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Synthetic peptides derived from the sequence around the plasmin cleavage site in vitronectin

Use in mapping the PAI-1 binding site

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A series of 8 peptides derived from the amino acid sequence accommodating the plasmin cleavage site in vitronectin were synthesized and used to map its binding site for the type I plasminogen activator inhibitor (PAI-1). This mapping assigned the inhibitor binding site to the K^{348}–R^{370} region with high affinity recognition elements within the K^{348}–R^{357} sequence. These results account for our previous finding that cleavage of the R^{361}–S^{362} bond by plasmin significantly reduces the affinity between PAI-1 and vitronectin, since it splits the PAI-1 binding site in two. Furthermore, in the case of the two-chain form of vitronectin, this cleavage detaches the S^{362}–R^{379} peptide which provides some of the affinity elements for the binding of PAI-1.

PAI-1; Peptides; Plasmin; Vitronectin

1. INTRODUCTION

The activation of plasminogen by its natural activators (e.g. tPA) provides a multipurpose biological tool for the proteolytic dissolution of barriers by plasmin. This activation has been reported to play an important role not only in fibrinolysis but also in inflammation, ovulation, tissue remodeling and development, angiogenesis, nerve regeneration, malignancy and tumor cell invasion [1–4]. In view of this diversity of functions it is obvious that plasmin activity must be under a strict regulatory control to secure a localized, site-restricted action and to limit its duration.

In order to stop plasmin action it is not sufficient only to inactivate the excess of already-formed plasmin (by α_2-antiplasmin [5]). It is also necessary to arrest further production of plasmin from plasminogen. This is achieved through the inhibition of plasminogen activator(s) by PAI-1, which was shown to be bound to vitronectin [6,7] and thus to become stabilized in its active form both in circulating blood and in the ECM [8–11].

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Abbreviations. BP, basic peptide (for the amino acid sequence of individual peptides see Scheme 1); ECM, extracellular matrix; ELISA, enzyme-linked immunosorbent assay; ONPG, *ortho*-nitrophenyl-β-D-galactopyranoside; PAI-1, plasminogen activator inhibitor - 1; tPA, tissue plasminogen activator; Vn, vitronectin.

Using the specific phosphorylation of vitronectin at S^{378} by platelet-released PKA [12–16], we recently showed [17] that plasmin specifically cleaves vitronectin at the R^{361}–S^{362} bond, 18 amino acids upstream from the site of the endogenous cleavage which gives rise to the two-chain form (V_{65+10}) of this protein. We also reported that as a result of the plasmin cleavage, the affinity between vitronectin and PAI-1 is significantly reduced [17] and that this cleavage is stimulated by glycosaminoglycans which anchor vitronectin to the ECM, thus favouring the cleavage of the vitronectin molecules found in this matrix. On the basis of these findings, we proposed a mechanism through which plasmin can arrest its own production by feedback signaling (Scheme I in [17]). At the initial stage of fibrinolysis, the plasminogen activator converts plasminogen to plasmin. This is made possible since PAI-1 is then anchored (trapped) by the vitronectin molecules (which were previously shown to be immobilized in the ECM [18], presumably through glycosaminoglycans). This anchoring of PAI-1 locally depletes the inhibitor by preventing it from reaching and inhibiting the plasminogen activator. When plasmin levels become too high, the excess plasmin can clip preferentially the vitronectin molecules immobilized in the subendothelium. Consequently, the equilibrium between anchored PAI-1 and the detached (mobile) PAI-1 is displaced, thus unleashing PAI-1 to reach and inhibit the plasminogen activator and arrest plasmin production. This unleashing actually represents a translocation, a transfer of the anchored PAI-1 from the ECM-bound (plasmin-clipped) vitronectin to solu-

