Interaction of Recombinant Procollagen and Properdin Modules of Thrombospondin-1 with Heparin and Fibrinogen/Fibrin*

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Many properties have been assigned to the procollagen and properdin (Type I) modules of thrombospondin-1 (TSP1) based on activities of large proteolytic fragments of TSP1 or peptides containing TSP1-derived sequences. To examine the activities of the modules more exactly, we expressed the first properdin module (P1); the third properdin module (P3); the first and second properdin modules (P12); the first, second, and third properdin modules (P123); and the procollagen module with the first, second, and third properdin modules (CP123) in the GELEX expression vector (GE1) using the baculovirus system. GE1 encodes the pre-pro sequence, the transglutaminase cross-linking site(s), the protease-sensitive site, and the gelatin binding domain from the amino terminus of rat fibronectin. All five recombinant proteins were expressed in insect cells, secreted into the culture medium, and purified by gelatin-agarose affinity chromatography. P123 shared with TSP1 a resistance to trypsin unless reduced and alkylated. P12/GE1, P123/GE1, and CP123/GE1 bound poorly to heparin-agarose except in the absence of sodium chloride, whereas peptides based on P2 are known to bind to heparin in up to 150 mM sodium chloride. In cross-linking experiments employing activated recombinant factor XIII and the transglutaminase cross-linking site in the fibronectin-derived sequence, P12/GE1, P123/GE1, CP123/GE1, and P3/GE1 but not P1/GE1 became incorporated into a fibrin clot more than GE1 alone. Analysis of the complex indicated that cross-linking was to the portion of the fibrin α-chain remaining in the D-dimer of plasmin digests. P123 also cross-linked to the αβ-chain of uncleaved fibrinogen. P123 competed for 125I-TSP1 incorporated into a fibrin clot more than GE1 alone. Analysis of the complex indicated that cross-linking was to the portion of the fibrin α-chain remaining in the D-dimer of plasmin digests. P123 also cross-linked to the αβ-chain of uncleaved fibrinogen. P123 competed for 125I-TSP1 incorporation into the fibrin clot. P123 did not cross-link to plasminogen, histidine-rich glycoprotein, fibrinogen, or plasma globulins other than fibrinogen/fibrin. These results indicate that the properdin modules of TSP1 specifically interact with fibrinogen/fibrin but not with heparin under physiologic conditions.

Thrombospondin-1 (TSP1) is a component of the platelet α-granule that is released upon platelet degranulation (1). It is a member of a family of proteins that includes TSP2, TSP3, TSP4, and TSP5 (COMP) (reviewed in Ref. 2). TSP1 and TSP2 are both large trimeric glycoproteins composed of 150-kDa subunits covalently linked by interchain disulfides (3). From the amino to the carboxyl terminus, each TSP1 or TSP2 monomer contains a heparin-binding domain, oligomerization (heptad) domain, a procollagen module, three properdin (type I) modules, three epidermal growth factor (EGF)-like (type II) modules, a calcium binding domain, and a globular carboxyl-terminal domain (C-globe) (2). TSP3, TSP4, and TSP5 are pentameric proteins that are composed of subunits that do not contain procollagen or properdin modules (2). The disulfide-rich, central stalk region of TSP1, containing the interchain disulfides, the oligomerization domain, the procollagen module, the properdin modules, and the EGF modules, is protease-resistant (4). Therefore, assigning function to a discrete module or set of modules within the central stalk of TSP1 has been difficult. Functions have been assigned to the domains of TSP1 using proteolytic fragments, monoclonal antibodies, and synthetic peptides (2, 5). Functions also have been ascribed to recombinant properdin modules expressed as bacterial fusion proteins including activation of latent transforming growth factor β (6), attachment of Bowes melanoma cells (7), inhibition of decorin interaction with TSP1 (8), and inhibition of TSP binding to MDA-MB-231 breast cancer cells (9). No attempts have been made, however, to express the individual modules of the stalk region in their native disulfide-bonded state.

The heparin binding domain of TSP1 has been localized to the amino terminus of TSP1 (10). The 70-kDa core fragment of platelet TSP1, which includes the procollagen and properdin modules, does not bind to heparin (10, 11), sulfatides, or heparan sulfate proteoglycans (12). Peptides based on sequences from the properdin modules, however, do interact with heparin and sulfatides (13, 14).

Platelet TSP1 interacts with purified fibrinogen/fibrin (15, 16), fibronectin (17), plasminogen (18), and histidine-rich glycoprotein (19). Such interactions may occur during blood coagulation when TSP1 is released from platelet α-granules (20). Binding of TSP1 to fibronectin (21), plasminogen (11), fibrinogen (11), and histidine-rich glycoprotein (19) is through the central stalk region as assessed by solid phase binding assays or affinity chromatography. TSP1 becomes bound to fibrin both noncovalently and covalently via activated factor XII (FVIIa)-

tor; FXIII, factor XIII; FXIa, thrombin-activated FVIIa; GBD, gelatin-binding domain; GE1, GELEX expression vector or protein encoded by the vector without inserted modules; P1, first properdin module; P3, third properdin module; P12, first and second properdin modules; P123, first, second, third properdin modules; CP123 procollagen module with the first, second, and third properdin modules; PAGE, polyacrylamide gel electrophoresis; FPA, fibrinopeptide A.

