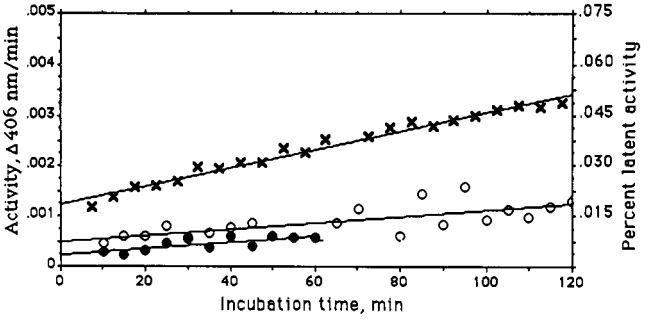

FIGURE 2: Continuous regeneration of amidolytic activity in DFP- and dansyl-EGRck-treated Sc-uPA preparations following removal of the Tc-uPA inhibitors. Sc-uPA was treated with the inhibitors to exhaustively inhibit all activity. The inhibitors were then dialyzed by microconcentration with buffer 3. The dialyzed preparations were incubated with buffer 1 containing S-2444 at 37 °C, and the amidolytic activity was followed by the change in absorbance at 406 nm. The activity is compared to the latent activity of control Sc-uPA samples that were not treated with the inhibitors but dialyzed with buffer 3. (×) Recombinant Sc-uPA; (○) fetal kidney cell Sc-uPA; (●) transformed kidney cell Sc-uPA.

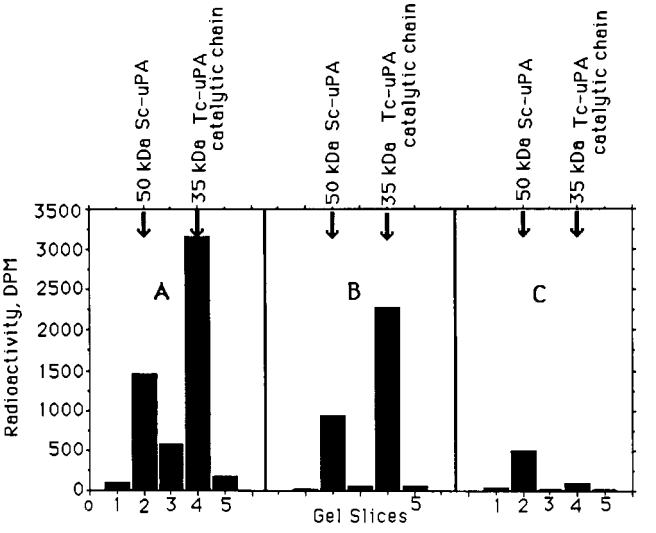

FIGURE 3: Tc-uPA contents of various Sc-uPA samples from incorporation of [^{3}H]DFP. Starting Sc-uPA or Sc-uPA treated with unlabeled DFP and dansyl-EGRck to sequester any preexisting Tc-uPA and then dialyzed against various buffers was treated with [^{3}H]DFP as described under Experimental Procedures. The treated proteins were analyzed by SDS–PAGE. (A) Starting Sc-PA; (B) DFP- and dansyl-EGRck-treated Sc-uPA dialyzed with buffer 2; (C) DFP- and dansyl-EGRck-treated Sc-uPA dialyzed with buffer 3.

corresponding to the 30-kDa catalytic chain of Tc-uPA and a minor band corresponding to Sc-uPA (Figure 3A). The minor band accounted for less than 0.1% of the Sc-uPA present in the reaction mixture.

Reaction of Sc-uPA with unlabeled DFP and dansyl-EGRck to exhaustively inactivate any Tc-uPA contaminant originally present in the preparation, followed by removal of the inhibitors and subsequent treatment with [^{3}H]DFP, showed that Tc-uPA was invariably regenerated from the Tc-uPA-free Sc-uPA. The amount of Tc-uPA determined from the SDS–PAGE analyses of the [^{3}H]DFP-treated Sc-uPA samples (Figure 3) paralleled the amidolytic activities of the unlabeled samples (Table III).

