Artificial Exon Shuffling between Tissue-Type Plasminogen Activator (t-PA) and Urokinase (u-PA): A Comparative Study on the Fibrinolytic Properties of t-PA/u-PA Hybrid Proteins†

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ABSTRACT: We constructed two human tissue-type plasminogen activator/urokinase (t-PA/u-PA) hybrid cDNAs which were expressed by transfection of mouse Ltk$^-$ cells. The properties of the secreted proteins were compared with those of recombinant t-PA (rt-PA) and high molecular weight (HMW) u-PA. The hybrid proteins each contain the amino-terminal fibrin-binding chain of t-PA fused to the carboxy-terminal serine protease moiety of u-PA but differ by a stretch of 13 amino acid residues between kringle 2 of t-PA and the plasmin cleavage site of u-PA. Hybrid protein rt-PA/u-PA I contains amino acids 1–262 of t-PA connected with amino acids 147–411 of u-PA, whereas hybrid protein rt-PA/u-PA II consists of the same t-PA segment and residues 134–411 of u-PA. We demonstrated fibrin binding for rt-PA, whereas the hybrid proteins bind to a lesser extent and HMW u-PA has no affinity for fibrin. Plasminogen activation by either one of the hybrid proteins in the absence of a fibrin substitute was similar to that by HMW u-PA, while rt-PA was much less active. The catalytic efficiency, in the presence of a fibrin substitute, increases more than 2000-fold for rt-PA, about 250-fold for hybrid proteins I and II, and 12-fold for HMW u-PA, respectively. Under these conditions the hybrid proteins are more efficient plasminogen activators than the parental ones. The hybrid molecules form a 1:1 molar complex with the human endothelial plasminogen activator inhibitor (PAI-1), analogous to that formed by rt-PA and HMW u-PA. The relative affinity of rt-PA for PAI-1 is 4.6-fold higher than that of HMW u-PA. The relative affinity of hybrid protein rt-PA/u-PA I for PAI-1 is comparable to that of rt-PA, while that of rt-PA/u-PA II is even less than the affinity of HMW u-PA for PAI-1. Our results are discussed in terms of the "exon shuffling" model that has been proposed for particular proteins, such as t-PA and u-PA.

The fibrinolytic system is responsible for dissolution of thrombi and thus ensures an unobstructed circulation of blood. Fibrin polymers, the main protein constituent of a thrombus, are degraded by plasmin. Plasmin is a serine protease with a broad specificity and is formed by proteolytic cleavage of a specific peptide bond within its precursor molecule, plasminogen. This conversion is mediated by plasminogen activators (PAs),[1] serine proteases belonging to the "trypsin-like" family (Neurath, 1985). Two distinct PAs have been described, tissue-type plasminogen activator (t-PA) and urokinase (u-PA), which can be distinguished by both biochemical and immunological criteria. t-PA displays a low activity in the absence of fibrin and is stimulated several orders of magnitude in its presence. In contrast, u-PA has a substantial plasminogen activator activity in the absence of fibrin and is not stimulated by its presence (Collen, 1980).

Both proteins are produced as single-chain polypeptides and can be converted by plasmin into two-chain molecules, having a unique disulfide bond to connect the two chains. The amino- and carboxy-terminal chains of these PAs are designated, H and L (t-PA) and A and B (u-PA), respectively. The primary structure of both PAs has been determined by biochemical methods (Günzler et al., 1982; Steffens et al., 1982; Pohl et al., 1984) and has also been deduced from the nucleotide sequence of constructed full-length cDNAs (Pennica et al., 1983; Heyneker et al., 1983). In addition, on the basis of homology between particular segments of t-PA and u-PA with other plasma proteins, models for the secondary structure have been proposed, depicting an array of distinct domains (Pennica et al., 1983; Bányai et al., 1983; Holmes et al., 1985). Consecutive domains are a signal peptide (SP), a pro-sequence (PS), a "finger" (F), an "epidermal growth factor like" domain (E), "kringle(s)" (K), and the serine protease moiety (P) located at the carboxy-terminal end. Hence, t-PA can be represented by the formula SP-PS-F-E-K1-K2-P and, similarly, u-PA by SP-E-K-P.

Considerable support for these secondary structure models came from two lines of evidence. First, the elucidation of the gene structure of t-PA and u-PA showed that exons or sets

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[1] Abbreviations: CNBr-Fbg, cyanogen bromide digested fibrinogen; ECCM, conditioned medium of cultured human endothelial cells; EDTA, sodium salt of ethylenediaminetetraacetic acid; HMW u-PA, high molecular weight urokinase; mt-PA, Bowes melanoma tissue-type plasminogen activator; PA, plasminogen activator; PAI-1, endothelial-type plasminogen activator inhibitor; PBS, phosphate-buffered saline [10 mM sodium phosphate (pH 7.4) and 150 mM NaCl]; rt-PA, recombinant tissue-type plasminogen activator; SDS, sodium dodecyl sulfate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; t-PA, tissue-type plasminogen activator; Tris, tris(hydroxymethyl)aminomethane; u-PA, urokinase.

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of adjacent exons encode a domain (Ny et al., 1984; Riccio et al., 1985; Degen et al., 1986). Second, we have constructed a series of t-PA cDNA deletion mutants lacking one or more of the proposed domains and expressed these mutant cDNAs in tissue culture cells (van Zonneveld et al., 1986a). These studies allowed us to assign specific functions to distinct domains: F and K2 mediate fibrin binding and subsequent stimulation of plasminogen activator activity by fibrin, while the catalytic center is located in the carboxy-terminal L chain (MacDonald et al., 1986; van Zonneveld et al., 1986a). Interaction of t-PA with the endothelial plasminogen activator inhibitor (PAI-1) is mediated by the L chain, although interaction with other t-PA domains could not be excluded (van Zonneveld et al., 1986b). For HMW u-PA the B chain contains the serine protease activity, as has been shown for the low molecular weight forms of u-PA (Günzler et al., 1982; Stump et al., 1986). These observations lend further support to the hypothesis that the PAs and other plasma proteins may have evolved by an evolutionary mechanism, called "exon shuffling" (Gilbert, 1978, 1985), that accounts for the creation of these genes. The exon shuffling theory predicts that an exon or a set of adjacent exons of particular genes encodes an autonomous, structural, and functional domain.

