Evidence that conformational changes upon the transition of the native to the modified form of vitronectin are not limited to the heparin binding domain

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Abstract Vitronectin (Vn) exists in vivo in at least two different conformational states, the native and the modified form, and these forms have different ligand binding properties. To characterize the molecular events associated with this conformational flexibility, modified Vn was analyzed by competitive ELISA using a panel of conformationally sensitive antibodies with known epitopes. These studies provided evidence for major molecular rearrangements upon the transition from the native to the modified form that are not limited to the C-terminal heparin binding domain, but also occur in the N-terminal part of the molecule.

Key words: Vitronectin; Adhesive glycoprotein

1. Introduction

Vitronectin (Vn), an adhesive glycoprotein present in the circulation and in a variety of tissues, is a conformationally labile molecule (reviewed in [1,2]). While only 2% of Vn in plasma binds to heparin-Sepharose, the relative amount increases to approximately 7% by the generation of serum [3]. Similarly, the formation of disulfide bonded complexes of Vn with thrombin–antithrombin III, as well as its interaction with either heparin or with complement CSb-7, induces a conformational change in the molecule, leading to the expression of the cryptic heparin binding domain and exposure of an epitope for a conformationally sensitive antibody (i.e. 8E6) [2], tentatively mapped to C-terminal Vn-fragments [4].

The structural basis for the conformational instability of Vn is only incompletely understood. It was suggested that heteropolar interactions between the N-terminal located acidic domain, and the pnlycationic heparin binding domain internally stabilize the entire molecule, which forces the heparin binding domain into a cryptic location within the native molecule as compared to an exposed position in the modified or denatured form [1]. Conformational changes in the Vn molecule are closely linked to a spontaneous formation of disulfide-linked multimers [5–7]. Interestingly, Vn present in the supernatant of stimulated platelets is conformationally altered and multimeric [6].

The conversion of native to the conformationally altered, multimeric form appears to be critical for some of the functions of Vn. For example, a number of its binding properties (e.g., interaction with collagen [8], β-endorphin [9], type 1 plasminogen activator inhibitor [10–12], urokinase receptor [13], incorporation into the extracellular matrix [14,15], cell binding [16]), depend on exposure of the cryptic heparin-binding region, and the modified form of Vn has been postulated to act as a scavenger for 'spent' molecules (e.g. thrombin–antithrombin III complexes [17]). Taken together, these results indicate that Vn in vivo exist in at least two distinct states, and these forms have different ligand binding properties. In the present study, we provide evidence that conformational changes upon the transition of the native to the modified form of the Vn molecule are not limited to the heparin binding domain.

2. Materials and methods

2.1. Proteins and antibodies

Native Vn was purified from pooled human plasma as described [18]. Modified Vn was obtained by incubation of native Vn (0.5 μM final concentration) in PBS at 55°C for 1 h as described [6]. For some experiments, solid urea was added to native Vn to a final concentration of 8 M, followed by incubation at 37°C for 2 h and extensive dialysation against PBS. This form of Vn was designated 'urea-treated' native Vn. Human Vn was also purified by heparin affinity chromatography in the presence of 8 M urea [19]. The latter form is referred to as 'denatured' Vn. CNBr cleavage and acid hydrolysis of denatured Vn were performed as described [20]. Monoclonal antibodies (MAbs) 153 (directed against amino acids (aa) 1–40) and 1244 (directed against aa 52–239) were obtained by standard hybridoma techniques using denatured Vn as immunogen [21]. The hybridoma clone producing MAb 8E6 (directed against aa 52–239; see Fig. 3) was kindly provided by Dr. D. Mosher (University of Wisconsin). MAbs were produced as ascites, and the resulting IgG was purified by using protein A-agarose (MAbs 153, 1244) or by the caprylic acid method (MAb 8E6) [22]. Selected MAbs were biotinylated with NHS-LC-Biotin according to the manufacturer (Pierce). Antibodies against purified native human Vn were also raised in rabbits and the IgG fraction was purified by using protein A-agarose as described [23]. Protein concentration was estimated either by the bicinchoninic acid method using bovine serum albumin (BSA) as a standard (Pierce) or by spectrophotometric methods using reported absorption coefficients [1,22].

