New insights into the size and stoichiometry of the plasminogen activator inhibitor type-1·vitronectin complex

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Plasminogen activator inhibitor-type 1 (PAI-1) is the primary inhibitor of endogenous plasminogen activators that generate plasmin in the vicinity of a thrombus to initiate thrombolysis, or in the pericellular region of cells to facilitate migration and/or tissue remodeling. It has been shown that the physiologically relevant form of PAI-1 is in a complex with the abundant plasma glycoprotein, vitronectin. The interaction between vitronectin and PAI-1 is important for stabilizing the inhibitor in a reactive conformation. Although the complex is clearly significant, information is vague regarding the composition of the complex and consequences of its formation on the distribution and activity of vitronectin in vivo. Most studies have assumed a 1:1 interaction between the two proteins, but this has not been demonstrated experimentally and is a matter of some controversy since more than one PAI-1-binding site has been proposed within the sequence of vitronectin. To address this issue, competition studies using monoclonal antibodies specific for separate epitopes confirmed that the two distinct PAI-1-binding sites present on vitronectin can be occupied simultaneously. Analytical ultracentrifugation was used also for a rigorous analysis of the composition and sizes of complexes formed from purified vitronectin and PAI-1. The predominant associating species observed was high in molecular weight (\(M_w = 320,000\)), demonstrating that self-association of vitronectin occurs upon interaction with PAI-1. Moreover, the size of this higher order complex indicates that two molecules of PAI-1 bind per vitronectin molecule. Binding of PAI-1 to vitronectin and association into higher order complexes is proposed to facilitate interaction with macromolecules on surfaces.

Vitronectin is a versatile glycoprotein that is found in circulating, in the extracellular matrix of endothelial cells, in platelets, and within various tissues of the human body. Circulating at micromolar levels, vitronectin participates in the regulation of humoral responses such as coagulation, fibrinolysis, and the complement cascade (reviewed in Refs. 1–4). Other functions of the protein that are confined to surfaces or tissues include cell-adhesion and regulation of pericellular proteolysis. Interactions with an assortment of biological molecules are responsible for the multiple functions exhibited by vitronectin. Defining the binding sites for these various biomolecules, along with determining the molecular mechanism of regulation, constitutes an active area of research on the protein. Work to date has focused on binding sites for several ligands, including heparin, PAI-1, and integrins; a working model representing the domain structure of vitronectin has been recently reported from this laboratory (5).

Arguably, one of the most important interactions known for vitronectin occurs with the serine protease inhibitor, PAI-1. PAI-1 is the physiological inhibitor of plasminogen activators, both tPA and uPA, the enzymes responsible for generating plasmin from its inactive zymogen precursor, plasminogen, and ultimately leading to clot lysis. In addition to contributing to the delicate balance required between coagulation and thrombolysis, PAI-1 plays a role in regulating the proteolytic processes responsible for tissue remodeling and metastasis (6). Although PAI-1 is inherently rather unstable, converting readily from an active to an inactive, latent form, vitronectin binds to PAI-1 and maintains the inhibitor in its active conformation for a longer period of time. Indeed, most circulating PAI-1 is thought to be complexed with vitronectin, so that the complex serves as a reservoir of the physiologically active form of PAI-1 (7).

The conversion of active PAI-1 to its latent form is appreciated at the structural level from x-ray crystallographic work by Goldsmith and colleagues (8). The active-to-latent transition of PAI-1 involves incorporation of a surface-exposed loop containing the reactive center into the central \(\beta\)-sheet of the protein. Using site-directed mutagenesis (9) and monoclonal antibodies (10), a vitronectin-binding site on the surface of PAI-1 has been localized to residues that lie within the central \(\beta\)-sheet and within adjacent secondary structures. An appealing interpre-

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1 The abbreviations used are: PAI-1, plasminogen activator inhibitor type 1; serpin, serine protease inhibitor; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; NBD, 4,4′-dimethyl-\(N\)-acetyl-\(N\)-\(\beta\)-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine; S338C, recombinant PAI-1 with cysteine substituted for serine 338 (the F9 position); NBD-p PAI-1, S338C mutant form of PAI-1 labeled with NBD; mAb, monoclonal antibody; BSA, bovine serum albumin; PBS, phosphate-buffered saline.