In this study we have investigated whether specific functions of two homologous genes, presumably both created by exon shuffling, can be combined into hybrid proteins. For that purpose, the cDNA encoding the amino-terminal H chain of t-PA, which mediates fibrin binding and stimulation of plasminogen activator activity by fibrin, has been fused to cDNA, encoding the B chain of u-PA exhibiting a higher "basal" plasminogen activator activity than t-PA.

Materials and Methods

Materials. Bowes melanoma t-PA (two chain, 570 000 IU/mg) was purchased from Biopool (Umea, Sweden), and human urinary HMW u-PA (two chain) was a kind gift of Dr. G. Cassani (Lepetit, Milano, Italy). The chromogenic substrate H-D-Val-Leu-Lys-*p*-nitroanilide (S2251), Glu-plasminogen, and cyanogen bromide digested fibrinogen (CNBr-Fbg) were obtained from Kabi Vitrum (Stockholm, Sweden). Human fibrinogen was purchased from Imco (Stockholm, Sweden), and human thrombin and hirudin were from Sigma Chemical Co. (St. Louis, MO). Restriction enzymes and DNA-modifying enzymes were from New England Biolabs (Beverley, MA). The synthetic oligonucleotides (36-mers) were prepared with an automated DNA synthesizer (Applied Biosystems Type 381A). Radioactive chemicals were obtained from The Radiochemical Centre (Amersham, UK). Conditioned medium of human, cultured endothelial cells (ECCM), containing approximately 2.4 ng of endothelial plasminogen activator inhibitor (PAI-1), was kindly provided by Dr. J. A. van Mourik (Department of Blood Coagulation, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service). All chemicals used were of analytical grade.

Construction of t-PA/u-PA Hybrid cDNAs. The plasmids pSV2/t-PA, containing full-length human t-PA cDNA (van Zonneveld et al., 1986a), and pHUK-1, which comprises a partially unspliced, human u-PA cDNA (Verde et al., 1984), were used to construct the t-PA/u-PA hybrid cDNAs. The numbering of the t-PA cDNA is according to Pennica et al. (1983), and for u-PA the cDNA numbering is as described by Verde et al. (1984). To construct the t-PA/u-PA hybrid cDNAs, a 511 base pair fragment of the full-length t-PA cDNA, extending from the *Eco*RI site (position 801) to the *Pst*I site (position 1312), and a 341 base pair fragment of the u-PA cDNA, from *Pst*I site (position 386) to the *Eco*RI site (position 727), were both subcloned into the *Eco*RI site of the multiple cloning site of M13mp8 (Vieira & Messing, 1982). Subsequently, we have employed the "M13 gapped-duplex outlooping" mutagenesis procedure (Kramer et al., 1984) using both recombinant M13mp8 (single stranded) and M13mp18 (double stranded, linearized) and two different synthetic oligonucleotides (36-mers) to fuse the t-PA cDNA and u-PA cDNA precisely at the two desired junctions (see Figure 1). Upon outlooping with oligonucleotide I, i.e., 5′-GAT GTG CCC TCC TGC TCC CAG TGT GGC CAA AAG ACT-3′, M13mp18 contained the t-PA cDNA sequence extending from the *Eco*RI site (position 801) to position 975 (corresponding with amino acid residue Ser-262) which was fused to the u-PA cDNA from position 679 (amino acid residue Gln-147) to the *Eco*RI site (position 727). The length of this "fusion fragment I" was 222 base pairs. Upon outlooping with oligonucleotide II, i.e., 5′-GAT GTG CCC TCC TGC TCC GGA AAA AAG CCC TCC TCT-3′, "fusion fragment II" was obtained, spanning 261 base pairs and again bracketed at both ends by *Eco*RI sites. This fusion fragment II harbors the same t-PA cDNA segment as fusion fragment I and has been fused to the u-PA cDNA from position 640 (corresponding to amino acid residue Gly-134) up to the *Eco*RI site (727). DNA sequence determinations were performed to verify correct fusion sequences (Sanger et al., 1977). Subsequently, the fusion fragments were extended at the 5′ side with t-PA cDNA derived from pSV2/t-PA, encoding the remaining amino-terminal H chain of the protein, and at the 3′ end with u-PA cDNA, encoding the remaining carboxy-terminal B chain of the protein. For that purpose, *Eco*RI fusion fragments I and II were isolated, and each of them was ligated both with the *Hin*dIII–*Eco*RI fragment of pSV2/t-PA DNA (positions 1–801) and with the *Eco*RI–*Pst*I fragment of pHUK-1 cDNA (positions 727–1969) and pUC19 (Yanisch-Perron et al.,1985) digested with *Hin*dIII and *Pst*I. It should be noted that both constructs contain sequences of the u-PA intron G (positions 860–1081), derived from pHUK-1 (Verde et al., 1984; Riccio et al., 1985). From the two pUC19 constructs the complete hybrid mutant sequences were isolated by using the *Hin*dIII and *Bam*HI sites in the pUC19 multiple cloning site, which bracket the entire t-PA/u-PA cDNA segments. Finally, these full-length hybrid mutant cDNAs were placed under the control of the SV40 early promotor by digesting pSV2/t-PA DNA with *Hin*dIII and *Bgl*II and replacing t-PA cDNA by either one of the aforementioned *Hin*dIII–*Bam*HI fragments containing the hybrid mutant cDNAs.

Tissue Culture and Transfection. Mouse Ltk$^-$ cells were maintained in Iscove's modified minimal medium, containing penicillin, streptomycin, and 10% (v/v) fetal calf serum. Transfection was carried out essentially as described (Lopata et al., 1984; van Zonneveld et al., 1986a). After transfection, the cells were incubated in serum-free Iscove's medium, containing penicillin and streptomycin. Five days after transfection, the cell media were harvested and Tween 80 and sodium azide [final concentrations 0.01% (v/v) and 0.02% (w/v), respectively] were added. Samples were dialyzed overnight at 4 °C against 50 mM sodium phosphate (pH 7.4), containing 0.01% (v/v) Tween 80 and 0.02% (w/v) sodium azide, and stored at 4 °C until use.

