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Position of γ-Chain Carboxy-Terminal Regions in Fibrinogen/Fibrin Cross-Linking Mixtures

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Position of γ-Chain Carboxy-Terminal Regions in Fibrinogen/Fibrin Cross-Linking Mixtures†

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Abstract

There are conflicting ideas regarding the location of the carboxyl-terminal regions of cross-linked γ-chain dimers in double-stranded fibrin fibrils. Some investigators believe that the chains are always oriented longitudinally along each fibril strand and traverse the contacting ends of abutting fibrin D domains ("DD-long" cross-linking). Other investigations have indicated instead that the chains are situated transversely between adjacent D domains in opposing fibril strands (transverse cross-linking). To distinguish between these two possibilities, the γ dimer composition of factor XIIIa-cross-linked fibrin/fibrinogen complexes that had been formed through noncovalent D/E interactions between fibrinogen D domains and fibrin E domains was examined. Two factor XIIIa-mediated cross-linking conditions were employed. In the first, fibrin/fibrinogen complexes were formed between ^{125}I-labeled fibrinogen 2 ("peak 2" fibrinogen), each heterodimeric molecule containing one γ_A and one larger γ' chain, and nonlabeled fibrin 1 molecules ("peak 1" fibrin), each containing two γ_A chains. If DD-long cross-linking occurred, ^{125}I-labeled γ_A–γ_A, γ_A–γ', and γ'–γ'dimers in a 1:2:1 ratio would result. Transverse cross-linking would yield a 1:1 mixture of ^{125}I-labeled γ_A–γ_A and γ_A–γ' dimers, without any γ'–γ' dimers. Autoradiographic analyses of reduced SDS−PAGE gels from protocol 1 revealed ^{125}I-labeled γ_A–γ_A and γ_A–γ' dimers at a ratio of ∼1:1. No labeled γ'–γ' dimers were detected. Protocol 2 used a converse mixture, ^{125}I-fibrin 2 and nonlabeled fibrinogen 1. DD-long cross-linking of this mixture would yield only nonradioactive γ_A–γ_A dimers, whereas transverse cross-linking would yield a 1:1 mixture of ^{125}I-labeled γ_A–γ_A and γ_A–γ' dimers. Autoradiographic analyses of this mixture yielded ^{125}I-labeled γ_A–γ_A and γ_A–γ' dimers in a 1:1 ratio. These findings provide no evidence that longitudinal (DD-long) γ chain positioning occurs in cross-linked fibrin and indicate instead that most, if not all, γ-chain positioning in an assembled fibrin polymer is transverse.

Following thrombin-catalyzed conversion of fibrinogen to fibrin, polymer assembly commences with noncovalent intermolecular interactions between the D domains and the E domains of neighboring molecules (*1*−*6*), resulting in double-stranded fibrils composed of a half-staggered overlapping array of molecules (*7*−*13*). In the presence of factor XIIIa, fibrin assembly is accompanied by intermolecular covalent cross-linking, in which ε-amino-(γ-glutamyl)lysine isopeptide bonds are introduced between appropriately positioned donor lysine and acceptor glutamine residues (*14, 15*). Cross-linking occurs rapidly between γ chains, forming γ dimers, more slowly between α chains to form α polymers (*16, 17*), and between α and γ chains (*18*−*21*).

Positioning of D domains in the fibrin polymer facilitates intermolecular covalent cross-linking between γ chains, resulting in reciprocal isopeptide bonds between paired donor−acceptor sites in the carboxy-terminal regions of γ chains (*22*−*25*). The orientation of the carboxy-terminal γ-chain dimers in cross-linked fibrin fibers remains in dispute. Several investigators have suggested that the chains are oriented longitudinally, parallel to each fibril strand, traversing the contacting ends of abutting fibrin D domains in a so-called DD-long arrangement (*11, 26*−*28*). This model draws support from ultrastructural studies of factor XIIIa-cross-linked fibrinogen molecules that showed dimeric structures aligned end-to-end (*11, 26, 27*), from studies of the morphology of plasmin degradation products of cross-linked fibrin (*28*), and from the appearance of fibrinogen molecules organized on a fibrin fragment E template (*29*). This model has also gained support from analyses of D-domain crystal structure, since theoretical calculations of

carboxy-terminal γ chain lengths suggested that only DD-long positioning was possible in such structures (*30, 31*).

