The cluster of basic amino acids in vitronectin contributes to its binding of plasminogen activator inhibitor-1: evidence from thrombin-, elastase- and plasmin-cleaved vitronectins and anti-peptide antibodies

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Derivatives of vitronectin obtained by specific cleavage at its cluster of basic amino acids with thrombin, elastase and plasmin are shown to have a decreased ability to bind plasminogen activator inhibitor-1 (PAI-1). The identification and localization of the segment involved in the binding of PAI-1 (Lys^{348}–Arg^{379}) were carried out by purification of these cleaved vitronectins and their subsequent structural characterization (sequence analysis, phosphorylation of Ser^{378} with cAMP-dependent protein kinase and immunostaining with peptide-specific antibodies), then measurement of the vitronectin–PAI-1 interaction by (a) a two-phase system (ELISA); (b) co-precipitation of the vitronectin–PAI-1 complex out of solution, and (c) analysis of the stereo-specific interaction between the active conformation of PAI-1 and a peptide derived from the above-mentioned cluster; this interaction occurs when the peptide is composed of all-L-amino acids but not when it is composed of all-D-amino acids. Our results explain why workers who have used immobilized vitronectin to study this interaction could not have observed the involvement of the cluster of basic amino acids in PAI-1 binding, since the immobilization of vitronectin is shown to render this cluster inaccessible for interaction. We propose that vitronectin binds active PAI-1 by interaction via amino acid residues that originate from distal locations in the N- and C-termini of vitronectin.

INTRODUCTION

Vitronectin was originally discovered by Holmes [1] as a protein that induces the immediate growth of unadapted cells *in vitro*. It is now well known as an adhesive protein with multiple functions associated with haemostasis and pericellular proteolysis (for reviews, see [2–5]), and it is found in the extracellular matrix [6] and in circulating blood [3,4]. With the discoveries that vitronectin is a carrier protein for plasminogen activator inhibitor-1 (PAI-1) [7–13] and that active PAI-1 is readily converted into a latent form, it became evident that vitronectin may play a role in the control of plasminogen activation, and thus participate in a variety of physiological regulatory processes. This is due to the fact that vitronectin was found not only to stabilize the active conformation of PAI-1 [7,10,14], but also to endow it with thrombin-inhibitory activity [15,16]. It should be noted that latent (i.e. non-inhibitory) PAI-1 can be re-activated *in vitro* by denaturants [17], even though it seems unlikely that the resulting activated PAI-1 is identical in structure to its native functional form.

The three-dimensional structure of latent PAI-1 has been elucidated using X-ray crystallography [18]. An important step towards the elucidation of the structural rearrangements in PAI-1 associated with the transition from the active to the latent state was made in a recent mutational study [19]. Nevertheless, the exact molecular mechanism through which the active structure of PAI-1 is stabilized by vitronectin is still not clear. The interaction between vitronectin and PAI-1 has attracted great interest, since the control of plasminogen activation plays a key role not only in fibrinolysis but also in other physiological and pathological processes that involve the degradation of extracellular components [20].

In blood, vitronectin occurs in two molecular forms: a single-chain 75 kDa polypeptide (VN_{75}), and a clipped form composed of two chains of 65 kDa and 10 kDa (VN_{65+10}) held together by a disulphide bridge [21]. The amino acid sequence of human vitronectin has been deduced from its cDNA sequence [22,23]. The cluster of basic amino acids located within the stretch Lys^{348}–Arg^{379} was proposed to accommodate the heparin-binding site of vitronectin [24]. The heparin-binding domain, although cryptic in the monomeric vitronectin circulating in plasma, becomes available upon exposure of vitronectin to denaturing conditions [25,26].

Our interest in vitronectin originated from the finding that, upon physiological stimulation of platelets by thrombin, these cells release cAMP-dependent protein kinase (PKA) [27], which singles out and phosphorylates vitronectin in human blood [28]. This phosphorylation was found to occur at one residue (Ser^{378}) [28–31] located at the C-terminal edge of the cluster of basic amino acids mentioned above, and very close to the endogenous proteolytic cleavage site (Arg^{379}–Ala^{380}) in vitronectin. Subsequent studies in our laboratory showed that the conditions prevailing at the locus of a haemostatic event permit phosphorylation of vitronectin by PKA [32].

We have shown previously that the cleavage of vitronectin by plasmin at the Arg^{361}–Ser^{362} bond results in a marked decrease in the binding of vitronectin to PAI-1 [33], suggesting that the integrity of the cluster of basic amino acids in vitronectin plays an important role in PAI-1 binding. Using a series of synthetic peptides with sequences identical to segments of the vitronectin

Abbreviations used: CKII, casein kinase II; PAI-1, plasminogen activator inhibitor-1; PKA, cAMP-dependent protein kinase; tPA, tissue-type plasminogen activator; VN_{75} and VN_{65+10}, single-chain (75 kDa) and two-chain (65 + 10 kDa) forms of vitronectin respectively.

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sequence around the plasmin cleavage site, we showed that this cluster (specifically the Lys^{348}–Arg^{370} segment) contains amino acid residues that are involved in PAI-1 binding [34]. Preissner and co-workers also provided evidence to show that this segment in vitronectin participates in its binding to PAI-1 [35]. On the other hand, studies conducted in the laboratories of Loskutoff [36–38] and Wiman [39] provided evidence to show that vitronectin binds PAI-1 only through one affinity site residing in the somatomedin B domain at the N-terminus of vitronectin. Furthermore, vitronectin has been suggested to contain an additional PAI-1-interacting site found within the sequence Gly^{115}–Glu^{121} [40].

The present study is aimed at clarification of this apparent discrepancy between the results obtained in the various laboratories, and reaches the conclusion that the PAI-1 binding site in vitronectin is composed of amino acid residues that originate from distal parts of the vitronectin sequence, including both the somatomedin B domain at its N-terminus and the cluster of basic amino acids at its C-terminus.

