Serine proteinase inhibitors, including plasminogen activator inhibitor type 1 (PAI-1) and antithrombin, are key regulators of hemostatic processes such as thrombosis and wound healing. Much evidence suggests that PAI-1 can influence such processes, as well as pathological events like tumor metastasis, through its ability to directly regulate binding of blood platelets and cells to extracellular substrata. One way that PAI-1 influences these processes may be mediated through its binding to the plasma protein vitronectin. Binding to PAI-1 results in the incorporation of vitronectin into a higher order complex with a potential for multivalent interactions (Podor, T. J., Shaughnessy, S. G., Blackburn, M. N., and Peterson, C. B. (2000) J. Biol. Chem. 275, 25402–25410). In this study, evidence is provided to support this concept from studies on the effects of PAI-1-induced multimerization on the interactions of vitronectin with matrix components and cell surface receptors. By monitoring complex formation and stability over time using size-exclusion high performance liquid chromatography, a correlation is made between PAI-1-induced multimerization and enhanced cell/matrix binding properties of vitronectin. This evidence indicates that PAI-1 alters the adhesive functions of vitronectin by converting the protein via the higher order complex to a self-associated, multivalent species that is functionally distinct from the abundant monomeric form found in the circulation.

The interactions that occur between cellular receptors and proteins that constitute the extracellular matrix are vital to physiological control of processes like cell adhesion and pericellular proteolysis. Events that alter these interactions can be deleterious, leading to pathological sequelae such as improper wound healing and tumor cell migration or metastasis. For example, components of the humoral response system known as fibrinolysis, which play a role in modulating various cell-binding properties of the extracellular matrix, can be exploited by cancerous cells. By altering the content and activity of proteins related to fibrinolysis, abnormally developing cells can acquire the ability to exit residing tissues and eventually invade and metastasize. An example involves the main regulator of fibrinolysis, PAI-1, in which increased levels of the protein in plasma correlate with the presence of malignant ovarian cancer and higher incidence of disease (1). PAI-1 is a member of the serpin family and represents a key regulatory protein in proteolytic processes responsible for tissue remodeling and tumor metastasis. This property is owed to the fact that PAI-1 is the main inhibitor of both plasminogen activators, uPA and tPA. Interesting features of PAI-1 include its structural lability and the propensity it exhibits to spontaneously adopt a more stable, but inactive conformation. This feature of PAI-1 is unique among the serpin family, with active PAI-1 exhibiting a half-life of ~90 min (2). In the body, however, this transition of PAI-1 into an inactive form is prevented by another important homeostatic protein, vitronectin, which binds to PAI-1 and increases the half-life of the active form approximately 2–4-fold (3). The fact that nearly all PAI-1 in the human vasculature is complexed with vitronectin underscores the importance of considering vitronectin when investigating PAI-1-related functions such as cell attachment and spreading (3).

Vitronectin is a versatile, multifunctional protein found both in circulation and the ECM. It is involved in several pathways relevant to such physiological events as blood coagulation and fibrinolysis, cellular immunity, and tumor metastasis (4–7). The diverse functionality of vitronectin arises from the ability of the protein to interact with many ligands in addition to PAI-1, including cellular receptors (such as integrins and uPA receptor), glycosaminoglycans (such as heparin), extracellular components (such as collagen), and complement complexes (such as C5b-9a). Interestingly, binding to some of these ligands, including PAI-1 and complement components, causes vitronectin to experience conformational changes that alter certain functions. Reports from this laboratory (8) and others (9) indicate that these conformational changes promote the formation of large oligomeric forms of vitronectin. These multimeric forms of vitronectin exhibit an apparent increase in heparin affinity because of multivalent binding sites for the polyanionic glycosaminoglycan (10). Are other ligand-binding
sites clustered in a similar fashion on the surface of the multimeric form of vitronectin, resulting in enhanced binding?

