Expression in *E. coli* of finger-domain lacking tissue-type plasminogen activator with high fibrin affinity

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Tissue-type plasminogen activator (t-PA) has a high affinity for fibrin and induces lysis of fibrin (fibrinolysis) on the surface of fibrin without degrading circulating fibrinogen. cDNA for t-PA which lacks the 'finger-domain' (the site for fibrin affinity) was isolated from Detroit 562 cells. Analysis of the nucleotide sequence revealed a lack of the sequences which code for the finger-domain. A plasmid (pDPAT 1) containing the *Escherichia coli tac* promoter/operator and the cDNA sequence coding for 'finger-domain lacking t-PA' was constructed for expression in *E. Coli*. The polypeptide so produced was a new type of t-PA lacking finger-domain, but revealed plasminogen activator activity with the function of fibrin affinity.

Tissue-type plasminogen activator *Finger-domain* *Fibrin affinity* *Plasmid*
E. coli tac *promoter/operator* *Detroit 562 cell*

1. INTRODUCTION

t-PA has a high affinity for fibrin and induces fibrinolysis on the surface of fibrin without degrading circulating fibrinogen [1,2]. Administration of t-PA to animals with experimentally induced thrombosis caused extensive thrombolysis without fibrinogenolysis [3,4]. Administration of t-PA in venous thrombosis [5] or acute myocardial infarction [6] in humans caused thrombolysis or reopening of the occluded vessels. Thus, t-PA induces a high ratio of fibrinolysis/fibrinogenolysis which is in contrast to the effect of the urinary plasminogen activator, UK.

The primary structure of t-PA is quite different from that of UK: t-PA has 2 Kringle structures, but UK only one [7,8]. Further, t-PA has a special domain termed the 'finger-domain' which is responsible for the fibrin affinity [9]. We report here the isolation of cDNA for 'finger-domain lacking t-PA' from Detroit 562 cells and the expression of finger-domain lacking t-PA in *Escherichia coli*, using a plasmid containing the *E. coli tac* promoter/operator.

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Abbreviations: t-PA, tissue-type plasminogen activator; UK, urokinase; S-2251, H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide

2. MATERIALS AND METHODS

2.1. *Cloning*

A cDNA library was prepared from Detroit 562 cells according to Okayama and Berg [10]. Approx. 100000 transformants of *E. coli* harboring the cDNA sequences were screened for complementarity to the following 2 synthetic oligonucleotide probes: probe I, 5'AATCGGGCATGGATTTCCTG3' and probe II, 5'GCCCCCGCACAGGAACCG3'. The nucleotide obtained from clones reacting to both probes, was analyzed.

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2.2. *Construction of pDPAT 1 which expresses finger-domain lacking t-PA*

Plasmid pDPAT 1 (4.2 kb) was constructed by ligating pKIT 1 (2.4 kb) with pDPA 3 (5.1 kb). The plasmid pKIT 1 obtained in our laboratory (full details to be published) contains the *tac* promoter/operator and the SD sequence of C230 (*Pseudomonas putida* metapyrocatechase). pKIT 1 (2.5 μg) was digested with 15 units *Cla*I and dephosphorylated with calf intestine phosphatase. At the same time, pDPA 3 (50 μg) was digested with 150 units *Bgl*II and a 1800 base pair DNA fragment was isolated. After incubating this DNA fragment with 5 units DNA polymerase I (Klenow fragment) plus 40 μM dGTP and dATP, 50 units S1 nuclease was reacted with the material. *Cla*I linker [d(CATCGATC)], whose 5′-terminal was phosphorylated, was then linked to the above DNA fragment with T4 DNA ligase and subsequently digested with *Cla*I. ATG sequence in the *Cla*I linker was used as initiative methionine, followed by serine residue at the 36th position. The final product derived from the DNA of pDPA 3 was ligated to the DNA fragment derived from pKIT 1 with T4 DNA ligase and pDPAT 1 was obtained. The recombinant plasmid pDPAT 1 was used for transformation of JM 103, the well characterized *lac* I^q strain of *E. coli*.