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† The abbreviations used are: TSP and TSP1 to -5, thrombospondin and thrombospondin-1 to -5, respectively; EGF, epidermal growth factor; α-granule that is released upon platelet degranulation (1). It is a member of a family of proteins that includes TSP2, TSP3, TSP4, and TSP5 (COMP) (reviewed in Ref. 2). TSP1 and TSP2 are both large trimeric glycoproteins composed of 150-kDa subunits covalently linked by interchain disulfides (3). From the amino to the carboxyl terminus, each TSP1 or TSP2 monomer contains a heparin-binding domain, oligomerization (heptad) domain, a procollagen module, three properdin (type I) modules, three epidermal growth factor (EGF)-like (type II) modules, a calcium binding domain, and a globular carboxyl-terminal domain (C-globe) (2). TSP3, TSP4, and TSP5 are pentameric proteins that are composed of subunits that do not contain procollagen or properdin modules (2). The disulfide-rich, central stalk region of TSP1, containing the interchain disulfides, the oligomerization domain, the procollagen module, the properdin modules, and the EGF modules, is protease-resistant (4). Therefore, assigning function to a discrete module or set of modules within the central stalk of TSP1 has been difficult. Functions have been assigned to the domains of TSP1 using proteolytic fragments, monoclonal antibodies, and synthetic peptides (2, 5). Functions also have been ascribed to recombinant properdin modules expressed as bacterial fusion proteins including activation of latent transforming growth factor β (6), attachment of Bowes melanoma cells (7), inhibition of decorin interaction with TSP1 (8), and inhibition of TSP binding to MDA-MB-231 breast cancer cells (9). No attempts have been made, however, to express the individual modules of the stalk region in their native disulfide-bonded state.

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mediated transglutamination during blood coagulation (16, 22). FXIII mediates formation of an $\varepsilon$-y-glutamyl cross-link between specific glutamine and lysine residues. Incorporation of TSP1 into the fibrin clot results in fibrin polymers that are finer and thinner (22, 23). The 70-kDa core fragment of TSP1 has the potential to contribute both lysine and glutamine residues to the cross-linking (24). Antibodies to the fibrinogen Aα-chain inhibit the fibrinogen-TSP1 interaction (25). In solid phase assays, TSP1 bound to both the Aα- and Bβ-chains of fibrinogen (26) and discrete peptides based on sequences in the Aα- and Bβ-chains (27). Binding of TSP1 to fibrinogen may account for the ability of TSP1 to alter platelet aggregation (28). Because the fibrin clot has an important role in wound healing, including promoting cell migration, cell adhesion, and endothelial tube formation (20, 29), the understanding of proteins incorporated into the clot may provide a better understanding of wound remodeling.

In order to explore specific interactions of procollagen and properdin modules of TSP1, we expressed the modules as fusion proteins with the gelatin-binding domain (GBD) of fibronectin in insect cells using baculovirus. The expression system allowed the modules to be processed by the secretory machinery and thus optimizes the chances that the modules will adopt their native fold and disulfides. The proteins were purified from conditioned media by gelatin-agarose affinity chromatography, examined for heparin binding activity, and studied in protein-protein interactions.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Recombinant Proteins**—Five constructs of the procollagen and properdin modules of human TSP1 cDNA were expressed in the GELEX (GE1) expression vector based on the amino acid sequence of fibronectin (30). The first, second, and third properdin modules were expressed in insect cells using baculovirus. The expression system included the gelatin-binding domain (GBD) of fibronectin in insect cells using baculovirus. The GBD was added to the expression vector at the NcoI position (positions 473–476) based on the same reasoning. The CP123 construct began at LRRP (positions 294–298), a choice based on a cleavage site in TSP1 that gives rise to the anti-angiogenic gp140 fragment of hamster TSP1 generated by baby hamster kidney cells (32). The carboxy-terminal sequences of the constructs were as follows: P1, QECQD, P12, DACFP, and P123, F3, and CP123, DCPI. TSP1 cDNA, a kind gift from Dr. Vishva Dixit (33), was amplified using PCR with appropriate primers to introduce a BstXI restriction site at each end of the PCR-amplified DNA. The PCR products were digested with BstXI and subcloned into GE1/pGEM4 producing module GE1 constructs (30). The DNA sequence of the cloned constructs was verified against errors introduced by polymerase chain reaction by dideoxy sequencing of double-stranded DNA (Sequenase kit, U.S. Biochemical Corp.). The module/GE1 constructs, P1/GE1, P12/GE1, P123/GE1, and CP123/GE1, were cut from pGEM4 using SfiI (Promega, Madison, WI). The constructs were treated with SfiI and XbaI. The SfiI to XbaI properdin/GEP sequence was isolated and subcloned into the transfer vector pVL1392 (Invitrogen, San Diego, CA) for production of recombinant baculovirus and expression in Sf9 insect cells. P3/GE1 was cut from pGEM4 (Promega) using BamHI and directly subcloned into pVL1392.

