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Endothelial plasminogen activator inhibitor (PAI): a new member of the Serpin gene family

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A human endothelial cDNA expression library, based on the *Escherichia coli* plasmid pUC9, was screened with a heterologous antibody raised against purified bovine aortic endothelial plasminogen activator inhibitor (PAI). A synthetic oligonucleotide, derived from a partial PAI cDNA expression clone, was used to select a full-length PAI cDNA, the size of which coincides with the length of PAI mRNA (approximately 2350 nucleotides) as determined by Northern blot analysis. The authenticity of full-length PAI cDNA is demonstrated by the expression of biologically active PAI both in lysates of transformed *E. coli* cells and in conditioned media of mouse Ltk$^-$ cells, transfected with PAI cDNA inserted into vector pSV2. Analysis of the *de novo* synthesized anti-plasminogen activator activity, employing reverse fibrin autography, shows that transfected mouse Ltk$^-$ cells synthesize a polypeptide with a mol. wt identical to that of the native PAI glycoprotein (M_r 52 000), whereas in *E. coli* an unglycosylated, active product with a mol. wt of 43 000 is made. The amino acid sequence, derived from the determined nucleotide sequence, shows that pre-PAI consists of 402 amino acids. It is proposed that the mature PAI is preceded by a signal peptide of 23 amino acid residues. The amino acid sequence of mature PAI includes three potential asparagine-linked glycosylation sites and lacks cysteine residues. The predicted amino acid sequence reveals significant homology with members of the serine protease inhibitor (Serpin) family, e.g. α_1-proteinase inhibitor and antithrombin III. This observation provides insight into the structure and function of PAI and into the mechanism of action with its target enzymes, tissue-type plasminogen activator, urokinase and Protein C.

Key words: anti-activator activity/full-length PAI cDNA/PAI nucleotide sequence/recombinant PAI/serine protease inhibitor/Serpin gene family

Introduction

Tissue-type plasminogen activator (t-PA) is a serine protease which converts the zymogen plasminogen into another serine protease, plasmin (Collen, 1980). Plasmin is the ultimate degradative enzyme of the fibrinolysis, the process directed at dissolving a clot. The endothelium may regulate this process, since it synthesizes and secretes t-PA as well as a fast-acting plasminogen activator inhibitor (PAI). PAI has been purified to homogeneity from conditioned medium of bovine aortic endothelial cells and partially characterized (Loskutoff *et al.*, 1983; Van Mourik *et al.*, 1984; Erickson *et al.*, 1985). It represents an unusually stable glycoprotein which is resistant to treatments with chaotropic and reducing agents as well as to detergents. These properties indicate that disulfide bonds do not contribute to the secondary structure of the active protein, whereas the framework either has a very rigid or a loose conformation.

The synthesis of PAI is regulated at the level of transcription of the PAI gene. PAI mRNA synthesis has been shown to vary depending on the conditions employed for culturing endothelial cells (Sawdey *et al.*, 1986), while stimulation of the PAI production by endotoxin or interleukin-1 may also be induced at the transcriptional level (Colucci *et al.*, 1985).

Regulation of the activity of the PAI protein is intimately linked with the physiological role of PAI and its mechanism of action. Inhibition of plasminogen activators [both t-PA and urokinase (uPA)] by PAI is accomplished by the formation of a 1:1 complex of the inhibitor and the 'target' serine protease. Hence, it is conceivable that PAI belongs to the family of serine protease inhibitors (Serpins). Serpins are crucial regulators of various processes, e.g. coagulation, complement activation, inflammatory reactions, and share structural features and a common functional principle (Travis and Salvesen, 1983; Carrell and Travis, 1985; Carrell and Boswell, 1986). In general, Serpins can interact with several serine proteases (Travis and Salvesen, 1983). However, the tissue/cell distribution of a Serpin and its 'target' protease, the concentration and the second-order rate constant determine the specificity of a Serpin. PAI reacts both with the plasminogen activators t-PA and u-PA and with the anticoagulant Protein C (Sakata *et al.*, 1985; Van Hinsbergh *et al.*, 1985). The second-order rate constant with t-PA and u-PA is approximately 10^7 $M^{-1}.s^{-1}$ (Kruithof *et al.*, 1983; Thorsen and Philips, 1984), the value of which strengthens the importance of PAI as the physiological regulator of the fibrinolytic system. Kinetic studies have not been reported for the interaction with Protein C as yet.

