Incorporation of Vitronectin into Fibrin Clots

EVIDENCE FOR A BINDING INTERACTION BETWEEN VITRONECTIN AND γA/γ FIBRINOGEN*

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Vitronectin is an abundant plasma protein that regulates coagulation, fibrinolysis, complement activation, and cell adhesion. Recently, we demonstrated that plasma vitronectin inhibits fibrinolysis by mediating the interaction of type 1 plasminogen activator inhibitor with fibrin (Podor, T. J., Peterson, C. B., Lawrence, D. A., Stefansson, S., Shaughnessy, S. G., Foulon, D. M., Butcher, M., and Weitz, J. I. (2000) J. Biol. Chem. 275, 19788–19794). The current studies were undertaken to further examine the interactions between vitronectin and fibrinogen. Comparison of vitronectin levels in plasma with those in serum indicates that ~20% of plasma vitronectin is incorporated into the clot. When the time course of biotinylated-vitronectin incorporation into clots formed from 125I-fibrinogen is monitored, vitronectin incorporation into the clot parallels that of fibrinogen in the absence or presence of activated factor XIII. Vitronectin binds specifically to fibrin matrices with an estimated $K_D$ of ~0.6 μM. Additional vitronectin subunits are assembled on fibrin-bound vitronectin multimers through self-association. Confocal microcopy of fibrin clots reveals the globular vitronectin aggregates anchored at intervals along the fibrin fibrils. This periodicity raised the possibility that vitronectin interacts with the γA/γ fibrinogen (ogen) variant of fibrinogen that represents about 10% of total fibrinogen. In support of this concept, the vitronectin which contaminates fibrinogen preparations co-purifies with the γA/γ fibrinogen fraction, and clots formed from γA/γ fibrinogen preferentially bind vitronectin. These studies reveal that vitronectin associates with fibrin during coagulation, and may thereby modulate hemostasis and inflammation.

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1 The abbreviations used are: PAI-1, type 1 plasminogen activator inhibitor; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PPACK, D-phenyl Pro-Arg-chloromethyl ketone-HCl; PFP, platelet-free plasma; FITC, fluorescein isothiocyanate; SAHVn, affinity-purified sheep anti-vitronectin IgG; TBS, Tris-buffered saline.
vitronectin interaction with the fibrinogen γ-chains, and particularly the γA/γ′ variant form of fibrinogen that represents about 5–10% of the total fibrinogen in plasma (36). The fibrinogen γA/γ′ arises from alternative mRNA processing, and differs structurally in that the carboxyl-terminal sequences 408–411 of the γA/γ′ chain are replaced in the γA/γ′ variant by an anionic 20 residue sequence.

The purpose of this current study was to further characterize the interactions between vitronectin and fibrin by: (a) comparing total vitronectin antigen levels in plasma with those in serum, (b) quantifying the binding of vitronectin to purified fibrin, and (c) pursuing a morphological analysis of vitronectin in clots formed from plasma or purified fibrinogen. We present evidence that vitronectin associates with fibrin clots due to its preferentially binding to the carboxyl-terminal γ′ chain of the fibrinogen γA/γ′ chain variant.

**EXPERIMENTAL PROCEDURES**

Chemicals, Proteins, and Reagents—Bovine serum albumin (Fraction V), p-nitrophenyl phosphate, alkaline phosphatase-conjugated streptavidin, soybean trypsin inhibitor, and phenylmethylsulfonyl fluoride were purchased from EMD Chemicals (Gibbstown, NJ). Reduced glutathione, Tween 80, ethanalamine, diethanolamine, caprylic acid, and mouse IgG were obtained from Sigma. Affinity purified sheep anti-human vitronectin IgG (SAHVn) and normal (non-immune) sheep IgG were obtained from Affinity Biologicals (Hamilton, ON, Canada). Plastic inoculation loops were obtained from Fisher Scientific (Nepean, ON).

TWEEN 20, Coomassie Brilliant Blue R-250, urea (electrophoresis grade), acrylamide/bis 37.5:1 (2.8% C), molecular weight markers, glycine, Tris-HCI, SDS, G-25M Sepharose, and gelatin were purchased from Bio-Rad (Mississauga, ON). Human α-thrombin (3,083 NIH units/mg), factor XIII (1,948 Loewy units/mg), and plasminogen-free fibrinogen were purchased from Enzyme Research Laboratories Inc. (South Bend, IN), and depleted of fibronectin and factor XIII by gelatin-Sepharose affinity chromatography using ion-exchange chromatography on DEAE-Sepharose (36).

