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Conformational Lability of Vitronectin: Induction of an Antigenic Change by α-Thrombin–Serpin Complexes and by Proteolytically Modified Thrombin†

Bianca R. Tomasini,‡,§ Maurice C. Owen,‖ John W. Fenton II,⊥ and Deane F. Mosher*,‡

Departments of Medicine and Physiological Chemistry, University of Wisconsin, 1300 University Avenue, Madison, Wisconsin 53706, Department of Pathology, The Christchurch School of Medicine, Christchurch, New Zealand, and Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201

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ABSTRACT: We previously showed that the α-thrombin–antithrombin III complex causes an antigenic change in vitronectin as monitored by the monoclonal anti-vitronectin antibody 8E6 (Tomasini & Mosher, 1988). We have extended these studies to other protease–serpin complexes and to γ-thrombin, a proteolytic derivative of α-thrombin. In the presence of heparin, recognition of vitronectin by 8E6 was increased 64- or 52-fold by interaction with the complex of α-thrombin and heparin cofactor II or the Pittsburgh mutant (Met_{358} → Arg) of α_1-proteinase inhibitor, respectively. This was comparable to the value obtained with the α-thrombin–antithrombin III complex. Factor Xa–serpin complexes were approximately 4-fold less effective than the corresponding thrombin complexes. α-Thrombin–serpin complexes but not Xa–serpin complexes formed disulfide-bonded complexes with vitronectin. Antigenic changes and disulfide-bonded complexes were not detected when trypsin- or chymotrypsin–serpin complexes were incubated with vitronectin. γ-Thrombin caused 7- and 34-fold increases in recognition of vitronectin by MaVN 8E6 in the absence and presence of heparin, respectively. In contrast, α-thrombin by itself had no effect. The antigenic change induced by γ-thrombin was maximal when γ-thrombin and vitronectin were equimolar, was not dependent on cleavage of vitronectin, and was abolished by inhibition of γ-thrombin with Phe-Pro-Arg-chloromethyl ketone but not with diisopropyl fluorophosphate. These data indicate that α-thrombin is the component in α-thrombin–serpin complexes that induces the antigenic change in vitronectin, probably via a region that is preferentially exposed in γ-thrombin.

Vitronectin is a plasma and serum glycoprotein that promotes cell–substratum adhesion (Hayman et al., 1985b; Silnutzer & Barnes, 1985). The binding of vitronectin to activated platelets (Thiagarajan & Kelly, 1988) and endothelial cells in suspension (Preissner et al., 1988) can be partially inhibited by peptides containing an Arg-Gly-Asp sequence, suggesting binding to the integrin receptors for vitronectin on these cells (Pytela et al., 1986; Suzuki et al., 1987). Vitronectin is also involved in the coagulation and complement systems. As S-protein (Jenne & Stanley, 1985; Preissner et al., 1986; Tomasini & Mosher, 1986), vitronectin incorporates into the membrane attack complex of complement, C5b-9, thus preventing binding of the attack complex to bystander cells not

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* To whom correspondence should be addressed at the Department of Medicine, University of Wisconsin.
‡ University of Wisconsin.
§ Present address: La Jolla Cancer Research Foundation, 10901 N. Torrey Pines Rd, La Jolla, CA 92037.
‖ The Christchurch School of Medicine.
⊥ New York State Department of Health.

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tagged for lysis by antibody (Podack & Müller-Eberhard, 1979). Vitronectin binds to thrombin–antithrombin complexes (Ill & Ruoslahti, 1985; Preissner et al., 1987). It acts as a heparin scavenger and thus modulates the inhibition of thrombin and factor Xa by antithrombin III (Preissner & Müller-Berghaus, 1986, 1987; Podack et al., 1986).

Vitronectin does not bind heparin avidly unless it has been denatured (Barnes et al., 1985; Hayashi et al., 1985), is otherwise modified (Preissner & Müller-Berghaus, 1987), or is in serum (Tomasini & Mosher, 1988). We have shown previously (Tomasini & Mosher, 1988) that vitronectin in serum, vitronectin complexed to thrombin–antithrombin III, vitronectin treated with urea, vitronectin adsorbed to polystyrene dishes, and vitronectin subjected to Western blotting are in conformations that allow recognition by the monoclonal anti-vitronectin antibody (MaVN)[1] 8E6. Vitronectin in plasma and vitronectin purified without denaturation are not recognized well by MaVN 8E6. Disulfide-bonded complexes between vitronectin and thrombin–antithrombin III were observed to form in association with the induction of the antigenic change in vitronectin; disulfide bonding, however, was not required for recognition by MaVN 8E6. We proposed that the interaction with the thrombin–antithrombin III complex results in a conformational change in vitronectin manifested by the exposure of the MaVN 8E6 epitope, the heparin-binding region, and free sulfhydryl group(s).

