The Cell Adhesion Domain in Plasma Vitronectin Is Cryptic*

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Vitronectin (Vn), an adhesive glycoprotein present in blood and in a variety of tissues, belongs to a group of molecules that contain the RGD cell adhesion sequence and play key roles in the attachment of cells to their surrounding matrix (1, 2). In addition, Vn has several other functions in the complement, coagulation, and fibrinolytic system. For example, Vn is identical to the S protein of the complement system and thus inhibits complement-mediated cell lysis (3, 4). Vn competes with heparin binding to antithrombin III, thereby preventing the rapid inactivation of thrombin and factor Xa by this protease inhibitor (5, 6). Vn also 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 (7, 8). Thus, Vn provides unique regulatory links between cell adhesion and proteolytic enzyme cascades, although it is not clear how the multiple functions of Vn are coordinately regulated.

Vn is a conformationally labile molecule, and this lability is likely to play a large role in regulating the functions of the protein. This notion is based on the observation that different preparations of Vn exhibit differing abilities to bind to the glycosaminoglycan heparin. Only 2% of the Vn in plasma is capable of binding to heparin-Sepharose, whereas this fraction is increased to 7% by the generation of serum (9). In addition, the formation of complexes of Vn with PAI-1 (10), thrombin-antithrombin III (11), or complement C5b-C9 (12) induces conformational changes in the molecule, which expose the heparin binding domain. Moreover, conformational changes are also induced by denaturation with chaotropic agents, heat-treatment and acidification. These changes are accompanied by the spontaneous formation of disulfide-linked multimers of Vn. Using a panel of conformationally sensitive antibodies, we provided evidence that conformational changes within Vn are not limited to the heparin binding domain, but also occur in the N-terminal half of the molecule, including the N-terminal somatomedin B (SMB) domain (13), a site that encompasses the RGD sequence.

Understanding the interrelationship between the binding domains of Vn has been complicated, because the exposure and function of these sites depends on the conformational state of this molecule. For example, a number of ligands interact preferentially with the conformationally altered and multimeric (i.e., denatured form) of Vn. These include collagen (14), glycosaminoglycans (15, 16), β-endorphin (17), PAI-1 (18), and urokinase receptor-urokinase complex (19, 20). In contrast, thrombin-antithrombin III complexes appear to preferentially interact with native, plasma Vn (21). The function of native and denatured Vn in promoting cell adhesion is even more complicated. Native Vn was reported to bind specifically and saturably to glycoprotein (GP) IIb/IIIa on stimulated platelets (22–24). The binding of denatured Vn to platelets are conflicting (25). In addition, Vn has been reported to both block (26) and stimulate (23) platelet aggregation. The reasons for these discrepancies remain unclear.

Vn is one of many adhesive proteins that bind to cell surface receptors called integrins (27, 28). Each integrin is a noncovalently associated αβ heterodimer that spans the plasma membrane. To date, nine integrin α subunits and 12 β subunits have been identified. Since the ligand binding specificity of each receptor is conferred by its subunit composition, functional diversity within the family of integrins is achieved by heterologous pairing between subunits. Three different integrins have been identified as receptors for Vn, GPIIb/IIIa (also known as αIIβ3 (29)), αvβ3, expressed by endothelial cells and implicated in angiogenesis (30), and αvβ5 (31). Vn contains a single RGD sequence located adjacent to the SMB domain (3, 4). Site-directed mutagenesis of the RGD motif shows that this sequence is required for cell adhesion and can not be compensated for by other parts of the molecule (32, 33). A number of
reports indicated that the cell adhesion domain is exposed on both native and denatured Vn (1, 34). Consequently, the adhesiveness of the protein is not thought to be regulated by the conformational liability of the molecule. However, this concept is challenged by two observations. First, only denatured Vn induces tyrosine phosphorylation in endothelial cells by binding to the αβ₃ integrin on the cell (35). Second, αβ₃-dependent endocytosis of Vn by skin fibroblasts was only observed using denatured Vn, the native form of Vn is not internalized (36, 37).

