Plasminogen activator inhibitor type-1: reactive center and amino-terminal heterogeneity determined by protein and cDNA sequencing

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Both the urokinase-type and tissue-type plasminogen activator can convert their ~54 kDa type-1 inhibitor (PAI-1) to an inactive form with a lower apparent molecular mass. We have determined the amino-terminal amino acid sequences of human native and converted PAI-1, and isolated PAI-1 cDNA and determined the nucleotide sequence in regions corresponding to the amino-terminus and the cleavage site. The data show that the conversion of the inhibitor consists of cleavage of an Arg–Met bond 33 residues from the carboxy-terminus, thus localizing the reactive center of the inhibitor to that position. In addition, a heterogeneity was found at the amino-terminus, with a Ser-Ala-Val-His-His form and a two-residue shorter form (Val-His-His-) occurring in approximately equal quantities.

Plasminogen activator *Plasminogen activator inhibitor* *Amino acid sequence* *Nucleotide sequence* *Serpin*

1. INTRODUCTION

Plasminogen activators are serine proteases that catalyze the conversion of the abundant proenzyme plasminogen to the active protease plasmin by specific cleavage of a single peptide bond. There are two known types of plasminogen activators, the urokinase-type (u-PA) and the tissue-type (t-PA); they are products of different genes (reviews [1,2]). Two types of specific and fast-acting inhibitors of plasminogen activators have been distinguished by immunochemical criteria; both inhibit u-PA as well as t-PA [3–21]. They may be designated PAI-1 and PAI-2, respectively. [Nomenclature recommended by the Subcommittee on Fibrinolysis of the International Committee on Thrombosis and Hemostasis, June 8, 1986 (Kruithof, E.K.O., personal communication).]

We previously demonstrated that incubation of human PAI-1 with catalytic amounts of u-PA or t-PA resulted in the conversion of the inhibitor to an inactive form, which, as judged from its electrophoretic mobility, had a slightly smaller M_r than the native form [22]. This suggests a proteolytic attack by the activators at the reactive center of PAI-1, similar to what has been observed with in-

Abbreviations: PAI-1, type-1 plasminogen activator inhibitor; PAI-2, type-2 plasminogen activator inhibitor; u-PA, urokinase-type plasminogen activator; t-PA, tissue-type plasminogen activator; PBS, 0.01 M sodium phosphate, pH 7.4, 0.15 M NaCl; PAGE, polyacrylamide gel electrophoresis; SSC, 0.15 M NaCl, 0.015 M sodium citrate, pH 7.6; Denhardt's solution, 0.02 mg/ml Ficoll, 0.02 mg/ml polyvinylpyrrolidone, 0.02 mg/ml bovine serum albumin; DTT, dithiothreitol; PTH, phenylthiohydantoin

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hibitors of other serine proteases [23,24]. We now report amino acid sequence data of PAI-1, and isolation and partial sequencing of PAI-1 cDNA, localizing the site of proteolytic cleavage and identifying an amino-terminal heterogeneity.

2. MATERIALS AND METHODS

PAI-1 was purified to apparent homogeneity from serum-free conditioned culture fluid of the human HT-1080 fibrosarcoma cell line, essentially as described [20]; Tris and glycine, however, were replaced with potassium phosphate and acetic acid in order to allow subsequent amino acid sequence analysis. Purified preparations were dialyzed extensively against PBS. For cleavage of PAI-1, it was first activated with 0.1% SDS for 1 h at room temperature at a concentration of approx. 100 μg/ml (see [15,22,25]); Triton X-100 was then added to a concentration of 1%, and u-PA or t-PA to a concentration of 0.5 μg/ml (molar ratio PAI-1:plasminogen activator equal to approx. 200:1). This mixture was incubated for 4 h at 37°C (see [22]). PAI-1 conversion by either u-PA or t-PA was better than 90%, as judged by SDS-PAGE analysis.