**Vitronectin Concentrations in Platelet-poor Plasma (PPP) and Serum**—Blood was collected from 6 healthy volunteers into 0.1 volume of 0.13 M trisodium citrate. After centrifugation at 1800 × g for 15 min at 4 °C, PPP was harvested and stored in aliquots at −70 °C until used, whereas serum was prepared by adding 3 M NaCl (40), centrifugation (12,000 ×g, 15 min) and allowing the samples to clot for 2 h at 37 °C. Prior to quantifying plasma and serum levels of vitronectin using an immunoassay for vitronectin (6), all samples and vitronectin standards were dialyzed against 6 M urea for 18 h at 23 °C to denature vitronectin (37–39), and diluted with PBS containing 3% BSA, 0.1% Tween 80, 5 mM EDTA, 20 units/ml aprotinin, and 0.05% sodium azide (Dilution buffer). The standard curve for vitronectin concentrations ranging from 2.0 to 125 ng/ml (r2 = 0.982), and the inter- and intra-assay coefficients of variation are 13.5 and 8.4%, respectively. As controls, plasminogen, fibrinogen, and albumin concentrations in plasma and serum also were measured. Plasminogen and fibrinogen concentrations were quantified using the IL Test kit (Instrumentation Laboratory Co., Lexington, MA) and STA-TEM assay (Diagnostica Stago, Asnières-sur-Seine, France), respectively, on an Automated Coagulation Analyzer (model MDA-180, Organon Teknika Inc., Scarborough, ON). Albumin was quantified with the Spectrum kit using an EPX Analyzer (Abbott Laboratories Ltd., Mississauga, ON).

**Immunoblotting for Vitronectin in Solubilized PPP Clots**—PPP clots were extensively washed with PBS, and solubilized in 0.2 ml of 1% Laemmli sample buffer comprised of 50 mM Tris, pH 6.8, 2% SDS, 10% glycerol, and 0.001% bromophenol blue in the presence or absence of N-ethylmaleimide and 5% 2-mercaptoethanol (40). After boiling for 1 h, the undissolved material was sedimented by centrifugation, and 0.1 ml aliquots of the supernatants were subjected to SDS-PAGE analysis on 7.5% slab gels. Separated proteins were transferred to nitrocellulose membranes, and after blocking with 1% casein, 0.05% Biotinylated Vitronectin Incorporation into Purified Fibrin Clots—Purified fibrin clots formed at 37 °C from a TBS buffer solution (0.2 ml) containing 3 μM fibrinogen, 100,000 cpm of 125I-fibrinogen, 150 mM biotinylated native vitronectin, and 2 units/ml thrombin. At intervals, α-Phe-Pro-Arg-chloromethyl ketone (PPACK) was added to 0.05 mM, and fibrin was pelleted by centrifugation at 16,000 × g for 20 min. The radioactivity in the fibrin clot supernatant was measured in a γ-counter, whereas the amount of biotinylated-vitronectin in the clot supernatant at each time point was quantified relative to the amount of nontreated fibrin clot radioactivity at time 0 by titration. Dextran-coated Charcoal was then added to remove free radioactivity, and the clots were washed 3 times with PBS containing 2 M NaCl. All conditions were performed in triplicate. At time 0, 0.5 ml of the bathing buffer was put into four test tubes and clots added to one of these. All samples were counted, and then all clots and buffer were washed 3 times with the 50-ml tubes. The clots were monitored in the same fashion for various times, and the radioactivity in the bathing buffer was subtracted from the average radioactivity of clots, and the clot radioactivity at each time point was then expressed as a percentage of their initial radioactivity.