**Cotransfection of Recombinant DNA with Baculovirus DNA (Invitrogen)**—was done in Sf9 insect cells under serum-free conditions. Viruses that had undergone homologous recombination were plaque-purified and amplified to pass 2 or 3. For production of recombinant protein, Hi5 or Sf9 insect cells were infected at a multiplicity of infection of 10 in suspension or adherent cultures. Forty-eight to 72 h after infection, culture medium was spun down to remove cells and/or cellular debris, and phenylmethylsulfonyl fluoride was added to 2 mM. Protein was bound to gelatin–agarose via the GBD; unbound material was removed by washing with TBS (10 mM Tris, pH 7.4, 150 mM NaCl); and recombinant protein was eluted with 3 mM guanidine hydrochloride in TBS, dialyzed against TBS, quick-frozen, and stored at –80 or –15 °C. Purity was assessed by SDS-polyacrylamide gel electrophoresis (PAGE). Determination of protein concentration was based upon the following calculated extinction coefficients (mg/ml/cm) (34) and absorbance at 280 nm: CP123/GE1, 2.10; P123/GE1, 2.10; P12/GE1, 2.05; P1/GE1, 1.96; P2/GE1, 1.98; GE1, 1.90; and P123, 2.55. The fusion protein modules were designated with prefixes "GE1" in contrast to proteins cleaved from the GBD that contain no suffix.

To remove the GBD, protein was bound to gelatin–agarose (Sigma) at 0.25 μg of protein/μl of gelatin–agarose by batch adsorption, poured into a column, equilibrated with digestion buffer (50 mM Tris, pH 8.5, 150 mM NaCl, 2 mM CaCl2), and treated with trypsin (0.2%, w/v) for 30 min in a reciprocating mixing at room temperature. The cleaved protein was washed from the column in TBS and collected into tubes containing soybean trypsin inhibitor bound to Sepharose (Pierce). The soybean trypsin inhibitor–Sepharose was removed by applying the sample to a chromatography column. The protein was found to be 85–90% pure by SDS-PAGE with the primary contaminant being free GBD. For incorporation of amine into the transglutaminase site(s), either fluorescein-cadaverine (Molecular Probes, Inc., Eugene, OR) or monodansylcadaverine (Sigma) was incubated with the recombinant protein and blood coagulation FXIIIa under similar conditions to the cross-linking experiments described below. The proteins were separated by SDS-PAGE and examined for fluorescent bands with an ultraviolet light source (35).

To compare trypsin sensitivity between untreated P123 and reduced, denatured, and alkylated P123, P123 was denatured with 3 mM guanidine hydrochloride and reduced with 50 mM dithiothreitol for 30 min at 37 °C. Then the protein was treated with iodoacetic acid (100 mM) for 2.5 h at room temperature and dialyzed overnight against TBS. Samples were treated with 0.6% (w/v) 1-1-tosylamide-2-phenylthiocarbomethyl ketone–treated trypsin (Sigma) for 30 min at the indicated temperature. The proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with rabbit anti-TSP1 antibodies (36).

**Heparin Binding Assay—**NaCl was removed by dialysis of fusion proteins into 20 mM Tris with subsequent addition to the indicated NaCl concentration. Alternatively, P123 cleaved from GBD was dialyzed against TBS. Heparin binding was determined by batch adsorption of proteins for 2 h at 4, 22, or 37 °C to heparin–agarose (Sigma). After batch adsorption, the unbound protein was removed, the heparin–agarose was washed three times, and bound protein was eluted with 20 mM Tris containing 300 mM NaCl or sample buffer (3% SDS, 9 M urea). Bound and unbound protein were analyzed by SDS-PAGE.

**Cross-linking—**Properdin-Protein Interaction in Solution—FXIIIa cross-linking was performed to examine the interaction in solution of properdin with TSP1. A properdin of interest, Properdin/FXIIIa (generous gift of Paul Bishop, Zymogenetics, Seattle, WA) was preactivated with thrombin (generous gift of John Fenton II, New York State Department of Health, Albany, NY) for 30 min at 37 °C, and thrombin was inactivated with hirudin (Sigma). The amino-terminal 70-kDa fragment of fibrinectin (37), fibrinogen (38), histidine-rich glycoprotein (39), plasminogen (40), and H2 DSK cyanogen bromide fragment of fibrinogen (41) were treated with 0.1 μM FXIIIa for 1 h at 30 °C. The proteins were incubated in solution with P123 (0.1 μM) at 37 °C in the presence of FXIIIa (1 μg/ml) in the absence of Ca2+ in a total volume of 30 μl to allow binding in the absence of cross-linking. After 1 h, CaCl2 (3 mM) was added for 10 min for cross-linking, and the reaction was stopped with sample buffer containing EDTA (6 mM). Alternatively, in the case of using fibrinogen, the FXIIIa was inhibited with EDTA, and the complex was treated with thrombin to cleave fibrinopeptide A and fibrinopeptide B from the Aα- and Bβ-chains of fibrinogen, respectively.

