The COOH-TERMINAL two thirds of the fibrinogen Aα chain (Aα 208-610) plays a central role in maintaining hemostasis by serving as a substrate for factor XIII during the final stages of fibrin formation. In this process, referred to as fibrin stabilization, covalent cross-links are introduced between specific glutamine and lysine residues of neighboring fibrin chains, and these function to stabilize the lateral alignments created during the earlier polymerization phase of fibrin formation. Similar cross-links introduced by factor XIII, between α chains and α2 antiplasmin (α2PI) confer the resulting fibrin network with an enhanced resistance to lysis by plasmin. Although several laboratories have collectively identified a few of the reactive residues that participate in α-α and α-α2PI cross-linking, the fibrin stabilization process remains one of the least understood aspects of fibrinogen biochemistry.

The fact that this is so, despite years of collective research in this area, underscores the technical challenge inherent in biochemical studies of α-chain cross-linking. The difficulties stem, in part, from the intrinsic structural heterogeneity of the starting substrate, i.e., the α-chain cross-linking domain, which is represented in vivo both on intact fibrinogen molecules and on natural catabolites whose COOH-terminal Aα-chain regions exist in a variety of truncated forms. A second source of heterogeneity arises due to the complexity of the α-chain cross-linking process itself, wherein a single α chain can interact with α chains of one or two (or more) adjacent fibrin molecules, resulting in a variegated α polymer network comprised of partially cross-linked α chains as well as α chains that contain a full complement of cross-links.

As one approach towards circumventing the intrinsic heterogeneity that complicates biochemical studies of α-chain cross-linking, a variety of specific structural probes have been applied over the years to target glutamine acceptor residues associated with factor XIII functional activity or defined sequences within α-chain regions known to participate in cross-linking. Recently, a new series of structural probes has been introduced, represented by a family of small, synthetic, glutamine-containing peptides (modeled after the NH2-terminal cross-linking domain of fibronectin) that act as acceptor analogues to localize donor lysine residues within proteins that are transglutaminase substrates. To date, these reagents have been applied to show factor XIII and cellular transglutaminase acyl acceptor activity (lysine donor sites) in fibrin and fibrinogen, and in lens crystallins and plasmogen, respectively.

The fibrinogen structural variant, Marburg, which is comprised of normal β and γ chains but contains Aα chains that are missing 150 COOH-terminal residues, provides a unique reagent for learning more about the structure-function relationships involved in α-chain cross-linking. (Note that, based on DNA sequencing, the normal human Aα chain ends at pro 625; therefore, fibrinogen Marburg more accurately represents a deletion of residues Aα 461-425. However, as the Aα chain of circulating fibrinogen ends at val 610, based on protein sequencing, we have chosen to refer to this convention because the functional studies described...
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 XIII, 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 XIII 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.24 Human factor XIII 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 (NQEIQVSPWTLLK). 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 reverse-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, 1.01; His, 3.18; Pro, 0.99; Val, 0.94; 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 (NQEIQVSPWTLLKC). 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 immunosorbents using these purified IgGs have been previously described.29 Immunoadsorption was conducted in 0.05 mol/L TRIS-0.15 mol/L NaCl, pH 7.6 (TBS) for 2 hours at room temperature, using equal volumes of plasma and F-102 and F-103 Sepharoses. Approximately 67% of the applied 280 nm absorbance was recovered as the fibrinogen-depleted plasma preparation (in the flow-through pool) in a volume representing a 33% dilution of the starting plasma. Immunoblotted with MoAbs F-102 and F-103 confirmed the absence of fibrinogen in the processed plasma and its recovery in material that was eluted from the immunosorbent with 0.1 mol/L glycine HCl, pH 2.8 (data not shown). Aliquots of fibrinogen-depleted plasma were stored frozen at −80°C for single use.

Hypo fibrinogenenic 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; Immect, Pierce, Rockford, IL) via its COOH-terminal cysteine residue using the
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.60

**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 phosphate-0.14 mol/L NaCl, pH 7.2 (PBS), were included between each assay step. Conjugate (29.6 pmol peptide-0.07 pmol KLH/100 μL) in 0.1 mol/L sodium carbonate buffer, pH 9.0, was added to the wells of polystyrene (PS) 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 molar 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 0.05 M Na2HPO4/0.024 M citric acid/0.01% H2O2, pH 5.0. After this final 45-minute, room temperature incubation, 414 nm absorbance was read in a Multiskan MC microtiter plate reader (Flow-ICN, Costa Mesa, 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,26 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.24 Ascites were generated in pristane-primed mice,22 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 I (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 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 antirabbit IgG (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 pg/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 mM-L). Non–cross-linked controls were prepared with factor XIII that had been preincubated with EDTA (5 mM-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/3% urea) 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 mM-L) and human thrombin (1 U/mL). Non–cross-linked controls included EDTA (5 mM-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 liquors discarded. After three 1-mL washes in TBS containing 12 mM-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 μg/mL)], 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 microtiter 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-Aα 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.28

SDS-PAGE and Immunoblotting

Discontinuous SDS-PAGE was conducted as described by Laemmli and Favre35 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,34 and the transfers were processed also as described.35 In some cases, transfers were stained for total protein with MoAb (0.5 to 1.5 μg/mL) for 1.5 hours at 37°C, with the HRP substrate, 4-chloro-1-naphthol (Bio-Rad). Bound antibody was detected after a 3-hour incubation with HRP conjugates (1:500) of 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 (IgG1, 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), (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 fibrin(ogen) were applied to...
visualize the incorporation of $\alpha_2$PI 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 $\alpha$-chain species (seen best in the anti-(A)$\alpha$ chain immunoblot, Fig 2C) and $\gamma$ 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 $\alpha$ and $\gamma$ 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 $\alpha_2$PI 1-12 (lanes 3 through 5 in each panel of Fig 2), antipeptide immunoreactivity became associated primarily with the $\alpha$ chains of fibrin. This association was apparent at the lowest peptide concentration examined, ie, a twofold molar excess over (A)$\alpha$ chains in the cross-linking mixture, and as the peptide concentration increased, the size of the resulting $\alpha$ monomers also increased. At the highest peptide concentration tested, a 200-fold molar excess over (A)$\alpha$ chains in the cross-linking mixture, and as the peptide concentration increased, the size of the resulting $\alpha$ monomers also increased.
Table 1. Factor XIIIa Substrate Concentrations in Control and Marburg Plasmas