**Binding of 125I-Vitronectin to Fibrin Clots—50–µl aliquots of varying concentrations of a fibrinogen solution were added to wells of a 96-well plate, and fibrin matrices were formed by clotting the fibrinogen with 1.0 units/ml thrombin and 10 mM CaCl2. After incubation for 3 h at room temperature, the plates were stored at 4 °C until use. To block nonspecific binding, 100–µl aliquots of PBS containing 3% BSA and 0.05% Tween 20 were added to wells for 2 h at 37 °C, and after washing, increasing concentrations of 125I-vitronectin in Dilution buffer were added. After incubating for 1 h at 37 °C, the plates were washed, and the bound 125I-vitronectin quantified. To examine the specificity of binding, experiments were repeated in the presence of a 10-fold molar excess of unlabeled vitronectin. Control experiments were done using BSA-coated microtiter wells, and the 125I-vitronectin incorporation was blocked by applying 3 µg/ml of native fibrinogen to control for nonspecific binding.

**Confluent Microscopic Image Analysis**—For fluorescence confocal laser microscopic analysis of vitronectin distribution in plasma and purified fibrin clots, 150 µl of PPP or purified fibrinogen (3 µg/ml) were plated on APTEX-covered coverslips and clotted with 1 unit/ml of thrombin and 10 mM CaCl2. To directly visualize the fibrin fibrils, a 1:20 molar ratio of FITC-conjugated fibrinogen (Molecular Probes) per mole of native fibrinogen was added. Samples also were prepared with...
various concentrations of biotin-labeled vitronectin or BSA prior to clotting. After incubation at 37 °C for 2 h, the biotinylated proteins were removed by centrifugation and the antigen levels of albumin, vitronectin, plasminogen, and fibrinogen were quantified in the pre-clot (plasma) and post-clot (serum) supernatants. Serum supernatant values are expressed as percent of plasma levels. Data represent the mean of n = 6 (±S.E.). Panel B, aliquots of plasma, serum, and plasma clot extracts from above were prepared in the presence (+) or absence (−) of N-ethylmalamide, and/or reduction by 2-mercaptoethanol, and then fractionated by SDS-PAGE, transferred to nitrocellulose, and processed for immunoblotting with SAHVn IgG. Lane 1, purified vitronectin standard (100 ng); Lane 2, plasma; Lane 3, serum; Lane 4, solubilized plasma clot. The arrows designated a, represents vitronectin multimers (dimers-trimers). The arrow b, represents the mobility of the non-reduced, native monomeric form of vitronectin. The arrow c, represents the mobility of the 75- and 65-kDa reduced forms of vitronectin.

FIG. 1. Immunological analysis of plasma vitronectin pre- and post-clotting. Panel A, citrated human platelet-poor plasma samples were re-calcified to initiate clotting, and then incubated at 37 °C for 2 h. The resulting clots were removed by centrifugation and the antigen levels of albumin, vitronectin, plasminogen, and fibrinogen were quantified in the pre-clot (plasma) and post-clot (serum) supernatants. Serum supernatant values are expressed as percent of plasma levels. Data represent the mean of n = 6 (±S.E.). Panel B, aliquots of plasma, serum, and plasma clot extracts from above were prepared in the presence (+) or absence (−) of N-ethylmalamide, and/or reduction by 2-mercaptoethanol, and then fractionated by SDS-PAGE, transferred to nitrocellulose, and processed for immunoblotting with SAHVn IgG. Lane 1, purified vitronectin standard (100 ng); Lane 2, plasma; Lane 3, serum; Lane 4, solubilized plasma clot. The arrows designated a, represents vitronectin multimers (dimers-trimers). The arrow b, represents the mobility of the non-reduced, native monomeric form of vitronectin. The arrow c, represents the mobility of the 75- and 65-kDa reduced forms of vitronectin.

RESULTS

Coagulation-induced Reduction in Plasma Vitronectin Levels Reflects Its Incorporation into the Plasma Clot Matrix—Initial experiments were undertaken to determine whether clotting of plasma influences vitronectin antigen levels. Vitronectin, plasminogen, fibrinogen, and albumin antigens were quantified, and levels in plasma and serum from six healthy volunteers were compared (Fig. 1A). Affinity purified sheep anti-vitronectin IgG (SAHVn IgG) was used as a capture antibody in the vitronectin immunoassay. This antibody, like the conformationally sensitive mAb 8E6, preferentially binds to the oligomeric, heparin-binding form of vitronectin. Because samples can contain mixed vitronectin conformers, all the samples were denatured with urea to convert the vitronectin to its oligomeric form. The mean vitronectin concentration in serum was ~20% lower than that in plasma (paired t test; p < 0.05). Likewise, plasminogen and fibrinogen levels in serum are 40 and 98% lower in serum than in plasma. In contrast, the level of albumin in serum and plasma is similar.

