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# Selection of peptides that bind to plasminogen activator inhibitor 1 (PAI-1) using random peptide phage-display libraries

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**Abstract Large random hexa- and decapenta-peptide libraries were constructed and displayed on the surface of the filamentous phagemid pComb8. Panning of the hexa-peptide library on immobilized plasminogen activator inhibitor 1 (PAI-1) specifically selected a minor fraction of concatemers, indicating that binding to PAI-1 requires an extended amino acid sequence. Accordingly, the decapenta-peptide library exclusively yielded PAI-1 binding peptides of 15 amino acid residues. None of these phage-bound peptides prevented the interaction between PAI-1 and its target serine protease urokinase (u-PA). To isolate peptides that block the interaction between PAI-1 and u-PA, phages bound to immobilized PAI-1 were eluted by incubation with u-PA. Remarkably, this procedure resulted in elution of a unique phage type that harbors a concatemer of decapentamers, consisting of 49 amino acid residues with no obvious similarity to the primary sequence of PAI-1 or u-PA.**



*Key words:* Phage display; Plasminogen activator inhibitor 1; Peptide; Peptide library

## 1. Introduction

Plasminogen activator inhibitor 1 (PAI-1) is a major regulatory protein of fibrinolysis and possibly of other processes, including the degradation of extracellular matrices during cancer invasion [1,2]. Tissue-type (t-PA) and urokinase-type plasminogen activator (u-PA) are the initiators of these processes and the activity of these activators is rapidly neutralized upon complex formation with PAI-1. PAI-1 belongs to the serine protease inhibitor ('serpin') superfamily of homologous proteins (reviewed in [3]). It acts as a pseudo-substrate by mimicking the genuine substrate plasminogen. To that end, the inhibitor presents its reactive center P1 residue (arginine 346) to form a stable acyl-enzyme intermediate, ultimately leading to cleavage of the peptide bond [4,5].

Clinical studies and observations with transgenic mice have provided conclusive evidence for the in vivo regulatory function of PAI-1 in fibrinolysis and thrombolysis. Homozygous individuals with complete PAI-1 deficiency suffer from an increased bleeding tendency [6,7], whereas so-called PAI-1 knock-out mice display a greater resistance to venous thrombosis due to increased fibrinolysis [8]. On the other hand, mice overexpressing PAI-1 develop venous occlusions, conceivably as a result of decreased fibrinolysis [9]. In humans, high levels of PAI-1 have been associated with an increased risk for myocardial reinfarction and thrombosis [10]. These observations might be explained by the occurrence of platelet-rich thrombi, containing high levels of fibrin-bound PAI-1 [11,12]. Accordingly, the application of either inhibitory anti-PAI-1 antibodies or low-molecular-weight compounds either in a rabbit jugular vein model or in a canine coronary artery model for thrombosis and stenosis enhanced t-PA-mediated thrombosis and reduced the frequency of reocclusions [13–15].

In contrast to the well-defined role of PAI-1 in fibrinolysis, the mechanism of action of the inhibitor in metastasis has not been documented to the same extent. However, it has been reported that high levels of PAI-1 are found to be associated with a variety of cancers. PAI-1 is specifically localized to the tumor stroma and, in many cases, to areas surrounding the tumor vessels (reviewed in [16]). These observations may signify that high levels of PAI-1 would prevent the degradation of tumor tissue by u-PA-induced proteolysis of extracellular matrices [17]. Consequently, it is hypothesized that inhibition of PAI-1 activity under these circumstances may induce autodegradation of the tumor tissue [18]. These considerations on the function of PAI-1 in fibrinolysis and thrombolysis and its potential role in cancer led us to develop low-molecular-weight compounds to ultimately inhibit the activity of PAI-1 in vivo under a variety of pathological conditions. In this report, we constructed and analyzed random hexa- and decapenta-peptide libraries that are displayed on the surface of filamentous phages (reviewed in [19]) to search for PAI-1 binding peptides. Such peptides may be instrumental for future studies to delineate in detail the interaction between PAI-1 and other components, notably the serine proteases u-PA, t-PA and thrombin and the accessory factors vitronectin, heparin and fibrin [1]. Finally, these peptides may serve as lead compounds for a rational design of low-molecular-weight products to combat thrombotic complications and possibly tumorigenesis.