Activation of Plasminogen by Sc-uPA. To isolate plasminogen activation catalyzed by the putative activity of Sc-uPA from other side reactions, experiments were performed in the presence of DFP, dansyl-EGRck, and Trasylol. A combination of DFP, dansyl-EGRck, and Trasylol was used

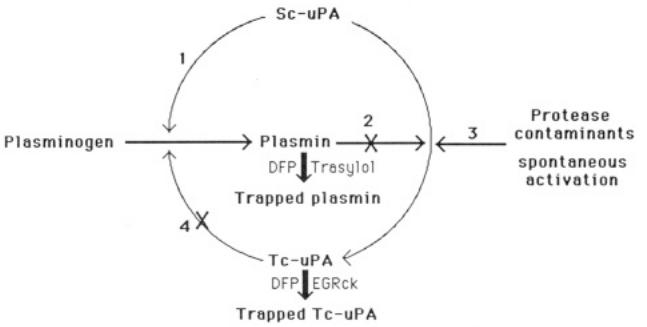

FIGURE 4: Diagrammatic representation of possible pathways in plasminogen activation initiated by Sc-uPA preparations. X signifies paths blocked by the added inhibitors. (Path 1) Activation of plasminogen initiated by the putative intrinsic activity of Sc-uPA. This route is not affected by the added inhibitors. (Path 2) Feedback conversion of Sc-uPA to Tc-uPA by plasmin. This route is blocked by entrapment of plasmin. (Path 3) Conversion of Sc-uPA to Tc-uPA by other proteases, possibly present as trace contaminants in Sc-uPA preparations. This route is not completely blocked by the added inhibitors. (Path 4) Conversion of plasminogen to plasmin by Tc-uPA. This route is blocked by entrapment of Tc-uPA.

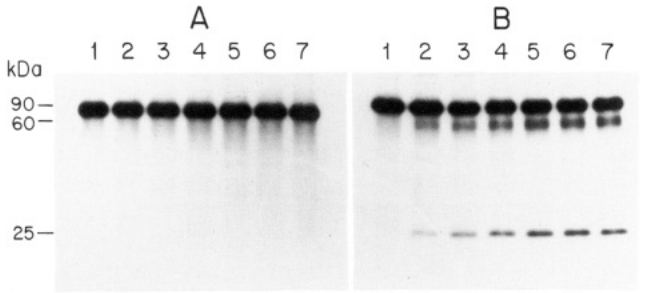

FIGURE 5: Plasminogen activation by Sc-uPA and Tc-uPA. Assays were performed as described under Experimental Procedures. (A) Sc-uPA (0.45 μM) in the presence of DFP, dansyl-EGRck, and Trasylol. (B) Tc-uPA (0.002 μM) in the presence of Trasylol. Lanes 1–7 represent samples analyzed after 0, 10, 20, 30, 40, 50, and 60 min of incubation.

to make sure that all traces of Tc-uPA and plasmin were completely removed. The effects of these inhibitors on various possible pathways of Sc-uPA-initiated plasminogen activation is shown in Figure 4. Since neither Trasylol nor DFP or dansyl-EGRck reacts with Sc-uPA, path 1, activation of plasminogen by the putative plasminogen activator activity of Sc-uPA is unaffected by these inhibitors. Any plasmin produced by this pathway is, however, effectively trapped by DFP or Trasylol. Consequently, path 2, conversion of Sc-uPA to Tc-uPA by plasmin, is blocked. Sc-uPA can still be possibly activated to Tc-uPA by path 3, spontaneous activation or action of proteases resistant to Trasylol, DFP, or dansyl-EGRck, which may be present in Sc-uPA preparations as contaminants. However, activation of plasminogen by any Tc-uPA generated in this manner, path 4, is aborted by effective trapping of Tc-uPA by dansyl-EGRck and DFP.