2.2. ELISA

Conformational changes in the Vn molecule were quantified by competitive ELISA as described [7,24]. Briefly, microtiter wells were coated with denatured Vn (1 μg/ml in PBS; 4°C, 16 h), and non-specific binding sites on the plastic dishes were blocked by incubating the washed wells for 1 h at 37°C in PBS containing 3% casein and 0.05% Tween 80. The wells were co-incubated for 1 h with a constant amount of IgG and the indicated concentration of Vn in PBS containing 0.1% casein and 0.1% Tween 80. The following final concentrations of antibodies were used: 153 and 1244, 50 ng/ml; and MAb 8E6, 300 ng/ml. These concentrations were required for half-maximal binding in the absence of soluble Vn competitor (not shown). Bound IgG was detected with biotin-conjugated goat anti-mouse or anti-rabbit IgG, followed by streptavidin alkaline phosphatase conjugate and the chromogenic substrate *p*-nitrophenyl phosphate (Zymed). The change of absorbance at

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Abbreviations: BSA, bovine serum albumin; MAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; Vn, vitronectin.

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405 nm of duplicate wells was determined and the absorbance of wells not incubated with antibodies but otherwise treated identically was subtracted. For cross-competition experiments, microtiter wells were coated with denatured Vn and blocked as above and incubated with a constant concentration of biotinylated MAb (at a concentration required for half-maximum binding) in the presence of increasing concentrations of unlabeled antibodies for 1 h at 37°C. After washing, bound biotinylated MAbs were detected with streptavidin alkaline phosphatase conjugate, followed by the chromogenic substrate. To determine the binding of Vn to heparin, microtiter wells were coated (1 mg/ml in PBS; 4°C, 16 h) with porcine heparin (Sigma), blocked as above, and incubated with the indicated concentrations of Vn in PBS containing 0.1% casein and 0.1% Tween 80. Bound Vn was detected with rabbit anti-human Vn IgG (10 μg/ml; 1 h, 37°C), followed by biotin-conjugated goat anti-rabbit IgG, streptavidin alkaline phosphatase complex and chromogenic substrate. Results of duplicate wells were averaged and corrected for absorbance at 405 nm from wells not incubated with Vn, but otherwise treated identically.

2.3. Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was performed in slab gels according to Laemmli [25]. The upper stacking gel contained 4% acrylamide, while the lower resolving gel contained 9% or 14% acrylamide. Native PAGE was performed on 3% acrylamide (stacking gel) and 7.5% acrylamide (resolving gel) in the absence of SDS using the Laemmli buffer system as described [6]. Reduction of samples for native PAGE and SDS-PAGE was carried out by the addition of DTT (50 mM) to the sample buffer. In control experiments, the success of the reduction for the native PAGE system was tested using human two-chain urokinase. Non-reduced urokinase, with a reported isoelectric point of 9.4 [26] did not enter the gel, as expected from the pH of the discontinuous native PAGE, whereas the acidic A-chain of urokinase was separated upon reduction (not shown). After electrophoresis, the proteins in the gel were transferred onto nitrocellulose membranes and analyzed by immunoblotting using MAb 1244 as described [21].