It has not been determined whether thrombin [see Podack et al. (1986)], antithrombin III [see Ill and Ruoslahti (1985)], or both interact with vitronectin. Thrombin and antithrombin III belong to the serine protease and serine protease inhibitor (serpin) families of proteins, respectively. Both families contain a number of proteins that have homologous structures and similar functions [see, among others, Ragg (1986), Ye et al. (1987), Furie et al. (1982), and Bing et al. (1986)]. In order to learn the specificity and generality of interactions of thrombin–antithrombin III with vitronectin, various combinations of proteases (α-thrombin, factor Xa, trypsin, chymotrypsin) and serpins [antithrombin III, heparin cofactor II, α_1-proteinase inhibitor, the Pittsburgh mutant of α_1-proteinase inhibitor ($Met_{358} \rightarrow Arg$; a mutation that greatly increases affinity for thrombin), and α_1-anti-chymotrypsin] were assayed for their ability to induce the conformational change in vitronectin recognized by MaVN 8E6 and to covalently complex with vitronectin. γ-Thrombin, a proteolytic derivative of α-thrombin, was also studied. The results suggest that the conformation of vitronectin is modulated by α-thrombin complexed to a generic serpin and by γ-thrombin alone.

Materials and Methods

Materials. Materials were as described previously (Tomasini & Mosher, 1988) with the following exceptions and additions. Benzamidine hydrochloride, diisopropyl fluorophosphate (DIP), and phenylmethanesulfonyl fluoride were from Sigma Chemical Co. (St. Louis, MO). Rabbit antisera to α_1-proteinase inhibitor, α_1-anti-chymotrypsin, and vitronectin (purified as S-protein) were from Calbiochem (San Diego, CA). Peroxidase-conjugated non-affinity-purified goat anti-mouse and anti-rabbit IgGs were from Organon Teknika (Malvern, PA). The monoclonal antibody to vitronectin (MaVN 8E6) was obtained from culture media of hybridoma cells (gift of Dr. E. Hayman and colleagues, La Jolla Cancer Research Foundation, La Jolla, CA) and was characterized as described previously (Hayman et al., 1983; Tomasini & Mosher, 1988). Rabbit antiserum to heparin cofactor II was a gift of Dr. D. M. Tollefsen (Washington University, St. Louis, MO).

Proteins. Vitronectin was purified by the method of Dahlbäck and Podack (1985). Antithrombin III was purified by using a modification of the method of Damus and Rosenberg (1976) as described (Tomasini & Mosher, 1988). α- and γ-thrombins (Fenton et al, 1977a,b; Fenton, 1986) were prepared as described previously and had 4035 and 2.24 units/mg (NIH) clotting activity, respectively. Esterase activity, as determined by titration with nitrophenyl guanidinobenzoate, was 100% for α-thrombin and 80% for γ-thrombin. Factor Xa was from Enzyme Research Laboratories (South Bend, IN). Tosylphenylalanine chloromethyl ketone (TPCK)-treated trypsin from bovine pancreas was purchased from Cooper Biomedical (Malvern, PA). Bovine pancreatic chymotrypsin was purchased from Sigma.

Heparin cofactor II was a geneous gift from Dr. Tollefsen. The Pittsburgh mutant ($Met_{358} \rightarrow Arg$) of α_1-proteinase inhibitor was prepared as described previously (Owen et al., 1983). Partially purified α_1-proteinase inhibitor was from Sigma. Pure α_1-proteinase inhibitor and α_1-anti-chymotrypsin were gifts from Dr. Salvatore Pizzo and Alan Mast (Duke University, Durham, NC).

Except for α_1-proteinase inhibitor and α_1-anti-chymotrypsin, which were stored at −20 °C, all proteins were aliquoted, snap-frozen, and stored at −70 °C. Protein concentration was determined spectrophotometrically by using absorption coefficients ($A_{280nm}^{1\%,1cm}$) of 9.0 for vitronectin (Preissner et al., 1985), 6.5 for antithrombin III (Nordeman et al., 1977), 11.7 for heparin cofactor II (Tollefsen et al., 1982), 5.3 for α_1-proteinase inhibitor (James & Cohen, 1978), 6.2 for α_1-anti-chymotrypsin (Travis et al., 1978), and 18.3 for thrombin (Fenton et al., 1977b). The molecular weight estimates used to determine molar concentrations were 75000 for vitronectin (Preissner et al., 1985), 58000 for antithrombin III (Travis & Salvesen, 1983), 65000 for heparin cofactor II (Tollefsen et al., 1982), 54000 for α_1-proteinase inhibitor (Lo et al., 1976), 62000 for α_1-anti-chymotrypsin (Travis et al., 1978), 36000 for thrombin (Fenton et al., 1977), 45000 for factor Xa (Fujikawa et al., 1975), 24000 for bovine trypsin (Buck et al., 1962), and 24000 for bovine chymotrypsin (Wilcox, 1970).