To determine whether the lower level of vitronectin in serum relative to plasma reflects vitronectin incorporation into the clot, equal volumes of plasma, serum, or solubilized plasma clots were subjected to SDS-PAGE, followed by immunoblot analysis and scanning densitometry. Samples fractionated under nonreducing conditions confirmed the presence of clot-associated native vitronectin and vitronectin multimers with electrophoretic mobilities similar to those of vitronectin in plasma and serum (Fig. 1B, arrows a and b). Under reducing conditions, multimeric vitronectin dissociates into 72- and 62-kDa reduced forms of vitronectin (Fig. 1B, arrows C). The presence of N-ethylmalamide had no effect on the mobility of vitronectin, indicating that the change in the apparent Mₖ of vitronectin after clotting was not the result of disulfide-mediated binding interactions. Thus, these experiments suggest that vitronectin binds to the clot matrix.

Time-dependent Association of Vitronectin with Fibrin—To monitor the time course of vitronectin incorporation into clots, 125I-fibrinogen (3 μM) was clotted with thrombin in the presence of biotinylated-vitronectin (0.15 μM). At different intervals after thrombin addition, fibrin was pelleted by centrifugation, and the concentrations of the labeled proteins in the clot and clot supernatant were quantified. Time-dependent incorpora-
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Kinetics of thrombin-induced polymerization of fibrinogen and vitronectin. Purified fibrin clots were formed from a TBS buffer solution (0.2 ml) containing 3 μg fibrinogen, 100,000 cpm of 125I-fibrinogen, and 0.15 μg biotinylated native vitronectin. At various times after the addition of thrombin, the thrombin was neutralized with PPACK, the insoluble fibrin clots precipitated by centrifugation, and the quantities of labeled proteins in the supernant and precipitate were determined. Panel A, time course of the incorporation of 125I-fibrinogen and biotinylated-vitronectin into fibrin clots. Dotted line represents point at which the molar ratio of precipitable vitronectin exceeds that of fibrinogen. Panel B, equal aliquots of solubilized fibrin clot extracts were fractionated by SDS-PAGE, the proteins transferred to nitrocellulose, and the membranes probed for biotinylated-vitronectin (Vn) using streptavidin-conjugated alkaline phosphatase, and processed as described. Arrow, a, interface between stacking and separating gels; arrows, b, M, range of vitronectin multimers; arrow, c, thrombin-cleaved vitronectin. This data represents one of three representative experiments.

FIG. 2 Kinetics of thrombin-induced polymerization of fibrinogen and vitronectin. Purified fibrin clots were formed from a TBS buffer solution (0.2 ml) containing 3 μg fibrinogen, 100,000 cpm of 125I-fibrinogen, and 0.15 μg biotinylated native vitronectin. At various times after the addition of thrombin, the thrombin was neutralized with PPACK, the insoluble fibrin clots precipitated by centrifugation, and the quantities of labeled proteins in the supernant and precipitate were determined. Panel A, time course of the incorporation of 125I-fibrinogen and biotinylated-vitronectin into fibrin clots. Dotted line represents point at which the molar ratio of precipitable vitronectin exceeds that of fibrinogen. Panel B, equal aliquots of solubilized fibrin clot extracts were fractionated by SDS-PAGE, the proteins transferred to nitrocellulose, and the membranes probed for biotinylated-vitronectin (Vn) using streptavidin-conjugated alkaline phosphatase, and processed as described. Arrow, a, interface between stacking and separating gels; arrows, b, M, range of vitronectin multimers; arrow, c, thrombin-cleaved vitronectin. This data represents one of three representative experiments.

Vitronectin Incorporation into Fibrin Clots Is Specific—To exclude the possibility that vitronectin is trapped in the fibrin clots, we compared the rates of 125I-labeled vitronectin diffusion from clots with that of albumin and thrombin. Over 95% of the 125I-labeled ovalbumin, a protein that does not bind to fibrin, diffuses out of the clot by 1 h (Fig. 4B). In contrast, at least 50% of the 125I-labeled vitronectin remains clot-associated at 2 h even in the presence of high salt (Fig. 4A). Thrombin, a protein known to bind to fibrin, also remains clot-associated, but unlike vitronectin, thrombin diffusion is accelerated in the presence of 2 M NaCl (Fig. 4C).

