Volume 210, number 1, 11–16 FEB 04307 January 1987

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

Tze-Chein Wun and Kuniko K. Kretzmer

Monsanto Co., AA3G, 700 Chesterfield Village Parkway, St. Louis, MO 63198, USA

Received 13 October 1986

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 λgt11 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 (λPAI 11.1 and λPAI 14.1) have been obtained. The cDNA insert of the longer isolate (λPAI 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 λgt 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.

Correspondence address: T.-C. Wun, Monsanto Co., AA3G, 700 Chesterfield Village Parkway, St. Louis, MO 63198, USA

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

2. EXPERIMENTAL

2.1. *Materials*

The λgt11 placental cDNA library constructed by the method of Millan [7] was obtained from Clonetech. The protoblot immunoscreening kit was purchased from Promega Biotech. Restriction enzymes, calf intestine alkaline phosphatase, DNA polymerase I (Klenow), exonuclease III, and S_1 nuclease were purchased from New England Biolabs; dNTPs, from P.L. Biochemicals; 5′-[α-^{35}S]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 $MgSO_4$, 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 λgt11 cDNA library

The λgt11 placental cDNA library, consisting of 10^6 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 λgt11 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 μl 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 abolishmen 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 ^{125}I-urokinase and recombinant proteins expressed by λ-phage isolates

Aliquots (0.4 ml) of λ-lysate or PBB buffer were mixed with 2 μl ^{125}I-urokinase (53 CTA units/ml) and incubated at room temperature for 30 min. 6 μl 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 μl 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 × 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 μl 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 λgt11 clones were digested with *Eco*RI 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 *Eco*RI and the cDNA insert isolated by electroelu-

tion. The insert was then subcloned in both directions into the *Eco*RI site of M13 mp18 [11]. Deletions were generated using exonuclease III [13]. Nucleotide sequence was determined by the dideoxy method [14] using [^{35}S]dATPαS 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 λgt11 was screened using rabbit antiserum against Hep G2 PAI. Initially, 10^6 pfu were screened yielding approx. 60 immunologically positive clones. Of these 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 ^{125}I-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 ^{125}I-urokinase with the lysates from immunologically positive clones followed by SDS-PAGE and autoradiography revealed that the same two clones (λPAI 11.1 and λPAI 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 λPAI 11.1 and λPAI 14.1 were digested with *Eco*RI and the cDNA inserts were found to be 1.4 and 1.9 kb, respectively.

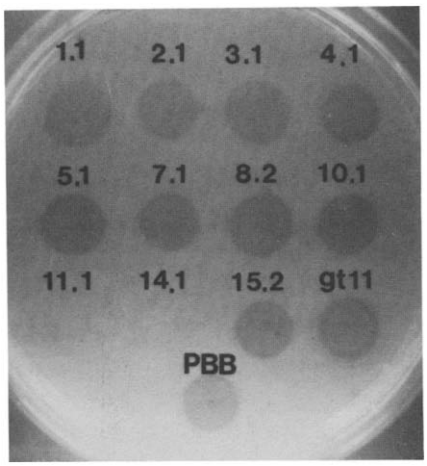

Fig.1. Urokinase inhibitory activities in λgt11 fusion proteins. Phage lysates were incubated with urokinase and the mixture spotted on a fibrin-agar plate. Urokinase activity is indicated by the appearance of a clear lytic zone and PAI activity is shown by the abolishment of the lytic zone. λ-lysates 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, λgt11 without insert, and a PBB control.

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

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

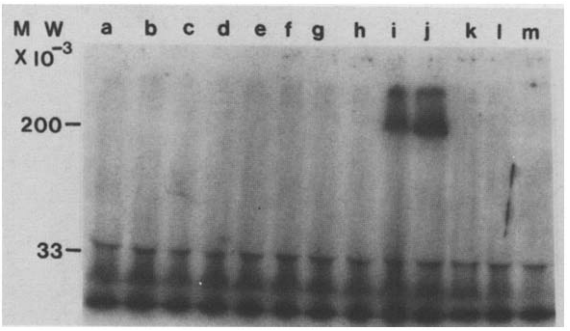

Fig.2. SDS-PAGE analysis of the complex formation between ^{125}I-urokinase and PAI inhibitors in the phage lysates. Phage lysates containing fusion proteins from 6 positive clones were incubated with ^{125}I-urokinase, immunoprecipitated by anti-Hep G2 PAI antibody, and electrophoresed on a 7.5% polyacrylamide gel as described in section 2. λPAI 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, λgt11; m, PBB control.

