Identification of the α Chain Lysine Donor Sites Involved in Factor XIIIa Fibrin Cross-linking*

(Received for publication, March 20, 1996, and in revised form, May 15, 1996)

Joan H. Sobel† and Mary Ann Gawinowicz

From the Department of Medicine, College of Physicians and Surgeons of Columbia University,
New York, New York 10032

Biochemical studies of fibrin cross-linking were conducted to identify the specific αα chain lysine residues that potentially serve as Factor XIIIa amine donor substrates during α polymer formation. A previously characterized Factor XIIIa fibrin labeling system was employed to localize sites of donor activity based on their covalent incorporation of a synthetic peptide acceptor substrate analog modelled after the NH₂-terminal cross-linking domain of α₂ antiplasmin. Peptide-decorated fibrin was prepared using purified fibrinogen as the starting material. Cyanogen bromide digestion, immunoaffinity chromatography, high pressure liquid chromatography (HPLC), and enzyme-linked immunosorbent assay (anti-peptide) methodologies were employed to isolate purified CNBr fibrin fragments whose structures included the acceptor probe in cross-linked form and, therefore, represented regions of (amine) donor activity. Five α chain CNBr fragments (within αα 208–610) and one γ chain CNBr fragment (γ 385–411) were the only portions of fibrin found associated with the acceptor peptide, based on collective sequencing, mass, and compositional data. Trypsin digestion, HPLC, and enzyme-linked immunosorbent assay (anti-peptide) methodologies were used to isolate smaller derivatives whose structures included an α chain tryptic cleavage product (the donor arm) cross-linked to the trypsin-resistant synthetic peptide (the acceptor arm). Biochemical characterization and quantitative peptide recovery data revealed that 12 of the 23 potential lysine donor residues within αα 208–610 had incorporated the peptide probe, whereas γ chain donor activity was due solely to peptide cross-linking at (γ) Lys406; the α chain lyses, Lys556 and Lys560, accounted for 50% of the total α chain donor cross-linking activity observed, with Lys539, Lys508, Lys518, and Lys446 contributing an additional 28% and Lys508, Lys527, Lys529, Lys509, Lys524, and/or Lys719 responsible for the remaining proportion (2–5% each). The collective findings extend current models proposed for the mechanism of α polymer formation, raise questions concerning the physiological role of multiple α chain donor sites, and, most importantly, provide specific information that should facilitate future efforts to identify the respective lysine and glutamine partners involved in native fibrin α chain cross-linking.

The structure-function relationships involved in fibrinogen's transition to the cross-linked fibrin gel that forms the hemostatically active portion of a thrombus has been the subject of intense investigation for more than two decades. During this time, the complete primary structure of this large molecule has been elucidated (1–6), its domainal architecture has been characterized (7–9), and the mechanisms involved in the initial events of the transition, i.e. thrombin cleavage and fibrin polymerization, have been defined (as reviewed in Ref. 10). Although these aspects of fibrinogen biochemistry are well understood, less is known about the final stages of fibrin formation in which Factor XIIIa cross-links are introduced between neighboring fibrin molecules to stabilize the alignments created during polymerization.

Factor XIIIa, or plasma transglutaminase, catalyzes the introduction of ε-(γ-glutamyl)lysine cross-links into its protein substrates via an acyl transfer reaction in which the γ carboxyamide group of glutamine serves as the acyl donor (amine acceptor) and the ε amino group of lysine serves as the acyl acceptor (amine donor).1 It has been known for some time, including the precise location of the single glutamine and lysine residues involved (Gln398 and Lys406) and the reciprocal nature of this cross-linking that results in γ chain dimerization (13, 14). The recent description of oligomeric forms of cross-linked γ chains in fibrin suggests, however, that our current understanding of the γ chain cross-linking process may be incomplete (15). More significantly, comparatively little is known about how α chains align with one another over the course of fibrin formation to ultimately produce the high molecular weight α polymers that are the hallmark of highly cross-linked fibrin (16). Efforts to define the respective glutamine and lysine partners within cross-linked α chains by traditional biochemical approaches have, as yet, proved unsuccessful. The unique primary structure of the αα chain cross-linking domain, its intrinsic COOH-terminal heterogeneity, and the complexity of the α chain cross-linking process itself all contribute to the challenge of isolating homogenous peptides capable of providing reliable sequence information. To date, two glutamine residues, Gln528 and Gln560, have been implicated as the major amine acceptors in α chain cross-linking based on the selective incorporation of small donor analogues at these sites (17). Biochemical studies of native fibrin cross-linking, conducted by several groups of investigators, indicate that donor regions are contained within the COOH-terminal portion of the αα chain, but these reports are conflicting with respect to the number of regions involved, and}
in no case was a specific lysine residue identified (18–21).

A variety of transglutaminase-sensitive proteins have recently been identified as donor cross-linking substrates based on their ability to covalently incorporate synthetic peptide acceptor analogues and form peptide-decorated products (22, 23). Several different Factor XIIIα-fibrin lysine labeling systems have been developed (24–27), but for the most part their reported application has been limited to the localization of donor activity based on the molecular weight changes observed in SDS-polyacrylamide gel electrophoresis (PAGE) as a result of peptide decoration. In this report, we describe a series of biochemical studies to identify the potential lysine donor sites involved in native α chain cross-linking, taking advantage of the fact that in the lysine labeling methodology susceptible lysine residues are all cross-linked to the same acceptor peptide partner. The studies feature a recently characterized Factor XIIIα-fibrin lysine labeling system comprised of a synthetic peptide modeled after the NH2-terminal acceptor cross-linking domain of α2 antiplasmin (α2PI) and an anti-peptide antibody that recognizes the probe even after it has become cross-linked to its lysine partner(s) (27). The collective findings provide new information to suggest that multiple α chain donor sites contribute to the mechanism of α polymer formation. In addition, the identification of the specific lysines involved in peptide-α chain cross-linking here should facilitate future efforts to characterize the respective glutamine and lysine partner residues involved in native fibrin cross-linking.

MATERIALS AND METHODS

Protein/Pep tide Preparations—Human fibrinogen (Pharmacia-He par, Franklin Lakes, OH) was further purified by successive chromatography on lysine-Sepharose and DEAE-Sepha cel (Pharmacia Biotech Inc.) as described previously (28). Plasma Factor XIII was isolated from plasmapheresis material by ammonium sulfate precipitation and purified according to reported procedure (29). The 12-residue peptide, NQEIVSPITLKL corresponding to the NH2-terminal cross-linking domain of α2PI (30) was synthesized and purified as described previously (27).

Peptide Fibrin Cross-linking and the Preparation of CNBr Derivatives—In a typical procedure, approximately 1 μmol of fibrinogen was converted to fibrin in the presence of an 18-fold molar excess of peptide relative to α chain (or γ chain) substrate. Fibrinogen (4 mg/ml) and peptide (0.66 mg/ml) in 0.05 M Tris, 0.14 M NaCl, pH 7.6, were incubated for 1 h at 37°C together with human thrombin (Calbiochem-Behring, La Jolla, CA) (1 unit/ml), Factor XIII (10 μg/ml), and CaCl2 (10 mM). The resulting fibrin was triturated to express the clot lutein and then exhaustively washed with 0.05 M Tris, 0.15 M NaCl, pH 7.6, containing 12 mM EDTA. The conversion from fibrinogen to fibrin was judged complete based on SDS-PAGE immunoblotting analysis with the anti-β chain monoclonal antibody, T2G1 (31), which revealed that the 56-kDa fibrinogen ββ chain population was replaced by a faster migrating component, consistent with fibrinopeptide B release. CNBr digestion was conducted in 70% formic acid (30 mg fibrin/ml) for 18 h at a 1000-fold molar excess of reagent relative to methionine content in the sample. The reaction was terminated by lyophilization, after the addition of 5 volumes of water, and the drying process repeated once to ensure removal of residual CNBr byproducts.

