Fibrinogen Contains Cryptic PAI-1 Binding Sites That Are Exposed on Binding to Solid Surfaces or Limited Proteolysis

Katarzyna Smolarczyk, Joanna Boncela, Jacek Szymanski, Ann Gils, Czeslaw S. Cierniewski

Objective—In this work, we identified the fibrinogen sequence that on exposure serves as the primary binding site for functionally active PAI-1 and to a lesser extent for its latent form. In contrast, this site only weakly interacts with PAI-1 substrate.

Methods and Results—The binding site is located in the N-terminal α (20-88) segment of fibrinogen, in the region exposed on (1) adsorption of fibrinogen to solid surfaces; (2) the release of fibrinopeptide A during thrombin conversion of fibrinogen to fibrin; and (3) plasmin degradation of fibrinogen. This region was first identified by the yeast 2-hybrid system, then its binding characteristics were evaluated using the recombinant α(16-120) fragment and its shorter version, the α(20-88) fragment, in a solid phase binding assay and plasmon surface resonance measurements. Because fibrinogen fragment E does not bind PAI-1, it suggests that sequences of αα chain interacting with PAI-1 are located in the N-terminal part of the α(20-88) segment.

Conclusions—Therefore, PAI-1 directly bound to the α(20-88) and thus concentrated in fibrinogen/fibrin, particularly at sites of injury and inflammation, may account for the recent observations that both its active and latent forms stimulate cell migration and wound healing. (Arterioscler Thromb Vasc Biol. 2005;25:2679-2684.)

Key Words: conformational changes fibrinogen PAI-1 binding sites proteolysis

PAI type-1 (PAI-1) is mainly identified as the primary physiological inhibitor of both urokinase-type (urokinase plasminogen activator) and tissue-type (tPA) plasminogen activators, and plays an important role in the regulation of the fibrinolytic system as well as in extra-cellular matrix remodeling. Such a broad range of biological activities of PAI-1 results from its unusual properties, ie, its inherent ability to self-inactivate and harboring interaction sites for a number of proteins. Under normal conditions, PAI-1 is present in plasma at low concentrations, although high levels are observed in a variety of clinical settings. The interaction of PAI-1 with fibrin has been extensively studied and is predominantly defined by reversible low-affinity binding. Initially, it was proposed that PAI-1 binds directly to fibrin, accumulates within human thrombi and protects fibrin clots from premature dissolution. However, recent studies provided the evidence that PAI-1 not only circulates in a complex with vitronectin but binds to fibrin via vitronectin which provides an intermolecular bridge facilitating high affinity interactions between PAI-1 and the fibrin network. (please see supplement I at http://atvb.ahajournals.org)

However, this concept does not take into consideration recent experimental observations. Local concentrations of PAI-1 can be much higher than those measured in blood plasma with levels almost equivalent to that of vitronectin. High local concentrations of fibrin-bound platelet PAI-1 were found to be 10 μg/mL or more, indicating that both high-affinity and low-affinity binding sites can be involved in anchoring PAI-1 within the fibrin network. However, this concept does not take into consideration recent experimental observations. Local concentrations of PAI-1 can be much higher than those measured in blood plasma with levels almost equivalent to that of vitronectin. High local concentrations of fibrin-bound platelet PAI-1 were found to be 10 μg/mL or more, indicating that both high-affinity and low-affinity binding sites can be involved in anchoring PAI-1 within the fibrin network. Hence, knowledge about medium or low affinity binding sites for PAI-1 and their cross-talk with high-affinity sites can be critical in understanding these mechanisms and explaining the complex biology of PAI-1. Therefore, in the present work we characterize binding sites for PAI-1 present in fibrinogen and describe their interaction with different molecular forms of PAI-1.
Materials and Methods

Proteins and Reagents

Human PAI-1 and vitronectin were purchased from Calbiochem (La Jolla, Calif). Different molecular forms of PAI-1 such as active, latent, and substrate as well as PAI-1 mutant Q123K, which does not interact with vitronectin obtained from Dr P. Declerck (Katholieke Universiteit Leuven, Belgium). Affinity-purified sheep anti-human vitronectin IgG (SAHVn) was obtained from Affinity Biologicals (Hamilton, Ontario, Canada). The BIACORE® biosensor system, reagents, and CM5 sensor chips (research grade), were from Biacore AB (Uppsala, Sweden). Vitronectin enzyme-linked immunosorbent assay kit HIVNK was purchased from Innovative Research Inc. (Southfield, Mich). Fragments D and E were kindly donated by Dr L. Medved (American Red Cross, Rockville, Md).

Fibrinogen

Fibrinogen was depleted from vitronectin by immunoaffinity chromatography using affinity-purified sheep anti-human vitronectin IgG immobilized on Sepharose 4B. Contamination of fibrinogen with vitronectin was analyzed by a sandwich-type immunoassay using the enzyme-linked immunosorbent assay kit HIVNK. Such a purification procedure reduced the vitronectin levels to approximately 100 pg per ml of 1 μmol/L fibrinogen solution.

Fibrinogen Recombinant Fragments

To obtain fibrinogen α(16-120), a 256-bp cDNA fragment corresponding to 64 to 320 bp was generated by digestion with EcoRI of pGAD-10 containing this fragment and selected after library screening. This fragment was subcloned into the EcoRI site of the pRecTA expression vector. Then, a 204-bp cDNA fragment encoding α(20-88) was generated from pRec A-αFG(16-120) and subcloned into the BamHI and Xhol sites of pRecA expression vector.

2D SDS PAGE and Microsequencing

Samples of fibrinogen and vitronectin-free fibrinogen were separated by 2D gel electrophoresis using ready-made gels with immobilized pH gradients (Amersham Biosciences) and spots expected to contain vitronectin were excised from the gel and subjected to in-gel digestion with trypsin and microsequenced using an electrospray (SSI-Q-TOF-Micromass) spectrometer as we described previously.