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tation of these results is that vitronectin contacts residues on adjacent structures in the vicinity of the central β-sheet of the protein, thus constraining movement of the strands that is necessary for incorporation of the reactive loop into the central β-sheet in latent PAI-1 (9). The limited freedom of movement of the β-strands to accommodate loop insertion accounts for the slower rate of conversion of PAI-1 to a latent form when complexed with vitronectin.

On the other hand, the localization of the binding site for PAI-1 on vitronectin has been more controversial. The PAI-1-binding site has been evaluated using proteolysis, synthetic peptides, and site-directed mutagenesis; however, conclusions from these studies are contradictory (reviewed in Ref. 11). The N-terminal 44 amino acids (known as the "somatomedin B" region) of the protein are proposed by some groups to constitute the PAI-1-binding site (12–15). Recent evidence that indicates this region of the protein is responsible for binding PAI-1 comes from work using isolated recombinant N-terminal fragments from vitronectin that bind and stabilize PAI-1 (14). Furthermore, site-directed mutagenesis within the recombinant somatomedin B region has identified all 8 cysteines and several other residues within the 44-amino acid stretch as important for binding (15).

Other investigators have identified sequences distal to the N-terminal somatomedin B region to be involved in binding of the serpin. Notably, some have localized PAI-1 binding to the positively charged region of the protein from amino acids 345–379 (16–20), the site known to be responsible for heparin binding to vitronectin. Proteases that cleave within the heparin-binding region have been shown to diminish PAI-1 binding to vitronectin, and a recent study demonstrated stabilization of PAI-1 activity in the presence of a synthetic peptide derived from the heparin-binding region (21). Also, Seiffert (22) suggests that ligand binding to the C-terminal heparin-binding region regulates the binding of PAI-1 to the N-terminal somatomedin B region. Seiffert and Smith (23) further argue that the N-terminal somatomedin B and cell-binding regions are cryptic in the native structure of vitronectin. Thus, reports in the literature support PAI-1 binding and stabilization via the N-terminal somatomedin B region of the protein are proposed by some groups to constitute the PAI-1-binding site (12–15). Recent evidence that indicates this region of the protein is responsible for binding PAI-1 comes from work using isolated recombinant N-terminal fragments from vitronectin that bind and stabilize PAI-1 (14). Furthermore, site-directed mutagenesis within the recombinant somatomedin B region has identified all 8 cysteines and several other residues within the 44-amino acid stretch as important for binding (15).

A major objective of this work was the direct demonstration of the size and stoichiometry of PAI-1-vitronectin complexes. These experiments are important for several reasons: (i) more than one binding site for PAI-1 has been reported on vitronectin; (ii) the binding stoichiometry for the interaction has not been carefully evaluated previously; (iii) a proposal has been made that PAI-1 binding induces self-association of vitronectin (25), and (iv) formation of the complex alters functional properties and stabilities of the two interacting proteins. Accurate methods for determining molecular weights and stoichiometries are needed to address these issues. In this study, analytical ultracentrifugation was used to directly observe complexes that form in solution. The sedimentation equilibrium method provides accurate determinations of molecular weights for mixtures of interacting components. Monoclonal antibodies for different epitopes on vitronectin provided evidence for two PAI-1-binding sites. The findings expand on the initial observation that PAI-1 promotes association of vitronectin by demonstrating a binding stoichiometry for PAI-1-vitronectin that is not 1:1, as well as a discrete associating species that is formed as the two proteins interact.
**RESULTS**

Monoclonal Antibodies Specific for Different Epitopes on Vitronectin Were Used to Evaluate the Models of One versus Two PAI-1-binding Sites on the Protein—Monoclonal antibodies against specific sites on vitronectin have been particularly useful over the years in localizing functions on the protein. However, in the case of PAI-1 binding to vitronectin, the results using different antibodies have been ambiguous. The monoclonal antibodies most frequently used in structure-function work relating to PAI-1 binding to vitronectin are mAb 8E6 (34, 35), specific for an epitope within the large central CNBr fragment from vitronectin (36), and mAb 153, which recognizes the PAI-1-binding epitope within the extreme N-terminal somatomedin B region (14). Both antibodies have been shown to block PAI-1 binding to vitronectin; in the case of mAb 8E6, the interference with PAI-1 binding was proposed to result from masking of the heparin-binding site (16). To further evaluate one versus two potential binding sites for PAI-1 on vitronectin, these antibodies were directly compared in this study for their ability to compete with PAI-1 for binding to vitronectin.