3. Results and discussion

Interaction of Vn with physiological ligands, or denaturation of Vn by heat-treatment or protein denaturants, induces conformational changes in the Vn molecule, followed by the formation of disulfide-bonded multimers and exposure of the C-terminal heparin binding domain [2,6]. To characterize further the nature of the conformational changes in the Vn molecule associated with the transition from the native to the modified form, Vn was mildly denatured by heat-treatment as described [6]. The resulting modified Vn was characterized by SDS-PAGE and native PAGE to document the success of the denaturation and compared to the electrophoretic mobility of native Vn (Fig. 1). Upon reduction, modified Vn co-migrated with the native form of the molecule on SDS-PAGE with the expected M_rs of 75,000 and 65,000 (Fig. 1, panel A). In contrast, when modified Vn was analyzed by SDS-PAGE under non-reducing conditions, the majority of the protein remained in the stacking gel and failed to enter the separating gel (Fig. 1, panel B). This electrophoretic mobility is characteristic for multimeric, modified Vn [6]. In contrast, native Vn migrated as a diffuse band between 75 and 65 kDa (Fig. 1, panel B, lane 1). The structure of the heat-induced Vn multimers was further analyzed by native PAGE. When modified Vn was fractionated by native PAGE under non-reducing conditions, the multimers remained in the stacking gel, whereas the majority of the native Vn entered the separating gel (Fig. 1, panel C) and co-migrated with Vn present in plasma (not shown). In a related assay system, modified Vn was fractionated on native PAGE gels under reducing conditions (Fig. 1, panel D). Also under these conditions, the majority of the modfied Vn failed to enter the

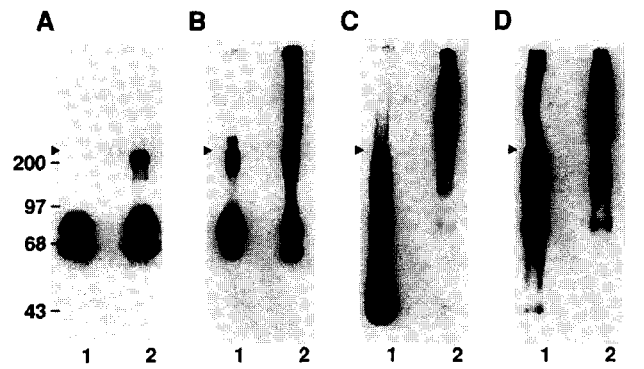

Fig. 1. Heat-treatment induces covalently- and non-covalently stabilized Vn multimers. Purified native Vn (lanes 1) was heated at 55°C for 1 h (lanes 2), fractionated by SDS-PAGE under reducing (panel A) and non-reducing (panel B) conditions, and by native PAGE under non-reducing (panel C) and reducing (panel D) conditions, and transferred to nitrocellulose membranes. The filters were analyzed by immunoblotting using MAb 1224 followed by ^{125}I-labeled sheep anti-mouse IgG, and subjected to autoradiography. The migration of M, standards for panels A and B are indicated to the left. Arrowheads denote the interface between stacking- and resolving gel.

separating gel. It should be noted that under reducing conditions, native Vn partially aggregated to lower M_r multimers that are not present in the non-reducing native PAGE gel (compare Fig. 1, panel C lane 1, and panel D lane 1). This observation may be related to a partial reversibility of the reduction during electrophoresis, resulting in free reactive cysteine residues that together with the expected high protein concentration at the interface between stacking and separating gel may facilitate intermolecular disulfide exchange. This conclusion is supported by the finding that Vn multimers are formed in a concentration-dependent manner [6]. Taken together, these results support previous observation that Vn multimers are stabilized by covalent- and non-covalent interactions [7] and indicate that the Vn molecule was successfully denatured by the mild heat-treatment.

In order to characterize conformational changes in the Vn molecule associated with the transition from the native to the modified form, relative reactivities of native and modified Vn with a panel of MAbs with known epitopes were determined using competitive immunoassays (Fig. 2). Increasing concentrations of either native or modified Vn were co-incubated with a constant concentration of MAbs on Vn coated plates and assessed for ability to compete for binding of MAbs to immobilized, denatured Vn. The reactivities of the modified Vn with MAb 8E6 and with immobilized heparin were used as positive controls since both binding sites are preferentially expressed in the denatured form [2]. Modified Vn reacted approximately 10-fold better than native Vn with MAb 8E6 (Fig. 2, panel C) and interacted with immobilized heparin in a dose-dependent manner, whereas only weak binding of native Vn to heparin-coated microtiter wells was detected (Fig. 2, panel D). In control experiments, modified Vn failed to bind to casein-blocked microtiter wells, indicating that the binding was specific (Fig. 2, panel D). The reactivity of modified Vn with MAbs directed to the N-terminal somatomedin B domain (Fig. 2, panel A) or the immediately C-terminal connecting region/first hemopexin-like repeat (i.e. amino acid 52–239) was compared to native Vn.