Other investigations have indicated that γ dimers are situated between D domains on opposite strands of the double-stranded fibrin fibrils (*18, 32−36*), in a transverse arrangement (DD-trans). This cross-linking pattern was first proposed by Selmayr et al. (*32*), who found that, in the presence of factor XIIIa, fibrinogen formed a covalently cross-linked complex with immobilized fibrin, an arrangement that could only have come about through transverse orientation of γ-chain cross-links. In later electron microscopic studies (*33*) they showed that cross-linked double-stranded fibrin fibrils were not depolymerized by 3 M urea, a solvent that disrupted noncovalent D/E interactions, thus reinforcing their conclusion that cross-linked γ chains were positioned transversely. Ultrastructural analyses of cross-linked fibrinogen polymers demonstrated double-stranded fibrils that had formed through cross-linked γ chains that traversed the strands (*34*), allowing the extrapolation that this situation holds for fibrin as well. More recently, high-resolution electron microscopic visualization of gold cadaverine-labeled γ chains in fibrinogen and fibrin molecules has shown that the C-terminal regions are most often oriented toward the central domain of the molecule, suitably aligned for transverse cross-linking (*36*). Even better evidence for transverse cross-link orientation comes from a biochemical study demonstrating that mixtures of fibrin and plasmic fragment D produced primarily covalently cross-linked D/fibrin/D complexes in the presence of factor XIIIa (*35*).

Human fibrinogen can be separated into two major chromatographic fractions, fibrinogen 1 ("peak 1" fibrinogen) and fibrinogen 2 ("peak 2" fibrinogen) (*37, 38*). The two fibrinogens differ from each other with respect to their γ-chain compositions. Fibrinogen 1 contains two γ_A chains, whereas heterodimeric fibrinogen 2 molecules each contain one γ_A and one γ′ chain. Factor XIIIa-treated mixtures of radioactively labeled fibrinogen 2 and fibrin 1 or radioactively labeled fibrin 2 and fibrinogen 1 will produce distinguishable combinations of radioactive γ-chain dimers depending upon whether cross-linking occurs in the DD-long or in the transverse orientation. By analyzing the γ dimer population in mixtures of ^{125}I-labeled fibrinogen 2 and fibrin 1 or ^{125}I-labeled fibrin 2 and fibrinogen 1, this situation was exploited to show that transverse cross-linking occurs in fibrinogen/fibrin complexes and underscores the conclusion that cross-linked γ chains are positioned transversely in an assembled fibrin polymer.

Materials and Methods

Tris, [1] glycine, Coomassie Brilliant Blue R250, and DTT were purchased from Aldrich Chemical Co., Milwaukee, WI. Trasylol (aprotinin) was obtained from Miles Inc., Kankakee, IL, and DE-52 cellulose was from Whatman Inc., Clifton, NJ. Human α-thrombin (3188 units/mg) was obtained from Enzyme Research Laboratories, South Bend, IN. Other chemicals were the highest purity available from commercial sources.

Human fibrinogen was isolated from pooled citrated plasma by glycine precipitation (*39*), further purified as previously described (*40*), and then subfractionated into fibrinogen 1 (γ_A−γ_A) and fibrinogen 2 (γ_A−γ′) by chromatography on DE-52 (*41*). Normal fibrinogen contains small amounts of an intramolecular cross-linked α−γ chain that migrates on reduced SDS−PAGE near the γ_A−γ′ dimer position (*42*). Radioactively labeled fibrinogen containing the α−γ dimer can obscure γ_A−γ′ quantification. This small fraction was removed from the fibrinogen preparations either by precipitating the fibrinogen 2

preparations with 20% ammonium sulfate (*43*) or by preparing I-9 fibrinogen 2 (*40*), which is devoid of α−γ dimer.

Fibrinogen 2 that was devoid of the α∠γ dimer was labeled with ^{125}I by the iodine monochloride method (*44*), resulting in a labeling ratio of 2 atoms of ^{125}I per fibrinogen molecule. Soluble fibrin 1 monomer and ^{125}I labeled I-9 fibrin 2 monomer (≥10 mg/mL in 20 mM acetic acid) were prepared by the method of Belitser et al. (*45*). Fibrinogen and soluble fibrin concentrations were determined spectrophotometrically at 280 nm using an absorbance coefficient ($A_{\mathrm{cm}}^{1\%}$) of 15.1 (*46*).