Molecular cloning of PAI cDNA, the establishment of the nucleotide sequence of the codogenic region and the prediction of the amino acid sequence, described here, provides new data on the structure and function of PAI, reveals that PAI belongs to the Serpin family and indicates the mode of action of PAI to control fibrinolysis.

Results

Molecular cloning of PAI cDNA

A human endothelial cDNA library was constructed, using *Escherichia coli* DH1 as a host and plasmid pUC9 as a vector (Vieira and Messing, 1982). Poly(A)$^+$ RNA, isolated from cultured human vascular endothelial cells derived from umbilical veins, was employed as substrate for cDNA synthesis and the final product was size-fractionated by Sephadex CL-4B chromatography. Sixty thousand independent colonies, containing pUC9-derivatives with inserts of at least 600 base-pairs (bp) bracketed with G-C tails, were screened with anti-bovine PAI IgG, which crossreacts with human endothelial PAI (Erickson *et al.*, 1985), and subsequently with ^{125}I-labelled sheep anti-rabbit IgG (Helf-

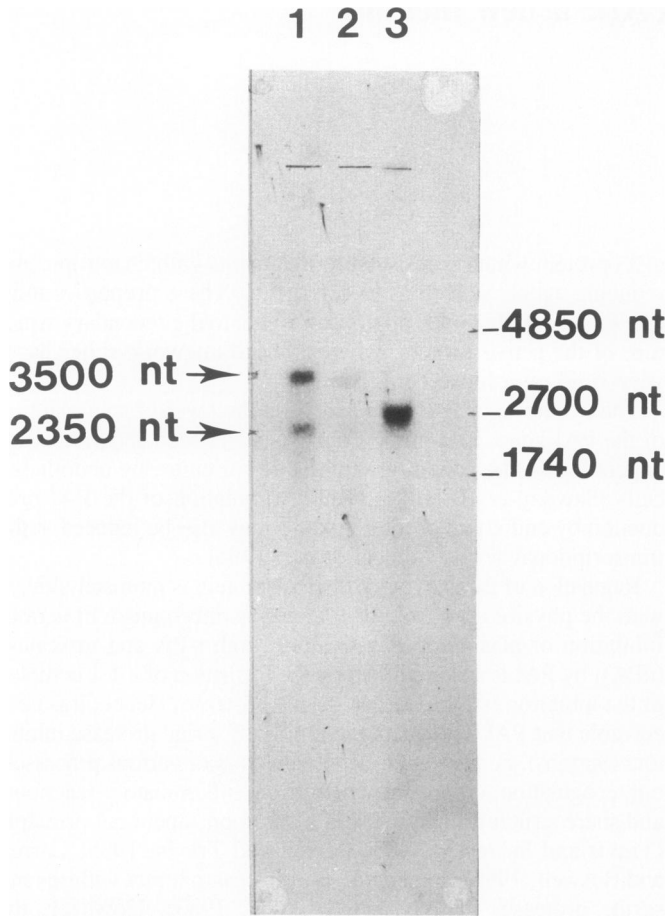

Fig. 1. Northern blotting of human endothelial poly(A)$^+$ RNA , probed with PAI2350 cDNA. **Lane 1**, 1 μg human endothelial poly(A)$^+$ RNA hybridized with ^{32}P-labelled PAI2350 cDNA. **Lane 2**, 20 μg total human endothelial RNA, hybridized with ^{32}P-labelled PAI2350 cDNA. **Lane 3**, 20 μg Bowes melanoma RNA hybridized with ^{32}P-labelled t-PA cDNA (length marker t-PA mRNA of ~2540 nt; Van Zonneveld *et al.*, 1986).

man *et al.*, 1983; Verweij *et al.*, 1985). Three positive clones were analyzed and appeared to harbour plasmids, the longest of them consisting of pUC9 and an insert of approximately 2150 bp. The coding capacity of the insert exceeds the length (~1400 bp) required to code for the (glyco)protein with a mol. wt of 52 000 (Sprengers *et al.*, 1984).

