Type 1 Plasminogen Activator Inhibitor Induces Multimerization of Plasma Vitronectin

A SUGGESTED MECHANISM FOR THE GENERATION OF THE TISSUE FORM OF VITRONECTIN IN VIVO

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The conformation and degree of multimerization of vitronectin (Vn) appears to be of critical importance for its functions, but little is known about the underlying mechanisms that control Vn multimerization. We report that Vn secreted by cultured hepatoma cells is present as a mixture of monomeric and multimeric forms. A single protein of Mr 45,000 co-purified with hepatoma cell-derived Vn, which was immunologically identified as type 1 plasminogen activator inhibitor (PAI-1). The possibility that PAI-1 may modulate Vn multimerization was investigated. The addition of active PAI-1 to unfractionated plasma containing Vn monomers resulted in the formation of covalently and noncovalently associated Vn multimers and expression of conformationally sensitive epitopes. In contrast, inactive forms of PAI-1 did not efficiently induce Vn multimerization and conformational change. Gel filtration analysis revealed that Vn remained multimeric after dissociation from PAI-1. Vn multimers were also assembled using purified monomeric Vn and PAI-1, suggesting that a plasma cofactor was not required to induce Vn multimerization. This study provides insights into physiological mechanisms responsible for the generation of homomultimeric Vn, a multimeric form of Vn that is not in complex with other proteins and which expresses a functional repertoire distinct from that of plasma Vn.

Vitronectin (Vn) is present in the blood circulation and in a variety of tissues. It plays key roles in the attachment of cells to their surrounding matrix (for reviews, see Refs. 1 and 2). In addition, Vn also functions in a number of proteolytic enzyme cascades, including the complement, coagulation, and fibrinolytic system. For example, Vn binds to and stabilizes the biological activity of type 1 plasminogen activator inhibitor (PAI-1), the physiological inhibitor of both tissue- and urinary-type plasminogen activators (tPA and uPA; Ref. 3). Taken together, these observations suggest that Vn provides a unique regulatory link between cell adhesion and proteolytic enzyme cascades.

Vn is a conformationally labile molecule. While only 2% of the Vn molecules in plasma binds to heparin-Sepharose, the relative amount increases to 7% through the generation of serum (4). This difference may be due to the formation of complexes between Vn and thrombin-antithrombin III, which results in Vn multimerization and increased affinity for heparin (5). Similarly, conformational changes in the Vn molecule are also induced by binding to the C5b-C7 complex of complement and highly sulfated glycosaminoglycans (5, 6). Under these physiological conditions, Vn is present in binary or ternary complexes. Finally, treatment of Vn with protein denaturants or subjection of Vn to acidification and/or heat treatment induces conformational changes in the molecule that are closely linked to the formation of noncovalently associated and disulfide-linked Vn multimers (1, 2). Using these experimental conditions, Vn homomultimers are formed. Conformational changes in the Vn molecule, induced either by ligand binding (e.g. interaction with thrombin-antithrombin III complexes (5) or by chemical or thermal denaturation, results in increased heparin binding and exposure of monoclonal antibody (mAb) epitopes that are not present in the native, plasma form (5). Epitope mapping of some of these antibodies demonstrates that conformational changes are not limited to the C-terminal heparin binding domain but also occur in the N-terminal half of the Vn molecule (7).

The conversion of the native, plasma form of Vn to the conformationally altered, disulfide-bonded multimeric form has been shown to be critical for its interaction with a number of molecules, including collagen (8), β-endorphin (9), and urokinase receptor (10, 11). In addition, multimeric Vn is required for binding to cells (12) and extracellular matrices (13), and only the multimeric form is degraded by fibroblasts (14, 15). The elucidation of these functional differences between native and multimeric Vn relies heavily on the use of Vn purified by heparin affinity chromatography in the presence of protein denaturants and reducing agents. This purification procedure results in the formation of disulfide-linked Vn aggregates with a molecular mass in excess of 1000 kDa (6). These aggregates are structurally and conformationally distinct from the Vn monomeric and multimeric forms present in plasma and platelets (16). It is not known whether Vn homomultimer (i.e. multimeric Vn that is not in complex with other molecules) can be generated under physiological conditions. This situation is even more complex in the case of PAI-1, since both monomeric and multimeric Vn were reported to bind active PAI-1. However, the latter form has an approximately 10-fold higher affinity for PAI-1 (17). In the present study, evidence is provided that PAI-1 mediates Vn multimerization and conformational changes.

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1 The abbreviations used are: Vn, vitronectin; CM, conditioned medium; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PAI-1, type 1 plasminogen activator inhibitor; PBS, phosphate-buffered saline; uPA, urinary-type plasminogen activator; tPA, tissue-type plasminogen activator; ELISA, enzyme-linked immunosorbent assay; PPP, platelet-poor plasma; TBS, Tris-buffered saline.

