Conformational States of Vitronectin: Preferential Expression of an Antigenic Epitope When Vitronectin Is Covalently and Noncovalently Complexed With Thrombin-Antithrombin III or Treated With Urea

By Bianca R. Tomasini and Deane F. Mosher

A difference in recognition of the adhesive glycoprotein vitronectin (also called S-protein, serum spreading factor, and epibolin) by monoclonal antibody 8E6 (Hayman EG, et al, Proc Natl Acad Sci USA 80:4003, 1983) was investigated using a competitive enzyme-immunosorbent assay and immunooaffinity chromatography. Recognition of vitronectin in serum was approximately 50-fold greater than recognition of vitronectin in plasma. Recognition of vitronectin incubated with heparin, thrombin-antithrombin III complex, or heparin and thrombin-antithrombin III complex together was 2.5-, 7-, or 32-fold greater, respectively, than recognition of vitronectin alone. Thrombin or antithrombin III by itself did not induce the antigenic change. Factor Xa-antithrombin III was less effective than thrombin-antithrombin III in induction of the change. Dextran sulfate and fucoidan were more potent than heparin in induction of the antigenic change, whereas dermatan sulfate, hyaluronic acid, heparan sulfate, chondroitin sulfate, or keratan sulfate were less effective. Immunoblotting analysis of serum and of vitronectin incubated with thrombin and antithrombin III demonstrated the presence of complexes composed of vitronectin and thrombin-antithrombin III that could only be dissociated with reducing agent. N-ethylmaleimide completely blocked the formation of the presumably disulfide-bonded complexes and partially blocked the antigenic change. Both non-disulfide-bonded and disulfide-bonded vitronectin bound to antibody-Sepharose from a mixture of vitronectin and thrombin-antithrombin III. Treatment of vitronectin with 8 mol/L urea resulted in enhanced recognition by the monoclonal antibody. Thus, the 8E6 antibody reacts with an epitope that is preferentially expressed by noncovalently and covalently linked vitronectin/thrombin-antithrombin III complexes and by urea-treated vitronectin. The change in vitronectin induced by thrombin-antithrombin III, therefore, is a physiological correlate of urea treatment and of adsorption of vitronectin into tissue culture plastic (as is done in cell adhesion assays). The change may be important for expression of vitronectin activity.

VITRONECTIN, also known as S-protein,1,3 serum spreading factor,4 and epibolin,5 is a glycoprotein present in human plasma and serum at concentrations of 200 to 400 μg/mL.6 Vitronectin rapidly adsorbs to glass7 and polystyrene plastic. It has been purified by several routes.5,8,9 It migrates on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), under reducing conditions, as two polypeptides with approximate molecular weights of 75,000 and 65,000. Two cDNA clones for vitronectin have been isolated and sequenced,11 and found to confer cell adhesion promoting activity. The fibroblast receptor that recognizes this sequence in vitronectin has been isolated,14 and found to belong to the “integrin” family of cell-adhesion receptors.15 The central domain of vitronectin is somewhat enriched in hydrophobic residues.11 Near the carboxyl terminus is a 12,000 molecular weight arginine-rich region that has been shown to be responsible for heparin binding.6,11 VITRONECTIN contains 14 cysteine residues, at least one of which is present as a free sulphydryl group.10,18

In addition to promoting the adhesion of various cells in culture,4,19 other activities have been described for vitronectin. Characterized as S-protein, vitronectin acts as an inhibitor of both the inactivation of thrombin19,20,22 and factor Xa23 by antithrombin III and of the membrane attack complex of complement.24 In addition, denaturant-treated vitronectin binds to collagen,24 and denatured or modified vitronectin,13,19-21 but not unmodified vitronectin,27 binds heparin.

In previous studies,3 we found that vitronectin in serum bound better than vitronectin in plasma to heparin-agarose and, unexpectedly, to an immunoadsorption column of the 8E6 monoclonal antibody of Hayman et al.8 In the present study, we examined expression of the 8E6 epitope in detail and found preferential expression in serum vitronectin and vitronectin exposed to the thrombin-antithrombin III complex or 8 mol/L urea. We postulate that this antigenic difference represents a change in the conformation of vitronectin that allows enhanced binding to heparin and possibly other substances of physiologic interest. We also found that vitronectin forms a complex with thrombin-antithrombin III that requires reducing agent to be broken apart.