Enzyme Immunoassay

To monitor exposure of fibrinogen α(16-120) epitopes induced by plasmin cleavage of fibrinogen, and the presence of vitronectin in fibrinogen in both samples of fibrinogen separated by 2D SDS-PAGE, a competitive inhibition enzyme-linked immunosorbent assay was used as we described previously.

Solid Phase Binding Assays

Direct binding assays were performed according to the procedure described in our previous work by adding increasing concentrations of PAI-1 (the active, latent, or substrate form, the stable PAI-1 mutant 14-1B and PAI-1 Q123K) to the wells of 96-well microtiter plates coated with fibrinogen, fibrin, and fibrinogen recombinant fragments α(16-120) and α(20-88) or fragments D and E, respectively. The same system was used to evaluate competitive inhibition of PAI-1 binding to fibrinogen produced by plasmin derived fragments of fibrinogen. Independently, binding of PAI-1 (the active, latent, and substrate forms as well as PAI-1 mutant Q123K) was performed in Leuven according to the procedure described by Gilis et al.

Surface Plasmon Resonance

The kinetic parameters (association and dissociation rate constants, $k_a$ and $k_d$, respectively) and the affinity constant ($K_a$) for binding of the stable PAI-1 mutant 14-1B to fibrin and fibrinogen fragments were measured by surface plasmon resonance using a BIAcore X (Biacore AB) as we described previously. The overall affinity constant, $K_a$, was derived from $k_a/k_d$.

Analytical Procedures

The protein content of fibrinogen fragments was determined by the bichinchonic acid method. The purity of proteins was analyzed by SDS-PAGE.

Results

PAI-1 Binding Sites in Fibrinogen α(16-120) Sequence Identified by the 2-Hybrid System

In our preliminary studies, several proteins were identified to interact with PAI-1 after using the yeast 2-hybrid system and screening human liver cDNA libraries cloned into the GAL4 activation domain, with PAI-1 fused to the GAL4 DNA binding domain used as the bait protein. Among His lacZ colonies isolated, there was also a colony giving a strong positive reaction, identified by DNA sequencing to encode the α(16-120) fragment of fibrinogen (Figure 1, available online at http://atvb.ahajournals.org). To verify this interaction, 2 recombinant fragments of the fibrinogen α chain, α(16-120), and its shorter version α(20-88), were expressed in Escherichia coli as His-tag fusion proteins, purified on nickel chelating columns in 6 mol/L urea, and refolded by sequential dialysis to remove the denaturant. The final products were soluble in aqueous buffers and were homogenous as assessed by SDS-PAGE and reacted with polyclonal antibodies to human fibrinogen (Figure 1).

Characterization of Vitronectin-Free Fibrinogen

Because PAI-1 binds tightly to vitronectin, it was important to establish that the fibrinogen used in these studies was devoid of vitronectin. For this purpose, fibrinogen was depleted of vitronectin using immunoaffinity chromatography with immobilized sheep anti human vitronectin antibodies. The fibrinogen-vitronectin mixture (1 μg of vitronectin added to 100 μg fibrinogen) (Figure 1A, upper gel) and vitronectin-free fibrinogen (Figure 1A, lower gel) were reduced and separated by 2D gel electrophoresis. After silver staining, the 2D protein patterns of fibrinogen enriched in vitronectin clearly shows the presence of this protein migrating as a double spot. As described before, the α chain was

Figure 1. Absence of vitronectin in the purified fibrinogen. A, 2D gel electrophoresis of a fibrinogen preparation supplemented with vitronectin (1 μg of vitronectin added per 100 μg of fibrinogen; upper gel) and vitronectin-free fibrinogen (lower gel). The presence of vitronectin in 2 spots shown on the upper gel (A) was confirmed by microsequencing. B, A competitive inhibition enzyme immunoassay analysis of fibrinogen to detect the presence of contaminating vitronectin. Vitronectin (Vn), the fibrinogen and vitronectin mixture (V-C), and vitronectin-free fibrinogen (B-J) shown in (A), upper and lower gels were used as competitors. Each experimental point represents the mean value of data obtained during three separate experiments performed in triplicate.
heterogeneous both in molecular mass and charge, whereas the Bβ chain appeared as 4 distinct spots of equivalent size. Identification of protein spots migrating with size and charge similar to vitronectin was performed by peptide sequencing and peptide mass fingerprinting using an electrospray (ISIQ-TOF-Micromass) spectrometer (Table I, available online at http://atvb.ahajournals.org). There was no vitronectin detectable in the fibrinogen purified by affinity chromatography (Figure 1A). To further determine whether vitronectin was present in the fibrinogen, a competitive inhibition enzyme-linked immunosorbent assay specific for vitronectin was used (please see supplement III). Figure 1B shows that fibrinogen, when used at 100,000-fold higher concentration than vitronectin, did not produce any competitive inhibition indicating that vitronectin, if present at all, is at very low levels in the fibrinogen preparation. This was further evidenced by using a commercial sandwich-type immunoassay kit for vitronectin, which showed that 1 mg of fibrinogen used in these experiments contained <300 pg of vitronectin.

Interaction of Different Molecular Forms of PAI-1 With Fibrin and Its Fragments

In the next set of analyses, the 2 α-chain fragments were immobilized onto microtiter wells, and their binding of the stable PAI-1 mutant (14-1B) was compared with that of fibrinogen, fibrin and its 2 major cleavage products, fragments D and E. Surprisingly, PAI-1 bound to the same extent to both fibrinogen and fibrin. Both recombinant fragments, α(16-120) and α(20-88), displayed the capacity to bind PAI-1 at levels only slightly lower than those observed with fibrinogen. Both fragments D and E showed only residual ability to interact with PAI-1 (Figure 2A). Furthermore, the binding of PAI-1 to fibrinogen and fibrin was inhibited by 80% in the presence of 100-fold molar excess of the α(16-120) fragment, indicating that the latter contains a major interaction site of fibrinogen for the inhibitor (Figure 2B). To evaluate which molecular forms of PAI-1 interact with these sites we analyzed binding of active, latent, and substrate PAI-1. Figure 2C shows that these binding sites preferentially interact with active form, to a lesser extent with latent PAI-1, and only weakly with substrate PAI-1. Furthermore, PAI-1 Q123K, which has dramatically reduced binding affinity to vitronectin,26 showed unchanged binding properties toward fibrinogen when compared with the wild-type active PAI-1 (Figure 2C). Essentially the same binding characteristics were obtained when binding to fibrin was analyzed (not shown). Because PAI-1 14-1B showed the same binding characteristics as the active form of wild PAI-1, it was used in all remaining experiments to maintain PAI-1 functional activity during the extended incubations required for different assays.