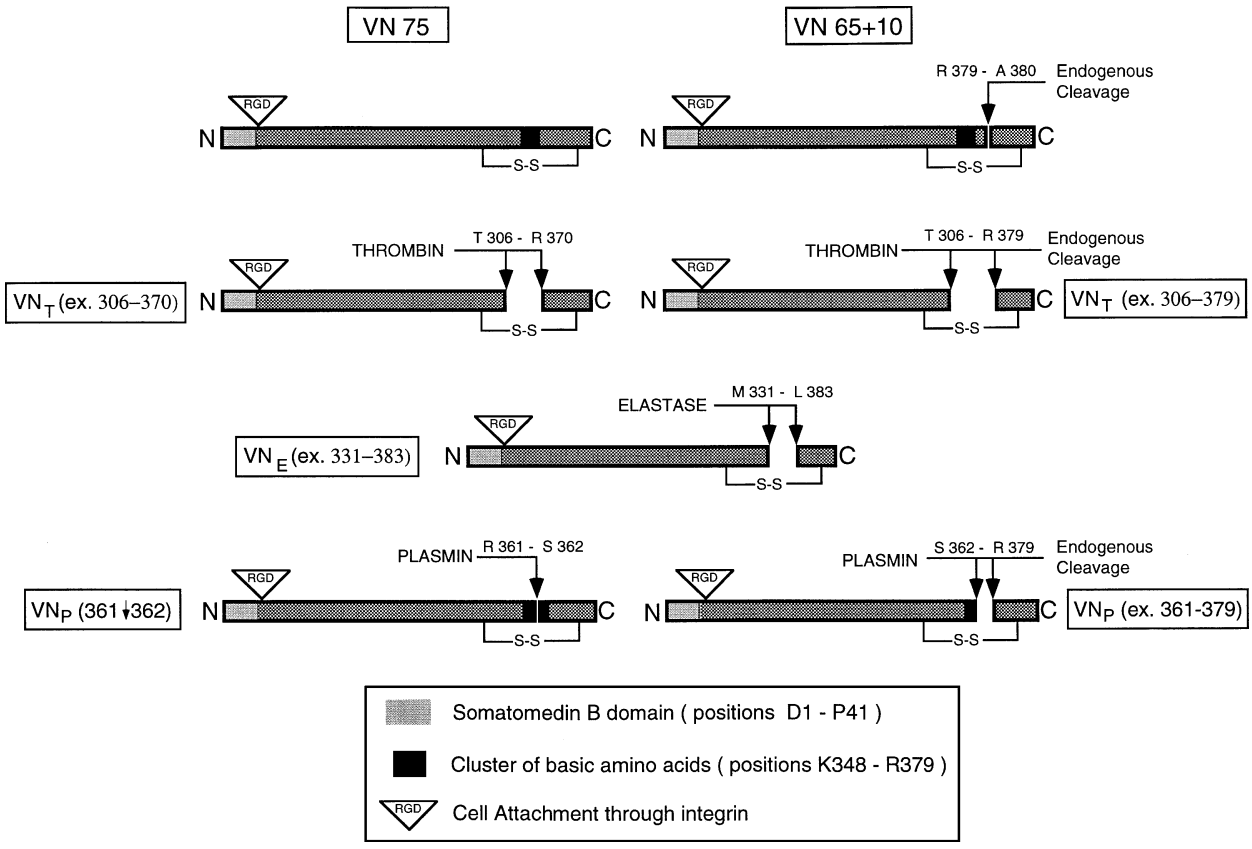
MATERIALS AND METHODS

Materials

PMSF, polyclonal anti-(rabbit IgG) conjugated to horseradish peroxidase, α-thrombin, pancreatic elastase, heparin (average molecular mass 15 kDa) and BSA were from Sigma. Polyclonal anti-vitronectin antibodies were from Calbiochem; PAI-1 (originating from the conditioned medium of the HT-1080 fibrosarcoma cell line) was from American Diagnostica; and *o*-nitrophenyl β-D-galactopyranoside, β-galactosidase-linked Protein A and [γ-^{32}P]ATP were from Amersham. Recombinant human casein kinase II (CKII) expressed in *Escherichia coli* was from Boehringer Mannheim. Protein A–Sepharose CL-4B was from Pharmacia, and PVDF membranes were from Millipore. Rabbit anti-(human PAI-1) antiserum was generously donated by Professor Israel Vlodavsky (Hadassah Medical School, Jerusalem, Israel). All other reagents were of the highest grade available from commercial sources.

Proteins

Vitronectin was purified from freshly frozen human plasma as described previously [21], with modifications [27,29]. The pure catalytic subunit of PKA was prepared from rabbit skeletal muscle [41]. Thrombin-cleaved, plasmin-cleaved and elastase-cleaved vitronectins (see Scheme 1) were purified on a Mono Q column (HR 5/5, 1 ml; Pharmacia) connected to an HPLC system (Hewlett–Packard 1050). The column was equilibrated with 50 mM Tris/HCl (pH 7.5)/30 mM NaCl and developed with an NaCl gradient from 0 to 1 M in the same buffer, at a flow rate of 0.5 ml/min. Fractions of 250 μl each were collected and analysed.



Scheme 1 Characterization of proteolytically modified vitronectins

Schematic representation of the thrombin-, plasmin- and elastase-cleaved vitronectins that were characterized, isolated and used in this study. The exact positions of the fragments excised in each case are indicated by arrows. Alongside each proteolytically modified vitronectin, the structural changes are indicated (ex., excised; an arrow indicates a cleaved peptide bond without removal of amino acids).

Synthetic peptides derived from the vitronectin sequence

The synthetic peptides used were derived from the vitronectin sequence and were prepared as described previously [34]. The peptides Ser-Gln-Arg-Gly-His-Ser-Arg-Gly-Arg-Asn-Gln-Asn-Ser-Arg-Arg-Pro-Ser-Arg-Ala and Lys-Lys-Gln-Arg-Phe-Arg-His-Arg-Asn-Arg-Lys-Gly-Tyr-Arg-Ser-Gln, corresponding to amino acids Ser^{362}–Ala^{380} and Lys^{348}–Gln^{363} respectively in vitronectin, were designated BP4 and BP6, respectively [34]. These peptides are derived from the stretch of basic amino acids in vitronectin, and are located around the plasmin-cleavage site (Arg^{361}–Ser^{362}) [33]. An optical enantiomer of BP6, which is identical to BP6 in size and sequence but is composed of D-amino acids, was designated BP8. The sequence of the vitronectin-derived synthetic peptide designated AP is Pro-Glu-Asp-Glu-Tyr-Thr-Val-Tyr-Asp-Asp-Gly-Glu-Glu, corresponding to residues Pro^{52}–Glu^{64}. Each of the peptides was purified by reverse-phase HPLC, and its structure and purity were established by amino acid analysis and sequencing.

Anti-peptide antibodies

Antibodies against the synthetic peptides BP4, BP6 and AP were raised in rabbits by subcutaneous injection of the peptide cross-linked to keyhole limpet haemocyanin as a carrier protein, and using the hetero-bifunctional cross-linker 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide. A standard immunization protocol was utilized. The rabbits were subsequently boosted with carrier-free peptides. For immunoblotting analyses, the antisera were usually diluted 1:200.

Cleavage of vitronectin by elastase, thrombin and plasmin

The reaction mixture (total volume 260 μl) contained the following constituents at the indicated final concentrations: vitronectin (50–200 μg/ml), Hepes buffer (50 mM), pH 7.5, and elastase (50 ng/ml), thrombin (30 units/ml) or plasmin (1 μg/ml). Where indicated, heparin (final concentration 50 μg/ml) was included. The reaction was allowed to proceed at 37 °C for the time periods indicated in each Figure, and were terminated by transferring aliquots (40 μl each) to another tube, boiling for 3 min and immediately chilling on ice, prior to phosphorylation with PKA. For immunoblotting, similar aliquots were transferred to tubes containing 10 μl of Laemmli sample buffer [42], boiled for 3 min and subjected to SDS/PAGE and subsequent analysis. The cleavage of vitronectin samples that were used for the measurement of PAI-1 binding was arrested by addition of PMSF (final concentration 1 mM) rather than by boiling.

Phosphorylation of vitronectin by PKA

The phosphorylation assay (total volume 50 μl) contained the following constituents at the indicated final concentrations: vitronectin (50–200 μg/ml), the C-subunit of PKA (3 μg/ml), magnesium acetate (10 mM), [γ-^{32}P]ATP (10 μM; 6 Ci/mmol) and Hepes buffer (30 mM, pH 7.5). The reaction was allowed to proceed at 22 °C for 5–15 min and was arrested by the addition of 12 μl of 5× concentrated Laemmli sample buffer [42] and boiling for 3 min. Where specified, the vitronectin samples contained heparin (50 μg/ml).

SDS/PAGE analysis

Proteins and peptides were resolved by SDS/PAGE using a linear gradient of acrylamide (7–20%) and the buffer system of Laemmli [42]. For fixation of the polypeptides on to the gel before Coomassie Blue staining and autoradiography, acetic acid was omitted from the fixing solution to avoid loss of the small basic vitronectin fragments generated after cleavage with plasmin, thrombin or elastase.

Western blot analysis of vitronectin and its proteolytic fragments

The cleavage products resolved by SDS/PAGE were transferred from the gels on to nitrocellulose paper using a Pharmacia semi-dry blotter. The buffer used contained Tris base (48 mM), glycine (39 mM), 0.037% SDS and 10% methanol. The products were electroblotted for 40 min at a constant voltage of 25 V. The blots were treated with polyclonal anti-vitronectin antiserum (1:1000 dilution), or with rabbit anti-BP4 or anti-BP6 antiserum (1:200 dilution). Staining was performed with an anti-(rabbit IgG) conjugated to horseradish peroxidase (1:1000 dilution) and using diaminobenzidine as a chromogenic substrate.