## 2. Materials and methods

### 2.1. Reagents

Fully active human recombinant PAI-1 was a generous gift from Dr. T.M. Reilly (DuPont Merck Pharmaceutical Comp., Wilmington, DE, USA). Human u-PA was purchased from Serono (Aubonne, France). The phage-display vectors pComb8 and pJuFo were gifts from Dr. C.F. Barbas (The Scripps Research Institute, La Jolla,

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*Abbreviations:* BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; LB, Luria broth; PAI-1, plasminogen activator inhibitor 1; SMB, somatomedin B; TBS, Tris-buffered saline; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator


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CA, USA) and Dr. M. Suter (University of Zurich, Biochemical Institute, Zurich, Switzerland), respectively.

*2.2. Construction of a phage displaying the somatomedin B domain of vitronectin*

To establish conditions for a specific interaction between immobilized PAI-1 and phage-displayed peptides, a phagemid was constructed that displays the high affinity PAI-1 binding site of vitronectin, i.e. the somatomedin B (SMB) domain [20]. The cDNA encoding this domain was inserted by overlap PCR into *Xba*I- and *Kpn*I-digested pJuFo DNA [21], a vector derived from the pComb series of phagemids [22]. To that end, the following primers were used, based on the vitronectin cDNA sequence (EMBL accession number X03168): forward 5′-AGATCTAGAGACCAAGAGTCATGCAAG (*Xba*I site underlined) and reverse 5′-ACCGGTACCTTACATAGTGAACACATCCCC (*Kpn*I site underlined). The resulting phagemid was denoted pJuFo/SMB.

*2.3. Construction of phage-displayed random peptide libraries*

Peptide libraries were constructed by using the multivalent phage-display vector pComb8 that had been originally developed to express the Fab part of immunoglobulins on the phage surface [22]. Hexa- and decapenta-peptides ($LE(X_6)TS$ or $LE(X_{15})TS$) are expressed as fusion proteins, preceded by a penta-peptide (AQVKL) representing a consensus of the 'fairly constant region' (FR1) of murine immunoglobulin heavy chains, and followed by the major phage coat protein VIII (gp8). The dipeptides LE and TS are encoded by sequences corresponding to restriction sites for *Xho*I and *Spe*I that are used to insert random oligonucleotides (18-mer or 45-mer, respectively) into pComb8. Double-stranded DNA inserts, encoding randomized hexa- and decapenta-peptides were prepared as follows. Oligonucleotides 5′-GTGCAGCTCGAG$(NNN)_6$ACTAGTGGTGGCGGTGGC-3′ and 5′-GTGCAGCTCGAG$(NNS)_{15}$ACTAGTGGTGGCGGTGGC-3′ and primer 5′-GCCACCGCCACCACTAGT-3′ (N = A, T, G or C and S = G or C) were made by automated DNA synthesis. Complementary DNA strands were made after annealing of the respective oligonucleotide with the primer, followed by incubation with Sequenase 2.0 (USB, Cleveland, OH, USA) in the presence of dNTPs. Double-stranded DNA was inserted into pComb8 after digestion with *Xho*I and *Spe*I. High efficiency transformation of *Escherichia coli* strain XL1-Blue (Stratagene, La Jolla, CA, USA) was done by electroporation. After transformation, SOC medium was added and the cultures were shaken at 220 rpm for 1 h at 37°C. Then, aliquots were taken and serial dilutions were plated on LB agar plates, containing ampicillin and tetracycline, to determine the library size ($2.8\times10^8$ and $1.6\times10^7$ for the hexa- and decapenta-peptide library, respectively). Determination of the frequency of inserts of the two libraries was done by DNA sequencing of several independent clones. Ten out of 10 clones of the hexa-peptide library were found to contain the expected insert of 18 random bp and nine out of 10 clones of decapenta-peptide library contains the expected 45 random bp insert, whereas one clone did not contain an insert. Hence, the percentage of inserts in the hexa- and decapenta-peptide libraries approaches 100% and will be about 90%, respectively. Finally, phage libraries were made by infection of transformed *E. coli* XL1-Blue cells with VCSM13 helper phage (Stratagene).