MATERIALS AND METHODS

Materials The following materials were purchased from Sigma Chemical Co (St Louis): heparin and all other polysaccharides, protein A-agarose, bovine serum albumin, Tween-20, SDS, N-ethylmaleimide1 (NEM), and alkaline phosphatase substrate 104 (p-nitrophenyl phosphate). Peroxidase substrate systems were: 4-chloro-1-naphthol/H2O2 from Kirkegaard and Perry (Gaithersburg, Maryland). From the Departments of Medicine and Physiological Chemistry, University of Wisconsin, Madison.


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Address reprint requests to Deane F. Mosher, MD, Department of Medicine, University of Wisconsin, 1300 University Ave, Madison, WI 53706.

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Vitronectin complexes. Samples to be used in the indirect enzyme-linked immunosorbent assay system (ELISA) or immunoblotting were prepared as follows. Equimolar amounts, typically 340 mmol/L, of vitronectin, antithrombin III, and thrombin were incubated in the presence or absence of 8 U/mL (50 \mu g/mL) heparin for 30 minutes at 37°C. The incubation was started by the addition of thrombin and ended by the addition of the irreversible thrombin inhibitor, PPACK (10 \mu mol/L, unless otherwise noted). Before SDS-PAGE, samples were treated with 1.6 mol/L urea, 0.6% SDS in the presence or absence of 2% beta-mercaptoethanol (alternatively, 2% glycerol, 2% SDS in the presence or absence of 2% beta-mercaptoethanol was used). Samples were boiled for three minutes and run on slab gels with 8% running gels and 3% stacking gels. Addition of 1 mmol/L NEM to nonreduced samples before addition of denaturant and boiling gave the same results as those shown.

Plasma samples were obtained from platelet-poor fresh frozen citrated plasma by incubating plasma in glass tubes with 20 mmol/L CaCl$_2$ for one hour at 37°C and extracting serum from the clot after syneresis. Whole blood serum was from blood obtained by venipuncture of a human volunteer, collected in glass tubes, incubated at 37°C for two hours, and spun at 2,000 rpm. The supernatant was aliquoted and stored at -70°C.

Treatment of vitronectin with 8 mol/L urea. Vitronectin (275 \mu g/mL) was incubated with or without 8 mol/L urea in the presence or absence of 1 mmol/L NEM (to block free sulphydryl groups) in Tris-buffered saline for two hours at room temperature with gentle mixing. Samples were dialyzed over 24 hours against four changes of 1 L each of Tris-buffered saline. Following dialysis, vitronectin concentration was verified spectrophotometrically; samples were then examined by SDS-PAGE and immunoblotting and assayed by the indirect ELISA system.

Vitronectin indirect or competition ELISA. A direct ELISA was used to determine the relative dilution (concentration) of antibody to be used in the indirect ELISA system.\textsuperscript{32} Microtiter wells were coated with 200 \mu L of a 2 \mu g/mL solution of vitronectin in 0.1% bovine serum albumin in Tris-buffered saline. Samples to be assayed were diluted in Tris-buffered saline, and 250 \mu L were incubated for 30 minutes with 250 \mu L of antibody diluted 1:200, for MaVN culture medium, or 1:1000 for MaS antibody in 0.5% bovine serum albumin/Tris-buffered saline (all incubations were performed in polypropylene tubes). Duplicate wells received 200 \mu L of sample-antibody mixture and were incubated for 45 minutes. Plates were washed four times with 0.05% Tween-20 in Tris-buffered saline and incubated with 200 \mu L/well of a 1:800 dilution of rabbit anti-mouse IgG conjugated to alkaline phosphatase in Tween-20/Tris-buffered saline. Plates were washed and incubated at 37°C with phosphate substrate (200 \mu L/well at 1 mg/mL in Tris-buffered saline, pH 9.5) until maximum A$_{max}$ was at least 0.7.

Data are expressed as a percent of the maximum absorbance unit obtained in each assay, ie, the absorbance obtained when MaVN was incubated with Tris-buffered saline instead of antigen. Data are presented as the average of duplicate determinations. Results were also analyzed for relative change in recognition by MaVN of untreated and treated vitronectin, ie, the ratio of the amount of plasma or untreated vitronectin necessary to compete for 50% of antibody activity in solution divided by the amount of serum or treated vitronectin required for a similar effect.