The nucleotide sequence of the region encoding the N-terminal part of the 2150 bp insert, which is proximal to the *lac* promoter on the vector, was determined (Maxam and Gilbert, 1977). The data showed one translation reading frame which contains no nonsense codons from the ATG initiator codon on pUC9 towards the C-terminal part, whereas the remaining reading frames harboured numerous nonsense codons. Based on these results, we could not decide whether the 2150 bp insert represents full-length PAI cDNA. To search for inserts with a more extended 5′ region than the 2150 bp insert, the human endothelial cDNA library was screened with a ^{32}P-labelled oligonucleotide, derived from the 2150 bp insert (24-mer: 5′GCACCAGCCGTGTCAGCTGGTCCA). Fifteen hybridizing clones were found and analyzed with restriction enzymes and Southern blotting. Several pUC9-based plasmids contained similar, more extended 5′ regions relative to the 2150 bp insert, suggestive for cDNA being derived from the extreme 5′ end of PAI mRNA. These plasmids have an insert of about 2350 bp and were designated pUC9/PAI2350. To decide whether the insert represents full-length PAI cDNA we performed Northern blot analysis of endothelial poly(A)$^+$ RNA and expressed biologically active polypeptides in various systems.

Northern blotting of endothelial RNA

Total RNA or poly(A)$^+$ RNA from cultured endothelial cells was subjected to agarose gel electrophoresis and subsequently blotted. Hybridization with [^{32}P]PAI2350 cDNA (Maniatis *et al.*, 1982), revealed two discrete hybridizing mRNA species with a length of about 2350 nucleotides (nt) and 3500 nt, respectively (Figure 1). The mRNA species of 2350 nt matches the length of PAI cDNA, indicating that plasmid pUC9/PAI2350 represents full-length or nearly full-length PAI cDNA. The nature of the mRNA species extending approximately 3500 nt, and which is revealed upon stringent hybridization and washing conditions, remains to be clarified.

Expression of biologically active PAI

Expression of PAI2350 cDNA could provide further evidence on whether this cDNA contains the entire genetic information to encode biologically active PAI. For that purpose, recombinant expression plasmids were constructed both for transfecting mammalian cells and for transforming *E. coli* strain DH1. The vectors for these expression experiments were, respectively, pSV2 (Mulligan and Berg, 1981) and pUC8 (Vieira and Messing, 1982). The construction of the PAI-expression plasmids, denoted pSV2/PAIex and pUC8/PAIex, is outlined in the legend to Figure 2a. Transfection of mouse Ltk$^-$ cells with pSV2/PAIex DNA, followed by transient expression for 5 days, yielded conditioned medium that was assayed for secreted biologically active PAI, employing the reverse fibrin autography (RFA) technique (Loskutoff *et al.*, 1983) (Figure 2). Clearly, mouse Ltk$^-$ cells transfected with pSV2/PAIex synthesize and secrete a product which displays a lysis-resistant zone by RFA. The mobility of this product is identical with that of PAI present in conditioned medium of cultured human vascular endothelial cells. These data demonstrate that the PAI cDNA of 2350 bp encodes biologically active PAI with an apparent mol. wt indistinguishable from that of the natural, human endothelial glycoprotein PAI (52 000) (Sprengers *et al.*, 1984). The observation that PAI is secreted by transfected mouse Ltk$^-$ cells demonstrates that pSV2/PAIex encodes a functional signal peptide and consequently harbours full-length PAI cDNA. Furthermore, these data indicate that PAI programmed by pSV2/PAIex DNA is faithfully glycosylated by mouse Ltk$^-$ cells. It can also be observed upon transfection with control DNA (pSV2 DNA) that mouse Ltk$^-$ cells themselves produce a relatively small amount of an endogenous plasminogen activator inhibitor with a similar apparent mol. wt to the human inhibitor.

Transformation of *E. coli* strain DH1 with pUC8/PAIex DNA should result in the synthesis of a fusion protein with a continuous translation reading frame, composed of 14 N-terminal amino acid residues of β-galactosidase, six glycines, 41 amino acids from the 5′ untranslated region of PAI, the PAI-signal peptide and the coding sequence for mature PAI. Electrophoresis of lysates of *E. coli* transformed with pUC8/PAIex DNA on SDS-polyacrylamide gels, and a subsequent analysis by RFA, showed a lysis-resistant zone with an apparent mol. wt of 43 000 and smaller than that of natural PAI or PAI synthesized in transfected mouse Ltk$^-$ cells. This observation suggests that the presumed fusion protein, produced in *E. coli*, is proteolytically processed at the site between the signal peptide and the mature protein. Furthermore, this experiment demonstrates that glycosylation of the PAI protein, which does not occur in *E. coli*, is not a prerequisite