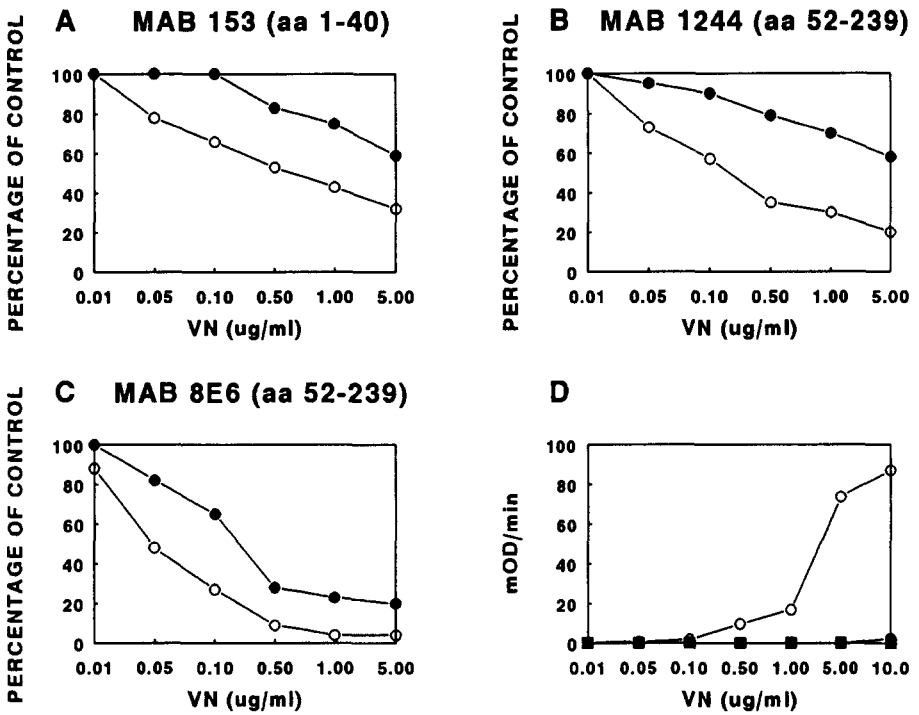

Fig. 2. Exposure of conformationallyv sensitive epitopes in modified Vn. The indicated concentrations of native Vn (closed circles) or modified Vn (open circles) were co-incubated with a constant concentration of anti-Vn MAbs on microtiter wells coated with denatured Vn. The binding of the MAbs to immobilized Vn was detected with biotin-labeled goat anti-mouse IgG, followed by streptavidin alkaline phosphatase complex and substrate (see section 2). (A) MAb 153 (directed against amino acids 1–40). (B) MAb 1244 (directed against amino acids 52–239). (C) MAb 8E6 (directed against amino acids 52–239; compare Fig. 4). In panel (D), the binding of native (closed circle) or modified Vn (open circle) to immobilized heparin was determined (see section 2). The binding of modified Vn to immobilized casein is indicated by the closed squares.

