

cDNA cloning and expression in *E. coli* of a plasminogen activator inhibitor (PAI) related to a PAI produced by Hep G2 hepatoma cell

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The human hepatoma line Hep G2 produces an acid- and SDS-sensitive plasminogen activator inhibitor (PAI). This protein has been previously purified and used to raise polyclonal antibodies. This antiserum has been used to isolate cDNA clones from a human placental λ gt11 cDNA library. The immunologically positive clones were screened for expression of recombinant proteins which inhibit urokinase activity and form an inhibitor-enzyme complex with 125 I-urokinase. Two positives (λ PAI 11.1 and λ PAI 14.1) have been obtained. The cDNA insert of the longer isolate (λ PAI 14.1) consists of 1962 base pairs encoding the entire mature Hep G2 PAI and a 3'-noncoding region of 801 base pairs. The clone apparently lacks portions of 5'- and 3'-untranslated sequences. The translated amino acid sequence matches the sequence obtained for the mature Hep G2 PAI and consists of 379 amino acids with a molecular mass of 42 770 Da. Interestingly, this PAI clone is quite different from the placental-type PAI-2 sequence as expected, but matches the sequence of the endothelial-type PAI (PAI-1) reported to be acid-insensitive and SDS-enhancible.

Plasminogen activator inhibitor; cDNA sequence; Phage λ gt 11; (Hep G2 hepatoma cell)

1. INTRODUCTION

The mammalian serine protease, plasminogen activator (PA), catalyzes the conversion of the protease zymogen plasminogen to the active protease, plasmin. This enzyme system plays an important role in a variety of biological functions (reviews [1-3]). Two distinct types of plasminogen activator have been identified, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA).

Both uPA and tPA are regulated by specific plasminogen activator inhibitors (PAIs). At least three immunologically distinct PAIs are known to exist in a variety of cells, tissues and biological

fluids. These include protease nexin [4], endothelial cell-type PAI (PAI-1) (the term PAI-1 refers to the plasminogen activator inhibitor of endothelial cells, platelets, and HT1080 cells, while PAI-2 refers to the plasminogen activator inhibitor of placenta, monocytes and U-937 cells, in accordance with the recommendations adopted at the 32nd annual meeting of the International Committee on Thrombosis and Haemostasis, June 8, 1986) [5], and placental-type PAI (PAI-2) [6]. Recently, we have isolated two immunologically unrelated PAIs from human placenta (Wun, T.-C. and Reich, E., submitted) and Hep G2 hepatoma cells (Wun, T.-C. and Palmier, M., in preparation). To facilitate further biochemical studies, we have undertaken molecular cloning of the respective genes. The cloning of the the cDNA for PAI-2 is reported elsewhere (Ye, R.D. et al., submitted). Here we describe the cloning of the cDNA corresponding to PAI-1.

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2. EXPERIMENTAL

2.1. Materials

The λ gt11 placental cDNA library constructed by the method of Millan [7] was obtained from Clontech. The protoblot immunoscreening kit was purchased from Promega Biotech. Restriction enzymes, calf intestine alkaline phosphatase, DNA polymerase I (Klenow), exonuclease III, and S₁ nuclease were purchased from New England Biolabs; dNTPs, from P.L. Biochemicals; 5'-[α -³⁵S]thio-dATP (600 Ci/mmol) and M13 sequence kit, from Amersham. The purifications of urokinase and plasminogen and iodination of urokinase were carried out as in [8]. Purification of PAI from Hep G2 conditioned medium and the development of antibody are described elsewhere (Wun, T.-C. and Palmier, M., in preparation).

The following buffers were used: PBB – phosphate-buffered saline containing 5 mg/ml bovine serum albumin and 2.5 mg/ml bovine γ -globulin; SM buffer – 0.1 M NaCl, 8 mM MgSO₄, 50 mM Tris-HCl (pH 7.5), 0.1 mg/ml gelatin; LB medium – medium containing 10 g Bacto-tryptone, 5 g Bacto-yeast extract, and 5 g NaCl per l.