Factor XIII was purified from pooled human plasma (*47*) and assayed by the method of Loewy et al. (*48*). The specific activity of the preparation was 2000 Loewy units/mg. Human plasma contains up to 120 Loewy units of factor XIII/mL. Factor XIII was activated to factor XIIIa by incubation with thrombin (10 units/mL) (*49*) for 30 min at 37 °C and the thrombin then inactivated by incubating with a 5-fold excess of hirudin (50 units/mL) (*34*).

Fibrinogen:fibrin ratios ranging from 2:1 to 15:1 at a final protein concentration of 1 mg/mL and factor XIIIa levels ranging from 5 to 100 Loewy units/mL were evaluated to determine the optimal conditions for fibrin/fibrinogen γ-chain dimer cross-linking. Cross-linking of experimental mixtures was initiated by adding factor XIIIa and incubating at 37 °C. The cross-linking reaction was terminated at 5-min intervals by adding an equal volume of double-strength Laemmli sample buffer containing 1% β-mercaptoethanol. The samples were analyzed by reduced SDS−PAGE. It was determined that a fibrinogen:fibrin ratio of 8:1 or higher yielded completely solubilized fibrin and that a factor XIIIa level of 20 Loewy units/mL permitted incubations of up to 15 min before significant fibrinogen−fibrinogen γ-chain cross-linking became evident.

Fibrin/fibrinogen reaction mixtures for protocol 1 were prepared by mixing fibrin 1 monomer solutions with ^{125}I-fibrinogen 2 in a buffer of 50 mM Tris, 100 mM NaCl, 10 mM $CaCl_2$, and 0.2 mM PMSF, pH 7.4. For protocol 2, ^{125}I-labeled I-9 fibrin 2 monomer solutions were mixed with fibrinogen 1 in a buffer of 50 mM Tris, 100 mM NaCl, 10 mM $CaCl_2$, and 0.2 mM PMSF, pH 7.4. In both instances the ratio of fibrinogen to fibrin was 8:1 at a final protein concentration of 1 mg/mL, the factor XIIIa level was 20 Loewy units/mL, and the mixtures were incubated at 37 °C. Samples were taken at 5, 10, or 15 min as described above. Control fibrinogen/fibrinogen mixtures contained fibrinogen 1 and ^{125}I-fibrinogen 2 at the same molar ratios and were treated with factor XIIIa under the identical conditions.

The products of the cross-linking reaction were analyzed by SDS−PAGE employing the discontinuous buffer system of Laemmli (*50*) on 9% polyacrylamide gels. Gels were stained with 0.5% Coomassie Brilliant Blue R250 in methanol/water/acetic acid (5:5:1), destained in methanol/water/acetic acid (5:5:1) with continuous shaking, and dried. Dried gels were subjected to autoradiography for up to 15 days with Kodak X-Omat X-ray film.

To evaluate how much of the available fibrin had been utilized during the cross-linking reaction, mixtures of reduced cross-linked fibrin 2 and fibrinogen 1 were prepared with fibrinogen:fibrin ratios ranging from 4:1 to 32:1. These mixtures were then subjected to reduced SDS−PAGE as described above, and the gels were stained with Coomassie Brilliant Blue. Stained gels were digitized on a UMAX Astra 2400S flat-bed scanner (UMAX Technologies, Fremont, CA), and bands in the γ dimer position (γ_A−γ_A, γ_A−γ′, and γ′−γ′) and Bβ-chain regions of the gel were quantified by use of NIH Image (v1.62). A calibration curve was constructed by plotting the fibrinogen:fibrin ratio against the amount of protein in

the γ dimer region normalized against the Bβ-chain region. Based upon this calibration curve, if all the fibrin in the experimental mixtures became incorporated into cross-linked complexes, 18% of the total protein would be found in the γ dimer region. This amount of the total protein is defined as the theoretical maximum amount of γ dimers. Stained experimental gels were digitized and the calibration curve was used to determine the amount of available fibrin that had undergone cross-linking. The amount of protein found in the γ dimer region of the control and experimental gels is reported as a percentage of this theoretical maximum amount, i.e., (amount in control or experimental samples ÷ theoretical maximum) × 100. Autoradiographs of the experimental samples were likewise digitized and the ratio of γ_A–γ_A, γ_A–γ‘, and γ‘–γ‘ dimers in these samples was calculated from these images.