Quantification of Expression Products. The presence of the kringle 1 domain on rt-PA as well as on both hybrid proteins and the availability of two monoclonal antibodies CLB-t-PA 16 and CLB-t-PA 72 (van Zonneveld et al., 1987) directed against two different epitopes on the kringle 1 domain

of t-PA allowed us to use these two monoclonal antibodies in a sandwich assay to determine the concentration of expression products in the conditioned media (van Zonneveld, unpublished data). The conditioned media containing the hybrid proteins were first concentrated with Amicon Centricon 30 filters, because the concentration of secreted products was below the detection level of this sandwich assay (1 ng/mL). We concentrated these media 40–50-fold with a recovery of 20–30%, as determined by the activity of samples in the S2251 assay (see paragraph on plasminogen activation). Sepharose-coupled CLB-t-PA 72 antibodies were incubated in 10 mM sodium phosphate (pH 7.4) and 150 mM NaCl (PBS), containing 1% (w/v) bovine serum albumin and 0.1% (v/v) Tween 20, with serial dilutions of the (concentrated) expression media. ^{125}I-Labeled CLB-t-PA 16 IgG (15 000 cpm) was added to this mixture to measure the binding of the recombinant proteins to the monoclonal anti-t-PA Sepharose. Incubations were performed for 18 h at room temperature in a final volume of 0.5 mL with head-over-head rotation. The Sepharose beads were washed 5 times with 1.5 mL of 0.15 M NaCl, 0.1% (v/v) Tween 20, and 10 mM EDTA, and bound radioactivity was determined with a γ counter. Serial dilutions of Bowes melanoma t-PA (mt-PA) were used as a standard.

Gelatin–Plasminogen Gel Electrophoresis and Fibrin Overlay. Electrophoresis on gelatin gels, containing copolymerized plasminogen (13 μg/mL), was performed essentially as described (Heussen & Dowdle, 1980). Alternatively, nonreduced PA samples were subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970), and fibrinolytically active bands were subsequently visualized by the appearance of lysis zones according to the fibrin overlay technique prepared as described by Granelli-Piperno and Reich (1978).

Fibrin-Binding Assay. The binding of PAs to fibrin was determined essentially as described (Loskutoff & Mussoni, 1983). The reaction mixtures (500 μL) contained 1 mg/mL human fibrinogen, 10 mM EDTA, 0.01% (v/v) Tween 80 in PBS, and either 0.2 ng of HMW u-PA, rt-PA/u-PA I, or rt-PA II or 2 ng of rt-PA. Then 1 NIH unit of human thrombin was added to clot the mixtures. After 15 min at 37 °C, thrombin was inactivated by adding 2 NIH units of hirudin, and the fibrin matrices were pelleted by centrifugation. The pellets were solubilized at 37 °C in 250 μL of PBS containing 0.5% (w/v) SDS, 0.01% (v/v) Tween 80, and 2 NIH units of hirudin. For each PA, samples of the supernatant, the pellet, and the controls [total reaction mixtures, either without fibrinogen (−Fbg) or without thrombin (−IIa)] were analyzed by gelatin–plasminogen gel electrophoresis.

Kinetics of Plasminogen Activation. The activation of Glu-plasminogen to plasmin by rt-PA, rt-PA/u-PA I and II, and HMW u-PA was measured at 37 °C either in the absence or in the presence of cyanogen bromide digested fibrinogen (CNBr-Fbg) (120 μg/mL) (Verheijen et al., 1982) in a buffer containing 0.1 M Tris-HCl (pH 7.4), 0.1% Tween 80, and 0.57 mM of the chromogenic substrate S2251. The reactions were performed in 96-well microtiter plates in a volume of 250 μL. A Titertek Multiscan spectrophotometer, equipped with a thermostat (37 °C), was used to follow the reaction for 5–6 h by determining the optical density at 405 nm (A_{405}) every 10 min. Initial rates of plasminogen activation were obtained from plots of A_{405}/min versus the time of the reaction. Under these conditions an A_{405}/min of 1 corresponds with 40 pmol of plasmin per 250 μL (160 nM). In the absence of CNBr-Fbg, the Glu-plasminogen concentration varied from 0.11 to 0.55 μM for both rt-PA/u-PA I and II (0.1–0.2 ng/mL), from 0.55 to 4.4 μM for rt-PA (1.6–3.2 ng/mL), and from 0.11 to 3.3 μM for HMW u-PA (0.1–0.2 ng/mL). In the presence of CNBr-Fbg (120 μg/mL), the Glu-plasminogen concentration ranged from 1.7 to 27.5 nM for both rt-PA (0.32–0.64 ng/mL) and rt-PA-uPA I/II (0.04–0.12 ng/mL), whereas for HMW u-PA (0.1–0.2 ng/mL) the Glu-plasminogen concentration ranged from 1.7 to 110 nM. For each Glu-plasminogen concentration the experiment was performed 4 times (in duplicate). From the subsequent Lineweaver–Burk plots, we determined the K_m and v_{max} values by weighted linear regression. The k_{cat} values were calculated by using M_r 70 000, 54 000, 70 000, and 72 000 for rt-PA, HMW u-PA, rt-PA/u-PA I, and rt-PA/u-PA II, respectively. It is assumed that the amounts of antigen of the various PAs employed, determined as outlined in a previous paragraph, are fully active.

Complex Formation of the Endothelial Plasminogen Activator Inhibitor (PAI-1) and Plasminogen Activators. Complex formation between PAI-1 and either rt-PA, HMW u-PA, or the rt-PA/u-PA mutants was shown by a shift in the molecular weights of the PAs subjected to SDS–PAGE after incubation with PAI-1 (van Mourik et al., 1984; Thorsen & Philips, 1984). For this purpose, the PAs (0.2 ng) were incubated with an excess of PAI-1 (75 μL of ECCM, containing approximately 180 ng of PAI-1) for 30 min at room temperature. Subsequently, "sample buffer" was added [final concentrations 2% (w/v) sucrose, 2.5% (w/v) SDS, 4 μg/mL bromphenol blue], and after another 15 min at room temperature the samples were subjected to SDS–PAGE (Laemmli, 1970), followed by the fibrin overlay technique.