Our studies thus far demonstrate that vitronectin binds to fibrin during the process of fibrinogen polymerization. To further characterize the vitronectin-fibrinogen interaction, we quantified the binding of a fixed concentration of 125I-labeled vitronectin (100 nM) to microtiter wells coated with increasing concentrations of fibrin (0.09–60.0 μM). Fig. 5A illustrates that both native (monomeric) and urea-treated (oligomeric) vitronectin conforms bind to fibrin in a dose-dependent, saturable fashion. Oligomeric vitronectin binds with an estimated Kd of 0.52 μM, while the native vitronectin conform binds fibrin with an estimated Kd of 0.9 nM, and a Kd of ~0.61 μM, indicating similar binding affinities for the two forms. The stoichiometry between vitronectin-fibrin is difficult to quantify in these experiments with pre-formed fibrin because the homogeneity, and exposure of binding sites in the microtiter plate is uncertain. To further examine the specificity of 125I-fibrinogen) into the clots was mirrored by an increase in the level of biotinylated-vitronectin incorporation (Fig. 2A). 125I-Fibrinogen incorporation reaches a plateau by 30 min, with 50% of the maximum incorporation achieved at ~12 min. In contrast, vitronectin incorporation reaches a plateau within 7.5 min, with 50% of the maximum vitronectin incorporation occurring at <2.5 min. Thus, the rate of vitronectin incorporation into the clots appears to exceed the rate of fibrin incorporation in the initial 5-min period so that the vitronectin:fibrin ratio is initially over 1 (Fig. 2A, left of dashed line).

The non-reduced SDS-PAGE gels in Fig. 2B suggest that, as early as 1 min after the addition of thrombin, vitronectin multimers and monomers are incorporated within the solubilized fibrin clots. A portion of the oligomeric vitronectin is too large to enter the separating gel (Fig. 2B, arrow a). After reduction, the majority of fibrin-associated vitronectin oligomers dissociate into native subunit forms, although there is evidence of residual vitronectin that migrates with the same apparent Mr as vitronectin dimers (Fig. 2B, arrow b), as well as thrombin-cleaved forms of vitronectin (Fig. 2B, arrow c). The rate of 125I-fibrinogen incorporation into clots was similar in the absence or presence of vitronectin, indicating that vitronectin does not influence fibrinogen incorporation (not shown).

Vitronectin Incorporation into Fibrin Clots Is Not Factor XIII-dependent—Studies were used to determine whether factor XIII-mediated cross-linking influences this interaction because both fibrinogen and vitronectin contain potential factor XIII-mediated cross-linking sites (43, 44). Clots were formed with 3 μg fibrinogen in the presence of increasing concentrations of 125I-labeled vitronectin, and in the absence or presence of factor XIII and calcium. Vitronectin incorporation into clots was similar in the absence or presence of activated factor XIII (Fig. 3). Moreover, the biphasic nature of the dose-response curves is consistent with the presence of two binding interactions between vitronectin and fibrin.

Although the binding isotherms in Fig. 3 indicate that factor XIII cross-linking activity does not govern the quantity of vitronectin incorporated into fibrin clots, it is still possible that factor XIII could mediate vitronectin cross-linking to fibrin. To address this issue, we analyzed clots and clot supernatants by SDS-PAGE analysis followed by autoradiography (Fig. 3, insert). Native 125I-labeled vitronectin consists of predominately monomeric vitronectin forms (Fig. 3, inset, lane 1, arrow c). After clotting in the presence or absence of factor XIII, the 125I-labeled vitronectin in reduced samples of clot supernatants and clot extracts migrates predominately as native vitronectin (Fig. 3, inset, lanes 2–5, arrow c), although small amounts of oligomeric vitronectin are also detected (Fig. 3, inset, lanes 2–5, arrow b). Minor amounts (<5%) of cross-linked vitronectin multimers that remain in the stacking gel are found in the extracts of clots formed in the presence of factor XIII (Fig. 3, inset, lane 5, arrow a), but not in its absence.
of the vitronectin interactions with fibrin, we quantified the binding of increasing concentrations of oligomeric 125I-labeled vitronectin (0.5–270 nM) to wells pre-coated with a fixed concentration of fibrin (375 nM) (Fig. 5B). A 10-fold molar excess of unlabeled vitronectin inhibited binding of 125I-labeled vitronectin by 75%. These results are consistent with the presence of a limited number of saturable vitronectin-binding sites on fibrin. Moreover, the Scatchard plot of the specific binding curve yields an upward-convex curve that is consistent with a non-linear, cooperative binding process (Fig. 5B, inset).