Results

The rationale for the cross-linking experiments employing radioactive fibrinogen/fibrin mixtures is depicted in Figures 1 and 2. D domains from two fibrinogen molecules bind noncovalently to the available complementary polymerization sites in the fibrin E domain (D/E) to form a trimolecular complex. In the presence of factor XIIIa, covalent cross-links are introduced between the carboxy-terminal regions of two appropriately positioned γ chains. γ_A and γ‘ chains are easily distinguishable by differences in their sizes, but they undergo cross-linking with one another nonselectively (*51*). When the fibrinogen/fibrin mixture consists of ^{125}I-labeled fibrinogen 2 (γ_A, γ‘) and fibrin 1 (γ_A, γ_A) (protocol 1), DD-long cross-linking would yield ^{125}I-labeled γ_A–γ_A, γ_A–γ‘, and γ‘–γ‘ dimers in a 1:2:1 ratio. Transverse cross-linking of this same mixture would result in ^{125}I-labeled γ_A–γ_A and γ_A–γ‘ dimers in a 1:1 ratio; γ‘–γ‘ dimers would not form. Alternatively, when the fibrinogen/fibrin mixture consists of ^{125}I-fibrin 2 and fibrinogen 1 (protocol 2), DD-long cross-linking would yield only nonradioactive γ_A–γ_A dimers, whereas transverse cross-linking would yield ^{125}I-labeled γ_A–γ_A and γ_A–γ‘ dimers at a 1:1 ratio.

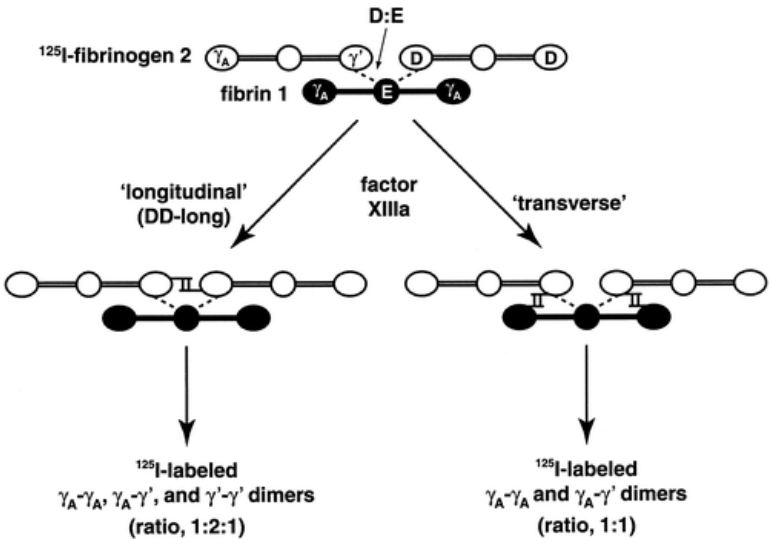

Figure 1 Diagrammatic representation of protocol 1, in which ^{125}I-fibrinogen 2 (γ_A, γ‘) and fibrin 1 (γ_A, γ_A) are incubated with factor XIIIa. The outcome of DD-long cross-linking versus transverse cross-linking is depicted in terms of γ dimer formation.