Inhibition of Plasminogen Activators with PAI-1. PAI-1, present in conditioned medium of cultured human endothelial cells (ECCM), was activated by SDS [final concentration 0.1% (w/v)] for 15 min at room temperature (Hekman & Loskutoff, 1985). Serial dilutions of activated PAI-1 (25 μL) were made in 96-well microtiter plates (25 μL), and the SDS was "neutralized" by adding Triton X-100 to a final concentration of 3% (v/v) in a total volume of 75 μL [0.1 M Tris-HCl (pH 7.4), 0.1% (v/v) Tween 20]. Subsequently, 60-μL samples of either one of the PAs (containing 0.35 fmol; i.e., 0.024 ng of rt-PA, 0.024 ng of rt-PA/u-PA I, 0.025 ng of rt-PA/u-PA II, 0.019 ng of HMW u-PA) were added, mixed well, and incubated for 30 min at room temperature. The measurement of the residual activity in a final volume of 250 μL was started by adding 115 μL of a buffer containing 0.1 M Tris-HCl (pH 7.4), 0.1% (v/v) Tween 20, human Glu-plasminogen (0.11 μM final concentration), the chromogenic substrate S2251 (0.54 mM final concentration), and CNBr-Fbg (120 μg/mL). Absorption measurements and determination of initial reaction rates were performed as described in the previous paragraph. The residual activity in the presence of different amounts of PAI-1 was expressed as percentage of the activity without PAI-1, but with SDS and Triton X-100, and dose–response curves of inhibition were plotted. From these plots we determined the amount of PAI-1 (expressed as microliters of ECCM) at which 50% of the control plasminogen activator activity is retained.

Results

Construction of t-PA/u-PA Hybrid cDNAs and Synthesis of Hybrid Proteins. We have created hybrid proteins to test if the stimulation of plasminogen activator activity, mediated by the amino-terminal H chain of t-PA, can be combined in one polypeptide with the relatively high basal plasminogen activator activity exhibited by the carboxy-terminal u-PA B chain. For that purpose, two different cDNA constructs were made, designated t-PA/u-PA I and t-PA/u-PA II. Both of

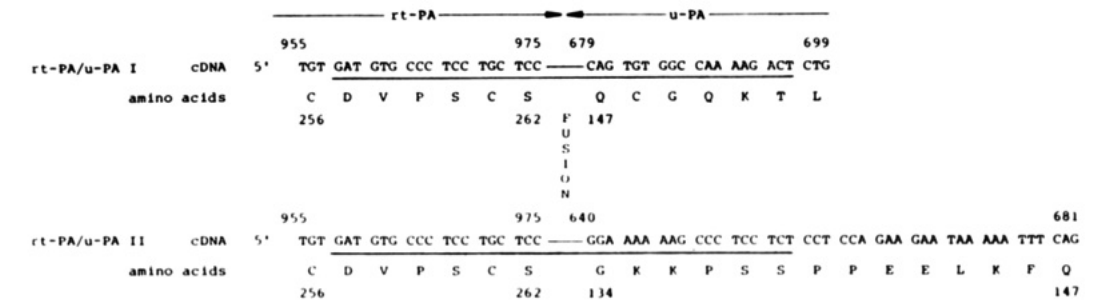

FIGURE 1: Nucleotide sequence of rt-PA/u-PA I and rt-PA/u-PA II around the fusion site of t-PA cDNA and u-PA cDNA. The numbering of t-PA cDNA is according to Pennica et al. (1983), and that of u-PA cDNA is as given by Verde et al. (1984). The corresponding amino acid residues (one-letter code) are given on a lower line. The hybrid cDNAs harbor an identical t-PA cDNA segment (extending to nucleotide 975) and contain u-PA cDNA extending either from nucleotide 679 to 1969 (I) or from 640 to 1969 (II). The nucleotide sequences of the synthetic oligonucleotides, which were used for the "M13-outlooping mutagenesis", are underlined.

these constructs contain the 5′ t-PA cDNA sequences, corresponding with exon II up to the 3′ end of exon IX of the t-PA gene. This t-PA cDNA segment encodes all of the H-chain domains, including kringle 2. On t-PA/u-PA II the 3′ end of this t-PA cDNA segment is fused to u-PA cDNA, corresponding precisely with the 5′ end of exon VII of the u-PA gene and continuing beyond the 3′ end of the last exon of this gene. The resulting hybrid protein thus consists of amino acids 1–262 of t-PA and residues 134–411 of u-PA (Figure 1). The rationale for the construction of t-PA/u-PA I cDNA is not based on the respective gene structures but on a striking difference between the composition of the t-PA and u-PA polypeptides. Specifically, the distance between the carboxy-terminal chain (arbitrarily defined by the plasmin cleavage site) and the proximal kringle is 13 amino acid residues shorter for t-PA than for u-PA. Consequently, on t-PA/u-PA I the 5′ portion of the u-PA cDNA, corresponding with the u-PA exon VII, has been truncated with 39 base pairs. Hence, hybrid protein rt-PA/u-PA I consists of amino acid residues 1–262 of t-PA and 147–411 of u-PA.

Full-length t-PA cDNA and the t-PA/u-PA mutant cDNAs were inserted into the eukaryotic expression vector pSV2 (Mulligan & Berg, 1980). The cDNAs are positioned downstream of the SV40 "early" promotor and are followed by appropriate splice and polyadenylation signals. Mouse Ltk$^-$ cells were transfected with various t-PA cDNA-containing constructs, and transient expression was allowed in serum-free medium. The concentration of the different expression products in the conditioned media was determined by an immunoradiometric assay, based on two monoclonal antibodies (CLB-t-PA 16 and CLB-tPA 72) directed against two different epitopes on the kringle 1 domain. This domain is present both on full-length rt-PA and on the t-PA substitution mutants, rt-PA/u-PA I and rt-PA/u-PA II. The concentration of rt-PA and rt-PA/u-PA I and II in the media of the transfected cells was 16, 0.6, and 0.4 ng/mL, respectively.