To analyze complex formation, the proteins were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted. The recombinant TSP1 modules were probed with anti-human TSP1 (36). The GBD of the fusion proteins and the tails from GE1 that remain on the amino and carboxy terminus of the inserted protein after cleavage with trypsin (Fig. 1) were probed with rabbit anti-GE1 (expressed without any insert) antibodies. These antibodies were specific for rat fibronectin and did not recognize human fibronectin in immunoblots. The 27-kDa fragment of fibrinectin was detected with rabbit anti-27 kDa (37). An ovalbumin conjugate of a synthetic peptide corresponding to human fibrinopeptide A (FPA, 1-16) was used for preparation of antibody FPA 19/7 (1g). In contrast to antibodies generally identified previously (42), antibody FPA 19/7 not only reacts with native and synthetic Aα (1-16) and synthetic Aα (7-16) but also with intact fibrinogen (Western blotting and enzyme-linked immunosorbent assay, data not shown). In fact, the reactivity of this antibody with fibrinogen is significantly greater than with any of the free peptides (IC50 fibrinogen) = 100 pmol/ml; IC50 Aα(1-16)/Aα(7-16) = 2000
pmol/ml). This antibody does not react with any chain of fibrin or FXIIIa cross-linked fibrin. Three other anti-As monoclonal antibodies were used: 1D4 to residues 349–406, 1C2-2 to residues 529–539, and 1T03 to residues 308–318 of fibrinogen. The epitope for 1T03 was defined by synthetic peptides similar to what was described previously for the epitopes of 1D4 and 1C2-2 (43). Negative controls, i.e., cross-linking in the presence or absence of P123, were included in each experiment to be certain there was no antibody cross-reactivity to other proteins. Cross-linking of P123 or the fusion proteins to self was assessed in each experiment and was negative under all conditions used. Chemiluminescence reagents (NEL Life Science Products) were used to detect horseradish peroxidase-conjugated goat secondary antibodies (Cappel/Organon Teknika Corp., West Chester, PA) bound to the rabbit or mouse primary antibodies.

**Procollagen and Properdin: Fibrin Interaction during Clot Formation**—To study the incorporation of the 27-kDa fragment of fibronectin (purified as described (44)) and procollagen and properdin modules of TSP1 into fibrin clots, the various proteins (0.1 μg) were incubated with purified fibrinogen (1.47 mg/ml) in 10 mM Tris with 150 or 300 mM NaCl in a total volume of 20 or 40 μl. The clots were formed for 60 min as described (45). Lysis of fibrin clots was done at 37 °C using 5–10 ng/μl concentration of urokinase-activated plasminogen (40). To analyze P123 cross-linking to fibrin in a more complex milieu, platelet poor plasma was prepared, and the globulin proteins were precipitated with 40% saturated ammonium sulfate. The precipitate was resuspended and dialyzed against TBS containing 0.1 mM EDTA. After the addition of P123, the plasma globulin fraction was clotted with thrombin and CaCl₂ at 37 °C. Purified fibrinogen supplemented with FXIII was used as a control. For plasma clots and the respective control, the clot was separated from the supernatant but not washed. The clot was reduced, denatured, and loaded onto the gel. For fibrin clots, the entire clot and supernatant were denatured and in some cases reduced, with the total volume loaded onto the gel. Proteins were separated by SDS-PAGE and analyzed by Western blot as described above. Alternatively, for 125I-TSP cross-linking experiments, fibrin clots were formed with purified fibrinogen (20 μg/ml) and 125I-TSP (3.5 μg/ml) in 30 μl in 1 h at 37 °C as described previously (16). P123 was tested at several concentrations (10–54 μg/ml). The negative controls, the 70-kDa fragment of fibronectin and GE1, were tested at equimolar concentration of 2.4 μg (equivalent to 54 μg/ml P123). The entire clot and supernatant were reduced and denatured, the total volume was loaded onto the gel, and the proteins were separated on 5% SDS-PAGE, in some cases in the absence of a stacking gel. Amounts of cross-linked 125I-TSP were quantitated by PhosphorImager analysis (Molecular Dynamics, Inc., Sunnyvale, CA). P123/GE1 was iodinated as described previously for the 70-kDa fragment of fibronectin (37), and binding of 125I-P123/GE1 to fibrinogen (1–3 μg/ml) coated onto plastic (Falcon Probind 96-well plate, Becton Dickinson, Franklin Lakes, NJ) was determined in duplicate as described for collagen V (46).

**RESULTS**

**Expression and Stability of the Procollagen and Properdin Fusion Proteins**—To explore the structure/function of the procollagen and properdin modules of TSP1, the modules (Fig. 1A) were expressed in insect cells using the GE1 expression vector that encodes the pre-pro secretion signal, the transglutaminase cross-linking site(s), the protease-sensitive region, and GBD (Fig. 1B) (30). The modules of the GBD must be in their native conformation to bind to gelatin (47). This property allows the GBD to serve as a quality control for proper folding of the fusion proteins during processing in the rough endoplasmic reticulum and Golgi apparatus. After trypsin cleavage, the expressed module(s) retain(s) from the expression vector the transglutaminase cross-linking sites in the amino and carboxyl-terminal tails from the expression vector. The primary transglutaminase acceptor site (Gln⁴) is amino-terminal to the module(s) (Fig. 1B), while a putative additional site, Gln¹⁴, is carboxyl-terminal to the expressed modules (35, 48).