Fig. 1A presents data from an experiment in which vitronectin is coated on microtiter plates, followed by incubation with a fixed concentration of PAI-1 in solution with varying concentrations of either mAb 8E6 or 153. The results show that both of the monoclonal antibodies tested interfere with PAI-1 binding to vitronectin. However, it is notable that neither of the antibodies fully blocks binding. In fact, each reduces binding of PAI-1 to vitronectin only by about half. This is the first time that effects of these antibodies on PAI-1 binding have been directly compared in the same assay, and the results are compelling in their support of two distinct PAI-1-binding sites on vitronectin.

Fig. 1B summarizes results from a similar experiment in which vitronectin is immobilized on the solid-phase, followed by incubation with varied concentrations of PAI-1 in the presence of a fixed concentration of one of the two mAbs. Bound PAI-1 is detected in this experiment using a functional assay based on inactivation of uPA by the serpin. Both monoclonal antibodies interfere with the binding of PAI-1, with a decreased amount of active PAI-1 bound. Moreover, neither of the mAbs inhibited 100% of the PAI-1 binding. The addition of both antibodies in these experiments inhibited PAI-1 binding to a greater extent than observed with either antibody alone, with inhibition to >90%.

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**Fig. 1. Effects of anti-vitronectin mAbs on PAI-1 binding to vitronectin.** Panel A, microtiter plate wells were coated overnight with 50 µl of vitronectin (1 µg/ml). After washing, the wells were blocked with 3% BSA in PBS and then incubated with PAI-1 (2 µg/ml) in the presence of the indicated concentrations of either mAb 153 (open triangles) or mAb 8E6 (closed circles). Bound PAI-1 was detected with 125I-labeled MAI-12. The amount of PAI-1 bound is expressed on the y-axis as a percentage of the PAI-1 bound to vitronectin in the absence of any competing antibodies. Panel B, microtiter plate wells were coated overnight with 50-µl solutions of vitronectin (1 µg/ml). After washing, the wells were blocked with 3% BSA in PBS. Then varied concentrations of PAI-1 were incubated with the bound vitronectin in the presence of fixed concentrations of mAb 153 (5 µg/ml; closed squares), mAb 8E6 (5 µg/ml; closed triangles), or buffer only (open circles). After washing, the wells were incubated with 160 units/ml urokinase for 30 min at room temperature; remaining urokinase activity was determined by monitoring hydrolysis of chromogenic substrate for uPA, S-2444 (1 mM concentration), at 405 nm. The concentration of functional PAI-1 bound to vitronectin on the solid-phase was calculated relative to a titration curve using known concentrations of PAI-1 and uPA in solution. The amount of active PAI-1 is expressed relative to the maximal amount bound in these experiments at saturating PAI-1 concentrations (≈0.5 µg/ml) in the absence of either competing antibody.
In light of the observation that PAI-1 may bind at active PAI-1 and 2

\[ 10^3 \text{M} \]

Microtiter wells coated with either mAb 153 (open triangles) or mAb 8E6 (closed circles) were incubated with vitronectin at a saturating concentration (2 μg/ml in PBS). Subsequently, the bound vitronectin was incubated with varying concentrations of the antibody that was not used in the capture phase, i.e. mAb 8E6 or mAb 153, respectively. The wells were then washed thoroughly and incubated with PAI-1 (2 μg/ml concentration in PBS), and bound PAI-1 was detected using [125I]-labeled MAI-12. Bound radioactivity is expressed as a percentage of the amount of PAI-1 bound to vitronectin in the absence of the second competing anti-vitronectin mAb.