In the present report, we compare the binding of native and denatured Vn to purified GPIIb/IIIa and αβ₃. Evidence is provided that native Vn is incapable of binding to integrin adhesion receptors and that its RGD sequence is not exposed. Conformational changes in Vn, induced by chemical or thermal denaturation, or by complexation with PAI-1, expose the integrin binding site on Vn, allowing it to bind tightly to both αβ₃ and GPIIb/IIIa.

MATERIALS AND METHODS

Proteins and Antibodies—Denatured Vn was purified by heparin affinity chromatography in the presence of 8 M urea (38). Vn was also isolated in denaturing conditions by borodic acid-urea-published procedures (39). Protein concentrations were determined by the bicinchoninic acid method (Pierce). The final Vn preparations were devoid of fibrinogen and fibronectin immunoreactivity as judged by immunoblotting using rabbit anti-human fibrinogen or fibronectin (Calbiochem). Denatured Vn was biotinylated using NHS-LC-biotin (Pierce). Briefly, 1.5 mg of Vn was dissolved in 50 mM sodium phosphate buffer, pH 8.5, at 20 µM and incubated with 3 mg of biotinylation reagent dissolved in 100 µL of Me₂SO for 3 h at 37 °C, followed by extensive dialysis against phosphate-buffered saline (PBS). GPIIb/IIIa was obtained from Enzyme Research Laboratories, and αβ₃ was isolated from human placenta extracts by affinity chromatography (40). Human fibrinogen was obtained from Calbiochem. PAI-1 was purified as described (41), activated with 4 a grindunian hydrochloride (42), dialyzed against 5 mM sodium phosphate, 1 mM EDTA, 0.345 M NaCl, pH 6.5, to stabilize the biological activity of PAI-1 (43), and the specific activity was determined by titration against urinary-type plasminogen activator (Calbiochem). Prior to each experiment, PAI-1 was dialyzed into ice-cold PBS. Latent PAI-1 was prepared by incubation of activated PAI-1 at 37 °C for 24 h. After this incubation step, the structural integrity of the resulting PAI-1 preparation was confirmed by SDS-polyacrylamide gel electrophoresis followed by staining with silver nitrate. The remaining PAI-1 activity after this incubation was determined by binding to immobilized tissue-type plasminogen activator (Calbiochem) (44) and was less than 0.5% of the starting material (not shown). Monoclonal antibodies (mAbs) 611, 153, and 1244 were obtained by standard hybridoma techniques using denatured Vn as immunogen (40). IgG was produced in mice as ascites fluid and purified by using protein A-Sepharose. The generation of rabbit anti-human Vn was described previously (41). Biotin-labeled secondary antibodies, streptavidin alkaline phosphatase, and substrate were from Zymed.

Conformational changes in the Vn molecule were quantified by competitive enzyme-linked immunosorbent assay (13). Briefly, microtiter wells were coated with denatured Vn (1 µg/ml in PBS, 4 °C, 16 h), and specific binding sites on the plastic dishes were blocked by incubating the binding to wells incubated with the same concentration of Vn in the presence of 10 mM EDTA. For competitive ligand binding studies, biotin-labeled denatured Vn (1 µg/ml; a concentration required to obtain half-maximal binding) was co-incubated (3 h, 37 °C) with unlabeled native Vn, denatured Vn, or fibrinogen on integrin-coated plates (see above). After washing, bound Vn was detected using streptavidin alkaline phosphatase and substrate. Specific binding was determined by subtracting the binding of biotin-labeled wells incubated in the presence of 10 mM EDTA from that incubated in the presence of divalent cations. Results are expressed as percentage binding in the absence of soluble competitor.