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Activated with 4 M guanidinium hydrochloride (21), and dialyzed to published procedures (19). PAI-1 was purified as described (20), revealing that Vn was completely eluted with 2 M NaCl (not shown). For elution attempts by boiling the beads in SDS-containing sample buffer (21), IgG or preimmune IgG (derived from the same rabbit) was removed by centrifugation as above. Affinity-purified rabbit anti-human vitronectin (Sigma), washed twice with PBS, blocked for 1 h at 37°C for 24 h. After this incubation step, the structural integrity of the resulting PAI-1 preparation was confirmed by SDS-PAGE followed by staining with silver nitrate. For some experiments, PAI-1 was incubated (37°C; 30 min) with a 2-fold molar excess of tPA (Calbiochem) or uPA. The remaining PAI-1 activity was determined by binding to immobilized tPA (23). Antisera to Vn and PAI-1 were raised in New Zealand White rabbits using standard procedures (24). IgG fragments were prepared by protein A affinity chromatography (Bio-Rad) and further purified by affinity purification using immobilized purified antigens as described (25). mAbs 153 and 1244 were obtained by standard hybridoma techniques using denatured Vn as immunogen (20). The hybridoma clone producing mAb 8E6 was kindly provided by Dr. D. Mason (University of Wisconsin). IgG was produced in mice ascites fluid and purified by using protein A-Sepharose beads (mAbs 153 and 1244) or employed without further fractionation (mAb 8E6).

**Cell Culture and Affinity Purification of Vn—** Hep 3B human hepatoma cells were maintained as described (26). Confluent cultures were washed three times with PBS and incubated with serum-free medium for 24 h. The CM was harvested, centrifuged at 900 × g for 10 min to remove cellular debris, concentrated approximately 10-fold using Centriprep concentrators (molecular mass cutoff 10 kDa; Amicon), and stored at –80°C until assayed. For affinity purification experiments, tissue culture dishes were coated at 4°C for 16 h with 2.5 μg/ml human fibronectin (Sigma), washed twice with PBS, blocked for 1 h at 37°C with PBS containing 3% bovine serum albumin, and washed again with PBS. Confluent Hep 3B cells were detached from the culture dishes by treatment with trypsin/EDTA (Bio Whittaker), washed once with complete medium to neutralize remaining trypsin, and washed three times with serum-free medium. Cells were plated in serum-free medium on the fibronectin-coated dishes for 24 h. The CM was harvested and concentrated approximately 10-fold as above, and cellular debris was removed by centrifugation as described. Affinity-purified rabbit anti-human Vn IgG or preimmune IgG (derived from the same rabbit) was coupled to a ratio of 1 mg of IgG/ml of settled CNBr-activated Sepharose 4B beads according to the instructions of the manufacturer (Pharmacia Biotech Inc.). Nonspecific protein binding sites on the beads were blocked by incubation with Tris-buffered saline (TBS) containing 0.05% Tween 20 and 3% casein for 1 h at room temperature. The washed beads (TBS, 0.05% Tween) were incubated with the CM for 16 h at 4°C, washed with 10 bead volumes of TBS, 0.05% Tween, and followed by 10 ml of TBS, 0.05% Tween 20, 0.5 M NaCl, and finally eluted with 5 μl of ImmunoPure Gentle Ag/Ab elution buffer (Pierce). The eluted fractions were dialyzed against Tris-buffered saline and concentrated by ultrafiltration as above prior to analysis.

**Heparin Affinity Chromatography—** The binding of Vn to glycans was tested by heparin affinity chromatography as described (26). Briefly, Vn containing samples (1 ml) were batch-absorbed, and the beads were washed three times with 10 bead volumes of PBS/Tween and eluted with 1 ml of PBS/Tween containing 2 M NaCl. Further elution attempts by boiling the beads in SDS-containing sample buffer revealed that Vn was completely eluted with 2 M NaCl (not shown). For some experiments, the ability of Vn to bind to heparin immobilized onto microtiter wells was determined as described (7).

**Induction of Vn Multimerization—** Platelet-poor plasma (PPP) was prepared under conditions aimed at preventing platelet activation as described (16). Briefly, Vn containing samples (1 ml) were batch-absorbed, and the beads were washed three times with 10 bead volumes of PBS/Tween and eluted with 1 ml of PBS/Tween containing 2 M NaCl. Further elution attempts by boiling the beads in SDS-containing sample buffer revealed that Vn was completely eluted with 2 M NaCl (not shown). For some experiments, the ability of Vn to bind to heparin immobilized onto microtiter wells was determined as described (7).