In a different approach to evaluate whether the two monoclonal antibodies compete for PAI-1-binding sites on vitronectin, separate sets of microtiter plates were coated with either of the two antibodies. Subsequently, vitronectin was specifically bound via the corresponding epitope recognized by the solid-phase antibody. Immobilizing vitronectin in an immunospecific fashion such as this avoids conformational perturbations that are known to occur when vitronectin is adsorbed to plastic surfaces (37–39). This was reasoned to be a better approach for testing effects of blocking both epitopes on vitronectin because all vitronectin bound to the solid-phase is, by design, blocked at one epitope. Thus, the bound vitronectin retains its native conformation for binding to PAI-1 or to the second antibody. Homogeneity of both antibodies binding to solid-phase vitronectin would not be expected due to conformational changes that are known to affect immunoreactivity. PAI-1 was then incubated with the bound vitronectin in the presence of varying concentrations of the other mAb that was not used in the capture phase. As the results in Fig. 2 illustrate, PAI-1 binding to vitronectin is severely impaired (>90%) in the presence of both antibodies. That is, PAI-1 can be bound to vitronectin if only one of the epitopes is occupied by antibody, but its binding is negligible when both epitopes are blocked specifically with the corresponding monoclonal antibodies. Interestingly, the percentage of PAI-1 bound is nearly equivalent in both scenarios, regardless of which antibody is used in the capture or competing phases in the study.

**Sedimentation Equilibrium Measurements Were Used to Observe the Sizes of Complexes Formed between Vitronectin and PAI-1**—In light of the observation that PAI-1 may bind at either or both of two potential binding sites on vitronectin, a set of experiments was designed using various ratios of vitronectin to PAI-1 to evaluate the molar equivalence of complexes. Table I summarizes the efforts to characterize these two interacting proteins using ultracentrifugation, including experiments that were conducted with altered ratios of the two proteins, different protein preparations and variations in rotor speeds. Representative results from a sedimentation equilibrium experiment with \( 2 \times 10^{-6} \text{ M} \) active PAI-1 and \( 2 \times 10^{-6} \text{ M} \) vitronectin are shown in Fig. 3. Experimental data points representing the radial distribution of total absorbance at 280 nm are shown in the solid squares. The smooth curve through the data represents a non-linear fit composed of the sum of the radial distributions for the contributing species. The best fit to the data required a model with more than one interacting component, with a distribution of the two species along the radial length of the cell. Mathematical deconvolution of the data into the contributing species is shown by the two exponential traces below the experimental data in Fig. 3. This analysis of the data is rigorous and treats complex formation as a function of the concentrations of the interacting proteins which varies along the length of the cell at equilibrium as a function of rotor speed, temperature, and total protein concentration.

At equilibrium, only two protein species are detected; one has a molecular weight of 72,000, corresponding to the molecular weight of vitronectin, and a second species is observed with a molecular weight of 317,000. Negligible free PAI-1 (molecular weight of 43,000) is detected. Similarly there is no evidence for a protein species with intermediate molecular weight, i.e. between 72,000 and 320,000. The high molecular weight species clearly represents a complex formed by vitronectin and PAI-1. The analysis for the size of the complex indicates a species with a calculated mean molecular weight of 324,000 ± 14,000 that was fairly consistent among a number of analyses (Table I) with mixtures of the interacting proteins tested under a variety of conditions. The analysis of the data is not indicative of a broad distribution of oligomeric species in these experiments.