*2.4. Panning of peptide libraries displayed on phages*

Selection of PAI-1 binding phages was performed in a biopanning assay. Briefly, 0.5 μg of PAI-1 in 0.1 sodium bicarbonate (pH 8.6) was coated for 16 h at 4°C onto wells of microtiter plates (Costar, Cambridge, MA, USA). Wells were blocked for 1 h at 37°C with TBS, 3% (w/v) bovine serum albumin (BSA). Then, approximately $10^{10}$ phages in TBS containing 1% BSA (w/v) were incubated for 1 h at room temperature in BSA-coated wells. Subsequently, non-bound phages were transferred to PAI-1-coated wells ('panning'). After incubation for 2 h at room temperature, the wells were extensively washed with TBS, 0.5% (v/v) Tween-20. Phages that bind to immobilized PAI-1 were recovered by adding exponentially growing *E. coli* XL1-Blue cells to the wells to allow infection and amplification of phages. The procedure for panning, washing and amplification was repeated three times. As indicated for a particular experiment, the elution of phages that bind to immobilized PAI-1 was done for 1 h at room temperature by adding 5 μg/well of u-PA. The procedure for panning, washing, elution with u-PA and amplification was repeated three times.

*2.5. Screening of phage binding to PAI-1 by phage sandwich ELISA*

Whole phage populations or small-scale phage preparations, obtained from single colonies of the subsequent rounds of affinity selection, were analyzed for binding to PAI-1 by phage sandwich ELISA. Briefly, $5\times10^9$ phages in TBS, 1% (w/v) BSA were incubated for 2 h at room temperature in PAI-1- or BSA-coated wells. For competition experiments, preincubation of immobilized PAI-1 with synthetic peptides, dissolved in TBS containing 1% (w/v) BSA, or with u-PA (control glu-plasminogen) was performed for 15 or 30 min, respectively. Phages bound to immobilized PAI-1 and to immobilized BSA were detected by incubation with polyclonal anti-M13 biotin conjugate (5 Prime→3 Prime, Inc., SanverTech, Heerhugowaard, The Netherlands), followed by incubation with horseradish peroxidase-coupled streptavidin and staining.

*2.6. Peptide synthesis*

The phage-derived peptide LLLWPLSEKPVVLPE and a control peptide AGEELTSFMQWAVLK were made by solid-phase synthesis, using Fmoc chemistry. The peptides were purified by gel filtration and the products were found to be more than 90% pure, according to HPLC analysis. The identity was verified by MALDI-TOF MS.

## 3. Results and discussion

*3.1. Selection of PAI-1 binding phages from a random hexa-peptide library*

First, conditions were established for specific binding (panning), washing and elution of phage-displayed peptides to immobilized PAI-1. For that purpose, a control phage was constructed that displays the SMB domain of vitronectin on its surface in a biologically active configuration [20]. The SMB domain constitutes the high-affinity interaction site that exclusively binds to active PAI-1, while no binding occurs to latent or inactivated PAI-1. The specificity of the binding of the SMB domain to PAI-1 is illustrated by the following observation. pJuFo/SMB phages were mixed with a 100 000-fold excess of the parental pJuFo phages, devoid of the SMB domain. After three rounds of panning with immobilized PAI-1, washing, elution and amplification, PCR was performed on 10 colonies using the primers outlined before. Nine out of 10 colonies contained the SMB cDNA insert, indicating an enrichment factor of about $10^5$, demonstrating the specificity of the procedure. Subsequently, a phage-displayed random hexa-peptide library, consisting of $2.8\times10^8$ independent clones, was constructed and used for the selection of PAI-1 binding

Table 1
PAI-1 binding peptides selected from random hexa-peptide library

| Amino acid sequence insert | Number of clones |
|---|---|
| GQF**W**HLTSMSGSYFLEPFDLISTSQQRNIPLEIRDADT | 17/22 |
| WLLGQDTSGVL**W**AELESIEGLS | 2/22 |
| **WW**LSHVTSTFEFQTLETLSD**W**S | 2/22 |
| LD**W**GQ**W**TSLILSRHLEQLALIV | 1/22 |