Formation of Protein Complexes. Samples to be assayed in the indirect enzyme-linked immunosorbent assay system (ELISA) and immunoblotting were prepared as described previously (Tomasini & Mosher, 1988). Briefly, in most cases, equimolar amounts (340 nM) of vitronectin, serpin, and protease were incubated in the presence or absence of 4 units/mL heparin (25 μg/mL) for 45 min at 37 °C. The incubation was started by the addition of the protease and ended by the addition of 10 μM D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) to samples containing thrombin, 128 μM benzamidine hydrochloride to samples containing factor Xa, and 4 mM phenylmethanesulfonyl fluoride to trypsin- or chymotrypsin-containing samples, in order to inhibit any remaining protease activity. In some experiments, a 2–5-fold molar excess of serpin was preincubated with the protease for 30 min or 2 h at 37 °C prior to incubation with vitronectin in order to increase serpin–protease complex formation.

[1] Abbreviations: DIP, diisopropyl fluorophosphate; ELISA, enzyme-linked immunosorbent assay; MaVN, monoclonal anti-vitronectin; NEM, *N*-ethylmaleimide; PPACK, phenylalanylprolylarginine chloromethyl ketone; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Table I: Differential Recognition of Vitronectin, As Measured by Competition ELISA with MaVN: Effect of Various Serpin–Protease Complexes on Native Vitronectin[a]

	serpin									
protease	ATIII	ATIII + heparin	HCII	HCII + heparin	α_1-Pitt	α_1-Pitt + heparin	α_1-PI	α_1-PI + heparin	α_1-ACT	α_1-ACT + heparin
α-thrombin	9 (7)	56 (7)	6 (2)	64 (2)	4 (4)	52 (4)	4[b] (2)	33[b] (2)	4 (2)[c]	9 (2)[c]
factor Xa	2 (3)	16 (3)	3 (2)[c]	12 (2)[c]	2 (2)	16 (2)	3 (2)	11 (2)	ND	ND
trypsin	1 (2)	5 (2)	1 (2)[c]	4 (2)[c]	ND	ND	1 (2)	3 (2)	1 (2)	5 (2)
chymotrypsin	1 (2)[c]	5 (2)[c]	ND[d]	ND	ND	ND	1 (2)	4 (2)	1 (2)	3 (2)

[a] The values represent the average obtained from the number of determinations given in parentheses. The relative change in recognition indicated by these values was obtained by calculating the ratio of the concentration of treated vitronectin necessary to compete for 50% of antibody in solution compared to the concentration of untreated vitronectin which gave the same competition. Abbreviations: ATIII, antithrombin III; HCII, heparin cofactor II; α_1-Pitt, Pittsburgh mutant of α_1-proteinase inhibitor; α_1-PI, normal α_1-proteinase inhibitor; α_1-ACT, α_1-anti-chymotrypsin. [b] Serpin in 5-fold excess of thrombin and vitronectin. [c] As discussed by Travis and Salvesen (1983) and Parker and Tollefsen (1985), formation of thrombin–α_1-anti-chymotrypsin, Xa–heparin cofactor II, trypsin–heparin cofactor II, or chymotrypsin–antithrombin III complexes should be minimal. [d] ND, not determined.

Prior to preparation of complexes for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), 300-500 μM *N*-ethylmaleimide (NEM) was routinely added to prevent further reactivity of free sulfhydryl groups. Samples were treated with 2% glycerol and 2% SDS in the presence and absence of 2% β-mercaptoethanol. Reduced samples were boiled for 3 min. Samples were analyzed on slab gels with 8% running gels and 3% stacking gels (Laemmli, 1970; Ames, 1974). Approximately 0.7 μg/well of vitronectin was analyzed by immunoblotting.

Indirect ELISA of Soluble Vitronectin. An ELISA was carried out in which soluble vitronectin competed for MaVN 8E6 binding to substrate adsorbed vitronectin as described (Tomasini & Mosher, 1988). Data are expressed as a percent of the maximum absorbance unit obtained in each assay and represent the average of duplicate determinations. Data presented in Tables I–III were analyzed for the relative change in recognition by MaVN of untreated versus treated vitronectin, i.e., the ratio of the amount of untreated vitronectin necessary to compete for 50% of antibody activity in solution divided by the amount of treated vitronectin required for a similar effect.