Isolation and Purification of Peptide-cross-linked CNBr Fibrin Derivatives—Peptide-cross-linked fragments were isolated from the CNBr-treated fibrin preparation by successive immunofluorometry chromatography, employing three different monoclonal antibodies as immunosorbents. These included the anti-fibrinogen mAbs, F-103 and F-102, that bind to epitopes within the COOH-terminal two-thirds of the Aα chain (Aα 259–276 and Aα 563–578, respectively) (32, 33) and the anti-peptide mAb, AP-102 (27). F-103- and F-102-Sepharoses were prepared as previously detailed (34), and these same procedures were used for the construction of AP-102-Sepharose; approximately 3 mg of IgG were coupled per ml of gel (38–58 nmol antibody combining sites/ml).

Immunoadsorption was conducted in 0.1 M NH4HCO3, pH 7.8, at a 3:1–5:1 ratio (antibody combining sites:epitope) based on amino acid analysis or immunologic measurement of the load(s) (see below). Following an 18-h incubation at 4°C, non-bound fragments were collected, and bound components were then released from the immunosorbent with 5% acetic acid.

For the initial isolation of CNBr peptide-fibrin derivatives, the dried CNBr digest was resuspended in 100 ml of 0.1 M NH4HCO3 and insoluble components were pelleted by centrifugation. (These included the disulfide-rich CNBr fibrinogen derivatives, N-DSK and the more hydrophobic disulfide knots, Hol-Ho3 DSKs; these fragments origin from portions of the E and F domains and are not crosslinked with Factor XIIIα cross-linking activity (35).) Immunoblotting analysis of the pellet confirmed that there was no anti-COOH (Aα chain) or anti-peptide immunoreactivity associated with this material, and it was not, therefore, pursued further. The soluble extract, containing α, β, and γ CNBr derivatives originating from regions outside the disulfide knots, was taken for successive immunofluorometry chromatography as shown schematically here and detailed in the legend to Fig. 1 (Scheme 1).

The peptide-cross-linked fibrin components recovered in the bound fractions (labeled A, B, and C in Fig. 1, left) were further purified by reversed phase high performance liquid chromatography (HPLC). HPLC was performed on either a Waters chromatographic system (Waters/Millipore, New Milford, MA) or on a Hewlett Packard model HP 1050 “Chemstation” (Palo Alto, CA). Peptide cross-linked CNBr derivatives were purified on a Vydac analytic C-4 column (0.45 × 25 cm; The Separations Group, Hesperia, CA) using 0.1% trifluoroacetic acid (Buffer A) and 80% acetonitrile in Buffer A (Buffer B) as the mobile phase. Chromatography was conducted at 42°C, and the column was developed with a linear gradient of increasing acetonitrile concentration to 100% B (1%/min) at a flow rate of 1 ml/min. Fractions (0.5 or 1 ml) were dried, in some cases after pooling corresponding fractions from multiple injections of the same material, and then resuspended in water for subsequent localization of anti-peptide immunoreactivity in the AP-102 enzyme-linked immunosorbent assay (ELISA) (see below). Fractions were pooled based primarily on the immunoreactivity profiles obtained, and material from each pool was taken for biochemical characterization prior to further processing (see below and the legends to Tables I-III for additional details).

Preparation and Purification of Peptide-cross-linked Tryptic Derivatives—Peptide-cross-linked CNBr fragments contained within the HPLC-purified pools indicated in Fig. 1 were digested with trypsin (Sigma) in 0.2 M NH4HCO3, pH 8.0, at protein concentrations of approximately 50–200 μg/ml and a substrate/enzyme ratio of 50:1 (w/w). Incubations were conducted for 1 1/2 or 3 h at 37°C, and proteolysis was interrupted by acidification (to 5% in acetic acid) and lyophilization. Peptide-cross-linked CNBr fragments (Fig. 1, right; pools A1 and A11) were reduced and alkylated prior to trypsin digestion because these derivatives contained the intrachain disulfide formed by residues Aα 442 and 472. Reduction with dithiothreitol (10 mM) was conducted for 2 h at 37°C in 0.5 M Tris-6 mM guanidine, pH 8.5, at a protein concentration of 0.1% (w/v). Iodoacetamide (15 mM) was added, and alkylation was allowed to proceed for 1 h in the dark. The reduced and alkylated derivatives were recovered following desalting on reverse phase C-18 Sep-Pak cartridges (Millipore, New Milford, MA) and elution with a mixture of 60% isopropanol, 30% acetonitrile, 10% H2O in Buffer A.

Tryptic peptides contained in each of the eight digests were separated by reversed phase HPLC. Chromatography was conducted as
described above except that a Vydac analytic C-18 column (0.46 × 25 cm) was employed, and peptides were eluted with a linear gradient of acetonitrile concentration increasing at a rate of 0.5% B/min. Cross-linked derivatives were localized either based on their immunoreactivity in the anti-peptide ELISA or by compositional analysis. Material from each peak of interest was taken for biochemical characterization to identify the lysine residues involved in cross-linking to the peptide probe (see below and the legends to Fig. 2 and Tables IV and V for additional details).

AP-102 (anti-peptide) ELISA—A solution phase competitive ELISA for the detection of the peptide probe in both free and cross-linked forms was performed using the same general assay procedures previously described for a direct binding AP-102 ELISA (27). In the application here, wells were coated with 0.11 μg of the 13-residue COOH-terminal cysteinylated derivative of α2PI 1–12 (i.e. α2PI 1–12 + C), whereas the parent 12-residue peptide served as the solution phase standard for quantitation. Equal volumes (125 μl) of standard or unknown and purified mAb AP-102 IgG (76 ng/ml) were pre-incubated for 1.5 h at 37°C, and then 100 μl of the mixtures was incubated on the solid phase for 18 h at 4°C. Bound antibody was detected as 414-nm absorbance following successive incubations with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako, Carpenteria, CA), diluted 1:500, and the substrate, ortho-phenylenediamine. The percentage of antibody binding in the presence and the absence of competitor was calculated for each data point, and apparent peptide concentration in unknown samples was quantified based on an eight-point, logit-transformed, standard curve where molar concentration (dose) was determined from amino acid analysis of a solution of the purified peptide standard. In some cases AP-102 immunoreactivity was expressed simply as the percentage of antibody "displaced" compared with binding in the absence of competitor.

Immunoblotting—SDS-PAGE was conducted on Laemmli gels (36), and electrophoresed components were transferred to nitrocellulose (Schleicher & Schuell) for immunoblotting according to standard methodology (37, 38) as previously detailed (27). Prestained molecular weight markers (Amersham Corp.) were included on each run. Transfers were incubated with the anti-Aα chain mAbs, F-102 and F-101, and the anti-peptide mAb, AP-102, (0.5–2 μg IgG/ml), and immunoreactive components were subsequently visualized following successive incubations with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins, diluted 1:500, and the substrate 4-chloro-1-naphthol (Bio-Rad).

