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\textsuperscript{166} and Lys\textsuperscript{168} were substituted with threonine, thus preventing cleavage by thrombin and plasmin; 2) hybrid molecules in which the NH\textsubscript{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\textsuperscript{168} 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)\textsuperscript{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\textsuperscript{166}-Ile\textsuperscript{167} bond and single-chain u-PA (scu-PA), primarily, between Lys\textsuperscript{166} and Ile\textsuperscript{168} residues. Secondary cleavages in the u-PA molecule occur at the Arg\textsuperscript{166}-Phe\textsuperscript{167} (with thrombin) (10) and Lys\textsuperscript{168}-Lys\textsuperscript{169} 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\textsubscript{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

\[ \text{ker}, \text{amino-terminal region of t-PA, homologous to the finger-like domains in fibronectin; \textit{aa}, amino acid(s).} \]

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

\[ \text{ Mutant and Chimeric Recombinant Plasminogen Activators PRODUCTION IN EUKARYOTIC CELLS AND PRELIMINARY CHARACTERIZATION*} \]

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domains are highly homologous to equivalent structures of plasminogen involved in fibrin-binding (25, 26). In addition, the NH₂-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 (k₉ ~ 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

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 procarboxypeptidase A₂ cDNA (9) and pDSPl.TPA25.BGH carries a t-PA precursor cDNA. The recombinant molecules derived from the manipulation of these DNAs share common features: they were all obtained by HindII-SacI 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 HindIII and SacI 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 HindIII-SacI 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 preprocarboxypeptidase A₂ 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 preprocarboxypeptidase A₂ (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 (Arg¹⁰⁶ and Lys¹⁰⁸ 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) (16-20), 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 (Lys¹⁰⁶-Arg¹⁰⁷) and to replace lysine and arginine in the fibrinopeptide A of 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 activeable prourokinase, whereas Scupa n.c.(410/366/131)del, coded for by pULB9152, is deleted and non-activeable. Finally, for comparison purposes, we constructed a recombinant prourokinase 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 DNA 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. Fgt.PA/UK.410 and Fgt.PA/UK.410/368 (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 356 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.410/368, 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 moiety (49, 50). A similar product, tPPUK.410 (coded for by pULB9125), consists of the NH₂-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
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TABLE II

General description of recombinant plasminogen activators

| Plasmid          | Product denomination | Amino acid residue position* | Description                                      |
|------------------|----------------------|----------------------------|--------------------------------------------------|
| pULB1000         | ppUK                 | 131 Trp, 366 Cys, 410 Val  | Preprourokinase cDNA clone (9)                    |
| pULB1135         | ppUK                 | 131 Trp, 366 Cys, 410 Val  | Preprourokinase cDNA clone (9)                    |
| pDSP1.1TPA25BGH  | t-PA                 | 131 Trp, 366 Cys, 410 Val  | t-PA cDNA clone                                  |
| pULB9122         | pUK.410              | 131 Trp, 366 Cys, 410 Val  | ppUK where Val<sup>131</sup> has been replaced by Ala |
| pULB9134         | pUK.410/366          | 131 Trp, 366 Cys, 410 Val  | ppUK.410 where Cys<sup>366</sup> has been replaced with Gly |
| pULB9154         | pUK.410/366/131      | 131 Trp, 366 Cys, 410 Val  | ppUK.410/366/131 where aa 132-147 have been replaced by Ser-Thr |
| pULB9139         | pUK.410.(131)del     | 131 Trp, 366 Cys, 410 Val  | pUK.410.(131)del where aa 156 and 158 have been replaced by Thr in order to produce an uncleavable ppUK |
| pULB9129         | Scupa n.c.410        | 131 Trp, 366 Cys, 410 Val  | Scupa n.c.410 where Cys<sup>410</sup> has been replaced by Gly |
| pULB9135         | Scupa n.c.410/366    | 131 Trp, 366 Cys, 410 Val  | Scupa n.c where aa 132 and 147 have been replaced by Ser-Thr |
| pULB9152         | Scupa n.c.(131)del   | 131 Trp, 366 Cys, 410 Val  | Scupa n.c.(131)del where aa 132 and 147 have been replaced by Ser-Thr |
| pULB9120         | FG.t-PA/UK.410       | 131 Trp, 366 Cys, 410 Val  | FG.t-PA/UK.410 where aa 156 and 158 have been replaced by Thr |
| pULB9124         | FG.t-PA/UK.410/366   | 131 Trp, 366 Cys, 410 Val  | FG.t-PA/UK.410 where aa 156 and 158 have been replaced by Thr |
| pULB9151         | tPPUK.410/366        | 131 Trp, 366 Cys, 410 Val  | tPPUK.410/366 where aa 156 and 158 have been replaced by Thr |
| pULB9125         | tPKUK.410            | 131 Trp, 366 Cys, 410 Val  | tPKUK.410 where aa 156 and 158 have been replaced by Thr |
| pULB9137         | UK-K2.410/366        | 131 Trp, 366 Cys, 410 Val  | UK-K2.410/366 where aa 156 and 158 have been replaced by Thr |

* 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 III). 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 (data not shown). As previously described by Drapier et al. (47), plotting of A<sub>405</sub> as a function of squared time (t<sup>2</sup>) enables us to linearize the assay as long as initial conditions are valid (see Miniprint Section). The slope of these straight lines is almost proportional to the total u-PA concentration present in the experimental standard incubation mixtures and 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 sci-PA appeared biphasic. As shown for three different dilutions of the recombinant pUK.410/366 (pULB-
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9134), the linear phase was preceded by an exponential lag phase (Fig. 4A). 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. 4B). 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. 4A, 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. 4A) 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 fibrinolytic/fibrinogenolytic 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 NH2 terminus the finger domain of t-PA; tPKUK.410 contains the A-chain of t-PA with u-PA (in part or in toto). 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.