RESULTS

Characterization of mAbs That Block Cell Adhesion to Vn—The effects of three different anti-Vn mAbs on cell adhesion to immobilized Vn were determined (Fig. 1A). Microtiter wells were coated with denatured Vn and incubated with the indicated concentration of antibody and metabolically labeled HT 1080 cells. Cells were allowed to attach for 1 h. Bound cells were quantified by β-counting. mAb 153 and 611 inhibited cell adhesion to Vn in a dose-dependent manner, whereas mAb 1244 was significantly (approximately 20-fold) less effective. The observed difference was apparently not due to a lower affinity of mAb 1244 for denatured Vn, since this antibody and clone 153 differ in the affinity for denatured Vn only by factor 2 (41). In contrast, no inhibition was observed using normal mouse IgG (data not shown). The mAbs 153 and 611 bind to Vn polypeptides encompassing amino acids 1–51, a fragment of Vn containing the RGD sequence, whereas the control antibody interacts with amino acids 52–239 (41) (data not shown).

We previously provided evidence that an immunoeptope (mAb 153) located in the SMB domain of Vn (amino acids 1–51) is cryptic in plasma Vn (15). Experiments were performed to understand whether lack of reactivity with native plasma Vn is a general feature of antibodies derived to this region of the Vn molecule. Competitive enzyme-linked immunosorbent assays were employed to determine the reactivity of mAb 611 with purified preparations of native and denatured Vn and Vn present in unfractionated plasma (Fig. 1B). Results were compared with mAb 153. Microtiter wells coated with denatured Vn were incubated with a constant amount of anti-Vn IgG and increasing concentrations of different types of Vn (Fig. 1B). Denatured Vn competes with the binding of both antibodies to immobilized denatured Vn in a dose-dependent manner, whereas neither antibody reacted well with Vn present in unfractionated plasma. The reactivity with purified native Vn with these two conformationally sensitive antibodies was at least 50-fold lower.
Characterization of mAbs that block cell adhesion to vitronectin. A, effect of mAbs to Vn on HT 1080 cell adhesion. Microtiter wells coated with denatured Vn were preincubated with the indicated concentration of mAb 153 (open circles), mAb 611 (closed circles), or mAb 1244 (closed squares), followed by the addition of 35S-labeled HT 1080 cells for 1 h. After gentle washing, bound cells were quantified by β-counting. Results are expressed as percentage of cells specifically bound (see “Materials and Methods”). B, expression of conformationally sensitive epitopes in Vn. The indicated concentration of denatured Vn (squares), native Vn (triangles), or unfractionated platelet-poor plasma (circles) were incubated with a constant concentration of anti-Vn IgG (closed symbols, mAb 153; open symbols, mAb 611) in microtiter wells coated with denatured Vn. The wells were washed, and bound antibodies were detected (see “Materials and Methods”). Results are expressed as percentage binding of antibodies in the absence of soluble competitor.

Binding of Native and Denatured Vn to GPIIb/IIIa and αvβ3—Based on the strong reactivity of the inhibitory antibodies with denatured Vn, and the inability of these antibodies to bind native Vn, we hypothesized that the RGD sequence in native Vn is cryptic. This hypothesis was tested by measuring the binding of native and denatured Vn to purified integrins (Figs. 2 and 3). To measure binding, microtiter wells were coated with purified integrin and then incubated with a range of either native or denatured Vn. Bound Vn was detected with rabbit anti-human Vn, followed by biotin anti-rabbit IgG, streptavidin alkaline phosphatase, and substrate. Results are expressed as mOD/min (change of absorbance at 405 nm). B, competitive binding studies and compared with fibrinogen. Native Vn showed little binding. Again, results were confirmed in competitive binding studies and compared with fibrinogen. Native Vn failed to compete with the binding of denatured Vn for binding to αvβ3. Both denatured Vn and fibrinogen competed for the binding of biotinylated Vn to αvβ3. Little difference was observed in the extent of competition between the latter two ligands.