The proteins were expressed at concentrations of 2.5–20 μg/ml, secreted into the culture medium, and purified by gelatin-agarose affinity chromatography (Fig. 2A). CP123/GE1, P123/GE1, and P12/GE1 were approximately 68, 61, and 55 kDa, respectively, as estimated from SDS-PAGE. P1/GE1 and P3/GE1 were similar in size, approximately 48 kDa. The estimated sizes by SDS-PAGE were consistent with the calculated masses. All proteins migrated more rapidly in the absence of reducing agent (data not shown), indicating the presence of intramolecular disulfide bonds, and disulfide-linked oligomers were not detected (data not shown). All proteins incorporated monodansylcadaverine or fluorescein-cadaverine when reacted with FXIIIa (data not shown), indicating that the transglutaminase site(s) were functional. There were no apparent differences in amine incorporation among constructs. The protein preparations shown are representative in that all contained GBDs of approximately 41 kDa, presumably due to cleavage between the expressed modules and the GBD fusion proteins during expression or purification. The stalk region of TSP1 is resistant to trypsin digestion (4, 11); therefore, we tested P123 for trypsin sensitivity. P123, cleaved from the GBD, resisted trypsin digestion (Fig. 2B) at 25 but not 37 °C after a 30-min incubation with 0.6% (w/v) trypsin. P123 that was reduced, denatured, and alkylated with iodoacetic acid shifted in molecular weight due to modification of the Cys residues with iodoacetic acid and was sensitive to trypsin digestion at 25 or 37 °C (Fig. 2B).
Fig. 2. Expression of procollagen and properdin modules of human TSP1. A, fusion proteins of the indicated modules in GE1 were expressed by insect cells using the baculovirus expression system. All five proteins were secreted into the culture medium and purified by gelatin-agarose affinity chromatography. The contaminating 41-kDa band in each protein is the GBD. Equal amounts of proteins were stained with Coomassie Brilliant Blue. B, purified P123 (cleaved from GBD) was untreated (−) or reduced, denatured, and alkylated (RDA) as indicated. The protein (30 μg/ml) was treated at 25 °C (25) or 37 °C (37) for 30 min with trypsin (0.6%, w/w) or left untreated (−) as indicated. The protein was resolved on 12% SDS-PAGE under reducing conditions, and protein was detected by anti-TSP1 antibodies on Western blot. The molecular mass markers (kDa) are as indicated.

Fusion Proteins—Peptides based on the properdin modules of human TSP1 (13) and a reduced and alkylated fragment that includes the first properdin module (46) bind to heparin. To test whether properdin modules of TSP1 expressed in insect cells also bind to heparin, we incubated the GE1 fusion proteins at 4 °C with heparin-agarose at three different salt concentrations: no added NaCl, 50 mM NaCl, or 150 mM NaCl. CP123/GE1, P123/GE1, and P12/GE1 all bound to heparin in the absence of added NaCl but did not bind heparin in the presence of 150 mM NaCl (Fig. 3, A–C). In the presence of 50 mM NaCl, a small fraction of CP123/GE1, P123/GE1, or P12/GE1 bound to heparin-agarose. P1/GE1 did not bind to heparin-agarose in the absence of added NaCl, 50 mM NaCl, or 150 mM NaCl (Fig. 3D). The majority of the P3/GE1 did not bind heparin-agarose in the absence of added NaCl, and none bound at 50 and 150 mM NaCl (Fig. 3E). The 41-kDa GBD, contaminating all proteins, was found in the unsolved fraction. Similarly, GE1 without any insertion did not bind to heparin-agarose (data not shown). P123 (cleaved from GBD) did not bind to heparin-agarose in the presence of 150 mM NaCl at 4, 22, or 37 °C (data not shown). To summarize, CP123, P123, and P12 modules bound to heparin at 4 °C but only at nonphysiologic, low salt concentrations, and P1 and P3 did not bind.

Cross-linking of P123 to TSP1-binding Proteins—TSP1 has been reported to bind numerous proteins, including fibronectin, fibrin, plasminogen, histidine-rich glycoprotein, and the amino-terminal 70-kDa portion of fibronectin (11, 16, 49). The transglutaminase cross-linking sites remaining in P123 after cleavage from the GBD (Fig. 1B) were utilized to analyze protein-protein interactions in solution. This strategy was employed previously to demonstrate that the EGF modules of blood coagulation factor IX interact specifically with zymogen but not with activated factor X (35). The primary transglutaminase acceptor site, Gln4, is contained within a flexible region of fibronectin and thus should be available for cross-linking to a variety of adjacent lysines (50). Proteins were allowed to interact in solution with P123 and then cross-linked using FXIIIa. Covalent complexes were analyzed by Western blotting with anti-TSP1. No cross-linking of P123 to itself was detected (Fig. 4), as was the case under all experimental conditions. P123 cross-linked to fibrinogen but not to plasminogen, albumin, histidine-rich glycoprotein, or the 70-kDa amino-terminal fragment of fibronectin (Fig. 4). 125I-P123/GE1 bound fibrinogen in a solid phase assay as well (data not shown).

The covalent complexes of P123 (23 kDa when not cross-linked) and fibrinogen had estimated sizes of 93 kDa (major band) and 83 kDa (minor band) under reducing conditions (Fig. 4) and were larger than fibrinogen in the absence of reducing agent (>330 kDa) (data not shown). The 93- and 83-kDa bands under reducing conditions were recognized by antibodies to both TSP1 and the fibrinogen fibrinopeptide A (Fig. 5). When the complex was treated with thrombin after cross-linking to remove fibrinopeptide A, the 93- and 83-kDa bands, as recognized by antibodies to TSP, shifted in molecular weight (Fig. 5A), and recognition by the monoclonal antibody to fibrinopeptide A (FPA 19/7) was lost (Fig. 5B). These data indicate that P123 cross-linked to the α-chain of fibrinogen, which has a size of 70 kDa. The 83-kDa band presumably represents the 23-kDa protein cross-linked to the approximately 60-kDa remnant of the α-chain seen in Fig. 5B.