Amino Acid Analysis—Samples were hydrolyzed in 6 N HCl for 24 h under vacuum at 110°C in a Pico Tag workstation (Waters, Milford, MA). Amino acid analysis was conducted on a Beckman model 6300 amino acid analyzer (Palo Alto, CA) or, when higher sensitivity was required, on an Applied Biosystems (Foster City, CA) model 420 instrument.

NH2-terminal Sequencing—Automated Edman degradation was conducted on an Applied Biosystems model 470A sequencer equipped with an on-line model 120A PTH analyzer. When higher sensitivity was required, a model 477A sequencer by the same manufacturer was employed.

Mass Spectrometry—Matrix-assisted laser desorption ionization time-of-flight mass spectrometry was performed on a Perspectve Bio-systems Voyager RP spectrometer (Framingham, MA) operated in both linear and reflector mode. Samples were prepared in either sinapinic acid or α-cyano-4-hydroxy cinnamic acid matrices and data collected with a UV nitrogen laser set at 337 nm and various accelerating voltages, generally 25–30 kV. Mass-assisted laser desorption ionization mass spectrometry was also performed at the Howard Hughes Medical Institute Protein Structure Facility (Harvard Medical School, Boston, MA) on a Fisons/VG Analytical TofSpec spectrometer as previously detailed (39). Predicted masses were calculated from the reported primary structure of fibrinogen (4–6), employing the mass analysis software, GPMAW (General Protein Mass Analysis for Windows, Lighthouse Data, Odense SV, Denmark).

RESULTS Isolation of Peptide-cross-linked CNBr Fibrin Derivatives—Fig. 1 illustrates the results of the purification scheme employed for the first phase of these studies, which was to isolate Aα chain fragments that by virtue of their association with the glutamine acceptor peptide probe could be identified as lysine donor cross-linking regions. Because previous work had implicated Aα 518–584 (CNBr X) and Aα 241–476 (CNBr VIII) as potential donor cross-linking regions (18–20, 34), initial steps were designed to extract these two components from the total mixture of (soluble) CNBr derivatives. Immunosorbents specific for epitopes within each of these Aα chain fragments were employed (F-102-Sepharose, anti-(Aα) 563–578; F-103-Sepharose, anti-(Aα) 253–268). Other, as yet unidentified, cross-linking regions were subsequently isolated from the partially depleted mixture of CNBr fragments by utilizing an immunosorbent that selected for the peptide component, as distinct from the fibrin component, of cross-linked derivatives (AP-102-Sepharose, anti-peptide α2PI 1–12).

The left portion of Fig. 1 shows the results of immunoblotting studies to monitor the recovery of peptide-cross-linked CNBr fibrin products at each of the immunoaffinity steps. The anti-peptide monovalent antibody (AP-102) was employed to visualize all cross-linked fragments based on the anomalous migration of the small 12-residue probe once it had become covalently bound to its various donor cross-linking partners (even-numbered lanes). The anti-Aα chain monovalent antibodies specific for epitopes within CNBr VIII (F-103) and CNBr X (F-102) were used to visualize peptide-cross-linked and non-cross-linked forms of these two CNBr derivatives (odd-numbered lanes in the top and middle panels, respectively). Coincident bands of specific anti-Aα chain and anti-peptide immunoreactivities revealed the presence of cross-linked CNBr VIII and CNBr X fragments, whereas their non-cross-linked counterparts exhibited Aα chain immunoreactivity only. The collective findings indicated that the successive immunosorption steps resulted in the specific recovery of three groups of peptide-cross-linked CNBr fibrin derivatives (identified as A, B, and C in Fig. 1, left), each exhibiting considerable size heterogeneity. These included 28.5–36 kDa peptide-cross-linked CNBr VIII fragments (Pool A), 13–18 kDa peptide-cross-linked CNBr X fragments (Pool B), and <10–25 kDa fragments comprised of the peptide covalently bound to other regions of fibrinogen that function with lysine donor cross-linking activity (Pool C).

The right portion of Fig. 1 illustrates the reversed phase HPLC elution profiles obtained for the purification of the three pools of peptide-cross-linked CNBr derivatives isolated as described above. In this analysis, the various fragments contained within each pool were separated based on their comparative hydrophobicity, and the relative peptide contribution to each was then determined by assaying the column effluent in the AP-102 ELISA. As shown in A and B of Fig. 1, right, cross-linked CNBr VIII and CNBr X fragments were each resolved into two major species (A-I, A-II; B-I, B-II; C-I, C-II) with the more hydrophobic material associated with enhanced levels of anti-peptide immunoreactivity in each case. (Note that the most hydrophilic fragments contained in Pool B, B-I, were not immunoreactive and reflected the presence of non-cross-linked CNBr X derivatives that were recovered together with peptide-cross-linked CNBr X fragments in the F-102 immunosorption step). As shown in Fig. 1C, five pools of anti-peptide immunoreactivity (some containing more than one component based on the accompanying 229 nm absorbance profile) were resolved when the fragments recovered as Pool C were separated by reversed phase HPLC (C-I, C-II, C-III, IV, and V).

Identification of Lysine Donor Cross-linking Regions—Tables I, II, and III summarize the biochemical data used to define the structure of the various peptide-cross-linked fragments separated by HPLC (Fig. 1, A, B, and C, respectively). As shown in Table I for fragments included in A-I and A-II, amino acid compositional data indicated that these were comprised of the Aα chain region, CNBr VIII, and the acceptor peptide probe; fragments in A-I contained approximately 1 mol of peptide cross-linked/mol of CNBr VIII, and those in A-II contained 2
FIG. 1. Isolation and purification of peptide-cross-linked CNBr fibrin derivatives by successive immunoadsorption and reversed phase (C-4) HPLC. Top left, fragments (135 mg) contained in the soluble extract of the CNBr fibrin preparation (see text) were applied to F-103-Sepharose (anti-(A)\textsubscript{241–476; CNBr VIII}). Bound components, including non-cross-linked and cross-linked forms of CNBr VIII (18.4 mg; see lanes 3 and 4, highlighted by a single asterisk), were subsequently processed on AP-102-Sepharose (anti-peptide) to specifically isolate peptide-cross-linked CNBr VIII fragments. Load (LD), eluted (EL), and flow-through (FT) pools (0.005–0.01% of the total recovered in each pool) were subjected to 10% SDS-PAGE under reducing conditions. Duplicate transfers were analyzed by immunoblotting with mAbs F-103 (odd-numbered lanes) and AP-102 (even-numbered lanes) to visualize CNBr VIII and peptide-associated components, respectively. The migration of standard molecular mass marker proteins (ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 21.5 kDa; and lysozyme, 14.3 kDa) is indicated at the extreme left for reference. Fragments eluted from the anti-peptide immunosorbent (see the lanes labeled A) were taken for HPLC purification. Right, approximately 18% of the total material recovered as pool A (1.6 mg) was chromatographed in 4 separate runs (see "Materials and Methods"); 74 \mu g (5%) were applied in the illustration shown; 220 nm absorbance (solid line) and anti-peptide immunoreactivity as determined in the AP-102 ELISA (dashed line) are indicated. Pools A-I and A-II were taken for further study. Middle left, CNBr fragments (95 mg) that were not retained in the first immunoadsorption step (see top left, lanes 5 and 6, highlighted by two asterisks) were applied to F-103-Sepharose (anti-(A)\textsubscript{518–584; CNBr X}), and the resulting pools were characterized using mAbs F-102 (odd-numbered lanes) and AP-102 (even-numbered lanes) for immunoblotting. Non-cross-linked and peptide cross-linked CNBr X derivatives that eluted in the bound fraction (see the lanes labeled B) were separated by HPLC purification. Right, approximately 17% of the total material recovered as pool B (2.9 mg) was chromatographed in 3 separate runs; 180 \mu g (6%) were applied in the illustration shown. Pools B-II and B-III were taken for further study. (Pool B-I was not associated with AP-102 immunoreactivity; see text). Bottom left, CNBr fragments (69 mg) that were not retained in the second immunoadsorption step (see middle left, lanes 5 and 6, highlighted by two asterisks) were applied to AP-102-Sepharose (anti-peptide), and the resulting pools were characterized using
Identification of lysine donor cross-linking regions in Pool A fragments (Fig. 1A)