In the indirect ELISA, the induction of an antigenic change in vitronectin that results in better competition for MaVN 8E6 is represented by a decrease in absorbance at 405 nm, reflecting the amount of antibody left to bind the adsorbed vitronectin. This result would also be obtained if the "inducer" molecule was displacing vitronectin from the substratum or binding to vitronectin on the substratum and blocking the MaVN 8E6 epitope. This possibility was tested by preincubating vitronectin-coated wells with "inducer" molecules, washing away the molecules, and then incubating with MaVN 8E6, secondary antibody, and substrate as was done for the indirect ELISA. No inhibition of the reaction of MaVN 8E6 with substrate-bound vitronectin was seen in wells preincubated with γ-thrombin or the thrombin–antithrombin III complex.

Immunoblotting of Disulfide-Bonded Complexes. Following SDS–PAGE, immunoblotting was carried out according to the method of Towbin et al. (1979) as described previously (Tomasini & Mosher, 1988). Blots of nonreduced samples were examined for bands of the appropriate size for a disulfide-linked complex of vitronectin and protease–serpin which were recognized by antibodies to both vitronectin and the serpin. Immunoblots of reduced samples were also used to detect formation of the SDS-stable serpin–protease complex as determined by staining with anti-serpin antibody of a band migrating with the size expected for the sum of the molecular weights of serpin and protease.

Inhibition of α- and γ-Thrombins. The inhibition of α- and γ-thrombins with a 50- or 1000-fold excess of PPACK or DIP, respectively, was carried out for 15 min at room temperature immediately before the ELISA experiment performed to test their interaction with vitronectin. The effect of inhibitor alone on the vitronectin ELISA system was tested in parallel. Control experiments were done to show that DIP and PPACK inhibited the clotting activity of α-thrombin and cleavage by α- and γ-thrombins.

Results

Table I shows the relative ratios of recognition by MaVN 8E6 of vitronectin alone as compared to vitronectin in combination with a series of serpin–protease complexes, without or with heparin. Complexes of α-thrombin with heparin cofactor II and α_1-proteinase inhibitor induced an antigenic change in vitronectin similar to that promoted by the thrombin–antithrombin III complex. Heparin enhanced the change in recognition of vitronectin promoted by all three complexes. Factor Xa–serpin complexes were approximately 4-fold less effective than their thrombin counterparts. Thrombin and α_1-anti-chymotrypsin did not form an SDS-stable complex (Travis & Salvesen, 1983) and did not induce an antigenic change in vitronectin. Complexes of serpin and trypsin or chymotrypsin did not induce the antigenic change.

The importance of serpin–protease complex formation was evident in the case of the thrombin–α_1-proteinase inhibitor complex. Thrombin and the Pittsburgh mutant of α_1-proteinase inhibitor, which is as effective an inhibitor of thrombin in the absence of heparin as antithrombin III in the presence of heparin (Owen et al., 1983; Courtney et al., 1985), promoted an increase in recognition of vitronectin comparable to that promoted by thrombin and antithrombin III. Thrombin and normal α_1-proteinase inhibitor, when tested at equimolar concentrations, did not (not shown). However, when a 5-fold excess of normal α_1-proteinase inhibitor was incubated with thrombin prior to incubation with vitronectin, more SDS-stable thrombin–α_1-proteinase inhibitor complexes formed, with the concomitant induction of the antigenic change in vitronectin (Table I).

Like α-thrombin complexed with antithrombin III (Tomasini & Mosher, 1988), α-thrombin complexed with the Pittsburgh mutant of α_1-proteinase inhibitor and with heparin cofactor II promoted binding of vitronectin to a MaVN–Sepharose column (not shown).

Vitronectin and the complexes of α-thrombin with antithrombin III, heparin cofactor II, and the Pittsburgh mutant of α_1-proteinase inhibitor migrated in SDS–PAGE in the same high molecular weight (145 000–160 000) bands under unreducing conditions as ascertained by staining with MaVN (Figure 1) and with the appropriate anti-serpin antiserum (not shown). Incubation of vitronectin and Xa–serpin complexes