**Analytical Gel Filtration—** Plasma was incubated at 37°C for 16 h in the presence or absence of PAI-1 (equimolar concentrations of Vn present in PPP and exogenously added activated PAI-1) and then applied to a Sephacryl S-300 superfine column (125 × 1.5 cm) equilibrated in PBS, 0.1% polyethylene glycol 3,350 (Sigma). Fractions (6 min) were collected at a flow rate of 25 ml/h, and the Vn and PAI-1 concentrations were determined in every second column fraction by sandwich ELISA as described (26, 27). The gel filtration column was calibrated with the following standards (Pharmacia): blue dextran 2,000 (M, 2,000,000), ferritin (M, 440,000), aldolase (M, 158,000), and albumin (M, 67,000). The primary antibody for immunoblotting was mouse anti-human Vn ascites (1:5000 dilution of mAb 1244 ascites) (20), affinity-purified rabbit anti-human Vn IgG (200 ng/ml IgG; Ref. 29), or affinity-purified rabbit anti-human PAI-1 IgG (100 ng/ml IgG; Ref. 27), followed by peroxidase-labeled secondary antibodies (Amersham Corp.) and ECL Western blotting detection system (Amersham). It should be noted that mAb 1244 fails to interact with bovine Vn present in the culture medium (not shown). Tissue-type plasminogen activator was coupled to CNBr-activated Sepharose 4B beads at a ratio of 1 mg of tPA/ml of settled beads according to the instructions of the manufacturers. Protein concentrations were determined by the bicinchoninic acid method (Pierce). Conformational changes in the Vn molecule were quantified by competitive ELISA exactly as described (7). Results were expressed as the percentage of binding of antibodies in the absence of soluble competitor. The Vn concentration in biological samples used for competitive binding assays was estimated by semiquantitative immunoblotting (mAb 1244) as described (16).

**RESULTS**

**Structural, Immunological, and Functional Comparison of Plasma Vn with the Vn Populations Secreted by Human Hepatoma Cells—** The electrophoretic mobility of Vn synthesized by the human hepatocarcinoma cell line Hep 3B or present in plasma was compared by PAGE, followed by immunoblotting using mAb 1244 raised to human Vn (Fig. 1). On nonreducing SDS-PAGE, Hep 3B CM contained a broad band between M, 65,000–75,000, broad immunologically Vn-reactive and, in addition, high molecular weight (M, 150,000–200,000) bands that barely entered the separating gel. In addition, Vn immunoreactivity was also present in the stacking gel (Fig. 1, lane 1). In contrast, under the same conditions, the majority of plasma Vn migrated as a broad band between M, 75,000 and 65,000, and only traces of the higher molecular weight forms of Vn were detected. No Vn immunoreactivity was present in the stacking gel of plasma samples (Fig. 1, lane 2). To exclude the possibility that the prolonged incubation of the CM (i.e., up to 24 h at 37°C) accounted for the formation of the high molecular weight Vn...
forms in Hep 3B CM, plasma was incubated on bovine serum-coated tissue culture dishes for 24 h. The electrophoretic mobility of the incubated plasma Vn was relatively unchanged in comparison with the nonincubated control (Fig. 1, compare lanes 2 and 3).

Vn is a substrate for tissue transglutaminase, resulting in the formation of reduction-insensitive, covalently linked Vn multimers (30). SDS-PAGE under reducing conditions was employed to test the potential role of transglutaminases in the formation of the high molecular weight Vn forms by Hep 3B cells. Under these conditions, Hep 3B Vn migrated as a doublet of Mr 75,000 and 65,000, and no high molecular weight forms were detected (Fig. 1, lane 4). This pattern is similar to that of plasma-derived Vn (Fig. 1, lane 5), indicating that hepatoma cell-derived Vn was not covalently linked by intermolecular ε-(γ-glutamyl)lysine bonds but rather was present in disulfide-bound multimers or present in disulfide-bonded complexes with other polypeptides present in the CM (see below).

The structure of Hep 3B-derived Vn was further analyzed by native PAGE. Plasma Vn (Fig. 1, lane 7) and plasma Vn incubated for 24 h on serum-coated dishes (Fig. 1, lane 8) migrated as single major bands toward the anode. This electrophoretic mobility is characteristic for monomeric Vn (16). In contrast, Hep 3B-derived Vn contained both monomeric Vn and a number of additional bands that barely entered into the resolving gel (Fig. 1, lane 6). Taken together, these observation suggest that the hepatoma cell-derived Vn was a mixture of both monomeric and higher molecular weight Vn species.

Multimerization of Vn, as well as complex formation with molecules of the coagulation and complement cascade, results in the exposure of immunoeptopes in the Vn molecule that are not present in the monomeric plasma form (1, 2). To determine whether the Vn forms produced by Hep 3B cells contain these neoeptopes, plasma Vn was compared with Vn present in Hep 3B CM by competitive ELISA using mAbs that are derived from these conformationally sensitive sites (Fig. 2). Hep3B-derived Vn (filled bars) competed with the binding of each mAb (i.e. mAb 153, epitope located between amino acids 1 and 40; mAbs 1244 and 8E6, nonoverlapping epitopes located between amino acids 52 and 239) to immobilized denatured Vn (Fig. 2A).

