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Mutant and Chimeric Recombinant Plasminogen Activators

PRODUCTION IN EUKARYOTIC CELLS AND PRELIMINARY CHARACTERIZATION*

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Mutant urokinase-type plasminogen activator (u-PA) genes and hybrid genes between tissue-type plasminogen activator (t-PA) and u-PA have been designed to direct the synthesis of new plasminogen activators and to investigate the structure-function relationship in these molecules. The following classes of constructs were made starting from cDNA encoding human t-PA or u-PA: 1) u-PA mutants in which the Arg^{156} and Lys^{158} were substituted with threonine, thus preventing cleavage by thrombin and plasmin; 2) hybrid molecules in which the NH_2-terminal regions of t-PA (amino acid residues 1–67, 1–262, or 1–313) were fused with the COOH-terminal region of u-PA (amino acids 136–411, 139–411, or 195–411, respectively); and 3) a hybrid molecule in which the second kringle of t-PA (amino acids 173–262) was inserted between amino acids 130 and 139 of u-PA. In all cases but one, the recombinant proteins, produced by transfected eukaryotic cells, were efficiently secreted in the culture medium. The translation products have been tested for their ability to activate plasminogen after *in situ* binding to an insolubilized monoclonal antibody directed against urokinase. All recombinant enzymes were shown to be active, except those in which Lys^{158} of u-PA was substituted with threonine. Recombination of structural regions derived from t-PA, such as the finger, the kringle 2, or most of the A-chain sequences, with the protease part or the complete u-PA molecule did not impair the catalytic activity of the hybrid polypeptides. This observation supports the hypothesis that structural domains in t-PA and u-PA fold independently from one to another.

The fibrinolytic system plays a major role in the removal of insoluble fibrin from the vascular bed. It is triggered by the conversion of an inactive proenzyme, plasminogen, into the active enzyme, plasmin, which will degrade fibrin clots into soluble components (1).

Among several plasminogen activators, two immunologically distinct enzymes, tissue-type plasminogen activator (t-PA)[1] and urokinase-type plasminogen activator (u-PA), have been extensively studied (for a review, see Ref. 2). The first one, t-PA, found to be identical to blood plasminogen activator (3), has been isolated from human uterus (4). The second enzyme, u-PA, has been identified in human urine and kidney cells (5, 6).

Both proteins are serine proteases of 70,000 and 54,000 daltons, respectively, synthesized as single-chain polypeptides including a signal sequence involved in secretion (7–9). Single-chain plasminogen activators are processed by plasmin to form active enzymes composed of two disulfide-linked polypeptides. t-PA is cleaved at the Arg^{278}-Ile^{279} bond and single-chain u-PA (scu-PA), primarily, between Lys^{158} and Ile^{159} residues. Secondary cleavages in the u-PA molecule occur at the Arg^{156}-Phe^{157} (with thrombin) (10) and Lys^{135}-Lys^{136} bonds, the latter event producing the low molecular size form of the enzyme (33,000 daltons) which has similar properties as the 54,000-dalton species (11).

Although both enzymes activate plasminogen, t-PA and u-PA present different fibrinolytic properties. Indeed, plasminogen activation by t-PA is highly fibrin-specific because the activator binds to the fibrin clot. Plasminogen then binds to both t-PA and fibrin, thus forming a cyclic ternary complex with increased stability (12). Both single-chain and two-chain t-PA have very similar fibrinolytic efficacy; this implies that the conversion of single-chain to two-chain t-PA at the surface of the fibrin clot (13) has no physiological significance. On the contrary, two-chain u-PA displays little affinity for fibrin and activates free and fibrin-bound plasminogen equally well. Single-chain urokinase (scu-PA), which has been isolated recently by several groups (14–17), is a plasminogen activator with better fibrin specificity than u-PA (18–20). scu-PA thus displays intrinsic plasminogen activator properties (21, 22).

A comparison of the amino acid and nucleotide sequences of t-PA and u-PA reveals extensive homology between their B-chains (COOH-terminal regions) which carry the active site. The A-chains (NH_2-terminal regions), however, differ in some significant aspects (7–9, 23, 24). t-PA contains two kringle domains, whereas u-PA has only one. These kringle

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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; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; SV40, simian virus 40; BGH, bovine growth hormone; bp, base pairs; kringle, triple loop disulfide-bonded structures occurring in t-PA (twice), u-PA (once), and plasminogen (five times); finger, amino-terminal region of t-PA, homologous to the finger-like domains in fibronectin; aa, amino acid(s).

domains are highly homologous to equivalent structures of plasminogen involved in fibrin-binding (25, 26). In addition, the NH_2-terminal region of t-PA contains a finger-like domain similar in structure to the fibrin-binding regions of fibronectin (27, 28). The high affinity of t-PA for fibrin has been attributed to the presence of both the finger and kringle domains in the enzyme (29, 30). Because plasminogen activation by t-PA occurs with a low catalytic rate constant ($k_2 \sim 0.5\ s^{-1}$) (31), new plasminogen activators with both a high fibrin specificity and a high turnover rate constant might constitute improved thrombolytic agents. Therefore, we have constructed, using recombinant DNA techniques, sequences coding for t-PA/scu-PA hybrids and for mutant scu-PA molecules resistant to the cleavage by plasmin. In the present study, we found that the recombinant proteins, produced by transfected eukaryotic cells, are efficiently secreted in the culture medium and, in most instances, display specific activities for the activation of plasminogen comparable to that of natural u-PA.

EXPERIMENTAL PROCEDURES[2]

RESULTS

Construction of Mutant u-PA and of Chimeric t-PA/u-PA Coding Sequences—In an effort to improve thrombolytic selectivity and fibrin specificity of plasminogen activators, a family of vectors carrying sequences encoding new plasminogen activators have been created through recombinant DNA technology. As outlined in the Miniprint Section, the starting material for the new constructs is carried by three plasmids: pULB1000 and pULB1135 carry a preprourokinase cDNA (9) and pDSP1.1TPA25.BGH carries a t-PA precursor cDNA. The recombinant molecules derived from the manipulation of these DNAs share common features: they were all obtained as *Hin*dIII-*Sac*I cassettes carrying a 5′-terminal sequence coding for a signal peptide and a 3′-terminal sequence corresponding to the whole or partial B-chain of u-PA. In all cases, the u-PA catalytic site has been maintained. The new constructs, however, differed either in the nature of the A-chain or in the sequence coding for the activation site of the proenzymes.

Full-length recombinant DNA molecules were obtained by subcloning various DNA fragments into the *Hin*dIII and *Sac*I sites of plasmid pULB1221 (42). When necessary, sequences joining DNA fragments of different origins were synthesized chemically and added to the ligation mixtures. The conformity of the recombinant DNAs to the expected sequences was then checked by DNA sequencing before proceeding to the insertion of *Hin*dIII-*Sac*I coding sequences into the eukaryotic transient expression vector pDSP1.1BGH (35), between the SV40 early promoter and the BGH polyadenylation signal (see Miniprint Section). Upon transfection in Chinese hamster R1610 or/and Cos I monkey cells, recombinant plasminogen activators were produced and secreted in the culture medium.