Under these stringent conditions, there was no measurable plasmin formation (generation of 60- and 25-kDa bands of plasmin from 90-kDa plasminogen) by 0.45 or 2.25 μM Sc-uPA in 1 h (Figure 5A). In contrast, detectable amounts of 60- and 25-kDa bands of plasmin were generated in the presence of Trasylol by 0.002 μM Tc-uPA in 10 min (Figure 5B).

To investigate if the presence of Trasylol alone in the reaction mixture is sufficient to block the cycle of plasminogen activation, reaction of DFP- and dansyl-EGRck-treated Sc-uPA with plasminogen was examined in the presence of Trasylol (600 μM). Under this condition, substantial amount

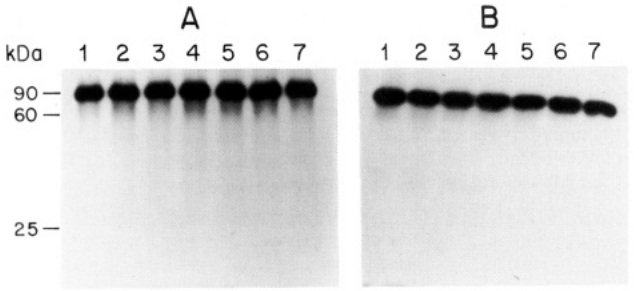

FIGURE 6: Effect of fibrin on plasminogen activation by Sc-uPA in the presence of DFP and dansyl-EGRck: (A) Sc-uPA (0.45 μM) without fibrin; (B) Sc-uPA (0.45 μM) in the presence of fibrin. Lanes 1–7 represent samples analyzed after 0, 10, 20, 30, 40, 50, and 60 min of incubation.

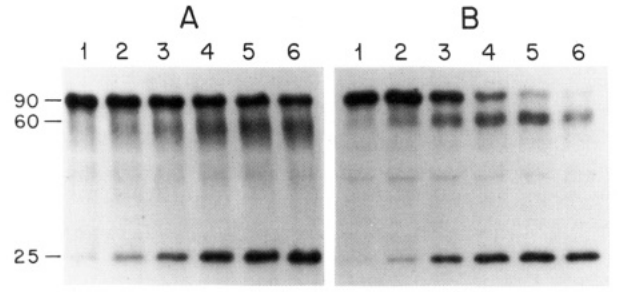

FIGURE 7: Effect of fibrin on plasminogen activation by Sc-uPA in the absence of DFP and dansyl-EGRck. Experiments were performed with DFP- and dansyl-EGRck-treated, dialyzed Sc-uPA in the presence of Trasylol as described under Experimental Procedures. (A) Sc-uPA (0.45 μM) without fibrin; (B) Sc-uPA (0.45 μM) in the presence of fibrin. Lanes 1–6 represent samples analyzed after 0, 10, 20, 30, 40, and 50 min of incubation.

of plasmin was formed (compare Figures 5A and 7A).

In an earlier section, it was shown that the minimum amount of DFP or dansyl-EGRck found from titration experiments to completely inactivate Tc-uPA also blocked the amidolytic activity of Sc-uPA. DFP and dansyl-EGRck present at near these minimum concentrations during the plasminogen activator assay were sufficient to block the plasminogen activator activity of the Sc-uPA preparation, suggesting that the inhibitors acted by trapping the active proteases and not by interfering with a putative plasminogen activator activity of Sc-uPA. To further exclude any possibility that DFP or dansyl-EGRck inhibit the putative intrinsic activity of Sc-uPA by a reversible competitive or noncompetitive mechanism, the effect of hydrolyzed DFP and dansyl-EGRck, molecules closely related to the parent inhibitors, on plasminogen activation by Sc-uPA preparations and Tc-uPA was investigated in the presence of Trasylol. The hydrolyzed inhibitors, added at a concentration 5 times higher than the parent inhibitors used with Sc-uPA, had no inhibitory effect on plasminogen activation by Sc-uPA preparations or Tc-uPA.