PAI-1 Binding Sites and α(16-120) Sequence Are Exposed on Fibrinogen Binding to Solid Surfaces

Because PAI-1 binding to fibrinogen could be detected by a solid phase binding assay, we further tested the mechanism by which the binding sites are exposed in fibrinogen molecule. Therefore, to further characterize this reaction, the real-time bimolecular interactions in vitro during complex formation between PAI-1 and fibrinogen, fibrin or its α chain fragments, were next monitored by surface plasmon resonance. For this purpose, limiting amounts of fibrin monomers, fibrinogen, α(16-120) or α(20-88) were immobilized on separate sensor chips through amine coupling. Injection of active PAI-1 as the analyte to the sensor chip containing fibrinogen produced a typical surface plasmon resonance binding signal (Figure 3A). Similar response was produced when PAI-1 binding to fibrin was analyzed. The binding of PAI-1 was dose-dependent and the maximum response monitored at the end of the protein injection phase was 325 and 330 for 5000 RU of initially immobilized fibrinogen and fibrin monomers, respectively. The specific signal produced by binding of PAI-1 to α(16-120) or α(20-88) was equal to 300 and 290 RU for 3200 and 3100 RU, respectively, of the immobilized peptides. There was no binding of PAI-1 to the immobilized fragment D or fragment E thus supporting data produced by enzyme-linked immunosorbent assay. When PAI-1 was immobilized on a sensor chip and α(16-120) or α(20-88) were used as analytes, they showed almost the same values of analytic parameters, including $K_p$, as those estimated in the reversed system (Table). In contrast, under such conditions, soluble fibrinogen showed only residual binding to PAI-1, characterized by a $K_p$ 2 orders of magnitude higher than that calculated for binding of PAI-1 to the immobilized fibrinogen (Figure 3B). As is shown in the Table, PAI-1

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Figure 2. Binding of PAI-1 to fibrinogen and its fragments assayed by enzyme-linked immunosorbent assay. A, Increasing doses of PAI-1 mutant 14-1b were incubated in wells coated with fibrinogen, fibrin, α(16-120), α(20-88), fragment D, or fragment E, respectively. Data represent mean±SD obtained during three separate experiments. B, Inhibition of PAI-1 binding to fibrinogen- or fibrin-coated wells produced by increasing concentrations of α(16-120). C, Binding of different molecular forms of wild-type PAI-1 to fibrinogen, namely active (○–○), latent (■ □), and substrate PAI-1 (▲◆▲). In the same system binding of constitutively active mutant 14-1B (▽▽▽), as well as PAI-1 mutant Q123K, which does not interact with vitronectin is shown (●●●).
Figure 3. Exposure of the sites for PAI-1 in fibrinogen on its binding to solid surfaces. The interaction of fibrinogen with PAI-1 was assayed in 2 systems, namely when fibrinogen was immobilized on the sensor and used to bind PAI-1 (A) and in the reversed system, with PAI-1 immobilized on the sensor and fibrinogen as the soluble ligand (B). Data are representative of several runs and are expressed as relative responses after subtraction of the background signal recorded on a reference surface made up of ethanolamine-substituted dextran matrix. C, Expression of \( \alpha(16-120) \) epitopes on fibrin and fibrinogen immobilized in the plastic wells as analyzed by binding of the affinity-purified anti \( \alpha(16-120) \) polyclonal antibodies in a solid phase immunoassay.

interacts with fibrin and \( \alpha(16-120) \) with the same binding affinity. Furthermore, binding of vitronectin or PAI-1 to fibrin is described by the same values of \( K_D \), indicating the same binding affinity of fibrin for both proteins. To analyze whether binding of fibrinogen to solid surfaces results in exposure of the \( \alpha(16-120) \) sequence, we next analyzed expression of \( \alpha(16-120) \) epitopes in both fibrin and fibrinogen by a solid phase enzyme immunoassay. For this purpose, polyclonal antibodies to \( \alpha(16-120) \) were isolated from anti-fibrinogen A\(\alpha\) antiseraum by affinity chromatography using the recombinant \( \alpha(16-120) \) fragment immobilized on Sepharose 4B. Figure 3C shows binding of affinity-purified polyclonal antibodies to \( \alpha(16-120) \) epitopes on fibrinogen and fibrin but lack of binding to FgD. This experiment indicates that adsorption of fibrinogen to a solid plastic surface results in exposure of the \( \alpha(16-120) \) sequence to the same extent as that observed in fibrin.