In contrast, little or no competition was observed using plasma as the Vn source (Fig. 2A, open bars). In these experiments, purified denatured Vn was used as a positive control (Fig. 2A, hatched bars). Thus, conformationally sensitive immunoeptopes were expressed in Hep3B-derived Vn that were not present in plasma Vn.

While plasma Vn lacks significant heparin binding (1, 2), multimeric Vn binds to and can be specifically eluted from heparin-Sepharose (18). The heparin binding domain is located in the C-terminal half of the Vn molecule (i.e. between amino acids 341 and 379) and is believed to be partially buried in the native, monomeric Vn form (1, 2). Thus, heparin affinity chromatography can be used to determine conformational changes in the C terminus of Vn. While Hep 3B-derived Vn bound to microtiter wells coated with heparin in a dose-dependent manner, little binding of plasma Vn was detected, and no specific binding of Hep 3B-derived Vn to casein-coated wells was observed (Fig. 2B). It should be noted that the polyclonal anti-Vn serum used to detect heparin-bound Vn has a higher affinity for multimeric Vn (7) and thus may underestimate the extent of binding of plasma Vn to heparin. To confirm and extend the ELISA results, the binding of Hep 3B and plasma Vn to heparin-Sepharose beads was compared. The resulting fractions were analyzed by SDS-PAGE under reducing conditions followed by immunoblotting using mAb 1244. Semiquantitative immunoblotting studies using purified denatured and native Vn standards revealed that under these conditions, no preferential detection of multimeric Vn occurred (data not shown). Fig. 2B (inset) indicates that a significant portion of the Hep 3B Vn bound to heparin-Sepharose and could be eluted from the column by an increase in the ionic strength. In comparison, no binding of plasma Vn was detected under these conditions. In control experiments, neither Hep 3B nor plasma Vn bound to unconjugated Sepharose beads (not shown), indicating that the binding of Hep 3B-derived Vn was specific. Taken together, these results indicate that Vn present in Hep 3B CM was structurally, immunologically, and functionally distinct from the plasma form of this molecule.

Affinity chromatography experiments were performed to identify molecules associated with Hep3B-derived Vn (Fig. 3). Hep3B-derived Vn (Fig. 3, lane 1) bound specifically to and was eluted (lane 2) from the anti-Vn IgG-Sepharose, whereas no binding to the preimmune IgG was detected (lane 3). The elution fractions were analyzed by silver staining to identify proteins that may co-purify with Vn. Two major polypeptides of Mr 65,000 and 45,000 and a number of minor polypeptides were detected in the elution fraction of the anti-Vn column (Fig. 3, lane 4). In contrast, no polypeptides were isolated using preimmune IgG (Fig. 3, lane 5). The upper band of Mr 65,000

![Fig. 2 Immunological and functional comparison of Hep 3B and plasma Vn.](image_url)
co-migrated with the Vn immunoreactive band, whereas the lower band of $M_r$ 45,000 was immunologically unrelated to Vn (compare Fig. 3, lanes 2 and 4). Since Vn binds to PAI-1 and Hep 3B cells are known to produce PAI-1 (31), the immunological relationship of the $M_r$ 45,000 polypeptide and PAI-1 was tested. PAI-1 immunoreactivity was detected in the starting material (not shown), the flow-through (not shown), and the elution fraction of the anti-Vn IgG (Fig. 3, lane 6). The PAI-1 immunoreactive band co-migrated with the $M_r$ 45,000 polypeptide in the silver-stained gel (Fig. 3, compare lanes 4 and 6). In contrast, PAI-1 immunoreactivity was not detected in the elution fraction of the preimmune IgG (not shown). Thus, Hep 3B-derived Vn was present, at least in part, in complex with PAI-1, whereas no other major polypeptides were detected in association with Vn under these experimental conditions. These observations raised the possibility that PAI-1 may influence the multimeric state and conformation of Vn.

**PAI-1 Shifts the Electrophoretic Mobility of Plasma Vn—**

Increasing amounts of activated PAI-1 were added to a constant amount of plasma, and after incubation for 2 h at 37 °C, the resulting samples were analyzed by SDS-PAGE under non-reducing (Fig. 4A) or reducing (Fig. 4B) conditions, followed by immunoblotting using mAb 1244 (A–C) or rabbit anti-human PAI-1 (D). A (SDS-PAGE, nonreduced) and C (native PAGE): lanes 1, incubation in the absence of PAI-1; lanes 2, 100 ng of PAI-1; lanes 3, 500 ng of PAI-1; lanes 4, 1 μg of PAI-1; lanes 5, 5 μg of PAI-1. B (SDS-PAGE, reduced): lane 1, PPP incubated in the absence of PAI-1; lane 2, incubation in the presence of 5 μg of PAI-1; D, lane 1 (SDS-PAGE, nonreduced) and lane 2 (native PAGE): PPP incubated in the presence of 1 μg of PAI-1. The mobility of $M_r$ standards for A, B, and D (lane 1) is indicated to the right, and the interfaces between separating and stacking gels are denoted by arrowheads.