The underlined sequences indicate amino acids encoded by DNA sequences corresponding to restriction sites for *Xho*I (LE) and *Spe*I (TS) that have been used to insert 18 random bp into pComb8. Tryptophan (W) residues are indicated in bold.

peptides. It should be noted that sequence analysis had shown that approximately 100% of the clones harbor the expected insert of 18 bp. After four rounds of panning, washing and elution, using identical conditions as for the binding of pJuFo/SMB phages to PAI-1, individual clones were amplified and serial dilutions of phage stocks were assayed, using the phage sandwich ELISA with either immobilized PAI-1 or immobilized BSA. Twenty-two phages that displayed binding to PAI-1, but not to BSA, were sequenced (Table 1). Remarkably, each of the phages contains a concatemer, encoding three or five copies of in-frame hexa-peptides. Notably, four different phage types were encountered: (i) 17 out of 22 contain the same insert, encoding 38 amino acid residues, (ii) two, two and one out of 22 harbor an insert, encoding 22 amino acid residues. The amino acid sequences of these four different phage types do not reveal an obvious consensus, except for an apparent enrichment of tryptophan residues in the amino-terminal half of these peptides, in accordance with data of others (summarized in [19]). These findings are in contrast to other observations with our random hexa-peptide library. In that case, the library was employed to map the epitope of a monoclonal antibody (EF1-4), directed against elastase-degraded human fibrinogen. These experiments yielded exclusively hexa-peptides carrying phages with a clear consensus sequence (H. Gårdsvoll, R. Bos and H. Pannekoek, unpublished data). As mentioned before, a limited analysis of the library revealed exclusively inserts that encode six contiguous amino acid residues. Nonetheless, the current procedure to isolate PAI-1 binding phages specifically selects a fraction of phages that is present at a low frequency in the library. This conclusion supports the assumption that the structural and/or conformational requirements for a ligand to bind to PAI-1 extend beyond those comprised in a hexa-peptide.

### 3.2. Selection of PAI-1 binding phages from a random decapenta-peptide library

In view of the findings above, we constructed a phage-displayed, random decapenta-peptide library. This library consists of $1.6\times10^7$ independent clones of which approximately 90% contain a single insert of 45 bp, whereas about 10% of the clones do not harbor an insert. Again, selection of phages was performed using immobilized PAI-1. All of the clones recovered from the final round of selection contain inserts encoding a decapenta-peptide (Table 2). The inserts of 17 independent clones were sequenced: four out of 17 appeared to have an identical sequence (#1) as well as two others (#13). An alignment of 10 different sequences (#1–10), using a multiple alignment program (MAP) [23], revealed a moderate consensus sequence, namely LLlWPg–lsd/e–PVvLP, which may indicate that these peptides bind to the same site on PAI-1. Consequently, the remaining peptides (#11–13) may bind to different sites on PAI-1. Furthermore, an evident enrichment is observed for the occurrence of tryptophan (W) residues as noted before (see Table 1 and [19]). To confirm the specificity of the selected peptides and to exclude that binding of the peptides to PAI-1 is only observed in the context of a fusion protein, a soluble peptide corresponding to the most frequently encountered sequence (LLLWPLSEKPVVLPE) was synthesized. Indeed, binding of the corresponding decapenta-peptide-carrying phages to immobilized PAI-1 was inhibited in a dose-dependent manner by this particular synthetic peptide, whereas no inhibition was observed with a control peptide (Fig. 1). Subsequently, we assessed whether the binding of the LLLWPLSEKPVVLPE-containing phage to PAI-1, as well as the other phages mentioned in Table 2, would interfere with the interaction between PAI-1 and u-PA. However, in none of the cases we found that the addition of u-PA prevented the binding of the phages to PAI-1 (results not shown). Although the recovered decapenta-peptide phages may be instrumental in the analysis of the interaction between PAI-1 and other components, in this study we primarily focused on the identification of peptides that prevent the binding of u-PA to PAI-1.