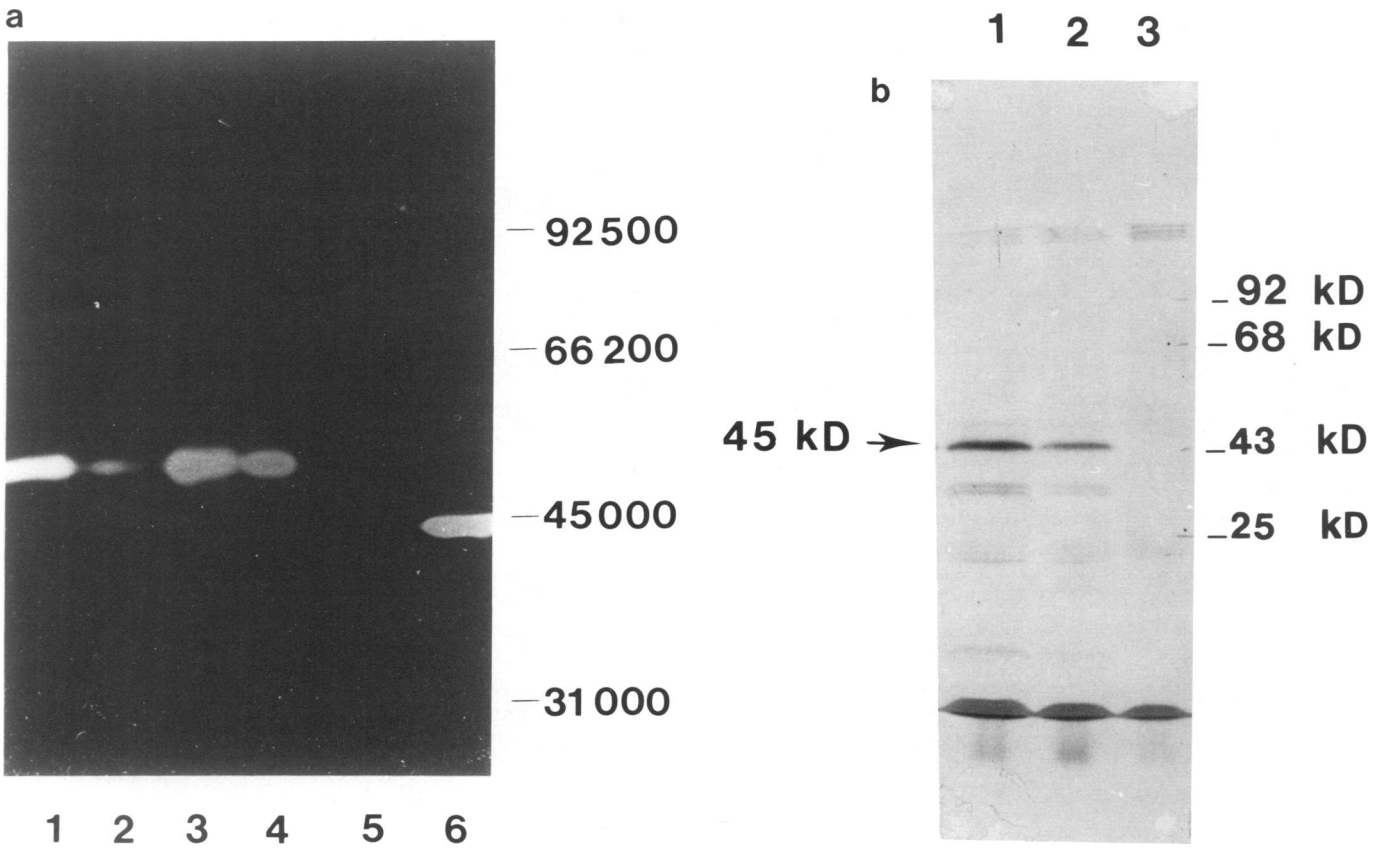

Fig. 2. **(a)** *In vivo* synthesis of biologically active PAI in transfected mammalian cells and in transformed bacteria. **Lanes 1** and **4**, 0.05 ml conditioned medium derived from cultured vascular human endothelial cells. **Lane 2**, 0.16 ml conditioned medium of mouse Ltk$^-$ cells transfected with pSV2 DNA. **Lane 3**, 0.16 ml conditioned medium of mouse Ltk$^-$ cells transfected with pSV2/PAIex DNA. **Lane 5**, 0.16 ml lysate of *E. coli* DH1 transformed with pUC8 DNA. **Lane 6**, 0.16 ml lysate of *E. coli* DH1 transformed with pUC8/PAIex DNA. The position of mol. wt marker proteins is indicated. **(b)** *In vitro* synthesis of PAI-like polypeptides. Transcription of linear pSP65/PAIex DNA was initiated at the SP6 promoter and 'runs off' the template, yielding PAI mRNA with a length of about 2350 nt. This RNA preparation was used to encode the *in vitro* synthesis of polypeptides labelled with [^{35}S]methionine. The procedures for transcription and translation are outlined in Materials and methods. The polypeptides were analyzed by electrophoresis on a 10% SDS-polyacrylamide gel (Laemmli, 1970), followed by fluorography. **Lane 1**, polypeptides encoded by 0.5 μg pSP65/PAIex RNA. **Lane 2**, polypeptides encoded by 0.1 μg pSP65/PAIex RNA. **Lane 3**, no RNA added. The position of mol. wt protein markers is indicated.