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Normal</th>
<th>Marburg</th>
<th>Hypofibrinogenemic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fbg</td>
<td>16.26 ± 1.04</td>
<td>3.41 ± 0.17</td>
<td>5.24 ± 0.27</td>
</tr>
<tr>
<td>a2PI</td>
<td>1.24 ± 0.01</td>
<td>1.24 ± 0.02</td>
<td>0.82 ± 0.04</td>
</tr>
</tbody>
</table>

Abbreviation: Fbg, fibrinogen.
* Prepared from normal plasma diluted with fibrinogen-depleted plasma as described in Materials and Methods.
† Expressed as micromolar Aα-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 Aα-chain concentration of the fibrinogen-depleted plasma was 0.05 μmol/L.
‡ Expressed as micromolar a2PI 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 a2PI 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 XIIIa-dependent, as incubations conducted in the presence of a 20-fold excess of peptide and EDTA, an inhibitor of factor XIIIa activity, produced fibrin monomers with no antipeptide immunoreactivity (lane 6, Fig 2B, right panel). 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.

Aα-Chain Lysine Donor Sites in Control and Marburg Fibrinogens

Quantitative estimates for the number of lysine donor sites in structurally normal and Marburg Aα 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
CROSS-LINKING IN FIBRIN(0GEN) MARBURG

Fig 3. Cross-linking of α2PI 1-12 peptide to control and Marburg fibrins in a plasma system. (A) Control (hypofibrinogenemic) 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-(A)α 259-276 (MoAb F-103 IgG; left panel), anti-α2PI 1-12 peptide (MoAb AP-101 IgG; middle panel), and anti-γ/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 (Aαα-chain equivalents). The migration of standard molecular weight markers is indicated at the extreme left, and the positions of cross-linked and native (Aαα) chains, as well as γ chain monomers and dimers, are labeled for reference. Note that in the right panel other anti-γ/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 Aαα-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 (Aαα-chain equivalents). The migration of standard molecular weight markers is indicated at the extreme left. Marburg non-cross-linked and cross-linked (Aαα) chains as well as γ chain monomers and dimers are labeled for reference.

value, 14 sites, was obtained for the control plasma system. This disparity can be explained from the apparent plateau in incorporation observed for the purified system, suggesting that the effective factor XIII concentration was limiting, perhaps due to a decrease in the potency of the factor XIII preparation with age. This is supported by data from additional experiments in which the same preparation of purified factor XIII was used to monitor the time course of peptide incorporation at a 100-fold molar peptide excess (data not shown). The values obtained, 12.3 and 15.2 mol of cross-linked α2PI 1-12 per mole of α chain after 4 and 24 hours, respectively, are consistent with 14.1 mol determined for the control plasma system (Fig 3A), where endogenous plasma factor XIII was responsible for cross-linking activity. A maximum of three to four lysine donor sites was calculated for Marburg Aα chains based on the findings in Fig 3B.

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)α chain of fibrinogen were used to visualize and distinguish among the various cross-linked products formed.

Figures 4 and 5 show the results of immunochromical 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 the known behavior of a2PI. As shown in Fig 5, when the antibody binding to the small peptide was negligible (less than 2%), despite the 100-fold molar excess of plating antigen used (relative to a2PI). 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%). As shown in the first two panels of Fig 6, cross-linking between Marburg α chains (48 kD) and a2PI (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 a2PI were also observed, showing that in addition to their interaction with a2PI, 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 a2PI, an indication of extensive α-chain cross-linking, were not a significant component of Marburg fibrin based on the negligible anti-(A)a 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. 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. 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. Several lines of evidence, including biochemical, electron microscopic, and immunologic data, 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. The immunohistochemical 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 XIII activity. The first of these, a dodecapeptide...
Fig 4. Anti-α2PI (MoAb AP-101): immunogen characterization (Western blotting). Partially purified α2PI (the source of the immunogen used to generate MoAb F-101) and purified preparations of α2PI (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 α2PI, which stained weakly with Amido Black at the loads applied to the gels.

Fig 5. Anti-α2PI (MoAb AP-101): immunochemical characterization (direct-binding ELISA). Purified MoAb AP-101 IgG was titrated on solid phase antigens as described in the text. Plating antigen concentrations were as follows: (1) purified α2PI (■), 1.5 μg/mL; (2) α2PI 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.
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 XIII 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 XIII 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)α-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, ie, the COOH-terminal portions of the α and β chains within the D domain and α 241-424 within the αC 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 crosslinking reaction demonstrated for Marburg α chains and α2PI (Fig 6) is consistent with the reported involvement of lysine Ace 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. 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 thromboembolitis, severe varicosis, and ulcerac cruris. In vivo, Marburg thrombi would be expected to be small and mechanically fragile as a result of the hypofibrinogenemia associated with the structural variant (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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REFERENCES

1. Takagi T, Doolittle RF: Amino acid sequence studies of the α chain of fibrinogen. Location of four-plasmin attack points and a covalent cross-linking site. Biochemistry 14:5149, 1975


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