Confocal Microscopic Examination of Vitronectin in Fibrin Clots—We have previously reported that fibrin-bound vitronectin mediates the binding of PAI-1 to fibrin clots (35). Confocal immunofluorescence microscopic imaging of fibrin-associated PAI-1 in clots formed from normal plasma reveals an intense punctate staining for PAI-1 that is distributed with a notably periodicity along the length of the fibrin fibrils (Fig. 6A, arrows), and at sites of fibrin fibrils branching. To visualize vitronectin interactions with fibrin, and determine how it may regulate the periodic distribution of PAI-1 on fibrin, we used confocal microscopy to examine the structure of fibrin-associated vitronectin in unfixed, wet-mounted clots formed in the presence of FITC-conjugated fibrinogen and Texas Red rhodamine/biotin-labeled vitronectin. As controls, clots were formed in the presence of FITC-fibrinogen but without labeled vitronectin (Fig. 6, B and C), or in the presence of biotin-labeled vitronectin but without FITC-fibrinogen (Fig. 6, D and E). These images illustrate that there is no fluorescence emission crossover between the two fluorochrome capture channels, and underscore the morphological differences between the linear network of fibrin fibrils versus the globular vitronectin aggregates.

Fig. 6A is a dual red/green overlay image of a FITC-fibrinogen-labeled clot formed in the presence of Texas Red rhodamine/biotin-BSA. The lack of any significant red fluorescence in these images confirms that the BSA does not associate with fibrin. In contrast, purified fibrin clots formed in the presence of similar molar ratios of biotin-labeled vitronectin...
Fibrinogen and vitronectin reveal distinct foci of variable-sized vitronectin aggregates that cluster around fibrin fibrils, particularly at points of fibril branching and overlap (Fig. 7B, large arrows). Closer inspection of the vitronectin distribution within successive Z-plane optical sections reveals fibrin-bound, biotin-labeled vitronectin is distributed at regular intervals along the length of FITC-fibrin fibrils (Fig. 7B, small arrows). These periodic points of vitronectin on fibrin are seen to coalesce to form the branching, globular vitronectin clusters. To further investigate the phenomenon of periodicity, purified fibrin clots were formed with a lower molar concentration of biotin-labeled vitronectin to fibrinogen (1:30) so as to minimize formation of fluorescent aggregates. Under these conditions, more punctate forms of fibrin-bound vitronectin (red) are observed (Fig. 7C, arrows). Likewise, punctate forms of vitronectin also are observed when plasma containing trace amounts of biotin-labeled vitronectin is clotted (Fig. 7D, arrows). Table I represents the results from morphometric measurements of the interval distances between fibrin-bound vitronectin foci in purified and plasma clots. The average distance between adjacent vitronectin foci on purified fibrin fibrils is 1.07 μm (±0.25 μm), and is not significantly different from that measured in plasma clots (1.13 ± 0.19 μm). Interestingly, these measures of periodicity coincide with those observed for fibrin-bound PAI-1 (Fig. 6A, arrows).

Interaction of Vitronectin with γA/γFibrinogen—Our find-
ings that vitronectin associates with fibrinogen during coagulation, and that vitronectin is bound with a distinct periodicity along fibrin fibrils, suggest that vitronectin binds only to a subset of fibrin(ogen) molecules. The linear length of fibrin(ogen) is 45 nm (44, 45). Our measured periodicities of fibrin-associated vitronectin range from 0.8 to 1.4 /H9262 M. Taken together, these data suggest that vitronectin binds to approximately one in every 18–31 fibrin(ogen) molecules that could be randomly incorporated along the length of fibrin protofibrils.