for its anti-activator activity. This conclusion is enforced by the observation that expression of PAI cDNA in mouse Ltk$^-$ cells in the presence of the asparagine-linked glycosylation inhibitor tunicamycin yields an active product with a mol. wt (43 000) similar to the *E. coli* product (results not shown). Furthermore, this mol. wt estimation is in good agreement with the apparent mol. wt of the unglycosylated precursor polypeptide (approximately 45 000), obtained after *in vitro* translation of capped PAI mRNA made with SP6 RNA polymerase using PAI2350 cDNA inserted in pSP65 DNA (Melton *et al.*, 1984) (Figure 2b). Both the results of the Northern blot analysis and of the expression in various systems of anti-activator activity, encoded by PAI2350 cDNA, justify the conclusion that this cDNA harbours the complete genetic information for PAI.

Nucleotide and amino acid sequence

The nucleotide sequence of the entire codogenic region of PAI-2350 cDNA was established both by the chemical degradation and by the dideoxy method (Maxam and Gilbert, 1977; Sanger *et al.*, 1977) (Figure 3). Inspection of the potential reading frames reveals that one of them is free of nonsense codons. The first ATG codon of this 'open' frame is encountered 127 nt downstream of the 5′ terminus of the mRNA, followed by 1206 nt till the TGA translation termination codon. Hence, the precursor protein constitutes 402 amino acid residues. The polypeptide is secreted by transfected mammalian cells and has therefore been preceded by a signal peptide, which will be removed during transport through the endoplasmic reticulum. A comparison of the N-terminal sequence of PAI with the consensus sequence for signal peptides (Von Heijne, 1983) indicates that the signal peptide may extend from methionine (M; −23) until valine (V; +1). Consequently, mature PAI would be composed of 379 amino acids with a calculated mol. wt of 42 800. The predicted amino acid sequence contains three potential asparagine-linked glycosylation sites which could account for an increase of the mol. wt until about 52 000 after glycosylation. It is remarkable that the mature form of PAI is devoid of cysteine residues, which implies that disulfide bonds do not contribute to the secondary structure of the protein. This conclusion is consistent with the observation that the PAI activity is resistant to reducing agents (Loskutoff *et al.*, 1983).

Homology of PAI with Serpins

A search for homology of PAI with other proteins, employing the NIH Data Bank included in the MicroGenie program (Beckman, Inc.), showed a significant homology of PAI with members of the Serpin family (Travis and Salvesen, 1983; Carrell and Travis, 1985; Carrell and Boswell, 1986). Identical amino acid residues, predominantly present within the carboxyl-terminal half of α_1-proteinase inhibitor, antithrombin III, α_1-antichymotrypsin