Characteristics of recombinant plasminogen activators are shown in Table 2 and the new enzymes schematically represented in Fig. 2. The products can be classified in three main groups. The first one consists of modified preprourokinase molecules (Table 2). Two of these enzymes, ppUK.410 and ppUK.410/366, coded for by pULB9122 and pULB9134, carry amino acid substitutions in the B-chain as compared to pULB1000 and pULB1135. These modifications were introduced to assess their effect on enzymatic activity, in view of the reported discrepancies between the deduced amino acid sequence of cloned preprourokinase (9) and the sequence of the purified natural enzyme (23, 24). Another set of constructs from the same group, Scupa n.c.410 and Scupa n.c.410/366 (coded for by pULB9129 and pULB9135), derives from the former molecules; additional amino acid substitutions have been introduced at the physiological activation site in the proenzyme (Arg^{156} and Lys^{158} are respectively replaced by threonine). The purpose of these constructions was to obtain prourokinase molecules with similar enzymatic properties as the natural single-chain species (scu-PA) (18–20), but resistant to cleavage by plasmin. The last constructs belonging to the first group, pULB9139 and pULB9152 (coding for ppUK.(410/366/131)del and Scupa n.c.(410/366/131)del), were designed to eliminate the secondary cleavage site of urokinase (Lys^{135}-Lys^{136}) and to replace amino acid 131, tryptophan in pULB1000 (9), by the cysteine residue found in the natural protein (23). This was achieved by deleting a stretch of amino acids (132–147) and replacing it with a shorter link (Ser-Thr) identical to the one found in t-PA at comparable positions of the enzyme. The product ppUK.(410/366/131)del, coded for by pULB9139, consists thus of a deleted but activable prourokinase, whereas Scupa n.c.(410/366/131)del, coded for by pULB9152, is deleted and non-activable. Finally, for comparison purposes, we constructed a recombinant preprourokinase DNA (pULB9154) identical to that described by Heyneker *et al.* (8).

The second group of constructions comprises four chimeric molecules. Taking into account the hypothesis of exon shuffling as a mechanism for protein evolution (48), we tried to recombine cDNA fragments, derived from t-PA and u-PA, corresponding as precisely as possible to exons in the genes and to structural domains in the corresponding proteins. Fg.t-PA/UK.410 and Fg.t-PA/UK.410/366 (coded for by pULB9120 and pULB9124) result from the fusion of the finger domain of t-PA to the COOH-terminus of scu-PA. The two species are identical, except for the amino acid at position 366 in urokinase (glycine in pULB9124 and cysteine in pULB9120). Both molecules were designed to explore the potential role of the t-PA finger domain (29) in fibrin binding when associated with scu-PA. Another molecule, tPPUK.410/366, encoded by plasmid pULB9151, combines a larger portion of t-PA, the A-chain, to the B-chain of u-PA; it is designed to confer to scu-PA the fibrin specificity of t-PA, which appears to be associated to the A-chain moeity (49, 50). A similar product, tPKUK.410 (coded for by pULB9125), consists of the NH_2-terminal part of t-PA containing the activation site, up to amino acid 313 in the B-chain, fused to the remaining COOH-terminal part of the B-chain of u-PA.

The single representative of the third group, UK-K2.410/366, coded for by plasmid pULB9137, is a nearly complete scu-PA polypeptide wherein the kringle 2 region of t-PA has been inserted between the single kringle domain and the B-chain. It was designed to test the hypothesis that the kringle 2 region of t-PA behaves as an autonomous domain conferring fibrin binding ability to the enzyme (30).

Expression of Recombinant Plasminogen Activators in Cell Cultures—Eukaryotic cells transfected with the recombinant plasmids described above were cultivated for 3–5 days in the presence of aprotinin to prevent conversion of the recombi-

[2] Portions of this paper (including "Experimental Procedures," Fig. 1, and Table 1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M 4391, cite the authors, and include a check or money order for $3.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

TABLE II

General description of recombinant plasminogen activators

Plasmid	Product denomination	Amino acid residue position[a]			Description
		131	366	410	
pULB1000	ppUK	Trp	Cys	Val	Preprourokinase cDNA clone (9)
pULB1135	ppUK	Trp	Cys	Val	Preprourokinase cDNA clone (9)
pDSP1.1TPA25BGH	t-PA				t-PA cDNA clone
pULB9122	ppUK.410	Trp	Cys	Ala	ppUK where Val^{410} has been replaced by Ala
pULB9134	ppUK.410/366	Trp	Gly	Ala	ppUK.410 where Cys^{366} has been replaced with Gly
pULB9154	ppUK.410/366/131	Cys	Gly	Ala	ppUK.410/366 where Trp^{131} has been replaced with Cys; identical to preprourokinase previously published (8, 23, 24)
pULB9139	ppUK.(410/366/131)del	Cys	Gly	Ala	ppUK.410/366/131 where aa 132–147 have been replaced by Ser-Thr
pULB9129	Scupa n.c.410	Trp	Cys	Ala	ppUK.410 where aa 156 and 158 have been replaced by Thr in order to produce an uncleavable ppUK
pULB9135	Scupa n.c.410/366	Trp	Gly	Ala	Scupa n.c.410 where Cys^{366} has been replaced by Gly
pULB9152	Scupa n.c.(410/366/131)del	Cys	Gly	Ala	Scupa n.c. where aa 132 and 147 have been replaced by Ser-Thr
pULB9120	Fg.t-PA/UK.410		Cys	Ala	t-PA sequence from the ATG codon to aa 67 followed by the sequence coding for aa 136 to the stop codon of prourokinase
pULB9124	Fg.t-PA/UK.410/366		Gly	Ala	Fg.t-PA/UK.410 where aa corresponding to position 366 of prourokinase has been replaced by Gly
pULB9151	tPPUK.410/366		Gly	Ala	t-PA sequence from the ATG codon to aa 262 followed by the sequence coding for aa 139 to the stop codon of prourokinase
pULB9125	tPKUK.410		Cys	Ala	t-PA sequence from the ATG codon to aa 313 followed by the sequence coding for aa 195 to the stop codon of prourokinase
pULB9137	UK-K2.410/366		Gly	Ala	Sequence coding for kringle 2 of t-PA (aa 173–262) inserted between aa 130 and 139 of preprourokinase

[a] Amino acid (aa) positions relative to preprourokinase sequence; aa 131 belongs to the A-chain and aa 366 and aa 410 to the B-chain.

nant plasminogen activators secreted in the medium. Dosage of the recombinant polypeptides by ELISA using two monoclonal antibodies, AAU2 and AAU6 (46), reveals that all recombinant plasmids, except pULB9125, direct the transitory expression of urokinase-like material (Table 3). Culture supernatants and extracts of cells transfected with pULB9125 (tPKUK.410) were consistently negative when assayed with anti-urokinase or anti-t-PA antibodies (data not shown).