**PAI-1 Binding Sites and \( \alpha(16-120) \) Sequence Are Exposed by Plasmin Cleavage of Fibrinogen**

Next, experiments were designed to characterize the interaction of fibrinogen with PAI-1 in a liquid phase. For this purpose, fibrinogen was first preincubated with PAI-1 in 10-fold molar excess and then aliquots of the mixture were added to the wells of 96-well microtiter plates coated with fibrinogen. Figure 4A shows that preincubation of PAI-1 with soluble fibrinogen did not influence its interaction with the immobilized fibrinogen. However, cleavage of fibrinogen with plasmin almost completely exposed PAI-1 binding sites in degradation products. Thus, 2 hours of fibrinogen digest, when preincubated with PAI-1 at a 10-fold molar excess, abolished the interaction of PAI-1 with the immobilized fibrinogen. SDS PAGE of such digests showed exclusively the presence of final degradation products of fibrinogen, namely FgD\(\alpha\), FgD\(\beta\), FgD\(\gamma\), and FgE\(\alpha\) (Figure 4A, inset). This ability to form a complex with PAI-1 in a liquid phase and thus inhibit its interaction with the immobilized fibrinogen was directly associated with a progressive exposure of \( \alpha(16-120) \) epitopes, as evidenced by a competitive inhibition enzyme immunoassay (Figure 4B). In this experiment, aliquots of the affinity-purified anti \( \alpha(16-120) \) antibodies were preincubated with increasing concentrations of a fibrinogen digest, withdrawn and quenched at different time points, and then introduced to the wells of 96-well microtiter plates coated with recombinant \( \alpha(16-120) \) fragment. The recombinant \( \alpha(16-120) \) fragment used in parallel was a control. These data indicate that after 2 hours of digestion with plasmin, \( \alpha(16-120) \) epitopes are almost fully exposed, thus supporting their role in binding of PAI-1. To further support location of the PAI-1 binding site in fibrin/fibrinogen, we next attempted to purify peptide fragments from the digestion mixture by an affinity chromatography on immobilized PAI-1. For this

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| Rate Constants for Binding of Active PAI-1 to Fibrin and Its Fragments |
|-----------------|-----------------|-----------------|
| Protein Immobilized on Sensor | Protein Ligand | \( k_{on} \) | \( k_{off} \) | \( K_D \) |
| No. | | | | M |
| 1 | Fibrin | PAI-1 | 2.11 \times 10^{4} | 3.75 \times 10^{-3} | 1.78 \times 10^{-7} |
| 2 | Fibrinogen | PAI-1 | 1.39 \times 10^{4} | 3.91 \times 10^{-3} | 2.81 \times 10^{-7} |
| 3 | \( \alpha(16-120) \) | PAI-1 | 8.33 \times 10^{3} | 1.78 \times 10^{-3} | 2.14 \times 10^{-7} |
| 4 | PAI-1 | Fibrinogen | 3.33 \times 10^{3} | 6.78 \times 10^{-3} | 2.04 \times 10^{-5} |
| 5 | PAI-1 | \( \alpha(16-120) \) | 6.23 \times 10^{3} | 1.42 \times 10^{-3} | 2.28 \times 10^{-7} |
| 6 | PAI-1 | \( \alpha(20-88) \) | 1.08 \times 10^{3} | 8.51 \times 10^{-3} | 7.88 \times 10^{-6} |
| 7 | PAI-1 | Vitronectin | 11.9 \times 10^{3} | 6.90 \times 10^{-5} | 5.79 \times 10^{-11} |
| 8 | Fibrin | Vitronectin | 5.45 \times 10^{3} | 1.51 \times 10^{-3} | 2.77 \times 10^{-7} |

The \( k_{on} \) and \( k_{off} \) were determined from the association and dissociation phases, respectively, with 4 different concentrations of \( \alpha_{1} \)-acid glycoprotein and vitronectin. Apparent \( K_D \) corresponds to the \( k_{off}/k_{on} \) ratio.
purposely, the 120 minutes of plasmic digest of fibrinogen was subjected into the recombinant PAI-1 immobilized on the Ni$^{2+}$-HiTrap column attached to fast protein liquid, then the column was washed extensively with 0.14 mol/L NaCl buffered with 0.1 sodium phosphate, pH 7.3, and peptides bound were eluted with 0.1 mol/L acetic acid. The bound fragments were identified by sequencing using an electrospray (ISI-Q-TOF-Micromass) spectrometer (please see supplement IV). There were several short fragments of fibrinogen identified in peptide material eluted from the column. Among them there were αN$^{16}$SLFEYQR$^{26}$, αG$^{20}$DFSSANNR$^{104}$, as well as αD$^{17}$YEDQQKLEQVIK$^{91}$. Two first peptides derived from the region of α chain which was originally identified by the yeast 2-hybrid system to bind PAI-1.

Discussion

In this work we provide evidence that PAI-1 interacts with the fibrinogen α(16-120) sequence (see supplement V), which is cryptic in the intact fibrinogen molecule but can be exposed either on its adsorption to solid surfaces or proteolytic cleavage by thrombin and plasmin. This conclusion is supported by the following observations. This fibrinogen segment first identified by the yeast 2-hybrid system to bind PAI-1, when obtained as the recombinant peptide, interacted predominantly with active PAI-1. The affinity-purified anti-α(16-120) polyclonal antibodies bound to fibrinogen immobilized on the plastic wells, indicating that their α(16-120) epitopes are well exposed (see supplement VI). Based on the recently published crystal structure, this fragment is located entirely in the α-helical coiled-coil domain region of fibrinogen. Tryptic digest of the peptide material eluted from the immobilized PAI-1 showed a presence of several short peptides including αN$^{16}$SLFEYQR$^{26}$, αG$^{20}$DFSSANNR$^{104}$, which are from the same region as α(16-120). Interestingly, these tryptic peptides are derived from the soluble fibrin degradation products released from a fibrin clot perfused with plasmin. Fibrinogen and fibrin, when immobilized on the plastic wells or covalently attached to BIACore CM5 chips, bound PAI-1 with the same binding capacity and affinity. They interacted the most efficiently with active PAI-1, to lower extent with its latent form, and weakly with cleaved PAI-1 (see supplement VII). Soluble fibrinogen did not bind to BIACore CM5 chips with immobilized PAI-1 and did not compete for PAI-1 with the immobilized fibrinogen even when used in a large molar excess. It proves that PAI-1 binds to fibrinogen only when the latter is adsorbed to solid surfaces but does not interact with soluble fibrinogen (see supplement VIII). Degradation of fibrinogen with plasmin progressively exposes cryptic α(16-120) sequences, and plasmic fragments obtained after 2 hours showed almost complete expression of both α(16-120) epitopes and PAI-1 binding sites. Vitronectin and PAI-1 individually bind to different regions of fibrin but show the same binding affinity for fibrin, with measured $K_D$ of $2.58 \times 10^{-7}$ and $1.88 \times 10^{-7}$ M, respectively. PAI-1 directly binds to the N-terminal region of the α chain, which is in close vicinity to fibrinopeptide A being released by thrombin during conversion of fibrinogen to fibrin. Thus, this region is exposed in fibrin monomer, explaining why PAI-1 preferentially recognizes fibrin over soluble fibrinogen. Binding sites for vitronectin were suggested to be localized in the C-terminal part of the fibrinogen γ chain, namely the γAγ′ variant. The apparent binding affinity estimated by SPR ($K_D$ of 0.26 μmol/L) is higher but consistent with that previously determined by solid-phase binding assay ($K_D$ of ~0.60 μmol/L) in which radiolabeled vitronectin was bound to pre-formed fibrin matrices.