Immunoblotting. SDS-PAGE\textsuperscript{19} followed by immunoblotting was carried out as previously described.\textsuperscript{1} Briefly, following separation of samples on 8% SDS-PAGE slab gels and electroblotting onto nitrocellulose filters, blots were soaked in Tris-buffered saline containing 3% bovine serum albumin for one hour at 37°C. Blots were then rinsed in Tris-buffered saline and incubated overnight in 10% spent media from MaVN hybridoma cells or 1% appropriate rabbit

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MD), and dianibenzidine tetrahydro-chloride/H$_2$O$_2$ from Polysciences (Warrington, PA). Heparin-Sepharose CL-6B, Sepharose S-200, and BrCN-activated Sepharose were from Pharmacia (Piscataway, NJ). Microtiter 96-well flat-bottom plates (no. 3596) were purchased from COSTAR (Cambridge, MA). YM-30 and PM-10 filters used in protein concentration were from AMICON (Danvers, MA). Nitrocellulose membrane and reagents for PAGE were from Bio-Rad (Richmond, CA). The irreversible thrombin inhibitor, D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) was from Calbiochem (San Diego, CA).

Cells producing the 8E6 monoclonal antibody to vitronectin (MaVN)\textsuperscript{6} were a gift of Dr Ed Hayman and colleagues, La Jolla Cancer Research Foundation, CA. This antibody was raised against vitronectin purified by glass bead chromatography and selected for its ability to recognize vitronectin strongly by immunoblotting and enzyme immunoassorbent assays (Dr Michael Pierschbacher, La Jolla Cancer Research Foundation, personal communication). Both conditioned medium and ascites were used as sources of the antibody. A second monoclonal antibody, MaS-protein, was to vitronectin purified as S-protein by the method of Dahlback and Podack (Boehringer Mannheim, Indianapolis, personal communication). Rabbit antisera to antithrombin III and prothrombin were purchased from Calbiochem. Peroxidase conjugated affinity purified goat anti-mouse and anti-rabbit IgG were purchased from Cappel Laboratories (Westchester, PA). Alkaline phosphatase conjugated rabbit anti-mouse IgG was purchased from Sigma. Normal goat serum was obtained from GIBCO (Grand Island, NY).

Vitronectin was purified by the method of Dahlback and Podack.\textsuperscript{18} Briefly, fresh frozen citrated human plasma (obtained from the Badger Red Cross, Madison, WI) was treated with reduced glutathione and benzamidine-HCl. Following precipitation with barium chloride and polyethylene glycol, the supernatant was consecutively chromatographed over DEAE-Sephasel, Blue Sepharose, and Sephacryl S-200 columns. The resulting vitronectin-containing peak was dialyzed against Tris-buffered saline (10 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.4). In some preparations, albumin was removed by passage over an anti-albumin Sepharose column. Antithrombin III was purified by batch absorption on Heparin-Sepharose CL-6B as follows, using a modification of a previously described procedure.\textsuperscript{30} Fresh frozen citrated human plasma (90 mL) was incubated with 20 mL Heparin-Sepharose resin for 30 minutes at room temperature with constant mixing. The mixture was centrifuged at 1,000 g for ten minutes and the pelleted resin was resuspended in 50 mmol/L Tris, 1 mol/L NaCl, 2 mmol/L EDTA, 2 mmol/L benzamidine-HCl, 1 mmol/L reduced glutathione, pH 7.4 or, with 10 mmol/L Tris-HCl, 1 mol/L NaCl, pH 7.4. The appropriate fractions were pooled, dialyzed against Tris-buffered saline and concentrated on an Amicon PM 10 filter. After gel filtration, antithrombin III (mol wt 58,000) was >90% pure by SDS-PAGE. Contamination with heparin cofactor II (mol wt 65,000) was not detected by protein staining. Alpha-thrombin (2.2 mg/mL, 3,000 U/mL) was a generous gift from John Fenton II (New York State Department of Health, Albany). Factor Xa was also analyzed for relative change in recognition by MaVN of antithrombin III, 9.5) by treatment with 20 mmol/L 200 tL of a 2 tg/mL solution of vitronectin in 0.1% bovine serum albumin in Tris-buffered saline. Samples to be assayed were diluted in Tris-buffered saline, and 250 \mu L were incubated for 30 minutes with 250 \mu L of antibody diluted 1:200, for MaVN culture medium, or 1:1000 for MaS-protein in 0.5% bovine serum albumin/Tris-buffered saline (all incubations were performed in polypropylene tubes). Duplicate wells received 200 \mu L of sample-antibody mixture and were incubated for 45 minutes. Plates were washed four times with 0.05% Tween-20 in Tris-buffered saline and incubated with 200 \mu L/well of a 1:800 dilution of rabbit anti-mouse IgG conjugated to alkaline phosphatase in Tween-20/Tris-buffered saline. Plates were washed and incubated at 37°C with phosphate substrate (200 \mu L/well at 1 mg/mL in Tris-buffered saline, pH 9.5) until maximum A$_{max}$ was at least 0.7.