The importance of covalent interactions for the formation of the high molecular weight Vn forms was evaluated. Under reducing conditions, plasma Vn migrated as a doublet of $M_r$ 75,000 and 65,000 (Fig. 4A, lane 1). The addition of activated PAI-1 resulted in a dose-dependent appearance of high molecular weight Vn immunoreactive bands (Fig. 4A, lanes 2–5). The molecular weight of the predominant high molecular weight Vn form was estimated, based on analyses on different percentage SDS-PAGE, to be 150 kDa. Similar results were obtained using a polyclonal antiserum to Vn as the detection step in the immunoblot procedure (not shown). The electrophoretic mobility of these high molecular weight Vn forms was similar to those observed in the supernatant of Hep 3B CM (compare Fig. 1), except that no Vn immunoreactivity was present in the stacking gel. The high molecular weight Vn immunoreactive bands were first observed at a 2-fold molar excess of activated PAI-1 over plasma Vn and increased further in intensity at a 10-fold molar excess of PAI-1 over plasma Vn (Fig. 4A). It should be noted that the half-life of the biological activity of PAI-1 is 1–2 h at 37 °C and increases approximately 2-fold in the presence of Vn (32). Accordingly, the molar ratio between activated PAI-1 and Vn is expected to decrease during the course of the experiment. Thus, the concentration of PAI-1 required for the induction of SDS-stable high $M_r$ Vn immunoreactive bands should only be viewed as an approximation. However, it is clear from these experiments that PAI-1 does not induce Vn multimerization at catalytic concentrations.
PAI-1 Induces Vitronectin Multimerization

Unfractionated plasma (1 μl containing 500 ng of Vn) was incubated (37 °C) for increasing periods of time in the presence of activated PAI-1 (equimolar concentrations of active PAI-1 and Vn) in a total volume of 50 μl of PBS. At indicated time points, the reaction was stopped by the addition of 5,5′-dithiobis(nitrobenzoic acid) (1 mM) and tPA-Sepharose (2-fold molar excess over initial active PAI-1) and incubated for 30 min at 37 °C. The beads were pelleted by centrifugation, and the resulting supernatant was co-incubated with the remaining samples for 24 h prior to fractionation by SDS-PAGE under nonreducing conditions (A) or by native PAGE (B). Vn immunoreactivity was detected by immunoblotting with mAb 12/44 (see Fig. 1). The mobility of Mr standards for A is indicated, and the interfaces between separating and stacking gels are denoted by arrowheads. A, lane 1, 0 h; lane 2, 1 h; lane 3, 3 h; lane 4, 6 h; lane 5, 24 h. B, lane 1, 0 h; lane 2, 1 h; lane 3, 24 h.

The possibility that nondissociated PAI-1-Vn complexes contribute to the altered electrophoretic mobility of plasma Vn treated with PAI-1 was analyzed. The sample presented in Fig. 4A, lane 4, was analyzed by immunoblotting using antibodies to PAI-1. While recombinant PAI-1 was easily detectable with the expected Mr of 40,000 upon fractionation by SDS-PAGE under nonreducing conditions, no PAI-1 was detected in the molecular size of the high molecular weight Vn immunoreactive bands (Fig. 4D, lane 1). This observation confirms previous studies in that the PAI-1-Vn complexes are SDS-sensitive (33). Analysis under nondenaturing conditions revealed that while PAI-1 immunoreactivity partially overlapped with areas of Vn immunoreactivity (Fig. 4, compare C, lane 4, and D, lane 2), the Vn immunoreactivity present in the stacking gel in panel C did not include PAI-1. Two lines of evidence further support the notion that the altered Vn mobility on native PAGE is not due to the presence of PAI-1-Vn complexes. First, the electrophoretic mobility of PAI-1-treated plasma on native PAGE was unchanged after a 24-h incubation at 37 °C prior to electrophoretic separation (not shown). Second, the samples were fractionated at room temperature for 16 h, which is substantially longer than the half-life of PAI-1-Vn complexes (32).