PAI-1 release can be induced from a number of cells (platelets, monocytes, macrophages, endothelial cells, and smooth vessel cells) by several cytokines during different processes including thrombosis, wound healing, and inflammation. Thus, it is conceivable to expect accumulation of PAI-1 at sites of injury and inflammation and fibrin seems to be the best candidate to concentrate this inhibitor. This effect may be particularly important in the case of platelets, which release large amounts of PAI-1. Although, there is a continuous production of active PAI-1 in platelets, only a small proportion of PAI-1 was reported to be functionally active. Thus, PAI-1 released from platelet α-granules is mostly in the latent state and it cannot interact with vitronectin, which is known to exclusively bind the active inhibitor. It suggests that large amounts of PAI-1, which are found in fibrin clots and that originated from activated platelets are directly bound to the α(20-88) region of fibrin. Previous studies described 2 classes of binding sites in fibrin that interact with the activated PAI-1. The first class consisted of a small number of high-affinity sites with a $K_D$ < 1 nM, whereas the second class, recognizing both active and latent PAI-1, contained a large number of low-affinity sites with an approximate $K_D$ of 3.8 μmol/L. Our present studies identified the nature of the second class and verified its binding affinity by measuring the apparent dissociation constant to be >10-fold lower than that published previously. Interestingly, the PAI-1/vitronectin complex can potentially interact with the same binding affinity with 2 sites of fibrin, the first primarily recognizing PAI-1 (α52–88) and the second one interacting with vitronectin (γAγ′). In the thrombus, all these sites are likely to be saturated because of the high concentration of fibrin-bound platelet PAI-1$^{18,9}$ (see supplement IX).

Acknowledgments

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Vitronectin is the mediator of fibrin-associated PAI-1 activity and plays a significant role in the regulation of PAI-1-mediated inhibition of clot lysis.\(^1\) Thus, the role of fibrin binding sites directly interacting with PAI-1 was proposed to be diminished due to the fact that plasma concentrations of PAI-1 are in the nanomolar range, whereas the concentration of vitronectin is in the micromolar range. Therefore, all active plasma PAI-1 should be expected to be in complex with vitronectin.

**Supplement II**

**MATERIAL AND METHODS**

**Proteins and reagents**

Human PAI-1 and vitronectin were purchased from Calbiochem (La Jolla, CA). The stable PAI-1 mutant (14-1B) and vitronectin previously characterized by Lawrence et al.\(^2\) was purchased from Calbiochem. Different molecular forms of PAI-1 such as active, latent, and substrate as well PAI-1 mutant Q123K which does not interact with vitronectin obtained from Dr. P. Declerck (Katholieke Universiteit Leuven, Belgium). Human glu-plasminogen, \(\alpha\)-thrombin, and plasminogen-free fibrinogen were purchased from Enzyme Research Laboratories, Inc. (South Bend, IN). Mouse monoclonal antibody to vitronectin was from Chemicon International, Inc. (Temecula, CA). Affinity-purified sheep anti-human vitronectin IgG (SAHVn) was obtained from Affinity Biologicales (Hamilton, Ontario, Canada). Mouse monoclonal antibodies to PAI-1 (MAI-12 and clone 7) were purchased from Biopool (Umea, Sweden). Anti-rabbit IgG-HRP Conjugate, 5-bromo-4-chloro-3-indolyl-\(\beta\)-D-galactopyranoside, and Wizard Miniprep and Maxiprep kits for isolation of plasmid DNA were purchased from Promega Corp. (Madison, WI). Protein A/G was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). High binding 96-well microtiter plates were obtained...
from Costar Science Corp. (Cambridge, MA). Alkaline phosphatase-conjugated goat anti-rabbit and anti-mouse IgG were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). The BIACORE® biosensor system, reagents, and CM5 sensor chips (research grade), were from Biacore AB (Uppsala, Sweden). Vitronectin ELISA assay kit HVNKT was purchased from Innovative Research Inc. (Southfield, MI, USA). Fragments D and E were kindly donated by Dr. L. Medved (American Red Cross, Rockville, USA). MATCHMAKER Two-Hybrid System together with cDNA library from human liver, were obtained from Clontech Laboratories (Palo Alto, CA).