Table 2
PAI-1 binding peptides selected from random decapenta-peptide library

| No. | Amino acid sequence insert | Number of clones |
|---|---|---|
| 1 | LLL**W**PLSEKPVVLPE | 4/17 |
| 2 | LLG**W**MGLVEHD**W**LPL | 1/17 |
| 3 | SL**W**SLVLGPESILGP | 1/17 |
| 4 | LL**W**GRAVDLQESLVV | 1/17 |
| 5 | **W**LGMPMRDYSPVLLP | 1/17 |
| 6 | **W**L**W**PAESLVSVPYMV | 1/17 |
| 7 | **W**LELMSTRADPTLPL | 1/17 |
| 8 | **W**FGVAGG**W**DLGPQLG | 1/17 |
| 9 | **W**FMGREEVVGMSLVP | 1/17 |
| 10 | **W**IMLD**W**PGRLSDIPV | 1/17 |
| 11 | YLMFLDMLDPEQSVL | 1/17 |
| 12 | MLGLVAPRPLLQEVV | 1/17 |
| 13 | CILFLRCGDIQLVTP | 2/17 |

Homology searches were performed using the multiple alignment program (MAP; [19]), comparing the amino acid sequences of clones 1–10. These inserts share a moderate consensus sequence, i.e. LLlWPg–lsd/e–PVvLP (capital letters signify high probability, whereas lowercase letters correspond to lower probability). Tryptophan residues are indicated in bold.

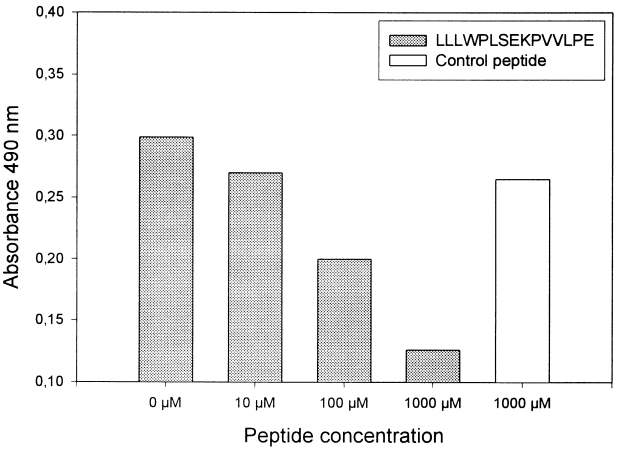


Fig. 1. Competition between phage-bound and soluble peptide LLLWPLSEKPVVLPE for binding to PAI-1. $5\times10^9$ pComb8/LLLWPLSEKPVVLPE phages were preincubated with the indicated concentrations of the soluble LLLWPLSEKPVVLPE decapenta-peptide or a control decapenta-peptide as outlined in Section 2. Subsequent binding of phages to immobilized PAI-1 was monitored by the administration of a polyclonal anti-M13 biotin conjugate, followed by incubation with horseradish peroxidase-coupled strepavidin. The experiments were performed in duplicate.

### 3.3. Selection of a peptide that prevents PAI-1/u-PA interaction

For the aim outlined above, we developed a specific competition elution procedure to isolate peptides that share a

Table 3
PAI-1 binding peptide selected from random decapenta-peptide library, eluted with u-PA

| 49 amino acid sequence of the insert | Number of clones |
|---|---|
| PVSQFVFLCGHQPCFTSEHAHDVPDPAPPHHPLELITGRQATPISVGMS | 12/12 |

The underlined sequences indicate amino acids encoded by DNA sequences corresponding to restriction sites for *Xho*I (LE) and *Spe*I (TS) that have been used to insert 45 random bp into pComb8.

