\textbf{α-Chain Cross-Linking in Fibrin(ogen) Marburg}

By Joan H. Sobel, Ilya Trakht, Hong Qi Wu, Sergei Rudchenko, and Rudolf Egbring

The fibrinogen structural variant, Marburg (Aα,444Bβγ), is comprised of normal Bβ and γ chains but contains severely truncated Aα chains that are missing approximately one half of their factor XIII, cross-linking domain. Immunochromatography studies of fibrinogen Marburg were conducted to characterize the degree to which deletion of a defined Aα-chain segment, Aα 461-610, can affect the process of fibrin stabilization, i.e., the factor XIII-mediated covalent interaction that occurs between α chains of neighboring fibrin molecules and between α chains and αβ antiplasmin (αβPI). The ability of Marburg (and control) α chains to serve as a substrate for factor XIII, and undergo cross-linking was examined in an in vitro plasma clotting system. The capacity for α-chain cross-linking was evaluated both as the covalent incorporation of the small synthetic peptide, NQEVSPLTLLK (which represents the first 12 amino acids of αβPI and includes the factor XIII-sensitive glutamine residue responsible for the cross-linking of αβPI to fibrin), and as the appearance of native, high-molecular-weight, cross-linked α-chain species. Antibodies specific for the (Aα and γ/γ-γ chains of fibrinogen) and for the peptide and its parent protein, αβPI (68 kD), were used as immunoblotting probes to visualize the various cross-linked products formed during in vitro clotting. Recalcification of Marburg plasma in the presence of increasing concentrations of peptide resulted in the formation of peptide-decorated Marburg α-chain monomers. Their size at the highest peptide concentration examined indicated the incorporation of a maximum of 3 to 4 mol of peptide per mole of α-chain. In the absence of αβPI 1-12 peptide, the α chains of Marburg fibrin cross-linked to form oligomers and polymers, as well as heterodimers that included αβPI. Both the peptide-decorated monomers and the native cross-linked α-chain species of Marburg fibrin were smaller than their control plasma counterparts, consistent with the truncated structure of the parent Marburg Aα chain. Collectively, the findings indicate that, although deletion of the Aα chain region no. 461-610 in fibrinogen Marburg prevents formation of an extensive α polymer network (presumably due to the absence of critical COOH-terminal lysine residues), it does not interfere with initial events in the fibrin stabilization process, namely, factor XIII binding and the ability of α chains to undergo limited cross-linking to one another and to αβPI.

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in this report deal with the processed gene product, and not its original transcript.) Unlike the COOH-terminal Aα chain heterogeneity characteristic of normal fibrinogen, the Marburg variant represents a relatively homogenous preparation of truncated fibrinogen molecules whose genetically programmed remnant Aα-chain population (Aα 1-460) uniformly lacks a COOH-terminal region (Aα 461-610) believed to be responsible for significant lysine donor activity during α polymer formation.2,4 In this report, we describe the application of fibrinogen Marburg (plasma) in immunochemical studies that explore the extent to which the molecular process of fibrin stabilization, α-α and α-α,PI cross-linking, is compromised by deletion of a defined segment of the α-chain cross-linking domain, ie, Aα 461-610. These studies feature a newly developed factor XIIIa-lysine donor-labeling system, comprised of a glutamine-containing peptide modeled after the NH₂-terminal cross-linking domain of human α-PI and a monoclonal antibody (MoAb) that detects this peptide in cross-linked form. The results obtained indicate that the truncated structure of fibrin and fibrinogen Marburg can bind factor XIIIa and form a cross-linked α-chain network that, although restricted in size, includes α,PI. Collectively, the findings not only extend our understanding of the structural features required for normal fibrin stabilization but also suggest that a partially competent fibrin stabilization system may contribute to the thrombotic complications associated with the Marburg dysfibrinogenemia.

MATERIALS AND METHODS

Purified Proteins

Human fibrinogen (Kabi, Stockholm, Sweden; grade L) was further purified by sequential chromatography on lysine-Sepharose and diethyl aminoethyl (DEAE)-Sephacel (Pharmacia-LKB, Piscataway, NJ) as previously detailed.26 Human factor XIIIa was isolated from citrated plasma obtained from patients undergoing plasmapheresis (Columbia-Presbyterian Medical Center (CPMC), New York, NY). The transglutaminase was purified by successive ammonium sulfate precipitations followed by ion exchange chromatography on DEAE-cellulose (Whatman, Clifton, NJ) according to reported methodology.25 Human α,PI was isolated from plasma recovered after centrifugation of platelet concentrates (obtained from the CPMC blood bank) and purified essentially as described.26 For the studies reported here, the partially purified preparations obtained after affinity chromatography on plasminogen-Sepharose and preparations isolated after a further purification on DEAE-Sephacel (to isolate the inhibitor free of the bulk of contaminating α,PI-plasmin complex) were used. Purified proteins were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing and reducing conditions, and their molar composition and concentration were determined by amino acid analysis.

Synthetic Peptides

α,PI 1-12 (NQQEQVSPALTLLKC). This peptide, which corresponds to the NH₂-terminus of α,PI based on the protein’s reported genomic structure,27 was synthesized on a model 431A synthesizer (Applied Biosystems, Foster City, CA) using t-Boc-protected L-amino acid derivatives and the standard chemistry programs provided by the manufacturer. The crude peptide was purified by reversed-phase high-performance liquid chromatography (HPLC) using a Dynamax preparative C8 column (21.4 × 250 mm; Rainin, Woburn, MA) and a mobile phase comprised of 0.1% trifluoroacetic acid (TFA) in water (buffer A) and 100% acetonitrile (buffer B). Under analytic HPLC conditions (4.6 × 250 mm C8 column; 1 mL/min flow rate; 4% to 60% buffer B in 30 minutes) the purified peptide emerged as a single homogeneous peak with a retention time of 18.10 minutes. Amino acid analysis indicated the following molar composition: Asp, 0.93; Thr, 1.06; Ser, 3.18; Pro, 0.99; Leu, 3.04; and Lys, 1.01. NH₂-terminal sequencing confirmed the identity and purity of the isolated peptide, which was recovered in 41.7% yield. A standard solution of the purified peptide was prepared for use in cross-linking studies, and its molar concentration was determined by amino acid analysis.