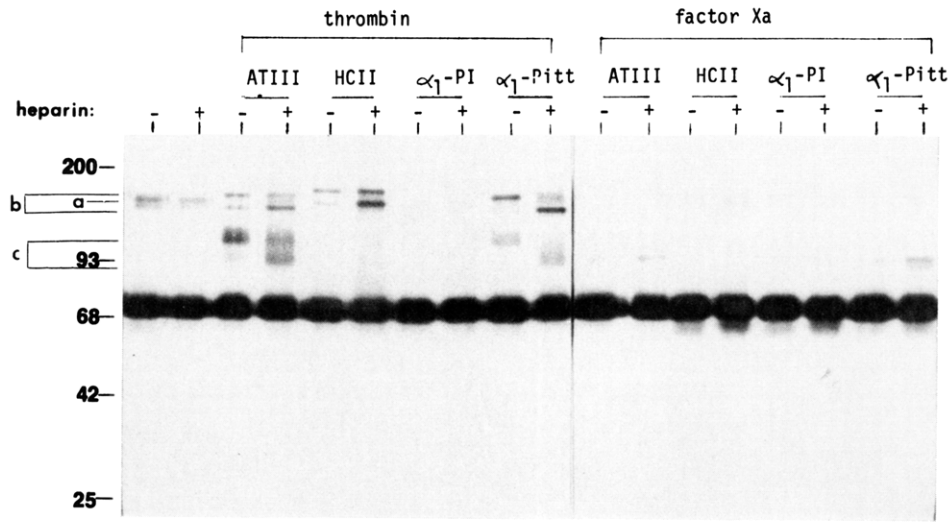

FIGURE 1: Immunoblots of unreduced vitronectin and vitronectin incubated with various serpins and proteases. Equimolar amounts (340 nM) of antithrombin III (ATIII), heparin cofactor II (HCII), α_1-proteinase inhibitor (α_1-PI), or the Pittsburgh mutant of α_1-proteinase inhibitor (α_1-Pitt), in combination with α-thrombin or factor Xa, were tested for their ability to form disulfide-bonded complexes with vitronectin. Samples were incubated in the absence (−) or presence (+) of 25 μg/mL heparin for 45 min at 37 °C. Following addition of 300 μM NEM, samples were processed for SDS–PAGE and immunoblotting as described under Materials and Methods. Molecular weights are depicted on the left margin. These areas are highlighted: (a) vitronectin dimers; (b) bands that also stained with anti-serpin antisera (immunoblots not shown); and (c) bands that do not stain with anti-serpin antisera (immunoblots not shown); the nature of these bands is unclear (see Discussion). Note disulfide-bonded complex formation with the Pittsburgh mutant but not the normal form of α_1-proteinase inhibitor. When the concentration of normal α_1-proteinase inhibitor was increased to 1700 nM, complexes were seen (immunoblot not shown). Also note the lack of disulfide-bonded complex formation with factor Xa–serpin complexes.

Table II: Differential Recognition of Vitronectin by MaVN, As Measured by Competition ELISA: Effect of α- and γ-Thrombin and Factor Xa on Vitronectin[a]

	serpin			
protease	none	none + heparin	ATIII	ATIII + heparin
none		3 (2)	1 (2)	3 (2)
α-thrombin	1 (5)	5 (5)	9 (7)	56 (7)
γ-thrombin	7 (4)	34 (4)	6 (2)	21 (2)
factor Xa	2 (2)	5 (2)	2 (3)	16 (3)

[a] Values were calculated as described in Table I.

did not result in formation of the high molecular weight bands. Neither trypsin–serpin nor chymotrypsin–serpin complex was found in disulfide-bonded complexes with vitronectin (not shown).

In order to further study the specificity of thrombin in reactions involving serpins and vitronectin, γ-thrombin, a proteolytic derivative of α-thrombin, was tested. γ-Thrombin by itself induced the antigenic change in vitronectin, whereas α-thrombin and factor Xa induced, at most, only a slight increase in antigenicity (Table II). Heparin enhanced the change in MaVN recognition induced by γ-thrombin. An equimolar amount of γ-thrombin was necessary for induction of the half-maximal antigenic change in 85 nM vitronectin (Figure 2).

During the course of our studies with α-thrombin, we observed cleavage of vitronectin by thrombin which was enhanced