2.3. *Measurement of fibrinolytic activity*

The fibrinolytic activity of the cell homogenate was examined by 3 methods. (i) Amidolytic activity [11]. S-2251, which is a plasmin substrate, was reacted with a mixture of the sample and purified plasminogen in the presence of fibrinogen-fragment. (ii) Fibrinolytic activity [12]. The fibrinolytic activity was estimated by the fibrin film method, for which bovine plasminogen-rich fibrinogen and bovine thrombin were employed. (iii) Electrophoretic enzymography [13]. To identify the molecular mass of the enzymatically active component, the sample was first electrophoresed under non-reduced conditions in SDS-polyacrylamide gel and the fibrin film produced as above was then overlayered on the gel.

2.4. *Binding of tissue-type plasminogen activator lacking finger-domain to a fibrin-celite column*

Transformed *E. coli* JM 103 containing the plasmid of pDPAT 1 was incubated in LB medium and the cell pellet obtained by centrifugation was sonicated in an ultrasonifier. After centrifugation, the supernatant produced was dissolved in 50% $(NH_4)_2SO_4$ and precipitated at 10000 rpm for 30 min. The pellet was dissolved in 50 mM Tris-HCl (pH 7.4), with 0.01% Tween 80, and acidified to pH 5.2. After 30 min incubation at 4°C and centrifugation, the neutralized solution was applied to anti-t-PA IgG Sepharose which was produced with a monoclonal antibody to t-PA [14]. The bound protein was eluted with 6 M guanidine hydrochloride and the plasminogen activator activity was measured. The bound t-PA was eluted completely with 6 M guanidine hydrochloride and no activity was eluted with 2 M KSCN. The fraction with t-PA activity was pooled and applied to a fibrin-celite column. The unbound portion and the protein eluted with 0.2 M arginine were used for measurement of the plasminogen activator activity.

3. RESULTS

3.1. *Cloning and expression of finger-domain lacking t-PA*

Among 100000 clones, 15 showed a positive hybridized signal to one of the probes and only one clone, χ1776 (pDPA 3), to both probes. The nucleotide sequence of pDPA 3 was determined and the amino acid sequence was deduced from it (fig.1). The cDNA sequence comprised 2459 base

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Fig.1. Sequence of human tissue-type plasminogen activator lacking the finger-domain, with nucleotides numbered in the 5′- to 3′-direction, beginning with the dC residue. The predicted protein sequence is shown above the DNA. The numbers in the right-hand column indicate the amino acid residues, beginning with the first residue of the ATG triplet encoding the initial methionine. The sequences homologous to the synthetic oligonucleotide probes I and II are underlined (nucleotides 225–244 for probe I, and 1032–1049 for probe II). The complete DNA sequence of the protein coding region of the tissue-type plasminogen activator was in part determined from genomic clones. Capital letters indicate putative amino acids; small letters indicate the putative 'prepro' region. Arrows indicate the cleavage sites for *Bgl*II.