2.2. Methods

2.2.1. Screening of λ gt11 cDNA library

The λ gt11 placental cDNA library, consisting of 10⁶ independent recombinant phages, was screened immunologically using antiserum against Hep G2 PAI as probe. The antiserum was preadsorbed with the lysate prepared from BNN97 lysogen to reduce the background. Screening was carried out using the Protoblot immunoscreening kit (Promega Biotech) according to the manufacturer's recommendation. Positive isolates were plaque purified and rescreened.

2.2.2. Preparation of λ -phage lysates containing recombinant proteins

Each plaque-purified λ gt11 was plated with *E. coli* Y1090 in 8 ml LB-10 mM isopropylthiogalactoside (IPTG)-0.7% agar onto 15-cm LB-agar plates at a density of 50000 pfu/plate [9]. The plates were incubated at 42°C for 3 h and then at 37°C for 5 h. 10 ml SM buffer was added to each plate and the plates gently shaken overnight at

4°C. The lysate was recovered and bacterial debris removed by centrifugation.

2.2.3. Screening of urokinase inhibitory activity by fibrin-agar spot assay

9 vols phage lysate was mixed with 1 vol. urokinase (5 CTA units/ml, dissolved in PBB) and incubated for 30 min at room temperature. 5 μ l aliquots were removed and spotted on a fibrin-agar plate. The plate was then incubated in a humidified chamber at 37°C. Urokinase activity causes the appearance of a clear lytic zone and PAI activity causes the abolishment of the lytic zone. The fibrin-agar plate was prepared as in [8].

2.2.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the complex formation between ¹²⁵I-urokinase and recombinant proteins expressed by λ -phage isolates

Aliquots (0.4 ml) of λ -lysate or PBB buffer were mixed with 2 μ l ¹²⁵I-urokinase (53 CTA units/ml) and incubated at room temperature for 30 min. 6 μ l rabbit anti-Hep G2 PAI serum and 0.1 ml of 0.1 M benzamidine were added and the mixture incubated at 4°C overnight. 60 μ l goat anti-rabbit Ig (1 ml precipitates 1.5 mg Ig, Sigma product) was then added and the mixture incubated at room temperature for 3 h. The mixture was centrifuged for 5 min in a microfuge to collect the immunocomplex. The precipitate was washed with 3 \times 1 ml aliquots of a PBS solution supplemented with 0.4 M NaCl and 0.1 M benzamidine and then with 1 ml PBS. The washed precipitate was then dissolved in 30 μ l of a buffer containing 0.1 M glycine/HCl, pH 2.2, 0.1% SDS, 6 M urea, 20 mM dithiothreitol and 0.001% bromophenol blue for SDS-PAGE in a 7.5% polyacrylamide gel [10]. The gel was stained, destained, and dried for autoradiography.

2.2.5. Nucleic acid methods and DNA sequence analysis

Phage suspensions were prepared using a plate lysate method [9]. DNA from λ gt11 clones were digested with *EcoRI* endonuclease. The cDNA insert was subcloned into the pUC19 plasmid [11]. Plasmid DNA was isolated by the method of Birnboim and Doly [12]. The plasmid was digested with *EcoRI* and the cDNA insert isolated by electroelution.

tion. The insert was then subcloned in both directions into the *EcoRI* site of M13 mp18 [11]. Deletions were generated using exonuclease III [13]. Nucleotide sequence was determined by the dideoxy method [14] using [³⁵S]dATP α S and the M13 sequence kit from Amersham.

2.2.6. Amino acid sequence analysis

Purified Hep G2 PAI was subjected to amino acid sequencing on an Applied Biosystems gas-phase sequenator. The inhibitor was also digested with trypsin and the tryptic peptides separated using a C-8 reverse-phase column (Browlee) on an Applied Biosystems model 13A separation system. Two tryptic peptides were randomly selected for sequence analysis.