It is intriguing to consider the potential effects of PAI-1 binding and induced multimerization of vitronectin on other functional properties of vitronectin such as cell adhesion and migration. Several lines of evidence indicate that PAI-1 and vitronectin may serve as co-factors in these processes. Vitronectin becomes incorporated into the matrix from a circulating pool by unknown mechanisms, and its deposition into the ECM of many tissues may be a means of localizing PAI-1 function to specific sites, i.e. regions of tissue injury or tumor invasion. Vitronectin contains an important integrin-binding site (an RGD sequence) near its amino-terminal end that is capable of binding cellular integrins (11, 12). Current understanding dictates that PAI-1 binding to vitronectin antagonizes the cell-binding functions of vitronectin. This effect is proposed to result from steric hindrance because of the proximity of the PAI-1-binding site near the N terminus and an adjacent RGD site comprising residues 45–47 (13–15). On the other hand, the effects on cell binding activity that result from PAI-1-induced changes in the oligomeric state of vitronectin have not been carefully studied. Does self-association of vitronectin into multimers, as a consequence of PAI-1 binding, result in an enhanced binding to integrins and components of the ECM because of its multivalent nature? The main objective of this study was to investigate the effect that PAI-1-induced multimerization has on the adhesive functions of vitronectin.

MATERIALS AND METHODS

Proteins and Antibodies—Native vitronectin was purified from human blood plasma using a modified protocol of the method developed by Dahlback and Podack (16, 17). Human GPIbIbIIIa was obtained from Enzyme Immunoassay Systems Inc. Native vitronectin was phosphorylated using [32P]ATP (Amersham Biosciences, Inc.) and protein kinase A (catalytic subunit, 20 (to prevent nonspecific binding), and the samples were incubated for 24 hours at 37 °C, or it was purchased from Molecular Innovations. Latent PAI-1 was either prepared by 24-hour incubation of wild-type PAI-1 at 37 °C, or it was purchased from Molecular Innovations. The S119C mutant of PAI-1 was labeled with NBD as previously described (18). The labeled PAI-1 was a generous gift from Duane Day and Joseph Shore (Henry Ford Hospital, Detroit, MI). Human ECM and Matrigel were purchased from Becton Dickinson. Polyclonal antibodies against human vitronectin were generated in rabbits by contract with Rockland Laboratories. A monoclonal antibody specific for PAI-1 was the kind gift of Dr. Tom Podor (Hamilton Civic Hospitals Research Center, Hamilton, Ontario, Canada). Peroxidase-labeled secondary antibodies (anti-rabbit and anti-mouse) were obtained from Vector Laboratories. Protein standards used for HPLC came from Amersham Biosciences Inc. and protein kinase A (catalytic subunit, product of Sigma). Unincorporated radiolabel was removed by a desalting centrifugation step using Bio-Spin 30 columns. Specific radioactivity was determined directly by quantifying vitronectin in a BCA (Amersham Biosciences, Inc.) and protein kinase A (catalytic subunit, product of Sigma). Unincorporated radiolabel was removed by a desalting centrifugation step using Bio-Spin 30 columns. Specific radioactivity was determined directly by quantifying vitronectin in a BCA assay (Pierce) and measuring radioactivity in a Beckman LS5801 Scintillation Counter. Specific radioactivity was 100,000 cpm/pmol of vitronectin.

Size Exclusion HPLC—Chromatography of vitronectin-PAI-1 mixtures was carried out using a Phenomenex Biosep SEC-S3000 (300 × 7.8 mm) column attached to a Hewlett-Packard 1100 series HPLC system. PBS containing 0.05% sodium azide was used for the reaction and isotropic mobile phase. Equimolar concentrations of vitronectin and PAI-1 were mixed and incubated for various times at 37 °C. In some experiments, an equimolar amount of tPA was added at the end of the incubation period to inactivate PAI-1 and dissociate it from vitronectin. On occasion, NBD-S119C PAI-1 was used in the experiment to explicitly monitor the absorbance of NBD at 494 nm as a probe for PAI-1. Samples (50 μl) were injected onto the pre-equilibrated size-exclusion column and chromatographed at room temperature at a flow rate of 1 or 0.5 ml/min. Absorbance was detected directly in the flow cell at 280 or 494 nm.

SDS-PAGE and Western Blotting—Samples from HPLC (100 μl) were analyzed by PAGE on 10% polyacrylamide SDS gels (19). Proteins were transferred to nitrocellulose filters using a Semi-Dry Blotter (Bio-Rad) in transfer buffer (0.35 M glycine, 25 mM Tris, containing 20% methanol). The filters were blocked as with 10% nonfat dry milk in PBS.