One possible candidate for specific vitronectin binding is the highly conserved fibrinogen variant that represents 5–10% of the total circulating fibrinogen. The more anionic fibrinogen variant can be isolated from fibrinogen by ion exchange chromatography on DEAE-Sepharose (36). Western blot analysis of fractionated fibrinogen indicates that most of the plasma vitronectin which contaminates commercial fibrinogen preparations (0.1 g of vitronectin/mg of fibrinogen) co-purifies with the /H9253 A/H9253 fibrinogen (Fig. 8A). A smaller portion of vitronectin also is detectable in the peak I fraction, but is only visible when the gel is overloaded. To explore the possibility that /H9253 A fibrinogen preferentially binds to vitronectin, we quantified the incorporation of 40 nM 125I-labeled vitronectin into clots formed in from increasing concentrations of either /H9253 A or /H9253 A fibrinogen.

![Fig. 7. Periodicity of vitronectin binding to fibrin fibrils in purified and plasma clots.](http://www.jbc.org/)

**TABLE 1**

| Type of clots analyzed | Number of measurements | Total surface area (mm²) | Mean interval (μm) ± S.D. |
|------------------------|------------------------|--------------------------|--------------------------|
| Purified fibrin clots  | 163                    | 15.4                     | 1.07 ± 0.29              |
| PPP clots              | 147                    | 13.7                     | 1.13 ± 0.28              |

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Fig. 8. Vitronectin association with the γA/γ fibrinogen. Purified fibrinogen was fractionated by ion exchange chromatography using DEAE-cellulose, and resulted in two distinct fractions, known as peak I and peak II, which differ with respect to their γ chains. The peak I fibrinogen contains two γA chain dimers, whereas peak II fibrinogen is a heterodimer containing one γA chain, and one γ chain. Panel A, fibrinogen samples were fractionated by SDS-PAGE (reduced), and the gels were either processed for Coomassie Blue staining for visualizing the fibrinogen chains, Aα, Bβ, and γA or γ (total fibrinogen, 15 μg/lane; peaks I and II fibrinogen, 9 μg/lane), or the samples (9 μg/lane) were transferred to nitrocellulose membranes, and processed for Western blot analysis using rabbit antisera directed against either human γ fibrinogen, or vitronectin. Panel B, purified fibrin clot samples were formed from a TBS buffer solution (0.2 ml) containing various concentrations of either peak I or peak II fibrinogen, and 40 nM cpm of 125I-vitronectin. After 2 h at 37 °C, the thrombin was neutralized with PPACK, the insoluble fibrin clots precipitated by centrifugation, and the quantities of radioactivity in the supernatant and precipitate were determined. Data represents the concentration of fibrin-bound vitronectin versus concentration of fibrinogen added from one of three representative experiments.

Discussion

This report is the first comprehensive examination of the direct binding interactions between vitronectin and fibrinogen. The notion that plasma vitronectin binds to fibrin clots is supported by our observations of lower levels of vitronectin in serum than in plasma, of the presence of vitronectin in solubilized plasma clot extracts, of the localization of vitronectin on fibrin fibrils in plasma clots, as well as of the vitronectin-dependent binding of plasma PAI-1 to fibrin (35). To further characterize the nature of these binding interactions, we used direct binding measurements and morphological studies with purified vitronectin and fibrinogen in solution, and on microtiter plates.

Vitronectin Is Incorporated into Fibrin Clots—Vitronectin incorporation into clots, like that of thrombospondin (46), another adhesive glycoprotein, is non-saturable and factor XIII-independent. Kinetic studies indicate that the incorporation of vitronectin into clots is not necessarily dependent on the presence of pre-formed fibrin as the initial rate of precipitable vitronectin incorporation into clots exceeds the rate of fibrinogen incorporation during the early phases (<5 min) of coagulation. Also, additional vitronectin incorporation is not observed even after fibrinogen polymerization is complete. However, this does not exclude the possibility that vitronectin interacts with pre-formed fibrin because oligomeric vitronectin binds specifically to fibrin-coated microtiter wells. Additional binding of vitronectin to the fibrin matrix occurs via cooperative binding interactions between the fibrin-bound vitronectin and native vitronectin subunits. This type of positive cooperativity binding process may account for the proposed two binding site interactions, and is consistent with the previously described mechanism of the concentration-dependent, urea-induced formation of vitronectin polymers (47).