Plasminogen Activation by Immobilized Recombinant Activators—The assay for plasminogen activation consisted of a two-step procedure. First, standard urokinase or cell culture supernatants were incubated with matrix-bound monoclonal antibody AAU2. Specific complexes were then exposed to plasminogen and to a plasmin-specific chromogenic substrate, D-Ile-Pro-Arg-*p*-nitroanilide. Any plasmin resulting from the activation of plasminogen will thus react with the substrate and release the paranitroaniline chromophor which can be monitored at 405 nm. In all cases, typical sigmoidal curves were observed when plotting absorbance as a function of time (t) (data not shown). As previously described by Drapier *et al.* (47), plotting of A_{405} as a function of squared time (t^2) enables us to linearize the assay as long as initial conditions are valid (see Miniprint Section) (Fig. 3). The slope of these straight lines is almost proportional to the total u-PA concentration present in the experimental standard incubation mixtures at the moment of enzyme immobilization onto matrix-bound antibody (Fig. 3, *inset*). From this relationship it is concluded that, within the experimental range tested (0–8 IU/ml), the amount of immobilized enzyme is proportional to the u-PA concentration in the upstanding solution. The dose-dependent plasminogen activation thus enables us to evaluate the enzymatic activity present in initial incubation mixtures.

Compared to the curves obtained with control u-PA (Fig. 3), those for all recombinant plasminogen activators and for purified natural scu-PA appeared biphasic. As shown for three different dilutions of the recombinant ppUK.410/366 (pULB-

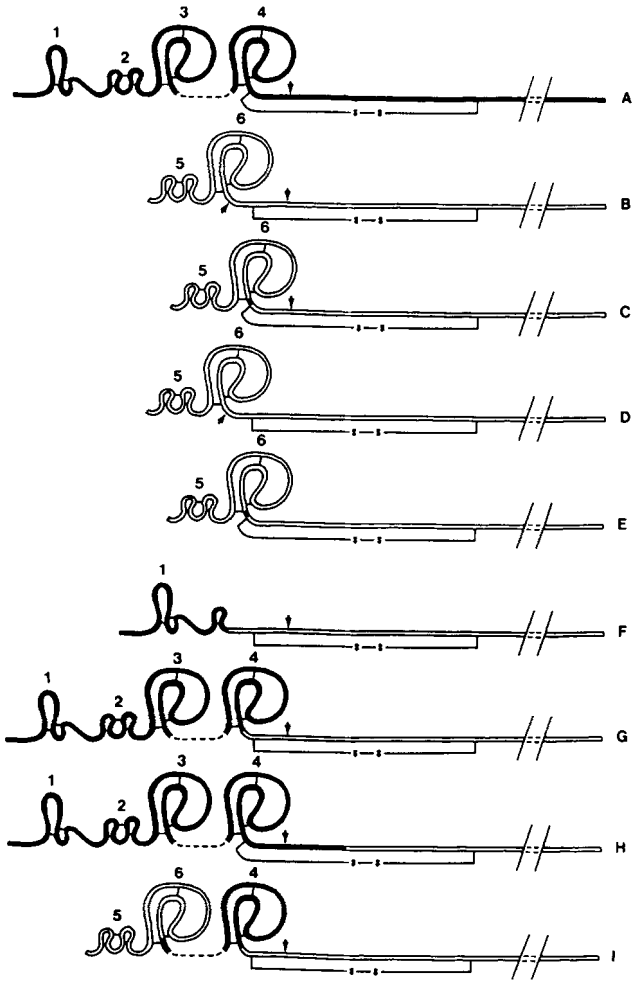

FIG. 2. **Schematic representation of the A-chain structural domains in recombinant plasminogen activators.** *Open* and *solid lines* correspond to sequences originating from scu-PA and t-PA, respectively. The different recombinant plasminogen activators are indicated by the following letters: *A*, t-PA; *B*, u-PA, ppUK.410, ppUK/410/366, or ppUK/410/366/131; *C*, ppUK.(410/366/131)del; *D*, Scupa n.c.410 or Scupa n.c.410/366; *E*, Scupa n.c.(410/366/131)del; *F*, Fg.t-PA/UK.410 or Fg.t-PA/UK.410/366; *G*, tPPUK.410/366; *H*, tPKUK.410; *I*, UK-K2.410/366. *Arrows* point to cleavage sites. Relevant disulfide bridges, in the A-chain and between the A- and B-chains, are shown. For simplification purposes, B-chains are only partially represented. Numbers refer to structural domains of A-chains: *1*, finger domain of t-PA; *2*, epidermal growth factor domain of t-PA; *3*, kringle 1 of t-PA; *4*, kringle 2 domain of t-PA; *5*, epidermal growth factor domain of u-PA; *6*, kringle domain of u-PA.

9134), the linear phase was preceeded by an exponential lag phase (Fig. 4*A*). Pretreatment of immobilized recombinant plasminogen activators with plasmin (or with trypsin) and careful elimination of the converting enzyme completely abolished this initial lag phase in subsequent plasminogen activation experiments (Fig. 4*B*). Slopes measured in both experimental conditions were identical, considering the steep part of the curves only. The data thus indicate that the initial phases, as observed in Fig. 4*A*, correspond to the activation of the immobilized recombinant activators which, under the experimental conditions, are harvested essentially as single-chain molecules. This was confirmed by the fact that the same lag phase was observed with standard one-chain urokinase purified from Calu-3 cell line (data not shown). Therefore, the enzymatic activity present in cell culture supernatants was determined by comparing the slopes of the linear part of the curves obtained for recombinant enzymes (Fig. 4*A*) to the standard u-PA system (Fig. 3). The enzyme activities are presented in Table 3 for all recombinant plasminogen activators tested; activities ranged from 0 to 4.5 IU/ml of culture supernatant. Apparent specific activities of the recombinant plasminogen activators were obtained by the ratio of measured activities to the amount of antigen (assuming that they display similar affinities for the monoclonal antibodies AAU2 and AAU6 as the standard 54,000-dalton u-PA). As seen in Table 3, values range from 35,000 and 100,000 IU per mg of 54,000-dalton activator, except for non-activable scu-PA molecules. From these data, it can be concluded that two-chain recombinant enzymes activate plasminogen with catalytic efficiencies comparable to that of u-PA and, thus, that they have maintained a correct three-dimensional active site. On the other hand, no or only very weak activity has been found in the supernatants of respectively R1610 and Cos I cells although they expressed efficiently the non-activable scu-PA molecules. As expected, the modification of the activation site resulted in a single-chain product which cannot be transformed into the two-chain active species in the presence of plasminogen and chromogenic substrate or by plasmin (data not shown). Whether non-convertible scu-PA-like and natural scu-PA will be able to activate plasminogen directly in a freely diffusing system remains to be determined.

DISCUSSION

Thrombolytic agents lacking fibrin specificity, such as u-PA or streptokinase, induce thrombolysis but in association with generalized plasminogen activation and fibrinogen breakdown. t-PA induces thrombolysis with a high degree of clot selectivity due to a markedly higher rate of plasminogen activation at the surface of the fibrin clot, as compared to rates observed in the absence of fibrin (12). Efficient and fibrin-selective thrombolysis has also been obtained with scu-PA, the single-chain precursor of u-PA (22). The mechanism of this selectivity is not fully understood but appears distinct from that of t-PA.

One way to design improved thrombolytic agents would consist of the combination, in a single molecule, of two essential characteristics: high fibrin-mediated plasminogen activation and low fibrin-independent plasminogen activation. Such agents would be expected to display, *in vivo*, a fibrinolysis/fibrinogenolysis ratio at least equal or superior to that of t-PA or scu-PA.