3. RESULTS

3.1. Screening of cDNA library and characterization of recombinant proteins

The placenta cDNA library in λ gt11 was screened using rabbit antiserum against Hep G2 PAI. Initially, 10⁶ pfu were screened yielding approx. 60 immunologically positive clones. Of these

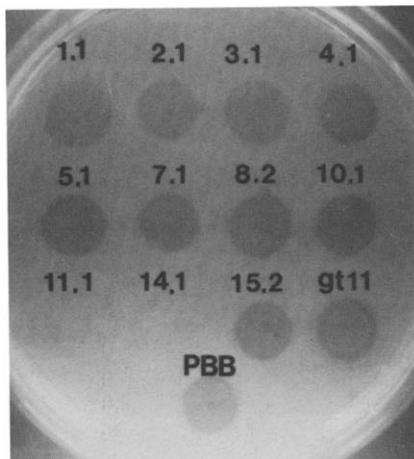


Fig.1. Urokinase inhibitory activities in λ gt11 fusion proteins. Phage lysates were incubated with urokinase and the mixture spotted on a fibrin-agar plate. Urokinase activity is indicated by the appearance of a clear lytic zone and PAI activity is shown by the abolishment of the lytic zone. λ -lysates tested were from clones 1.1, 2.1, 3.1, 4.1, 5.1, 7.1, 8.2, 10.1, 11.1, 14.1, 15.2, λ gt11 without insert, and a PBB control.

positives, 15 were randomly selected for plaque purification and secondary screening. Plate lysates of these purified clones were tested for inhibitory activity against urokinase and the presence of recombinant protein capable of complexing with ¹²⁵I-urokinase. As shown in fig.1, 2 out of 11 immunologically positive clones exhibit inhibitory activity against urokinase on the fibrin-agar spot assay. Incubation of ¹²⁵I-urokinase with the lysates from immunologically positive clones followed by SDS-PAGE and autoradiography revealed that the same two clones (λ PAI 11.1 and λ PAI 14.1) formed complexes (~200 kDa) which are immunoprecipitable by antibody against Hep G2 PAI. Since the natural Hep G2 PAI and urokinase heavy chain have molecular masses of 50 and 33 kDa respectively, the high molecular mass of the complex (200 kDa) supports the notion that the recombinant inhibitor is being made as a fusion protein with β -galactosidase. The clones λ PAI 11.1 and λ PAI 14.1 were digested with *EcoRI* and the cDNA inserts were found to be 1.4 and 1.9 kb, respectively.

3.2. Nucleotide sequence of PAI cDNA and its translated protein sequence

The λ PAI 14.1 clone was subjected to M13 subcloning and sequencing by the strategy shown in fig.3. The entire sequence was determined on

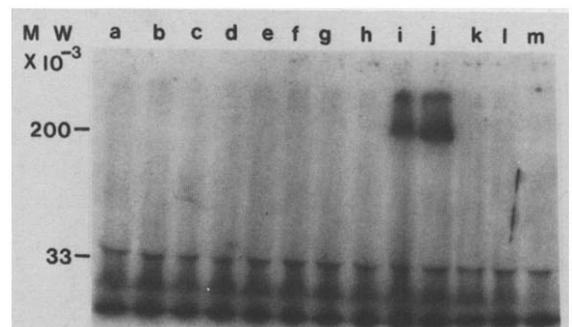


Fig.2. SDS-PAGE analysis of the complex formation between ¹²⁵I-urokinase and PAI inhibitors in the phage lysates. Phage lysates containing fusion proteins from 6 positive clones were incubated with ¹²⁵I-urokinase, immunoprecipitated by anti-Hep G2 PAI antibody, and electrophoresed on a 7.5% polyacrylamide gel as described in section 2. λ PAI clones tested were: a, 1.1; b, 2.1; c, 3.1; d, 4.1; e, 5.1; f, 7.1; g, 8.2; h, 10.1; i, 11.1; j, 14.1; k, 15.1; l, λ gt11; m, PBB control.

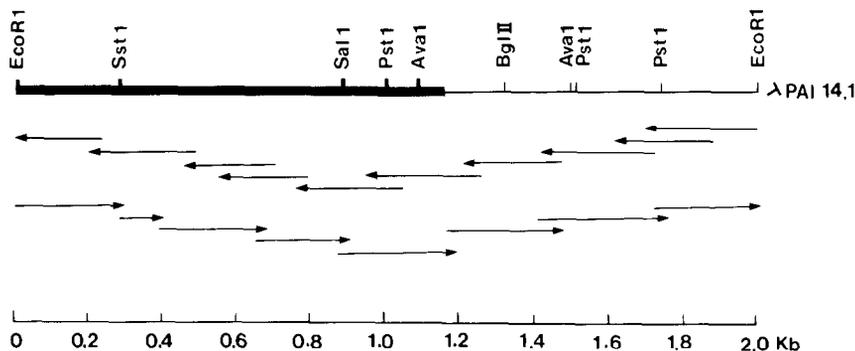


Fig.3. Partial restriction map and sequencing strategy for λ PAI 14.1 insert. The scale at the bottom indicates the nucleotide position. The thick bar represents the coding region. The thin bar represents the 3'-noncoding region. The restriction endonuclease sites were confirmed by digestion. The arrows show the overlapping M13 clones used to sequence the cDNA.