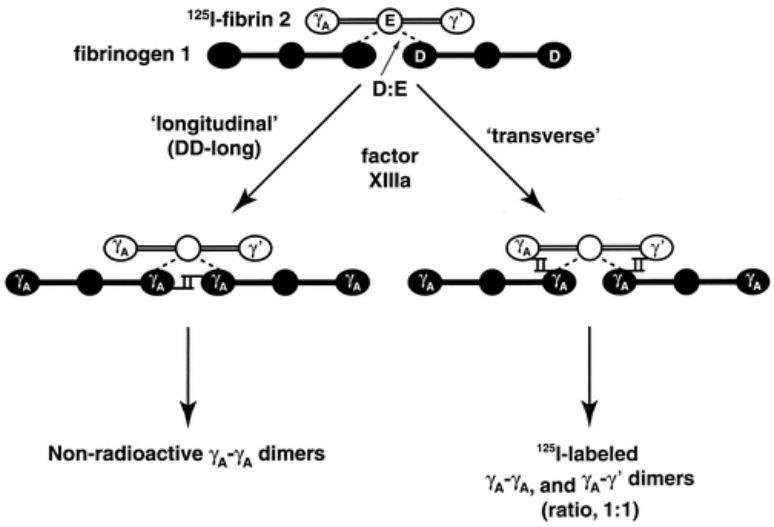

Figure 2 Diagrammatic representation of protocol 2 in which ^{125}I-fibrin 2 (γ_A, γ') and fibrinogen 1 (γ_A, γ_A) are incubated with factor XIIIa. The outcome of DD-long cross-linking versus transverse cross-linking is depicted in terms of γ dimer formation.

When mixtures of ^{125}I-labeled fibrinogen 2 (γ_A, γ') and fibrin 1 (γ_A, γ_A) were cross-linked by factor XIIIa (protocol 1, Figure 3), two γ dimer bands migrating in the γ_A–γ_A and γ_A–γ' positions were present by the 5-min sample. The γ_A–γ_A band contained 44% of the protein in the γ dimer region of the gel, and the γ_A–γ' band contained 56% (mean of three trials). No band was ever detected in the γ'–γ' region of the gel. Based upon scans of the stained gels, 25% of the possible fibrin/fibrinogen cross-linked dimers were in the γ dimer region of the gel. Few, if any, γ dimers were present in the 5 min fibrinogen 2/fibrinogen 1 control sample. As the time of incubation increased, the intensity of the two γ dimer bands increased in the experimental sample. At the 15 min time point, 67% of the possible fibrin/fibrinogen cross-linked dimers were present in the γ dimer region (mean of three trials). The γ_A–γ_A band contained 49% of the protein in the γ dimer region and the γ_A–γ' band contained 51%, a 1:1 molar ratio. A γ'–γ' dimer band was never detected. In the 15 min fibrinogen/fibrinogen control sample, small amounts of γ dimers were found. Compared to the fibrin/fibrinogen mixtures, the γ dimers present in the control sample amounted to 8% of the theoretically possible amount of γ dimers. This low level of γ dimers in the control sample was no doubt due to cross-linking among non-fibrin-complexed fibrinogen molecules. Overall, the results are consistent only with transverse positioning of γ chains.