In the present study, three main groups of plasminogen activators have been produced; first, we constructed scu-PA-like molecules (Scupa n.c.410 and Scupa n.c.410/366) wherein conversion to two-chain urokinase was prevented by substituting two amino acids involved in the cleavage of the natural scu-PA molecule. This approach finds its rationale in the fact that the conversion of scu-PA into u-PA, *in vivo*, is not a prerequisite for thrombolysis, but leads to a loss in clot selectivity.

In a second approach, we recombined several domains derived from the A-chain of t-PA with u-PA (in part or *in toto*). Fg.t-PA/UK.410 and Fg.t-PA/UK.410/366 consist of the low molecular weight scu-PA carrying on its NH_2 terminus the finger domain of t-PA; tPPUK.410/366 contains the A-chain of t-PA fused to the low molecular weight scu-PA, and tPKUK.410 is similar to tPPUK.410/366 but contains the cleavage site of t-PA. We hypothesized that some of these molecules might not only induce clot selectivity for plasminogen activation, via the mechanism of scu-PA, but also enhance fibrin specificity by binding to the clot via their t-PA structures. Another hybrid product, UK-K2.410/366, carries the putative fibrin binding domain of t-PA (kringle 2) inserted

TABLE III

Expression levels and activity determinations of recombinant plasminogen activators

Plasmid	Gene product	Cell type: R1610 ELISA[a]	R1610 Activity in supernatant	R1610 Apparent specific activity[a]	Cos I ELISA[a]	Cos I Activity in supernatant	Cos I Apparent specific activity[a]
		ng/ml	*IU/ml*	*IU/mg*	*ng/ml*	*IU/ml*	*IU/mg*
I[b]							
pULB9122	ppUK.410	3.6	0.23	62,400			
pULB9134	ppUK.410/366	3	0.2	66,000			
pULB9139	ppUK.(410/366/131)del	3.2	0.22	68,900			
pULB9120	Fg.t-PA/UK.410	12	0.43	35,300			
pULB9124	Fg.t-PA/UK.410/366	11.4	0.49	43,000			
pULB9151	tPPUK.410/366	1.9	0.21	109,900			
pULB9125	tPKUK.410	0	0	0			
pULB9137	UK-K2.410/366	1.1	0.08	69,000			
II[b]							
pULB9134	ppUK.410/366	74.3	2.64	35,500	48	2.28	47,000
pULB9129	Scupa n.c.410	18	0	0	40	0.12	2,900
pULB9135	Scupa n.c.410/366	29	0	0	96	0.57	5,900
pULB9151	tPPUK.410/366	6.6	4.44	66,300	21.5	1.34	62,000
pULB9137	UK-K2.410/366	13.8	0.43	31,250	7.3	0.38	52,200
III[b]							
pULB9122	ppUK.410	7.5	0.48	63,500	8.4	0.62	73,700
pULB9154	ppUK.410/366/131	7.5	0.36	48,000	6.9	0.52	75,000
pULB9135	Scupa n.c.410/366	9	0	0	6.5	0.02	3,100
pULB9152	Scupa n.c.(410/366/131)del	3.8	0	0	4	0.01	2,500

[a] Concentrations and apparent specific activities are given in 54,000-dalton urokinase equivalents.

[b] I, II, and III refer to three independent transfection experiments.

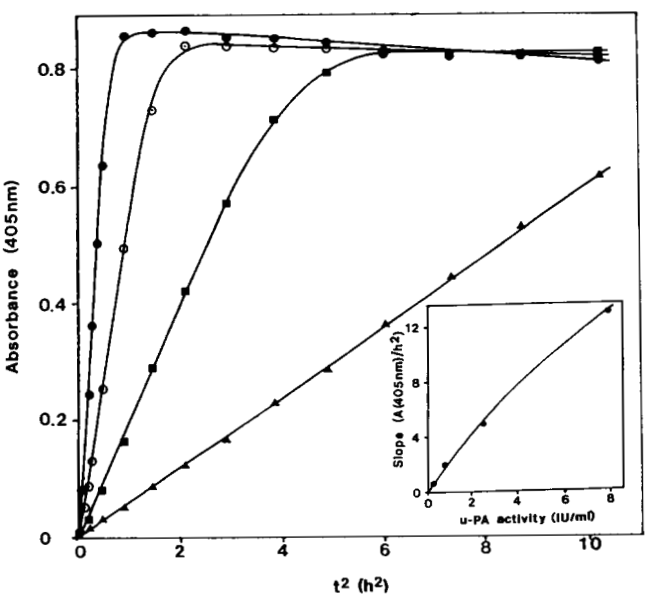

FIG. 3. **Kinetic analysis of plasminogen activation by purified u-PA.** Formation of paranitroanilide was recorded at 405 nm. The system contains purified u-PA linked to the monoclonal antibody AAU2, plasminogen (1 μM) and the plasmin-specific chromogenic substrate D-Ile-Pro-Arg-*p*-nitroanilide (0.5 mM). Data were plotted *versus* squared time. Slopes of the linear phases represent the acceleration of paranitroanilide formation. Four concentrations of purified u-PA were assayed: ●, 7.9 IU/ml; ○, 2.5 IU/ml; ■, 0.79 IU/ml; ▲, 0.25 IU/ml. *Inset*, acceleration of pNA formation plotted *versus* u-PA activities. The curve can be used to evaluate the activity present in cell culture supernatants assayed on microtiter plates.

into the nearly complete scu-PA molecule; this form is expected to yield a potent urokinase-like plasminogen activator showing high fibrin affinity if, indeed, the kringle 2 behaves as an autonomous domain.

All recombinant plasminogen activators, except tPKUK.410, were efficiently produced in cell cultures. In addition, specific activities of recombinant two-chain u-PA and of chimeric polypeptides were comparable to that of natural u-PA, indicating that the catalytic site carried by the urokinase moiety of the molecules has been maintained and is fully functional for plasminogen activation.

The recombinant uncleavable scu-PA molecules (Scupa n.c.410 and Scupa n.c.410/366), derived from transfected R1610 cells, did not show any activity in our assay system. However, the supernatants derived from transfected Cos I cells exhibited a slightly higher level of activity than the control. This is apparently due to the secretion by the cells of an endogenous plasminogen activator. Indeed, pretreatment of Cos I cell supernatant with plasmin confirmed this hypothesis (data not shown). We showed also that plasmin was unable to convert uncleavable scu-PA derived from transfected R1610 cells into an amidolytically active species. This observation supports the conclusion that Scupa n.c. proteins are effectively stable one-chain molecules. Whether the absence of the activation site in the Scupa n.c. molecule has any decisive influence on its biological *in vitro* and *in vivo* activities will be investigated in more detail once the recombinant product is obtained in large amounts and purified.