Similar amounts of the mutant proteins, HMW u-PA, rt-PA, and Bowes melanoma t-PA (mt-PA) were analyzed by gelatin–plasminogen gel electrophoresis. In Figure 2 it is shown that the basal plasminogen activator activity of the t-PA substitution mutants is comparable to that of HMW u-PA and at least 10-fold higher than the basal plasminogen activator activity of either rt-PA or mt-PA. Furthermore, the relative mobility of the mutant proteins rt-PA/u-PA I and II under these conditions corresponds to the designed length of the glycoproteins of 527 and 540 amino acid residues, respectively. The observed heterogeneity of the polypeptides is mainly due to differential asparagine-linked glycosylation, as demonstrated before for rt-PA (deletion mutants) (van Zonneveld et al., 1986a). Furthermore, we have chosen an extensive period to develop this indicator gel in order to visualize the relatively low activity of t-PA, thereby simultaneously revealing some minor active degradation products of the hybrid proteins and HMW u-PA.

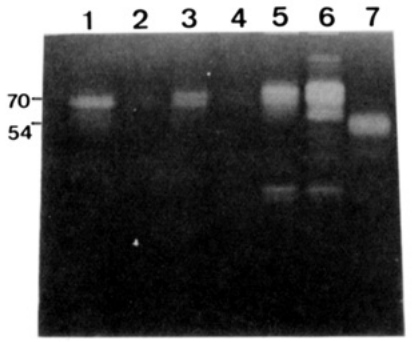

FIGURE 2: Gelatin–plasminogen gel electrophoresis of the expression products: lane 1, 1 ng of mt-PA; lane 2, 0.1 ng of mt-PA; lane 3, 1 ng of rt-PA; lane 4, 0.1 ng of rt-PA; lane 5, 0.1 ng of rt-PA/u-PA I; lane 6, 0.1 ng of rt-PA/u-PA II; lane 7, 0.1 ng of HMW u-PA. Apparent molecular weights ($\times 10^3$) for mt-PA (70) and HMW u-PA (54) are indicated.

Fibrin Binding of the rt-PA/u-PA Hybrid Proteins. Fibrin matrices were formed in the presence of either rt-PA or HMW u-PA or one of the mutant proteins and subsequently pelleted. The plasminogen activator activity of the controls, supernatants, and the solubilized pellets was analyzed by gelatin–plasminogen gel electrophoresis (Figure 3). This semiquantitative analysis shows that most of the rt-PA input fraction is bound to the fibrin matrix, whereas no significant binding of HMW u-PA is observed. The hybrid proteins exhibit affinity for the fibrin matrices, although to a lesser extent than rt-PA. It should be noted that the activity of HMW u-PA and the hybrid proteins is somewhat decreased due to the incubation with thrombin, as has been reported for HMW u-PA before (Ichinose et al., 1986; Gurewich & Pannell, 1986). We conclude that novel PAs have been created that display a u-PA-like plasminogen activator activity and are equipped with the fibrin-binding property of t-PA.

Kinetics of Plasminogen Activation by t-PA/u-PA Hybrid Proteins. We determined the initial reaction rates of plasminogen activation by the various PAs, using an indirect assay with the plasmin-specific chromogenic substrate S2251. Initial reaction rates were established both in the presence and in the absence of CNBr-digested fibrinogen (CNBr-Fbg), a soluble preparation known to mimic the stimulatory effect of fibrin on the plasminogen activator activity of t-PA (Verheijen et al., 1982). Lineweaver–Burk plots were used to determine the apparent Michaelis constants (K_m) and the catalytic rate constants (k_{cat}). The results are given in Table I. rt-PA

Table I: Kinetic Parameters of Plasminogen Activation by rt-PA, HMW u-PA, and the rt-PA/u-PA Hybrid Proteins in the Absence and in the Presence of Cyanogen Bromide Digested Fibrinogen[a]

	−CNBr fibrinogen			+CNBr fibrinogen			
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} μM^{-1})	stimulation
rt-PA	>50	0.49	<0.01	0.016	0.34	21	>2000
HMW u-PA	2.2	0.65	0.29	0.073	0.26	3.6	12
rt-PA/u-PA I	0.61	0.25	0.41	0.005	0.45	90	219
rt-PA/u-PA II	0.88	0.30	0.34	0.004	0.39	98	287

[a] K_m, apparent Michaelis constant; k_{cat}, catalytic rate constant; k_{cat}/K_m, catalytic efficiency. Stimulation indicates the increase in k_{cat}/K_m ratio due to addition of CNBr-Fbg.