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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-K410/366. 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.
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TABLE III

Expression levels and activity determinations of recombinant plasminogen activators

| Plasmid       | Gene product            | R1610 | Cos I |
|---------------|-------------------------|-------|-------|
|               |                         | activity in supernatant | apparent specific activity | activity in supernatant | apparent specific activity |
| pULB9122      | ppUK.410                | 3.9   | 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      | F.g.t-PA/UK.410        | 12    | 0.43  | 35,300 |
| pULB9124      | F.g.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 |
| pULB9134      | ppUK.410/366           | 74.3  | 2.64  | 35,500 |
| pULB9129      | Scupa n.c.410          | 18    | 0     | 0      |
| pULB9135      | Scupa n.c.410/366      | 29    | 0     | 0      |
| pULB9151      | tPPUK.410/366          | 6.5   | 4.44  | 66,000 |
| pULB9137      | UK-K2.410/366          | 13.8  | 0.43  | 31,250 |
| pULB9122      | ppUK.410               | 7.5   | 0.48  | 63,600 |
| pULB9154      | ppUK.410/366/131       | 7.5   | 0.36  | 48,000 |
| pULB9135      | Scupa n.c.410/366/131  | 3.8   | 0     | 0      |

* Concentrations and apparent specific activities are given in 54,000 dalton urokinase equivalents.

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

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-
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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: 1, undiluted supernatant; 2, 5-fold dilution; 3, 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.

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 (FgUK.410), and the data are presented in the accompanying paper (51).

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SUPPLEMENTARY MATERIAL

EXPERIMENTAL PROCEDURES

Construction of expression plasmids:

Plasmids pULB 1000 (32), pULB 1135 (33), pJR0184 (33) and pLM381 (34) have been described earlier. Plasmids pULB 1000 (32), pULB 1135 (33) and pJR0184 (33) were derived from pULB 1000 (32), pULB 1135 (33) and pJR0184 (33), respectively. The restriction fragment coding for the amino acid sequence of the plasminogen activator gene was ligated into the appropriate sites of pULB 1000 (32), pULB 1135 (33) and pJR0184 (33) to yield pULB 1000 (32), pULB 1135 (33) and pJR0184 (33), respectively.

Expression of plasminogen activator:

Plasmids pULB 1000 (32), pULB 1135 (33) and pJR0184 (33) were introduced into E. coli K12 strain MM 294 (endA, thr-, thr-). The plasmids were prepared by digestion with Hind III and Sac I and ligated to yield pULB 1000 (32), pULB 1135 (33) and pJR0184 (33), respectively.

Characterization of recombinant plasminogen activators:

The recombinant plasminogen activators were characterized by sequencing the DNA and comparing it with the DNA sequence of the plasminogen activator gene. The amino acid sequence of the plasminogen activator was determined by amino acid analysis and by amino acid sequencing of the recombinant plasminogen activator.

The amino acid sequence of the plasminogen activator was compared with the DNA sequence of the plasminogen activator gene. The DNA sequence of the plasminogen activator gene was determined by DNA sequencing and by DNA synthesis. The DNA sequence of the plasminogen activator gene was compared with the DNA sequence of the plasminogen activator coding region.

The DNA sequence of the plasminogen activator coding region was compared with the DNA sequence of the plasminogen activator gene. The DNA sequence of the plasminogen activator gene was determined by DNA synthesis and by DNA sequencing. The DNA sequence of the plasminogen activator gene was compared with the DNA sequence of the plasminogen activator coding region.
New Recombinant Plasminogen Activators

Table 1: Sequences of the synthetic DNA substrates.

| ADAPTOB 5' | ADAPTOB 3' |
|------------|------------|
| TGC TAC TCT CCC GAC G | GGC TCA CTG TCG TTC CTT CTC TTA CCG GAC GAG ACT C |
| TAC TCT CCC GAC G | GAC TCA CTG TCG TTC CTT CTC TTA CCG GAC GAG ACT C |
| (P)ro Leu Val Gin Clu Cya Het Val Hla Aap Cya Ser Tlir CfS CI(y) |
| 5' CC CTA CTA CAA CAC TCC ATC CTC CAT GAC TGC TCT CAC GG T |
| 5" GA CCC CTC GTC TGT TCC CTC CAA CGC |
| ADAPTOR 5: |
| (Cl)y Gin |
| CGG |
| 3" GCG TCA CTG TCG TTC CTT CTC TTA CCG GAC GAG ACT C |
| (Tr)p Il* Arg Ser Hia Thr |

Construction involves IIKIIIC peptide Ser-Thr as it is also in the t-PA mutator (7). The cytokine was reactivated to Src until it is not identical to MIU-IU-I. The peptide resulted in MIU-IU-I in vivo.


drures described above were ligated to pILB 1221 (42) coding for the ATG initiating codon, for the signal peptide and for the ATG initiating codon up to a 300 bp Hind III fragment.

The second fragment, a 164 bp Hco I-Fnu4H I piece coding for prourokinase. Sequences separating these two 5' regions originate from pULB 9139 coding for an H2KIIIC cassette codon.

For plasminogen activators or a single-stranded oligonucleotide and phosphorylated to link to prourokinase.

New Recombinant Plasminogen Activators

Deiermination of enzymatic activity in cell culture supernatants

The cell supernatants were recovered to assess the production level and the activity of a single-stranded oligonucleotide and phosphorylated to link to prourokinase.

For plasminogen activators or a single-stranded oligonucleotide and phosphorylated to link to prourokinase.

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