α,PI 1-12 + C (NQQEQVSPALTLLKC). The α,PI 1-12 peptide, with an added COOH-terminal cysteine residue, was synthesized on a model 431A synthesizer (Applied Biosystems) using F-moc-protected L-amino acid derivatives and the standard chemistry programs provided by the manufacturer. Peptide purification was conducted exactly as described above for α,PI 1-12. Analytic HPLC of the purified material showed one major component with a retention time of 23.02 minutes. Amino acid analysis indicated the following molar composition: Asp, 0.97; Thr, 1.02; Ser, 0.98; Glu, 3.12; Pro, 1.11; Cys, 0.23; Val, 0.91; Leu, 2.88; and Lys, 0.88. NH₂-terminal sequencing confirmed the identity and purity of the peptide, which was recovered in 13.7% yield. A standard solution of the purified peptide was prepared for immunologic studies, and its molar concentration was determined by amino acid analysis.

Plasmas

Marburg plasma was obtained from the homozygous propositus in whom the fibrinogen structural variant was first discovered.20 Blood (nine parts) was collected into 3.8% sodium citrate (one part) and then centrifuged at 3,000 rpm for 10 minutes at 4°C. Plasma aliquots were stored frozen at −80°C for single use. Normal plasma was obtained from an individual donor. Blood was collected and processed as described above for Marburg plasma. Fibrinogen-depleted plasma was prepared from normal plasma by immunoaffinity chromatography on MoAb-102/MoAb F-103 Sepharose. MoAbs F-102 and F-103 recognize epitopes within the COOH-terminal two thirds of the fibrinogen Aα chain, in the vicinity of Aα 563-578 and Aα 259-276, respectively.28,29 Details for the purification of F-102 and F-103 IgGs from ascites and for the construction of immunosor- 

Hypofibrinogenenic control plasma. A plasma sample containing a low concentration of structurally normal fibrinogen (and slightly decreased concentrations of all other plasma components) was created in vitro, as needed, by combining normal plasma (one part) with fibrinogen-depleted plasma (two parts).

Hybridoma Development: Anti-α,PI 1-12 Peptide (MoAb AP-102)

Peptide conjugation. The 13-amino acid peptide, α,PI 1-12 + C, was coupled to keyhole limpet hemocyanin (KLH; Immun, Pierce, Rockford, IL) via its COOH-terminal cysteine residue using the

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maleimide cross-linker, sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC; Pierce). Conjugation was performed as described by the manufacturer, except that 2 mol/L guanidine was included during the peptide reduction and desalting steps to maximize solubility. Conjugation efficiency was estimated by subtractive analysis based on amino acid compositional data obtained for the carrier, the free peptide, and the conjugate. Two different conjugate preparations exhibited molar ratios of 424:1 and 497:1 (peptide:KLH).

**Immunization.** Mice were immunized over a 23-day period with repeated (nine total) 35-μg doses of the peptide-KLH conjugate in complete Freund’s adjuvant. Significant titers (1:10,000 to 1:20,000) were observed after the 12th day in all the immunized mice, based on a direct-binding screening enzyme-linked immunosorbent assay (ELISA) on immunogen-coated plates (see below). Fusion was conducted on the 23rd day, and hybridomas were grown according to standard methodology.

**Selection of antipeptide cell lines.** Fusion products secreting antipeptide immunoglobulins were identified in a direct-binding screening ELISA on conjugate-coated plates, with excess KLH in the assay buffer to block the reactivity of antibodies directed at the carrier component of the immunogen. All reagents were added to the wells in a 100-μL volume, and three washes in 0.01 mol/L sodium carbonate buffer, pH 9.0, was added to the wells of polyvinylchloride (PVC) microtiter plates (Dynatech, Chantilly, VA) and allowed to bind for 18 hours at 4°C. Nonspecific binding sites were blocked by a 1-hour room temperature incubation with 5% bovine serum albumin (BSA; ELISA grade; Sigma, St Louis, MO) in PBS. Hybridoma supernatants, diluted 1:3 in assay diluent (1% BSA-PBS) containing 0.2 mg/mL KLH (100-fold mol excess over KLH on the solid phase), were preincubated for 1.5 hours at 37°C; added to the wells, and allowed to bind for 18 hours at 4°C. Antipeptide antibodies were detected after a 3-hour, room temperature incubation with horseradish peroxidase-conjugated goat-antimouse IgG (GAM-IgG-HRP; Kierkegaard & Perry, Gaithersburg, MD), diluted 1:2,000 in assay diluent, and subsequent reaction with the HRP substrate, o-phenylenediamine (OPD), 5.6 mmol/L in 0.051 mol/L Na2HPO4/0.024 mol/L citric acid/pH 5.0. After this final 45-minute, room temperature incubation, 414 nm absorbance was read in a Multiskan MC microtiter plate reader (Flow, Irvine, CA). One cell line, designated AP-102, was taken for further study based on its immunoglobulin’s strong binding to the peptide on the solid phase.

**Hybridoma Development: Anti-α2PI (MoAb AP-101)**

**Immunogen.** Partially purified α2PI obtained from the equivalent of 1 L of starting material was subjected to preparative SDS-PAGE (see below) on 12.5% gels (16 cm × 20 cm × 1.5 mm) under nonreducing conditions. A major band at 68 kD, consistent with the reported molecular weight of α2PI,28 was identified in a Coomassie Blue-stained guide strip, and its position was used to excise the portion of the gels containing α2PI free of contaminating proteins. This material was macerated repeatedly and then used as the immunogen for the generation of MoAbs directed at epitopes within denatured α2PI.