both strands by the exonuclease III deletion method [13] and was found to consist of 1962 bases in length. The sequence is shown in fig.4. It contains an open reading frame of 1158 nucleotides, a stop codon, and 3'-noncoding region of 801 nucleotides. The proper reading frame and the translated amino acid sequence are indicated under the nucleotide sequence. These were based on the N-terminal amino acid sequence of the purified Hep G2 PAI matching the predicted amino acid sequence (underlined) in fig.4. We have also subjected the purified Hep G2 PAI to tryptic digestion and separated the tryptic peptides. Two tryptic peptides were randomly selected for sequencing and the sequences were found to correspond to two separate segments of the coding regions as underlined in fig.4. These data strongly indicate that the λ PAI 14.1 clone corresponds to the Hep G2 PAI and that the clone contains the entire coding region of the mature protein, 379 amino acids long with a calculated molecular mass of 42770 Da excluding carbohydrate. There are three potential *N*-glycosylation sites at amino acid positions 209, 265 and 329. The λ PAI 14.1 clone lacks part of the 5'-untranslated region and ATG initiation codon. It also lacks an identifiable poly(A) tail at the 3'-end and hence probably at least a portion of the untranslated 3'-end.

4. DISCUSSION

The present data collectively suggest that the cDNA clone isolated from a λ gt11 placental cDNA

library corresponds to the PAI produced by the Hep G2 hepatoma cell. This includes: (i) the recombinant protein coded by the cDNA expresses inhibitory activity against urokinase; (ii) the recombinant protein forms a complex with urokinase not dissociable in SDS-PAGE; (iii) the expressed protein reacts with the antibody against Hep G2 PAI; and (iv) the amino acid sequences of the N-terminal segment and two tryptic peptides exactly match those deduced from the cDNA sequence.

Several other groups have also cloned endothelial type PAI. Ny et al. [15] used an antibody against bovine aortic endothelial PAI to screen a human placenta λ gt11 cDNA library and obtained cDNA clones with two different size classes – 1.9 and 3.0 kb. The 3.0 kb cDNA has been sequenced [15]. Comparison of the cDNA sequence of Ny et al. and our own reveals a total match except that our clone is shorter at the 3'-untranslated end and that our clone is 9 bases longer at the 5'-end. By Northern hybridization analysis, two distinct transcripts of 2.2 and 3.0 kb have been detected [15]. Therefore, the 1.9 kb cDNAs may have been copied from the shorter mRNA transcript. H. Pannekoek et al. and D. Ginsburg et al. have cloned cDNAs for PAI-1 from human umbilical vein endothelial cell (personal communication). The comparison of the endothelial clones with the placental clones has not yet been reported.

As discussed elsewhere (Ye, R.D. et al., submitted), comparison of the translated protein sequences for PAI-1 and PAI-2 to other serine