Binding of vitronectin to immobilized PAI-1

Intact vitronectin, or vitronectin clipped by either elastase or thrombin, was serially diluted in buffer containing 15 mM sodium phosphate, pH 7.4, 150 mM NaCl, 0.01% Tween 20 and 4% (w/v) poly(ethylene glycol) (average molecular mass 8 kDa). These serially diluted vitronectin samples were added to microtitre wells (50 μl in each well) coated with SDS-activated PAI-1, prepared as described previously [17]. After incubation for 2 h at 37 °C, the bound vitronectin was determined by reaction with specific rabbit anti-(human vitronectin) polyclonal antibodies (1:1000 dilution), followed by binding of β-galactosidase-linked Protein A, and using *o*-nitrophenyl galactopyranoside as a chromogenic substrate. The enzymic reaction was allowed to proceed for 60 min (22 °C), and was then arrested by the addition of 50 μl of 1 M Na_2CO_3. The absorbance at 405/490 nm was measured by an ELISA reader (TITERTEK multiscan MCC-340).

Determination of the binding of vitronectin and of its cleaved derivatives to PAI-1 by their co-precipitation from solution as immune complexes

Intact vitronectin ($VN_{75/65+10}$), or vitronectins clipped by either thrombin or plasmin (5 μg each), purified on a Mono Q column (HR 5/5, 1 ml; Pharmacia), were enzymically radiolabelled by phosphorylation with CKII (20 ng) in 30 mM Tris/HCl buffer, pH 7.5, in the presence of [γ-^{32}P]ATP, which labels selectively the N-terminal moiety of vitronectin (Z. Gechtman and S. Shaltiel, unpublished work). The labelling was allowed to proceed for 40 min at 22 °C, and was then arrested by the addition of heparin (a CKII inhibitor) to a final concentration of 0.5 μg/ml. Increasing amounts (0.15–0.90 μg) of vitronectin samples diluted in 50 mM Tris/HCl, pH 7.5, were incubated with a constant amount (0.45 μg) of guanidine-activated PAI-1 [17] for 20 min at 22 °C, in a total volume of 50 μl. At the end of the incubation period, the reaction volume was adjusted to 600 μl by the addition of ice-cold buffer containing 30 mM Tris/HCl, pH 7.5, 150 mM NaCl (TBS buffer) and 1.5% (w/v) BSA. The samples were pre-cleared with normal rabbit IgG coupled to Protein A–Sepharose beads [Pharmacia; 15 μl of beads suspended at 50% (v/v) in TBS] for 2 h at 4 °C. The beads were then spun down and the supernatants were transferred to new tubes. This was followed by immunoprecipitation with rabbit anti-PAI-1 polyclonal antibodies coupled to Protein A–Sepharose beads in TBS. Immunoprecipitation was carried out for 10–16 h at 4 °C, at the end of which time the immune complexes were spun down and washed

five times with ice-cold TBS. The proteins were dissolved in Laemmli sample buffer (40 μl), the solution was boiled for 3 min and the proteins were resolved on SDS/PAGE (10 % acrylamide). The gels were dried and then exposed to Kodak X-Omat AR films for autoradiography. The quantification of the immune complexes was carried out using a phosphorimager (Fujix; BAS1000). Non-specific binding of vitronectin was determined with control samples in which PAI-1 was omitted from the immunoprecipitation reaction. Non-specific binding (in most cases less than 10 % of total vitronectin binding) was subtracted from the total binding values obtained.

Determination of the effects of vitronectin and of a vitronectin-derived synthetic peptide on PAI-1 activity

Inherently active PAI-1 (non-re-activated; 0.2 μM) was pre-incubated for 5 h at 37 °C without any additions, in the presence of 1 μM vitronectin or of the synthetic peptides BP6 or BP8 (10 μM each) in buffer containing 50 mM Tris/HCl, pH 7.4, 100mM NaCl and 0.01 % Tween (TST buffer). Subsequently, the various PAI-1 samples were diluted 10-fold in TST buffer by bringing the total volume of the reaction mixture to 800 μl. Tissue-type plasminogen activator (tPA; 1 μg/ml final concentration) was then added to all samples, followed by an additional incubation for 1 h at 24 °C. The residual tPA activity was measured by the addition of 150 μl of developing solution containing 50 μg/ml plasminogen, 200 mM KP_i, pH 7.5, 200 mM KCl, 0.1 % Triton X-100, 220 μM benzyloxycarbonyl-lysine thiobenzyl ester and 220 μM 5,5′-dithiobis-(2-nitrobenzoic acid). The plasmin produced was then assayed at the times indicated in the Figures, by measuring its thioesterase activity [11]. The absorbance of the reaction product was recorded at 405 nm with a spectrophotometer (Spectronic 601; Milton Roy). Under these assay conditions, the peptides themselves had no detectable effect on the activities of tPA and plasmin.

RESULTS

Anti-peptide antibodies against sequence stretches of vitronectin

In order to study the possible role(s) of the cluster of basic amino acids in vitronectin, we prepared sequence-specific antibodies against peptide segments in this cluster, which surrounds its plasmin cleavage site (Arg^{361}–Ser^{362} [33]), and against a segment from the N-terminal moiety of the protein. Polyclonal antibodies were raised in rabbits against the synthetic peptides BP4, BP6 and AP [34] (see the Materials and methods section). The antibodies raised against these peptides were designated anti-BP4, anti-BP6 and anti-AP, respectively. These sequence-oriented antibodies were used: (i) to identify the exact sites of proteolytic cleavage in vitronectin by the proteinases used; (ii) to assess the availability of the cluster of basic amino acids for interaction in vitronectin-coated plates (and consequently the validity of ELISAs based on such vitronectin immobilization for assessing the involvement of this cluster in PAI-1 recognition and binding); and (iii) to study the consequences of such cleavages on the function of vitronectin, specifically on its binding and stabilization of PAI-1.

Making use of the specific phosphorylation of Ser^{378} of vitronectin by PKA [29], we have previously shown that the bonds Arg^{361}–Ser^{362} and Arg^{305}–Thr^{306} are the primary sites of vitronectin cleavage by plasmin and thrombin, respectively [30,33]. As expected from the location of this plasmin cleavage site and the specificity of the anti-BP6 and anti-BP4 antibodies, when vitronectin was cleaved by plasmin for increasing periods of time and the reaction products monitored after reduction of disulphide bridges and SDS/PAGE, a cleavage product of approx. 12 kDa was observed that was immunoreactive with anti-BP4, but not with anti-BP6 (Figure 1C). However, upon cleavage of vitronectin by thrombin for increasing periods of time followed by similar analysis, two cleavage products were formed, with molecular masses of ~ 17 kDa and ~ 7 kDa, that were immunoreactive with both anti-BP4 and anti-BP6 antibodies (Figures 1B and 1C).

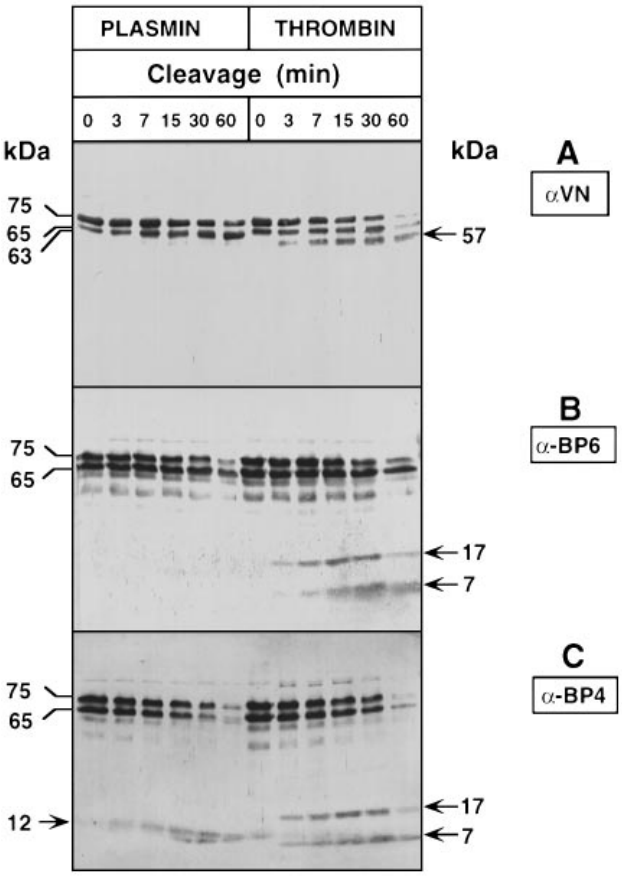


Figure 1 Monitoring of the cleavage of vitronectin by plasmin and by thrombin using anti-vitronectin and sequence-directed anti-peptide antibodies