The nitrocellulose membrane was washed three times after this and subsequent incubation steps with PBS containing 0.05% Tween 20. Immunoblotting was performed to detect vitronectin and PAI-1 simultaneously by incubating the filter with a 1:2500 dilution of polyclonal anti-vitronectin rabbit serum and a 1:2500 dilution of polyclonal anti-PAI-1 in a 2.0% solution of nonfat dry milk in PBS. The blot was then incubated with a 1:1000 dilution of both HRP-linked goat anti-rabbit and HRP-linked rabbit anti-mouse antibodies in a 20-ml solution of 2.0% milk in PBS. Immunostained protein bands were visualized by developing in a freshly prepared solution of 30 ml of PBS containing 50 mg/ml 4-chloronapthol and a 1:3,000 dilution of 30% H2O2.

Fluorescence Measurements—Changes in the fluorescence emission of NBD-S119C-PAI-1 were measured using the PerkinElmer LS580B luminescence spectrometer with an excitation wavelength of 480 nm and emission wavelength of 525 nm. Excitation and emission slits were set at 4 and 7 nm, respectively. Solutions of NBD-PAI-1 (200 nm) in HEPES buffer (100 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.1% (w/v) polyethylene glycol 8000, pH 7.4) were titrated with small aliquots of monomeric human vitronectin to a final concentration of 550 nm. Samples were mixed in a total volume of 2.0 ml in acrylic cuvettes (Starstedt) that were pre-coated with a 1.0% (w/v) solution of polyethylene glycol 20,000, according to the procedure of Lalatto and Hall (20). Titrations were performed in triplicate. All data were mathematically corrected for dilution change in emission. Effects of nonspecific binding were determined by quantifying vitronectin in a BCA (Amersham Biosciences, Inc.) and protein kinase A (catalytic subunit, 20 (to prevent nonspecific binding), and the samples were incubated for various times at 37 °C. Following washing with buffer containing BSA and Tween, blocked wells were layered with vitronectin-PAI-1 mixtures and incubated at 37 °C for 1 h. Following incubation, mixtures were removed, washed with PBS, and bound vitronectin was detected by two different methods. For the human ECM binding assays, detection was immunochromically, using a polyclonal anti-vitronectin IgG and a peroxidase-conjugated anti-rabbit IgG. The plates were then developed with a 0.2 mg/ml solution of ABTS in 50 mM sodium citrate, pH 5.5, containing a 1:2000 dilution of 3% hydrogen peroxide. ELISA plates were read (λ = 405) on a Wallac Victor® ELISA plate reader. For the Matrigel-binding experiments, 32P-labeled vitronectin was used and detected by scintillation counting. Nonspecific binding was monitored in all experiments and found to be minimal. Effects of nonspecific binding were subtracted from the reported results. Assays were also performed to test the effects of ionic strength and tPA-mediated release of PAI-1, using equimolar concentrations of tPA and PAI-1.

Integrin Binding Assays—ELISA plates (polystyrene, CoStar) were coated with 500 ng of human ECM or 2.5 μg of Matrigel in PBS. Wells were washed three times with PBS and then blocked with 3.5% BSA for 1 h at 37 °C. Vitronectin and PAI-1 were incubated in integrin-binding buffer containing 0.2% BSA and Tween 20 (to prevent nonspecific binding), and the samples were incubated for various times at 37 °C. Following washing with buffer containing BSA and Tween, blocked wells were layered with vitronectin-PAI-1 mixtures and incubated at 37 °C for 1 h. Following incubation, mixtures were removed, washed, and bound vitronectin was detected by two different methods. For the human ECM binding assays, detection was immunochromically, using a polyclonal anti-vitronectin IgG and a peroxidase-conjugated anti-rabbit IgG. The plates were then developed with a 0.2 mg/ml solution of ABTS in 50 mM sodium citrate, pH 5.5, containing a 1:2000 dilution of 3% hydrogen peroxide. ELISA plates were read (λ = 405) on a Wallac Victor® ELISA plate reader. For the Matrigel-binding experiments, 32P-labeled vitronectin was used and detected by scintillation counting. Nonspecific binding was monitored in all experiments and found to be minimal. Effects of nonspecific binding were subtracted from the reported results. Assays were also performed to test the effects of ionic strength and tPA-mediated release of PAI-1, using equimolar concentrations of tPA and PAI-1.