P123 Incorporation into a Plasma Clot—We also analyzed cross-linking of P123 to fibrin after clot formation of purified fibrinogen. For these studies, FXIII was added as the zymogen, and thrombin was added as the final reagent to initiate clotting that proceeded for 60 min. Using a constant concentration of P123/GE1 (55 μg/ml) and increasing concentrations of fibrinogen (30 μg/ml to 1 mg/ml), there was a dose-dependent increase in incorporation of P123/GE1 into fibrin clots (data not shown). To test the specificity of the interaction of P123 with fibrin, we incubated P123 with the fibrinogen-rich globulin fraction of platelet-poor plasma containing FXIII. The P123 was detected in the clot of the plasma globular fraction on Western blot by antibody to the GE1 fusion protein as a single band with a molecular mass of 93 kDa after reduction (Fig. 6). Although the bands were distorted due to high concentrations of 60–100-kDa proteins in the plasma globulin fraction of the unwashed clot, the molecular weight of the cross-link product was the same as the cross-link product found in a clot formed with purified fibrinogen and FXIII. The 93-kDa band was recognized by a monoclonal antibody to fibrin α-chain (1D4) (data not shown). Higher α-chain multimers also were recognized by 1D4 (data not shown). With longer exposure times, P123 could be detected in higher multimers as well (data not shown; see Fig. 8). Inclusion of unlabeled P123 (54 μg/ml) resulted in a decrement in cross-linking of 125I-TSP (3.8 μg/ml) to fibrin (fibrinogen, 20 μg/ml) with 60 ± 10% decrease (mean ± S.D. of three experiments) that was dose-dependent with a 25% decrease at 20 μg/ml and 40% decrease with 30 μg/ml. Neither the 70-kDa fragment of fibronectin nor GE1 caused a decrease in cross-linking of 125I-TSP into the fibrin clot (<10% decrease).

Plasmin Digest of Fibrin Clots Containing P123—Fibrin clots containing P123 were digested with plasmin to identify the segment(s) of the α-chain that cross-link(s) to P123. Under nonreducing conditions, the cross-link material containing P123 as detected by anti-TSP1 was in a >200-kDa band (Fig. 7A). After 40 or 80 min of plasmin digestion (5 μg/ml) at 37 °C, P123 was detected in a band at approximately 180 kDa, the expected size of the D-dimer fragment of cross-linked fibrin.

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Higher molecular weight bands persisted, reflecting the plasmin resistance of cross-linked fibrin (52). Protein staining of the digests revealed that about 85% of the fibrin was converted to D-dimer by 80 min (data not shown). In reduced samples, P123 was present in a band of 70 kDa at 20 and 40 min and 56 kDa at 80 min (Fig. 7 B). The 180- and 56-kDa bands were not recognized by monoclonal antibodies 1C2–2, 1D4, and T103, which recognize residues 529–539, 349–406, and 308–318 of the Aα-chain, respectively (43). P123 protein did not cross-link to the monomeric Hi2DSK fragment of fibrin encompassing residues 241–476 of the Aα-chain (data not shown). As a control, the amino-terminal 27-kDa fragment of fibrinogen (Fig. 8A) or with fibrinogen (Fbg), plasminogen (Plg), bovine serum albumin (BSA), histidine-rich glycoprotein (HRGP), or the amino-terminal 70-kDa fragment of fibronectin (Fn). After cross-linking, proteins were separated by 8% SDS-PAGE under reducing conditions, and complexes containing P123 were detected by anti-TSP1 antibodies on Western blot. P123 formed covalent complexes with fibrinogen (arrow) but not with other proteins. Non-cross-linked P123 (P123) and molecular mass markers (kDa) are as indicated.

FIG. 4. Cross-linking of P123 to TSP1 binding proteins. Cross-linking conditions were as described under “Experimental Procedures.” The P123 was incubated alone (C) or with fibrinogen (Fbg), plasminogen (Plg), bovine serum albumin (BSA), histidine-rich glycoprotein (HRGP), or the amino-terminal 70-kDa fragment of fibronectin (Fn). After cross-linking, proteins were separated by 8% SDS-PAGE under reducing conditions, and complexes containing P123 were detected by anti-TSP1 antibodies on Western blot. P123 formed covalent complexes with fibrinogen (arrow) but not with other proteins. Non-cross-linked P123 (P123) and molecular mass markers (kDa) are as indicated.

FIG. 5. P123 cross-links to the fibrinogen Aα-chain. P123 was cross-linked to fibrinogen (lane 1), or P123 was cross-linked to fibrinogen and then treated with thrombin (lane 2). The complexes were separated on 8% SDS-PAGE under reducing conditions and detected by Western blotting. The cross-linked complex (arrow) was detected with polyclonal anti-thrombospondin (α-TSP) (A) or with monoclonal antibody FPA 19/7 directed to fibrinopeptide A at the amino terminus of fibrinogen Aα-chain (B). Non-cross-linked P123 (P123) and molecular mass markers (kDa) are as indicated.
fibronectin was cross-linked to fibrin (38) and plasmin treated. After 20, 40, or 80 min of plasmin digestion, the majority of the 27-kDa fragment was contained in cross-linked material migrating between 35 and 60 kDa under nonreducing conditions (data not shown).