Denaturation of Native Vn Exposes the RGD Sequence—Only about 7% of the total Vn present in plasma is recovered using a heat denaturation procedure that maintains the protein in its native conformation (39). This raised the possibility that a non-integrin-binding subpopulation of native Vn could have been purified and employed in the binding studies. To address this issue, a heat denaturation procedure was employed to convert for GPIIb/IIIa. Fibrinogen was 10–50-fold more efficient in competing with the binding of labeled Vn for GPIIb/IIIa than denatured Vn. More importantly, no competition was observed using native Vn, confirming the inability of the native form of Vn to bind to GPIIb/IIIa.

GPIIb/IIIa is only one of several integrins that are reported to bind Vn. The αvβ3 integrin is also considered a receptor for Vn and was originally named the “vitronectin receptor.” To determine whether the αvβ3 integrin also exhibited preferential binding to the denatured form of Vn, similar binding studies were performed using immobilized αvβ3 (Fig. 3A). Denatured Vn bound to αvβ3 in a dose-dependent manner and again approached saturation at 5 μg/ml Vn. In contrast, native Vn showed little binding. Again, results were confirmed in competitive binding studies and compared with fibrinogen. Native Vn failed to compete with the binding of denatured Vn for binding to αvβ3. Both denatured Vn and fibrinogen competed for the binding of biotinylated Vn to αvβ3. Little difference was observed in the extent of competition between the latter two ligands.
native VN into conformationally altered, multimeric VN (Fig. 4). Heat treatment of native VN resulted in the exposure of the mAb 611 epitope (Fig. 4A) and mAb 153 epitope (not shown) to a similar extent as in denatured VN. In addition, the electrophoretic mobility of heat-treated native VN on native polyacrylamide gel electrophoresis was indistinguishable from denatured VN (i.e. heat treatment resulted in the formation of high molecular weight multimers that failed to enter the separating gel) (not shown). The interaction of heat-treated native VN with \( \alpha_\beta_3 \) was compared with that of purified denatured VN (Fig. 4B). Heat-treated VN binds to immobilized \( \alpha_\beta_3 \) in a dose-dependent manner similar to denatured VN (Fig. 4B). In addition, heat-treated native VN competes with labeled denatured VN for binding to this integrin to an extent comparable with that of denatured VN (Fig. 4C). Similar results were obtained when binding to GPIIb/IIIa was examined (not shown). In addition, an alternative denaturation procedure (i.e. treatment of native VN with 8 M urea) also induced integrin binding (not shown). These observations indicate that denaturation of native VN results in the exposure of the cryptic cell binding domain and that the inability of native VN to bind to \( \alpha_\beta_3 \) or GPIIb/IIIa was not due to the purification of a nonintegrin-binding subpopulation of VN from plasma.