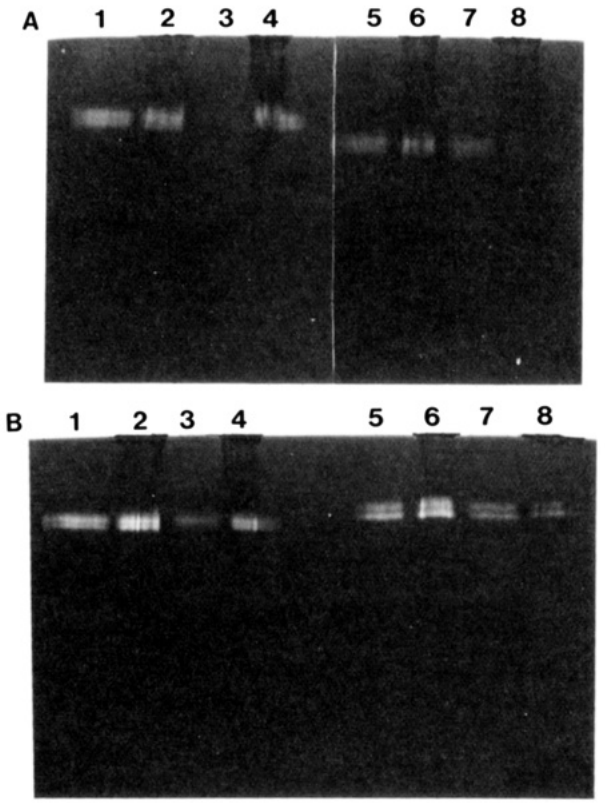

FIGURE 3: Binding of rt-PA, HMW u-PA, and the rt-PA/u-PA hybrid proteins to fibrin matrices. Equivalent samples of both the bound (pellet) and the nonbound fractions (supernatant) and of controls either without fibrinogen (−Fbg) or without thrombin (−IIa) were electrophoresed on gelatin–plasminogen gels. (Panel A) Lane 1, rt-PA (control, −Fbg); lane 2, rt-PA (control, −IIa); lane 3, rt-PA (supernatant); lane 4, rt-PA (pellet); lane 5, HMW u-PA (control, −Fbg); lane 6, HMW u-PA (control, −IIa); lane 7, HMW u-PA (supernatant); lane 8, HMW u-PA (pellet). (Panel B) Lane 1, rt-PA/u-PA I (control, −Fbg); lane 2, rt-PA/u-PA I (control, −IIa); lane 3, rt-PA/u-PA I (supernatant); lane 4, rt-PA/u-PA I (pellet); lane 5, rt-PA/u-PA II (control, −Fbg); lane 6, rt-PA/u-PA II (control, −IIa); lane 7, rt-PA/u-PA II (supernatant); lane 8, rt-PA/u-PA II (pellet).

exhibits in the absence of CNBr-Fbg a relatively low plasminogen activator activity, due to a high K_m (higher than 50 μM) as observed before (Rånby, 1982; Hoylaerts et al., 1982). The affinity of rt-PA/u-PA I, rt-PA/u-PA II, and HMW u-PA for plasminogen in the absence of CNBr-Fbg is more than 20–80-fold higher than that of rt-PA, as reflected by K_m values of 0.61, 0.88, and 2.2 μM, respectively. Under these conditions, the k_{cat} of the various PAs is not significantly different. The catalytic efficiency of rt-PA, represented by the ratio k_{cat}/K_m, is more than 29-fold lower than that of HMW u-PA and more than 34–41-fold lower than that of either one of the t-PA substitution mutants.

In the presence of CNBr-Fbg, the catalytic efficiency of rt-PA is substantially increased (more than 2000-fold), in accordance with previous observations (Rånby, 1982; Hoylaerts et al., 1982). This increase is mainly due to an enhanced affinity for the substrate plasminogen, as seen by a change in the K_m value of more than 50 μM to 0.016 μM, whereas a 1.4-fold decrease in k_{cat} is observed. In the presence of CNBr-Fbg, the catalytic efficiency of HMW u-PA also increases (12-fold). However, this effect has been attributed to a conformational change of Glu-plasminogen induced by CNBr-Fbg, rather than to a distinct effect on the enzyme (Lijnen et al., 1984). The presence of CNBr-Fbg also affects the kinetic parameters of the rt-PA/u-PA hybrid proteins, albeit in an intermediate fashion. The affinity for the substrate increases 120–220-fold (K_m from 0.61 and 0.88 μM to 0.005 and 0.004 μM), while the catalytic rate constants increase 1.8- and 1.3-fold (k_{cat} from 0.25 and 0.30 s^{-1} to 0.45 and 0.39 s^{-1}) for rt-PA/u-PA I and II, respectively. From these measurements we conclude that the catalytic efficiency of the mutants is stimulated about 18–24-fold more by the fibrin substitute than that of HMW u-PA and about 10-fold less than that of rt-PA. Furthermore, since the mutant proteins rt-PA/u-PA I and II display in the presence of CNBr-Fbg k_{cat}/K_m values that are 4–5-fold higher than the corresponding value of rt-PA, we conclude that under these conditions the mutants are more potent PAs than rt-PA.

Interaction of rt-PA/u-PA Hybrid Proteins with the Endothelial Plasminogen Activator Inhibitor (PAI-1). PAI-1 is generally considered to be the physiological inhibitor of t-PA and inhibits u-PA presumably equally well (Kruithof et al., 1986; Sprengers & Kluft, 1987). PAI-1 belongs to the family of serine protease inhibitors ("serpins"), as shown by homology of its amino acid sequence with that of other serpins, e.g., α_1-antitrypsin (Ny et al., 1986; Pannekoek et al., 1986; Ginsburg et al., 1986; Andreasen et al., 1986). Serpins, such as PAI-1, form a 1:1 molar complex with their "target" protease, thereby preventing the proteolytic activity (Travis & Salvesen, 1983).

We have investigated the interaction of human PAI-1 with rt-PA/u-PA I, rt-PA/u-PA II, and the control PAs rt-PA and HMW u-PA. First, the various PAs were incubated with an excess of PAI-1 and complex formation was visualized by the fibrin overlay technique after SDS–PAGE. The data are presented in Figure 4. The results clearly show that the mutant proteins form a 1:1 molar complex with PAI-1, like rt-PA and HMW u-PA. Even without the addition of PAI-1, rt-PA/u-PA I and II already display a lysis zone with an apparent molecular weight corresponding with PA–PAI-1 complexes (see also Figure 2, lanes 5 and 6). We attribute this to the presence of an endogenous PA inhibitor secreted by mouse Ltk$^-$ cells (Pannekoek et al., 1986). Such a high molecular weight lysis zone is not displayed by rt-PA, presumably because the expression level of rt-PA is more than 20-fold higher than that of the mutant proteins.

We have also determined the extent of inhibition of the various PAs by PAI-1. For that purpose, the latent fraction of the PAI-1 preparation was activated by SDS treatment (Hekman & Loskutoff, 1985). Subsequently, increasing amounts of PAI-1 were preincubated with a fixed amount

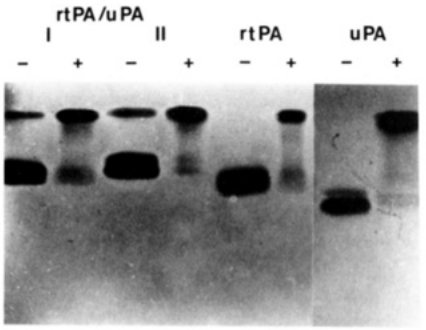

FIGURE 4: Complex formation of the hybrid proteins, rt-PA and HMW u-PA, with PAI-1. Each of the PAs (0.2 ng) was preincubated with 75 μL of ECCM before the samples were subjected to SDS–PAGE. Specific bands which contain plasminogen activator activity were visualized by the fibrin overlay technique. From left to right: rt-PA/u-PA I without PAI-1 (−) and with PAI-1 (+); rt-PA/u-PA II without PAI-1 (−) and with PAI-1 (+); rt-PA without PAI-1 (−) and with PAI-1 (+); HMW u-PA without PAI-1 (−) and with PAI-1 (+).