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antiserum in Tris-buffered saline containing 1% normal goat serum. Filters were again rinsed and incubated for one hour at room temperature in 1% or 0.1% peroxidase-conjugated goat anti-mouse or anti-rabbit IgG. Following rinsing, blots were washed in substrate solution: 4-chloro-1-naphthol/H2O2 or diaminobenzidine tetrahydrochloride/H2O2. The rabbit antiserum to human prothrombin recognized thrombin well but showed diminished reactivity toward thrombin-antithrombin III and some cross-reactivity against vitronectin. This cross-reactivity was abolished by including 3 μg/mL vitronectin in the diluted rabbit anti-prothrombin used for immunoblotting.

Nonreduced/reduced two-dimensional SDS-PAGE and immunoblotting. Vitronectin, antithrombin III, and thrombin, all 1.7 μmol/L, were incubated with 50 μg/mL heparin for 30 minutes at 37°C. The incubation was started by the addition of thrombin and ended with 200 μmol/L PPACK. Before electrophoresis, samples were diluted with an equal volume of 3.2 mol/L urea and 1.2% SDS with or without 4% beta-mercaptoethanol. A set of unreduced samples, 10 μL each, were separated on a 0.5-mm thick gel on a Hoefer mini-gel apparatus. A lane containing a reduced sample and one of the lanes containing unreduced samples were cut and stained for protein, while the other lanes, containing unreduced samples, were incubated in 12.5 mmol/L Tris-Cl, 0.1% SDS, pH 6.8 buffer containing 4% beta-mercaptoethanol for two hours at room temperature. Strips were then cut and placed on a second set of 8% slab gels with 1 cm 3% stacking gels.25 Strips were sealed in by layering 1% agarose in barbital buffer. Following the second electrophoretic run, gels were immunoblotted as described above, by incubating with MaVN, rabbit anti-human antithrombin III, or rabbit anti-human prothrombin. After peroxidase reaction with 4-chloro-1-naphthol/H2O2, which gives a purple precipitate, blots previously treated with MaVN were incubated with one of the rabbit antibodies or vice versa. The blots were processed as described above except that the substrate system was changed to one that gives a brown precipitate (diaminobenzidine tetrahydrochloride/H2O2), thereby overlaying purple spots with brown and allowing detection of two antigens.

Immunoaffinity chromatography of vitronectin and vitronectin complexes. MaVN was purified from ascites by chromatography on Protein A-Sepharose CL 4B and coupled to BrCN-activated Sepharose according to the manufacturer's instructions. MaVN-Sepharose columns (1 mL) were prestripped with 8 mol/L urea in Tris-buffered saline and equilibrated in Tris-buffered saline. Vitronectin and vitronectin/thrombin-antithrombin III complexes (0.67 μmol/L) made in the presence or absence of 50 μg/mL heparin in 0.5% bovine serum albumin were incubated for 30 minutes at 37°C. Samples of each (0.5 mL) were chromatographed on MaVN-Sepharose; bound material was eluted with 8 mol/L urea in Tris-buffered saline. Unbound and bound material was analyzed by SDS-PAGE without reduction, followed by immunoblotting with MaVN, rabbit anti-human antithrombin III, or rabbit anti-human prothrombin.