**Effects of PAI-1 on the Adhesive Functions of Native and Denatured VN**—VN binds to a number of plasma proteins, and these interactions alter the conformation of this molecule (10–12). Experiments were performed to test whether physiological ligands of native VN can induce the exposure of the cell adhesion domain. The binding of native VN to active PAI-1 results in the formation of VN multimers that express epitopes for conformationally sensitive antibodies like mAbs 153 (10) and 611 (data not shown). Interestingly, PAI-1-VN complexes are relatively labile and PAI-1 readily dissociates from VN, but VN remains multimeric and conformationally altered (10). We reasoned that the binding of PAI-1 to native VN is likely to expose the integrin binding site on VN. Native VN was incubated with a 2-fold molar excess of active PAI-1 for 16 h at 37 °C. After this incubation period, more than 99% of the PAI-1 was converted into the latent conformation as based by binding to immobilized tissue-type plasminogen activator (not shown). Little competition of native VN (Fig. 5A, left closed bar) for mAb 153 binding to immobilized denatured VN was observed. Incubation of native VN with active PAI-1 (Fig. 5A, left open bar) resulted in the exposure of the conformationally sensitive mAb 153 epitope. In contrast, no effects of latent PAI-1 on the exposure of this epitope was observed (Fig. 5A, left hatched bar). The mAb 153 does not detect VN in complex with PAI-1 (41), con-
modulation of the adhesive functions of Vn by PAI-1. A, PAI-1 induces the exposure of conformationally sensitive epitopes and $\alpha_\beta$, binding site in native Vn. Native Vn (20 $\mu$g/ml, closed bars) was incubated with active PAI-1 (40 $\mu$g/ml, open bars) or latent PAI-1 (40 $\mu$g/ml, hatched bars) for 16 h at 37 °C and tested for the exposure of the conformationally sensitive mAb 153 epitope as in Fig. 1 or for binding to immobilized $\alpha_\beta$, as in Fig. 3 (10 $\mu$g/ml final concentration of Vn). Results are expressed as percentage inhibition (mAb 153) or binding ($\alpha_\beta$,) in comparison with denatured Vn (10 $\mu$g/ml). Identical results were obtained in three independent experiments with a standard error of less than 10%. B, effects of PAI-1 on the binding of denatured Vn to $\alpha_\beta$. Increasing concentrations of active (closed circles) or latent (open circles) PAI-1 were co-incubated with denatured Vn (500 ng/ml) on $\alpha_\beta$,-coated plates. Bound Vn was detected with rabbit anti-human Vn IgG, followed by biotin-labeled anti-rabbit IgG, streptavidin alkaline phosphatase, and substrate. Results are expressed as percentage binding in the absence of PAI-1. Closed squares, $\alpha_\beta$,-coated wells were preincubated with denatured Vn (500 ng/ml), washed, and then incubated with the indicated concentration of active PAI-1 for 1 h at 37 °C. Bound Vn was detected as above.

Confirming that the majority of the Vn was present in an uncomplexed, conformationally altered form. In parallel experiments, the effects of pretreatment of native Vn with PAI-1 on its ability to bind to $\alpha_\beta$ (Fig. 5A, right bars) was analyzed. Again, while little binding of native Vn (Fig. 5A, right closed bar) to $\alpha_\beta$, was observed, PAI-1-induced Vn multimers (A, right open bar) demonstrated increased binding to $\alpha_\beta$. In contrast, latent PAI-1 (Fig. 5A, right hatched bar) had failed to induce $\alpha_\beta$ binding of native Vn.

The PAI-1 and integrin binding sites in denatured Vn are structurally distinct but located in close proximity, raising the possibility that PAI-1 may modulate the adhesive functions of denatured Vn. To test this hypothesis, microtiter wells were co-incubated with a constant amount of denatured Vn (which binds to $\alpha_\beta$) and increasing concentrations of either active or latent PAI-1 in this binding assay (Fig. 5B). Active PAI-1 blocked the binding of denatured Vn to immobilized $\alpha_\beta$, in a dose-dependent manner. At the highest concentration of PAI-1, approximately 80% inhibition was observed. The competition reached a plateau at a 10-fold molar excess of active PAI-1 over denatured Vn. In contrast, latent PAI-1 has little effect on the binding of Vn to $\alpha_\beta$. These findings are consistent with the observation that latent PAI-1 lacks high-affinity binding to denatured Vn (41). However, when Vn-$\alpha_\beta$ were preformed on microtiter wells, active PAI-1 failed to dissociate these complexes (data not shown).