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CCACCGACCCCACCCCCTGCCTGGAAACTTAAAGGAGGCCGGAGCTGTGGGGAGCTCAGAGCTGAGATCCTACAGGAGTCCAGGGCTGGAGAGAAAACC
1                                                  50
                                                                met asp ala met lys arg gly leu cys cys val 11
TCTGCGAGGAAAGGGAAGGAGCAAGCCGTGAATTTAAGGGACGCTGTGAAGCAATCATG GAT GCA ATG AAG AGA GGG CTC TGC TGT GTG
100                                                150
leu leu leu cys gly ala val phe val ser pro ser gln glu ile his ala arg phe arg arg gly ala arg SER 36
CTG CTG CTG TGT GGA GCA GTC TTC GTT TCG CCC AGC CAG GAA ATC CAT GCC CGA TTC AGA AGA GGA GCC AGA TCT
                200                                                             250
TYR GLN GLY CYS SER GLU PRO ARG CYS PHE ASN GLY GLY THR CYS GLN GLN ALA LEU TYR PHE SER ASP PHE VAL 61
TAC CAA GGT TGC AGC GAG CCA AGG TGT TTC AAC GGG GGC ACC TGC CAG CAG GCC CTG TAC TTC TCA GAT TTC GTG
                                                300
CYS GLN CYS PRO GLU GLY PHE ALA GLY LYS CYS CYS GLU ILE ASP THR ARG ALA THR CYS TYR GLU ASP GLN GLY 86
TGC CAG TGC CCC GAA GGA TTT GCT GGG AAG TGC TGT GAA ATA GAT ACC AGG GCC ACG TGC TAC GAG GAC CAG GGC
                350                                                             400
ILE SER TYR ARG GLY THR TRP SER THR ALA GLU SER GLY ALA GLU CYS THR ASN TRP ASN SER SER ALA LEU ALA 111
ATC AGC TAC AGG GGC ACG TGG AGC ACA GCG GAG AGT GGC GCC GAG TGC ACC AAC TGG AAC AGC AGC GCG TTG GCC
                                                450
GLN LYS PRO TYR SER GLY ARG ARG PRO ASP ALA ILE ARG LEU GLY LEU GLY ASN HIS ASN TYR CYS ARG ASN PRO 136
CAG AAG CCC TAC AGT GGG CGG AGG CCA GAC GCC ATC AGG CTG GGC CTG GGG AAC CAC AAC TAC TGC AGA AAC CCA
                500                                                             550
ASP ARG ASP SER LYS PRO TRP CYS TYR VAL PHE LYS ALA GLY LYS TYR SER SER GLU PHE CYS SER THR PRO ALA 161
GAT CGA GAC TCA AAG CCC TGG TGC TAC GTC TTT AAG GCG GGG AAG TAC AGC TCA GAG TTC TGC AGC ACC CCT GCC
                                                600
CYS SER GLU GLY ASN SER ASP CYS TYR PHE GLY ASN GLY SER ALA TYR ARG GLY THR HIS SER LEU THR GLU SER 186
TGC TCT GAG GGA AAC AGT GAC TGC TAC TTT GGG AAT GGG TCA GCC TAC CGT GGC ACG CAC AGC CTC ACC GAG TCG
                650                                                             700
GLY ALA SER CYS LEU PRO TRP ASN SER MET ILE LEU ILE GLY LYS VAL TYR THR ALA GLN ASN PRO SER ALA GLN 211
GGT GCC TCC TGC CTC CCG TGG AAT TCC ATG ATC CTG ATA GGC AAG GTT TAC ACA GCA CAG AAC CCC AGT GCC CAG
                                                750
ALA LEU GLY LEU GLY LYS HIS ASN TYR CYS ARG ASN PRO ASP GLY ASP ALA LYS PRO TRP CYS HIS VAL LEU LYS 236
GCA CTG GGC CTG GGC AAA CAT AAT TAC TGC CGG AAT CCT GAT GGG GAT GCC AAG CCC TGG TGC CAC GTG CTG AAG
                800                                                             850
ASN ARG ARG LEU THR TRP GLU TYR CYS ASP VAL PRO SER CYS SER THR CYS GLY LEU ARG GLN TYR SER GLN PRO 261
AAC CGC AGG CTG ACG TGG GAG TAC TGT GAT GTG CCC TCC TGC TCC ACC TGC GGC CTG AGA CAG TAC AGC CAG CCT
                                                900
GLN PHE ARG ILE LYS GLY GLY LEU PHE ALA ASP ILE ALA SER HIS PRO TRP GLN ALA ALA ILE PHE ALA LYS HIS 286
CAG TTT CGC ATC AAA GGA GGG CTC TTC GCC GAC ATC GCC TCC CAC CCC TGG CAG GCT GCC ATC TTT GCC AAG CAC
                950                                                             1000
ARG ARG SER PRO GLY GLU ARG PHE LEU CYS GLY GLY ILE LEU ILE SER SER CYS TRP ILE LEU SER ALA ALA HIS 311
AGG AGG TCG CCC GGA GAG CGG TTC CTG TGC GGG GGC ATA CTC ATC AGC TCC TGC TGG ATT CTC TCT GCC GCC CAC
                                                1050
CYS PHE GLN GLU ARG PHE PRO PRO HIS HIS LEU THR VAL ILE LEU GLY ARG THR TYR ARG VAL VAL PRO GLY GLU 336
TGC TTC CAG GAG AGG TTT CCG CCC CAC CAC CTG ACG GTG ATC TTG GGC AGA ACA TAC CGG GTG GTC CCT GGC GAG