**Immunization.** Mice were injected subcutaneously with a 0.5-mL dose of the SDS-polyacrylamide-α2PI suspension each week for 2 weeks, followed by a 0.5-mL intraperitoneal boost during the fourth week. Significant titers (1:20,000 to 1:40,000) were observed at this time in all the mice tested, based on a direct-binding screening ELISA on α2PI-coated plates (see below). Fusion was performed after 2 months without additional boosts because of the immunogen’s apparent toxicity.

**Selection of anti-α2PI cell lines.** Hybridoma cell lines secreting anti-α2PI IgGs were selected in a direct-binding screening ELISA on α2PI-coated plates (0.2 μg per well) using highly purified preparations of α2PI as the plating antigen. General assay procedures were as described above for the antipeptide ELISA. One line of interest, designated AP-101, was selected for further study based on the strong binding of its IgG to α2PI on the solid phase.

**Antibody Production and Characterization**

The cell lines AP-102 and AP-101 were cloned by limiting dilution according to standard methodology.1 Ascites were generated in pristane-primed mice,29 and MoAbs AP-102 and AP-101 IgGs were isolated by affinity chromatography on Protein A Sepharose (Bio-Rad, Richmond, CA) as described by the manufacturer. The protein concentration of standard solutions of the purified IgGs was determined by amino acid analysis. Isotyping was performed with the Monoclonal Antibody Isotyping Kit 1 (Immunopure; Pierce), using the antigen-independent method as described by the manufacturer. The comparative binding of MoAbs AP-102 and AP-101 to specific and irrelevant antigens was evaluated in direct-binding ELISA’s as similar to the ones already described, except that KLH was omitted from the assay diluent (in the case of the AP-102 ELISA) and peroxidase-conjugated rabbit antitoxin IgGs (RAM Ig-HRP; Dako, Carpeneria, CA), diluted 1:500, was used in place of GAM-IgG-HRP for the detection of bound MoAbs (see Figs 1 and 5 for additional details).

**Fibrin-α2PI 1-12 Peptide Cross-linking**

**Purified system.** Mixtures of purified fibrinogen (0.826 mg/mL; 4.86 μg/mL Aa chain equivalents based on amino acid analysis) and increasing amounts of α2PI 1-12 peptide [0, 12.1, 121, and 1,212 μg/mL (0, 10.9, 109, 1,090 μg/mL)] in TBS were clotted under cross-linking conditions in the presence of purified factor XIII (10 μg/mL) and CaCl2 (10 mmol/L). Non-cross-linked controls were prepared with factor XIII that had been peptidated with EDTA (5 mmol/L). Fibrin formation was initiated by the addition of human thrombin (1 U/mL; Sigma). The reactions were conducted in a total volume of 250 μL; final concentrations are indicated. Cross-linking was allowed to continue for 1 hour at 37°C, after which a 250-μL vol of electrophoresis sample buffer (0.125 mol/L Tris, pH 6.8/20% glycerol (vol/vol)/2.5% SDS (w/vol)/10% β-mercaptoethanol (vol/vol)/0.003% bromphenol blue;95 mL vol) was added, and the samples were boiled.

**Marburg and control plasmas.** Aliquots of Marburg plasma (200 μL; 2.73 μg/mL Aa chain equivalents determined immunologically in the F-103 ELISA, see below) containing 250 U/mL Trasylol (Mobay Pharmaceuticals, New York, NY) were clotted in the presence of increasing amounts of α2PI 1-12 peptide [0, 3.9, 39, and 392 μg/mL (0, 2.9, 289, and 289 μg/mL)] by the addition of CaCl2 (13 mmol/L) and human thrombin (1 U/mL). Non-cross-linked controls included EDTA (5 mmol/L) in place of the calcium. The reactions were conducted in TBS in a 250-μL total volume; final concentrations are indicated. Fibrin formation was allowed to proceed for 1 hour at 37°C, after which the clots were triturated and the clot liquids discarded. After three 1-mL washes in TBS containing 12 mmol/L EDTA, the clots were dispersed in a 200-μL volume of electrophoresis sample buffer (see above) and boiled. Aliquots (200 μL; 4.19 μg/mL Aa chain equivalents determined immunologically in the F-103 ELISA) of a hypofibrinogenemic control plasma (see Materials and Methods) containing 250 U/mL Trasylol were clotted in the presence of increasing amounts of α2PI 1-12 peptide [0, 4.8, 48, and 479 μg/mL (0, 3.5, 35, and 353 μmol/L)], and the resulting fibrins were processed exactly as described above for Marburg plasma.

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**Fibrin Cross-linking in Marburg and Control Plasmas**

Aliquots of Marburg plasma and the hypofibrinogenemic control plasma (200 µL, containing 250 U/mL Trasylol, as above) were each clotted under cross-linking conditions for increasing periods of time (15 minutes, 1 hour, 4 hours, and 18 hours) at 37°C. Fibrin formation was initiated by the addition of CaCl₂ (13 mmol/L) and thrombin (1 U/mL). At the indicated times, the clots were triturated, washed, then dispersed in a 200-µL vol of electrophoresis buffer, and boiled (see above).

**Functional α₂PI Assay**

Plasma concentrations of α₂PI were determined in a microtitre plate assay based on the inhibition, by α₂PI, of plasmin's amidolytic activity toward the chromogenic substrate, H-D-Val-Leu-Lys-pNA (S-2251; Pharmacia-Hepar, Piscataway, NJ). The assay was performed exactly as described by the manufacturer, using a pooled reference plasma preparation as the assay standard (Pharmacia-Hepar).