The temporal events of PAI-1-induced Vn multimerization were further studied in time course experiments (Fig. 5). Based on the observations that 5,5′-dithiobis(nitrobenzoic acid) pre-

vents disulfide-linked PAI-1-induced Vn multimerization,2 that tPA-PAI-1 complexes fail to induce Vn multimers (see Fig. 7), and that tPA dissociates preformed PAI-1-Vn complexes (33), the reaction was stopped by the addition of 5,5′-dithiobis(nitrobenzoic acid) and tPA-Sepharose. Analysis of the time course experiment by nonreducing SDS-PAGE revealed that PAI-1 induced the formation of disulfide-linked Vn dimers in a time-dependent manner. First covalently stabilized Vn dimers appeared within 1 h of incubation and increased in intensity up to 24 h (Fig. 5A). In contrast, the formation of noncovalently associated Vn multimers was maximal already at 1 h and did not further change by incubation of up to 24 h (Fig. 5B). Thus, PAI-1 apparently initially induces the formation of non-disulfide-linked Vn multimers that are secondarily stabilized by interchain disulfide bonds between Vn molecules. It should be noted that similar conclusions have been derived for thrombin-antithrombin III complex-induced Vn multimerization (1, 2).

Gel Filtration Analysis of PAI-1-treated Plasma—Molecular sieving chromatography was employed as an independent method to confirm the presence of PAI-1-induced Vn multimers. Plasma was incubated for 16 h at 37 °C in the absence or presence of an equimolar concentration of exogenously added activated PAI-1. This prolonged incubation resulted in a reduction of PAI-1 activity to less than 0.1% of the starting material (determined by binding to immobilized tPA; Ref. 23), indicating that the majority of PAI-1 added to plasma had converted into the latent form. Plasma incubated in this way was then size-fractionated by gel filtration chromatography. The protein elution profiles (i.e. A280) were very similar in the presence or absence of PAI-1 (Fig. 6A (top)). The Vn content in the column fractions was determined by specific ELISA (Fig. 6A (bottom)). The elution of plasma Vn incubated in the presence of PAI-1 was partially shifted to higher molecular weight fractions and extended to the void volume (Mr2 × 106) of the Sephacryl S-300 column. In contrast, plasma Vn incubated in the absence of PAI-1 eluted with the expected molecular weight of approximately 67,000, indicative of monomeric Vn (19, 34, 35; Fig. 6A (bottom)). Analysis of the column fractions by the PAI-1 ELISA revealed that little or no PAI-1 immunoreactivity was present in the high molecular weight Vn-containing fractions (Fig. 6A (bottom)). Control experiments revealed that the addition of plasma to activated PAI-1 did not affect the detection of PAI-1 by the ELISA assay (not shown), indicating that PAI-1 immunoreactivity was not masked by plasma Vn or other molecules present in plasma. Analysis of the gel filtration fractions derived from PAI-1-treated plasma by nonreducing SDS-PAGE revealed that while the starting material contained both high and low Mr Vn immunoreactive bands (Fig. 6B, lane 1), the high molecular weight Vn fractions (37–48; panel A, top) contained predominantly disulfide-linked Vn multimers (lane 2), whereas the low molecular weight fractions (49–55; panel A, top) contained both high and low molecular weight Vn forms (lane 3). Vn and PAI-1 immunoreactivity were present in the starting material (Fig. 6B, lane 4), whereas the high molecular weight fractions (lane 5) and low molecular weight fractions (lane 6) were devoid of PAI-1 immunoreactivity within the detection limit of our immunoblotting procedure. PAI-1 was only detected in later elution fractions (Fig. 6B, lane 7). In addition, no Vn immunoreactivity was present in the corresponding high molecular weight fractions of plasma incubated in the absence of PAI-1 (not shown). Thus, the immunoblotting studies confirmed the results of the PAI-1 and Vn ELISAs. Moreover, the shift in the elution profile of PAI-1-treated plasma Vn was not due to remaining PAI-1-Vn complexes but

2 D. Seiffert, unpublished observation.
rather indicated that Vn remained in a high molecular weight form after dissociation from PAI-1.

Evidence That Active PAI-1 Is Required to Induce Vn Multimerization in Plasma—To begin to understand the structural features of PAI-1 required to induce the formation of Vn multimers in plasma, activated PAI-1 was converted into the latent form of the inhibitor by prolonged incubation at 37 °C or was inactivated by the addition of PAs and the formation of PA-PAI-1 complexes. The remaining PAI-1 activity after these treatments was less than 0.1% of the starting material (as determined by binding to immobilized (PA). While activated PAI-1 induced the formation of SDS-stable Vn multimers (Fig. 7A, lane 2), neither PAI-1 in complex with PAs (lanes 3 and 4) nor latent PAI-1 (lane 5) shifted the Vn mobility. To test whether the formation of noncovalently stabilized Vn multimers also requires active PAI-1, parallel samples were analyzed by native PAGE (Fig. 7B). Again, while activated PAI-1 shifted the mobility of plasma Vn toward the cathode (Fig. 7B, lane 2), the electrophoretic mobility of plasma Vn was unchanged using the inactive forms of PAI-1 (Fig. 7B, lanes 3–5). These results indicate that the active form of the inhibitor was required for both the induction of disulfide-bonded and noncovalently stabilized Vn multimers in plasma.