                1100                                                            1150
GLU GLU GLN LYS PHE GLU VAL GLU LYS TYR ILE VAL HIS LYS GLU PHE ASP ASP ASP THR TYR ASP ASN ASP ILE 361
GAG GAG CAG AAA TTT GAA GTC GAA AAA TAC ATT GTC CAT AAG GAA TTC GAT GAT GAC ACT TAC GAC AAT GAC ATT
                                                1200
ALA LEU LEU GLN LEU LYS SER ASP SER SER ARG CYS ALA GLN GLU SER SER VAL VAL ARG THR VAL CYS LEU PRO 386
GCG CTG CTG CAG CTG AAA TCG GAT TCG TCC CGC TGT GCC CAG GAG AGC AGC GTG GTC CGC ACT GTG TGC CTT CCC
                1250                                                            1300
PRO GLU ASP LEU GLN LEU PRO ASP TRP THR GLU CYS GLU LEU SER GLY TYR GLY LYS HIS GLU ALA LEU SER PRO 411
CCG GAG GAC CTG CAG CTG CCG GAC TGG ACG GAG TGT GAG CTC TCC GGC TAC GGC AAG CAT GAG GCC TTG TCT CCT
                                                1350
PHE TYR SER GLU ARG LEU LYS GLU ALA HIS VAL ARG LEU TYR PRO SER SER ARG CYS THR SER GLN HIS LEU LEU 436
TTC TAT TCG GAG CGG CTG AAG GAG GCT CAT GTC AGA CTG TAC CCA TCC AGC CGC TGC ACA TCA CAA CAT TTA CTT
                1400                                                            1450
ASN ARG THR VAL THR ASP ASN MET LEU CYS ALA GLY ASP THR ARG SER GLY GLY PRO GLN ALA ASN LEU HIS ASP 461
AAC AGA ACA GTC ACC GAC AAC ATG CTG TGT GCT GGA GAC ACT CGG AGC GGC GGG CCC CAG GCA AAC TTG CAC GAC
                                                1500
ALA CYS GLN GLY ASP SER GLY GLY PRO LEU VAL CYS LEU ASN ASP GLY ARG MET THR LEU VAL GLY ILE ILE SER 486
GCC TGC CAG GGC GAT TCG GGA GGC CCC CTG GTG TGT CTG AAC GAT GGC CGC ATG ACT TTG GTG GGC ATC ATC AGC
                1550                                                            1600
TRP GLY LEU GLY CYS GLY GLN LYS ASP VAL PRO GLY VAL TYR THR LYS VAL THR ASN TYR LEU ASP TRP ILE ARG 511
TGG GGC CTG GGC TGT GGA CAG AAG GAT GTC CCG GGT GTG TAC ACC AAG GTT ACC AAC TAC CTA GAC TGG ATT CGT
                                                1650
ASP ASN MET ARG PRO XXX
GAC AAC ATG CGA CCG TGA CCAGGAACACCCGACTCCTCAAAAGCAAATGAGATCCCGCCTCTTCTTCTTCAGAAGACACTGCAAAGGCGCAGT
                1700                                                            1750
GCTTCTCTACAGACTTCTCCAGACCCACCACACCGCAGAAGCGGGACGAGACCCTACAGGAGAGGGAAGAGTGCATTTTCCCTGATACTTCCCATTTTG
                1800                                                            1850
GAAGTTTTCAGGACTTGGTCTGATTTCAGGATACTCTGTCAGATGGGAAGACATGAATGCACACTAGCCTCTCCAGGAATGCCTCCTCCCTGGGCAGAA
                1900                                                            1950
GTGGCCATGCCACCCTGTTTTCGCTAAAGCCCAACCTCCTGACCTGTCACCGTGAGCAGCTTTGGAAACAGGACCACAAAAATGAAAGCATGTCTCAAT
                2000                                                            2050
AGTAAAAGATAACAAGATCTTTCAGGAAAGACGGATTGCATTAGAAATAGACAGTATATTTATAGTCACAAGAGCCCAGCAGGGCTCAAAGTTGGGGCA
                2100                                                            2150
GGCTGGCTGGCCCGTCATGTTCCTCAAAAGCACCCTTGACGTCAAGTCTCCTTCCCCTTTCCCCACTCCCTGGCTCTCAGAAGGTATTCCTTTTGTGTA
                2200                                                            2250
CAGTGTGTAAAGTGTAAATCCTTTTTCTTTATAAACTTTAGAGTAGCATGAGAGAATTGTATCATTTGAACAACTAGGCTTCAGCATATTTATAGCAAT
                2300                                                            2350
CCATGTTAGTTTTTACTTTCTGTTGCCACAACCCTGTTTTATACTGTACTTAATAAATTCAGATATATTTTTCACAGTTTTTCC
                2400                                                            2450
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pairs except the dG-dC and dA-dT stretches, and one long open reading frame of 1548 base pairs in which a code of 516 amino acids was included. The sequences were very close to that reported previously for t-PA [7], but the molecular mass of the sequence was similar to that of UK (53 kDa) and not to that of t-PA (70 kDa). The smaller molecular mass reflected the lack of 138 base pairs which code for the finger-domain (fig.2).