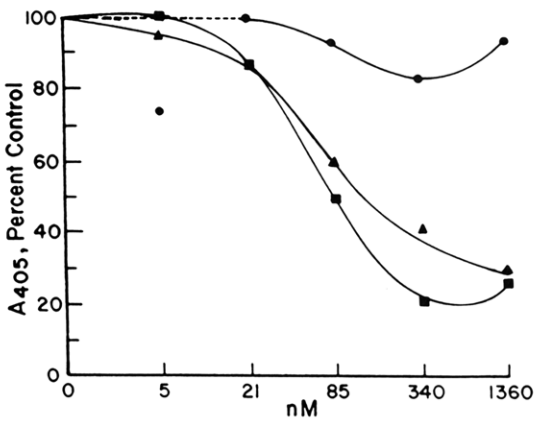

FIGURE 2: Recognition of vitronectin incubated with increasing concentrations of α-thrombin, γ-thrombin, and α-thrombin–antithrombin III as detected by MaVN in competition ELISA. Solutions of α-thrombin (circles), γ-thrombin (squares), and α-thrombin–antithrombin III complex (triangles) were mixed at concentrations indicated on the abscissa with 85 nM (6.4 μg/mL) vitronectin. After incubation for 45 min at 37 °C, mixtures were assayed by ELISA as described under Materials and Methods. Note that α-thrombin–antithrombin III and γ-thrombin had about half-maximal effects at 85 nM, the concentration of vitronectin.

in the presence of heparin (Tomasini & Mosher, 1988). γ-Thrombin was assayed for its ability to cleave vitronectin to test the possibility that the effect of the protease is due to cleavage of vitronectin. γ-Thrombin was not as active as

Table III: Effect of Proteases and Active-Site-Inhibited Proteases on Vitronectin: Recognition by MaVN 8E6 Relative to Native Vitronectin (ELISA)[a]

	inhibitor							
protease	none	none + heparin	ATIII	ATIII + heparin	PPACK	PPACK + heparin	DIP	DIP + heparin
α-thrombin	1 (5)	5 (5)	9 (7)	56 (7)	2	6	3	6
γ-thrombin	7 (4)	34 (4)	6 (2)	21 (2)	2	2	4	64

[a] Values were calculated as described for Table I.

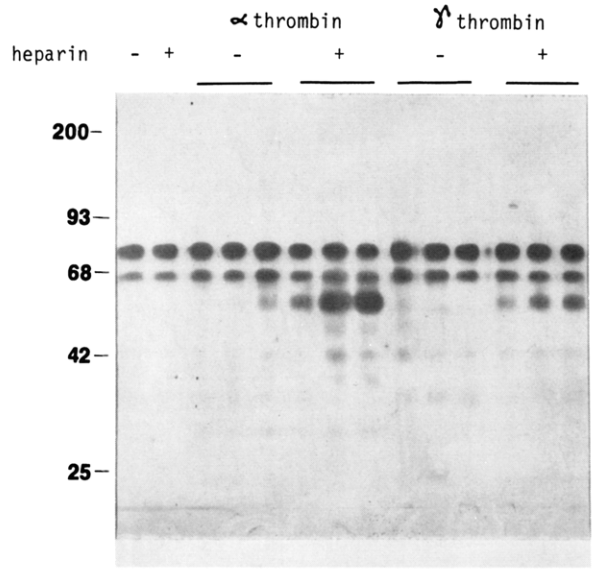

FIGURE 3: Time course of cleavage of vitronectin by an equimolar concentration of α-thrombin or γ-thrombin in the presence and absence of heparin. Immunoblot of reduced samples reacted with rabbit antiserum to vitronectin purified as S-protein. Vitronectin (340 nM) was incubated with α-thrombin or γ-thrombin (340 nM) in the absence or presence of a 4 units/mL heparin for 0, 5, 25, and 45 min at 37 °C. Time periods are shown consecutively in blot underline depicting each condition. At each time point, an aliquot was removed to which was added SDS with 2% β-mercaptoethanol. Samples were boiled for 3 min and frozen at −20 °C. Samples were processed for SDS–PAGE and immunoblotting as described. Note the 57 000 molecular weight fragment described previously (Barnes et al., 1985; Tomasini & Mosher, 1986, 1988).

α-thrombin at cleaving vitronectin (Figure 3). For both thrombins, however, cleavage was enhanced in the presence of heparin.

α- and γ-thrombins were incubated with inhibitors that modify the active-site serine (DIP) or histidine (PPACK). The inhibited thrombins were tested for their ability to induce the antigenic change in vitronectin (Table III). Both inhibitors were effective at prevention of cleavage of vitronectin by both thrombins in the presence and absence of heparin (not shown). DIP-inhibited γ-thrombin was effective at inducing the change in vitronectin, whereas the PPACK-inhibited γ-thrombin was not. Incubation of the inhibitors alone with vitronectin did not have any effect on the MaVN-based ELISA.

When a mixture of γ-thrombin and vitronectin was applied to a column of MaVN–Sepharose, both proteins bound to the column (not shown). No disulfide-bonded complexes between vitronectin and γ-thrombin could be demonstrated by SDS–PAGE and immunoblotting (not shown).