RESULTS

Since substratum-adsorbed or SDS-treated and electroblotted vitronectin is recognized avidly by MaVN 8E6, we used a competitive ELISA system to detect antigenic changes of vitronectin in solution under various conditions. Serum vitronectin was recognized 50-fold better by MaVN than plasma vitronectin (Table 1), thereby confirming results obtained previously by immunoaffinity chromatography.3

Because vitronectin has been shown to interact with the thrombin-antithrombin III complex,18,20,22,36,37 we examined the possibility that the serum-induced antigenic change in vitronectin could be due to its interaction with thrombin-antithrombin III. Incubation of vitronectin with equimolar amounts of antithrombin III and thrombin in the absence or presence of heparin promoted a seven- or 32-fold increase, respectively, in recognition by MaVN as compared with untreated vitronectin (Fig 1A and Table 1). Heparin alone induced a 2.5-fold increase in recognition by MaVN. Addition of the thrombin inhibitor PPACK to the mixture before addition of thrombin, in the presence or absence of heparin, resulted in inhibition of the induction of the antigenic change. Incubation of vitronectin with antithrombin III or thrombin alone resulted in recognition profiles similar to that obtained for untreated vitronectin. In the presence of heparin, however, thrombin did promote an increase in recognition (Table 1). As shown in Fig 1B, the MaS-protein monoclonal antibody (raised against vitronectin purified as S-protein) did not preferentially recognize vitronectin incubated with heparin and/or the thrombin-antithrombin III complex. Incubation of vitronectin with factor Xa and antithrombin III in the absence and presence of heparin promoted an increase in recognition by MaVN; this increase, however, was approximately half of that induced by thrombin-antithrombin III (Table 1).

Enhancement of antigenicity by thrombin-antithrombin III was found no matter what the order of addition of reagents was (Table 2). In particular, the antigenic change was induced by thrombin that had been preincubated with antithrombin III to form thrombin-antithrombin III complexes before addition of vitronectin. The relative stoichiom-
Comparison of the relative potencies of various polysaccharides in the absence of thrombin-antithrombin III is shown in Fig 3. Vitronectin was incubated with the indicated range of polysaccharide concentrations and then processed for competition ELISA with MaVN. Highly sulfated polysaccharides, such as dextran sulfate, fucoidan, and heparin were most effective at inducing the antigenic change in vitronectin. Dermatan sulfate, hyaluronic acid, heparan sulfate, chondroitin sulfate, and keratan sulfate had little or no effect on vitronectin antigenicity.

In order to determine whether the interaction of the thrombin-antithrombin III complex with vitronectin involves any modification of vitronectin, samples similar to those assayed in competition ELISA experiments were analyzed by SDS-PAGE followed by immunoblotting with MaVN. Figures 4A and B show immunoblots of reduced and unreduced samples, respectively. Under reducing conditions, some fragmentation of vitronectin by thrombin-antithrombin III was apparent, particularly in the presence of heparin. This cleavage occurred even in the presence of antithrombin III, albeit to a lesser extent. Under unreducing conditions, the 65,000 and 75,000 molecular weight forms migrated together due to the disulfide bridge holding the 10,000 molecular weight carboxy-terminal cleavage fragment to the rest of the mole-
The nature of the disulfide-bonded complexes was studied further by un-reduced/reduced two-dimensional SDS-PAGE. Replica gels were immunoblotted with MaVN, rabbit anti-human antithrombin III, and rabbit anti-human prothrombin. As shown in Fig 6A, immunoblott-ting with MaVN showed three forms of vitronectin polypeptides (mol wt 65,000 to 75,000, 145,000 to 160,000, and >200,000) in the unreduced dimension, all of which migrated as polypeptides of mol wt 65 to 75,000 in the reduced dimension. Immunoblotting with rabbit anti-prothrombin (Fig 6B) showed two forms of thrombin migrating with mol wt 80,000 to 90,000 and 160,000 in the unreduced dimension and polypeptides of mol wt 80,000 to 90,000 in the reduced dimension. No free thrombin was apparent. Immunoblotting with rabbit anti-antithrombin III (Fig 6C) demonstrated various forms of antithrombin III with mol wt 90,000, 145,000 to 160,000, and 200,000 in the unreduced dimen-sion; these all migrated as polypeptides of mol wt 80,000 to 90,000 when reduced. Free antithrombin III, mol wt 58,000, migrated identically in unreduced and reduced dimensions.
fragmented antithrombin III, mol wt 45,000, was apparent only in the reduced dimension. MaVN and rabbit anti-antithrombin III or anti-prothrombin were co-localized by a double staining technique. Shown in Fig 6C′ is the immunoblot obtained after restaining the antithrombin III blot (Fig 6C) with MaVN. The arrowheads indicate the positions of the bands that stained brown due to MaVN. Vitronectin, antithrombin III, and thrombin migrated together as the mol wt 110,000 band is unclear. It was seen variably in experiments with purified vitronectin, thrombin, and antithrombin III (see above) but not with vitronectin alone.