Effect of Fibrin on Plasminogen Activation by Sc-uPA. Fibrin did not promote any plasminogen activator activity in Sc-uPA when the assay was performed in the presence of DFP and dansyl-EGRck (Figure 6). However, when the assay was performed in the presence of Trasylol alone, plasminogen activation by Sc-uPA was enhanced by fibrin (Figure 7).

DISCUSSION

The findings presented in this paper show that preparations of Sc-uPA treated with DFP or dansyl-EGRck to exhaustively inactivate contaminating Tc-uPA, followed by assay in the presence of these inhibitors to effectively trap any nascent Tc-uPA generated in the assay mixture, do not express

measurable amidolytic or plasminogen activator activities above the detectable limits of 0.001% or 0.01%, respectively, of the corresponding Tc-uPA activities. As evidenced by the lack of significant incorporation of [^{3}H]DFP in Sc-uPA and the expression of full latent activity after activation of DFP- or dansyl-EGRck-treated Sc-uPA with plasmin, the Tc-uPA inactivators do not affect the molecular properties of Sc-uPA. Since the loss of amidolytic activity of Sc-uPA preparations, like that of Tc-uPA, on treatment with DFP or dansyl-EGRck is time dependent and irreversible on dilution, any possibility that the inhibitors interfere with an intrinsic activity of Sc-uPA by a reversible inhibition is unlikely. Inactivation of the amidolytic activity of Sc-uPA preparations by DFP at about the same rate as that of Tc-uPA suggests that the loss of activity of Sc-uPA on treatment with DFP is due to inactivation of Tc-uPA contaminant. This is further supported by the finding that the minimum amount of DFP or dansyl-EGRck found from titration experiments to completely inactivate Tc-uPA also blocks the amidolytic activity of Sc-uPA. Control experiments showing that high concentrations of hydrolyzed DFP and dansyl-EGRck, which only differ from the parent molecules in the substitution of the halide groups by hydroxyl groups, do not interfere with the amidolytic or plasminogen activator activities of Sc-uPA preparations rule out any reasonable possibility of DFP or dansyl-EGRck being reversible inhibitors of an intrinsic activity of Sc-uPA.

The present investigations using highly purified preparations of Sc-uPA, derived from a variety of different sources and purified by different procedures, suggest that it is virtually impossible to exclude traces of Tc-uPA from Sc-uPA preparations by passage through a benzamidine–Sepharose column or treatment with DFP or dansyl-EGRck, followed by separation of the Tc-uPA-free Sc-uPA from the inhibitors. Analysis of [^{3}H]DFP-labeled products of Sc-uPA that had been previously treated with unlabeled DFP and dansyl-EGRck to exhaustively inactivate any preexisting Tc-uPA suggests that traces of Tc-uPA are regenerated after the inhibitors are removed from Sc-uPA. To maintain a Tc-uPA- and activity-free status, it is therefore essential that inhibitors, such as DFP or dansyl-EGRck, that effectively trap any newly generated Tc-uPA are continually present during determination of intrinsic activity of Sc-uPA. The problem of excluding Tc-uPA contamination during determination of the putative intrinsic activity of Sc-uPA is reminiscent of the difficulty encountered in excluding active two-chain factor VII from one-chain inactive factor VII (Williams et al., 1989). The definitive evidence that factor VII is not an "active zymogen" was obtained only when assays were performed in the presence of dansyl-EGRck or other chloromethyl ketone inhibitors that effectively trap two-chain factor VII.