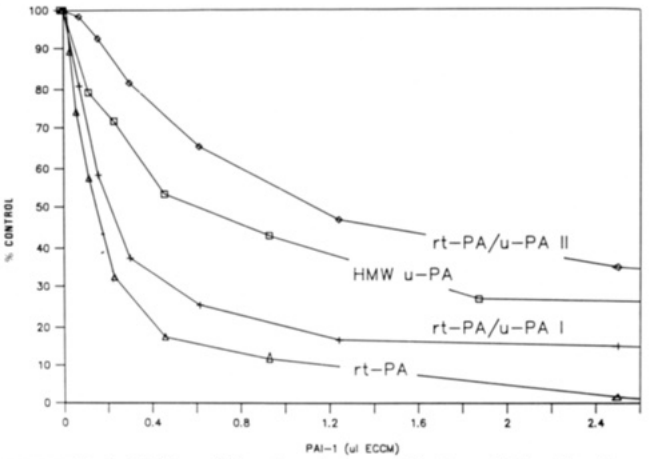

FIGURE 5: Inhibition of the plasminogen activator activity of various PAs by PAI-1. PAI-1 was activated with SDS and subsequently treated with Triton X-100. A constant amount of either one of the PAs (0.35 fmol) was preincubated with various amounts of activated PAI-1. The residual plasminogen activator activity was measured as described under Materials and Methods. The data are presented as the average of three experiments, performed in duplicate. Symbols: rt-PA (△), rt-PA/u-PA I (+), HMW u-PA (□), and rt-PA/u-PA II (◇).

(0.35 fmol) of either one of the hybrid proteins or rt-PA or HMW u-PA, and the residual plasminogen activator activity was monitored with the indirect assay with the plasmin-specific chromogenic substrate S2251. The results, presented as percentage of the activity without PAI-1, are shown in Figure 5. Surprisingly, the amount of PAI-1 required to inhibit 50% of the original activity of each of the various PAs is significantly different. For rt-PA, rt-PA/u-PA I, HMW u-PA, and rt-PA/u-PA II 50% inhibition is obtained with 0.15 ± 0.05, 0.22 ± 0.05, 0.69 ± 0.24, and 1.15 ± 0.10 μL of ECCM (containing approximately 2.4 ng/μL PAI-1), respectively. Consequently, the relative affinity of the parental PAs, rt-PA and HMW u-PA, for PAI-1, differs 4.6-fold. The dose–response curve of rt-PA/u-PA I with PAI-1 is similar to that of rt-PA, whereas the relative affinity of rt-PA/u-PA II for PAI-1 is even less than that of HMW u-PA. Strikingly, the affinity of these PAs for PAI-1 correlates with the presence of a particular polypeptide segment on these respective proteins. On rt-PA/u-PA I the distance between kringle 2 and the plasmin cleavage site resembles that of t-PA, whereas this region on rt-PA/u-PA II is comparable to HMW u-PA. From these results we conclude that either the distance between particular structures on the t-PA H chain and the catalytic center of u-PA or the specific amino acid sequence between kringle 2 and the plasmin cleavage site affects the efficiency of the interaction with PAI-1.

Discussion

The aim of this study was to investigate the possibility to combine functional properties of different PAs into novel hybrid molecules. These properties are encoded by defined sets of exons of the t-PA and u-PA genes, respectively (Ny et al., 1984; Riccio et al., 1985). On the hybrid molecules t-PA cDNA, encoding the L chain, was substituted by u-PA cDNA, encoding the B chain of u-PA. Using this approach, one might expect to create a protein, harboring the relatively high basal plasminogen activator activity characteristic for u-PA and the fibrin-binding feature of t-PA, resulting in fibrin-induced stimulation of activity. A successful additive combination of properties into novel hybrid proteins would be the ultimate test for the validity of the exon shuffling theory, which explains the evolutionary creation of particular gene families (Gilbert, 1978, 1985; Patthy, 1985). We observed that the plasminogen activator activity of the rt-PA/u-PA hybrid proteins in the absence of fibrin (i.e., CNBr-Fbg) is not affected by the H chain of t-PA. Clearly, both the K_m and k_{cat} values of the mutant proteins are very similar to those of HMW u-PA, even though they have a different amino-terminal chain. Thus, the amino-terminal chain dose not affect the interaction of the catalytic center of the mutants or HMW u-PA with the substrate plasminogen. We conclude that the serine protease moiety of u-PA, encoded by a defined set of adjacent exons (Riccio et al., 1985), is able to execute its function independently of the presence and/or of the nature of other associate domains. Furthermore, the hybrid proteins have acquired properties, dictated by t-PA. Both hybrid proteins were shown to bind to fibrin, and their plasminogen activator activity is stimulated by fibrin, as examplified by a 120–220-fold decrease in their K_m value. So far, our observations strongly support the predictions made from the exon shuffling model, which implies that domains (encoded by an exon or a set of adjacent exons) are autonomous, functional entities. However, the combination of properties is not entirely additive. First, the hybrid proteins bind to fibrin matrices but to a lesser extent than rt-PA. Second, the catalytic efficiency of both hybrid proteins, as shown by the k_{cat}/K_m ratios, increases about 10-fold less by the addition of the fibrin substitute than that of rt-PA. It is, however, conceivable that the fibrin binding exerted by the hybrid proteins, even though to a lesser extent than rt-PA, causes an increase in the affinity for the substrate to a level which is optimal for the catalytic center of u-PA (i.e., K_m values of 0.004–0.005 μM). It should be noted that under these circumstances the hybrid proteins have a catalytic efficiency 4–5-fold higher than rt-PA and about 25-fold higher than HMW u-PA, exclusively due to a lower K_m value.