The scu-PA molecule encoded by the cDNA described in Jacobs *et al.* (9) differs at three positions from the amino acid sequence of the natural protein (23, 24) and from the deduced sequence derived from an independently isolated cDNA clone (8). We showed that none of these differences had a significant effect on the production levels and on the plasminogen acti-

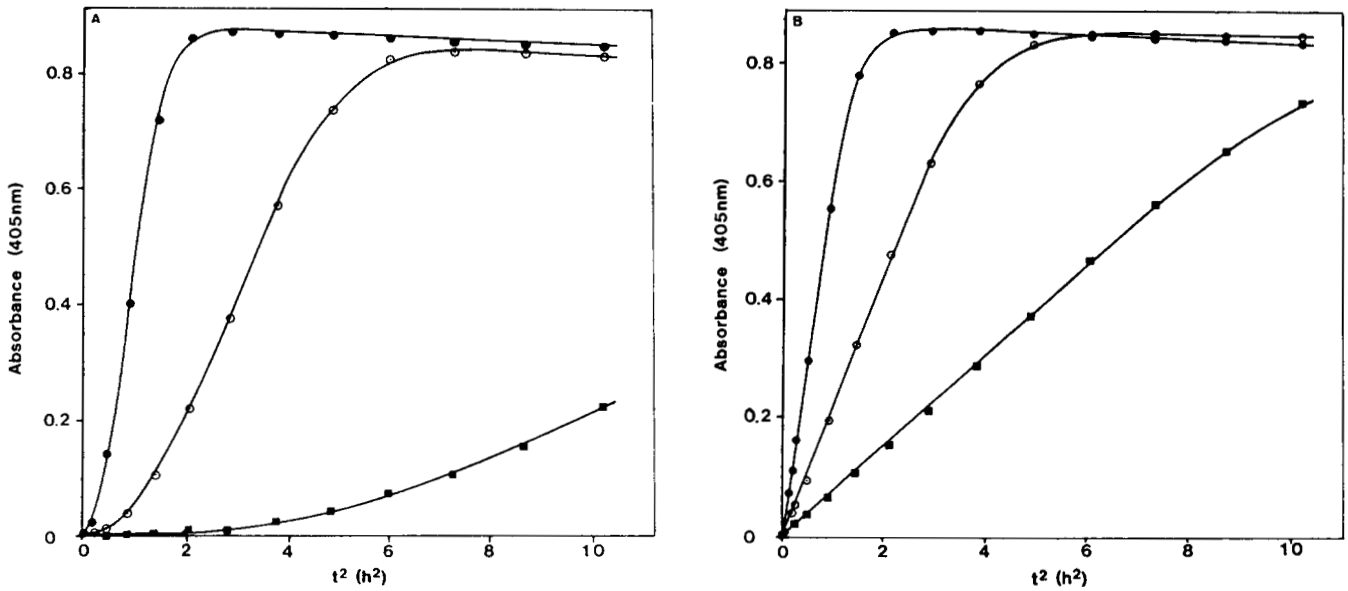

FIG. 4. **Kinetic analysis of plasminogen activation by recombinant ppUK.410/366 secreted in culture medium of transfected Cos I cells.** In *A*, procedure was as for Fig. 3. Three dilutions of the cell culture supernatant were tested: ●, undiluted supernatant; ○, 3-fold dilution; ■, 10-fold dilution. Slopes of linear phases were used to evaluate the activity of the supernatants by comparison to the standard curve shown in the *inset* of Fig. 3. In *B*, procedure and dilutions of cell culture supernatant were as for *A* except that ppUK.410/366 linked to the monoclonal antibody AAU2 was converted to its two-chain form, prior to the assay, by exposure to plasmin as described under "Experimental Procedures." The data show that the lag phases observed in *A* are due to the conversion of one-chain ppUK.410/366 to its two-chain form.

vation capability. In addition, deleting the cleavage site responsible for conversion of 54,000-dalton u-PA to 33,000-dalton u-PA (Lys^{135}-Lys^{136}) was equally without effect.

A number of hypotheses might be tested once these mutant and chimeric plasminogen activators have been produced in efficient host/vector systems, extensively purified, and characterized. A first step in this direction has been already achieved for two of our constructs, pULB9122 (ppUK.410) and pULB9120 (Fg.t-PA/UK.410), and the data are presented in the accompanying paper (51).

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SUPPLEMENTARY MATERIAL

to

Mutant and chimaeric recombinant plasminogen activators

-Production in eukaryotic cells and preliminary

characterization

by

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EXPERIMENTAL PROCEDURES

Materials:

Restriction endonucleases, T4 DNA polymerase, T4 DNA ligase, Escherichia coli DNA polymerase I (Klenow fragment) and T4 polynucleotide kinase were purchased from Boehringer-Mannheim Biochemicals, Amersham or New England Biolabs. Aprotinin was from Sigma, fetal calf serum from Flow laboratories and D-Val-Leu-Lys-p-nitroanilide (S2251) from Kabivitrum. Human plasminogen was purified as described (32). One and two-chain urokinase were prepared from human lung adenocarcinoma line Calu-3 (ATCC, HTB-55) as described by Stump et al.(17). Their activities were determined using the reference standard urokinase of Calbiochem-Behring Corp. These activities were 70,000 IU/mg and 79,300 IU/mg respectively for one-chain and two-chain urokinase.

Construction of expression plasmids:

Plasmids pULB 1000 (9), pULB 1135 (9), pJRD184 (33) and pJRD158 (34) have been described earlier. Plasmid pDSP1.1TPA25BGH,provided by Dr. Reff, is a pDSP1.1BGH-like plasmid wherein a t-PA cDNA has been inserted. Plasmid pDSP1.1BGH (35) is a transient expression vector for eukaryotic cells. Genes cloned in this plasmid are flanked by the SV40 early promoter and the BGH polyadenylation site. All recombinant genes described in this paper have been introduced in pDSP1.1BGH as Hind III-Sac I cassettes (Fig. 1). The procedures for DNA preparation and restriction analysis were as published by Maniatis et al. (36). Oligodeoxynucleotides were synthesized on a Applied Biosystem Synthesizer model 380 A via the solid-phase phosphoramidite method as previously described (37,38,39). Ligation and bacterial transformation of Escherichia coli K12 strain MM 294 (endA, thr$^-$,hsr$^-$, hsm$^+$) were performed according to Maniatis et al (36). The methods of Maxam and Gilbert (40) and Sanger et al. (41) were used for DNA sequence analysis.

pULB 9122

This recombinant plasmid codes for preprourokinase. It has been constructed as follows: a 1440 bp Bgl I-Bal I DNA fragment was derived from plasmid pULB 1000. It encompasses the urokinase cDNA molecule coding for aa 16 in the signal sequence to the stop codon and includes in its 3' terminus, the poly C extension and 124 bp of pBR322 sequences. This fragment wss treated with T4 DNA polymerase (36) and ligated on its 5' end to a 21 bp Hind III-Bgl I (blunt ended site) double-stranded synthetic DNA adaptor (adaptor 1, Table 1) to construct the sequence corresponding to the ATG initiating codon and the urokinase signal peptide up to aa -16. The molecule coding for preprourokinase, ppUK, was then ligated (36) to the intermediate E. coli plasmid, pULB 1221 (42), cut with Hind III and Bal I. The resulting plasmid was further manipulated firstly to remove exceeding 3' poly C and pBR322 sequences, and secondly to substitute the codon for Val 410 present in pULB 1000 or pULB 1135 by a codon specifying Alanine. This was done by replacing a 211 bp BamH I-Sac I DNA fragment by a 21 bp double-stranded synthetic DNA adaptor (adaptor 2,Table 1) coding for amino acid 398 to the stop codon followed by a Sac I protruding end. A 1309 bp Hind III-Sac I cassette coding for ppUK.410 was excised from the resulting recombinant plasmid pULB 9117 and inserted into the vector pDSP1.1BGH, to produce the final recombinant plasmid pULB 9122.