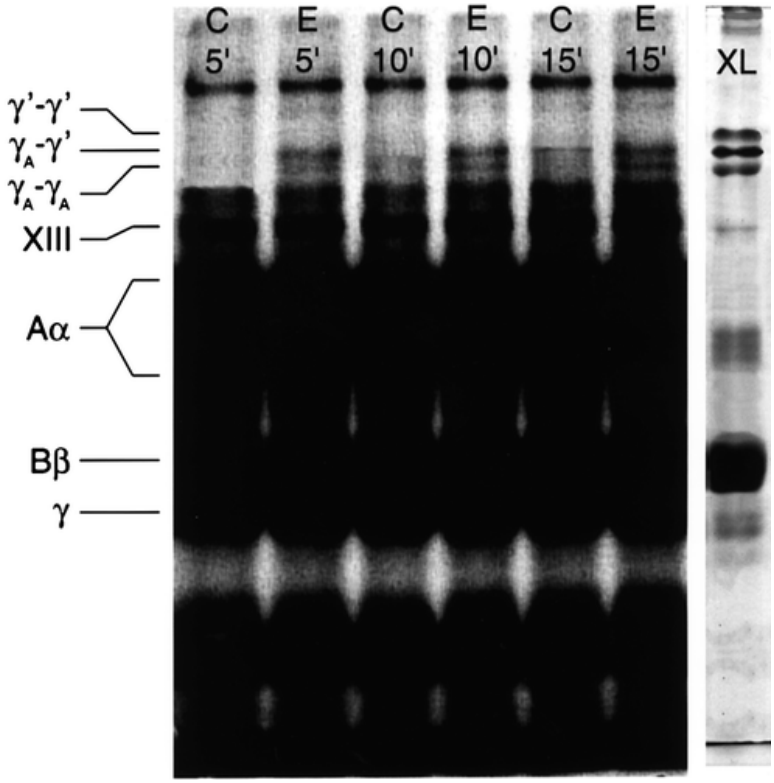

Figure 3 Autoradiograph of a mixture of ^{125}I-fibrinogen 2 (γ_A, γ') and fibrin 1 (γ_A, γ_A) that had been incubated with factor XIIIa. ^{125}I-Fibrinogen 2 and fibrin 1 at an 8:1 molar ratio were incubated with factor XIIIa (20 units/mL) at 37 °C for the indicated times. A control sample consisted of ^{125}I-fibrinogen 2 and fibrinogen 1 incubated at the same molar ratio of 8:1 in the presence of factor XIIIa. The positions of the γ_A–γ_A, γ_A–γ', and γ'–γ' dimer bands were assigned by reference to a marker lane containing cross-linked ^{125}I-fibrin 2. The bands in the marker lane that was included in this run were readable and usable but unsightly. Accordingly, the marker lane from another gel run, which had been run under the same conditions, has been substituted. C, control sample; E, experimental sample; XL, cross-linked ^{125}I-labeled I-9 fibrin 2.

In the converse experiment, autoradiographs of ^{125}I-labeled I-9 fibrin 2 and fibrinogen 1 cross-linking mixtures revealed at the 5-min time point γ dimer bands migrating in the γ_A–γ_A and γ_A–γ' positions (protocol 2, Figure 4), amounting to 17% of the possible fibrin/fibrinogen cross-linked dimers (mean of three trials). Cross-linking among ^{125}I-fibrin 2 molecules did not occur under these conditions because no γ'–γ' dimer band was evident at any time. As the incubation time increased, the intensity of the γ dimer bands increased. At 15 min the γ dimer region contained 74% of the theoretically possible fibrin/fibrinogen γ dimers. The γ_A–γ_A band accounted for 52% of the protein and the γ_A–γ' area contained 48%, a 1:1 ratio (mean of three trials). In the fibrinogen/fibrinogen controls, radioactive γ dimers did not become apparent until the 15 min time point. Compared to the fibrin/fibrinogen mixtures, the γ dimers in this control sample amounted to only 14% of the possible fibrin/fibrinogen dimers. Radioactive γ dimer bands above the levels observed in controls could have formed only if γ-chain cross-linking had occurred from a transverse orientation.