Native vitronectin (40 μg) was cleaved by plasmin (1.3 μg) or by thrombin (3.5 units) at 22 °C in the presence of heparin (13 μg) in a reaction mixture (260 μl) containing 50 mM Hepes, pH 7.5. The reaction was allowed to proceed for the indicated times, and was terminated by the removal of aliquots (40 μl each) into tubes containing 10 μl of 5 × concentrated Laemmli sample buffer [42] and boiling for 3 min. The reaction products were subjected to SDS/PAGE (7–20% acrylamide), then electroblotted on to nitrocellulose membranes. These membranes were then treated with polyclonal anti-vitronectin antiserum (αVN) diluted 1:1000 (**A**), with anti-BP6 antiserum (α-BP6) diluted 1:200 (**B**) or with anti-BP4 antiserum (α-BP4) diluted 1:200 (**C**). The bands were visualized with polyclonal anti-(rabbit IgG) antibodies (1:1000) conjugated to peroxidase, using diaminobenzidine as substrate.

The ~ 12 kDa peptide that appeared after cleavage of vitronectin with plasmin is derived from the one-chain form of vitronectin (VN_{75}). Cleavage by plasmin of the two-chain form of vitronectin (VN_{65+10}, which results from the endogenous cleavage of vitronectin at its Arg^{379}–Ala^{380} bond) yields a peptide of ~ 2 kDa which could not be detected in this system due to its low molecular mass. The 17 kDa and 7 kDa peptides are the products of cleavage by thrombin of the one-chain (VN_{75}) and the two-chain (VN_{65+10}) forms, respectively (Figures 1B and 1C).

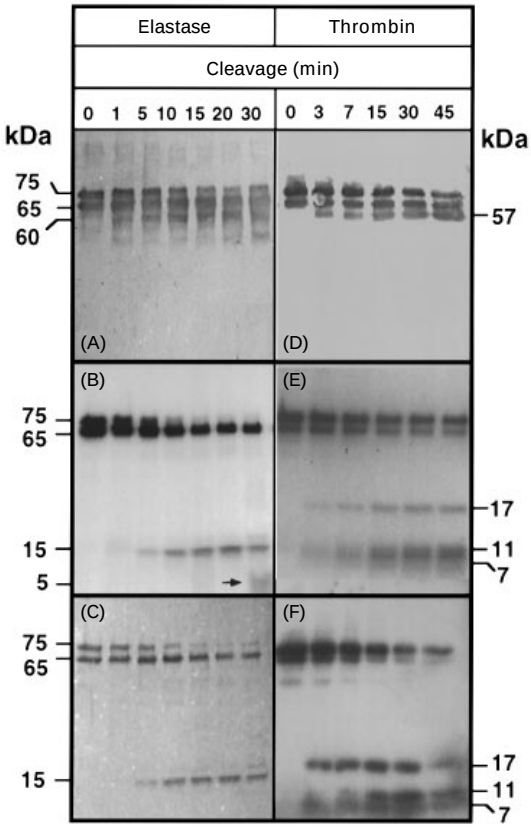


Figure 2 Monitoring of the cleavage of vitronectin by elastase and by thrombin using anti-vitronectin and anti-BP4 antibodies, and by measuring Ser^{378} phosphorylation by PKA

Native vitronectin (50 μg) was cleaved by elastase (**A**–**C**) or by thrombin (**D**–**F**) in the presence of heparin (12 μg) in a total volume of 260 μl. The reaction was allowed to proceed for the time periods indicated, and was then arrested by boiling, as described in the legend to Figure 1. The reaction products were resolved on SDS/PAGE (7–20% acrylamide) and the gel was then electroblotted on to nitrocellulose membranes. These membranes were treated with polyclonal anti-vitronectin antiserum (dilution 1:1000; **A** and **D**) or with anti-BP4 antiserum (dilution 1:200; **C** and **F**). Alternatively (**B** and **E**), cleavage was arrested by boiling the sample, which was then chilled on ice and subjected to phosphorylation by PKA and [γ-^{32}P]ATP, as described in the Materials and methods section. Phosphorylation was terminated by boiling, as described in the legend to Figure 1. The phosphorylated products were resolved on SDS/PAGE (7–20% acrylamide) and visualized by autoradiography.

The 12, 17 and 7 kDa peptides reacted with anti-BP4 antibodies, since all three contain the stretch Ser^{362}–Ala^{380} recognized by anti-BP4. On the other hand, the anti-BP6 antibodies reacted only with the 17 and 7 kDa thrombin cleavage fragments (Figure 1B), but not with the 12 kDa plasmin cleavage product (Figure 1B), because the former contains the stretch Lys^{348}–Gln^{363} that is recognized by the anti-BP6 antibodies, whereas the latter does not. These low-molecular-mass cleavage products are not seen when the cleavage of vitronectin by plasmin or by thrombin is monitored using polyclonal anti-vitronectin antibodies (Figure 1A). This result supports our previous observation that the bond Arg^{361}–Ser^{362} is the primary site in vitronectin for cleavage by plasmin [33]. It also demonstrates that the anti-peptide antibodies (anti-BP4 and anti-BP6) specifically recognize the vitronectin stretches against which they were raised.

Identification of the fragments formed from vitronectin by elastase or thrombin cleavage, using Ser^{378} phosphorylation and anti-BP4 antibodies

Elastase and thrombin have been implicated in physiological processes that involve vitronectin: elastase in the degradation of extracellular matrix components during inflammation [43–46], and thrombin in several facets of haemostasis, including an interaction leading to the formation of the thrombin–antithrombin III complex [47–49]. Therefore we undertook to analyse in detail the cleavage of vitronectin by these proteinases with the aim of determining the primary cleavage site(s), if any, and hopefully using the resulting clipped vitronectins for the identification of amino acid residues that may be involved in the recognition and binding of PAI-1 by vitronectin. The characterization of vitronectin in many blood samples before and after thrombin action led us to believe that, in addition to the initial cleavage site previously identified in vitronectin (Arg^{305}–Thr^{306} [30]), thrombin might also cleave vitronectin at an additional specific site, and thus provide us with an additional modulated vitronectin form. It should be noted that all proteolytic cleavages were carried out in the presence of heparin, as we found previously that heparin enhances the rate of cleavage of vitronectin by plasmin and thrombin [30,33] (see also below).

The cleavage of vitronectin by elastase resulted in the time-dependent appearance of a 60 kDa protein band, as is clearly evident on SDS/PAGE under reducing conditions and staining with polyclonal anti-vitronectin antibodies to visualize the cleavage products (Figure 2A). On the other hand, when the cleavage was monitored by the PKA phosphorylation of Ser^{378} (Figure 2B) or by using anti-BP4 antibodies (Figure 2C), the elastase cleavage of vitronectin was seen to generate two low-molecular-mass fragments of approx. 15 and 5 kDa. The appearance of these low-molecular-mass fragments was accompanied by the concomitant loss of radioactive phosphate from the vitronectin doublet (VN_{75} and VN_{65+10}) normally found in human plasma, and by a concomitant decrease in the immunoreactivity of this doublet with anti-BP4 antibodies (Figures 2B and 2C). These results show that both proteinases selectively remove a C-terminal fragment from the vitronectin molecule which bears the PKA phosphorylation site and the epitope recognized by anti-BP4.