**Two-hybrid procedures, library screening and evaluation of protein-protein interactions**

Cloning of plasmids was done in *E. coli* strain DH5α while the yeast strain Y190 was used to assay protein-protein interactions and for library screening. PAI-1 cDNA was polymerase chain reaction amplified using Pfu polymerase (Stratagene, La Jolla, CA) and subcloned into the EcoRI and BamHI restriction sites of pAS2-1 to produce pAS2-1-PAI-1. DNA sequencing of the resulting product by the use of Sequaterm kits (Epicentre) revealed an in-frame fusion of PAI-1 to the 3’ end of the GAL4 DNA binding domain. The GAL4-PAI-1 fusion protein was stably expressed in yeast as evidenced by immunoblotting using monoclonal antibody to PAI-1. Concentrations of PAI-1 were determined by ELISA using the Imulyse PAI-1 kit from Biopool (Umea, Sweden). Furthermore, PAI-1 in this construct was able to inhibit t-PA-induced plasminogen conversion to plasmin. The two-hybrid assay using the GAL4 system was performed according to the instructions of the manufacturer (Clontech). For library screening, Y190 yeast cells were transformed with a liver two-hybrid library inserted into the activation vector pGAD10, and pAS2-1-PAI-1 was used as bait. Doubly transformed cells were plated on Leu’, Trp’, His’ deficient plates. Colonies were picked, restreaked onto double minus plates, and assayed for the *LacZ* phenotype. Yeast transformants were permeabilized in liquid nitrogen and transferred on nitrocellulose soaked
in 60mM Na$_2$HPO$_4$, 40mM NaH$_2$PO$_4$, 10mM MgCl$_2$, 50mM β-mercaptoethanol and containing 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside at 30°C. Positive blue colonies appeared in 1 hour (strong interaction) to 6 hours (weak interaction). Library clones from colonies His$^+$, Lac$^+$ were isolated and re-transformed alone with pAS2-1, with pAS2-1-PAI-1 and pLAM5' and checked again for β-galactosidase activity.

**Fibrinogen digestion with plasmin**

Fibrinogen was depleted from vitronectin by immunoaffinity chromatography using affinity-purified sheep anti-human vitronectin IgG immobilized on Sepharose 4B. Contamination of fibrinogen with vitronectin was analyzed by a sandwich-type immunoassay using the ELISA assay kit HVNKT (Innovative Research Inc., Southfield, MI, USA). Such a purification procedure reduced the vitronectin levels to approximately 100 pg per ml of 1 µM fibrinogen solution. Vitronectin-free fibrinogen (2 mg/ml) was digested with plasmin (0.3 U/ml) in PBS, pH 7.4. At the selected time points, aliquots were withdrawn and the reaction stopped by adding a cocktail of protease inhibitors (Complete) from Roche, 4x SDS sample buffer and boiling. Then, samples of fibrinogen digest were used in functional assays and competitive inhibition ELISAs as well as in SDS-PAGE performed under non-reducing conditions.

**Fibrinogen recombinant fragments**

To obtain fibrinogen α(16-120), a 256 bp cDNA fragment corresponding to 64-320 bp was generated by digestion with EcoRI of pGAD-10 containing this fragment and selected after library screening. This fragment was subcloned into the EcoRI site of the pRsetA expression vector. A 204 bp cDNA fragment encoding α(20-88) was generated from pRset A-αFg16-120 and using primers: forward with BamHI site (5’CACAGCAGGATCCGCAGATAG3’) reverse with XhoI site (5’GTGAATTCTCGAGCTTTATTATTCG3’). This fragment was subeloned into the
BamHI and XhoI sites of pRset A expression vector. The inserts were verified by DNA sequencing and then the relevant plasmid was used to transform E. coli BL21 competent cells.

Expression of recombinant proteins was induced by adding isopropyl thio-β-D-galactoside to E. coli BL21/Fgα16-120 culture, grown in TB medium with 100 µg/ml ampicillin. After 4 h at 37°C the bacterial culture was harvested by centrifugation at 1000 x g at 4°C for 30 min. The bacterial pellet was frozen at -70°C overnight and subsequently suspended in binding buffer (20mM Tris-HCl pH 7.9, 0.5M NaCl, 0.1% Triton X-100 (v/v)). Cells were sonicated on ice for four 45 s bursts with 2 min intervals. The sonication mixture was centrifuged for 30 min at 27,000 x g at 4°C. The sonication and centrifugation were repeated. The lysate was incubated 12 h at 4°C in binding buffer containing 6M urea then centrifuged for 30 min 50,000 x g at 16°C, the supernatant was recovered and subjected to Ni²⁺-HiTrap column purification. The recombinant fragments were isolated under denaturing conditions on a Ni2+ HiTrap column as described by the manufacturer (Amersham Biosciences). The eluate was then dialyzed against TBS buffer, pH 7.4, containing 0.06M urea.

2D SDS PAGE and microsequencing

Samples of fibrinogen and vitronectin-free fibrinogen were separated by 2D gel electrophoresis using ready-made gels with immobilized pH gradients (Amersham Biosciences). For the first dimension, samples containing 5 µg of fibrinogen in Lysis Buffer were mixed with the IPG Reswelling Solution (8 M urea, 1% CHAPS, 0.4 % DTT, 0.5 % Pharmalyte) to obtain a final volume of 450 µl. Then they were loaded onto 24 cm immobilized pH linear gradient strip gels (pH 3-10). IEF strips were allowed to rehydrate for 5 h, and isoelectric focusing was performed according to the manufacturer’s protocol by a gradual increase of voltage (30 V for 5 h, 500 V for 1 h, 1000 V for 1h, followed by 70 kVh at 8000 V) using an IPGphor system (Amersham Biosciences). SDS electrophoresis was
performed on 12.5% polyacrylamide gels using the Ettan Dalt vertical system. Protein spots were visualized by staining with silver according to the method compatible with the analysis of proteins by mass spectrometry. Proteins spots expected to contain vitronectin were excised from the gel and subjected to in-gel digestion with trypsin and microsequenced using an electrospray (ISI-Q-TOF-Micromass) spectrometer as we described previously.  

**Enzyme immunoassay**

To monitor exposure of fibrinogen α(16-120) epitopes induced by plasmin cleavage of fibrinogen, and the presence of vitronectin in fibrinogen in both samples of fibrinogen separated by 2D SDS PAGE, a competitive inhibition ELISA was employed as we described previously.  