DISCUSSION

The observations in this report demonstrate that native Vn, present in blood, is not an adhesive glycoprotein. This conclusion is based on the findings that antibodies against Vn that block its adhesive function fail to bind to the native form of Vn. These blocking antibodies are capable of binding to denatured Vn or PAI-1-induced Vn multimers. In addition, direct binding studies prove that denatured Vn binds to both GPIIb/IIIa and $\alpha_\beta$, but that native Vn is unable to bind to these integrins. Moreover, native Vn, which is not adhesive, could be converted into an adhesive glycoprotein by chemical and thermal denaturation. It should be noted that at high concentration, binding of native Vn to integrins was observed. This finding is probably related to the presence of some denatured Vn in the native Vn preparation. This conclusion is supported by the observation that unfractionated plasma was unreactive with conformationally sensitive antibodies, whereas the native Vn employed in this study showed exposure, although limited in comparison with denatured Vn, of conformationally sensitive epitopes. Based on the observations presented here, the majority of Vn present in vivo (i.e. in plasma) is not expected to bind to integrins. A number of consideration suggest that the adhesive functions of Vn are mediated through the N-terminal SMB domain rather then the C-terminal glycosaminoglycan binding domain. First, site-directed mutagenesis studies revealed that the RGD motif located between amino acids 45 and 47 is required for cell adhesion to Vn (32, 33). Second, heparin does not affect the adhesion of HIT 1080 or a number of other cell lines to immobilized Vn. Similarly, heparin was without effect in the purified receptor binding assay. Third, recent studies from our laboratory indicate that native Vn binds heparin, implying that the glycosaminoglycan binding domain is exposed in the native conformation.

Interestingly, PAI-1 can have opposing effects on the adhesive properties of Vn. First, active PAI-1 blocked the binding of denatured Vn to $\alpha_\beta$ in a dose-dependent manner. The binding site for active PAI-1 has been localized to amino acids 1 to 40 (41), a region in close proximity to the RGD sequence at residues 45–47 of Vn (3, 4). RGD-containing peptide has no effect on the binding of PAI-1 to Vn (41), suggesting that PAI-1 is not binding directly to the cell adhesion domain in Vn. The close proximity of both sides makes it likely that the competition of PAI-1 for Vn is due to steric hindrance. In contrast, latent PAI-1, which does not bind to the N-terminal high affinity PAI-1 binding site in Vn (41), failed to compete with integrin binding of Vn. In addition, PAI-1 failed to dissociate pre-existing Vn-$\alpha_\beta$, complexes. Thus, PAI-1 is not expected to detach anchored cells, but may modulate biological processes dependent on de novo formation of adhesive contacts between Vn and $\alpha_\beta$, including cell migration. These observations have been confirmed in a recent publication (47).

Second, active PAI-1 had an additional affect on Vn binding to integrins. PAI-1 induced the formation of conformationally altered Vn multimers, and these multimers present, after dissociation from PAI-1, an exposed cell adhesion domain. The Vn concentration in plasma is between 2.5 and 5 $\mu$M (1, 2), whereas normal PAI-1 levels are approximately 0.4 nM (48). Plasma levels of PAI-1 are regulated under pathophysiological condi-
...and for example endotoxinemia results in a dramatic up-regulation of plasma PAI-1 levels to 20 nM (48). However, these concentrations are still 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 (10). Thus, PAI-1 induced Vn multimerization in plasma is expected to have little affects on the adhesive properties of Vn. The scenario may be quite different in tissues and in platelets. PAI-1 and Vn are both contained within platelet α-granules (49, 50), and platelets contain 26 nM Vn and 12 nM PAI-1 per 10^9 platelets (49, 51, 52). Interestingly, platelet Vn is present in high molecular weight multimers that express the mAB 153 epitope (50). It should be noted that the expression of the cryptic mAB 153 epitope appears to correlate with the adhesive properties of Vn. These observation suggest that platelet Vn is adhesion competent. Platelet-derived proteins concentrate up to 200-fold in areas of thrombosis, suggesting that platelet-derived adhesion competent Vn will accumulate in areas of tissue injury and thrombosis. Similarly, increased tissue-associated levels of PAI-1 have been reported in a number of pathophysiological conditions, including endotoxinemia (48), raising the possibility that PAI-1 will regulate the adhesive properties of Vn in inflamed tissues. Taken together, these observation support the concept that plasma Vn is in a latent form. Interactions with either free proteases inhibitors or inhibitors in complex with the respective target protease results in the exposure of the cryptic RGD motif.

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