**Immunologic Measurement of Plasma Fibrinogen Concentrations (F-103 competitive ELISA)**

Plasma fibrinogen was measured in a solution phase competitive ELISA on fibrinogen-coated plates, using MoAb F-103 (anti-Aa 259-276) as the basis for detection and purified fibrinogen as the assay standard for quantitation. Serial dilutions of test plasmas or the fibrinogen standard were preincubated (125 µL) with an equal volume of MoAb F-103 IgG (10.6 ng/mL) for 1.5 hours at 37°C, and the mixtures were then added (100 µL, in duplicate) to blocked wells that had been previously coated with purified fibrinogen (1 µg/100 µL). All subsequent steps in this assay methodology, including the final calculation of plasma fibrinogen concentration, have been previously detailed.µ

**SDS-PAGE and Immunoblotting**

Discontinuous SDS-PAGE was conducted as described by Laemmli and Favre on large format or mini gels (Protein II, Mini Protein; Bio-Rad) with prestained molecular weight markers included on each run (Amersham, Arlington Heights, IL; GIBCO-BRL, Gaithersburg, MD). (See the appropriate figure legends for additional details.) Electrophoresed components were transferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH) according to reported methodology, and the transfers were processed also as described.µ In some cases, transfers were stained for total protein with Amido Black. The following antibody preparations were used for immunoblotting reagents: (1) α₂PI 1-12 peptide (MoAb AP-102 IgG), (2) anti-α₂PI (MoAb AP-101 IgG), (3) antifibrinogen(ogen) (Aα 259-276 MoAb F-103 IgG,) and (4) antifibrinogen(ogen) γ/γ-γ 385-411 [antiserum R-581, raised against the 27-residue, γ-chain COOH-terminal cyanogen bromide (CNBr) peptide].µ Incubation with MoAb (0.5 to 1.5 µg/mL) or antiserum (1:2,500) was conducted for 18 hours at room temperature. Bound antibody was detected after a 3-hour incubation with HRP conjugates (1:500) of RAM- Ig or swine antirabbit Igs (SAR-Ig; Dako), as appropriate, and subsequent reaction with the HRP substrate, 4-chloro-1-naphthol (Bio-Rad).

**Molecular Weight Determinations**

Molecular weights of electrophoresed components were determined from scanned images of stained nitrocellulose transfers using a ScanJet IIcx scanner (Hewlett Packard, Santa Clara, CA). Plots of band intensity versus molecular weight were derived from these images, based on the migration of standard molecular weight markers, using the scientific imaging and graphing software, SigmaScan and SigmaPlot (Jandel Scientific, San Rafael, CA).

**Amino Acid Analysis**

Samples were hydrolyzed in 6 N HCl for 24 hours under vacuum at 110°C in a Pico Tag workstation (Waters, Milford, MA), and amino acid analysis was conducted on a Beckman Model 6300 amino acid analyzer (Beckman, Palo Alto, CA).

**RESULTS**

**Immunovisualization of α₂PI 1-12 Peptide-Fibrin Cross-linking in a Purified System**

Figure 1 shows the titration curves obtained when MoAb AP-102 IgG (IgGα, kappa), an antibody raised against a peptide conjugate that represents the first 12 residues of α₂PI, was assayed in a direct-binding ELISA on antigen-coated plates. Comparative binding to three different solid phase antigens was evaluated: (1) the peptide component of the immunogen (α₂PI 1-12 + C), (2) α₂PI (68 kD), and (3) fibrinogen. At approximately equimolar plating antigen concentrations, the antibody selectively bound to the small peptide. Less than 4% and 6% cross-reactivity was observed for α₂PI and fibrinogen, respectively, once the plateau antibody concentration was achieved. These findings show the specificity of the antibody for the small peptide against which it was raised and, importantly, indicate that its antigenic determinant is not shared by fibrinogen.

Figure 2 shows the immunoblotting profiles obtained when the antipeptide antibody and antibodies specific for the (A)α and γ/γ-γ chains of fibrinogen(ogen) were applied to
visualize the incorporation of α2PI 1-12 into fibrin chains during in vitro cross-linking (Fig 2B, C, and D, respectively; total protein staining is shown in Fig 2A). In this analysis, cross-linking between the peptide and fibrin was inferred from an observed change in the molecular weight of one or more fibrin chains, coincident with the appearance of antipeptide immunoreactivity.

As shown in the second lane of each panel of Fig 2, when peptide was omitted from the incubation mixture, fibrin cross-linking produced the expected array of high-molecular-weight α-chain species (seen best in the anti-(A)α chain immunoblot, Fig 2C) and γ dimers (Fig 2D). There was no peptide immunoreactivity associated with any fibrin component in this control lane (Fig 2B). A high proportion of residual monomeric α and γ chains was observed, but this is consistent with the partial cross-linking expected for a 1-hour in vitro incubation and also reflects the extreme sensitivity of the immunoblotting technique. When cross-linking was conducted in the presence of increasing concentrations of α2PI 1-12 (lanes 3 through 5 in each panel of Fig 2), antipeptide immunoreactivity became associated primarily with the α chains of fibrin. This association was apparent at the lowest peptide concentration examined, i.e., a twofold molar excess over (A)α chains in the cross-linking mixture, and as the peptide concentration increased, the size of the resulting α monomers also increased. At the highest peptide concentration tested, a 200-fold molar excess over (A)α chains in the cross-linking mixture, and as the peptide concentration increased, the size of the resulting α monomers also increased. At the highest peptide concentration tested, a 200-fold molar excess over (A)α chains in the cross-linking mixture, and as the peptide concentration increased, the size of the resulting α monomers also increased. At the highest peptide concentration tested, a 200-fold molar excess over (A)α chains in the cross-linking mixture, and as the peptide concentration increased, the size of the resulting α monomers also increased. At the highest peptide concentration tested, a 200-fold molar excess over (A)α chains in the cross-linking mixture, and as the peptide concentration increased, the size of the resulting α monomers also increased.
Figure 3 shows the comparative immunoblotting profiles obtained when the studies described for a purified cross-linking system were conducted using the hypofibrinogenemic control (Fig 3A) and Marburg (Fig 3B) plasmas. As shown in Fig 3A, the results obtained for the control plasma in the absence of α-PI 1-12 (lane 2) and in the presence of increasing amounts of peptide (lanes 3 through 5) paralleled, for the most part, the findings observed for the purified system. Namely, as peptide-decorated α-chain monomers appeared and increased in size, native α-chain cross-linking (ie, α oligomers and polymers) decreased in intensity. Cross-linking of γ-chain appeared less sensitive to peptide incorporation in a plasma milieu, because at α-PI 1-12 levels that inhibited γ dimer formation in a purified cross-linking system (84-fold molar excess), there was no accumulation of γ-chain monomers with coincident antipeptide immunoreactivity.