Table I

Amino acid composition of key peptides in the BP series*

Peptides	A	R	N	Q	G	H	K	F	P	S	Y
BP4	0.85	5.32	1.98	2.14	2.0	0.98	–	–	1.0	2.4	–
	(1)	(6)	(2)	(2)	(2)	(1)	–	–	(1)	(4)	–
BP4-1	–	2.95	–	1.06	2.0	1.12	–	–	–	1.32	–
	–	(3)	–	(1)	(2)	(1)	–	–	–	(2)	–
BP5	–	4.1	–	1.10	3.0	1.02	0.86	–	–	1.56	1.05
	–	(4)	–	(1)	(3)	(1)	(1)	–	–	(2)	(1)
	–	5.0	1.02	2.18	1.0	0.97	3.38	0.90	–	0.81	1.10
	–	(5)	(1)	(2)	(1)	(1)	(3)	(1)	–	(1)	(1)

*Values given are the number of nanomoles obtained in the analyis, while the values in brackets represent the integer numbers expected for the correct structure.

ble vitronectin molecules which have not been clipped by plasmin and thus possess a higher affinity for PAI-1. Since the plasmin cleavage of vitronectin at the R^{361}–S^{362} bond attenuates its PAI-1 binding, we assumed that the key PAI-1 binding elements could be located either at a distant site, the conformation of which is significantly affected by the integrity of the R^{361}–S^{362}bond, or that these binding elements are located in the immediate vicinity of this bond. This paper shows that the latter is the case.

2. MATERIALS AND METHODS

2.1. *Materials purchased*

Freshly frozen human plasma was obtained from the Tel-Hashomer Medical Center, Ramat-Gan, Israel. Rabbit anti-human vitronectin polyclonal antibodies were obtained from Calbiochem. β-Galactosidase-linked Protein A and *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) were purchased from Amersham, UK. PAI-1 was obtained from American Diagnostica, New-York. All other chemicals were of the best available grade from commercial sources.

2.2. *Vitronectin*

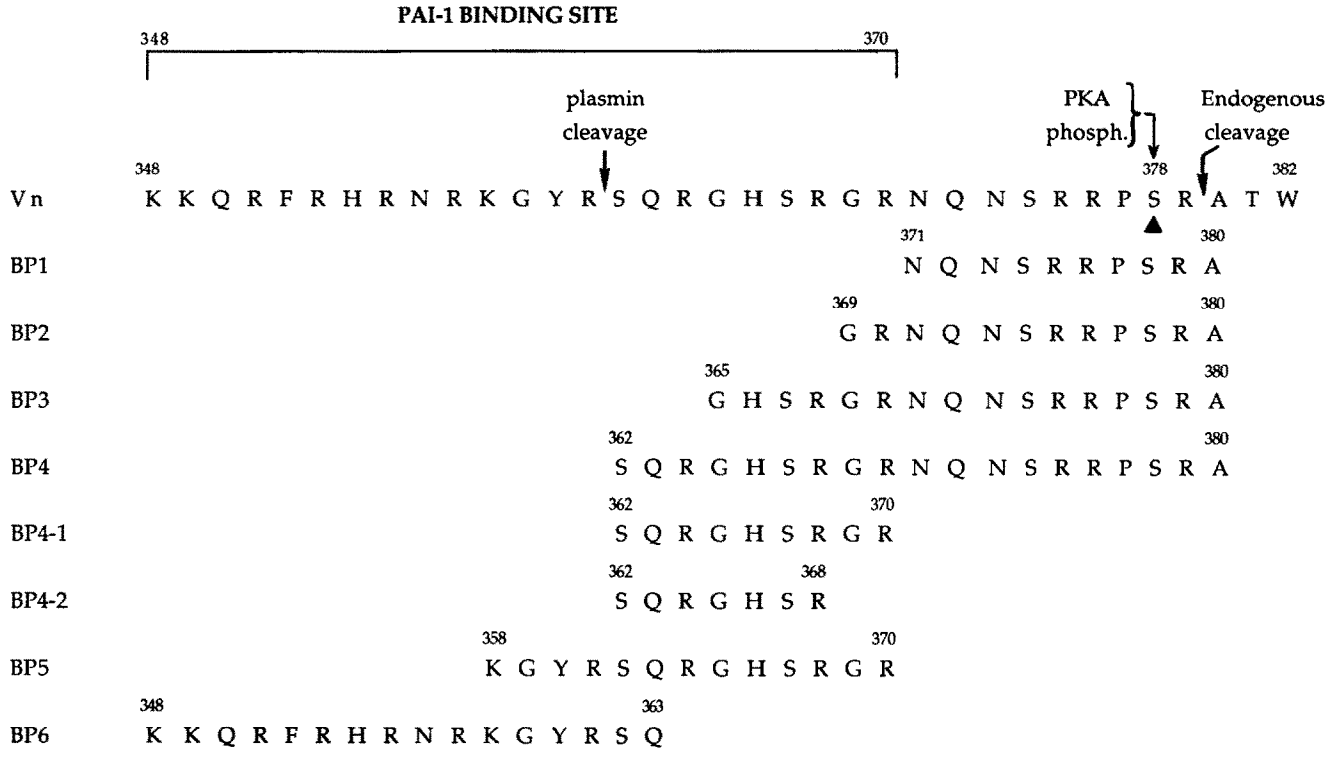
Vitronectin was prepared from freshly frozen human plasma using