Similarly, the cleavage of vitronectin by thrombin resulted in the time-dependent appearance of a 57 kDa protein band, clearly seen on SDS/PAGE under reducing conditions and staining with anti-vitronectin antibodies (Figure 2D). On monitoring the cleavage by the phosphorylation of Ser^{378} (Figure 2E) or by using anti-BP4 antibodies (Figure 2F), we found that the cleavage of vitronectin by thrombin generated three low-molecular-mass fragments of approx. 17, 11 and 7 kDa, accompanied by the concomitant loss of radioactive phosphate from the vitronectin doublet and by a decrease in the immunoreactivity of this doublet with anti-BP4 antibodies (Figures 2E and 2F).

Elastase cleavage sites in vitronectin

The products of vitronectin cleavage by elastase, visualized by phosphorylation and by anti-BP4 antibodies, were subjected to N-terminal sequence analysis. As seen in Table 1, the 60 kDa polypeptide obtained after elastase cleavage of vitronectin (Figure 2A) still contained the N-terminus of vitronectin, since its sequence matches that of the first 15 amino acids of vitronectin. The N-terminal sequence of the 15 kDa fragment (Figures 2B and 2C) matched that of vitronectin, starting from Met^{331}. Judging by its molecular size, this fragment is a cleavage product of the one-chain vitronectin form (VN_{75}), ending at its C-

Table 1 N-terminal sequence analysis of the vitronectin cleavage products generated by elastase and thrombin

Vitronectin cleaved by elastase or thrombin was subjected to SDS/PAGE. The resolved cleavage products were then electroblotted on to PVDF and the indicated bands (which were identified by protein staining, by phosphorylation with PKA or immunochemically) were excised and subjected to sequence analysis. The numbering of amino acid positions is from the sequence of vitronectin deduced by Jenne and Stanley [23].

Elastase

60 kDa				15 kDa				9 kDa			
Cycle no.	Amino acid	Yield (pmol)	Position in vitronectin	Cycle no.	Amino acid	Yield (pmol)	Position in vitronectin	Cycle no.	Amino acid	Yield (pmol)	Position in vitronectin
1	D	30	1	1	M	24	331	1	S	88	384
2	Q	15	2	2	A	26	332	2	L	150	385
3	E	4	3	3	G	8	333	3	F	95	386
4	S	6	4	4	R	7	334	4	S	8	387
5	–*	–	5 (C)	5	I	16	335	5	S	11	388
6	K	2	6	6	Y	8	336	6	E	18	389
7	G	10	7	7	I	12	337	7	E	27	390
8	R	11	8	8	S	3	338	8	S	0.2	391
9	–*	–	9 (C)	9	G	10	339	9	N	12	392
10	T	8	10	10	M	7	340	10	L	5	393
11	E	4	11	11	A	9	341	11	G	4	394
12	G	2	12	12	P	6	342	12	A	5	395
13	F	2	13	13	R	7	343	13	N	4	396
14	N	1.5	14	14	P	4	344	14	N	6	397
15	V	4	15					15	Y	5	398

Thrombin

57 kDa				11 kDa				7 kDa			
Cycle no.	Amino acid	Yield (pmol)	Position in vitronectin	Cycle no.	Amino acid	Yield (pmol)	Position in vitronectin	Cycle no.	Amino acid	Yield (pmol)	Position in vitronectin
1	D	10	1	1	N	12	371	1	T	26	306
2	Q	8	2	2	Q	11	372	2	S	8	307
3	E	16	3	3	N	15	373	3	A	19	308
4	S	13	4	4	S	2	374	4	G	12	309
5	–*	–	5 (C)	5	R	3	375	5	T	14	310
6	K	9	6	6	R	3	376	6	R	10	311
7	G	11	7	7	P	7	377	7	Q	12	312
8	R	6	8	8	S	2	378	8	P	10	313
9	–*	–	9 (C)	9	R	2.5	379	9	Q	11	314
10	T	7	10	10	A	2.5	380	10	F	10	315
11	E	6	11	11	M	3	381	11	I	8	316
12	G	10	12					12	S	3	317

* The cysteines were not determined in this analysis because they were not pre-modified.

terminus. It should be noted that cleavage by elastase would yield from VN_{65+10} a fragment that would begin with Met^{331} and end at Arg^{379}, i.e. at the endogenous cleavage site of the two-chain form. This fragment would have a molecular mass of ~ 5 kDa, it should contain the PKA phosphorylation site (Ser^{378}) and, in view of the sensitivity of this method of detection, it should be seen in the autoradiogram after phosphorylation with [γ-^{32}P]ATP. Indeed, such a band was clearly seen in the autoradiogram monitoring cleavage by elastase (Figure 2B). However, we failed to purify this fragment in sufficient amounts to sequence it.

When cleavage by elastase was monitored by SDS/PAGE and Coomassie Blue staining (results not shown), we identified and sequenced an additional vitronectin fragment that appeared after cleavage for longer time periods. This fragment has a molecular mass of ~ 9 kDa and its N-terminus begins at Ser^{384} (Table 1). As expected, it is not seen in either Figure 2(B) or Figure 2(C), as it does not contain either Ser^{378} or the anti-BP4 epitope. We conclude, therefore, that elastase cleaves vitronectin at two sites, initially attacking the Ala^{330}–Met^{331} bond and then the Leu^{383}–Ser^{384} bond. These cleavage sites are consistent with the known specificity of pig pancreatic elastase [50].

Thrombin cleavage sites in vitronectin

Upon cleavage of vitronectin by thrombin, an ~ 57 kDa protein was obtained (Figure 2D) that has the original N-terminal sequence of vitronectin, as clearly seen from its sequence analysis (Table 1). In addition, an ~ 17 kDa fragment was formed (Figures 1B and 1C; Figures 2E and 2F), the sequence of which begins at Thr^{306}. This fragment originates from the thrombin cleavage of VN_{75}, and was previously identified by sequence analysis in our laboratory [30]. Cleavage by thrombin of VN_{65+10}

at the same site resulted in a 7 kDa fragment (Figures 2E and 2F) that is phosphorylated at Ser^{378}, the sequence of which also begins at Thr^{306}, but ends at Arg^{379} (on the basis of its molecular size and the location of the endogenous cleavage site). Careful analysis of the autoradiograms revealed that, in addition to the 7 kDa phosphorylated fragment (Figure 2, panels E and F), there was also a phosphorylated fragment with a molecular mass of ~ 11 kDa which appeared after the thrombin cleavage of vitronectin. N-terminal sequencing of this fragment revealed that it begins at Asn^{371}. We conclude, therefore, that thrombin cleaves vitronectin at two sites: Arg^{305}–Thr^{306} and Arg^{370}–Asn^{371}.

The alignment of the sites of cleavage of vitronectin by elastase and thrombin is depicted in Scheme 1. These results indicate that the cleavage of vitronectin by both elastase and thrombin leads to the excision of the cluster of basic amino acids in vitronectin containing the PKA phosphorylation site and the epitope recognized by anti-BP4. Earlier work has suggested that this cluster accommodates the heparin-binding site in vitronectin [24,35], its plasminogen binding site [35] and amino acid residues that interact with PAI-1 [33–35].

Protective effect of heparin on elastase and thrombin cleavage products

Since vitronectin has been reported to bind to glycosaminoglycans *in vitro* [24–26,51] and *in vivo* [52,53], and since this binding was shown to have a distinct effect on its conformation, exposing the cluster of basic amino acids (reflected in cleavage by plasmin [33] and phosphorylation by PKA on Ser^{378} [29,30]), we tested the effect of heparin on the cleavage of vitronectin by elastase and by thrombin. Analysis of the effects of heparin on each of these cleavages (based on the disappearance of VN_{75} and VN_{65+10}) showed that heparin has a minor and a moderate effect on the rate of vitronectin cleavage by elastase and thrombin respectively. However, whereas in the absence of heparin no detectable amounts of the 15 kDa elastase cleavage product could be seen, the formation of this fragment was clearly seen in the presence of heparin. In the case of thrombin, heparin slowed down the cleavage but it also prevented the further degradation of the 17 kDa product (not shown). Thus heparin protects the resulting clipped products of vitronectin cleavage that contain the heparin binding site (Lys^{348}–Arg^{370}) from being degraded further by these proteinases (see also [24,35]), a finding that led us to use heparin during the isolation of such low-molecular-mass products and in establishing the initial elastase and thrombin cleavage sites in vitronectin.