SDS–PAGE analysis of [^{3}H]DFP-treated Sc-uPA indicates that, besides incorporation into the Tc-uPA contaminant present in the preparation, a trace amount of the label is also incorporated into a band corresponding to the position of Sc-uPA. This band may arise from a slow reaction of DFP with Sc-uPA. However, the possibility that this band originates from a proteolyzed derivative of Sc-uPA that has a M_r close to Sc-uPA and is reactive toward DFP cannot be ruled out. The amount of DFP incorporated, approximately 0.1% in 2 h at room temperature, however, is too insignificant to cause any depletion in Sc-uPA during assays performed in the presence of the inhibitor. The present finding that trace amounts of labeled DFP are incorporated into recombinant Sc-uPA confirms an earlier observation of similar incorporation into urinary Sc-uPA (Husain et al., 1983). Trace incorporation of DFP into Sc-uPA, however, is not necessarily indicative, as suggested by Pannel and Gurewich (1986), of a low level of intrinsic enzymatic activity in Sc-uPA. Factor VII, for example, is fully reactive toward DFP (Broze & Majerus, 1980; Zur et al., 1982) but is completely devoid of enzymatic activity (Williams et al., 1989).

The generation of Tc-uPA in Sc-uPA preparations is most likely catalyzed by traces of contaminating proteases, possibly originating from Sc-uPA-producing cells or derived from the tissue culture medium. These proteases are apparently resistant to high concentrations of serine protease inhibitors, DFP, dansyl-EGRck, and Trasylol, as well as to the acid protease inhibitor pepstatin, suggesting that they might belong to other classes of proteolytic enzymes. The inhibitory effect of EDTA on activation of Sc-uPA suggests that the activating protease may be a metalloprotease. In this context, it is interesting to note that chelation of metal ions in blood plasma inhibits Sc-uPA-mediated clot lysis but not Tc-uPA-mediated clot lysis, suggesting a role for metal ions in the activation of Sc-uPA in the plasma milieu (Zaidi & Husain, 1990). Sc-uPA is unique among zymogens of fibrinolytic and coagulation proteases in being susceptible to proteolytic cleavage at the Lys^{158}–Ile^{159} peptide bond, not only by trypsin-like serine proteases but also by other classes of proteases whose specificity is not normally determined by carboxyl side Lys or Arg residues of peptide bonds. Highly purified preparations of thermolysin, a bacterial metalloprotease that cleaves at peptide bonds on the N-terminal side of hydrophobic residues, for example, effectively activates Sc-uPA by cleavage at the Lys^{158}–Ile^{159} bond (Marcotte & Henkin, 1990). The protease apparently recognizes Ile^{159}, rather than Lys^{158}, of the peptide bond. Other reports suggest that low concentrations of neutrophil elastase, cathepsin G, and collagenase also cause activation of Sc-uPA (Scully & Kakkar, 1990).

Earlier conclusions that Sc-uPA possesses an intrinsic amidolytic activity of 0.1–0.2% (Pannell et al., 1984; Pannell & Gurewich, 1986) and plasminogen activator activity of 0.5–1% (Ellis et al., 1987; Pannell & Gurewich, 1987; Lijnen et al., 1989, 1990) compared to the corresponding activities of Tc-uPA were based on studies of Sc-uPA that had been depleted of contaminating Tc-uPA by passage through benzamidine column or treatment with DFP or dansyl-EGRck followed by removal or decomposition of the inhibitors. In view of the pervasive regeneration of Tc-uPA from Sc-uPA, even in preparations treated with comparatively high concentrations of DFP, dansyl-EGRck, and Trasylol, it is likely that the low level of putative intrinsic activities attributed to Sc-uPA in the previous studies originated from contaminating Tc-uPA. This view is supported from the present finding that high concentrations of Trasylol alone, which was used in several of the previous studies to isolate Sc-uPA-mediated plasminogen activation from other side reactions (Ellis et al., 1987; Pannell & Gurewich, 1987; Lijnen et al., 1990), is not fully effective in preventing plasminogen activation by Sc-uPA preparations. In a recent investigation of activation of plasminogen, Sc-uPA was treated with α_2-PI and subsequently assayed in the presence of this inhibitor (Lijnen et al., 1990). The findings suggested that Sc-uPA possesses an intrinsic plasminogen activator activity of 0.5% of that of Tc-uPA. However, α_2-PI, a very slow inhibitor of Tc-uPA (Moroi & Aoki, 1976), may not have been effective in assuring absence of Tc-uPA in the reaction mixture. A recent account (Orfeo et al., 1990) suggests that activation of plasminogen in the presence of PAI-1, a more effective inhibitor of Tc-uPA (Van Mourik et al., 1984), is an order of magnitude lower than the activation