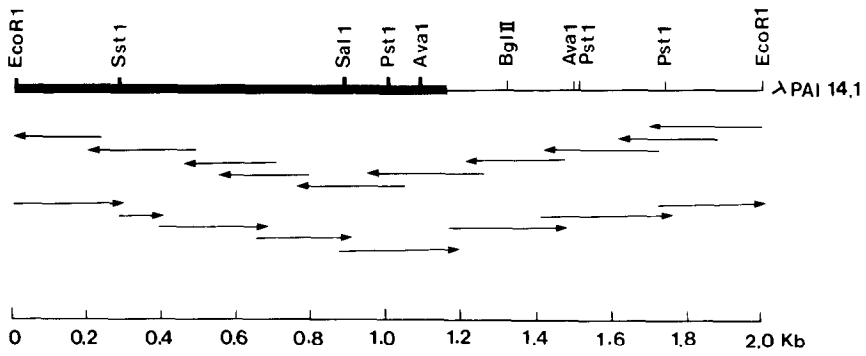

Fig.3. Partial restriction map and sequencing strategy for λPAI 14.1 insert. The scale at the bottom indicates the nucleotide position. The thick bar represents the coding region. The thin bar represents the 3′-noncoding region. The restriction endonuclease sites were confirmed by digestion. The arrows 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 λPAI 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 λPAI 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 λgt11 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 λgt11 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