Table II

Sequence analysis of BP4, BP5 and BP6

BP4			BP5			BP6		
Cycle number	Sequence determined		Cycle number	Sequence determined		Cycle number	Sequence determined	
	Residue	(yield*)		Residue	(yield*)		Residue	(yield*)
1	S	(58)	1	K	(64)	1	K	(62)
2	Q	(97)	2	G	(47)	2	K	(67)
3	R	**	3	Y	(61)	3	Q	(68)
4	G	(48)	4	R	**	4	R	(13)
5	H	(45)	5	S	(20)	5	F	(69)
6	S	(37)	6	Q	(44)	6	R	(19)
7	R	**	7	R	**	7	H	(14)
8	G	(34)	8	G	(26)	8	R	(22)
9	R	**	9	H	(16)	9	N	(52)
10	N	(28)	10	S	(8)	10	R	(24)
11	Q	(20)	11	R	**	11	K	(40)
12	N	(15)	12	G	(16)	12	G	(34)
13	S	(15)	13	R	**	13	Y	(44)
14	R	**				14	R	(24)
15	R	**				15	S	(13)
16	P	(25)				16	Q	(17)
17	S	(9)						
18	R	**						
19	A	(12)						

*Number of pmols of amino acid phenylthiohydantoin in derivative recovered.
**Qualitatively identified (yield too low for quantitative determination).



Scheme I

a purification procedure described elsewhere [15]. Our preparations usually contained the two molecular forms of this protein: V_{75}, a one-chain (75 kDa) form; and V_{65+10}, a proteolytically clipped, two-chain protein (65 kDa and 10 kDa) held together by a disulfide bridge [19,20].

2.3. *Peptide synthesis*

The synthesis of peptides was carried out manually, in a mechanical shaker, using the solid phase methodology [21], on a chloromethylated polystyrene – 1% divinylbenzene resin. α-Amino groups were protected by the *t*-butyloxycarbonyl group. Side chain groups were masked by the following protecting groups: S, by benzyl; Y, by 2.6-dichlorobenzyl; K, by 2-chlorobenzyloxycarbonyl; H, by benzyloxycarbonyl; and R, by *p*-toluene sulfonyl. The synthesis was initiated by coupling the C-terminal amino acid to the resin. Peptide elongation (coupling) was performed with a 3-fold molar excess of the protected amino acid and an equimolar mixture of *N,N'*-dicyclohexylcarbodiimide and 1-hydroxybenzotriazole. Deprotection and detachment of the peptides from the polymeric carrier was carried out with anhydrous HF. The crude peptides were purified to homogeneity by preparative HPLC (Lichrosorb RP-8, Merck) using a linear gradient between 0.1% trifluoroacetic acid in water and 0.1% of this acid in 75% acetonitrile/water. The structure of each of the peptides was confirmed by amino acid composition and sequence. The analyses of key peptides are given in Tables I and II.