pULB 9134

This plasmid carries the sequences coding for preprourokinase modified to substitute the codon for Cys 366, present in pULB 1000 or pULB 1135, by a codon specifying glycine. Starting from pULB 9117, the 2661 bp Hind III-BamH I fragment was produced. This fragment carries, 5' to 3', the sequence coding for aa 398 to the stop codon of the urokinase DNA and sequences derived from pULB 1221. On the other hand, a 1140 bp Hind III-Ava II fragment coding for the ATG initiating codon, the signal peptide and aa 1 to aa 358 of urokinase molecule was also excised from pULB 9117. A third natural DNA fragment was prepared by digestion of pULB 9117 with the enzymes Fnu4H I and BamH I. It is a 95 bp long stretch encoding aa 367 to aa 398 of urokinase. At last, a double-stranded 26 bp long Ava II-Fnu4H I fragment coding for aa 358 to aa 366 including the single amino acid substitution described above, was chemically synthesized (adaptor 5, Table 1). These fragment were ligated together and, from the resulting plasmid, pULB 9119, the Hind III-Sac I cassette coding for ppUK.410/366 was recovered as a 1309 bp fragment and introduced into pDSP1.1BGH yielding the final plasmid, pULB 9134.

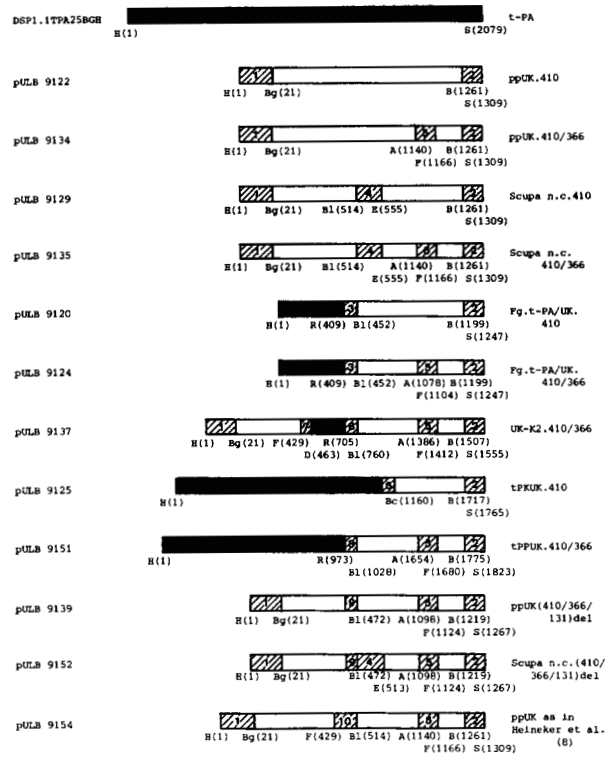

Figure 1 : Schematic representation of Hind III-Sac I sequences coding for recombinant plasminogen activators.
Open and dotted bars correspond to sequences originating respectively from scu-PA and t-PA. Hatched boxes correspond to synthetic DNA adaptors ; numbers refer to sequences in Table 1. Plasmid number and product denomination are indicated on the left and right side of the figure respectively. Restriction sites are indicated by the following abbreviations : A: Ava I, B: Bam HI, Bc: Bcl I, Bg: Bgl I, Bl: Bal I, D: Dde I, E: Eco RI, F: Fnu4 HI, H: Hind III, N: Nco I, R: Rsa I and S: Sac I.

pULB 9129

This plasmid carries the sequence coding for preprourokinase in which the activation site (Arg 156-Phe 157-Lys 158) is replaced by Thr 156-Phe 157-Thr 158. Starting from plasmid pULB 9117, a 3175 bp Bal I-BamH I fragment was recovered. This fragment carries on its 3' end, the ATG initiating codon, the sequence coding from aa-19 to aa149 of preprourokinase and, on its 5' end, the sequence coding for aa 398 to the stop codon, those two regions being separated by the sequences of pULB 1221 present in the plasmid pULB 9117. On the other hand, a 292 bp EcoR I-Xho II fragment coding for aa 163 to aa 260 of preprourokinase was derived from pULB 9117. A third fragment , a 414 bp Xho II -BamH I piece from pULB 9117, encoding aa 260 to aa 398 of the preprourokinase, was purified. At last, a double-stranded 41 bp Bal I-EcoR I adaptor coding for aa 149 to aa 163 including the two amino acid substitutions described above (adaptor 4, Table 1) was synthesized. The four fragments were ligated to generate the recombinant plasmid pULB 9128. The Hind III-Sac I cassette coding for Scupa n.c.410 was recovered from pULB 9128 as a 1309 bp fragment and introduced into pDSP1.1BGH, resulting in pULB 9129.

pULB 9135

pULB 9135, is identical to pULB 9129 except that the codon for Cysteine 366 has been replaced by a codon for Glycine. The procedure used to construct pULB 9135 is identical to the one described for pULB 9134. The recombinant plasmid pULB 9131 coding for unactivable preprourokinase (Scupa n.c.410/366) has been amplified in E. coli as described above. A 1309 bp Hind III-Sac I cassette was then excised from pULB 9131 and introduced in pDSP1.1BGH to generate pULB 9135.

Table 1: Sequences of the synthetic DNA adaptors.

ADAPTOR 1:

```
                Met Arg Ala Leu L(eu)
5'  AG CTT ACC ATG AGA GCC CTG C  3'
3'     A TGG TAC TCT CGG GAC G  5'
    Hind III                Bgl I blunt
```

ADAPTOR 2:

```
(Tr)p Ile Arg Ser His Thr Lys Glu Glu Asn Gly Leu Ala Leu STOP
5'  G ATC CGC AGT CAC ACC AAG GAA GAG AAT GGC CTG GCC CTC TGA GAG CT 3'
3'     GCG TCA GTG TGG TTC CTT CTC TTA CCG GAC CGG GAG ACT C     5'
    BamHI                                                  Sac I
```

ADAPTOR 3:

```
(T)yr Lys Pro Ser Ser Pro Pro Glu Glu Leu Lys Phe Gln Cys Gl(y)
5' AC AAG CCC TCG AGT CCT CCA GAA GAA TTA AAA TTT CAG TGT GG 3'
3' TG TTC GGG AGC TCA GGA GGT CTT CTT AAT TTT AAA GTC ACA CC 5'
    Rsa I                                              Bal I
```

ADAPTOR 4:

```
(Gl)y Gln Lys Thr Leu Arg Pro Thr Phe Thr Ile Ile Gly Gly G(lu)
5'  C CAA AAG ACT CTG AGG CCC ACC TTT ACC ATT ATT GGG GGA G     3'
3'  G GTT TTC TGA GAC TCC GGG TGG AAA TGG TAA TAA CCC CCT CTT AA 5'
  Bal I                                                Eco RI
```

ADAPTOR 5:

```
(G)ly Pro Leu Val Cys Ser Leu Gln Gly
5' GA CCC CTC GTC TGT TCC CTC CAA GGC   3'
3'     GG GAG CAG ACA AGG GAG GTT CCG G 5'
   Ava II                       Fnu4H I
```