Discussion

The specificity of the interaction between the thrombin–antithrombin III complex and vitronectin was examined by testing other serpin–protease complexes for their ability to induce the conformational change in vitronectin detected by the monoclonal antibody MaVN 8E6. α-Thrombin in complex with three different serpins induced the antigenic change in vitronectin. Factor Xa–serpin complexes were less potent, and trypsin and chymotrypsin complexes lacked any conformational change-inducing activity. Thrombin and normal α_1-proteinase inhibitor, a poor thrombin inhibitor (Travis & Salvesen, 1983), were not as effective at induction of the antigenic change as thrombin and the Pittsburgh mutant, an excellent thrombin inhibitor (Owen et al., 1983; Courtney et al., 1985).

It is noteworthy that heparin enhanced the ability of the thrombin–α_1-proteinase inhibitor (Pittsburgh) complex to induce the antigenic change in vitronectin just as heparin enhanced the effects of the thrombin–antithrombin III and thrombin–heparin cofactor II. The reaction of thrombin with the Pittsburgh mutant is not sensitive to heparin (Owen et al., 1983; Courtney et al., 1985). Thus, the heparin effect is likely due to the enhancement or stabilization of the conformational change in vitronectin and not to the acceleration of serpin–protease complex formation as happens with antithrombin III and heparin cofactor II (Rosenberg & Damus, 1973; Tollefsen et al., 1982). Heparin rendered vitronectin more susceptible to cleavage by α- or γ-thrombin. γ-Thrombin, in contrast to α-thrombin, does not bind heparin (Olson et al., 1986). It is probable, therefore, that the partial antigenic change induced by heparin reports a conformational state in which vitronectin is a better substrate for the thrombins.

The α-thrombin–serpin combinations that induced the antigenic change in vitronectin formed disulfide-bonded complexes with vitronectin. The present results provide insight into which component in the serpin–thrombin complexes lends the reactive disulfide or sulfhydryl group(s). Neither antithrombin III (Kurachi et al., 1976) nor thrombin (Elion et al., 1977) has a free sulfhydryl group. Thus, thiol–disulfide exchange must be postulated, with the thiol(s) coming from vitronectin (Dahlbäck & Podack, 1985). There is a precedent for this process in that vitronectin induces formation of disulfide-bonded dimers of C9, a molecule which does not have free sulfhydryls (Dahlbäck & Podack, 1985). Heparin cofactor II has three cysteines of unknown oxidation status (Ragg, 1986). α_1-Proteinase inhibitor contains a single cysteine (Laurell & Jeppsson, 1975), and thus, although it could presumably form disulfide-bonded complexes with conformationally changed vitronectin by oxidation, it could not participate in the postulated exchange. Although there are invariant residues scattered throughout the serpins, no cysteine residues are common to all three serpins (Ragg, 1986; Ye et al., 1987). Given the lack of a conserved disulfide in the serpin, it is reasonable to surmise that the disulfide comes from thrombin. This conclusion is supported by the fact that factor Xa–serpins did not form disulfide-bonded complexes with vitronectin. Interestingly, these are data suggesting disulfide-bonded complex formation between thrombin and thrombospondin, with thrombospondin providing the free sulfhydryl (Danishefsky et al., 1984; Detwiler et al., 1987).

We have noted variable amounts of a smaller disulfide-bonded complex (110 000 molecular weight) when vitronectin is incubated with thrombin–antithrombin III (Tomasini & Mosher, 1988) or thrombin–α_1-proteinase inhibitor complex (Figure 1) or is present in serum (Conlan et al., 1988). This band was not observed with thrombin–heparin cofactor II complexes. The band stains with anti-vitronectin but not with anti-serpin antisera. The size of the band suggests it may consist of vitronectin disulfide bonded to thrombin perhaps arising due to decay of the SDS-stable association between thrombin and serpin. This suggestion is supported by the fact that ^{125}I-thrombin, when incubated with serpin and vitronectin, was detected in both the 145 000–160 000 and 110 000 molecular weight complexes (not shown).

The results of studies with γ-thrombin were a surprise to us. γ-Thrombin has been well studied (Berliner, 1984; Fenton, 1986; Berliner et al., 1986; Elion et al., 1986). It is missing two fragments and consists of three noncovalently bound chains, each containing one member of the catalytic triad (Boissel et al., 1984). It retains esterolytic activity toward

small substrates comparable to that of α-thrombin but has reduced proteolytic activity toward large substrates and lacks the ability to clot fibrinogen. It forms complexes with antithrombin III, although more slowly than α-thrombin (Chang et al., 1979; Bezeaud et al., 1985), and has no heparin-binding capacity (Olson et al., 1986). As determined by electron spin resonance studies with a series of rigid spin-labeled analogue of phenylmethanesulfonyl fluoride, γ-thrombin seems to have an altered active site with a hydrophobic region that is not present in α-thrombin (Berliner et al., 1986). No positive function has been found for γ-thrombin. It is probable that γ-thrombin forms from the α-thrombin retained in fibrin clots (Fenton, 1986).