2.4. *Procedures carried out as described in the literature*

Protein concentration was determined by the method of Bradford [22]. The binding of vitronectin to immobilized PAI-1 was conducted as described by Salonen et al. [9]. The activation of PAI-1 by sodium dodecylsulfate was carried out as described by Katagiri et al. [23]. Amino acid sequencing was performed with an Applied Biosystem 475A sequencer.

3. RESULTS AND DISCUSSION

In order to map the PAI-1 binding site in vitronectin, we synthesized a series of peptides, the structure of which was derived from the amino acid sequence surrounding its plasmin cleavage site (R^{361}–S^{362}) [17]. Each of these synthetic peptides (Scheme I) was tested by a competition ELISA assay for its ability to inhibit the binding of vitronectin to immoblized activated PAI-1. The first screening (Fig. 1) was carried out with an identical molar concentration (25 μM) of the peptides. In view of a possible variability in such tests (mainly due to some variability in the activated PAI-1 coating of the plates), a control of vitronectin alone was run alongside in each plate for reference.

As seen in Fig. 1, all of the peptides BP1, BP2, BP3 and BP4 (panels A and B) which cover the vitronectin sequence between the plasmin cleavage site (R^{361}–S^{362}) and the endogenous cleavage site (R^{379}–A^{380}) inhibit to some extent the binding of vitronectin to PAI-1. This inhibition is mainly due to the N-terminus moiety of BP4, since BP4–1 (S^{362}–R^{370}) was a rather effective inhibitor (Fig. 1, panel C) while BP1 (N^{371}–A^{380}) exhibited a very minor inhibition, if any (Fig. 1, panel A). It should be noted that BP4–2 (S^{362}–R^{368}) which is only two amino acids shorter than BP4–1, was a less effective inhibitor (Fig. 1, panel C) suggesting that G^{369} and/or R^{370} may contain an important biorecognition element. Furthermore, the inhibition capacity of the various pep-