The plasmin digestion pattern of P123 cross-linked to fibrinogen was different from the digestion of fibrin. After digestion with plasmin (10 μg/ml) for 3 min, all P123-antigen was in a band of 23 kDa, indistinguishable from the size of non-cross-linked P123, although monomeric D-fragment remained intact as ascertained by protein staining (data not shown).

Incorporation of Procollagen and Properdin Fusion Proteins into the Fibrin Clot—Additional studies were conducted to characterize the interaction of the procollagen and each properdin module of TSP1 with fibrin clots. Fibrinogen was incubated with CP123/GE1, P123/GE1, P12/GE1, P1/GE1, P3/GE1, GE1, or P123 in the presence of FXIII, and clotting was initiated with thrombin. The incorporation of the proteins into the fibrin clot was analyzed by Western blot with antibodies to GE1. CP123/GE1, P123/GE1, P12/GE1, and P3/GE1 were incorporated into the fibrin clot and were present in a band of the expected size for cross-linking to a single α-chain as well as larger complexes consistent with α-chain multimers (Fig. 8). The unincorporated protein is present on the blot and labeled “module/GE1.” A larger proportion of the added P123 or P123/GE1 was incorporated into the fibrin clot than cross-linked with fibrinogen (compare Fig. 4 with Fig. 8). These data are consistent with 30–50% of added TSP1 incorporated into a fibrin clot (16). P1/GE1 and GE1 did not incorporate into the fibrin clot to the same extent as other proteins, although in some experiments a small amount of GE1 or P1/GE1 was cross-linked into the fibrin clot. The small incorporation is consistent with previously published data showing that a portion of the GE1 is incorporated into the fibrin clot (53). These data indicate that the second and third properdin modules, but not the first, mediate the interaction of the GE1 fusion constructs with fibrin.

FIG. 6. Incorporation of P123 into plasma or fibrin clots. The plasma globulin fraction (Plasma) or purified fibrinogen (Fbg) was clotted in the presence of P123 as described under “Experimental Procedures.” The clots were solubilized in reducing gel sample buffer and separated using 8% SDS-PAGE, and complexes were detected by Western blotting. The P123 complex (arrow, approximately 93 kDa) was detected by antibodies (anti-GE1) to the fusion protein tails that do not recognize human proteins. Molecular mass markers (kDa) are as indicated.

FIG. 7. Plasmin digests of fibrin clots containing P123. Fibrin clots were formed with P123 for 60 min and then treated with plasmin (5 μg/ml) at 37 °C. At the indicated times (0, 20, 40, and 80 min) the clots were solubilized with nonreducing (A) or reducing (B) sample buffer, separated by 8% SDS-PAGE, and detected by Western blotting with anti-TSP1 polyclonal antibodies. The arrow indicates the primary cross-link product. Non-cross-linked P123 (P123) and molecular mass markers (kDa) are as indicated.

FIG. 8. Incorporation of the procollagen and properdin modules of TSP1 into fibrin clots. Fibrin clots were formed with purified fibrinogen alone (C) or in the presence of CP123/GE1, P123/GE1, P12/GE1, P1/GE1, P3/GE1, GE1, or P123. The clots were solubilized in reducing sample buffer, separated by 8% SDS-PAGE, and detected by Western blotting with GE1 antibodies. The cross-linked material is bracketed as fibrin/module cross-links and non-cross-linked material is indicated as module/GE1 or P123. Molecular masses (kDa) are as indicated.

DISCUSSION

We expressed recombinant procollagen and properdin modules of TSP1 as chimeras with the GBD of fibronectin in insect cells. The GE1 system has previously been used to probe the disulfide pattern of module I-12 of fibronectin (30), binding
activities of the EGF modules of factor IX (35), and binding activities of the modules I-1 to I-5 of fibronectin (53). The present fusion proteins were folded and disulfide-bonded correctly as assessed by gelatin binding, resistance to trypsin, and shift in molecular weight upon reduction. Functional studies indicate that the binding properties of the properdin modules of TSP1 are more restricted than would be inferred from studies of larger proteolytic fragments of TSP1 or of TSP1-based peptides.

Constructs containing all three properdin modules or the first and second properdin modules bound to heparin at 4 °C but not in the presence of physiological concentrations of NaCl (150 mM). P123 cleaved from GE1 did not bind heparin at physiological salt concentration, indicating that the lack of binding is not due to the presence of the GDP of the fusion protein. These findings are consistent with reports that the thrombin or chymotryptic fragments of TSP1 containing the procollagen and properdin modules do not bind heparin, sulfatide, or proteoglycans under physiologic conditions (11, 12). The data contrast with results showing that peptides based on the properdin modules of TSP1 bind to solid phase heparin at 4 °C in physiologic salt (13, 14) similar to an endopeptidase-derived fragment of bovine TSP1 that is reduced and alkylated and binds to solid phase heparin at 22 °C (46). Therefore, the recombinant procollagen and properdin modules of TSP1 expressed as disulfide-bonded proteins in baculovirus act similarly to the native 70-kDa fragments of TSP1 rather than a smaller reduced and alkylated fragment or short peptide. Individual properdin modules P1 and P3 did not bind heparin even in the absence of NaCl, while properdin modules in series, P12, P123, and CP123, did bind. A likely explanation is that the KRFK sequence between P1 and P2, which causes enhanced heparin binding when introduced into synthetic peptides (13), binds to heparin under conditions that favor ionic interactions.