The nucleotide sequences coding for the putative mature finger-domain lacking t-PA were expressed in *E. coli*. Briefly, a 1.8 kb *Bgl*II fragment which codes for the putative mature polypeptide, was excised from pDPA 3, and ligated with the plasmid pKIT 1 which is an expression vector derived from pBR 322 containing the *tac* promoter/operator system. Since microheterogeneity was observed in the N-terminal region of previously reported amino acid sequences for t-PA [15,16], the amino acid in the 36 position, Ser, was tentatively adopted as the N-terminal amino acid of the expressed finger-domain lacking t-PA.

3.2. *Plasminogen activator activity of finger-domain lacking t-PA*

The fibrinolytic activity of the cell homogenate, *E. coli* JM 103, was examined by 3 methods, as outlined above. The cell homogenate revealed S-2251 catalytic activity in a dose-dependent manner in the presence of purified plasminogen and fibrinogen-fragment. Plasminogen activator was

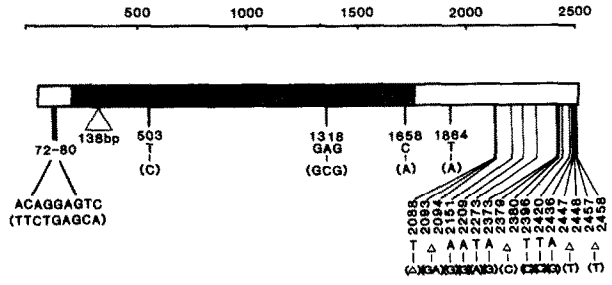

Fig.2. Specific regions of tissue-type plasminogen activator. The cross-hatched region indicates the coding sequence of the putative mature tissue-type plasminogen activator (481 amino acids). The stippled region represents the putative 35-residue 'prepro' peptide coding sequences. (Δ) Deletion of base pairs; capital letters, substituted bases from those of t-PA obtained from melanoma cells (parentheses).