As shown in Fig 3B (lane 1, left panel), the Aa chains of Marburg plasma fibrinogen migrated as 49.8-kD immunoreactive bands, consistent with their reported structure, which includes Aa 259-276, the region of the F-103 epitope, but is missing residues 461-470. When cross-linking was conducted in the absence of peptide, Marburg α chains became incorporated into a variety of higher-molecular-weight cross-linked species that were smaller than their control fibrin counterparts, in keeping with the truncated structure of their parent Aa chains (compare lane 2 in Figs 3B & A). As shown in lanes 3 through 5, when cross-linking was conducted in the presence of increasing concentrations of α-PI 1-12, peptide-decorated Marburg α-chain monomers were observed (left and middle panels). At the highest peptide concentration examined, a 106-fold molar excess over (A)a chains, native Marburg α-chain cross-linking was inhibited based on the decreased intensity of high-molecular-weight products (compare lanes 5 and 2). Profiles of γ chain cross-linking in Marburg fibrin, produced in both the absence and the presence of peptide, were similar to those observed for the control plasma, in keeping with the structurally normal γ-chain component of Marburg fibrinogen (compare Fig 3B and A, right panels).

The collective findings in Fig 3, which show the formation of peptide-decorated α-chain monomers as well as larger cross-linked α-chain species in Marburg fibrin, imply that Marburg α chains can serve as a substrate for factor XIII, despite the absence of an approximately 20-kD COOH-terminal segment.

### Aa-Chain Lysine Donor Sites in Control and Marburg Fibrinogens

Quantitative estimates for the number of lysine donor sites in structurally normal and Marburg Aa chains are shown in Table 2. These data were derived from the SDS-PAGE mobility shifts observed for peptide-decorated α-chain monomers (Figs 2 and 3) and assume that addition of 1.35 kD to the apparent molecular weight of an α-chain monomer reflects cross-linking at one donor lysine site. Data for the purified fibrinogen cross-linking system indicated incorporation at a maximum of six to seven sites under the in vitro conditions used. A significantly higher

### Table 1. Factor XIII, Substrate Concentrations in Control and Marburg Plasmas

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Normal</th>
<th>Marburg</th>
<th>Hypofibrinogenemic Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fbg Az1</td>
<td>16.26 ± 1.04</td>
<td>3.41 ± 0.17</td>
<td>5.24 ± 0.27</td>
</tr>
<tr>
<td>αPI</td>
<td>1.24 ± 0.01</td>
<td>1.24 ± 0.02</td>
<td>0.82 ± 0.04</td>
</tr>
</tbody>
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Abbreviation: Fbg, fibrinogen.
* Prepared from normal plasma diluted with fibrinogen-depleted plasma as described in Materials and Methods.
† Expressed as micromolar Aa-chain equivalents, determined immunologically in the F-103 ELISA (see Materials and Methods). Data are presented as the mean (±SEM) of at least seven separate determinations. The Aa-chain concentration of the fibrinogen-depleted plasma was 0.05 μmol/L.
‡ Expressed as micromolar αPI concentration, determined in a functional assay (see Materials and Methods). A concentration of 1 μmol/L was assumed for the reference plasma. Data are presented as the mean (±SEM) of at least two separate determinations. The αPI concentration of the fibrinogen-depleted plasma was 0.08 μmol/L.

peptide immunoreactivity accumulated at the expense of dimeric forms (lanes 4 and 5, Fig 2B and D). Peptide incorporation was factor XIII-dependent, as incubations conducted in the presence of a 20-fold excess of peptide and EDTA, an inhibitor of factor XIII activity, produced fibrin monomers with so antipeptide immunoreactivity (lane 6, Fig 2B, C, and D).

These findings for a purified cross-linking system are consistent with the formation of peptide-decorated α monomers, and to a lesser extent, γ monomers, as a result of the factor XIII-mediated interaction between the known acceptor glutamine residue at position 2 in Aa 1-12 and selected lysine donor sites within the (α and γ) chains of fibrin.

**Cross-linking of α-PI 1-12 Peptide to Marburg (and control) Plasma Fibrin**

Based on the collective observations in Figs 1 and 2, we next applied the peptide and its antibody probe to visualize peptide-decorated products of factor XIII activity in a plasma cross-linking system. Specifically, we were interested to learn whether or not Marburg plasma fibrin, with its truncated α-chain structure, could incorporate α-PI 1-12 peptide, and, if so, how the resulting products compared with those formed in a control plasma cross-linking system. Initial studies were conducted to determine the fibrinogen Aa chain and α-PI concentrations in normal and Marburg plasmas, as the levels of these native factor XIII substrates would contribute significantly (independently of structural considerations) to the extent of peptide incorporation. As shown in Table 1, Aa chain antigen was approximately five times higher in the normal plasma than in Marburg plasma, consistent with the hypofibrinogenemia associated with the structural variant; α-PI levels were the same (normal) in both plasmas. This information was used to create an artificial hypofibrinogenemic normal plasma that represented a true control, because its structurally intact Aa-chain substrate was present at the same low level as its truncated counterpart in Marburg plasma.
Xenotropic Influenza Virus: A Model for Understanding the Role of Emerging Viruses in Human Health