Integrin Binding Assays—ELISA plates (polystyrene, CoStar) were coated with 250 ng of GPIbIbIIIa or α5β1, in integrin-binding buffer (50 mM NaCl, 1 μM CaCl2, 1 mM EDTA, 1 mM Cys, and 0.5% BSA for 1 h at 37 °C. Equimolar or 1:2 mixtures of vitronectin and PAI-1 were incubated in integrin-binding buffer containing 0.2% BSA and 1% Tween 20 (to prevent nonspecific binding) for various times at 37 °C. Following washing with integrin-binding buffer containing BSA and Tween, blocked wells were labeled with vitronectin-PAI-1 mixtures...
and incubated at 37 °C for 1 h. Incubation with secondary antibody–enzyme conjugates and development with substrates were performed as described above for the matrix-binding assays. Assays were also performed to test the effects of metals, ionic strength, and tPA-mediated release of PAI-1, using equimolar concentrations of tPA and PAI-1.

**RESULTS**

*Size Exclusion HPLC Was Used to Monitor the Formation of Large Complexes Resulting from PAI-1 Binding to Vitronectin—*An underlying hypothesis of this work is that the interaction of vitronectin with circulating target ligands, such as PAI-1, results in the formation of large complexes that may have enhanced binding to other potential targets, including heparin and cell-surface receptors. The increased binding is because of the multivalent nature of the binding sites for these ligands on the self-associated form of vitronectin (10). For the purposes of this study, namely to investigate the effects of PAI-1 induced multimerization of vitronectin, an HPLC method of size-exclusion chromatography was employed to monitor the formation of large multivalent complexes.

Fig. 1 shows chromatograms of equimolar mixtures of vitronectin and PAI-1 that were incubated for various times. Separation immediately following mixing of vitronectin and PAI-1 demonstrated the fast formation of very large complexes (elution time = 6 min). From 1 to 4 h, significant amounts of the high molecular weight complexes accumulated. Analysis at long times (up to 36 h) demonstrated that high molecular weight forms of vitronectin persisted even after the disassociation of PAI-1 from the complex upon its conversion to a latent form.

In the HPLC experiments, NBD-S119C PAI-1 provided a chromophore to directly determine whether PAI-1 was an integral part of the high molecular complexes that form. As shown in Fig. 2A, the elution profile at 494 nm (the extinction maximum for NBD) confirms that PAI-1 is present in the high molecular weight complexes formed after 1 h of incubation. Furthermore, SDS-PAGE and Western blotting (Fig. 2B) demonstrated that these large complexes contained both vitronectin and PAI-1. The same approach indicated that the high molecular weight species observed at long times (e.g. 36 h) comprised oligomeric forms of vitronectin without associated PAI-1. As a control, latent PAI-1 did not cause the formation of large complexes when mixed with vitronectin. These results are in agreement with findings from Seiffert and Loskutoff (9), as well as with a recent study from this laboratory that demonstrated self-association of vitronectin when complexed with PAI-1 (8).

The information obtained from this HPLC gel filtration approach was instructive for the remainder of this study. By monitoring the formation of complexes upon vitronectin binding to PAI-1 over time, we could correlate this information with effects that PAI-1-induced multimerization has on the adhesive properties of vitronectin. We were especially interested in functions relative to matrix and/or cell interactions.
the matrix and which may be mediated by heparin-like glycosaminoglycans. The ability of vitronectin to interact with both integrins and matrix proteins places vitronectin at the boundary of cell-matrix interactions and activities. Results presented in Fig. 3 address this idea by demonstrating that vitronectin, when incubated with PAI-1, shows increased binding to matrix-coated ELISA plates. The enhanced binding of vitronectin upon incubation with PAI-1 was demonstrated using two commercially available matrices, human ECM and Matrigel. Binding of complexes is observed with both matrices and is detected using either immunochemical (panel A) or radiological detection (panel B), indicating a specific effect of PAI-1 on matrix incorporation of vitronectin. These findings support a model in which PAI-1 promotes formation of vitronectin multimers that have enhanced affinity for components that constitute the ECM.