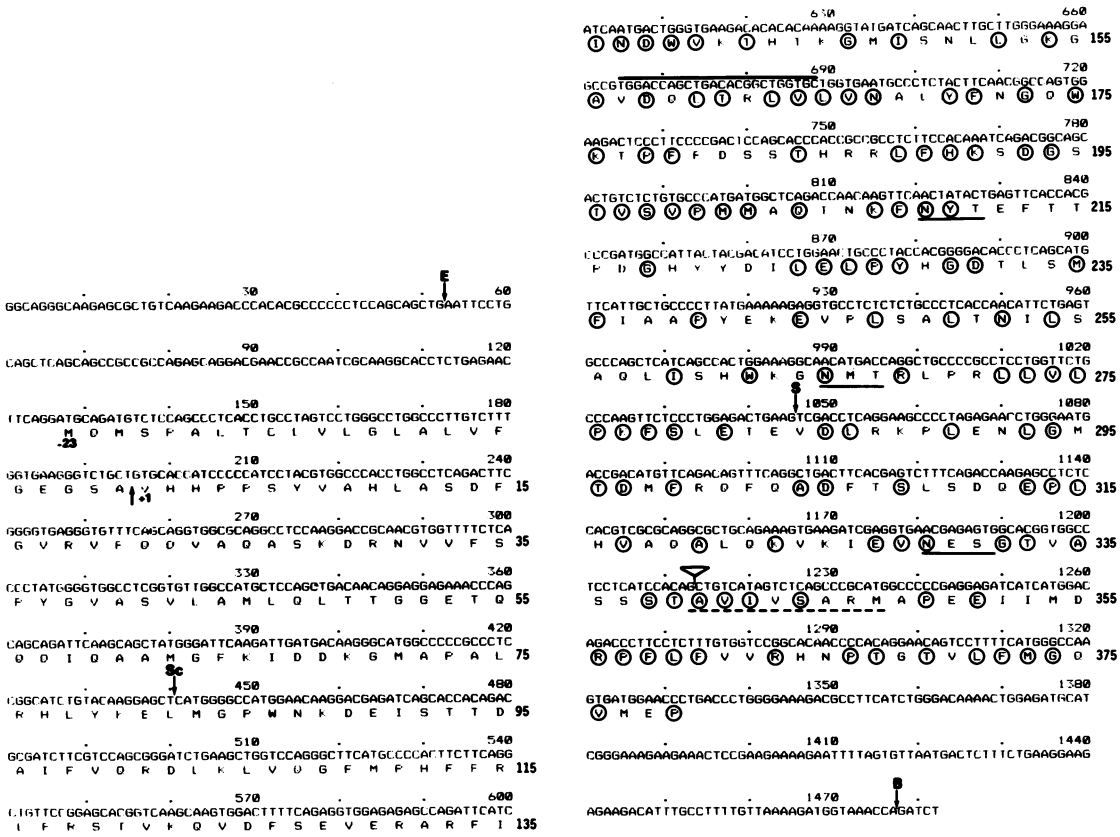

Fig. 3. Complete nucleotide sequence of the codogenic region of PAI2350 cDNA. The predicted amino acid sequence of PAI is indicated using the one-letter code. The residues from methionine (M; −23) until valine (V; +1) constitute the putative signal peptide of PAI, indicated with an arrow. Potential asparagine-linked glycosylation sites, specified by the sequences N-X-T or N-X-S, are underlined. The nucleotide sequence which served to synthesize a complementary oligomer (24-mer: 5'GCACCAGCCGTGTCAGCTGGTCCA) to detect full-length PAI cDNA inserts is overlined. Amino acids in the C-terminal part of PAI, which are identical to either antithrombin III or α_1-proteinase inhibitor and which occupy a similar position within the amino acid sequence, are encircled. Restriction sites for *Sac*I (Sc) and *Sal*I (S), used for subcloning fragments in M13 mps and 5' terminal labelling are indicated. The *Eco*RI (E) and *Bgl*II (B) restriction sites were used for the construction of the expression plasmid pSV2/PAIex. Another full-length PAI cDNA clone harbours an insertion of 21 bp between position 1213 and 1214, as indicated by a triangle (5'ACATTGTCCCTTCTCTTGCAG). The encoded seven amino acid residues (D-I-V-P-S-L-A) are 'in-frame' with the remaining C-terminal sequence.

and others have recently been recognized (Carrell and Boswell, 1986). PAI displays a 32% homology for its residues 136−379 with α_1-proteinase inhibitor, and 34 and 31% with antithrombin III and α_1-antichymotrypsin, respectively. In general, the homologous amino acids of these Serpins are hydrophobic residues. In many cases structurally similar residues also align between PAI and another Serpin, indicating that the overall structure of the carboxyl-terminal part of these proteins is alike.

Discussion

Consecutive screening of a human endothelial cDNA expression library by an immunological method and by hybridization with a synthetic oligonucleotide yielded a full-length PAI cDNA-containing plasmid. The authenticity of PAI cDNA was shown by expressing anti-plasminogen activator activity, encoded by this cDNA, displaying properties indistinguishable from native PAI.

The amino acid sequence, predicted from the determined nucleotide sequence, does not contain cysteine residues, the observation of which excludes disulfide bonds in the PAI protein. This conclusion agrees with biochemical data which showed that PAI is resistant to reducing agents (Loskutoff *et al.*, 1983).