We observed a higher relative affinity of rt-PA for its physiological inhibitor PAI-1 than of HMW u-PA for PAI-1. This difference might be due to the intrinsically different serine protease moieties of t-PA and u-PA and their accessibility for PAI-1. However, it has been indicated that the mechanism of action of PAI-1 on t-PA and u-PA might differ. Hekman and Loskutoff (1986) observed a mixed type of inhibition for t-PA and a competitive type of inhibition for u-PA with bovine PAI-1. Moreover, analogous to the interaction of α_2-antiplasmin with plasmin (Wiman & Collen, 1978), it has been proposed that PAI-1 initially interacts with kringle(s) of t-PA and subsequently with the serine protease moiety (Ehrlich et al., 1987). From our results, showing a higher relative affinity of rt-PA for PAI-1 than of HMW u-PA, it might be deduced

that the kringle(s) of t-PA exert a cooperative effect on the interaction of the serpin PAI-1 with the serine protease moiety of t-PA. This conclusion is substantiated by the observed affinity of the hybrid protein rt-PA/u-PA I for PAI-1, being considerably higher than that of HMW u-PA. Probably, the t-PA H chain on this molecule facilitates the interaction of PAI-1 with the u-PA catalytic center. In contrast, rt-PA/u-PA II is even less sensitive for PAI-1 than HMW u-PA, although the hybrid proteins differ only by a stretch of 13 amino acid residues. We conclude that either the distance between the t-PA kringle(s) and the serine protease moiety should be precisely tuned for the assumed cooperativity or the aforementioned amino acid sequence of 13 residues has an interfering role on the interaction with PAI-1. To further elucidate the mechanism of inhibition, we will have to determine the influence of PAI-1 on the initial rate of plasminogen activation by each of the described PAs.

In the foregoing paragraph we have concluded that, in the absence of fibrin, the presence of and/or the nature of the amino-terminal chain does not affect the interaction of the u-PA catalytic center with the substrate plasminogen. Inhibitors, such as PAI-1, which belong to the serpin family (Travis & Salvesen, 1983; Carrell & Bowell, 1986) act as a "pseudosubstrate" for their target protease. They present their P1 residue, which mimics the amino-terminal residue of the crucial peptide bond in the substrate, to the serine in the catalytic triad of the protease. However, if this were the only mechanism of interaction of PAI-1 with t-PA, then it would be expected that the pseudosubstrate PAI-1 would interact, in the absence of fibrin, similarly as the substrate plasminogen. Our observation on the effect of an amino-terminal chain on the interaction of PAI-1 with the u-PA catalytic center implicitly demonstrates the involvement of the amino-terminal chain in the interaction with PAI-1.

In conclusion, we have shown that in agreement with the exon shuffling model, proposed for mosaic genes such as t-PA and u-PA (Gilbert, 1978, 1985; Patthy, 1985), it is possible to exchange functional domains among proteins. Our data further indicate that the specific activities of "shuffled" domains are affected by the overall structure of the (hybrid) proteins, as well as by the distance between domains.

Added in Proof

During submission of this paper, a similar study was reported (Nelles et al., 1987). The conclusions of those authors on the properties of a t-PA/u-PA hybrid protein generally agree with ours as reported in this paper.

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Registry No. PAI, 105844-41-5; plasminogen, 9001-91-6.

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Thrombin Inactivates Acidic Fibroblast Growth Factor but Not Basic Fibroblast Growth Factor†

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ABSTRACT: Incubation of bovine brain derived acidic fibroblast growth factor (aFGF) with bovine or human thrombin, 0.5 NIH unit/mL, for 24 h at 37 °C results in cleavage of the mitogen, generating a 14-kilodalton fragment which has significantly reduced affinity for immobilized heparin as compared to aFGF, and is at least 50-fold less potent at stimulating mitogenesis. In addition, an 18 amino acid peptide, aFGF(123-140), is generated, identifying one of the thrombin cleavage sites as the Arg-122/Thr-123 bond. The peptide, aFGF(123-140), is neither mitogenic itself nor an inhibitor of the mitogenic activity of aFGF. The cleavage of aFGF by thrombin is inhibited by heparin (50 μg/mL) and is completely blocked by the irreversible thrombin inhibitors D-Phe-Pro-Arg chloromethyl ketone and hirudin. Incubation of aFGF with 50 units/mL thrombin at 37 °C results in rapid cleavage of the mitogen into several fragments. In contrast, incubation of bovine brain derived basic fibroblast growth factor with 1 unit/mL thrombin for 24 h, or 50 units/mL thrombin for 6 h, does not result in significant cleavage of mitogen. The results show that the C-terminal region of aFGF is of functional importance in both mitogenesis and heparin binding. Most importantly, a novel role for anionic heparin-binding growth factors and their fragments is indicated in physiologic and pathologic situations associated with thrombin generation.

Heparin-binding growth factors (HBGF's)[1] are a family of polypeptides with a wide range of mitogenic and nonmitogenic functions in vitro for cells of the vascular, neural, endocrine, and immune systems (Gospodarowicz et al., 1986a; Lobb et al., 1986a). In addition, they induce neovascularization, regeneration, and morphogenesis in vivo (Gospodarowicz, 1976; Gospodarowicz et al., 1986a; Risau, 1986; Slack et al., 1987), suggesting that they are of broad physiologic significance. HBGF's can be grouped into two classes (Lobb et al., 1986a,b): anionic mitogens found in high levels in neural tissue, typified by bovine brain derived acidic fibroblast growth factor (aFGF) (Thomas et al., 1984), and cationic mitogens found in virtually all tissues, typified by bovine pituitary derived basic fibroblast growth factor (bFGF) (Bohlen et al., 1984). Mitogens from both classes have been sequenced (Esch et al., 1985a,b; Gimenez-Gallego et al., 1985, 1986a; Harper et al., 1986; Strydom et al., 1986), and their genes and cDNAs

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[1] Abbreviations: HBGF, heparin-binding growth factor; aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; FPRC, D-Phe-Pro-Arg chloromethyl ketone; Z-Lys-SBzl, benzyloxycarbonyl-L-lysine thiobenzyl ester; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PBS, Dulbecco's calcium- and magnesium-free phosphate-buffered saline; BSA, bovine serum albumin; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Gd-NPF, glia-derived neurite-promoting factor; TCA, trichloroacetic acid; kDa, kilodalton(s); Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; PTH, phenylthiohydantoin.

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