Although equimolar mixtures of vitronectin and PAI-1 exhibited enhanced association with the ECM, as shown in Fig. 3, we considered whether nonstoichiometric mixtures of the two proteins would vary in matrix-binding behavior. This experiment was initiated in light of our recently published results that demonstrate the association of vitronectin and PAI-1 into complexes with a 2:4 stoichiometry (8). Thus, the concentration of vitronectin was fixed in the ECM-binding experiments, whereas the concentration of PAI-1 was varied from less than stoichiometric to a 4-fold excess relative to vitronectin. As shown in Fig. 4, matrix binding is dependent on the amount of PAI-1 present, saturating at a higher value for relative binding than observed for a simple 1:1 ratio of the two proteins in Fig. 3. For this reason, subsequent ECM-binding experiments were performed using a 2:1 ratio of PAI-1:vitronectin, and these results show a greater enhancement in ECM binding than the titrations shown in Fig. 3. Latent PAI-1, tested over a wide range in concentrations, had no effect on matrix binding by vitronectin.

The Time Course of PAI-1-induced Multimerization of Vitronectin Parallels Enhanced Association with the ECM—To correlate the observed effects of PAI-1 binding on vitronectin adhesive functions and the formation of large multimeric complexes as a result of this interaction, these properties were carefully monitored over time. Mixtures of vitronectin and PAI-1 were incubated for various times and then added to ECM-coated ELISA plates. Fig. 5A demonstrates the significant increase in the amount of vitronectin bound to matrix when PAI-1 is added versus native vitronectin alone. Strikingly, this increase in matrix deposition persists at long times after which PAI-1 is no longer present in the large complexes. These results indicate that PAI-1 binding causes a consistent enhancement of the adhesive form of vitronectin in a temporal pattern similar to that of complex formation and vitronectin multimerization observed in the HPLC time course described above.

For an explicit test of the requirement for PAI-1 in the complexes for the enhancement in matrix binding, tPA was added to the incubation mixtures at timed intervals. This protease is known to associate with PAI-1 to form a stable acylcomplex, thus disrupting the vitronectin-PAI-1 interaction (21). Dissociation of the vitronectin-PAI-1 complex by adding tPA at early times (~1 h) diminishes binding to the matrix, as shown in Fig. 5A. Note that the binding observed is nevertheless enhanced relative to the binding of monomeric vitronectin. Indeed, analysis by HPLC gel filtration (Fig. 5B) demonstrated that, by 1 h, a stable multimeric form of vitronectin that more readily associates with the matrix is present, and that it persists even after tPA-mediated release of PAI-1 from the complexes. The fact that tPA treatment reduces the amount of complexes observed indicates that active PAI-1 is present in these high molecular weight complexes formed at 1 h. From the decrease in peak height eluting at the void volume of the column, the findings also indicate that some of the complexes can disaggregate when tPA neutralizes PAI-1 and dissociates it.
vitronectin oligomers are apparent after PAI-1 is neutralized by tPA. As elution
pared with the chromatograms in Figs. 1 and 2 by expressing the data
for these experiments is 0.5 ml/min; these data can be directly com-
ination of an equimolar amount of tPA (a solid line), equimolar mixtures of
mixtures has little effect, indicating that the aged vitronectin
on PAI-1 (22). Binding isotherms for NBD-S119C-PAI-1 and
utated for serine 119, situated near the vitronectin-binding site
ing affinity for the interaction. Under both salt conditions,
a 70%. These results indicate that the
binding of vitronectin to ECM.
from vitronectin. However, at longer times, adding tPA to the
mixtures has little effect, indicating that the aged vitronectin
aggregates exhibit enhanced matrix binding that is independ-
ent of PAI-1. Presumably, this reflects the multimeric nature of
the large complexes that persist after PAI-1 dissociates.
Enhancement of Binding of PAI-1-Vitronectin Complexes to
ECM Is Dependent on Ionic Strength—As shown in Fig. 6,
increasing ionic strength can reverse the association of
vitronectin complexes with the matrix upon PAI-1 binding. At
a 1 mM NaCl concentration, the enhancement of vitronectin bind-
ing is decreased by ~70%. These results indicate that the
binding of vitronectin-PAI-1 complexes to components of the
extracellular matrix is also partly dependent on ionic interac-
tions, such as those known to occur between vitronectin and
glycosaminoglycans.
To ensure that these results are not because of an effect of
ionic strength on the interaction between PAI-1 and vitronectin,
the binding of PAI-1 and vitronectin was monitored at
varying ionic strengths using a fluorescence-based assay with
NBD-labeled PAI-1. The binding of the two proteins has been
previously measured using this approach with PAI-1 mutants
containing fluorescent labels within the reactive center loop
(18). These former measurements were challenging because of
low changes in fluorescence quantum yield upon vitronectin
binding. A more suitable form of PAI-1 for the fluorescence
 assay is the mutant with an NBD label at a cysteine substi-
tuted for serine 119, situated near the vitronectin-binding site
on PAI-1 (22). Binding isotherms for NBD-S119C-PAI-1 and
vitronectin, in the presence of both 150 and 500 mM NaCl, are
shown in Fig. 7. Cooperative binding isotherms were observed,
reflecting a complex binding/association phenomenon consist-
ent with the ultracentrifugation data that demonstrate a non-
stoichiometric binding of the two proteins accompanied by self-
association of vitronectin (8). Data fits to the Hill equation
were used to calculate $K_{0.5}$, which can be correlated with an average
binding affinity for the interaction. Under both salt conditions,
a $K_{0.5}$ of ~60 nM and a Hill coefficient of 2 were obtained.
Differences observed in the maximal fluorescence change ob-
erved with the two salt concentrations are presumably be-
cause of intrinsic properties (e.g. quantum yield, exposure to
solvent, quenching) of the fluorophores at the two ionic
strengths. Salt-dependent quenches of fluorescence are a well
established phenomenon in photobiophysics. These findings
demonstrate that similar complexes form regardless of the
ionic strength of the solution. Furthermore, they argue that the
ionic-strength dependent effects observed in Fig. 6 reflect disso-
ciation of the complexes from the matrix rather than disso-
ciation of PAI-1 from vitronectin.