γ-Thrombin by itself was effective at inducing the conformational change in vitronectin, a property not shared by α-thrombin. This interaction was enhanced by heparin and did not appear to be due to cleavage of vitronectin. PPACK (directed toward the essential histidine) ablated the ability of γ-thrombin to induce the antigenic change in vitronectin, whereas DIP (directed toward the essential serine) did not. PPACK interacts, via the phenylalanine side chain, with an apolar region near the active site of thrombin (Sonder & Fenton, 1984). Perhaps γ-thrombin binds to vitronectin via this unique apolar region, and the serpins, as part of the major conformational change that occurs during complex formation (Bruch et al., 1988), cause exposure of a similar region in complexed α-thrombin.

Inasmuch as induction of the conformational change in vitronectin seems to be a necessary consequence of thrombin generation, regardless of whether thrombin is inhibited by a serpin or degraded by partial proteolysis, it will be of considerable interest to learn the functional consequences of the altered conformation. One consequence of the interaction, described in the introduction, is acquisition of heparin-binding activity. A second consequence may be the clearance of the thrombin–serpin and factor Xa–serpin complexes by the liver (Shifman & Pizzo, 1982; Fair & Plow, 1986). However, serpin–protease clearance by the liver seems to be dependent on the identity of the serpin rather than the identity of the protease (Pratt et al., 1988). A third consequence is that the conformational change may allow vitronectin to interact differently under certain conditions with cell adhesion receptors. Preissner et al. (1988) reported that vitronectin and the vitronectin–thrombin–antithrombin III complex bound to a substratum did not differ in cell adhesive activity. Adsorbed vitronectin, however, probably has a similar conformation to that of vitronectin compled with thrombin–antithrombin III because it is recognized avidly by MaVN 8E6 (Tomasini & Mosher, 1988), and therefore a cell adhesion assay would not be expected to distinguish between conformers. Vitronectin by itself was 5–10-fold less effective than vitronectin incubated with thrombin–antithrombin III in competing for binding of radiolabeled vitronectin to endothelial cells in a suspension assay (Preissner et al., 1988). These results indicate, therefore, that the conformational change induced in vitronectin in thrombin–serpin complexes may indeed involve exposure of the Arg-Gly-Asp cell adhesion sequence. Additional consequences of the conformational change could be modulation of binding of vitronectin to extracellular matrix collagen (Gebb et al., 1986) and cell surface reportes such as thrombodulin (Bourin et al., 1988).

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Cell-Adhesive Immunoglobulin M in Human Plasma†

Shin-Ichiro Takahashi, Chiya Kuraishi, Mari Sakamoto, Toshihiro Tanabe, Takuma Nakajima, Takaaki Kosuge, Yoshiyuki Sakano, and Daisaburo Fujimoto*

Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Fuchu-shi, Tokyo 183, Japan

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ABSTRACT: Human plasma contains a cell-adhesive protein that has a structure related to immunoglobulins. This protein was purified by affinity chromatography on an elastin–Sepharose column and by Mono Q anion-exchange chromatography. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis under non-reducing and reducing conditions revealed that this protein is a kind of immunoglobulin M (IgM). Antibodies against the μ chain and against the Fc region of IgM inhibited the adhesion of cells to this protein. Addition of the peptide GRGDS into media inhibited the adhesion, too. These results suggest that this protein is a special subset of IgM having a cell-binding sequence in the Fc region. We propose the name "cell-adhesive immunoglobulin M (CA-IgM)" for this protein. CA-IgM binds to α-elastin and laminin, suggesting that it may play a role in the interaction between cells and the extracellular matrix.

It has been shown that cellular adhesion on extracellular matrix proteins plays an important role in events such as embryonic development and cancer metastasis (Alberts et al., 1983). It has also been shown that blood plasma contains several cell-adhesive proteins such as fibronectin, chondronectin, and vitronectin (Yamada, 1983). Cells adhere to and spread on solid surfaces that have been coated with these proteins.

Recently, the relationship between extracellular matrix and adhesive proteins was elucidated. That is, adhesive proteins can interact with extracellular matrix components. For example, fibronectin, laminin, and vitronectin interact with collagens and glycosaminoglycans (Yamada, 1983). These interactions may play important roles in cell–cell or cell–extracellular matrix communications. However, no cell-adhesive

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* Address correspondence to this author.

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