in the presence of α_2-PI. These investigations along with various other studies on the activity of Sc-uPA and the present findings show that the estimates of the putative plasminogen activator activity of Sc-uPA has gradually moved downward as more and more elaborate precautions have been taken to exclude Tc-uPA during assay of Sc-uPA activity.

Investigations of enzymatic activities of mutants of Sc-uPA in which Lys-158 has been replaced by Glu, Val, Met, etc., have suggested that they possess amidolytic and plasminogen activator activities ranging from 0.1% to 0.5% of that of Tc-uPA (Nelles et al., 1987; Gurewich et al., 1988; Lijnen et al., 1988a,b, 1990). Both the amidolytic and plasminogen activator activities of these mutants are at least 2 orders of magnitude larger than the detection limit of amidolytic and plasminogen activator activities of Sc-uPA in the present study, suggesting that the mutant preparations may have been contaminated with their two-chain active forms. In view of the present finding that Sc-uPA is slowly converted to Tc-uPA even after treatment with high concentrations of serine protease inhibitors, coupled with reports that Sc-uPA is highly susceptible to activation by proteases that are not specific for lysyl or arginyl residues, it is possible that the plasmin-resistant mutants of Sc-uPA may also undergo slow proteolysis to generate active two-chain forms by the action of traces of contaminating proteases. This possibility is supported by a survey of the published putative intrinsic amidolytic activities of various mutants of Sc-uPA which contain Glu, Gly, Met, or Val instead of Lys at position 158 and Pro or Gly instead of Ile at residue 159. The mutant with Pro at position 159 appears to be the least amidolytically active mutant (Lijnen et al., 1988a,b). Peptide bonds consisting of prolyl residues at the amino terminal side of a bond, incidentally, are usually more resistant to cellular proteases than those containing other amino acid residues at this position.

Activation of plasminogen by uPA is involved in a variety of physiological processes that require localized extracellular proteolysis. The stimulus that initiates activation of plasminogen in most of these processes is not well-defined. In blood clot lysis, however, fibrin provides the stimulus for activation of plasminogen by Sc-uPA. The initial generation of plasmin is followed by an effective feedback conversion of Sc-uPA to Tc-uPA by plasmin. However, the initiating event leading to activation of plasminogen is obscure. Two main hypotheses have been proposed to explain the role of fibrin leading to the initiation of plasminogen activation by Sc-uPA. Both these hypotheses are based on the assumption that Sc-uPA possesses a significant, albeit low, activity. According to one hypothesis (Lijnen et al., 1986), Sc-uPA circulates in blood as a complex with an inhibitor which is dissociated in the presence of fibrin, allowing initial slow conversion of plasminogen by Sc-uPA. Recent investigations (Lijnen et al., 1989) have, however, failed to substantiate the presence of such an inhibitor in blood plasma.

According to the second view (Pannell & Gurewich, 1986), Sc-uPA has a low activity in the absence of fibrin but converts fibrin-bound plasminogen more efficiently. Several investigations have suggested that Sc-uPA has no intrinsic activity (Kasai et al., 1985; Urano et al., 1988; Petersen et al., 1988). However, in these studies it was not possible to detect Sc-uPA plasminogen activator activity below 0.4% of Tc-uPA activity, the level of intrinsic activity that Sc-uPA has been shown to possess by other investigators (Pannell & Gurewich, 1987; Lijnen et al., 1989, 1990).