Immobilization of vitronectin on microtitre well plates renders its cluster of basic amino acids unavailable for interaction

In view of reports from two other laboratories [39,54,55] that the cluster of basic amino acids in vitronectin does not contribute to PAI-1 binding, whereas such a contribution was observed in our own laboratory [33,34,56] and in that of Preissner and co-workers [35], we attempted to discover the reason for this apparent discrepancy.

For this purpose, we used the anti-peptide antibodies raised against the cluster of basic amino acids in vitronectin (anti-BP6 and anti-BP4), and similar antibodies (anti-AP) raised against its N-terminal moiety (Pro^{52}–Glu^{64}). We first determined by dot-blot analysis the immunoreactivity of each of the three antisera towards vitronectin. We could then compare in immobilized vitronectin the accessibility of the cluster of basic amino acids (C-terminal moiety) with that of its N-terminal moiety, using a comparable titre ($\pm 5\%$) of each antibody. We carried out this accessibility test (Figure 3) because other workers have immobilized vitronectin for assaying the interaction of vitronectin with PAI-1, whereas we immobilized PAI-1 for this purpose. As clusters of basic amino acids (e.g. in poly-lysine) tend to bind to polystyrene plates, we suspected that the cluster of basic amino acids in vitronectin might be a major anchoring element in binding vitronectin to the plates.

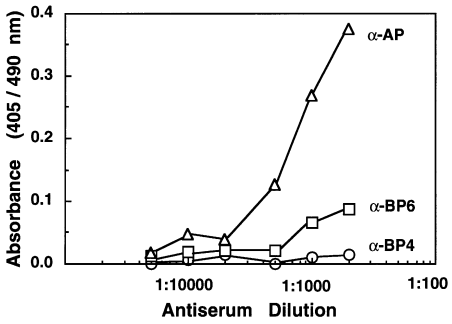


Figure 3 Availability of the cluster of basic amino acids for interactions of vitronectin immobilized on polystyrene wells

Vitronectin dissolved in PBS (10 μg/ml) was allowed to adsorb (16 h at 4 °C) on to microtitre wells (50 μl/well). Non-specific adsorption sites in the wells were blocked by BSA [3% (w/v) in PBS]. The relative availability of the N- and C-terminal moieties of vitronectin was determined (i) with anti-peptide polyclonal antibodies (anti-AP; $\triangle$) against the epitope Pro^{52}–Glu^{64} in vitronectin; and (ii) with anti-peptide polyclonal antibodies [anti-BP4 ($\bigcirc$) and anti-BP6 ($\square$)] directed against epitopes from the cluster of basic amino acids in the C-terminal moiety of vitronectin (epitopes Ser^{362}–Ala^{380} and Lys^{348}–Gln^{363} respectively; see Scheme 1). Solutions of these three antibodies at different dilutions (in PBS containing 0.5% BSA) were added to the vitronectin-coated wells, and the relative amount of antibodies that bound to the immobilized vitronectin was determined with Protein A-linked β-galactosidase using the substrate *o*-nitrophenyl β-D-galactopyranoside. The β-galactosidase reaction was allowed to proceed for 60 min (22 °C), then arrested by the addition of 50 μl of 1 M Na_2CO_3. The absorbance at 405/490 nm was measured to determine β-galactosidase activity. Each point in the graph represents the mean of triplicate determinations.

We assumed that, if this is so, the cluster in vitronectin would become inaccessible to interaction with PAI-1. This was indeed the case (Figure 3): upon immobilization of vitronectin on the plates, the segments containing the cluster of basic amino acids were essentially undetectable by anti-BP4 and anti-BP6 antibodies, whereas the N-terminal moiety was detected with anti-AP. These results support the original findings of Salonen et al. [11] regarding the different stoichiometry of binding of vitronectin to immobilized PAI-1 compared with the binding of PAI-1 to immobilized vitronectin. At the same time, our results account for the fact that workers who use immobilized vitronectin to measure the vitronectin–PAI-1 interaction would fail to observe the contribution of the cluster of basic amino acids in the binding of vitronectin to PAI-1, and consequently would not attribute to it the appropriate importance.

Vitronectin–PAI-1 interaction assessed in a two-phase system (ELISA) and in solution

Limited cleavage of vitronectin by elastase or by thrombin results in a significant decrease in its binding to PAI-1, as measured by ELISA in which PAI-1 (rather than vitronectin) was immobilized (Figure 4). Immunoblotting of the cleavage products with polyclonal anti-vitronectin antibodies revealed appreciable amounts of the 60 and 57 kDa vitronectin cleavage products formed by elastase and thrombin respectively (Figure 4, inset). This result is in agreement with our previous report on the cleavage of vitronectin by plasmin, which we have shown to

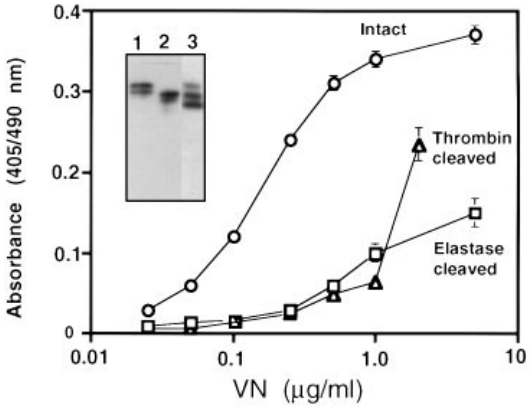


Figure 4 Effects of the cleavage of vitronectin by elastase or thrombin on its binding of PAI-1

Native vitronectin (40 μg) was cleaved by elastase (0.1 μg) for 15 min or by thrombin (3 units) for 30 min, as described in the Materials and methods section. The reaction was arrested by the addition of an excess of PMSF (final concentration 1 mM). The binding of native (○), elastase-cleaved (□) or thrombin-cleaved (△) vitronectin (VN) to PAI-1 immobilized on polystyrene was determined by ELISA, as described in the Materials and methods section. The antibody used in this experiment was polyclonal anti-vitronectin, which was shown to react equally (within 10%) with all vitronectins used. The results presented are representative of three independent experiments, and are means ± S.E.M. of triplicate determinations. The inset shows an immunoblot with polyclonal anti-vitronectin antiserum of the three vitronectin samples used. Lane 1, intact vitronectin (control); lane 2, elastase-cleaved vitronectin; lane 3, thrombin-cleaved vitronectin.

result in a similar decrease in the binding of vitronectin to PAI-1 [33].

To challenge further our earlier conclusion regarding the presence of PAI-1 binding amino acid residues within the cluster of basic amino acids in vitronectin [33,34], we used three excised derivatives of native human vitronectin (Scheme 1). These preparations, which contain all the naturally occurring post-translational modifications found in vitronectin, were purified (Figure 5), structurally analysed (Table 1), and shown to have an intact N-terminus and to be modified only in their cluster of basic amino acids. As seen in Scheme 1, in each of these modified vitronectins the cluster of basic amino acids was either completely removed [in VN_T (ex. 306–370), VN_T (ex. 306–379) and VN_E (ex. 331–383)] or split in two and partly removed [in VN_P (361 ↓ 362) and VN_P (ex. 362–379)].

The binding of vitronectin to PAI-1 was now compared in solution (no immobilization) by the co-precipitation of the vitronectin–PAI-1 complex with anti-PAI-1 antibodies, to ensure that all epitopes in both PAI-1 and the modulated vitronectins were available for interaction. As seen in Figure 6(A), vitronectin–PAI-1 binding is specific, since it occurred with the active but not with the latent form of PAI-1 [7,55,57,58], whereas the polyclonal antibodies against PAI-1 used to co-precipitate the vitronectin–PAI-1 complex immunoprecipitated equally (within 10%) the latent and active forms of PAI-1 (results not shown). However, upon removal of the cluster of basic amino acids by specific proteolysis with thrombin or plasmin, the resulting altered vitronectins were found to have a significantly decreased affinity for activated PAI-1 (Figure 6B).