PAI-1 Induces the Expression of Conformationally Sensitive Epitopes in the Vn Molecule—The effect of PAI-1 on the expression of denaturation-sensitive immunoeptopes was determined by competitive ELISA (Fig. 8). Increasing amounts of activated PAI-1 were added to unfractionated plasma, and the resulting samples were incubated at 37 °C for 16 h to facilitate dissociation of PAI-1-Vn complexes. While parallel incubated plasma only weakly competed with the binding of the antibodies to immobilized Vn (Fig. 8, A–C), the addition of activated PAI-1 to plasma induced the expression of three different immunoeptopes located in the N-terminal half of the Vn molecule (compare Fig. 2). The induction of these conformational changes was dose-dependent, and at the highest concentration of PAI-1, the extent of expression approached that of purified denatured Vn (Fig. 8, A–C).

The results presented in Fig. 7 indicated that active PAI-1 was required for the induction of Vn multimers. To test whether the same was true for the induction of conformational changes, the ability of plasma Vn treated with either activated (dotted bars) or latent PAI-1 (hatched bars) or incubated in the absence of PAI-1 (filled bars) to compete in the competitive ELISA assay was compared (Fig. 8D). While activated PAI-1 induced the expression of conformationally sensitive epitopes, conformational changes were drastically reduced using latent PAI-1 (containing less than 0.5% of the PAI-1 activity as determined by binding to immobilized tPA). These results suggest that active PAI-1 is required to alter both Vn conformation and...
Fig. 8. PAI-1 induces the expression of conformationally sensitive epitopes in plasma Vn. Plasma (diluted in PBS to 20 μg/ml Vn) was incubated in the absence or the presence of increasing concentrations of activated PAI-1 for 16 h at 37 °C. The PAI-1-dependent generation of conformationally sensitive epitopes was determined as in Fig. 2A. Closed circle, PPP incubated in the absence of PAI-1; open circle, PPP incubated with 50 μg/ml PAI-1; closed square, PPP incubated with 10 μg/ml PAI-1; open square, PPP incubated with 5 μg/ml PAI-1; closed triangle, PPP incubated with 1 μg/ml PAI-1; open triangle, purified denatured Vn. A, mAb 153; B, mAb 1244; C, mAb 8E6. Results are expressed as the percentage of antibody binding in the absence of soluble competitor. In D, the expression of conformationally sensitive immunoepitopes was compared using PPP incubated for 16 h at 37 °C in the absence of PAI-1 (filled bars) or in the presence of activated PAI-1 (40 μg/ml PAI-1; dotted bars) or latent PAI-1 (prepared by preincubation of activated PAI-1 (40 μg/ml) at 37 °C for 24 h; hatched bars). The final concentration of Vn employed in the competitive ELISA was 5 μg/ml.

Evidence That PAI-1 Induces Plasma Vn Multimerization—Unfractionated plasma incubated for 16 h at 37 °C failed to bind to immobilized heparin (Fig. 9, open circles). In contrast, the addition of PAI-1 to plasma resulted in the generation of a heparin-binding competent population of Vn in plasma (Fig. 9, closed circles). The binding was specific, since no binding of PAI-1-treated plasma to casein-coated microtiter wells was detected (Fig. 9, closed squares). These results were confirmed by heparin affinity chromatography followed by SDS-PAGE and immunoblotting (Fig. 9, inset). While a significant portion of Vn present in PAI-1-treated plasma bound to and was eluted from the heparin-Sepharose (Fig. 9, inset, lanes 1–3), no binding of control plasma incubated for 24 h in parallel was evident (lanes 4–6). It should be noted that heparin affinity chromatography has been employed to purify PAI-1 (36), raising the possibility that the binding of Vn to heparin was mediated by the PAI-1 moiety in nondissociated PAI-1-Vn complex but not by direct interactions of Vn with heparin. To discriminate between these possibilities, the interaction of Vn present in the PAI-1-deficient high molecular weight fractions from the gel filtration experiments (see Fig. 6) was determined. Vn bound to and was specifically eluted from heparin in the absence of PAI-1 (Fig. 9, inset, lanes 7–9). Thus, the interaction of PAI-1 with monomeric Vn results in the generation of a multimeric, conformationally altered Vn molecule that is endowed with new ligand binding functions.

DISCUSSION

The observations in this report demonstrate that PAI-1 induces Vn multimerization and associated conformational changes. These conclusions are based on the findings that the addition of active PAI-1 to unfractionated plasma resulted in a dose-dependent appearance of disulfide-linked Vn dimers and higher molecular weight, noncovalently stabilized Vn multimers, and concomitantly, plasma Vn also expressed epitopes for conformationally sensitive antibodies. Moreover, after dissociation from PAI-1, the Vn remains in a multimeric, conformational-similar changes in the electrophoretic mobility and expression of conformationally sensitive epitopes were observed using purified native Vn and PAI-1 (not shown). These results suggest that PAI-1 does not require a plasma cofactor to induce multimerization and conformational changes of the Vn molecule.