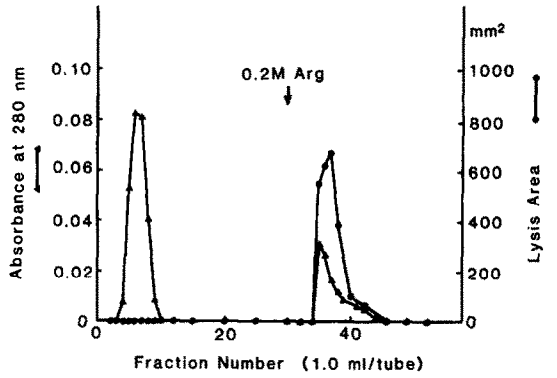

Fig.3. Binding of tissue-type plasminogen activator lacking finger-domain to a fibrin-celite column. The unbound protein had no plasminogen activator activity, but the eluted protein with 0.2 M arginine demonstrated clear t-PA activity.

also observed by the standard fibrin film method. Electrophoretic enzymography demonstrated a lytic zone at an M_r of 55000, which was close to the M_r of UK. This value for the molecular mass fitted well with that calculated on the basis of the number of amino acid residues (481).

3.3. *Fibrin affinity of finger-domain lacking t-PA*

The fibrin affinity was investigated by purifying the plasminogen activator using monoclonal antibody of t-PA, and the eluted plasminogen activator fraction was applied to fibrin-celite column. Although the unbound fraction did not reveal any fibrinolytic activity, the elution fraction with 0.2 M Arg demonstrated plasminogen activator activity (fig.3). Thus, the plasminogen activator which was lacking in the finger-domain showed an affinity for fibrin as well as plasminogen activator activity.

4. DISCUSSION

t-PA has a high affinity for fibrin [1,2] and the mechanism of expression of the fibrin affinity at the molecular level has been reported to involve a finger-domain in the N-terminal region which is responsible for such biological characteristics [9]. Since UK, another plasminogen activator, does not display any fibrin affinity, the molecular structure of t-PA has been extensively studied. This study was undertaken to clarify the mechanism of the fibrin affinity of the t-PA molecule by producing

finger-domain lacking t-PA in *E. coli*. A cDNA library was prepared from Detroit 562 cells and transformants of *E. coli* which showed a positive hybridization signal to 2 synthetic oligonucleotide probes were screened (pDPA 3). Analysis of the nucleotide sequence revealed one long open reading frame of 1548 base pairs which coded for 516 amino acids (fig.1), of which the sequence was close to t-PA [7]. However, one exceptional feature was that the nucleotide sequence lacked 138 base pairs which code for the finger-domain in the t-PA structure (fig.2). These missing base pairs corresponded exactly to the exon IV noted in the structure of the t-PA gene [17]. Exon V was thus directly connected to exon III. Such novel joining of exons III and V resulted in a glycine residue (amino acid number 39 in fig.1) at the junction site. The deletion of 138 base pairs may imply that alternative splicing of RNA occurs in Detroit 562 cells. However, the possibility cannot be excluded that these cells possess an altered genomic structure.

A plasmid containing the *E. coli tac* promoter/operator and the cDNA sequence coding for finger-domain lacking t-PA was constructed for expression in *E. coli*. The polypeptide produced in *E. coli* (which carries no carbohydrate) demonstrated plasminogen activator activity which was examined by 3 methods. The molecular mass of the plasminogen activator was 55 kDa, which was close to that of UK, and not to that of native t-PA, the carbohydrate content of which was 6.8% (w/w) [15]. This molecular mass also fitted well to that calculated on the basis of the number of amino acid residues (481). The fibrin affinity was examined using the purified plasminogen activator and it was found that the plasminogen activator which lacked the finger-domain still had fibrin affinity (fig.3).

Induction of fibrinolysis only on the surface of fibrin by t-PA represents one of the most significant findings in recent research on blood fibrinolysis [1–3]. The mechanism underlying the fibrin affinity of t-PA still remains unclear. The finger-domain has been reported to be responsible for the fibrin affinity of t-PA [9]. However, the expression of fibrin affinity of t-PA lacking the finger-domain as observed in this study suggests that the finger-domain is not a prerequisite for the expression of fibrin affinity in the t-PA molecule.

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