Vitronectin Associates with Fibrinogen during Coagulation—Several lines of evidence indicate that vitronectin co-polymerizes with fibrin. First, vitronectin incorporation into clots is non-saturable, and is directly related to the concentrations of vitronectin and fibrinogen. Second, disulfide-linked vitronectin multimers bind to purified fibrin clots in a cooperative manner, a characteristic consistent with that of an accreting polymer, like fibrinogen. Third, it is unlikely that the vitronectin is trapped in the clots because diffusion of clot-associated...
vitronectin is consistently lower than that of ovalbumin, and levels of binding of vitronectin remain stable. Albumin is not incorporated into clots formed from plasma or purified fibrinogen. Finally, direct morphological examination of clot-associated vitronectin structure reveals a branching network of globular polymeric aggregates.

A New Function of $\gamma A/\gamma F$ Fibrinogen in Hemostasis—Confocal microscopic studies reveal fibrin-bound vitronectin aggregates clustered at intervals along the length of fibrin fibers, and extending laterally between adjacent fibrin fibers, particularly at sites of fibril branching or overlap. Morphometric analysis reveals an average periodicity of fibrin-bound vitronectin of 1.1 ± 0.3 μm, suggesting that vitronectin binds to specific, repeating domains along the fibrin polymers. Our studies indicate that the vitronectin, which is a trace contaminant in commercial fibrinogen preparations (35), co-elutes with peak II fibrinogen, and clots formed from $\gamma A/\gamma F$ fibrinogen incorporate significantly more vitronectin than clots formed from peak I fibrinogen. These results strongly support the hypothesis that the anionic sequence within the carboxyl termini of the fibrinogen $\gamma$ chain contains the major vitronectin-binding site for fibrin.

The fibrinogen $\gamma A/\gamma F$ variant is found in 5–10% of circulating fibrinogen levels in humans (48–50), and has unique interactions with proteins that regulate fibrin formation. Fibrinogen $\gamma A/\gamma F$ accelerates thrombin-mediated factor XIII activation as a consequence of possessing binding sites for factor XIII (51) and thrombin (52). Moreover, the binding of vitronectin to fibrinogen $\gamma A/\gamma F$ may serve an anti-fibrinolytic function by localizing PAI-1 on fibrin fibrils. Furthermore, it remains speculative whether the presence of vitronectin in proximity of the thrombin-binding sites on the fibrinogen $\gamma A/\gamma F$ variant may also serve a regulatory role in the vitronectin-dependent interactions of thrombin with anti-thrombin (15) and PAI-1 (24).

Proposed Model for Coagulation-Induced Vitronectin Association with Fibrinogen—Our findings suggest plasma vitronectin interacts with circulating $\gamma A/\gamma F$ fibrinogen prior to clotting, and additional vitronectin incorporation occurs post-clotting. Moreover, the binding of vitronectin to fibrin may not be limited to its pre-clotting interactions with $\gamma A/\gamma F$ fibrinogen as vitronectin also binds to clots formed from peak I fibrinogen, and to pre-formed fibrin surfaces. Recent studies with vitronectin-deficient mice support the notion that plasma vitronectin has complex effects on thrombogenesis. Thus, investigators have identified a previously unanticipated antithrombotic effect of vitronectin at sites of platelet-rich thrombosis (53). These authors postulate that the effect is caused, at least in part, by vitronectin-mediated inhibition of thrombin-fibrin interactions, an phenomenon that may be related to the binding of vitronectin to fibrin. On the other hand, in another vascular injury model of occlusive thrombus formation, the absence of vitronectin inhibits reocclusion, and modulates endogenous fibrinolysis (54). This may be related to the recent findings that arterial thrombi in vitronectin-deficient mice are unstable and frequently embolize (55). Thus, incorporation of vitronectin into fibrin clots is likely to play a multifunctional role in regulating hemostasis, fibrinolysis, and cell adhesion/migration during thrombosis, angiogenesis, and wound healing.
Incorporation of Vitronectin into Fibrin Clots: EVIDENCE FOR A BINDING INTERACTION BETWEEN VITRONECTIN AND $\gamma$A/$\gamma$ FIBRINOGEN

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