ADAPTOR 6:

```
(Se)r Pro Cys Trp Va(l)
5'  C CCT TGC TGG GT     3'
3'  G GGA ACG ACC CAC TAG 5'
                 Bcl I
```

ADAPTOR 7:

```
(P)ro Leu Val Gln Glu Cys Met Val His Asp Cys Ser Glu Gl(y)
5' CG CTA GTA CAA GAG TGC ATG GTC CAT GAC TGC TCT GAG GG     3'
3'  C GAT CAT GTT CTC ACG TAC CAG GTA CTG ACG AGA CTC CCA GCT 5'
Fnu4H I                                          Sal I
```

ADAPTOR 8:

```
(T)yr Cys Asp Val Pro Ser Cys Ser Ser Pro Pro Glu Glu Leu Lys Phe Gln Cys Gl(y)
5' AC TGT GAC GTC CCC AGC TGC TCG AGT CCT CCC GAG GAA CTT AAG TTT CAG TGT GG 3'
3' TG ACA CTG CAG GGG TCG ACG AGC TCA GGA GGG CTC CTT GAA TTC AAA GTC ACA CC 5'
 Rsa I                                                              BAL I
```

ADAPTOR 9:

```
(P)ro Leu Val Gln Glu Cys Met Val His Asp Cys Ser Thr Cys Gl(y)
5' CA CTA GTC CAA GAG TGC ATG GTG CAT GAC TGC TCC ACC TGT GG 3'
3'  T GAT CAG GTT CTC ACG TAC CAC GTA CTG ACG AGG TGG ACA CC 5'
                                                    Bal I
```

ADAPTOR 10a:

```
(P)ro Leu Val Gln Glu Cys Met Val His Asp Cys Ala Asp
5' CG CTT GTC CAA GAG TGC ATG GTG CAT GAC TGC GCA GAT                  3'
3'  C GAA CAG GTT CTC ACG TAC CAC GTA CTG ACG CGT CTA CCT TTT TTC GGG 5'
 Fnu4H I
```

ADAPTOR 10b:

```
   Gly Lys Lys Pro Ser Ser Pro Pro Glu Glu Leu Lys Phe Gln Cys Gl(y)
5' GGA AAA AAG CCC TCC TCT CCT CCA GAA GAA TTA AAA TTT CAG TGT GG 3'
3'              AGG AGA GGA GGT CTT CTT AAT TTT AAA GTC ACA CC 5'
                                                    Bal I
```

Double-stranded DNA adaptors were synthesized chemically as single-stranded oligonucleotides and hybridized prior to ligation (36).

pULB 9139

pULB 9139 codes for a urokinase-like plasminogen activator with the following modifications: firstly, amino acid Trp 131 has been replaced by a cysteine residue. Secondly, the amino acid sequence expanding from residue 132 to residue 147 has been deleted from the molecule and replaced by the dipeptide Ser-Thr as it is the case in the t-PA molecule (7). The construction involves ligation of three fragments. A 3673 bp Bal I-Nco I fragment from pULB 9119 carries on its 3' end the ATG initiating codon, the signal sequence and the codons for aa 1 to aa 66 of preprourokinase and, on its 5' end, sequences coding for aa 149 to the stop codon . Both 3' and 5' ends are separated by sequences derived from pULB 1221. A 164 bp Nco I-Fnu4H I fragment from pULB 9119 codes for aa 66 to aa 121 of preprourokinase. The third fragment is a 43 bp Fnu4H I-Bal I adaptor (adaptor 9, Table 1) obtained by chemical synthesis. The three fragments described above were ligated, resulting pULB 9138. A 1267 bp Hind III-Sac I cassette encoding ppUK.(410/366/131)del was excised and introduced into pDSP1.1BGH, to generate pULB 9139.

pULB 9152

pULB 9152 carries the coding sequences of Scupa n.c.(410/366/131)del, a preprourokinase unactivable (aa 156 and 158 are Thr instead of Arg and Lys, see pULB 9129) and undegradable (the region from aa 132 to 147 has been deleted and replaced by the dipeptide Ser-Thr, see pULB 9139). This construction was performed by ligation of a 472 bp Hind III-Bal I fragment from pULB 9138 encoding the region from the ATG initiating codon to aa 135 of ppUK.(410/366/131)del, and of a 6975 bp Hind III-Bal I fragment from pULB 9135, encompassing the large Hind III-Sac I fragment from pDSP1.1BGH and the coding sequence for aa 149 to the stop codon of Scupa n.c.410/366.

pULB 9120

This plasmid carries the gene coding for Fg.t-PA/UK.410, a chimaeric low molecular weight urokinase having the finger module of t-PA on its NH_2 terminus. To construct this plasmid, a 409 bp Hind III-Rsa I fragment was derived from pDSP1.1TPA25BGH. This fragment encompasses the sequences corresponding to the ATG initiating triplet, the signal sequence, the so-called finger module and part of the so-called EGF module (up to aa 67) of the t-PA molecule (7). On the other hand, a 1219 bp Bal I-Sal I fragment was derived from pULB 1135. This fragment encompasses the cDNA coding for aa 149 to the stop codon of preprourokinase and includes 3' pBR322 sequences. At last, a 43 bp Rsa I and Bal I adaptor encoding aa 67 of t-PA and aa 136 to 149 of prourokinase (adaptor 3, Table 1) was synthesized. The three fragments described above were ligated to pULB 1221 (42) cut with Hind III and Sal I. The resulting plasmid was further manipulated as described for pULB 9117 to remove exceeding 3' pBR322 sequences. Finally, the 1247 bp Hind III-Sac I cassette coding for Fg.t-PA/UK.410 was excised from pULB 9115 and inserted into pDSP1.1BGH, giving pULB 9120.

pULB 9124

pULB 9124 is identical to pULB 9120 except that the codon corresponding to aa 366 of preprourokinase has been replaced by a codon for glycine. We ligated a 452 bp Hind III-Bal I fragment purified from pULB 9115, encoding the region from the ATG initiating codon to aa 67 of t-PA and aa 136 to 149 of preprourokinase to a 6975 bp Hind III- Bal I fragment from pULB 9134 encompassing the large Hind III-Sac I fragment of pDSP1.1BGH and the sequence coding for aa 149 to the stop codon of preprourokinase.