For determination of amino-terminal amino acid sequences of native or converted PAI-1, these proteins were precipitated with 20% trichloroacetic acid, washed with acetone and dried. The samples were dissolved in 1% SDS and brought to alkaline pH with ammonium hydroxide, or dissolved in 70% trifluoroacetic acid. Approx. 1 nmol protein was subjected to sequence analysis. To obtain internal partial amino acid sequence data for generating oligonucleotide probes, PAI-1 was reduced and carboxymethylated with iodoacetic acid, 1:40 weight additions of *Staphylococcus* V8 protease was made at 0 and 5 h, and the digestion was continued for a total of 24 h at 37°C. Peptides were separated by reversed-phase HPLC on a Vydac C18 column with a 0–80% acetonitrile gradient in 0.05% trifluoroacetic acid, and their amino acid sequences determined. Protein sequencing was performed with Applied Biosystems model 470A gas-phase sequencers [26,27]. A 39-mer oligonucleotide corresponding to a methionine-containing peptide was synthesized with an Applied Biosystems oligonucleotide synthesizer [28].

A cDNA library established from an SV40-transformed human fibroblast cell line, GM637, by the use of the pcD vector system [29], was screened with a ^{32}P end-labelled 39-mer oligonucleotide probe (see below) by the procedure of Verde et al. [30], hybridizing filters in 5×SSC, 10×Denhardt's solution, 0.1% SDS, 200 μg/ml of yeast tRNA and 200 μg/ml of salmon sperm DNA at 52°C for 16 h, followed by a wash in 5×SSC, 0.1% SDS at the same temperature. Positive clones were isolated, and their DNA analyzed by restriction enzyme mapping, hybridization to the synthetic oligonucleotide probe, and by nucleotide sequencing. Nucleotide sequencing was carried out by the dideoxy method [31], using restriction enzyme fragmented DNA cloned into M13 vectors [32,33].

Poly(A)$^+$ RNA from GM637 cells was analyzed by a Northern blot technique, hybridizing filters with the 39-mer oligonucleotide probe under the conditions described above.

PAI-1 was identified and quantitated by reverse zymography [15] and ELISA (Nielsen, L.S. et al., in preparation).

All reagents and enzymes were those described before [15,20–22,26,30,34], or of the best grade commercially available.

3. RESULTS AND DISCUSSION

Amino acid sequence analysis of five different native PAI-1 preparations showed 2 residues in each cycle. The analyses unambiguously identified the amino-terminal sequence Ser-Ala-Val-His-His-Pro-Pro-Ser-Tyr-Val-Ala-His-Leu-Ala-Ser-Asp-Phe-Gly-Val-Arg-Xaa-Phe-Gln-, accounting for approx. 45%, and the identical des(Ser-Ala) variety (Val-His-His-etc.) accounting for approx. 55% (see fig.1). Amino acid analysis of PAI-1 showed the presence of the amino sugar glucosamine and thus demonstrated that it is a glycoprotein (not shown).

Quantitative sequence analyses on converted PAI-1 showed, in addition to the above two sequences, a third one, Met-Ala-Pro-Glu-Glu-Ile-Ile-Met-Asp-Arg-Pro-Phe- (fig.1). Identical results were obtained with conversion by u-PA (2 runs) and t-PA (1 run). Control incubations without u-PA or t-PA did not contain this sequence. Thus, the conversion of PAI-1 to an inactive form ap-