**Fig 3.** Cross-linking of α2PI 1-12 peptide to control and Marburg fibrins in a plasma system. (A) Control (hypofibrinogeneric) plasma was clotted under cross-linking conditions in the presence of increasing concentrations of α2PI 1-12 peptide as described in the text. The resulting fibrins were subjected to SDS-PAGE on 5% gels (16 cm × 20 cm × 0.75 mm) under reducing conditions. Nitrocellulose transfers were processed for immunoblotting with anti-α2PI 259-276 (MoAb F-103 IgG; left panel), anti-α2PI 1-12 peptide (MoAb AP-102 IgG; middle panel), and anti-γ/γ 385-411 (antisera R-581; right panel), also as described. The following loads were applied to the five lanes in each panel: lane 1 (PL), 2 μL starting plasma (10.5 pmol αα-chain equivalents); lane 2 (XL), 10 μL (52.4 pmol) plasma fibrin cross-linked for 1 hour in the absence of α2PI 1-12 peptide; lanes 3, 4, and 5 (XL), 10 μL (52.4 pmol) plasma fibrins cross-linked for 1 hour in the presence of a 0.94-, 8.4-, and 84-fold molar excess, respectively, of peptide relative to fibrinogen (Ala-chain equivalents). The migration of standard molecular weight markers is indicated at the extreme left, and the positions of standard molecular weight markers are labeled for reference. Note that in the right panel other anti-γ/γ chain immunoblots revealed γ dimer immunoreactivity in lane 5 as well as lane 4; the blot shown was included here because it, in all other respects, produced the clearest profile for the collective data. (B) Marburg plasma was clotted under cross-linking conditions in the absence or presence of α2PI 1-12 peptide, and the resulting fibrins were analyzed by immunoblotting as detailed for panel A. The following loads were applied to the five lanes in each panel: lane 1 (PL), 2 μL starting plasma (6.8 pmol αα-chain equivalents); lane 2 (XL), 15 μL (51.2 pmol) plasma fibrin cross-linked for 1 hour in the absence of peptide; and lanes 3, 4, and 5 (XL), 15 μL (51.2 pmol) plasma fibrins cross-linked for 1 hour in the presence of a 1.06-, 10.6-, and 106-fold molar excess, respectively, of peptide relative to fibrin or fibrinogen (Ala-chain equivalents). The migration of standard molecular weight markers is indicated at the extreme left. Marburg non-cross-linked and cross-linked (Ala chains as well as γ chain monomers and dimers, are labeled for reference.

immunovisualization of Native α-chain Cross-linking in Marburg (and control) Plasma Fibrin

To further characterize the degree to which Marburg α chains can engage in the various covalent interactions involved in fibrin stabilization, ie, α-α as well as αα2PI cross-linking, immunoblotting studies of native α-chain cross-linking in control and Marburg plasmas were conducted. In this analysis, antibodies specific for α2PI and the (A)a chain were used to visualize and distinguish among the various cross-linked products formed.

Figures 4 and 5 show the results of immunoblotting studies to characterize the specificity of the anti-α2PI antibody, MoAb AP-101 (IgG1, kappa), which was raised against a purified but denatured form of 68-kD α2PI. As shown by the immunoreactivity profiles in Fig 4, anti-α2PI immunoreactivity was localized primarily within a 68-kD band whose apparent mobility did not change after reduction, consistent
the known COOH-terminal heterogeneity in the scans of the control lanes of Figs 2 and 3A (no peptide) reflects the antibody was titered in a direct-binding ELISA with 207: 1 84: 1 56.5 67.5 0.7 20.7: 1 63.5 72.5 6.7 59.5 68.0 6.3 56.5 63.5 5.2 207: 1 63.5 72.0 6.3 59.5 69.0 7.0 56.5 66.0 7.0 Control plasma cross-linking system (Fig 3A) 0.84: 1 63.0 64.5 1.1 60.0 60.5 0.4 8.4: 1 63.0 71.0 5.9 60.0 66.5 4.8 84: 1 63.0 82.0 14.1 60.0 71.5 8.5 Marburg plasma cross-linking system (Fig 3B) 1.06: 1 48.0 48.0 — 10.6: 1 48.0 50.5 1.9 106: 1 48.0 52.5 3.3

Table 2. Peptide Incorporation Into Fibrin α Chains Determined From Mobility Shifts in SDS-PAGE

<table>
<thead>
<tr>
<th>Peptide: α (molar ratio)</th>
<th>α Chain Mol Wt (kD)</th>
<th>XL Peptide (mol peptide/mol α chain)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide</td>
<td>α Chain</td>
<td>+Peptide</td>
</tr>
<tr>
<td>Purified cross-linking system (Fig 2) 2.07:1</td>
<td>63.5 66.0</td>
<td>1.9</td>
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<tr>
<td>20.7:1</td>
<td>63.5 72.5</td>
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<tr>
<td>207:1</td>
<td>63.5 72.0</td>
<td>6.3</td>
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<tr>
<td>Control plasma cross-linking system (Fig 3A) 0.84:1</td>
<td>63.0 64.5</td>
<td>1.1</td>
</tr>
<tr>
<td>8.4:1</td>
<td>63.0 71.0</td>
<td>5.9</td>
</tr>
<tr>
<td>84:1</td>
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<td>14.1</td>
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<tr>
<td>Marburg plasma cross-linking system (Fig 3B) 1.06:1</td>
<td>48.0 48.0</td>
<td>—</td>
</tr>
<tr>
<td>106:1</td>
<td>48.0 52.5</td>
<td>3.3</td>
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</table>

Molecular weights of α chains were derived from the scanned images of F-103 immunoblots (see Figs 2C, and 3A & B), as described under Materials and Methods. The detection of multiple α chains in the scans of the control lanes of Figs 2 and 3A (no peptide) reflects the known COOH-terminal heterogeneity in αα-chain structure. The extent of peptide cross-linking in the decorated monomers was calculated from the molecular weight differences observed, using 1.36 kD as the molecular weight of the α2PI 1-12 peptide.