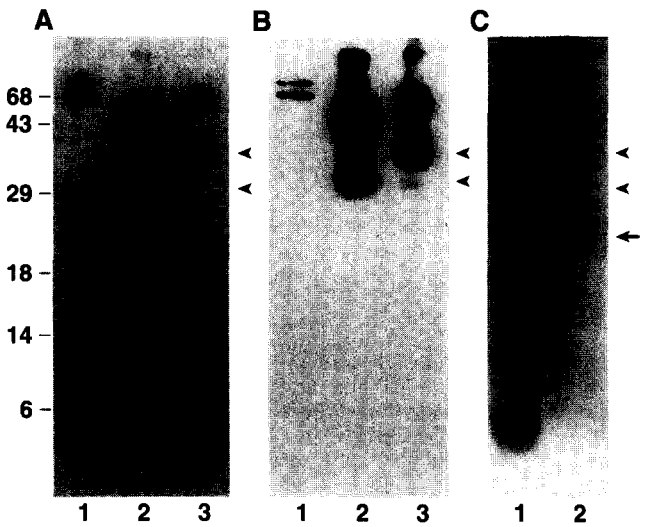

Fig. 3. Immunoblotting analysis of the binding of MAbs to Vn and Vn fragments. Vn was cleaved with cyanogen bromide or acid hydrolyzed and fractionated by SDS-PAGE (14% acrylamide in the separating gel) and transferred to nitrocellulose membranes. The filters were incubated with MAb 8E6 (panel A) or MAb 1244 (panel B), followed by ^{125}I-labeled sheep anti-mouse IgG. The membranes then were exposed to X-ray film for 16 h. (A and B) lanes 1 = uncleaved Vn; lanes 2 = cyanogen bromide cleaved Vn; lanes 3 = acid-hydrolyzed Vn. In panel (C), the Vn fragments (lane 1 = cyanogen bromide cleavage; lane 2 = acid-hydrolysis) were stained with Coomassie blue R250. The position of M, standards is indicated to the left. The arrowheads refer to the position of fragments of 40 and 35 kDa and the migration of the C-terminal 26 kDa fragment is indicated by the arrow.

The epitope mapping of these antibodies using defined Vn-fragments has been described previously [21]. Modified Vn reacted approximately 100-fold better than native Vn with both antibodies (Fig. 2, panels A and B). Similar results were obtained comparing the reactivity of native Vn with that of urea-treated native Vn (see section 2) or denatured Vn (not shown). Thus, conformational changes in the Vn molecule are not restricted to the heparin binding domain, but appear to occur also in the N-terminal somatomedin B domain and the connecting region/first hemopexin-like repeat. It should be noted that the regions in the Vn molecule recognized by the above antibodies have been recently reported to be destroyed upon denaturation and thus probably exposed in the native molecule [27]. At least two considerations may account for these differences. The Vn employed in this latter study was denatured by treatment with 8M urea and in addition reduced, whereas the Vn employed in our study was only denatured by either heat- or urea-treatment in the absence of reducing agents. Thus, it remains unclear whether the loss of epitope expression in the Morris study is actually due to the conversion of the native to the conformationally altered, denatured form, or general alterations in the protein structure due to reduction. Secondly, differences in epitope expression between native and denatured Vn were not determined in solution, but upon immobilization of Vn to plastic wells. The latter treatment is reported to induce conformational changes in the native Vn molecule [1].

The MAb 8E6 has been used as the standard to evaluate conformational changes in the Vn molecule upon ligand binding [2,5,7]. The epitope for this antibody has been tentatively

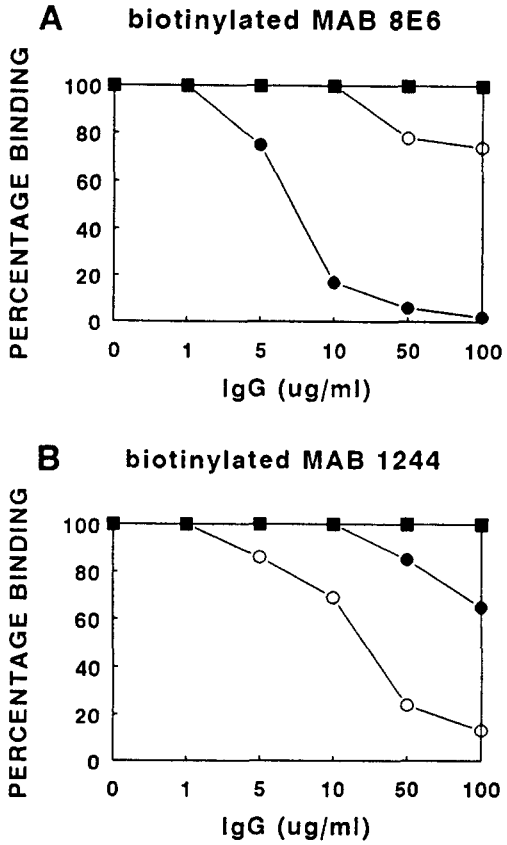

Fig. 4. Cross-competition of MAbs. Denatured Vn coated microtiter wells were incubated with a constant concentration of biotinylated MAb 8E6 (panel A) or biotinylated MAb 1244 (panel B) in the presence of increasing concentrations of unlabeled MAb 8E6 (closed circles), MAb 1244 (open circles), or MAb 153 (closed squares). Bound labeled antibody was detected with streptavidin alkaline phosphatase complex, followed by substrate. Results are expressed as percentage binding in the absence of unlabeled competitor.