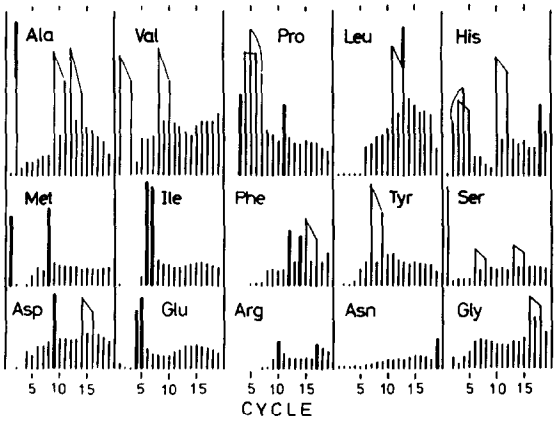

Fig.1. Quantitative amino acid sequence analysis of u-PA-converted PAI-1. For each PTH-amino acid, the yields are plotted as a function of the cycle number. Lys, Gln, Trp, Cys and Thr were absent in the first 19 cycles. Two samples, incubated in parallel with and without u-PA, as described in section 2, were compared. The heavy bars represent the differences between u-PA-treated PAI-1 and the incubation control which was identical to native PAI-1 (not shown). The amino-terminal sequence of the u-PA-released fragment therefore reads Met-Ala-Pro-Glu-Glu-Ile-Ile-Met-Asp-Arg-Pro-Phe-. The linked thin bars indicate that PAI-1 exists in two varieties which differ in length by two residues at the amino-terminus. The des(Ser-Ala) variety corresponds to the first of the linked bars (Val-His-His-Pro-Pro-Ser-Tyr-etc.; see text), and the longer variety to the second of the linked bars (Ser-Ala-Val-His-His-etc.).

pears to consist of a proteolytic cleavage of the molecule. We calculated that the new amino-terminal sequence was present in an amount corresponding to 25–30% (3 runs) of the sum of the two other sequences, probably reflecting only partial coprecipitation with trichloroacetic acid.

To confirm the protein sequence data and to localize the cleavage site, we have isolated and sequenced PAI-1 cDNA. An oligonucleotide 39 bases long was synthesized on the basis of the sequence of a peptide obtained by *Staphylococcus* V8 protease digestion of PAI-1. The amino-terminal amino acid sequence of the peptide was Xaa-Leu-Gly-Met-Lys-Asp-Met-Phe-Arg-Gln-Phe-Gln-Ala-Asp-, and the nucleotide sequence of the oligonucleotide, chosen according to human codon usage frequence [35], was 5′-GTCAGCCTGGA-ACTGTCGGAACATGTCCTTCATTCCTAG-3′.

An SV40-transformed human fibroblast cell line, GM637 [29], was found to produce high amounts of PAI-1 by reverse zymography and ELISA with specific monoclonal antibodies, and Northern blot analysis of poly(A)$^+$ RNA from these cells showed that the above 39-mer probe hybridized to two RNA species of approx. 3.4 and 2.4 kb (not shown). A cDNA library established from the GM637 cells [29] was therefore screened for PAI-1 DNA with the probe. Positive clones were identified with an abundance of approx. 1:1000. Several positive clones were isolated. Restriction enzyme analysis of the inserts showed that all clones fell into two classes, differing in the 5′- or 3′-ends. The clones with the longest inserts, A1 and A5, were chosen for further investigation. The insert from clone A1 was approx. 2200 and that from clone A5 approx. 3200 nucleotides long. Their restriction maps (fig.2) showed that they were likely to be identical, except for their 3′-end. This was confirmed by determination of the nucleotide sequence of portions of the DNA. No differences were observed in their 5′-noncoding and their coding regions (see below), but the extreme 3′-ends of their 3′-untranslated regions of 800 and 1800 nucleotides, respectively, were different (not shown). However, both had typical polyadenylation sites, this sequence being present three times in tandem in A5 and only once in A1. We therefore suggest that the PAI-1 mRNA heterogeneity may depend on the choice of different polyadenylation sites during post-transcriptional processing. However, alternative splicing within the 3′-un-