The observation of only two species at equilibrium indicates that an equimolar mixture of proteins does not give a stoichiometric complex or a depletion of free reactants under these conditions. Mixing equal, approximate micromolar concentrations of the two proteins would have been expected to yield a species of 115,000 molecular weight, with little free vitronectin or PAI-1, if a tight complex with 1:1 stoichiometry were formed. First, the complex formed is not a simple 1:1 complex of vitronectin and PAI-1, as it is larger than the expected 115,000; and second, there is apparently some free vitronectin “leftover” that is not found as part of the high molecular weight complex, although there are negligible amounts of free PAI-1, arguing against a 1:1 stoichiometry for the reactants. The findings are more consistent with the binding of two PAI-1 molecules to each molecule of vitronectin, with an overall stoichiometry of 4 PAI-1:2 vitronectin yielding a complex of 316,000 molecular weight. (The molecular weight for the 2:1 complex equals 158,000.) The analysis of equilibrium sedimentation data summarized in Table I, along with the immunochemical data, argue strongly for this 4:2 complex. Note that a 3:3 complex of PAI-1:vitronectin would have a predicted molecular weight of 345,000 that is near the upper limit of these measurements and may be within experimental error. However, the composition of a 3:3 complex is less compatible with the immunochemical data arguing for binding at two sites on vitronectin. Moreover, a stoichiometric n:n complex (with n equal to any number of vitronectin and PAI-1 monomers) is not compatible with the ultracentrifugation results that show a substantial amount of free vitronectin when equimolar concentrations of the two proteins are mixed.
As a test for association between vitronectin and the latent form of PAI-1, an equimolar mixture of the two proteins was analyzed by sedimentation equilibrium. As shown in Fig. 4, the exponential distribution of protein in the cell corresponded to the sum of both of the free proteins, with no evidence for association to give a complex. This result supports previous work that used other methods to show that latent PAI-1, as well as any form of PAI-1 with a rearranged central β-sheet containing the fifth β-strand, bound only weakly to vitronectin (40). The analytical ultracentrifugation experiment used here extends these previous findings by showing that micromolar concentrations of vitronectin and latent PAI-1 do not associate.

A Unique Label on PAI-1 Indicates That the Serpin Is Present in the High Molecular Weight Complex—A powerful approach that was used in these experiments to distinguish the two reactant proteins was to specifically incorporate a chromophore into PAI-1 (30). A mutant form of PAI-1 with a cysteine substituted at the P9 position was labeled with NBD, providing a probe specific to PAI-1 that is monitored in the visible wavelength range. The fact that the NBD-labeled PAI-1 absorbs maximum for the NBD probe on the inhibitor. Once again, the distribution of absorbing species at equilibrium cannot be adequately fit to only a single species. Measurements at this wavelength clearly show the presence of the high molecular weight (approximately 320,000) complex, and there is also evidence for some free labeled PAI-1 (due to a small amount of latent protein in the PAI-1 preparation). This experiment conclusively demonstrates the presence of PAI-1 as an integral component of the complex, as opposed to the complex being formed solely of self-associated vitronectin species.

Fig. 5B shows the results of the same sample mixture analyzed at 280 nm, which detects both PAI-1 and vitronectin, rather than at 501 nm for PAI-1 components only. The fits shown use the concentration of free PAI-1 and PAI-1-containing complex determined using the 501 nm data in Fig. 5A. The protein species observed are quite consistent in size and relative concentration with the results summarized in Table I. Note that the amount of free (latent) PAI-1 is the same in both panels, although it appears more prominently in data collected at 501 nm because vitronectin does not contribute to absorbance at this wavelength. The fact that the NBD-labeled PAI-1 forms the same ~320,000 molecular weight complex with vitronectin as does wild-type PAI-1 (Table I) in the ultracentrifuge studies confirms the observation that the label on PAI-1 does not perturb its function or interaction with vitronectin (30, 41). The ultracentrifugation data indicate that the composition of the complex formed from an equimolar mixture of PAI-1 and vitronectin is not equimolar. Free vitronectin in addition to the complex is observed in these mixtures of the two proteins, and the data support a model for a complex composed of two molecules of vitronectin and four molecules of PAI-1 with a predicted molecular weight of 316,000. Obviously, it is not possible from these data alone to distinguish between a model in which two PAI-1 molecules are bound to a single site versus a model...
with two distinct binding sites for PAI-1 on vitronectin.