mapped based on functional studies (i.e. interference with ligand binding in the heparin binding domain) to the C-terminal portion of the Vn molecule [4]. To reevaluate this assessment, the binding of MAb 8E6 to Vn-fragments derived from incomplete acid hydrolysis and incomplete CNBr-cleavage was analyzed by immunoblotting and compared to MAb 1244, previously mapped to amino acids 52–239 [21]. Surprisingly in view of the reported results, the migration of the fragments detected by MAb 8E6 were very similar if not identical to that stained by MAb 1244 (Fig. 3). Specifically, both antibodies detected the M_r 40,000 N-terminal Vn fragrnent derived from acid hydrolysis (compare Fig. 3, panels A and B, lanes 3, arrowheads) and the M_r 35,000 fragment (amino acid 52–239) derived from cleavage by CNBr (Fig. 3, panels A and B, lanes 2, arrowheads). It should be noted that the localization of these fragments in the intact Vn molecule was determined by N-terminal sequence analysis [20]. However, the M_r 26,000 C-terminal fragment (Fig. 3, panel C, lane 2, arrow) derived from acid hydrolysis was present in the digest as revealed by Coomassie blue staining, but failed to react with both antibodies. Moreover, none of these antibodies stained the 12 kDa fragment derived from CNBr cleavage encompassing the heparin binding domain (Fig. 3, panel C, lane 1) and incubation of the membranes without primary antibody abolished the specific signal (not shown).

The relationship between the 8E6 and 1244 epitopes was further evaluated in competitive binding assays (Fig. 4). Unlabeled MAb 8E6 competed with the binding of biotinylated MAb 8E6 to immobilized Vn in a dose-dependent manner, whereas MAb 1244 only weakly interfered with the binding at relatively high IgG concentrations (Fig. 4, panel A). Consistently, only weak competition of MAb 8E6 with the binding of labeled MAb 1244 to immobilized Vn was detectable (Fig. 4, panel B). Thus, although both antibodies interact with the CNBr cleavage-derived 35 kDa fragment (i.e. amino acid 52–239), this functional assay revealed that these two epitopes are clearly distinct.

It should be noted that the MAb 8E6 was recently reported to cross-react with a sulfated 30 kDa protein, and inhibition of sulfatation decreased the reactivity of this antibody for Vn and the 30 kDa protein, suggesting that sulfate, at least in part, may be part of the epitope recognized by MAb 8E6 [28]. The sites of tyrosine sulfatation have been mapped to residues 56 and 59 in Vn [29]. In agreement with these observation, MAb 8E6 failed to detect Vn polypeptides containing amino acid 52–239 expressed in *E. coli* using the pET 15b expression plasmid (Novagen), whereas MAb 1244 strongly bound to this molecule (not shown). Taken together, these experiments strongly suggest that the 8E6 epitope is localized immediately upstream of the somatomedin B domain of the Vn molecule.

Similar differences in the epitope expression between native and modified Vn were not restricted to the antibodies characterized in this study. For example, polyclonal antibodies derived from immunization with native Vn reacted approximately 100-fold more strongly with denatured Vn in comparison to native Vn, and we were unable to identify MAbs specific for native Vn using a panel of more than 250 different hybridoma clones. Taken together, these results suggest that the native form of the molecule is poorly immunogenic, consistent with the high extent of cross-species conservation of both functional domains and primary structure [30].

In summary, this study provides evidence that conformational changes in the Vn molecule upon denaturation are not limited to the heparin binding domain, suggestive of major molecular rearrangements upon the transition of native to modified Vn.

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