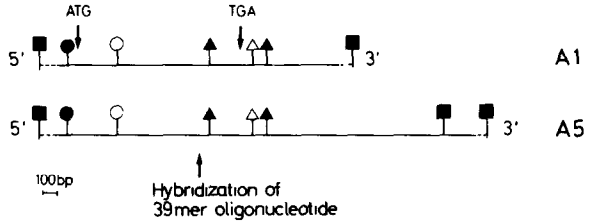

Fig.2. Restriction map of PAI-1 cDNA, clones A1 and A5. Restriction enzymes: ↑, *Ava*I; ↓, *Bam*HI; ⍋, *Bgl*II; ⚲, *Eco*RI; ⚲, *Sac*I. The positions of the start and stop codons, and the site of hybridization of the 39-mer oligonucleotide probe are indicated. The dashed parts of the lines at each end of the two clones indicate parts of the vector, i.e. the *Bam*HI sites at each end are situated a short distance inside the vector [29]. The distance between the start and the stop codon is in agreement with the M_r of the protein, as judged from its migration in SDS-PAGE [15].

translated region cannot be excluded. The presence of two genes can be excluded by preliminary Southern blot analysis (not shown).

Fig.3 shows the nucleotide sequence of the portions of the DNAs corresponding to the amino- and carboxy-termini of the proteins for which amino acid sequences were available (see above). In a portion containing part of the 5′-untranslated region and part of the coding region (fig. 3A), the first methionine codon ATG is found at nucleotide 146 and is preceded by a sequence which is consistent with the Kozak rule for translation initiation [36]. This identifies the possible start of the signal peptide. We find the Ser and Val residues which we identified as amino-terminal amino acids of the two forms of the mature protein, 21 and 23 residues downstream from that, respectively. The heterogeneity at the N-terminus of the mature protein is most likely due to two possible cleavage sites of signal peptidases, since these appear to cleave at the carboxyl side of small, neutral amino acids like glycine or alanine [37]; these are indeed the residues preceding the two observed amino-terminal amino acids of mature PAI-1 (fig.3A). However, we cannot exclude the possibility that further processing or degradation occurred after removal of the signal peptide.