A rigorous analysis of the composition of the complex comes from calculations on the sedimentation equilibrium data to determine actual concentrations of labeled PAI-1 and the high molecular complex as a function of radial distribution in the experiment. The data at 501 nm together with the data at 280 nm which were obtained from the same experiment, as shown in Fig. 6, can be used to directly assess the stoichiometry of labeled protein (NBD-PAI-1) within the high molecular weight complex. The absorbance due to the complex alone at 501 and 280 nm were obtained by subtracting the spectral contributions of free vitronectin and free PAI-1 (known values from the non-linear fits). This allows for calculation of absorbance at 501 and 280 nm that exclusively correspond to the proteins within the complex. From the extinction coefficients for NBD at 501 nm and for vitronectin and PAI-1 at 280 nm we determined the number of NBD-labeled PAI-1 molecules within the complex. As shown in Fig. 6, this ratio is not a function of radial position indicating that the number of NBD-PAI molecules in the complex is constant across the cell. The average stoichiometry of NBD-PAI-1 within the complex is approximately 3.5 ± 0.5, providing substantial support for the proposed composition of the complex.


discussion

The fundamental objective of this work was to determine the size and stoichiometry of the PAI-1-vitronectin complex. The technique of analytical ultracentrifugation was selected because it provides a rigorous approach to the measurement of protein molecular weights in solution under equilibrium conditions. Prior to this study, the binding data for PAI-1 and vitronectin had been interpreted assuming a single PAI-1-binding site with a unique affinity. Results from enzyme-linked immunosorbent-type assays that have primarily been used in the past are difficult to reconcile because variable binding affinities are observed, depending on which reactant is immobilized on the plastic surface (42). It is difficult to estimate stoichiometries of binding from these types of experiments, and attempts to quantify binding stoichiometries have varied from a ratio of 1:1 (PAI-1:vitronectin) with immobilized PAI-1 to a ratio of 1:3 (PAI-1:vitronectin) using immobilized vitronectin (42). In this report, a combination of immunoassays using two monoclonal antibodies specific for different epitopes on vitronectin, and the sensitive ultracentrifugation method to evaluate molecular weights of associating systems, has clarified stoichiometry issues and provided a new picture of the PAI-1-vitronectin complex.

a higher order complex is formed when PAI-1-vitronectin complexes associate—On first inspection, the sedimentation equilibrium data clearly indicated that a complex other than a simple 1:1 product formed when vitronectin and PAI-1 interact. The molecular weight for the complex was consistently found to equal about 320,000, and the species of equilibrium using equimolar mixtures of vitronectin and PAI-1 clearly included lower molecular weight material (free vitronectin or free latent PAI-1) in addition to the large complex. The ultracentrifuge data also show that at equilibrium there are no detectable intervening molecular weight species that would correspond to potential stable intermediates from the assembly process. Thus, in the time required to achieve sedimentation equilib-
**Stoichiometry of Vitronectin-PAI-1 Complexes**

A great deal of controversy surrounds the issue of PAI-1-binding sites on vitronectin, with sites localized to two distinct regions of vitronectin. The immunochemical studies performed here provide insight into the fundamental reason for the debate, namely that both of the regions appear to be functional PAI-1-binding sites. The mole ratio of 4:2 PAI-1:vitronectin suggested from the ultracentrifugation experiments was supported by the explicit evaluation of two anti-vitronectin monoclonal antibodies that had given apparently contradictory results in the past regarding PAI-1 binding to vitronectin. The problem had been that independent research groups had used these antibodies to consider only one PAI-1-binding site on vitronectin, residing in the vicinity of one or the other antibody epitope. This study demonstrates that PAI-1 binding to vitronectin occurs simultaneously with either of the antibodies, and PAI-1 binding is fully blocked only if the two binding sites on vitronectin are saturated with both monoclonal antibodies. Thus, the data from this study are consistent with binding of PAI-1 at both sites.