PAI-1 binding site with u-PA. To that end, we employed the random decapenta-peptide library that was bound to immobilized PAI-1, extensively washed and then incubated with 5 μg/well of u-PA. This procedure of panning, washing, elution with u-PA and, finally, amplification of the eluted phages was repeated three times. Subsequently, the inserts of 12 independent clones were sequenced. Surprisingly, only a single unique sequence was encountered that consists of a concatemer of inserts, encoding a peptide of 49 amino acid residues (Table 3). In contrast to the previous experiments (Tables 1 and 2), the 49 amino acid long peptide is devoid of tryptophan residues, however, we observed an obvious enrichment for proline (P) and histidine (H) residues. Furthermore, alignment of the 49 amino acid sequence with that of u-PA did not reveal a common sequence or motif. Again, it should be noted that a limited sequence analysis of the original decapenta-peptide library revealed only single inserts of 45 bp, indicating that concatemers constitute only a minor fraction of the library. However, as a consequence of the chosen selection procedure, a rare peptide-harboring phage is specifically recovered. Attempts to chemically synthesize a complete, soluble peptide of 49 amino acids, and truncated versions of it, have not been successful as yet and, in view of the extreme length, might present a considerable hurdle. Finally, we verified the specificity of the 49 amino acid peptide in the interaction between PAI-1 and u-PA. For that purpose, we performed a competition experiment with u-PA or with glu-plasminogen, a protein with a similar structure and amino acid composition. Our data demonstrate that u-PA inhibits the binding of the 49 amino acids carrying phage to PAI-1 in a dose-dependent manner, whereas glu-plasminogen did not (Fig. 2). A rational explanation for these findings is that the peptide and u-PA

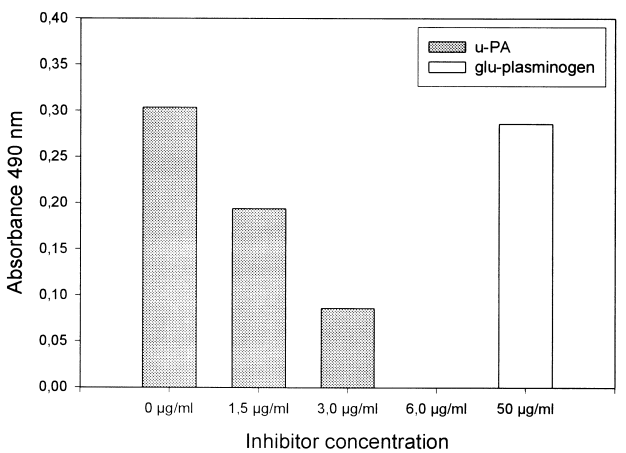


Fig. 2. Competition between phages, carrying the peptide of 49 amino acids, and u-PA for binding to PAI-1. $5\times10^9$ pComb8/'49 aa' phages were preincubated with the indicated concentrations of u-PA or with 50 μg/ml glu-plasminogen as outlined in Section 2. The quantification of PAI-1-bound phages was performed as indicated in the legend of Fig. 1. The experiments were performed in duplicate.

share (part of) a binding site on PAI-1, although at this point it cannot be excluded that complex formation between PAI-1 and u-PA induces a conformational change that subsequently prevents binding of the peptide. Future studies with a soluble preparation of the peptide should reveal whether it acts as a competitive or a non-competitive inhibitor.

Finally, it should be noted that none of the PAI-1 binding peptides and concatemers, selected from the hexamer and the decapentamer libraries, has an amino acid sequence with obvious homology to the 'linear' sequence of either PAI-1 or u-PA. Similar findings have been reported for peptides that interfere with other protein-protein interactions. For example, phage-displayed peptides have been isolated that block the interaction between the urokinase receptor and u-PA [24]. Similarly, these peptides reveal no homology with the amino acid sequence of these components. However, it cannot be excluded that the three-dimensional structure of these proteins encompasses quasi-linear arrays of amino acids that may be homologous to the selected peptides. In conclusion, by using phage display of random peptide libraries, we identified novel peptides that bind to PAI-1. These peptides may constitute useful tools for further studies on the interaction between PAI-1 and its various protein targets. In particular, we have designed a competitive elution protocol to enrich for peptides that interfere with the interaction between PAI-1 and u-PA. A single concatemer was identified, consisting of 49 amino acid residues, that may serve as starting point for shorter versions that will be assessed in experimental models that rely on u-PA activity.

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