pULB 9137

pULB 9137 carries the gene coding for an hybrid molecule where the amino acid sequence corresponding to the kringle 2 of t-PA is inserted between the kringle domain and the protease moeity of preprourokinase. The construction has been carried out by subcloning several DNA fragments into intermediate E. coli plasmid vectors. Firstly, a 164 bp Nco I-Fnu4H I fragment encoding aa 66 to aa 121 of preprourokinase was excised from pULB 9119 and a 40 bp Fnu4H I-Sal I adaptor (adaptor 7, Table 1) were prepared. Adaptor 7 codes, in a row, for aa 121 to aa 130 of prourokinase and for aa 173 to aa 175 of t-PA, this sequence being immediately followed by an additional Sal I site. The two fragments were ligated to a 2916 bp Sal I-Nco I fragment derived from pJRD184 to obtain pBA1. Secondly, a 258 bp Pst I-Rsa I DNA fragment derived from pDSP1.1TPA25BGH and coding for aa 169 to aa 255 of t-PA was purified. In addition, a 55 bp Rsa I-Bal I adaptor (adaptor 8, Table 1) was prepared. Adaptor 8 codes, in a row, for aa 255 to aa 262 of t-PA and for aa 139 to aa 149 of preprourokinase. The two fragments were ligated to a 3436 bp Bal I-Pst I fragment from pJRD158 giving pBA2. At last, a 198 bp Nco I-Dde I fragment from pBA1 coding for aa 66 to aa 130 of preprourokinase and for aa 173 to 174 of t-PA and a 297 bp Dde I-Bal I piece from pBA2 coding for aa 174 to 255 of t-PA and for aa 139 to 149 of preprourokinase were excised. These two fragments were ligated to a 3673 bp Nco I-Bal I DNA fragment derived from pULB 9119. This last piece of DNA carries on its 3' end the sequences for the ATG initiating codon, for the signal peptide and for aa 1 to 66 of preprourokinase. On its 5' end, it codes for aa 149 to the stop codon of preprourokinase. Sequences separating these two 3' and 5' regions originate from pULB 1221. From the resulting plasmid, pULB 9136, a 1555 bp Hind III-Sac I coding cassette corresponding to the product UK-K2.410/366 was excised and introduced into pDSP1.1BGH, resulting in pULB 9137.

pULB 9151

pULB 9151 carries the sequence coding for an hybrid plasminogen activator, tPPUK.410/366, comprising the A-chain of t-PA (from the ATG initiating codon to aa 262) on its N-terminus and the B-chain of preprourokinase (from aa 139 to the stop codon) on its C-terminus. The construction of pULB 9151 involves three DNA pieces. A 206 bp EcoR I-Bal I fragment was excised from pULB 9136; it provides the sequence joining the kringle 2 of t-PA and the protease moeity of urokinase. A second 822 bp Hind III-EcoR I DNA fragment, coding for the ATG codon, the signal sequence and aa 1 to 204 of t-PA , was obtained from pDSP1.1TPA25BGH. The last fragment, is a 6975 bp Hind III-Bal I stretch from pULB 9134. This fragment carries the coding sequence from aa 149 to the stop signal of preprourokinase and all the sequences of pDSP1.1BGH from the Hind III to Sac I. The three fragments were ligated to obtain pULB 9151 which carries a 1823 bp Hind III-Sac I cassette coding for tPPUK.410/366.

pULB 9125

pULB 9125 carries the sequences coding for an hybrid between t-PA and prourokinase , tPKUK.410, presenting the A-chain and the activation site (from the ATG initiating codon up to aa 313) of t-PA and the enzymatic moeity (aa 195 to the stop codon) of preprourokinase. The construction has been achieved by ligating 5 DNA fragments directly to the 6180 bp Hind III-Sac I fragment of pDSP1.1BGH. The 5 fragments are:

- a 822 bp Hind III-EcoR I fragment from pDSP1.1TPA25BGH. It contains the sequences coding for the initiating codon, the signal peptide and aa 1 to 204 of the t-PA molecule.
- a 326 bp EcoR I-Alu I fragment from pDSP1.1TPA25BGH and encoding aa 205 to 313 of t-PA.
- a 12 bp adaptor coding for aa 195 to 199 of preprourokinase (adaptor 6, Table 1).
- a 248 bp Bcl I-Taq I fragment from pULB 9117 coding for aa 199 to 282 of prourokinase.
- a 397 bp Taq I-Sac I fragment derived from pULB 9117 coding for aa 282 to the stop codon of prourokinase.

pULB 9154

pULB 9154 carries the sequence for ppUK.410/366/131, which is a preprourokinase which conforms previously published sequence (8, 23, 24). To construct this plasmid, four fragments have been prepared. Firstly, a large 3673 bp Nco I-Bal I fragment was excised from pULB 9119. It carries on its 3' end the sequences for the ATG initiating codon, for the signal peptide and for aa 1 to 66 of preprourokinase. On its 5' end, it codes for aa 149 to the stop codon of preprourokinase. Sequences separating these two 3' and 5' regions originate from pULB 1221. The second fragment, a 164bp Nco I-Fnu4H I piece from pULB 9119 encodes aa 66 to 121 of preprourokinase. Finally, adaptors 10a and 10b (Table 1) coding for aa 121 to 149 of preprourokinase were synthesized. These four DNA fragments were ligated to generate pULB 9153 and the 1309 bp Hind III-Sac I cassette coding for ppUK.410/366/131 was introduced into pDSP1.1BGH, resulting in pULB 9154.

Transfection and expression in mammalian cells

Chinese hamster R1610 cells ($Gal k^-$,$hgprt^-$) (43) or monkey COS I cells (44) were maintained in Dulbecco's modified minimal essential medium (DMEM) supplemented with 5% Fetal calf serum (FCS) and 2 mM L-glutamine. Transfections were performed as follows: 20 µg of recombinant plasmid DNA, twice purified by CsCl gradient centrifugation was added to 80 cm^2 dishes with approximately 2.10 cells in DMEM supplemented with 5% FCS, 2 mM L-glutamine and 50 µg/ml Gentamycin (Sigma) via the calcium phosphate coprecipitation procedure (45). After 4 hours of incubation, the cells were shocked with 10% glycerol in DMEM for 4 minutes. The cells were then further incubated in DMEM containing 5% FCS for 3 to 5 days in presence of Aprotinin. The cell supernatants were recovered to assay the production level and the biological activity of secreted recombinant enzymes.

Immunological detection of recombinant plasminogen activators in cell culture supernatants

Recombinant protein levels in cell culture medium were measured using a micro-ELISA system as described by Hérion et al. (46), involving the monoclonal antibodies AAU2 and AAU6 raised against 33000 daltons urokinase .

Determination of enzymatic activity in cell culture supernatants

Micotiter plates (NUNC Immunoplate I) precoated with monoclonal antiboby AAU2 (46) were incubated with increasing concentrations of the standard two-chain urokinase and with several dilutions of biological samples as performed for ELISA procedure. After rincing, 100 l of PBS buffer pH 7.5 containing 0.1% Tween 80, 1µM plasminogen and 0.5 mM S2251 were added to each well. Change of absorbance at 405 nm was then followed for 5 to 6 hours with a microELISA automatic reader (Dynatech MR 600) and plotted versus squared time (t^2) as described by Drapier et al. (47). This procedure enables to linearize the assay as long as plasminogen and S2251 concentrations remain constant.

Control incubations without plasminogen activator, with culture supernatants of cells producing no recombinant plasminogen activators or without plasminogen in the assay mixture did not show any significant change of absorbance at 405 nm during the period of the assay (data not shown).

When specified, plasminogen activators linked to monoclonal antibody AAU2 were incubated with PBS buffer pH 7.5 containing 5 nM plasmin and 0.1% Tween 80 for 1 hour. The plates were then briefly rinced and exposed to PBS buffer pH 7.5 containing 100 nM aprotinin and 0.1% Tween 80 for 3-4 minutes. Enzymatic activity assay was then directly performed after extensive rincing with PBS buffer pH 7.5 containing 0.1% Tween 80.