Multimerization Promoted by PAI-1 Binding Enhances the
Interaction of Vitronectin with Cell Surface Receptors—If
vitronectin-PAI-1 complexes have a propensity to become matrix-
associated, a plausible corollary is that adhesive properties
of the complexes will be affected. To test the effects on the
function of vitronectin upon PAI-1 binding, we applied a cell-
binding assay using rabbit SMCs and radiolabeled vitronectin.
Incubation of vitronectin with PAI-1 resulted in an increase in
the amount of vitronectin that adhered to SMCs (Table I).
Although active PAI-1 caused a nearly 2-fold increase in
vitronectin binding to SMCs, latent PAI-1 did not have any
effect. These findings coupled with the HPLC results described
above indicate that PAI-1 binding to vitronectin results in the
formation of large complexes that are more adhesive to cell
surfaces than the monomeric (circulating) form of vitronectin.
Fluorescence experiments using NBD-labeled PAI-1 demonstrate that PAI-1-binding to vitronectin is not influenced by ionic strength. Small volume aliquots of monomeric vitronectin were added to a 2-ml sample of NBD-labeled S119C-PAI-1 at a final concentration of 0.2 μM. Binding was measured by monitoring the increase in the fluorescence emission of NBD (emission at 525 nm) upon vitronectin binding to PAI-1. Experiments were performed at NaCl concentrations of 0.15 (open circles) and 0.50 (solid circles) M. Smooth curves show fits to the Hill equation.

**FIG. 7.** Effects of PAI-1 on binding of radiolabeled vitronectin to smooth muscle cells

![Graph showing binding of radiolabeled vitronectin to smooth muscle cells](Image)

**TABLE 1**

| Sample          | Counts/min<sup>a</sup> |
|-----------------|----------------------|
| Vitronectin     | 15,600 ± 2,250       |
| Vitronectin + PAI-1 | 27,200 ± 2,850      |
| Vitronectin + latent PAI-1 | 16,500 ± 1,280 |

<sup>a</sup> All proteins were diluted to a final concentration of 100 nM for incubation with cultured cells.

<sup>b</sup> Bound vitronectin was quantified using 32P-labeled vitronectin and is expressed as counts/min/100,000 cells. Results are presented as mean ± standard error.