The present findings conclusively show that Sc-uPA has no measurable amidolytic or plasminogen activator activities above the detection limits of 0.001% and 0.01% of the corresponding activities of Tc-uPA. These studies also indicate that fibrin does not promote any intrinsic activity in Sc-uPA. The present findings suggest that the initiation of plasminogen activation by the inactive zymogen Sc-uPA requires a proteolytic step to convert Sc-uPA to an active form. Although, under in vitro conditions or during thrombolytic therapy when relatively high concentrations of Sc-uPA are infused, the presence of plasmin or Tc-uPA in various preparations may initiate activation of plasminogen or Sc-uPA, thus fueling the feedback loop, the precise physiological mechanism whereby activation of Sc-uPA is achieved remains to be established.

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Yeast Nucleosomal Particles: Structural and Transcriptional Properties†

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ABSTRACT: Yeast nucleosomal core particles have been characterized by thermal denaturation, circular dichroism, and digestion with DNase I and with trypsin. Practically all nucleosomal DNA melts in one transition centered at 70 °C, and the circular dichroism spectrum is displaced to lower wavelengths as compared to that corresponding to chicken nucleosomal cores. The susceptibility of yeast nucleosomal particles to dissociation by salt is significantly higher than that of chicken nucleosomal cores, a substantial dissociation being observed at 0.5 M NaCl. Treatment of yeast nucleosomal particles with the amino group reagent dimethylmaleic anhydride is accompanied by selective release of histones H2A and H2B. The results indicate not large but significant structural differences between yeast and chicken nucleosomal cores. However, the in vitro transcription properties of complete and H2A·H2B-deficient nucleosomal cores are similar in the two kinds of particles: the histone octamer blocks RNA synthesis, this block being eliminated in part by the partial loss of histones H2A and H2B.

Yeast chromatin is of special interest to understand the relationships between structure and function in the nucleosome-complexed DNA. Important differences have been found between the chromatin of bakers' yeast and that of higher organisms. No typical condensed chromosomes have been observed in yeast (Gordon, 1977), which might be related to the apparent absence of histone H1 (Certa et al., 1984; Smith et al., 1984) and to the high transcriptional activity of yeast chromatin. The large proportion of DNA (about 40%) transcribed under normal growth conditions (Hereford & Rosbash, 1977) and the low level of repetitive sequences are properties which make yeast an attractive organism to study transcriptionally active chromatin.

Although yeast chromatin contains nucleosomal core particles basically similar to those found in higher eukaryotes, their shorter linker (Lohr & Van Holde, 1975; Thomas & Furber, 1976; Nelson et al., 1977), the apparent absence of histone H1, and the sequence changes present in the conservative core histones (Van Holde, 1988) might significantly affect its transcription-related dynamics. In spite of the high interest of yeast nucleosomal particles, there is a paucity of relevant structural information, probably because of the difficulty in obtaining suitable nucleosomal particles. The published data seem to indicate a relaxed structure of yeast nucleosomal particles as compared with those from chicken or calf thymus (Lee et al., 1982; Morse et al., 1987).

The purpose of the present work is the structural characterization of isolated nucleosomal particles from yeast and the evaluation of their transcriptional efficiency. By use of a simple in vitro transcription system, previous work from our laboratory shows that the loss of one H2A·H2B dimer from the chicken nucleosomal core is accompanied by partial elimination of the block to transcription and a substantial increase in the level of RNA synthesis (González et al., 1987; González & Palacián, 1989). The results described in the pesent paper show small but significant structural differences between yeast and chicken nucleosomal particles, which indicate a slight relaxation of those from yeast. Moreover, the yeast nucleosomal particles, like chicken nucleosomal cores, are inefficient

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