1	GTC TTT GGT GAA GGG TCT <u>GCT GTG CAC CAT CCC CCA TCC TAC GTG GCC CAC CTG GCC TCA GAC TTC GGG GTG AGG GTG TTT CAG CAG GTG</u> Val Phe Gly Glu Gly Ser Ala Val His His Pro Pro Ser Tyr Val Ala His Leu Ala Ser Asp Phe Gly Val Arg Val Phe Gln Gln Val	23
91	GCG CAG GCC TCC AAG GAC CGC AAC GTG GTT TTC TCA CCC TAT GGG GTG GCC TCG GTG TTG GCC ATG CTC CAG CTG ACA ACA GGA GGA GAA Ala Gln Ala Ser Lys Asp Arg Asn Val Val Phe Ser Pro Tyr Gly Val Ala Ser Val Leu Ala Met Leu Gln Leu Thr Thr Gly Gly Glu	53
181	ACC CAG CAG CAG ATT CAA GCA GCT ATG GGA TTC AAG ATT GAT GAC AAG GGC ATG GCC CCC GCC CTC CGG CAT CTG TAC AAG GAG CTC ATG Thr Gln Gln Gln Ile Gln Ala Ala Met Gly Phe Lys Ile Asp Asp Lys Gly Met Ala Pro Ala Leu Arg His Leu Tyr Lys Glu Leu Met	83
271	GGG CCA TGG AAC AAG GAT GAG ATC AGC ACC ACA GAC GGG ATC TTC GTC CAG CGG GAT CTG AAG CTG GTC CAG GGC TTC ATG CCC CAC TTC Gly Pro Trp Asn Lys Asp Glu Ile Ser Thr Thr Asp Ala Ile Phe Val Gln Arg Asp Leu Lys Leu Val Gln Gly Phe Met Pro His Phe	113
361	TTC AGG CTG TTC CGG AGC ACG GTC AAG CAA GTG GAC TTT TCA GAG GTG GAG AGA GCC AGA TTC ATC ATC AAT GAC TGG GTG AAG ACA CAC Phe Arg Leu Phe Arg Ser Thr Val Lys Gln Val Asp Phe Ser Ser Glu Val Glu Arg Ala Arg Phe Ile Ile Asn Asp Trp Val Lys Thr His	143
451	ACA AAA GGT ATG ATC AGC AAC TTG CTT GGG AAA GGA GCC GTG GAC CAG CTG ACA CGG CTG GTG CTG GTG AAT GCC CTC TAC TTC AAC GGC Thr Lys Gly Met Ile Ser Asn Leu Leu Gly Lys Gly Ala Val Asp Gln Leu Thr Arg Leu Val Leu Val Asn Ala Gln Tyr Phe Asn Gly	173
541	CAG TGG AAG ACT CCC TTC CCC GAC TCC AGC ACC CAC CGC CGC CTC TTC CAC AAA TCA GAC GGC AGC ACT GTC TCT GTG CCC ATG ATG GCT Gln Trp Lys Thr Phe Pro Phe Ser Thr His Ser Arg Arg Leu Phe His Lys Ser Thr Val Ser Val Pro Met Met Ala	203
631	CAG ACC AAG AAG TTC AAC TAT ACT GAG TTC ACC ACG CCC GAT GGC CAT TAC TAC GAC ATC CTG GAA CTG CCC TAC CAC GGG GAC ACC CTC Gln Thr Asn Lys Phe Asn Tyr Thr Glu Phe Thr Thr Pro Asp Gly His Tyr Tyr Asp Ile Leu Glu Leu Pro Tyr His Gly Asp Thr Leu	233
721	AGC ATG TTC ATT GCT GCC CCT TAT GAA AAA GAG GTG CCT CTC TCT GCC CTC ACC AAC ATT CTG AGT GCC CAG CTC ATC AGC CAC TGG AAA Ser Met Phe Ile Ala Ala Pro Tyr Glu Lys Glu Val Pro Leu Ser Ala Leu Thr Asn Ile Leu Ser Ala Gln Leu Ile Ser His Trp Lys	263
811	GGC AAC ATG ACC AGG CTG CCC CGC CTC CTG GTT CTG CCC AAG TTC TCC CTG GAG ACT GAA GTC GAC CTC AGG AAG CCC CTA GAG AAC CTG Gly Asn Met Thr Arg Leu Pro Arg Leu Leu Val Leu Pro Lys Phe Ser Leu Glu Thr Glu Val Asp Leu Arg Lys Pro Leu Glu Asn Leu	293
901	GGA ATG ACC GAC ATG TTC AGA CAG TTT CAG GCT GAC TTC ACG AGT CTT TCA GAC CAA GAG CCT CTC CAC GTC GCG CAG GCG CTG CAG AAA Gly Met Thr Asp Met Phe Arg Gln Phe Gln Ala Asp Phe Thr Ser Leu Ser Asp Gln Glu Pro Leu His Val Ala Gln Ala Leu Gln Lys	323
991	GTG AAG ATC GAG GTG AAC GAG AGT GGC ACG GTG GCC TCC TCA TCC ACA GCT GTC ATA GTC TCA GCC CGC ATG GCC CCC GAG GAG ATC ATC Val Lys Ile Glu Val Asn Glu Ser Gly Thr Val Ala Ser Ser Ser Thr Ala Val Ile Val Ser Ala Arg Met Ala Pro Glu Glu Ile Ile	353
1081	ATG GAC AGA CCC TTC CTC TTT GTG GTC CGG CAC AAC CCC ACA GGA ACA GTC CTT TTC ATG GGC CAA GTG ATG GAA CCC TGA CCC TGG GGA Met Asp Arg Pro Phe Leu Phe Val Val Arg His Asn Pro Thr Gly Thr Val Leu Phe Met Gly Gln Val Met Glu Pro End	
1171	AAG ACG CCT TCA TCT GGG ACA AAA CTG GAG ATG CAT CGG GAA AGA AGA AAC TCC GAA GAA AAG AAT TTT AGT GTT AAT GAC TCT TTC TGA	
1261	AGG AAG AGA AGA CAT TTG CCT TTT GTT AAA AGA TGG TAA ACC AGA TCT GTC TCC AAG ACC TTG GCC TCT CCT TGG AGG ACC TTT AGG TCA	
1351	AAC TCC CTA GTC TCC ACC TGA GAC CCT GGG AGA GAA GTT TGA AGC ACA ACT CCC TTA AGG TCT CCA AAC CAG ACG GTG ACG CCT GCG GGA	
1441	CCA TCT GGG GCA CCT GCT TCC ACC CGT CTC TCT GCC CAC TCG GGT CTG CAG ACC TGG TTC CCA CTG AGG CCC TTT GCA GGA CGG AAC TAC	
1531	GGG GCT TAC AGG AGC TTT TGT GTG CCT GGT AGA AAC TAT TTC TGT TCC AGT CAC ATT GCC ATC ACT CTT GTA CTG CCT GCC ACC GCG GAG	
1621	GAG GCT GGT GAC AGG CCA AAG GCC AGT GGA AGA AAC ACC CTT TCA TCT CAG AGT CCA CTG TGG CAC TGG CCA CCC CTC CCC AGT ACA GGG	
1711	GTG CTG CAG GTG GCA GAG TGA ATG TCC CCC ATC ATG TGG CCC AAC TCT CCT GGC CTG GCC ATC TCC CTC CCC AGA AAC AGT GTG CAT GGG	
1801	TTA TTT TGG AGT GTA GGT GAC TTG TTT ACT CAT TGA AGC AGA TTT CTG CTT CCT TTT ATT TTT ATA GGA ATA GAG GAA GAA AGG TCA GAT	
1891	GCG TGC CCA GCT CTT CAC CCC CCA ATC TCT TGG TGG GGA GGG GTG TAC CTA AAT ATT TAT CAT ATC CTT GCC	