An alignment of the amino acid sequence of PAI with that of other proteins revealed that PAI is a member of the Serpin family. It is conceivable that the mechanism of action of PAI with its 'target' protease t-PA and u-PA, and possibly Protein C (Sakata *et al.*, 1985; Van Hinsbergh *et al.*, 1985) will be based on the same principles as the other Serpins. In particular, the interaction of α_1-proteinase inhibitor with leukocyte elastase has been extensively studied (Travis and Salvesen, 1983; Loebermann *et al.*, 1984; Carrell and Boswell, 1986). The inhibitors serve as an ideal pseudo-substrate ('bait') for the serine protease. The serine (Ser) residues of the active center of the protease reacts with the P1 residue in the reactive site of the Serpin, resulting in a scission of the P1-P1' peptide bond and a possible covalent bond between the protease and the P1 residue (Laskowski and Kato, 1980). The structure of the reaction center, located near the carboxyl terminus, determines the 'target' specificity. In general, the P1 residue of the reactive center corresponds with the amino-terminal residue of the peptide bond to be cleaved within the genuine substrate. Hence, both t-PA and u-PA cleave the arginine (Arg-560)-valine(Val-561) bond of Lys plasminogen (Robbins *et al.*, 1967), whereas it has been proposed that Protein C digests the arginine (Arg-336)-methionine(Met-337) peptide bond of the coagulation Factor VIII (Eaton *et al.*, 1986). Accordingly, it is expected that the P1 residue of PAI would constitute an arginine (Arg) residue, and consequently that PAI is classified as an Arg-Serpin (Carrell and Boswell, 1986). Computer alignment of the amino acid sequence of PAI with other Serpins matched their respective P1-P1' residues with the PAI amino acids Arg(347)-Met(348) or with Val(343)-Ser(344). In view of the observation

that the P1′ residue is almost invariably a serine (Ser), we cannot unambiguously attribute a particular amino acid residue to a P1 position.

In the initial search of PAI cDNA-containing plasmids a full-length PAI cDNA was detected, which did not, however, program anti-plasminogen activator activity. Direct comparison of the nucleotide sequence of this cDNA with that of other investigators (Ny *et al.*, 1986) revealed identity, except for a 21 bp insertion situated just upstream of the putative reactive center (indicated in the legend to Figure 3) which is present in that PAI cDNA. An analysis of our PAI cDNA-containing clones showed that 14 out of 15 lacked the 21 bp insertion. Interestingly, the nucleotide sequence of the 21 bp insertion (5′ACATTGTCCCTTCTCTTGCAG) matches perfectly with the consensus for an acceptor splice site and, consequently, may represent alternative splicing. This event will yield a protein with an 'extra' seven amino acids in-frame with the remaining part of the sequence. With respect to the highly ordered structure of the Serpins (Loebermann *et al.*, 1984) an insertion of seven amino acids near the putative active center would conceivably alter the reactivity. Currently, we are investigating whether alternative splicing of PAI mRNA represents a functional mechanism in endothelial cells to change the specificity or to prevent the activity of this protein.

Materials and methods

Construction and screening of a cDNA library

The isolation of poly(A)$^+$ RNA from cultured human vascular endothelial cells, the synthesis of C-tailed double-stranded cDNA and annealing with G-tailed pUC9 were performed as previously described (Verweij *et al.*, 1985). Transformation of *E. coli* strain DH1 yielded an expression cDNA library of about 60 000 independent clones. Screening for bacterial synthesis of PAI-like antigen(s) was carried out with a heterologous IgG preparation, raised in rabbits against a purified preparation of PAI, isolated from conditioned medium of cultured bovine aortic endothelial cells (Van Mourik *et al.*, 1984). Subsequently, positive clones were detected with ^{125}I-labelled sheep anti-rabbit IgG. The protocol for the immunological screening has been outlined before (Verweij *et al.*, 1985). Hybridization of the endothelial cDNA library with a 5′ ^{32}P-labelled oligomer (24-mer: 5′GCACCAGCCGTGTCAGCTGGTCCA) was performed using a standard procedure (Maniatis *et al.*, 1982).