To further investigate the effects of PAI-1 binding on adhesive properties of vitronectin to cells, a solid-phase binding assay using purified integrins was employed. Similar to the binding to SMCs, vitronectin showed an increased binding to GPIIbIIIa- or αβ<sub>3</sub>-coated ELISA plates when incubated with active PAI-1 (Fig. 8, A and B). Over a wide range of concentrations, a consistent enhancement of vitronectin binding was observed; this finding was apparent at concentrations as low as 10 nM vitronectin and PAI-1. The binding was saturable with respect to vitronectin (Fig. 8A) or PAI-1 (Fig. 8B). This effect was not observed with latent PAI-1 (Fig. 8A). Ratios of PAI-1 to vitronectin that exceed 1:1 give maximal binding to integrins, presumably reflecting the nonstoichiometric association of the two proteins (8). These observations give further evidence that PAI-1-induced multimerization of vitronectin is correlated with enhancement of vitronectin binding to cells.

A cursory evaluation of the mechanism by which PAI-1 enhances association with the integrins involved experiments that measured several properties inherent to integrins and PAI-1 as partners with vitronectin in the binding phenomena. Integrin binding to ligands occurs through a variety of mechanisms, some of which involve metal-dependent interactions. Quite strikingly, the binding of vitronectin-PAI-1 complexes to GPIIbIIIa is abolished in the presence of EDTA (Fig. 9). Binding of uncomplexed vitronectin to the receptor was also diminished in the presence of EDTA. Additionally, an ionic strength dependence was observed for vitronectin binding to GPIIbIIIa. Binding of the vitronectin-PAI-1 complexes was diminished significantly when measured in the presence of 0.5 and 1.0 M NaCl, respectively. Furthermore, binding of the complexes to the receptors was reduced when tPA was added and the serpin-protease complexes dissociate from vitronectin. As shown in Fig. 9, this effect is notable at 1 h in experiments evaluating association with GPIIbIIIa. In fact, the dependence of integrin binding on the presence of PAI-1 in the complexes at the 1-h time point is more pronounced than was observed with the matrix binding experiments (Fig. 5A). However, these effects are minimized at longer times with both integrins (data not shown) and matrices (Fig. 5A) when latent PAI-1 accumulates and oligomeric vitronectin prevails.

**FIG. 8.** PAI-1-induced multimerization enhances the binding of vitronectin to GPIIbIIIa integrins. Panel A, vitronectin was pre-incubated with an equimolar amount of active PAI-1 (●) or with integrin-binding buffer alone (○), for 1 h at 37 °C. A control using a high concentration of latent PAI-1 is shown by ▲. These solutions were then incubated with GPIIbIIIa-coated ELISA wells as described under “Materials and Methods.” Following a 1-h incubation at 37 °C, wells were washed and bound vitronectin was detected using a polyclonal antibody and a peroxidase-labeled secondary antibody. Results are given in absorbance units (λ = 405 nm). Panel B, varying concentrations of PAI-1 were mixed with a fixed concentration of vitronectin (20 nM) and pre-incubated for 1 h at 37 °C. Protein samples were then incubated for 1 h with GPIIbIIIa-coated (○) or αβ<sub>3</sub>-coated (▲) ELISA wells as described under “Materials and Methods.” Wells were washed, and bound vitronectin was detected using a polyclonal antibody and a peroxidase-labeled secondary antibody. Binding was measured by absorbance at 405 nm. Results are expressed as an increase relative to the binding of vitronectin in the absence of PAI-1.

**FIG. 9.** PAI-1-induced multimerization enhances the binding of vitronectin to GPIIbIIIa integrins. A, binding of vitronectin-PAI-1 complexes to GPIIbIIIa is abolished in the presence of EDTA. Panel B,-binding was measured by monitoring the increase in the fluorescence emission of NBD (emission at 525 nm) upon vitronectin binding to PAI-1. Experiments were performed at NaCl concentrations of 0.15 (open circles) and 0.50 (solid circles) M. Smooth curves show fits to the Hill equation.

**TABLE 1**

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<sup>a</sup> All proteins were diluted to a final concentration of 100 nM for incubation with cultured cells.

<sup>b</sup> Bound vitronectin was quantified using 32P-labeled vitronectin and is expressed as counts/min/100,000 cells. Results are presented as mean ± standard error.

**DISCUSSION**

Proper cell adhesion and migration is required for the maintenance of homeostasis; alterations in these processes can lead to pathological situations such as impaired blood clotting and wound healing. In addition, tumor cells may be able to modify their own adhesive properties and acquire the ability to exit residing tissues and travel through circulation to other sites and metastasize. Investigation into both normal and pathological processes related to cell adhesion and migration represents an important area of clinical research and a focus of this work.
Vitronectin with integrin-coated wells (value normalized to 1). The relative binding compared with that of untreated monomeric effects of varied ionic strength and addition of tPA on the binding of represented standard errors.