Material from the two major peaks of anti-peptide immunoreactivity, A-I and A-II, was subjected to amino acid analysis as described in the text; 1% of each pool was analyzed. Data are presented as residues/mol. These were calculated from the averaged picomole contribution of CNBr VIII unique residues (His, Ala, Tyr, Ile, and Phe) based on the reported primary structure of the Aα chain obtained by cDNA sequencing (5). The amino acid composition expected for the region Aα 241–476 (CNBr VIII) is shown in column 4. The amino acid composition expected for the 12-residue peptide probe is shown in column 5.

| Amino Acid | A-I | A-II | Aα 241-476 Peptide |
|------------|-----|------|--------------------|
| Asp        | 17.3| 19.0 | 19 1               |
| Glu        | 24.2| 26.0 | 20 3               |
| Ser        | 40.5| 42.3 | 45 1               |
| Gly        | 38.9| 38.6 | 43 1               |
| His        | 3.9 | 4.1  | 4                  |
| Arg        | 11.1| 11.0 | 13 1               |
| Thr        | 31.8| 30.9 | 29 1               |
| Ala        | 5.7 | 5.6  | 6                  |
| Pro        | 18.8| 17.9 | 18 1               |
| Tyr        | 2.5 | 2.0  | 2                  |
| Val        | 10.1| 9.9  | 10 1               |
| Met*       | 0.4 | 0.4  | 1                  |
| Cys        | 0.7 | 0.3  | 1                  |
| Ile        | 1.9 | 1.9  | 2                  |
| Leu        | 6.7 | 7.8  | 3 3                |
| Phe        | 2.3 | 2.2  | 2                  |
| Lys        | 10.9| 10.3 | 10 1               |
| nmol/pool  |    |      |                    |
| peptide    | 88.8| 54.4 |                   |
| CNBr VIII  | 64.3| 25.7 |                   |
| Ratio (peptide/CNBr VIII) | 1.38:1 | 1.83:1 |

* Recovered as homoserine and homoserine lactone, not quantified here.

Identification of lysine donor cross-linking regions in Pool B fragments (Fig. 1B)

Material from the two major peaks of anti-peptide immunoreactivity, B-I and B-II, were subjected to amino acid analysis as in Table I. Data are presented as residues/mole (see Table I legend). Data were calculated based on the averaged picomole contribution of CNBr X-unique amino acids (gly, his, arg, tyr, ile and phe). The amino acid composition expected for the region Aα 518–584 is shown in column 4.

| Amino Acid | B-I | B-II | Aα 518-584 Peptide |
|------------|-----|------|--------------------|
| Asp        | 4.8 | 5.6  | 3 1                |
| Glu        | 11.5| 14.1 | 8 3                |
| Ser        | 17.1| 18.5 | 18 1               |
| Gly        | 6.2 | 6.4  | 6                  |
| His        | 2.0 | 2.1  | 2                  |
| Arg        | 3.0 | 3.2  | 3                  |
| Thr        | 7.6 | 8.7  | 6 1                |
| Ala        | 1.7 | 1.7  | 1                  |
| Pro        | 2.8 | 3.6  | 2 1                |
| Tyr        | 3.1 | 2.8  | 3                  |
| Val        | 2.3 | 3.1  | 1                  |
| Met*       | 0.1 | 0.2  | 1                  |
| Cys        | 0.1 | 0.0  | 1                  |
| Ile        | 2.0 | 2.0  | 2                  |
| Leu        | 3.9 | 6.1  | 1 3                |
| Phe        | 4.7 | 4.6  | 5                  |
| Lys        | 4.7 | 6.5  | 5 1                |
| nmol/pool  |    |      |                    |
| peptide    | 171.3| 196.7|
| CNBr X     | 125.3| 103.3|
| Ratio (peptide/CNBr X) | 1.37:1 | 1.90:1 |

* See Table I legend.

Identification of the amino acid composition of the Aα chain regions CNBr XI (585–610), CNBr V (208–235), and CNBr IX (477–517) also contained lysine donor cross-linking activity. (Note that for CNBr XI, fragments with 2 mol of peptide cross-linked/mol as well as fragments with only 1 mol of cross-linked peptide were identified based on compositional and mass analysis of the material in C-III and C-I, respectively). The relative amounts of each peptide-associated fragment recovered suggested that CNBr X and CNBr VIII represented the major Aα chain donor cross-linking regions (Tables I and II) while CNBr IX, XI, and V contributed auxiliary activity (Table III).

Apart from these five Aα chain cross-linking regions, the only other CNBr fibrin derivative found associated with the peptide probe was the COOH-terminal γ chain CNBr fragment corresponding to γ 385–421 (Table III; C-III and C-IV), a region previously reported to contain the single lysine residue (γ 406) involved in γ chain cross-linking (121).

Identification of Aα Chain Lysine Donor Cross-linking Sites—Based on the collective findings in Fig. 1 and Tables I-III, five Aα chain donor cross-linking regions were identified, as represented by unique peptide-cross-linked CNBr fragments that contained the small acceptor probe cross-linked at one site, and in some cases at two sites, within their respective structures. Among these five regions, CNBr IX was the only one that contained a single lysine residue (based on the reported primary structure of the Aα chain obtained by cDNA sequencing (5)).

mAb AP-102, only, for immunoblotting. Fragments that eluted in the bound fraction (see the lane labeled C) were present at low concentration and are only faintly visible in the figure given the levels applied to the gel. These fragments were taken for HPLC purification. Bottom right, the total material recovered as pool C (1.0 mg) was processed in three separate runs; 48 μg (5%) were applied in the illustration shown. Pool C-I, C-II, C-IV, and C-V were taken for further study. (Pool C-II contained the free peptide; see text).
Material from peaks C-I - C-V was taken for 10-step NH₂-terminal sequencing (5-7% of the total pool) and mass spectrometry (4-16 pmols), as described in the text; 1% of each pool was subjected to amino acid analysis and molar contribution determined as in Tables I and II. In the case of C-IV, the presence of multiple fragments, some without unique amino acids for normalization, allowed only an approximation for the molar content of each component in this pool.