To examine whether disulfide-bonded complex formation occurred during blood coagulation, plasma, plasma-derived serum, and whole blood serum was analyzed under reducing and unreducing conditions and immunoblotted against MaVN and rabbit anti-antithrombin III antiserum (Fig 7). Under reducing conditions, the vitronectin polypeptides in plasma and serum migrated the same (Fig 7, left panel), whereas under unreducing conditions there were two strongly staining bands of approximate mol wt 110,000 and 145,000 (and faint bands of mol wt > 160,000) that appear in plasma serum and whole blood serum, but not in plasma. The 145,000 molecular weight band co-migrated with a band of the same size (arrow) when separated serum samples were reacted with rabbit anti-antithrombin III (Fig 7, right panel). The MaVN-staining band of mol wt 110,000 did not stain with anti-antithrombin III. The nature of the mol wt 110,000 band is unclear. It was seen variably in experiments with purified vitronectin, thrombin, and antithrombin III (see above) but not with vitronectin alone.

Of the three components in the disulfide-bonded complex, only vitronectin contains a free sulfhydryl group. As shown in Fig 8, the sulfhydryl-blocking agent N-ethylmaleimide blocked the formation of the disulfide-bonded complex(es) of vitronectin with thrombin-antithrombin III. NEM also inhibited by 60% to 70% the antigenic change as detected by MaVN in the competitive ELISA system (Table 1). Similar results were obtained when vitronectin was treated with NEM before incubation with thrombin-antithrombin III and analyzed by ELISA.

In order to determine whether disulfide-bonding to thrombin-antithrombin III was necessary for enhanced recognition of vitronectin by MaVN, affinity chromatography of vitronectin and vitronectin complexes on MaVN IgG-Sepharose was performed, followed by analysis of the unbound and bound material by SDS-PAGE under unreducing conditions and immunoblotting. When vitronectin alone was chromatographed over MaVN-Sepharose, most of the protein was found in the unbound fraction (Fig 9). The trace amounts of vitronectin dimer were found in the unbound fraction as well. When vitronectin was incubated with thrombin-antithrombin III and passed over MaVN-Sepharose, most of the vitronectin was in the bound fraction. This increase in bound protein was more apparent when vitronectin was incubated
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Fig 7. Immunoblot of reduced and unreduced plasma and serum processed with MaVN and rabbit anti-antithrombin III. Plasma (lanes 1), plasma serum (lanes 2), and whole blood serum (lanes 3) were obtained as described in Materials and Methods, diluted 1:100 before addition of 300 μmol/L N-ethylmaleimide, 1.6 mol/L urea, 0.6% SDS in the presence (R) or absence (U) of 2% beta-mercaptoethanol and boiled for three minutes. The immunoblot shown in left panel was processed with MaVN, while the immunoblot in right panel was processed with rabbit anti-human antithrombin antiserum. Molecular weight standards are depicted on the left margin. Arrow points to a band that stained for both antigens in unreduced samples of plasma serum and whole blood serum, but not of plasma. The band corresponding to uncomplexed antithrombin III was obscured by the dense albumin (alb) band. Arrowhead points to thrombin-antithrombin III band present in both reduced and unreduced serum samples stained with rabbit anti-antithrombin III.

Fig 8. Effect of NEM on covalent complex formation of vitronectin exposed to thrombin-antithrombin III. Vitronectin (340 nmol/L) in Tris-buffered saline was incubated with no added macromolecules (lanes 1 and 5), 50 μg/mL heparin (lanes 2 and 6), 340 nmol/L thrombin-antithrombin III (lanes 3 and 7), or heparin and thrombin-antithrombin III (lanes 4 and 8) for 30 minutes at 37°C. The samples analyzed in lanes 5 through 8 were incubated in the presence of 340 μmol/L NEM. The samples were separated by SDS-PAGE without reduction and immunoblotted with MaVN as described in Fig 48. The samples were also analyzed by the competitive ELISA (see Table 1).

Free thiol groups of vitronectin in dimerization. The urea-treated vitronectin was recognized 20- to 30-fold more avidly by MaVN than untreated vitronectin (Table 1). Vitronectin that was treated with urea in the presence of NEM also was more antigenic.