**A Different Scheme for Association of PAI-1 and Vitronectin Complex Must Be Considered**—An important result from this study is the demonstration of a higher-order complex that contains molecules of both vitronectin and PAI-1. Although the idea that PAI-1 binding produces multimeric forms of vitronectin has been suggested previously (25), the possibility that the complex between vitronectin and PAI-1 is not a binary 1:1 species has not been considered or addressed experimentally. This is despite the observation that circulating PAI-1 is quantitatively bound to vitronectin, and that early size-exclusion chromatography on the plasma proteins produced a complex with a molecular weight larger than the 1:1 form (7). The multifaceted approach to address the questions of reaction...
FIG. 7. A model for the association of PAI-1 and vitronectin into large complexes with a stoichiometry of 4:2 (PAI-1-vitronectin). Putative intermediates in the assembly process that are not populated at equilibrium are shown in brackets. The model assumes binding of PAI-1 at two putative sites on vitronectin. Either the 1:1 or the 2:1 (PAI-1-vitronectin) intermediate associates with a second complex via self-association of the vitronectin components. Following dissociation of the complex and accumulation of latent PAI-1 at long times, the model proposes that vitronectin remains self-associated. It is postulated that even higher order oligomers form from the 4:2 complex or from associated vitronectin following conversion of PAI-1 to the latent form.

A model for the PAI-1-vitronectin complex that assumes binding of PAI-1 at two putative sites on vitronectin is shown in Fig. 7. In the model, PAI-1 binding leads to association of vitronectin. The self-association of vitronectin may be favored upon binding of one PAI-1 molecule, or the binding of PAI-1 to both sites may be required before the higher order complex is formed. PAI-1 is stabilized in the complex, although PAI-1 that is not associated rearranges to the latent form at long times. Latent PAI-1 and vitronectin do not interact, so the latent form of the inhibitor accumulates. The low temperature used for these experiments should be noted, as it allowed the observation of the 4:2 complex, with little conversion of PAI-1 to a latent form that would not associate, and with little propensity of the complex to form higher order aggregates that may be favored at higher temperature. The self-associated forms of vitronectin remain intact after conversion of all PAI-1 to the latent form, producing a stable altered form of vitronectin that is multivalent, as previously proposed (25). Under some conditions, even higher order oligomers may form from the 4:2 complex or from the associated vitronectin that remains after PAI-1 is released. Note that aged samples exhibit species of even higher molecular weight that may correspond to higher order aggregates that form after dissociation of the complex and conversion of PAI-1 to a latent form. \(^3\) The higher order complex observed in the ultracentrifugation experiments supports the hypothesis that PAI-1 binding promotes self-association of vitronectin.

At the micromolar protein concentrations used in this work, PAI-1 binds to both sites and gives large complexes. The PAI-1 concentration used experimentally is clearly higher than that in the circulation, which is approximately 0.5 nM. However, the configuration of PAI-1 in either venous or arterial thrombi is determined by a series of orders of magnitude greater than in the circulation (43–47). Recent immunohistochemical studies suggest that, like PAI-1, vitronectin is concentrated along fibrin fibrils within thrombi in situ (45). Also, vitronectin and PAI-1 are stored in relatively high concentrations within platelet α-granules, and the majority of vitronectin released upon platelet activation is high in molecular weight and at least partially associated with PAI-1 (48). Thus, it appears that the complexes observed in the ultracentrifugation experiments can readily form in the vicinity of a blood clot. In fact, recent studies by Podor and colleagues (49) indicate that vitronectin binds directly to fibrin clots, \(^4\) and the fibrin-associated vitronectin mediates the high-affinity binding of PAI-1 to fibrin.

The model in Fig. 7 proposes a physiological means to promote association of vitronectin into higher order species. Similar to the advantage offered by multiple heparin-binding sites on vitronectin, the self-association of vitronectin may aid in binding to other ligands, especially cell surface receptors. Other work has shown that multimeric forms of vitronectin are preferentially endocytosed via integrins in fibroblasts (50), and by non-integrin-mediated mechanisms in megakaryocytes (50), which target the internalized vitronectin to α-granule structures containing PAI-1 (51). In addition, only vitronectin multimers are functional in binding to the uPA receptor (52, 53). PAI-1 may be the physiological stimulus for association of vitronectin to achieve the "clustering" of receptor-binding sites required for biological activity.

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\(^3\) K. H. Minor, M. N. Blackburn, and C. B. Peterson, unpublished observations.

Stoichiometry of Vitronectin·PAI-1 Complexes