**Table III**

| NH₂-terminal sequence | ID   | Mass Peptide | CNBr Ratio peptide/CNBr |
|-----------------------|------|--------------|-------------------------|
| C-I ADEAGS N*EQVS    | exp  | 4083.4       | 8.4                     |
|                      | obs  | 4084.3       | 7.1                     |
|                      |      |              | 1.18:1                  |
| C-II NQEQVS          | peptide | 1369.6       | 65.0                    |
|                      |      |              | 1.51:1                  |
| C-III ADEAGS N*EQVS  | peptide | 1369.6       | 20.7                    |
|                      |      |              | 1.00:1                  |
| C-IV KIPFEN γ 385-411| exp  | 4237.9       | 20.4                    |
|                      | obs  | 4234.3       | 20.4                    |
|                      |      |              | 1.00:1                  |
|                      |      |              | 0.69:1                  |
| C-V DLGTLS N*EQVS    | peptide | 1369.6       |                         |

*The PTH amino acids released at the first six cycles are shown. The asterisk highlights the absence of PTH Gln, expected for the non-cross-linked peptide probe at cycle 2. Inclusive residues were assigned (column 3) based on the reported primary structures of the fibrinogen Aα, Bβ, and γ chains (4–6) and the sequence expected for the 12-residue peptide probe (30). CNBr Aα chain fragments, only, are designated by a Roman numeral (in parentheses), according to their relative position from the NH₂ terminus of the molecule.

Expressed as a molar ratio of mass units. In the case of internally derived CNBr fibrin fragments (i.e. COOH-terminal Met residue), homoserine and homoserine lactone forms were both observed, but only the data for the larger homoserine derivative is included here. The mass expected for each peptide-cross-linked CNBr fragment was determined from the summed masses of the fibrin component (identified by NH₂-terminal sequencing) and the peptide (1369.6 atomic mass units), less 17 atomic mass units to account for the release of one NH₃ group for each crosslink introduced. The observed masses were all within 0.08% of these summed, expected values.

Although the γ chain COOH-terminal CNBr fragment was identified by sequencing no component with the mass expected for any of its obvious peptide-cross-linked derivatives was observed. The same discrepancy was found when the γ chain tryptic peptides released from C-IV digests were analyzed. The reason for this remains unclear but may reflect abnormal (ionization)/volatilization due to a unique structural feature within this peptide-cross-linked fragment and its tryptic derivative. Given its minor contribution to the total population of crosslinked fragments isolated, its identity was not pursued further.

mary structure of the Aα chain (5)), thereby strongly implicating Lys508 as one of the Aα chain’s donor cross-linking sites. Because the other four regions contained multiple lysine residues, each of which could potentially serve as a donor site (CNBr V, 4; CNBr VIII, 10; CNBr X, 5; and CNBr XI, 2), the next series of studies sought to identify which of these had become associated with the peptide probe.

Fig. 2 illustrates the results obtained when the various pools of peptide-cross-linked CNBr fragments were digested with trypsin to release smaller non-cross-linked and cross-linked derivatives, which were then separated by reversed phase HPLC. Heterodimeric peptides that included the trypsin-resistant acceptor probe in cross-linked form were localized in each column effluent based on their associated anti-peptide immunoactivity or their increased retention time compared with non-cross-linked, control fingerprints obtained for each of the five CNBr Aα chain fragments (data not shown). 12 alpha chain cross-linked tryptic peptides were subsequently identified within the unique peaks observed, as indicated by arabic numeral on the various panels in Fig. 2.

Table IV summarizes the biochemical data used to characterize the Aα chain tryptic component of each of the 12 cross-linked peptides (column 3). Identification of the specific lysine residues involved in cross-linking (column 6) was inferred based on the unique sequencing pattern expected for two small peptides cross-linked by an ε-(γ-glutamyl)lysine bond (where Gln acceptor activity is contributed by the synthetic peptide probe and Lys donor activity is represented by the natural Aα chain tryptic peptide). Edman degradation of the cross-linked peptides is characterized by 1) the release of two common PTH amino acids in approximately equimolar yield at each cycle, except at the steps corresponding to the positions of the “active” Gln and Lys residues on the cross-linked peptide’s acceptor and donor arms, respectively, and 2) the release of the ε-(γ-glutamyl)lysine cross-link as a unique PTH derivative, identified based on its characteristic elution time, i.e. between PTH trp and PTH phe under the separation conditions employed in these studies. In addition, because a cross-linked lysine residue is inert to tryptic cleavage, peptides that include an ε-(γ-glutamyl)lysine bond are recovered as incomplete cleavage products, with Edman degradation continuing through the cross-link until the next susceptible lysine (or arginine) residue is reached. These features are illustrated for cross-linked peptide 5 in Table V, which summarizes the sequencing data used to implicate Aα 556, but not 562, as a donor cross-linking site. (In this particular case, the Gln and Lys residues involved in the cross-link occupied the same position, i.e. the second amino acid from the NH₂ terminus of the acceptor probe and donor arm, respectively).

The quantitative data obtained for each of the tryptic fingerprints (Table IV, column 7) indicated that the 12 cross-linked α chain peptides were recovered in yields consistent with the 1:1 or 2:1 molar ratios (acceptor probe:α chain cross-linking region) originally observed for their respective parent CNBr fragment. This was determined by comparing the nanomole recovery of a control peptide in each fingerprint (one not involved in cross-linking and, therefore, expected at maximum level) with the total nanomole contribution from cross-linked peptides in the same digest. These quantitative findings provided strong evidence that a full complement of Aα chain donor lysine residues had been recovered and that no significant cross-linking site had been missed. Collectively, the data obtained indicated that Lys508 and Lys508 together accounted for 50% (38 and 12%, respectively) of the total Aα chain donor cross-linking activity observed, with Lys508, Lys508, Lys508, and Lys508 contributing an additional 28% (6–8% each); the remaining activity was distributed among the six other lysines identified, each of which contributed approximately 2–5%.

In addition to the 12 α chain donor cross-linking sites characterized in Table IV, two peptides (labeled a and b in Fig. 2),
each representing γ chain donor activity at Lys^406, were identified in the C-III tryptic fingerprint. Peptide a accounted for 94% of the total peptide-cross-linked γ chain donor activity recovered. Mass analysis (within 0.15% of the expected atomic mass units) and sequencing data revealed that Peptide a contained the acceptor probe cross-linked to the partial tryptic cleavage product, γ 392–411. The mass data for Peptide b (within 0.37% of the expected atomic mass unit) suggested a tri-membered, hybrid structure that included the acceptor peptide, a γ 392–411 dimer, and two ε-(γ-glutamyl)lysine cross-links. The unique sequencing profile obtained for Peptide b identified the respective cross-linking partners as follows: 1) the two Lys^406 residues within the γ 392–411 dimer were each involved in a different cross-link and were the sole source of donor activity, based on the observed release of the cross-linked PTH derivative (in the absence of any PTH Lys) at the corresponding cycle, exclusively; 2) a 56.6% yield of PTH Gln at the cycle corresponding to Gln^398, the acceptor glutamine previously implicated in native γ chain cross-linking, indicated that one but not both of these sites was involved in joining the two γ 392–411 components within Peptide b; 3) other glutamine residues within this γ chain derivative, i.e. Gln^399 and Gln^407, did not participate in cross-linking, based on the release of PTH Gln at >85% yield at the corresponding cycles; 4) the absence