Nucleic acid procedures

The alkaline lysis method, followed by CsCl/ethidium bromide equilibrium centrifugation was employed for plasmid DNA isolations (Birnboim and Doly, 1979). A eukaryotic expression plasmid, based on the vector pSV2 (Mulligan and Berg, 1981) and full-length PAI2350 cDNA, was designated pSV2/PAIex and was constructed as follows: pUC9/PAI2350 DNA was digested with *Eco*RI and the cohesive ends were filled in with DNA polymerase I (large fragment). Subsequently, this preparation was digested with *Bgl*II and the PAI cDNA fragment was ligated into pSV2 DNA which was previously digested with *Hin*dIII, treated with DNA polymerase I (large fragment) to fill in cohesive ends and then cleaved with *Bgl*II. On the resulting plasmid, PAI cDNA is preceded by the SV40 'early' promoter and linked at its 3′ end to splice and polyadenylation signals. Purified pSV2/PAIex DNA was used to transfect mouse Ltk$^-$ cells as described (Lopata *et al.*, 1984) and transient expression in serum-free medium was allowed for 5 days. An *E. coli* expression plasmid, derived from the vector pUC8 (Vieira and Messing, 1982) and designated pUC8/PAIex, was constructed as follows: essentially, the vector part of plasmid pUC9/PAI2350 DNA was substituted for vector pUC8. To that end, pUC9/PAI2350 DNA was digested with both *Bam*HI and *Hin*dIII, which have unique cleavage sites within the polylinker of pUC9 on either side of the PAI cDNA insert. The relevant fragment was subsequently inserted into pUC8 digested with both *Hin*dIII and *Bam*HI. DNA sequencing was performed to verify that this construction resulted in an 'open' translation reading frame from the ATG codon of β-galactosidase until the TGA termination codon of mature PAI (Maxam and Gilbert, 1977). Cell lysates of *E. coli* DH1, transformed with pUC8/PAIex DNA, were prepared as follows: a culture of 10 ml (5×10^8 cells/ml) was concentrated 10-fold by centrifugation and the cells were suspended in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 M NaCl, 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Lysis was achieved by extensive sonification. The analysis of endothelial poly(A)$^+$ RNA by Northern blotting, after treatment with formamide plus formaldehyde and electrophoresis on an 0.8% agarose–formaldehyde gel, and radioactive labelling of probes by nick-translation, was performed as described (Maniatis *et al.*, 1982).

DNA sequencing, both by the chemical degradation (Maxam and Gilbert, 1977) and by the dideoxy method (Sanger *et al.*, 1977), was performed for both strands of PAI2350 cDNA. To that end, the unique *Bam*HI and *Hin*dIII sites on pUC9 on either side of the PAI2350 cDNA insert as well as internal *Sac*I and *Sal*I sites were employed for subcloning in M13 mps and for 5′ end-labelling (Maniatis *et al.*, 1982).

Determination of PAI activity

Aliquots of serum-free conditioned media of mouse Ltk$^-$ cells, transfected with pSV2/PAIex DNA or cell lysates of *E. coli* DH1, transformed with pUC8/PAIex DNA, were subjected to electrophoresis and a 10% polyacrylamide gel containing 0.1% SDS (Laemmli, 1970). Gels were treated with Triton X-100 and prepared for reversed fibrin autography as described (Loskutoff *et al.*, 1983). The fibrin overlay contained 25 μg/ml Glu-plasminogen and 50 mU u-PA/ml. This technique visualizes anti-plasminogen activator activity by the appearance of a zone which inhibits plasminogen activator-(t-PA or u-PA)-induced lysis of a fibrin overlay by generated plasmin. Concomitantly, this technique allows an estimation of the apparent mol. wt of PAI. Lysis-resistant zones were visible approximately 4 h after application of the overlay on the gel.

In vitro synthesis of polypeptides

PAI2350 cDNA, situated on a *Bam*HI–*Hin*dIII fragment, was inserted into pSP65 DNA (Melton *et al.*, 1984) digested with *Bam*HI plus *Hin*dIII, yielding plasmid pSP65/PAIex. This plasmid was linearized with *Bam*HI and transcribed *in vitro* with SP6 RNA polymerase, according to the manufacturer's instructions (New England Nuclear, Dreieich, FRG). The RNA preparation, with a uniform length of about 2350 nt, was used to program the *in vitro* synthesis of polypeptides labelled with [^{35}S]methionine employing a reticulocyte lysate.

Materials

Restriction enzymes, DNA polymerase I, Klenow fragment and polynucleotide kinase were from New England Biolabs (Beverley, MA, USA). Calf intestine alkaline phosphatase was from Boehringer (Mannheim, FRG). SP6 RNA polymerase was purchased from New England Nuclear, while reticulocyte lysates were from Promega-Biotec (Madison, WI, USA). Radioactive chemicals were purchased from Amersham (UK). A synthetic oligonucleotide (24-mer) was prepared with an automated DNA synthesizer (Applied Biosystems type 381A).

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