A

```
          .         .        30         .         .        60
ACAGCTGTGTTTGGCTGCAGGGCCAAGAGCGCTGTCAAGAAGACCCACACGCCCCCCTCC

          .         .        90         .         .       120
AGCAGCTGAATTCCTGCAGCTCCGGGCAGCCGCCGCCAGAGCAGGACGACCGCCAATCGC

          .         .       150         .         .       180
AAGGCACCTCTGAGAACTTCAGGATGCAGATGTCTCCAGCCCTCACCTGCCTAGTCCTGG
                          MetGlnMetSerProAlaLeuThrCysLeuValLeuG
                         -21
          .         .       210         .         .       240
GCCTGACCCTTGTCTTTGGTGAAGGGTCTGCTGTGCACCATCCCCCATCCTACGTGGCCC
lyLeuThrLeuValPheGlyGluGlySerAlaValHisHisProProSerTyrValAlaH
                         +1                                +10
          .         .       270         .
ACCTGGCCTCAGACTTCGGGGTGAGGGTGTTTCAGCAGGTGGCG
isLeuAlaSerAspPheGlyValArgValPheGlnGlnValAla
                         +20
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B

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          .         .         .         .         .         .
GTCATAGTCTCAGCCCGCATGGCCCCCGAGGAGATCATCATGGACAGACCCTTCCTCTTT
ValIleValSerAlaArgMetAlaProGluGluIleIleMetAspArgProPheLeuPhe
                  ↓
          .         .         .         .         .         .
GTGGTCCGGCACAACCCCACAGGAACAGTCCTTTTCATGGGCCAAGTGATGGAACCCTGA
ValValArgHisAsnProThrGlyThrValLeuPheMetGlyGlnValMetGluPro

          .         .         .         .         .         .
ACCCTGGGGAAAGACGCCTTCATCTGGGACAAAACTGGAGATGCATCGGGAAAGAAGAAA
          .         .         .         .
CTCCGAAGAAAAGAATTTTATGTTATGACTCTTTCTGAAGGAAA
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Fig.3. Nucleotide sequence of selected portions of PAI-1 DNA, clones A1 and A5, and the deduced amino acid sequence of the parts corresponding to the coding region. (A) The 5′-untranslated region and the amino terminus of the protein. (B) The carboxy-terminus and part of the 3′-untranslated region. The arrow indicates the site of cleavage of PAI-1 by u-PA and t-PA, as identified by the combination of the amino acid sequence deduced from the nucleotide sequence of the cDNA, and the actual amino acid sequence determined on converted PAI-1.

An additional sequenced DNA region covered the carboxy-terminal 39 amino acid residues of the protein, a TGA stop codon and 104 nucleotides of the 3′-untranslated region (fig.3B). The carboxy-terminal amino acid sequence derived from the DNA sequence overlaps and confirms the amino acid sequence identified as the amino-terminal end of the peptide released by cleavage with u-PA or t-PA (cf. figs 1 and 3B). The combination of the results from protein and DNA sequencing identifies the cleavage site at an Arg–Met bond 33 residues before the carboxy-terminus and thus localizes the active center of PAI-1 to that position [23,24]. The molecular mass of the released fragment is 3804 kDa.

The presence of an arginine residue on the carboxyl side of the bond cleaved in PAI-1 matches the substrate specificity of the activators, since both t-PA and u-PA cleave an Arg–Val bond in plasminogen. This is in good agreement with findings with the other inhibitors of serine proteases (serpins), since their inhibitory specificity is in each case primarily defined by a single amino acid, P_1, at the reactive center of the molecule (table 1). The inhibitor is thought to trap the proteases by presenting its reactive center as an ideal substrate, the P_1 residue acting as bait for the appropriate serine protease. There is formation of a tightly bound enzyme-inhibitor complex, in which the acyl bond of P_1 is being hydrolyzed. Cleaved inhibitor may subsequently be released from the complex (reviews [23,24]). The present data indicate that PAI-1 is an Argserpin, according to the terminology of Carrell and Travis [24].

There are no previous reports on direct amino acid sequence determination on human PAI-1.

Table 1

Comparison of the site of proteolytic cleavage of PAI-1 by u-PA and t-PA with the reactive centers of a variety of serpins and with the cleavage site of plasminogen

Inhibitor or substrate	Protease	Residues at reactive center or cleavage site					
		P_2	P_1	P_1'	P_2'	P_3'	P_4'
α_1-Antitrypsin [41]	elastase	Pro	Met	Ser	Ile	Pro	Pro
α_1-Antichymotrypsin [42]	chymase	Leu	Leu	Ser	Ala	Leu	Val
Mouse contrapsin [43]	trypsin	Arg	Lys	Ala	Ile	Leu	Pro
C1-inhibitor [44,45]	complement component C1	Ala	Arg	Thr	Leu	Leu	Val
Antithrombin [46–48]	thrombin	Gly	Arg	Ser	Leu	Asn	Pro
PAI-1	u-PA, t-PA	Ala	Arg	Met	Ala	Pro	Glu
Plasminogen [49,50]	u-PA, t-PA	Gly	Arg	Val	Val	Gly	Gly

The designation of the residues is as proposed by Schechter and Berger [51]. The P_1–P_1' bond is cleaved by the indicated proteases

Our data are, however, in good agreement with recently reported data on PAI-1 cDNA clones isolated from other sources [38–40]. The amino acid sequences presented here completely coincide with the data in those reports, with the exception of the threonine residue at position −7 in the signal peptide which was found to be an alanine residue in the other studies [39,40], and the lysine residue at position 5 of the peptide used as a basis for the oligonucleotide probe, which was found to be a threonine residue in the other studies [38–40]. It is noteworthy that the site of cleavage with u-PA and t-PA demonstrated in this paper is identical to that predicted by Ny et al. [38] on the basis of analogy with other serpins.

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