**Fig. 2. Isolation of peptide-cross-linked tryptic derivatives by reversed phase (C-18) HPLC.** The eight pools of peptide-cross-linked CNBr fragments (identified by roman numeral in Fig. 1, A–C, right) were digested with trypsin, and the resulting digests were subjected to C-18 reversed phase HPLC as described in the text. Although both pools of cross-linked CNBr VIII (A-I and A-II) and CNBr X (B-II and B-III) fragments were processed, only the fingerprints for A-I and B-III are illustrated. Anti-peptide immunoreactivity (dashed line), as determined in the AP-102 ELISA, and 220 nm absorbance (solid line) are indicated. For the C-I, C-IV, and C-V fingerprints, cross-linked derivatives were not localized by ELISA but by direct compositional analysis of material in each peak (C-I) or in selected peaks that eluted in more hydrophobic regions compared with control fingerprints (—58.2 min and >77.3 min for C-IV and C-V, respectively). Peptide-cross-linked tryptic derivatives are labeled in each panel by arabic numeral and were identified as summarized in Table IV. The asterisks indicate the elution positions of the non-cross-linked, control peptides used for quantitation (see Table IV). The following loads were applied for each fingerprint: A-I, 248 μg; B-III, 166 μg; C-I, 53 μg; C-III, 221 μg; C-IV, 121 μg; and C-V, 180 μg.
Identification of $\alpha_a$ chain lysine donor sites in cross-linked tryptic peptides (see Fig. 2).

Material from the peaks containing the 12 cross-linked peptides indicated in Fig. 2 was taken for NH$_2$-terminal sequencing (approximately 1 nmol; 12–20 cycles) and amino acid analysis (100–500 pmol). When inclusive residues could not be assigned based on these parameters alone, additional material was subjected to mass spectrometry (1–20 pmol). A control tryptic peptide (non-cross-linked) was identified for each fingerprint by compositional analysis (see peaks highlighted by an asterisk in Fig. 2) and used for yield determinations; these are listed following each set of numbered cross-linked derivatives. There was no control peptide for the $\alpha_a$ 585–610 component of the C-III fingerprint because its entire structure was represented by two cross-linked derivatives. (10.9 nmol is the average of their respective recoveries).

| Cross-linked peptide | Donor arm (inclusive residues)$^a$ | Mass$^b$ | XL Lys$^c$ | nmol/pool$^d$ |
|----------------------|-----------------------------------|---------|-----------|-------------|
| A-I$^e$              |                                   |         |           |             |
| 1                    | Aa 425–439                        | 2906.3  | Aa 427    | 13.9        |
| 2                    | Aa 425–439                        | 2906.3  | Aa 429    | 13.9        |
| 3                    | Aa 414–424                        | 2598.0  | Aa 418    | 13.0        |
| 4                    | Aa 445–457                        | 2705.2  | Aa 448    | 13.0        |
| B-III$^f$            |                                   |         |           |             |
| 5                    | Aa 555–562                        | 2598.0  | Aa 556    | 65.3        |
| 6                    | Aa 573–583                        | 2598.0  | Aa 580    | 21.1        |
| 7                    | Aa 529–554                        | 2598.0  | Aa 539    | 18.2        |
| C-I                  |                                   |         |           |             |
| 8                    | Aa 585–602                        | 2598.0  | Aa 601    | 9.6         |
| 9                    | Aa 603–606                        | 2598.0  | Aa 601    | 7.3         |
| 10                   | Aa 208–224                        | 2598.0  | Aa 601    | 9.3         |
| 11                   | Aa 208–230                        | 2598.0  | Aa 606    | 12.5        |
| C-IV$^g$             |                                   |         |           |             |
| 10                   | Aa 208–224                        | 2598.0  | Aa 224    | 8.0         |
| 12                   | Aa 231–235                        | 2598.0  | Aa 224    | 8.0         |
| C-V                  |                                   |         |           |             |
| 12                   | Aa 492–517                        | 2598.0  | Aa 508    | 7.4         |
|                      | Aa 477–491                        | 2598.0  | Aa 508    | 7.7         |

$^a$ Each of the cross-linked peptides exhibited two sequences, one representing the fibrin-derived donor arm and the other representing the sequence of the acceptor probe. The inclusive residues for the donor tryptic component only are indicated.

$^b$ Data are presented as the mass expected for the cross-linked $\alpha_a$ chain tryptic peptide identified by NH$_2$-terminal sequencing, assuming that 1) the donor arm would reflect an incomplete cleavage product due to the presence of a blocked lysine at the cross-link site and 2) that each peptide's mass would include the acceptor probe's contribution (1369.6 atomic mass units), less 17 atomic mass units for each epsilon-(gamma-glutamyl)lysine bond introduced (see text and Table III legend, footnote b, for additional details). The observed masses were within 0.16–0.22% of expected.

$^c$ Lysine donor sites were identified by direct NH$_2$-terminal sequencing as described in the text. In the case of cross-linked peptides 1 and 2, which coeluted as a single immunoreactive peak (see A-I), two different donor sites were represented on the same partial tryptic cleavage product. This was inferred from the observation that although mass spectrometry identified a single component, sequencing revealed two, based on the release of both PTH Lys and the cross-linked PTH derivative in roughly equimolar yield at two different cycles, i.e., those corresponding to Lys$^{427}$ and Lys$^{429}$.

$^d$ Determined by amino acid analysis and normalized to reflect the amount of each cross-link present in the parent CNBr pool (see Table III). Control peptide recoveries were based on the starting material used for trypsin digestion and generally ranged from 60 to 90%, consistent with the varied yields expected for peptide purification by HPLC. C-V peptide recoveries were particularly low (23%), but sequencing and mass data supported the assignment of Lys$^{508}$ as a donor site, initially inferred from the findings in Table III (see text).

$^e$ Pool A-I fragments contained the same four $\alpha_a$ chain donor sites: Lys$^{427}$ and Lys$^{508}$, 4.5 nmol each; Lys$^{418}$ and Lys$^{429}$, 15.1 nmol each.

$^f$ Pool B-II fragments contained the same three $\alpha_a$ chain donor sites: Lys$^{427}$, 68.5 nmol; Lys$^{508}$, 20.7 nmol; and Lys$^{429}$, 9.9 nmol. The small immunoreactive peaks eluting after peptides 6 and 7 in the B-II fingerprint contained, respectively: 1) cross-linked Lys$^{427}$, as represented within an anomalous chymotryptic cleavage product, Aa 573–582 (8.2 nmol), and 2) material that provided no clear sequence information and was present at insignificant level.

$^g$ Lys$^{508}$ was identified as a donor site based on the release of the cross-linked PTH derivative at cycle 1 in both peptides; its release at this cycle was unexpected because the acceptor Gln residue occupies position 2 but may be the result of anomalous coupling and cleavage due to the presence of the individual cross-link components at the initial sequencing steps. The coincident release of PTH Lys at cycle 1 (at 30% peptide 10) and 60% (peptide 11) the level observed for the PTH cross-linked derivative) indicated that at least one other Lys, Lys$^{427}$ (in peptide 10) and/or Lys$^{429}$ (in peptide 11), was also a donor site. However, because these two cross-linked peptides sequenced poorly (5–10 pmol of specific PTH amino acids were released at the first eight cycles with progressively decreased yields obtained thereafter), the definitive data required to implicate Lys$^{427}$ and/or Lys$^{429}$ as donor sites could not be obtained with the limited amount of material available. The control peptide for this fingerprint includes 4.3 nmol recovered as partially cleaved, Aa 225–235 (eluting at 40.98 min).

of PTH Gln at the cycle corresponding to the peptide probe's acceptor residue, Gln$^{2}$, indicated that this site contributed to the structure of the second cross-link. Thus, these collective findings for Peptide b reflect incorporation of the synthetic peptide into $\gamma$ chains that either before or after this cross-linking event (via donor activity at Lys$^{508}$) underwent native cross-linking with a second $\gamma$ chain, this time via acceptor activity at Gln$^{2}$.