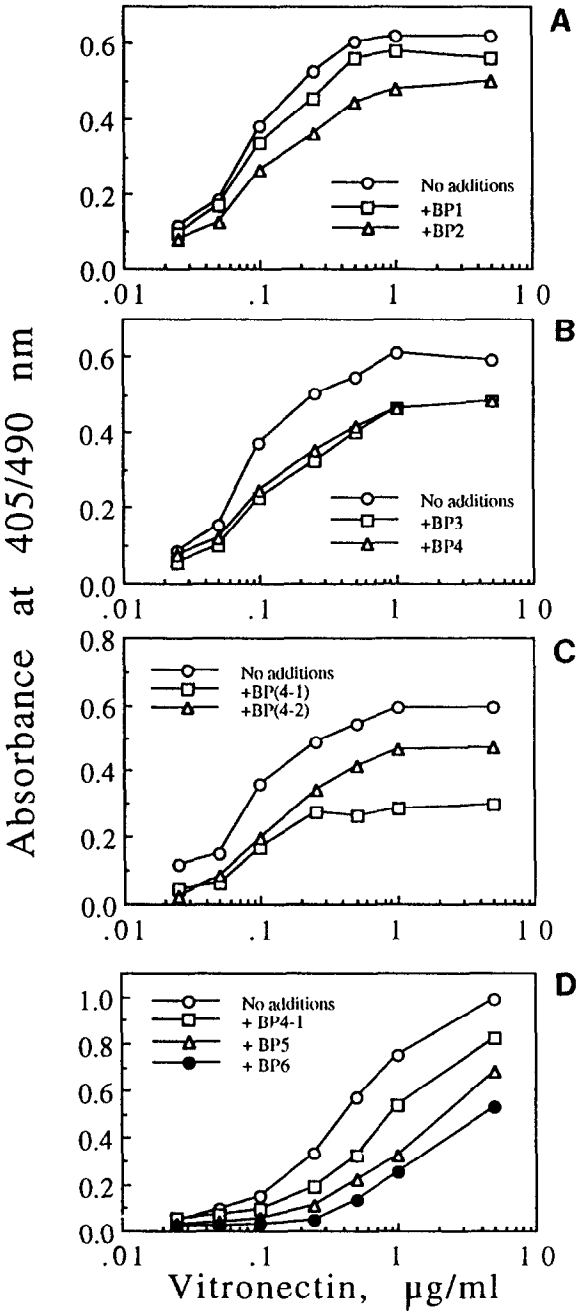


Fig. 1. Effect of synthetic peptides from the BP series on the binding of vitronectin onto immobilized PAI-1. The various synthetic peptides (all 50 μM) were dissolved in 50 μl of a buffer composed of PBS, Tween 20 (0.01%), polyethylene-glycol (average M.W. 8 kDa) (4% W/V). Their concentration was 50 μM in all cases. Each of the samples was added to a microtiter well coated with SDS-activated PAI-1 prepared as described in the literature [23]. After an incubation of 30 min at 22°C, aliquots (50 μl) of a vitronectin solution (at various concentrations) in the same buffer were added to each well. This was followed by an incubation for 2 h at 37°C. The amount of bound vitronectin was determined with specific rabbit-anti-human Vn antibodies (1:1000 dilution), followed by a β-galactosidase linked protein A and using ONPG as a substrate. The enzymatic reaction was allowed to proceed for 60 min (22°C) and was stopped by the addition of 50 μl of 1 M Na_2CO_3, and the absorbance at 405+490 nm was measured. Each point in the graph represents the mean of triplicate determinations. The final peptide concentrations in the incubation mixtures are 25 μM.

tides could not be attributed merely to their positive charge, since BP4–1 (net charge +4) was found to be more effective than BP3 (net charge +6) or BP4 (net charge +7) (Fig. 1, panels B and C).

The peptide BP5 (K^{358}–R^{370}), which contains all of BP4–1 and a further extension beyond the plasmin cleavage site, was found to be more effective than BP4–1 as an inhibitor (Fig. 1, Panel D). Furthermore, the peptide BP6 (K^{348}–Q^{363}) seemed to be the most effective in this series (Fig. 1, Panel D).

In view of these results, we assessed the relative inhibitory power of the peptides by measuring the concentration dependence of the inhibition. As seen in Fig. 2, all three of the peptides BP4, BP5 and BP6 achieved a