For this purpose, antibodies to fibrinogen α(16-120) were purified from rabbit anti-fibrinogen Aα antiserum by affinity chromatography using recombinant α(16-120) fragment immobilized on Sepharose 4B. The wells of 96-well microtiter plates were coated overnight at 4°C with recombinant α(16-120) fragment at 1.0 µg/ml in PBS. Unbound proteins were washed from the wells, and nonspecific binding sites were blocked by incubation with TBS, pH 7.5, containing 1% BSA and 0.01% Tween 20 for an hour at room temperature. In competitive inhibition experiments, the affinity-purified anti-α(16-120) polyclonal antibodies, producing 60% of the binding to the immobilized α(16-120), were preincubated overnight at 4°C with 50 µl of the serial dilutions of fibrinogen digest samples withdrawn at different time points and analyzed by ELISA using plastic wells coated with recombinant α(16-120). Then, 100 µl aliquots of the incubation mixtures were added to the wells coated with recombinant α(16-120) and incubated overnight at 4°C. Plates were then extensively washed with TBS and incubated with goat anti-rabbit secondary antibodies conjugated with horseradish peroxidase. The reaction was developed using ABTS (2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) from Sigma at 1mg/ml in 0.1 M sodium citrate, pH 4.5, and the change in color was determined at 405 nm. To detect nonspecific
binding, all assays were simultaneously done on plates coated with BSA alone and processed as described above. The background binding to BSA was subtracted from all samples before data analysis. Data were plotted as the percent of binding versus molar concentration of the competing protein. Experimental points were fitted to the sigmoidal curve by Graph PAD™ program, version 2.

Similarly, the presence of vitronectin in fibrinogen in both samples of fibrinogen separated by 2D SDS PAGE was analyzed, but the wells were coated with vitronectin, and anti-vitronectin polyclonal antibodies as well as the serial dilutions of either fibrinogen or vitronectin were used.

Solid Phase Binding Assays

The wells of 96-well microtiter plates were coated overnight at 4°C with fibrinogen, fibrin or fibrinogen fragment α(16-120) at 1 µg/ml in PBS. Unbound proteins were washed from the wells, and nonspecific binding sites were blocked by incubation with 3% BSA, 0.05% Tween 20 in PBS for an hour at room temperature. Direct binding assays were performed according to the procedure described in our previous work³ by adding increasing concentrations of PAI-1 (the active, latent or substrate form, the stable PAI-1 mutant 14-1B and PAI-1 Q123K) to the wells of 96-well microtiter plated coated with fibrinogen, fibrin, fibrinogen recombinant fragments α(16-120) and α(20-88) or fragments D and E, respectively, in TBS, pH 7.5, containing 1% BSA, 0.01% Tween 20, and 1mM CaCl₂ and then incubated for an hour at 37°C. Unbound PAI-1 was aspirated and the wells were washed three times with PBS containing 0.1% BSA and 0.05% Tween 20. The same system was used to evaluate competitive inhibition of PAI-1 binding to fibrinogen produced by plasmin derived fragments of fibrinogen. For this purpose PAI-1 (44 nM) was first preincubated for an hour at RT with 10-fold excess of fibrinogen digests in PBS, pH 7.4, containing 0.1% BSA, 0.002% Tween 80, then added to the wells coated with fibrinogen at 4 µg/ml in PBS.
To detect bound PAI-1, the plates were incubated with polyclonal antibodies to PAI-1 (American Diagnostica) for an hour at room temperature, and then washed with PBS containing 0.1% BSA and 0.05% Tween 20. The plates were incubated with followed by goat anti-rabbit secondary antibodies conjugated with horseradish peroxidase and the reaction was developed using ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) from Sigma at 1mg/ml in 0.1M sodium citrate, pH 4.5, and the change in color was determined at 405 nm.

To detect nonspecific binding, all assays were simultaneously done on plates coated with BSA alone and processed as described above. The background binding to BSA was subtracted from all samples before data analysis. Independently, binding of PAI-1 (the active, latent, and substrate forms as well as PAI-1 mutant Q123K) was performed in Leuven according to the procedure described by Gils et al.6

**Surface Plasmon Resonance**

The kinetic parameters (association and dissociation rate constants, $k_{on}$ and $k_{off}$, respectively) and the affinity constant ($K_d$) for binding of the stable PAI-1 mutant 14-1B to fibrin and fibrinogen fragments were measured by surface plasmon resonance (SPR) using a BIAcore X (Biacore AB) as described in our previous work.3 Briefly, fibrin, fibrinogen, PAI-1 or fibrinogen fragments were covalently attached to carboxymethyl dextran (CM5) chips (BIAcore) previously activated with N-hydroxysuccinimide and N-ethyl-N'-dimethylaminopropyl carbodiimide according to the manufacturer’s instructions. Experiments were performed at 23°C using as a running buffer HEPES-buffered saline/BIA (50mM HEPES pH 7.4, 150mM NaCl, 3.4mM EDTA, 0.005% P20). The sensor chip was regenerated with a short pulse of 200nM glycine-HCl, pH 2.2. The amount of the relevant protein ligand (PAI-1, fibrinogen, $\alpha_{16-120}$, $\alpha_{20-88}$) bound to immobilized proteins was monitored by measuring the variation of the surface plasmon resonance angle as a function of time. The data obtained for at least three different concentrations of protein ligands were
fitted to several models and the best fits (Chi$^2$ =1.4-2.0) were obtained assuming either a one-to-one or a one-to-two interaction for PAI-1 binding to monovalent (α chain fragments and vitronectin) or bivalent (fibrinogen and fibrin monomers) proteins, respectively. The association rate constant, $k_{on}$, and dissociation rate constant, $k_{off}$, were determined separately from individual association and dissociation phases, respectively. The overall affinity constant, $K_D$, was derived from $k_{on}/k_{off}$.

**Analytical procedures**

The protein content of fibrinogen fragments was determined by the BCA method. The purity of proteins was analyzed by SDS-PAGE.