Fig 9. Immunoblots of vitronectin and vitronectin complexes chromatographed on MaVN-Sepharose. Vitronectin (lanes 1 through 3) or a mixture of vitronectin, thrombin-antithrombin III and heparin (lanes 4 through 6) was chromatographed on MaVN-Sepharose as described (similar results were obtained in the absence of heparin). The starting mixture (lanes 1 and 4), unbound protein (lanes 2 and 5), and bound protein eluted with 8 mol/L urea (lanes 3 and 6) were analyzed by SDS-PAGE under unreducing conditions and immunoblotted with MaVN (left panel) and rabbit anti-human antithrombin III (right panel). Molecular weight standards are indicated on left margin.
Antithrombin III is a plasma proteinase inhibitor that inhibits thrombin by direct binding to the active site of the protease with formation of a 1:1 complex with a molecular weight of 90,000 that resists dissociation with SDS. The progressive inactivation of thrombin by antithrombin III can be accelerated by the sulfated polysaccharide, heparin, which is a catalyst of thrombin-antithrombin III complex formation. Vitronectin acts as a noncompetitive inhibitor of the heparin-catalyzed inactivation of both thrombin and factor Xa by antithrombin III. Dot-blotted or fragmented vitronectin, urea-treated vitronectin, and vitronectin in serum (ie, vitronectin exposed to thrombin-antithrombin) bind to heparin avidly. The data presented herein indicate that the thrombin-antithrombin III, once formed, binds to vitronectin, causing a conformational change that exposes the antigenic determinant recognized by MaVN 8E6. Augmentation by heparin of the recognition of this determinant probably represents stabilization of the induced conformation. These findings indicate that circulating vitronectin binds heparin avidly only after it has interacted with the thrombin-antithrombin III. In this respect, vitronectin is different from other heparin-neutralizing proteins, such as histidine-rich glycoprotein and platelet factor 4, which do not need to undergo a conformational change to acquire heparin-binding activity.

In the absence of thrombin-antithrombin III, heparin and other sulfated glycosaminoglycans, fucoidan and dextran sulfate, induced only a partial antigenic change in vitronectin, even at high concentration. The effects of fucoidan and chondroitin sulfate on the antigenicity of vitronectin in the presence of thrombin-antithrombin III have also been examined. Fucoidan, like heparin, enhanced the effect of thrombin-antithrombin III. In contrast, when vitronectin was incubated with thrombin-antithrombin III and chondroitin sulfate, the recognition profile was similar to that obtained when vitronectin was incubated with thrombin-antithrombin III alone (data not shown). As determined by SDS-PAGE and immunoblotting, the extent of thrombin-antithrombin III complex formation of our standard 30 minutes incubation at 37°C was equivalent in the absence and presence of heparin. This and the results discussed above suggest that the effect of the polysaccharide in the complexes is probably due to a direct interaction with vitronectin and not via its accelerating effect on thrombin-antithrombin III complex formation.

It was suggested that a conformationally changed form of antithrombin III was responsible for interaction of vitronectin with thrombin-antithrombin III. Hydrophobic sites are exposed on interaction of antithrombin III with thrombin, presumably due to a conformational change in antithrombin III. It could be that vitronectin binds to altered antithrombin III and undergoes a conformational change itself. Podack et al. however, presented electrophoretic evidence of a direct interaction of thrombin with vitronectin. In addition, vitronectin is cleaved by thrombin and, as shown in this study, this cleavage is enhanced in the presence of heparin. Interestingly, we found that thrombin-antithrombin III was more effective than factor Xa-antithrombin III in the induction of the antigenic change. A study of other serpin-protease complexes is underway to determine the relative importance of protease and serpin in mediation of the interaction with vitronectin.

One to two free sulfhydryl groups have been detected in vitronectin. The existence of an even number of cysteine residues would preclude the presence of only one free sulfhydryl group, unless the unpaired cysteine is bound as a mixed disulfide with, eg, cysteine or glutathione. The finding of complexes of vitronectin and thrombin-antithrombin III that can only be dissociated with reducing agent and inhibition of formation of these complexes by NEM suggest that the free sulfhydryl(s) of vitronectin participate in a thiol-disulfide exchange with a labile cysteine in thrombin-antithrombin III. Dimerization of purified C9 has also been proposed to occur as a result of thiol-disulfide exchange induced by vitronectin.
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BR Tomasini and DF Mosher