Evidence That PAI-1 Endows Plasma Vn with Increased Heparin Binding Affinity—Unfractionated plasma incubated for 16 h at 37 °C failed to bind to immobilized heparin (Fig. 9, open circles). In contrast, the addition of PAI-1 to plasma resulted in the generation of a heparin-binding competent population of Vn in plasma (Fig. 9, closed circles). The binding was specific, since no binding of PAI-1-treated plasma to casein-coated microtiter wells was detected (Fig. 9, closed squares). These results were confirmed by heparin affinity chromatography followed by SDS-PAGE and immunoblotting (Fig. 9, inset). While a significant portion of Vn present in PAI-1-treated plasma bound to and was eluted from the heparin-Sepharose (Fig. 9, inset, lanes 1–3), no binding of control plasma incubated for 24 h in parallel was evident (lanes 4–6). It should be noted that heparin affinity chromatography has been employed to purify PAI-1 (36), raising the possibility that the binding of Vn to heparin was mediated by the PAI-1 moiety in nondissociated PAI-1-Vn complex but not by direct interactions of Vn with heparin. To discriminate between these possibilities, the interaction of Vn present in the PAI-1-deficient high molecular weight fractions from the gel filtration experiments (see Fig. 6) was determined. Vn bound to and was specifically eluted from heparin in the absence of PAI-1 (Fig. 9, inset, lanes 7–9). Thus, the interaction of PAI-1 with monomeric Vn results in the generation of a multimeric, conformationally altered Vn molecule that is endowed with new ligand binding functions.

DISCUSSION

The observations in this report demonstrate that PAI-1 induces Vn multimerization and associated conformational changes. These conclusions are based on the findings that the
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... tionally altered form. The induction of Vn multimerization/ conformational changes is relatively specific and not a general function of serine protease inhibitors. This conclusion is based on the observation that plasma Vn is present in the monomeric form despite the presence of high concentrations of other members of this gene family (e.g., \( \alpha \)-protease inhibitor; \( \alpha \)-antichymotrypsin). Moreover, PAI-1 apparently does not require a plasma cofactor to induce Vn multimerization and conformational changes, since similar results were obtained in a purified protein system.

The data presented here describe a new mechanism for the formation of multimeric Vn. The Vn concentration in plasma is between 2.5 and 5 \( \mu \text{M} \) (1, 2), whereas normal PAI-1 levels in plasma are approximately 0.4 \( \text{nM} \) (3). While plasma PAI-1 levels are regulated under pathophysiological conditions and, for example, endotoxinemia results in a dramatic up-regulation of plasma PAI-1 levels up to 20 \( \text{nM} \) (3), these concentrations are still more than 100-fold lower than that of Vn. Using this ratio of PAI-1 and Vn, we were unable to detect PAI-1-induced Vn multimers in plasma. The scenario is quite different in platelets. Both PAI-1 and Vn are contained within platelet \( \alpha \)-granules (16), and platelets contain 26 \( \text{nM} \) Vn and 12 \( \text{nM} \) PAI-1/10^9 platelets (37, 38). Interestingly, platelet Vn is present in high molecular weight multimers and expresses epitopes for conformationally sensitive antibodies (16, 35), raising the possibility that the multimerization of platelet Vn is mediated by PAI-1.

The physiological significance of the PAI-1-induced Vn multimerization remains to be elucidated. PAI-1-multimeric Vn complexes are expected to accumulate in areas of tissue injury. This conclusion is based on the observation that the interaction of PAI-1 with monomeric Vn endows the Vn molecule with increased glycosaminoglycan binding functions and a reactive free sulphydryl group, which may mediate noncovalent and/or covalent incorporation of Vn into tissues. In addition, PAI-1 is produced by cells in areas of tissue injury (3), raising the possibility that locally produced PAI-1 will recruit plasma-derived monomeric Vn, increasing further the deposition of multimeric Vn in areas of tissue injury. As a result, this model predicts an initial fibrinolytic shutdown due to the accumulation of active PAI-1 via Vn, preventing fibrin dissolution in areas of tissue injury. Timely formation and dissolution of fibrin clots is of critical importance for wound healing. This model further predicts that after dissociation of the PAI-1-Vn complexes, the hemostatic balance will be shifted to a profibrinolytic state, facilitating local fibrin dissolution. In addition, tissue-associated multimeric Vn may be important for local cell recruitment (e.g., endothelial cells), resulting in reendothelialization of the denuded vessel wall. Thus, Vn is expected to regulate proteolytic enzyme cascades in areas of tissue injury, resulting in the halting of the process of injury and in the initiation of repair processes aimed at returning the body to normal function.

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