**Supplement III**

In this assay, the plates were coated with 0.2 μg of vitronectin and the anti-vitronectin antibodies used at the dilution producing 60% of the binding were preincubated with serial dilutions of either vitronectin or fibrinogen. Then, 100 μl aliquots of the incubation mixture were added to the wells and changes in absorbance at 405 nm were measured after developing the reaction.

**Supplement IV**

For this purpose, the eluted peptide fragments were subjected to reduction with 10 mM dithiothreitol, alkylation with 50 mM iodoacetamide, and tryptic digestion with the modified trypsin (10 μg/ml; Promega) at 37 °C for 14 h. The product peptides were extracted stepwise with three portions 60 μl 0.1% TFA- 2% acetonitrile and loaded on an RP-18 pre-column (LC Packings). Peptides were eluted to a nano-HPLC RP18 column (75 μm x15 cm capillary; LC Packings) by acetonitrile gradient in the presence of formic acid and directly applied into an electrospray (ISI-Q-TOF- Micromass) spectrometer. The spectrometer was working in the
regime of data dependent MS to MS/MS switch giving peptide sequencing data in addition to mass fingerprint data.

**Supplement V**

Numerous studies have indicated the critical role of molecular vehicles of plasma PAI-1 in modulation of fibrinolysis and reorganization of the clot network. PAI-1 circulates as a complex with the abundant plasma glycoprotein vitronectin, and this interaction stabilizes the inhibitor in its active conformation. A similar function can be displayed by plasma α₁-acid glycoprotein which, via binding PAI-1, prolongs its inhibitory activity and provides an alternative reservoir of the physiologically active form of the inhibitor, particularly during inflammation or other acute phase reactions. Unexpectedly, recent observations showed that both active and latent PAI-1 itself have profound effects on cells and their association with the extracellular matrix. Thus, it appears that proteins that contribute to the accumulation and concentration of PAI-1 at the cellular membranes or in the extracellular matrix can play a significant although indirect role in the regulation of PAI-1 mediated processes.

**Supplement VI**

The antibodies did not bind to fibrinogen used as a competitor in liquid phase competitive inhibition enzyme immunoassay, proving cryptic location of the α(16-120) sequence in the intact molecule. The complex with PAI-1 is more rapidly formed by α(16-120) and remains more stable when compared to that of α(20–88). This means that the release of the C-terminus of this fragment slightly impaired the binding site for PAI-1. Since fibrinogen fragment E containing Aα chain segment (1-51) does not bind PAI-1, it may be concluded that sequences of α chain interacting with PAI-1 are located within the N-terminal part of the α(20-88) segment.
Supplement VII

Furthermore, they bound PAI-1 Q123K mutant to the same extent as wild type PAI-1 proving that even if there were traces of vitronectin, which were not detectable by 2D SDS-PAGE but were estimated by a sandwich-type vitronectin enzyme immunoassay to be approximately 0.000003%, this binding was mediated via the fibrinogen α(16-120) sequence.

Supplement VIII

This is in agreement with previous observations suggesting that such unmasking of cryptic regions in fibrinogen upon its adsorption to solid surfaces, for example on implanted biomaterials, can be responsible for its conversion to a proinflammatory state.11

Supplement IX

Furthermore, under some conditions PAI-1 can bind to fibrin efficiently, particularly when vitronectin is engaged in the interaction with other proteins, for example with high molecular weight kininogen. Although having no direct effect on PAI-1 activity itself, high molecular weight kininogen was found to displace PAI-1 from the complex with vitronectin and markedly attenuated the vitronectin-dependent inhibition of uPA- or tPA-mediated plasminogen activation by PAI-1.11

In summary, PAI-1 recognizes at least two apparently medium affinity binding sites on fibrin localized in the N-terminal regions of the α chain adjacent to the thrombin sensitive peptide bond. These sites recognize both active and latent forms of PAI-1. Thus, PAI-1 concentrated in fibrin, particularly at sites of injury and inflammation, may account for the recent observations that both active and latent forms of this protein stimulate cell migration in chemotaxis, haptotaxis, chemokinesis and wound healing assays.
References


Table I

**Vitronectin in fibrinogen preparation separated by 2D PAGE.** Vitronectin was added to fibrinogen preparation (1µg of vitronectin per 340 µg of fibrinogen) and separated by 2D electrophoresis (Figure 2A, upper panel). Protein spots were identified after silver staining and analyzed by sequencing as described in Material and Methods. Table columns are as follows: gi, gi accession numbers of the identified proteins in the protein database; Mass, predicted molecular masses of identified proteins; Peptides matched, numbers of peptides identified from the amino acid sequences of assigned proteins; Total score, total probability scores from the Mascot search for identified proteins.

<table>
<thead>
<tr>
<th>Spot</th>
<th>gi</th>
<th>Protein</th>
<th>Mass</th>
<th>Peptides matched</th>
<th>Total score</th>
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<td>Vitronectin precursor</td>
<td>55069</td>
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Figure I  Fibrinogen α chain fragment provides binding sites for PAI-1.  Panel A shows a 312 bp cDNA fragment encoding fibrinogen α(16-120) identified by the yeast two hybrid system to interact with PAI-1.  After generation from the selected pGAD-10 by digestion with EcoRI, this fragment was subcloned into the EcoRI site of the expression plasmid pRsetA and the inserted sequences were verified by electrophoresis (lane 1, DNA ladder standard; lane 2 – 7, the inserted 312 bp fragment after EcoRI digestion of pRsetA isolated from different bacteria clones) and sequencing.  Panel B shows recombinant α(16-120) expressed in E. coli, purified by Ni²⁺ affinity chromatography and analyzed by SDS-PAGE (lanes 1-3, eluted fractions; lane 4, molecular weight markers).  Panel C shows recombinant α(20-88) produced as described above and separated by SDS-PAGE.  Panel D shows Western blotting of both α(16-120) and α(20-88) stained with rabbit serum anti-human fibrinogen (lane 1, α(20-88); lane 2, α(16-120); lane 3, reduced human fibrinogen).