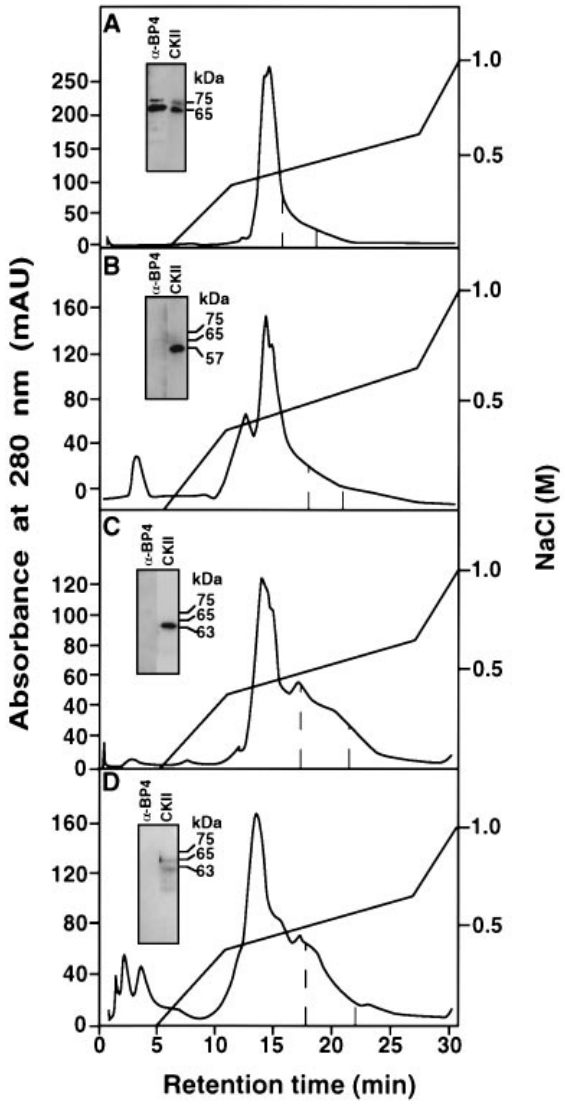


Figure 5 Purification of thrombin-cleaved, elastase-cleaved and plasmin-cleaved vitronectin by FPLC

(**A**) Native vitronectin (500 μg) was incubated in the presence of 5 μg of heparin at 22 °C for 40 min; (**B**) native vitronectin (400 μg) was cleaved with thrombin (50 units) at 22 °C for 1 h; (**C**) native vitronectin (500 μg) was cleaved by plasmin (2 μg) at 22 °C for 40 min in the presence of 5 μg of heparin; (**D**) native vitronectin (700 μg) was cleaved by elastase (0.8 μg) at 37 °C for 40 min in the presence of 5 μg of heparin. All reactions were carried out in 50 mM Tris, pH 7.5 (total volume 500 μl). Each of the four vitronectin samples was loaded on an FPLC Mono Q column equilibrated with 50 mM Tris, pH 7.5. The column was developed with an NaCl gradient in the same buffer, at a flow rate of 0.5 ml/min. Fractions (250 μl each) were collected and analysed for their content. The broken vertical lines indicate the fractions that were pooled in each case for further characterization. mAU, milli-absorbance units. The vitronectin content of the pooled fractions was determined with polyclonal anti-vitronectin antiserum by quantitative immunoblotting (not shown) using as a standard a pure vitronectin solution of known concentration (the yields of the purified cleaved vitronectins depicted in **B**, **C** and **D** were 10–20% of the applied material). The composition of these pools (inset in each panel) was determined by phosphorylation with CKII, which specifically labels Thr residues adjacent to the Arg-Gly-Asp site in the N-terminal moiety of vitronectin (Z. Gechtman and S. Shaltiel, unpublished work), and by staining with anti-BP4 (α-BP4), which interacts specifically with the C-terminal moiety of vitronectin (see above).

BP6 interacts stereospecifically with activated PAI-1, slowing down its conversion into the latent form

Another approach used for identification of the amino acid residues in vitronectin that contribute to its specific binding to PAI-1 was based on the interaction of synthetic peptides derived from the cluster of basic amino acids with this inhibitor. The most suitable peptide in this respect was found to be BP6

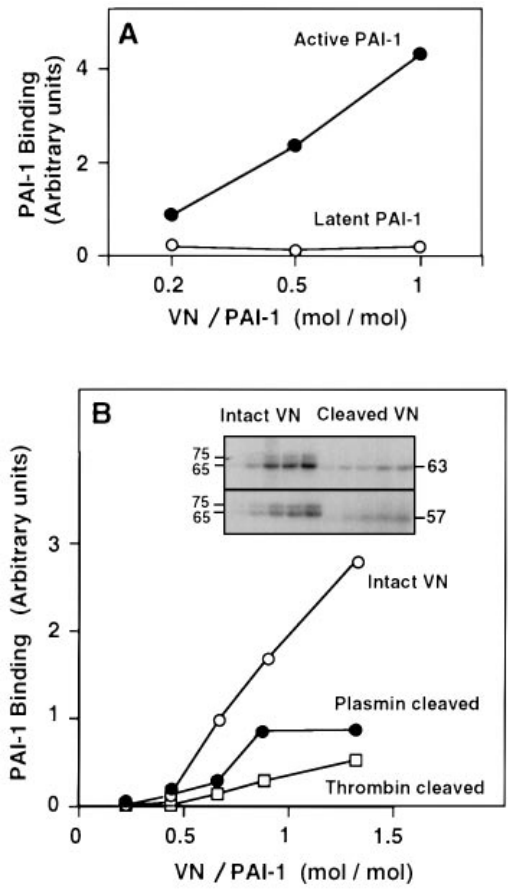


Figure 6 Binding of vitronectin to PAI-1 in solution: co-precipitation by anti-PAI-1 polyclonal antibodies

(**A**) Vitronectin co-precipitates with the active but not with the latent form of PAI-1. Native vitronectin (VN; 15 μg) was phosphorylated by CKII in the presence of [γ-^{32}P]ATP at 22 °C for 40 min. The reaction was arrested by the addition of heparin (a CKII inhibitor) to a final concentration of 0.05 μg/ml. Vitronectin was diluted in a buffer composed of 50 mM Tris (pH 7.5) and 150 mM NaCl (TBS), and incubated with a constant amount (0.45 μg) of either latent PAI-1 (○) or guanidinium hydrochloride-activated PAI-1 (●) for 30 min at 22 °C in a total volume of 50 μl. At the end of the incubation period, the vitronectin–PAI-1 complexes were immunoprecipitated with polyclonal antibodies to PAI-1 (rabbit) complexed to Protein A–Sepharose beads (for details, see the Materials and methods section). The samples were then subjected to SDS/PAGE and autoradiography. Values for the non-specific binding of vitronectin (less than 10%) were obtained by the omission of PAI-1 from the immunoprecipitation reaction (control). The relative amount of PAI-1 to bound vitronectin was measured by densitometric scanning of the CKII-phosphorylated vitronectin bands. (**B**) Binding of intact, thrombin-cleaved and plasmin-cleaved vitronectins to PAI-1 in solution. Intact (○), thrombin-cleaved (□) and plasmin-cleaved (●) vitronectins (40 μg/ml each) were phosphorylated by CKII in the presence of [γ-^{32}P]ATP at 22 °C for 40 min. The reaction was arrested by the addition of heparin to a final concentration of 0.05 μg/ml. The vitronectin samples (0.15–0.9 μg) were diluted in TBS and incubated with a constant amount (0.45 μg) of guanidinium hydrochloride-activated PAI-1 for 30 min at 22 °C in a total volume of 50 μl. Vitronectin–PAI-1 complexes were co-precipitated as in (**A**). The relative amount of PAI-1 bound to intact thrombin-cleaved and plasmin-cleaved vitronectins was measured by densitometric scanning of the CKII-phosphorylated vitronectin bands or quantified using a phosphoimager (Fujix, BAS1000). The inset shows autoradiograms of two independent experiments: upper panel, intact and plasmin-cleaved vitronectins; lower panel, intact and thrombin-cleaved vitronectins.