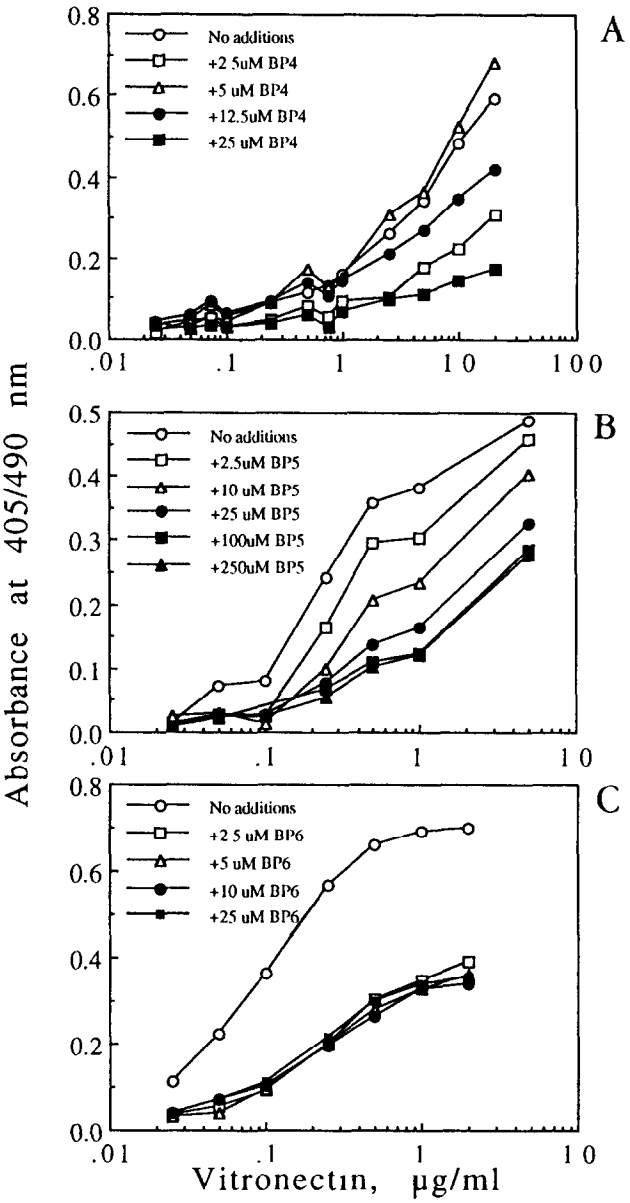


Fig. 2. Effect of the concentration of BP4 (panel A), BP5 (panel B) and BP6 (panel C) on the binding of vitronectin to immobilized PAI-1. The experiments were carried out as described in the legend to Fig. 1 with the indicated final concentrations of the peptides and of vitronectin.

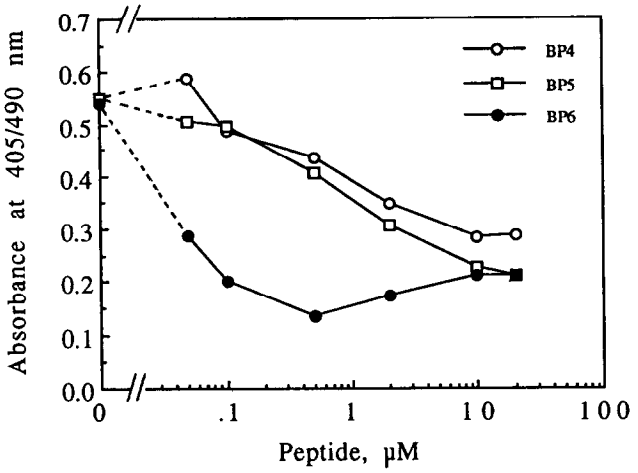


Fig. 3. Comparing the efficacy of BP4, BP5 and BP6 in impeding the association of vitronectin to PAI-1. The titration was carried out as described in the legend to Fig. 1, except for the fact that the final concentration of vitronectin was constant (0.5 μg/ml), while the concentration of the peptides was varied as indicated.

maximal inhibition with increasing concentration. However, while BP4 did so at a concentration of 25 μM (Fig. 2, panel A), and BP5 at a concentration of 100 μM (Fig. 2, panel B), the peptide BP6 was maximally effective already at a 2.5 μM concentration (Fig. 2, panel C). This is further illustrated in Fig. 3, where the relative inhibitory efficacy of these peptides is compared at one concentration of vitronectin (0.5 μg/ml) and with increasing peptide concentrations. It is apparent from this figure that all three peptides achieve the same level of inhibition (~65%). However, BP6 achieves this level of inhibition at a concentration which is 200- to 500-fold lower than BP4 and BP5.

The results presented above indicate that while some high affinity elements of the PAI-1 binding site reside within the K^{348}–R^{357} region, the complete PAI-1 binding site encompasses the K^{348}–R^{370} sequence. This positioning of the PAI-1 binding site can account for our previous finding [17]** that upon cleavage of the R^{361}–S^{362} bond by plasmin, the affinity between vitronectin and PAI-1 is attenuated to unleash the inhibitor, since the PAI-1 binding site is split in two by this cleavage. Furthermore, in the case of the two-chain (endogenously clipped) form of vitronectin, the plasmin cleavage detaches the S^{362}–R^{379} peptide, between the plasmin cleavage site and the endogenous cleavage site, which provides some of the affinity elements for the binding of PAI-1.

**While this manuscript was being prepared for publication, this finding was confirmed by Kost et al. [24] who also assigned the PAI-1 binding site within the sequence 348-370 in vitronectin, without reporting the relative contribution of the various biorecognition elements to the binding.

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