Abbreviations: Mol wt, molecular weight; XL, cross-linked.

with the known behavior of α2PI. As shown in Fig 5, when the antibody was titrated in a direct-binding ELISA with (1) α2PI (68 kD), (2) the peptide α2PI 1-12 + C, or (3) fibrinogen on the solid phase, it reacted specifically with α2PI. Less than 8% cross-reactivity was observed with fibrinogen at the highest antibody concentration tested (1,460 ng/mL), and antibody binding to the small peptide was negligible (less than 2%), despite the 100-fold molar excess of platelet antigen used (relative to α2PI).

As shown in the first two panels of Fig 6 for the control plasma fibrin, rapid cross-linking between structurally intact α chains (64 kD) and α2PI (68 kD) was visualized as a 130-kD band of coincident immunoreactivities, not present in the starting plasma or when factor XIII, activity was inhibited by the addition of EDTA before clotting. Cross-linked α-chain species (100 to 225 kD and higher molecular weight polymers) not associated with α2PI were also present within 15 minutes of in vitro fibrin formation, and these persisted throughout the entire incubation. Large α polymers that included α2PI formed slowly and were represented at 4 and 18 hours, by faint bands of anti-α2PI immunoreactivity at the top of the resolving gel. As shown in the last two panels of Fig 6, cross-linking between Marburg α chains (48 kD) and α2PI (68 kD) was visualized at the earliest time point examined as a doublet of coincident immunoreactivities whose increased mobility (approximately 112 kD, 120 kD) relative to the control fibrin’s profile reflected a cross-linked contribution by the smaller-sized, Marburg α chains. At least six cross-linked α-chain species not associated with α2PI were also observed, showing that in addition to their interaction with α2PI, Marburg α chains cross-linked to one another (and/or, possibly, to other plasma proteins that are factor XIII substrates). Large α polymers with or without α2PI, an indication of extensive α-chain cross-linking, were not a significant component of Marburg fibrin based on the negligible anti-(A)α chain immunoreactivity localized at the top of the resolving gel compared with the control fibrin lanes.

DISCUSSION

The study of fibrinogen structural variants, in which a defined mutation is found to correlate with clotting abnormalities in the propositus, has contributed significantly to our understanding of the biochemistry of fibrin formation.25 Although the majority of characterized dysfibrinogenemias result from mutations involving the NH2-terminal portions of the Aα or Bβ chains, several have been reported with a structural defect in the COOH-terminal two thirds of the Aα chain. Among these, fibrinogens Dusart (Aα 554 arg-cys) and Marburg (Aα 461 thr-stop) are associated with thrombotic tendencies in individuals homozygous for the respective genes.26-28,38-39 The lesion in fibrinogen Caracas II (Aα 432 ser-asn/glycosylation site) does not appear to produce any clinical ramifications, although in vitro studies do indicate impaired fibrin monomer aggregation.40 Several lines of evidence, including biochemical,41 electron microscopic,42 and immunologic data,43 indicate that the COOH-terminal two thirds of the Aα chain, also referred to as the αC domain, is involved in fibrin polymerization. In view of this, it has been suggested that the thrombotic tendencies observed in the Dusart and Marburg homozygotes originate from an abnormal clot architecture (the result of impaired fibrin polymerization caused by the altered primary structure of the αC domains in these variants) that cannot mediate the binding and/or activation of plasminogen required for effective fibrinolysis.31,44 The biochemical and immunological studies described in this report focus on fibrinogen Marburg as a tool for learning more about the biochemistry of fibrin formation and specifically address whether a second feature of the αC domain—namely, its role as a substrate for factor XIII, during fibrin stabilization—might also contribute to the thrombotic complications observed in the homozygous propositus.

In this study, α-chain cross-linking in Marburg fibrin was characterized in a series of immunoblotting experiments that used novel structural probes to detect cross-linked products of factor XIIIa activity. The first of these, a dodecapeptide
Fig 4. Anti-αPI (MoAb AP-101): immunogen characterization (Western blotting). Partially purified αPI (the source of the immunogen used to generate MoAb F-101) and purified preparations of αPI (see Materials and Methods) were each subjected to SDS-PAGE on 4% to 15% (gradient) and 10% (homogeneous) gels (10 cm × 7 cm × 0.75) under nonreducing and reducing conditions, respectively; 0.1% and 0.05% of the crude preparation and 2 μg and 1 μg of the purified material were applied for total protein staining (lanes 1 and 3) and immunoblotting (lanes 2 and 4). Nitrocellulose transfers were processed for immunoblotting with MoAb AP-101 IgG as described in the text. The migration of standard molecular weight markers is indicated at the extreme left of each set of lanes (the standards and their molecular weights, as defined by the manufacturer are 200 kD, myosin; 97.4 kD, phosphorylase B; 68 kD, BSA; 43 kD, ovalbumin; and 29 kD, carbonic anhydrase). The band of material excised for use as the immunogen for the development of MoAb AP-101 (see Materials and Methods) is highlighted with an asterisk. Arrows indicate the migration of αPI, which stained weakly with Amido Black at the loads applied to the gels.

Fig 5. Anti-αPI (MoAb AP-101): immunochemical characterization (direct-binding ELISA). Purified MoAb AP-101 IgG was titered on solid phase antigens as described in the text. Plating antigen concentrations were as follows: (1) purified αPI (■), 1.5 μg/mL; (2) αPI 1-12 + C peptide (○), 1.5 μg/mL; and (3) purified fibrinogen (▲), 8.6 μg/mL, corresponding to 1.5, 112.5, and 5.1 (as subunit equivalents) pmol per well, respectively. Data are expressed as the mean (±SEM) of at least two independent determinations.

modeled after the NH₂-terminal cross-linking domain of α₂PI, was originally shown by others to behave as a glutamine substrate analog for factor XIII, capable of covalently binding to multiple sites within the α chains of fibrin and of specifically inhibiting the native cross-linking that occurs between α chains and α₂PI. Because the small peptide indirectly serves as a structural probe for factor XIII-sensitive lysine residues, we sought to develop methods that would enhance its detection in cross-linked form and broaden its application as a tool for α-chain cross-linking studies. The antipeptide MoAb AP-102, developed here (Fig 1), recognizes its epitope at high sensitivity within α₂PI 1-12-decorated fibrin products formed in both purified and plasma cross-linking systems (Figs 2 and 3). Its availability, in conjunction with the dodecapeptide, thus provides a new labeling system for the identification of lysine donor cross-linking sites in factor XIII-sensitive (and possibly cellular transglutaminase-sensitive) proteins, a concept originally introduced by Lorand et al using an anti-dansyl MoAb and a dansylated peptide probe based on the acceptor cross-linking domain of fibronectin.