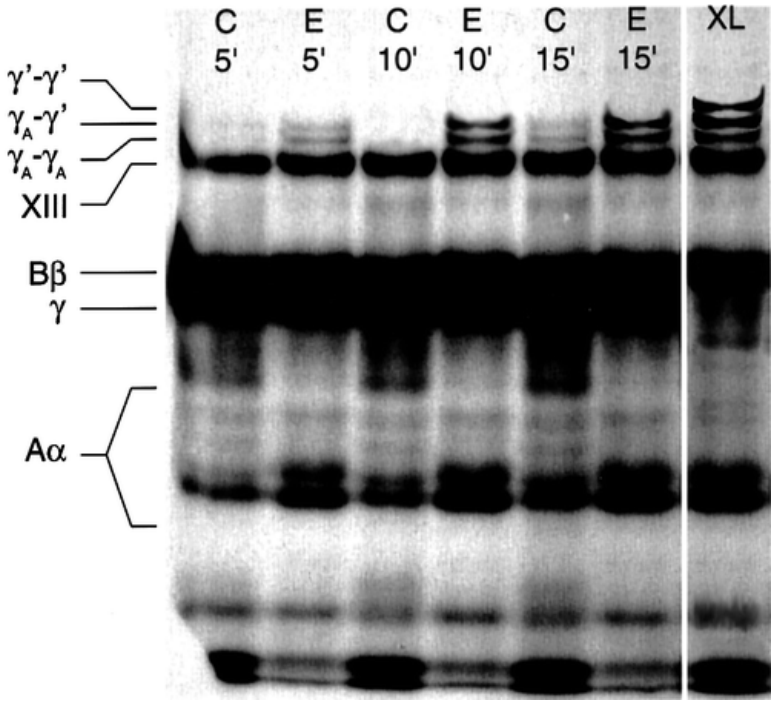

Figure 4 Autoradiograph of a mixture of ^{125}I-fibrin 2 and fibrinogen 1 (γ_A, γ_A) that had been incubated with factor XIIIa. Fibrinogen 1 and ^{125}I-fibrin 2 at an 8:1 molar ratio were incubated with factor XIIIa (20 units/mL) at 37 °C for the indicated times. A control sample consisted of ^{125}I-fibrinogen 2 and fibrinogen 1 incubated at the same molar ratio of 8:1 in the presence of factor XIIIa. The low level of radioactivity in the intact α chain position is due to the fact that intact α chains are absent in the fraction I-9 fibrinogen 2 that was used. The positions of the γ_A–γ_A, γ_A–γ', and γ'–γ' dimer bands were assigned by reference to a marker lane containing cross-linked ^{125}I-fibrin 2. C, control sample; E, experimental sample; XL, cross-linked ^{125}I labeled I-9 fibrin 2.

Discussion

An earlier study that was concerned with the question of γ-chain orientation employed mixtures of fibrin and plasmic fragment D to probe the orientation of fibrin γ chains (*35*) and was conceptually similar to the one presented here. Those findings led to the same conclusion as ours with regard to γ-chain orientation in fibrin. Coupled with other previous (*18, 32–34*) and subsequently published experiments (*36*), we ingenuously were led to believe that further experiments along these lines would not be necessary. That seems not to be the case, judging from conclusions drawn in recent publications concerned with the structure of fibrinogen molecules organized on a fragment E template (*29*) and the interpretations based upon X-ray crystallographic studies of the fibrin D domain (*30, 31*).

Conclusions regarding γ-chain orientation in fibrin based upon crystallographic analyses are worthy of comment. Based upon calculations of the length of the exposed carboxyl-terminal regions of the γ chains and the distance required to bridge the span between the D domains, Yee et al. (*30*) suggested that transverse cross-linking would be unlikely. Spraggon et al. (*31*) used similar logic based on their observations of a cross-linked fibrin D dimer fragment. Although they were unable to visualize the position of the cross-linked γ chains, they could easily surmise the general location of the cross-linked γ chains in the fragment. From this they evidently extended their reasoning to conclude that cross-linking in fibrin must occur in a DD-long orientation. Although crystallographic studies provide many important

details on the folding and conformation of the fibrinogen D domain, it must be recognized that for a variety of reasons the configuration and conformation of a structure in the crystalline state may differ significantly from that of the native molecule in solution. For example, in the crystal structure of the γ module the sequence γ381−390, which directly leads to the terminal cross-linking site of the γ chain, is inserted as the middle strand of a five-stranded antiparallel β sheet. Medved et al. (*52*) have shown that this strand can be removed from its insertion in the γ module without disrupting the compact structure of the module. This implies that the carboxyl-terminal cross-linking region can in turn become considerably more extended than it is in the crystal structure.

The different γ-chain compositions of fibrinogen 1 (γ_A, γ_A) and fibrinogen 2 (γ_A, γ′) (*37, 38*) provided a means for distinguishing between the DD-long and transverse γ-chain cross-linking arrangements. Analysis of the γ-chain dimers formed in cross-linking mixtures of fibrin 1 and ^{125}I-fibrinogen 2 yielded ^{125}I-labeled γ_A−γ_A and γ_A−γ′ dimers accounting for 67% of the total possible fibrin/fibrinogen γ-chain dimers before the appearance of significant fibrinogen/fibrinogen dimers. Mixtures of ^{125}I-fibrin 2 and fibrinogen 1 also yielded ^{125}I-labeled γ_A−γ_A and γ_A−γ′ dimers, accounting for 74% of the total possible fibrin/fibrinogen γ-chain dimers. This combination of γ dimers under both experimental conditions could only have resulted from cross-linking in the transverse orientation. The paucity of cross-linked γ-chain dimers in control fibrinogen/fibrinogen mixtures indicates that, under the conditions employed, factor XIIIa preferentially cross-linked assembled fibrinogen/fibrin complexes. This is expected since it is well-known that fibrinogen γ chains become cross-linked more slowly than do fibrin γ chains (*34,49*). While not eliminating the possibility of a DD-long orientation of γ chains under certain defined circumstances, such as in D dimer fragments, these experiments, in addition to a substantial body of existing data, strongly indicate that transverse positioning of γ chains occurs in fibrinogen/fibrin complexes and that cross-linked γ chains are oriented in the same manner in assembled fibrin.

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Abbreviations:

Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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