**DISCUSSION**

The biochemical studies in this report describe the application of a recently reported Factor XIII$\alpha_a$ lysine labeling system (27) to identify the specific $\alpha_a$ chain residues that serve as donor sites during fibrin cross-linking. The studies feature the use of three monoclonal antibodies, two specific for defined COOH-terminal ($\alpha_a$ chain sequences and a third that recognizes the synthetic peptide probe, to facilitate the isolation of regions within fibrin that contain transglutaminase-sensitive lysine residues. The collective findings obtained are significant because they provide new information about the precise location, number, and relative contribution of the $\alpha_a$ chain lysines potentially involved in cross-linking and, in so doing, begin to address the as yet poorly understood mechanism(s) surrounding the final stages in the conversion of fibrinogen to fibrin.

Several aspects of the lysine labeling system deserve comment. First, peptide incorporation was conducted in an in vitro fibrin cross-linking system, with no detectable fibrinogen found associated with the fibrin gel that formed after 1 h under the experimental conditions employed (see "Materials and Methods"). Thus, the $\alpha_a$ chain regions eventually identified as those cross-linked to the peptide probe originated from fibrin molecules and not from Factor XIII$\alpha_a$-mediated fibrinogen interactions. Second, peptide incorporation was specific for fibrin chains previously reported to be plasma transglutaminase sub-
strates, namely α and γ chains (12). Although the (B)β chain CNBr fragment, 315–354, was recovered at low level in the initial steps used to isolate peptide-cross-linked derivatives (Fig. 1), sequence and mass spectral analysis indicated that this fragment was not associated with the acceptor probe (Table I, C-1). Because there is no apparent primary structural homology between the small peptide and Bβ 315–354, nonspecific binding must have been responsible for this Bβ chain derivative's recovery during the anti-peptide immunosorption step. Third, peptide incorporation occurred exclusively via the small probe's glutamine acceptor activity in the Factor XIIIa cross-linking reaction. Even though its structure includes a glutamine adduct of dehydroalanine, which was calculated as in footnote i.e., the demonstration of the probe's functional Factor XIIIa substrates responsible for peptide incorporation (21). Identification of Lys539 cross-linked to the acceptor peptide at position 2 as the residue responsible for peptide incorporation (21). Identification of Lys539 cross-linked to the acceptor peptide at position 2 as the residue responsible for peptide incorporation.

---

**Table V**

| Cycle | PTH amino acid | Peptide | Aα 555–562 | PTH+γ-(γ-Glu)Lys |
|-------|----------------|---------|------------|------------------|
| 1     | Asn 296        | Gly 460 | 426e       |
| 2     | Glu 133        | Ser1b   | 118        |
| 3     | Gin 105        | Ser 126 |            |
| 4     | Val 68         | Ser 126 |            |
| 5     | Ser 67         | Tyr 137 |            |
| 6     | Leu 12         | Lys 56  |            |
| 7     | Thr (a)        | 3 Glu 133 Ser 97 |
| 8     | Leu 8          | 8 Leu 12 Lys 5 |
| 9     | Leu 8          |            |
| 10    | Leu 8          |            |
| 11    | Leu 8          |            |
| 12    | Lys 5          |            |

a. PTH Gin and PTH Lys were not detected. The amount of cross-linked PTH derivative released was approximated using the averaged integration value for 17 common PTH amino acid standards.
b. The sum of PTH Ser and its degradation product, the diethylthreitol adduct of dehydroalanine, which was calculated as in footnote a.
c. The cross-linked PTH derivative was not detected.
d. PTH Thr was detected but at insufficient levels for quantitation.

---

**Fig. 3. The Aα chain cross-linking domain.** The 12 Aα chain lysine donor sites identified by peptide labeling and their relative contribution to peptide-fibrin cross-linking are indicated (bars). The data are expressed as the total amount of each cross-link (e.g., A-I + A-II, etc.), corrected for HPLC losses based on control peptide recoveries for each fingerprint (see Table IV legend). The positions of the two reported Aα chain acceptor glutamine residues (17) are shown at the top of the figure (Q) together with the CNBr fragments that comprise the Aα chain cross-linking domain. For reference, the inclusive residues for these fragments are: 208–235 (CNBr V), 236–238 (CNBr VI), 239–240 (CNBr VII), 241–476 (CNBr VIII), 477–517 (CNBr IX), 518–584 (CNBr X), and 585–610 (CNBr XI).

---

Among the 23 potential lysine donor residues within the region Aα 208–610 (CNBr V-XI), 12 were identified as the functional Factor XIIIa substrates responsible for peptide incorporation. The combined sequencing, mass, and compositional data obtained for the various tryptic peptides used in this analysis (Fig. 2) provided unequivocal assignments for all but one of the 12 donor sites, with only the relative contribution of Aα 224 and/or Aα 219 requiring additional clarification (Table IV). In view of the reported role of Lys507 as the Aα chain donor site involved in α–Pl–α chain cross-linking (40), it was surprising to find that this residue was not represented among the peptide-labeled tryptic products recovered, particularly because the acceptor probe was modeled after this lysine's native cross-linking partner within α–Pl (41). One possible explanation for this discrepancy may be, as previously suggested, that the small acceptor analog is missing structural features, present in intact α–Pl, that are required for optimum alignment of the enzyme intermediate and its Aα chain donor substrate, Lys507 (24).

Recovery data for the various cross-linked peptides (Table IV) indicated that peptide incorporation was not random but rather reflected preferential activity at selected donor sites, as summarized in Fig. 3. Certainly, a major argument challenging the validity of the findings shown is that the small acceptor analog used in lysine labeling has greater flexibility than its native α chain (Factor XIII) substrates within the organized structure of the intact fibrin molecule. Although the present data may therefore represent an overestimate of cross-linking information, several lines of evidence indirectly support the specific α chain cross-linking findings obtained. Identification of Lys568 as a donor site by lysine labeling is consistent with the previously reported involvement of the region Aα 477–517 (CNBr IX), with its single lysine residue, in α polymer formation (21). Identification of Lys539 cross-linked to the acceptor probe is in keeping with the recent observation that a mono-
clonal antibody whose epitope is localized within Aα 529–539 (mAB 5A2) specifically inhibits α chain but not γ chain cross-linking (42). The γ chain lysine, Lys460, is the only donor site to date that has been identified by biochemical characterization of a cross-linked peptide isolated from native fibrin (13). Its recovery in peptide-cross-linked form here indicates that the conformational elements required for natural γ chain cross-linking and γ chain peptide labeling are similar and therefore strengthens the likelihood that the same is true for α chains. Finally, the peptide-associated derivative recovered as a hybrid product of native γ chain cross-linking and lysine labeling (Fig. 2; C-III, peptide b) supports the view that γ chain trimers and tetramers (15) comprise an integral part of fibrin’s structure.