Several features related to the specificity of the lysine labeling system and its application to quantify available Aα-chain donor sites deserve comment. First, peptide incorporation was restricted to known factor XIII substrates. There was no evidence of cross-linking to β chains, either as an observed change in apparent migration (Fig 2A) or as the appearance of antipeptide immunoreactivity in the vicinity of greater than 53.5 kD (Fig 2B). When BSA or ribonuclease was substituted for fibrinogen in the purified cross-linking system, peptide-decorated products were not seen (data not
shown). Second, although peptide incorporation into γ chains did occur, the number of lysines involved was apparently limited, with no significant molecular weight change observed for the peptide-decorated γ-chain monomers (Fig 2D); this is consistent with the reported single donor lysine residue involved in γ-chain cross-linking. Finally, how does the finding that 14 of the 22 available lysine residues within the αC domain of intact fibrinogen potentially function as donor sites (Table 2) reflect the true specificity of the α-chain cross-linking process? While the answer to this question will be realized only with the isolation and biochemical characterization of cross-linked dipeptides from both peptide-decorated and native cross-linking systems (studies that are in progress), reports identifying five different CNBr αC derivatives as lysine donor regions suggest that α-chain cross-linking may, in fact, involve a surprisingly large number of donor sites.

Application of the lysine labeling system to study fibrin formation in Marburg plasma showed that Marburg α chains could serve as factor XIII, donor substrates and incorporate up to 3 to 4 mol of α2PI 1-12 peptide in a dose-dependent manner (Fig 3B and Table 2). Thirteen lysine residues are contained within the primary structure of the truncated Marburg α-chain cross-linking domain (considered here to extend from Aα 208-460), and the findings suggest that, among these, three to four selectively function as factor XIIIα donor cross-linking sites. Therefore, although the shortened structure of Marburg Aα chains appears to involve the loss of COOH-terminal cross-linking sites present within intact Aα chains, other sites contained within the region Aα 208-460 are available to serve as factor XIIIα donor substrates and cross-link to their native acceptor glutamine partners at Aα 328 and 366. Data obtained for Marburg plasma fibrin formed in the absence of peptide confirmed this by showing that cross-linked α-chain species were a component of the Marburg fibrin clot (Fig 3B, left panel, lane 2; Fig 6). These same findings also suggested a specific role for COOH-terminal (Aα)-chain regions—or the cross-linking sites included within them—in the regulation of α polymer growth because Marburg α-chain networks were limited in size compared with the large polymers found for the control plasma fibrin preparation (Fig 3A, left panel, lane 2; Fig 6).

These data for the characterization of α-α chain cross-linking in a fibrinogen variant with a defined lesion in its αC domain extend the findings of previous reports that have localized NH₂-terminal cross-linking activity within the αC domains of naturally occurring catabolites of normal fibrinogen. Moreover, the fact that Marburg α chains (and γ chains; Fig 3B, right panel, lane 2) can serve as a substrate for factor XIII, implies that the truncated structure of this fibrinogen variant includes the conformational elements required for factor XIII binding and, subsequently, the enzyme’s catalytic efficiency. Two reports, each providing evidence for a different factor XIII binding site on fibrin, are of interest here. Because the regions implicated, i.e., the COOH-terminal portions of the α and β chains within the D domain, are both included within the structure of Marburg fibrinogen, these two reports may be conflicting or, alternatively, may reflect the presence of unique binding sites for γ- and α-chain cross-linking, respectively.

In addition to their capacity for (limited) α-α chain cross-linking, Marburg α chains effectively cross-linked to α2PI, shown here using a newly developed anti-α2PI antibody,
MoAb AP-101 (Figs 4 and 5), as an immunoblotting probe. The covalent interaction demonstrated for Marburg α chains and α2PI (Fig 6) is consistent with the reported involvement of lysine Aα 303 as the factor XIII, donor residue in the α-α2PI cross-linking reaction and indicates that the structure of this αC functional domain is also intact within the fibrinogen variant.

Cross-linking between α2PI and the α chains of fibrin—rather than the extent of α-chain cross-linking per se—is considered to be the primary factor governing the inhibition of clot lysis in vivo.4,5,46 Given this, the collective in vitro findings obtained here, which indicate that α2PI becomes covalently associated with a partially cross-linked Marburg α-chain network, suggest that this mechanism may contribute to the thrombotic complications observed in the Marburg homozygous propositus, which include recurrent thrombotic episodes, severe varicosis, and ulcerae cruris.39 In vivo, Marburg thrombi would be expected to be small and mechanically fragile as a result of the hypofibrinogenemia associated with the structural variant20,21 (confirmed here as a markedly decreased Aα-chain antigen concentration; Table 1) and the impaired capacity of its truncated α chain for α polymer formation. At the same time, the normal levels of factor XIII and α2PI (Table 1) found for Marburg plasma could impart these thrombi with an enhanced resistance to lysis by plasmin, leading to the thrombotic clinical ramifications observed.

The studies described in this report represent the first time that a defined fibrinogen structural variant has been applied to investigate the structure-function relationships involved in α-chain cross-linking. The information obtained, together with the development of a novel factor XIII, lysine donor labeling system, should facilitate future biochemical studies dealing with the process of fibrin stabilization.

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