Bars

-associated vitronectin into the extracellular matrix. This study addresses the following questions: 1) Is complex formation between vitronectin and PAI-1 also associated with increased deposition of vitronectin into the extracellular matrix? 2) Does this event result in enhanced binding of vitronectin to cell-surface receptors? 3) What is the physiological relevance of PAI-1-induced multimerization of vitronectin?

A time course was evaluated to monitor the formation of complexes between vitronectin and PAI-1, extending to long times sufficient for the release of latent PAI-1 from complexes formed. This method demonstrated the immediate formation of large complexes containing vitronectin and PAI-1, leading to observable vitronectin multimers after PAI-1 release. The picture that emerged correlated well with results from both matrix- and cell-binding studies, in that an enhancement in vitronectin binding to both ligands was associated with complex formation between vitronectin and PAI-1. Comparing the HPLC time course with a ligand-binding time course using ECM provides a nice correlation between augmentation of binding and formation of higher order species. In addition, it is evident that this enhancement of binding of vitronectin to ECM is observable long after the release of PAI-1, indicating that the effect is because of the conversion of vitronectin into a large, multivalent species.

A scheme shown in Fig. 10 helps in considering the dynamic processes that occur in the vitronectin-PAI-1 interaction over a fairly long time scale up to 24–36 h. At all times after mixing of the two proteins, there are assembly and disassembly processes that occur simultaneously. Immediately after mixing, PAI-1 binds to vitronectin and promotes formation of higher order complexes. Addition of tPA to bind and neutralize PAI-1, thus releasing it from vitronectin, yields a mixture of species including vitronectin multimers with some reversible dissociation into monomers. At extremely long times (e.g., 24 h), all PAI-1 has converted to a latent form and is no longer bound to the vitronectin multimers. As such, the addition of tPA has essentially no effect because it does not bind latent PAI-1. At intermediate times, represented by the 3-h time point in this figure, there are competing processes in which latent PAI-1 accumulates and tPA has less of an effect in binding PAI-1 and removing it from the complexes. These competing processes explain in part the curious observation at 3 h that tPA has little effect on association of the vitronectin multimers with the ECM.

The findings presented in this study demonstrate the effects of PAI-1-induced multimerization on the adhesive properties of vitronectin by correlating binding to cells, integrins, and matrix proteins. Variations in adhesive properties of monomeric and associated (multimeric) forms of vitronectin are reminiscent of the dependence of binding of the protein to heparin on the oligomeric state of vitronectin (10). With the polyvalent ligand, heparin, the conversion of vitronectin to a multimeric form is responsible for an apparent enhancement in binding that is attributed to changes in the stoichiometry of binding rather than to changes in affinity of heparin binding. In a similar fashion, changes in the binding of vitronectin to matrix ligands presumably result from a conversion of vitronectin into
in a cell culture system is glycosaminoglycan-dependent (24). Matrix molecules, possibly including heparin-like glycosaminoglycans, may be involved in these interactions. Ionic interactions with these higher order oligomers have formed to a considerable extent. Addition of tPA to bind and neutralize PAI-1, dissociating it from vitronectin, could lead to the formation of self-associated, higher order oligomers (8). Within 1 h, vitronectin-PAI-1 complexes, their association into higher order multimers, dissociation of latent PAI-1 over time, and the neutralization of vitronectin-PAI-1 by tPA. Vitronectin is depicted in blue ovals, PAI-1 by yellow ovals, and tPA by the red symbols. Previous work has shown that complexes of vitronectin and PAI-1 are formed with a 2:1 stoichiometry en route to the formation of self-associated, higher order oligomers (8). Within 1 h, all PAI-1 has converted to a latent form and is no longer bound to the vitronectin multimers. As such, the addition of tPA has essentially no effect because it does not bind latent PAI-1. At intermediate times, represented by the 3-h time point in this figure, there are competing processes in which latent PAI-1 accumulates and tPA has less of an effect at binding PAI-1 and removing it from the complexes. During this time course, multimeric adhesive complexes of vitronectin form in increasing amounts until a maximal level is reached.

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