How does the demonstration of multiple α chain donor sites contribute to our current understanding of the mechanism of a polymer formation, i.e., the process whereby one α chain is thought to interact with at least two others on different fibrin molecules to produce the large lattices that are the hallmark of “aged” fibrin? Recognizing that models based on lysine labeling data must remain speculative given the lack of information provided about the respective (native) glutamine partners involved in each cross-link, our findings (see Fig. 3) extend previously proposed models for a polymer formation (18, 19). The fact that there appear to be two sets of donor sites, one providing the greater proportion of cross-linking activity (represented by Lys556 and Lys580 within the CNBr X region) and a second contributing supplementary activity (as represented by the 10 chain cross-linking) raises an obvious question: why are there by Lys556 and Lys580 within the CNBr X region) and a second contributing supplementary activity (as represented by the 10 chain cross-linking) raises an obvious question: why are there significant COOH-terminal structural heterogeneity affecting the process whereby one α chain is thought to interact with at least two others on different fibrin molecules to produce the large lattices that are the hallmark of “aged” fibrin in both these earlier observations. Because fibrinolytic system activation leads to enhanced COOH-terminal Aα chain degradation, loss of cross-linking sites, a decreased capacity for fibrin stabilization, and disruption to normal hemostasis, it may be that multiple α chain donors have evolved to circumvent the intrinsic structural heterogeneity (and its functional ramifications) of Factor XIII’s primary substrate, fibrinogen.

Acknowledgments—We thank Ernie Petit at Perspectives Biosystems for performing many of the mass analyses and Andy Pound at the Howard Hughes Protein Core Facility at Columbia University for help with amino acid analysis. We also thank Dr. Bohan Kudryk for providing the monoclonal antibody, T2G-L. Dr. Steven Birken’s support throughout the course of this work is greatly appreciated. We acknowledge the preliminary efforts of Dr. Laszlo Lorand and co-workers in applying the lysine labeling technique to study α chain cross-linking and recognize their contribution to the studies reported here.

REFERENCES

1. Lottspeich, F., and Henschel, A. (1978) Hoppe-Seyler’s Z. Physiol. Chem. 359, 1611–1616
2. Lottspeich, F., and Henschel, A. (1978) Hoppe-Seyler’s Z. Physiol. Chem. 359, 1451–1455
3. Henschel, A., Lottspeich, F., and Hessel, B. (1979) Hoppe-Seyler’s Z. Physiol. Chem. 360, 1951–1956
4. Watt, K. W., Takagi, T., and Doolittle, R. F. (1979) Biochemistry 18, 68–76
5. Rixon, M. W., Chan, W. Y., Davie, E. W., and Chung, D. W. (1983) Biochemistry 22, 3237–3244
6. Chung, D. W., Chan, W. Y., and Davie, E. W. (1983) Biochemistry 22, 3250–3256
7. Hall, C., and Slattery, H. (1959) J. Biol. Chem. 235, 5–16
8. Fowler, W. E., and Erickson, H. P. (1979) J. Biol. Chem. 254, 241–249
9. Weisel, J. W., Staffacker, C., Bullitt, E., and Cohen, C. (1985) Science 230, 1389–1391
10. Mosesson, M. (1990) J. Lab. Clin. Med. 116, 8–17
11. Matalic, S., and Loewy, A. G. (1968) Biochem. Biophys. Res. Commun. 30, 36–36
12. Sturrock, P. A., Mattack, P., and Hill, R. L. (1970) Proc. Natl. Acad. Sci. U. S. A. 66, 738–744
13. Chen, R., and Doolittle, R. F. (1971) Biochemistry 10, 4486–4491
14. Doolittle, R. F., Chen, R., and Lau, F. (1971) Biochem. Biophys. Res. Commun. 44, 94–100
15. Mosesson, M. W., Siebenlist, K. R., Arnnari, D. L., and DiOrio, J. P. (1989) J. Biol. Chem. 264, 1113–1117
16. McDonald, R. P., Jr., McDonald, J., Blomback, M., and Blomback, B. (1971) FEBS Lett. 14, 33–36
17. Cottrell, B. A., Strong, D. D., Watt, K. W., and Doolittle, R. F. (1979) Biochemistry 18, 5405–5410
18. Doolittle, R. F., Cassman, K. G., Cottrell, B. A., and Friezer, S. J. (1977) Biochemistry 16, 1715–1719
19. Fretto, L. J., and McKee, P. A. (1978) J. Biol. Chem. 253, 6614–6622
20. Sobel, J. H., Ehrlich, P. H., Birken, S., Saffran, A. J., and Canfield, R. E. (1983) Biochemistry 22, 4175–4183
21. Corcoran, D. H., Ferguson, E. W., Fretto, L. J., and McKee, P. A. (1980) Thromb. Res. 19, 883–888
22. Lorand, L., Velasco, P. T., Murthy, N. P., Wilson, J., and Parameswaran, K. N. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11161–11163
23. Bendikson, E., Birth, W., and Harpel, P. C. (1993) J. Biol. Chem. 268, 21962–21967
24. Ichinoe, A., Tamaki, T., and Aoki, N. (1983) FEBS Lett. 153, 369–371
25. Parameswaran, K. N., Velasco, P. T., Wilson, J., and Lorand, L. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8472–8475
26. Lorand, L., Parameswaran, K. N., Velasco, P. T., and Murthy, S. N. P. (1992) Bioconjugate Chem. 3, 37–41
27. Sobel, J. H., Tracht, J., Wu, H. Q., Ruchdenko, S., and Ebringer, R. (1995) Blood 86, 989–1000
28. Koeln, J. A., and Canfield, R. E. (1981) Anal. Biochem. 116, 349–356
29. Rapaport, D., and Goth, T. (1978) Methods Enzymol. 19, 770–787
30. Hirajama, S., Nakamura, Y., Miura, M., Sugimori, Z., and Aoki, N. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 6836–6840
31. Kudryk, B., Rychova, A., Ahadi, M., Chin, J., and Wiebe, M. E. (1984) Mol. Immuno. 21, 89–94
32. Ehrlich, P. H., Sobel, J. H., Moustafa, Z. A., and Canfield, R. E. (1983) Biochemistry 22, 4184–4192
33. Sobel, J. H., Wu, H. Q., and Canfield, R. E. (1994) Blood 84, 535–546
34. Sobel, J. H., Thibodeau, C. A., Gawanowicz-Kolks, M. A., and Canfield, R. E. (1988) Thromb. Haemostasis 60, 160–169
35. Fung, D., Bessel, B., Marques, E., Murano, G., and Blomback, B. (1977) Eur. J. Biochem. 77, 595–610
36. Laemmli, U. K., and Fave, M. (1983) J. Biol. Chem. 258, 575–579
37. Tappern, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
38. Burnett, W. N. (1981) Anal. Biochem. 112, 195–203
39. Fath, M. J., Zhang, L. H., Rush, J., and Kolter, R. (1994) Biochemistry 33, 1911–1917
40. Kimura, S., and Aoki, N. (1986) J. Biol. Chem. 261, 15591–15595
41. Tamaki, T., and Aoki, N. (1982) J. Biol. Chem. 257, 14767–14772
42. Maitre, D., Doolittle, R. F., and Laszlo Lorand and co-workers in applying the lysine labeling technique to study α chain cross-linking and recognize their contribution to the studies reported here.