The central stalk of TSP1 has been shown to interact with numerous proteins (11). We were unable to detect solution phase interaction with plasminogen, histidine-rich glycoprotein, or the amino-terminal region of fibronectin by cross-linking of the module constructs to purified proteins. Furthermore, no interactions with plasma globulins other than clotting fibrin were detected in the complex milieu of a clot made from plasma globulins. The results indicate that plasminogen, fibronectin, and histidine-rich glycoprotein interact with TSP1 in regions outside of the properdin modules of TSP1. These results are in accord with solution phase studies that indicate that the fibronectin and fibrinogen binding sites in TSP1 are distinct (49) but not with solid phase binding assays in which the two proteins cross-compete for the binding sites in TSP1 (17). A weakness of the GE1 strategy is that binding partners may not have a lysine readily accessible for transglutaminase cross-linking despite the flexibility of the cross-linking site (50). Nonetheless, the FXIII cross-linking strategy has been used successfully to identify binding partners for the amino terminus of fibronectin (37) and for the EGF modules of coagulation factor IX (35).

The results suggest that the incorporation of TSP1 modules into the fibrin clot involves highly specific protein-protein interactions. Properdin modules cross-linked only to fibrin in a complex mix of plasma globulins. Further, the properdin modules competed for cross-linking of full-length 125I-TSP to fibrin. Cross-linking to fibrin was to the α-chain based on the size of the complexes, the higher order multimers, and reaction with monoclonal antibody to fibrin. Analysis of plasmin digests indicates that cross-linking was to a portion of the α-chain in D-dimer rather than to the COOH-terminal 60% of the α-chain. Finally, P1 did not cross-link to fibrin, whereas P2 and P3 did.

The amino-terminal tail of GE1 (EAQQIVQPSPFW) is similar to the EAQQIV fibronectin-based peptide (54) and NQEQVSPLTLLK α2-antiplasmin-based peptide (55) that have been used to probe for lysine cross-linking partners in α-chains. NQEQVSPLTLLK cross-links to at least 12 lysines in the α-chain (55). The glutamine-containing tails derived from GE1 are necessary for P123 (derived from GE1) cross-linking because P123 expressed in a different vector with a histidine tag does not cross-link into a fibrin clot.2 This result indicates that active glutamines within the type I modules of TSP1 do not mediate cross-linking, although the central stalk region of TSP1 is known to contain reactive glutamines (24), and also that a lysine in the properdin modules does not cross-link to a reactive glutamine in fibrin. P123 was found cross-linked to the D-dimer fragment of fibrin after plasmin digest, whereas the amino-terminal 27-kDa fragment of fibronectin, also with the EAQQIVQ sequence, was cross-linked to smaller fragments presumably derived from the αc domain (αc(220–610)) of the fibrin α-chain. These results show that the protein module associated with the cross-linking peptide directs the site of cross-linking.

P123-fibrinogen cross-linked material digested with plasmin for 3 min was indistinguishable from non-cross-linked P123 by SDS-PAGE. In contrast, plasmin digests of P123 cross-linked into fibrin clots indicated that P123 remains associated with D-dimer of fibrin (180-kDa band) after 80 min of plasmin treatment. The D-dimer includes the pieces of the D-dimer fragment of fibrin after plasmin digest, whereas the amino-terminal 27-kDa fragment of fibronectin, also with the EAQQIVQ sequence, was cross-linked to smaller fragments presumably derived from the αc domain (αc(220–610)) of the fibrin α-chain. These results show that the protein module associated with the cross-linking peptide directs the site of cross-linking.

The fibrin clot has a critical role in wound healing. Cells migrate into the area of tissue injury through multiple cell surface receptors that interact with proteins incorporated into the fibrin clot (1, 20). Other adhesive glycoproteins incorporated into the fibrin clot, like fibronectin, are highly susceptible to plasmin proteolysis (58). The central stalk region of TSP1 is protease-resistant (11) and may provide a scaffold for cell migration even as the clot begins to be dissolved by plasmin. Therefore, properdin modules would remain bound to the clot and may provide interaction with CD36 (59) or a breast cancer cell receptor (60) even after cleavage of the RGD sequence in the Ca2+-binding region (61). Furthermore, TSP1 alters the structure of the fibrin clot making finer, thinner fibrils and reducing the opacity of the clot (22, 23). Recent studies show that fibrin clots with a reduced opacity stimulate endothelial tube formation (29). Therefore, TSP1 in the fibrin clot could also regulate endothelial cell function independently of direct interactions with cell surface receptors.

2 T. S. Panetti, K. G. Hwiler, and D. F. Mosher, unpublished observations.
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Recombinant Properdin Modules of Thrombospondin-1