Fig.4. Nucleotide sequence and translated amino acid sequence of the human PAI-1 cDNA. Nucleotides are numbered on the left and amino acids on the right. The underlined sequences have been independently confirmed by amino acid sequence analysis of the purified Hep G2 PAI protein and two tryptic peptides. The valine designated as number 1 is the amino-terminus based on sequence match with the amino acid sequence obtained for the purified mature protein.

protease inhibitors clearly shows that they are members of the serpin gene family. Despite the fact that PAI-1 and PAI-2 possess an overlapping inhibitory profile and are rather specific inhibitors of uPA and tPA, PAI-1 is no more similar to PAI-2 than it is to any other mammalian serpin.

PAI-1 produced by endothelial cells [5,16,17], HT1080 fibrosarcoma cells [18] and Hep G2

hepatoma cells [19] in culture has been reported to be stable to acid treatment. These PAIs are immunologically related and are reported to exist predominantly in a latent form which can be activated by treatment with SDS and other denaturants. By contrast, our isolated Hep G2 PAI is essentially fully active without treatment with denaturant and its inhibitory activity is, on the

contrary, labile to SDS and acid treatments. Whether these discrepancies reflect a difference in the handling of the materials, in the differential post-translational processing and modification, or in alternative splicing of the mRNA is currently under investigation.

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