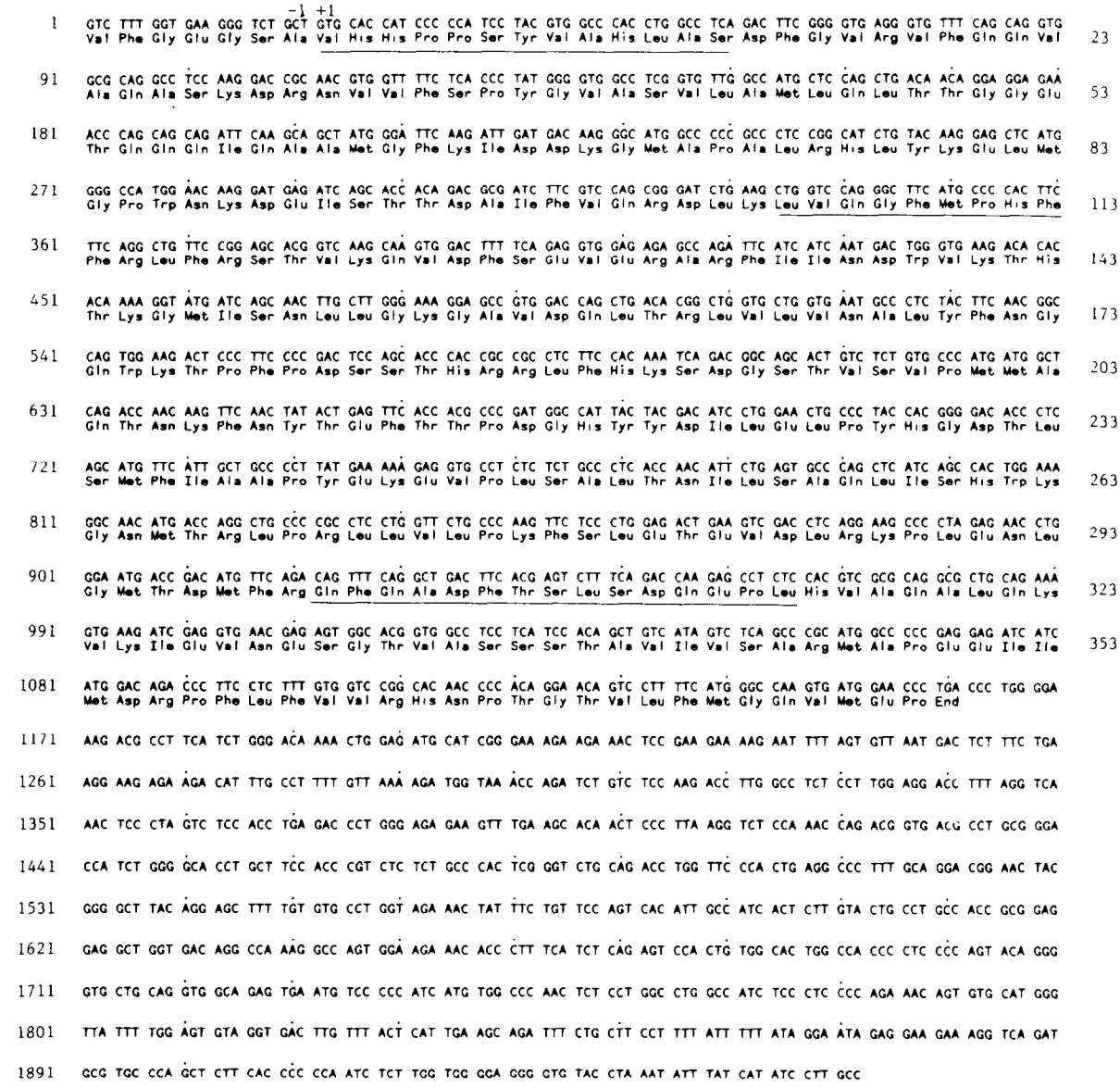

Fig.4. Nucleotide sequence and translated amino acid sequence of the human PAI-1 cDNA. Nucleotides are numbered on the left and amino acids on the right. The underlined sequences have been independently confirmed by amino acid sequence analysis of the purified Hep G2 PAI protein and two tryptic peptides. 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.

ACKNOWLEDGEMENTS

We would like to thank Dr D. Tiemeier for helpful discussions and encouragement, S. Aykent, C. Smith and N. Siegel for amino acid sequence analysis and V. Grant for help in the preparation of the manuscript. We acknowledge Drs T. Ny and D. Loskutoff for sending us a preprint of their paper.

REFERENCES

[1] Dano, K., Andreasen, P.A., Grondahl-Hansen, J., Kristensen, P., Nielsen, L.S. and Skriver, L. (1985) Adv. Cancer Res. 44, 139–266.
[2] Collen, D. (1980) Thromb. Haemostas. 43, 77–89.
[3] Astrup, T. (1978) Prog. Chem. Fibrinolysis 3, 1–89.
[4] Scott, R.W., Bergman, B.L., Bajpai, A., Hersh, R.T., Rodriguez, H., Johns, B.N., Barreda, C., Watts, S. and Baker, J.B. (1985) J. Biol. Chem. 260, 7029–7034.
[5] Van Mourik, J.A., Lawrence, D.A. and Loskutoff, D.J. (1984) J. Biol. Chem. 259, 14914–14921.
[6] Astedt, B., Lecander, I., Brodin, T., Lundblad, A. and Low, K. (1985) Thromb. Haemostas. 53, 122–125.
[7] Millan, J.L. (1986) J. Biol. Chem. 261, 3112–3115.
[8] Wun, T.-C., Ossowski, L. and Reich, E. (1982) J. Biol. Chem. 257, 7262–7268.
[9] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[10] Laemmli, U.K. (1970) Nature 227, 680–685.
[11] Norrander, J., Kempe, T. and Messing, J. (1983) Gene 26, 101–106.
[12] Birnboim, H.C. and Doly, J. (1979) Nucleic Acids Res. 7, 1513–1522.
[13] Henikoff, S. (1984) Gene 28, 351–359.
[14] Sanger, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
[15] Ny, T., Sawdey, M., Lawrence, D., Millan, J.L. and Loskutoff, D.J. (1986) Proc. Natl. Acad. Sci. USA 83, 6776–6780.
[16] Levine, E.G. (1986) Blood 67, 1309–1313.
[17] Sprengers, E.D., Verheijen, J.H., Van Hinsbergh, V.W.M. and Emeis, J.J. (1984) Biochim. Biophys. Acta 801, 163–170.
[18] Andreasen, P.A., Nielsen, L.S., Kristensen, P., Grondahl-Hansen, J., Skriver, L. and Dano, K. (1986) J. Biol. Chem. 261, 7644–7651.
[19] Sprengers, E.D., Princen, H.M.G., Kooistra, T. and Van Hinsberg, V.W.M. (1985) J. Lab. Clin. Med. 105, 751–758.