(corresponding to the Lys^{348}–Gln^{363} stretch in vitronectin), which was shown previously to inhibit the binding of PAI-1 to immobilized vitronectin in an ELISA, being significantly effective at a concentration of only 50 nM and maximally effective at 2.5 μM (see Figure 3 in [34]).

If we assume that BP6 interacts specifically with PAI-1 at its vitronectin-binding site, then this peptide might retard the conversion of active PAI-1 into its latent form. This is indeed the case (Figure 7). Although BP6 is a small and flexible peptide, it afforded protection from latency at a concentration as low as 10 μM. More indicative of a specific interaction is the fact that this stabilization is stereospecific, since it occurs to a much lower extent (if at all) with the optical enantiomer of BP6, namely the peptide BP8 (Figure 7), a peptide identical to BP6 in size and sequence but composed of D-amino acids.

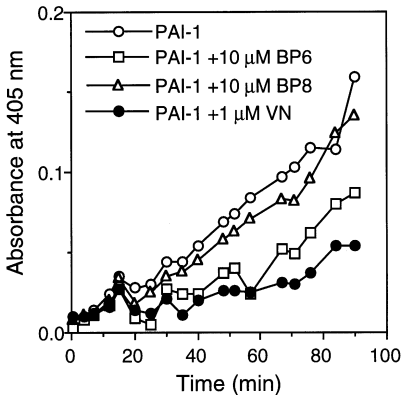


Figure 7 The PAI-1-stabilizing activity of vitronectin is reproduced by BP6 but not by its stereoisomer BP8

Inherently active PAI-1 (non-re-activated; 0.2 μM) was preincubated for 5 h at 37 °C without any additions in the presence of 1 μM vitronectin (VN) or of the synthetic peptides BP6 or BP8 (10 μM) in TST buffer. The PAI-1 activity towards tPA in all samples was measured with chromogenic substrate, as described in the Materials and methods section. The absorbance (405 nm) was recorded at the various times indicated. Note that in this experiment we used inherently active (rather than guanidine-activated) PAI-1, since the guanidine could not be removed completely after activation, and since the residual guanidine was found to interfere with quantification of the interaction with the synthetic peptides, which have several Arg residues each possessing a guanidino group.

DISCUSSION

In view of the observation that plasmin cleaves vitronectin at the Arg^{361}–Ser^{362} bond [33], within its cluster of basic amino acids, we attempted to find out whether this cleavage affects the interaction between PAI-1 and vitronectin, given that the latter has been shown by several groups of workers to be the PAI-1 binding protein in blood and in the extracellular matrix [7,9–13]. For this purpose we immobilized activated PAI-1 and measured its binding of both native and plasmin-clipped vitronectin by an ELISA [11], and showed that the cleavage of vitronectin by plasmin attenuates its binding of PAI-1[33]. The choice of the assay used (binding of vitronectin to immobilized PAI-1, rather than of PAI-1 to immobilized vitronectin) was based on the fact that the procedures reported previously for the activation of PAI-1 involved harsh denaturating agents (8–12 M urea and 3–4 M guanidine) [17,54], suggesting that activated PAI-1 may retain its inhibitory activity after immobilization. In addition, Salonen et al. had shown [11] that, whereas immobilized vitronectin binds PAI-1 with a K_d of 1.9×10^{-7} M, immobilized PAI-1 binds vitronectin with a K_d of 5.5×10^{-8} M, i.e. more tightly. Finally, Salonen et al. had also shown that, whereas the stoichiometry of binding of vitronectin to immobilized PAI-1 is (at saturation) one molecule of PAI-1 per molecule of vitronectin, the stoichiometry of binding PAI-1 to immobilized vitronectin is one molecule of PAI-1 per three molecules of vitronectin [11],

suggesting that about two-thirds of the immobilized vitronectin molecules are somehow unavailable for PAI-1 binding.

In more recent papers, Deng et al. [55,59] analysed the vitronectin–PAI-1 interaction; they provided further evidence for the involvement of the somatomedin B domain, and came to the conclusion that 'only fragments containing the N-terminus of vitronectin bind PAI-1'. Referring to the evidence that the cluster of basic amino acids in vitronectin contributes to PAI-1 binding (based on the cleavage of vitronectin by plasmin [33,60] and on the inhibitory effects of a series of peptides derived from this cluster [34]), these authors stated that this cluster has an uncertain physiological relevance [55].

It seems to us important to note that, for a comparison of the binding of differently modified vitronectins to PAI-1, the binding of vitronectins to immobilized PAI-1, rather than of PAI-1 to immobilized vitronectins, has an advantage, since it avoids the complication of having to secure an equal yield in the binding of the different vitronectins to the plate, especially if the anchoring of vitronectin to the plates may involve a putative site of interaction with PAI-1. Furthermore, a truncated vitronectin devoid of the cluster of basic amino acids was found to bind much less efficiently to ELISA plates (D. Seger and S. Shaltiel, unpublished work).

On the basis of our findings, we proposed [34] that the cluster of basic amino acids in vitronectin (more specifically, the segment Lys^{348}–Arg^{370}) contains 'affinity-contributing elements' for the PAI-1 binding site [33,34,56]. We used this term, rather than stating that this region accommodates the PAI-1 binding site, to emphasize that this finding in itself does not exclude the possibility that other parts of the molecule may also contribute to the binding and thus participate in creating the three-dimensional structure of the PAI-1 binding site in vitronectin. In fact, in most protein–protein interactions, amino acid residues from distal locations in the protein sequence participate in creating the three-dimensional conformation of the binding site. Studies carried out in Preissner's laboratory [35] also provided evidence that the cluster of basic amino acids in vitronectin participates in its PAI-1 binding site. Other workers have assigned the PAI-1 binding site to the somatomedin B domain in the N-terminus of vitronectin (positions 1–40) [36–39,55,59] or to the middle section of the molecule (positions 115–121) [40].

The results presented here provide several independent lines of evidence to establish the participation of the cluster of basic amino acids in the PAI-1 binding site of vitronectin, based on a set of peptides and proteins prepared specifically for this purpose. These included the following. (i) Proteolytically clipped vitronectin analogues, in which the cluster of basic amino acids is excised or split. These vitronectin analogues were characterized structurally by purification and sequencing of the proteolytic fragments, by their specific phosphorylation (radiolabelling) with PKA, and by specific immunostaining with sequence-orientated anti-peptide antibodies. (ii) Peptides derived from distinct segments of vitronectin within this cluster of basic amino acids and from the N-terminal moiety of the molecule. (iii) Sequence-orientated antibodies raised against these peptides.

By assessing the vitronectin–PAI-1 interaction, not only in a two-phase system but also in solution, we present evidence that the cluster of basic amino acids in vitronectin contains amino acid residues that participate in this interaction. This evidence includes ELISAs, co-precipitation from solution of vitronectin–PAI-1 complexes, and an interaction of PAI-1 with a peptide derived from the cluster of basic amino acids, an interaction that is shown to occur at a low peptide concentration and to be stereospecific. In addition, the present results provide an explanation as to why workers who used immobilized vitronectin to measure the vitronectin–PAI-1 interaction could not observe the contribution of the cluster of basic amino acids in vitronectin to this interaction, since the immobilization of vitronectin renders its cluster of basic amino acids inaccessible for binding of PAI-1.

In conclusion, we propose that PAI-1 interacts with both the cluster of basic amino acids at the C-terminal moiety of vitronectin, and the somatomedin B domain at its N-terminus.

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