Marquette University
e-Publications@Marquette

Biological Sciences Faculty Research and Publications

Biological Sciences, Department of

1-1-2002

Fibrinogen Assembly and Crosslinking on a Fibrin Fragment E Template

Kevin R. Siebenlist
Marquette University, kevin.siebenlist@marquette.edu

Michael W. Mosesson

Irene Hernandez

Joseph S. Wall

James F. Hainfeld

Thrombosis and Haemostasis, Vol. 87, (2002), pp. 651-58.
©2002 Schattauer

© 2002 Schattauer GmbH, Stuttgart

Fibrinogen Assembly and Crosslinking on a Fibrin Fragment E Template

Michael W. Mosesson[1], Kevin R. Siebenlist[2], Irene Hernandez[1], Joseph S. Wall[3], James F. Hainfeld[3]

[1]The Blood Research Institute of the Blood Center of Southeastern Wisconsin, Milwaukee, WI,
[2]Department of Biomedical Science, College of Health Sciences, Marquette University, Milwaukee, WI,
[3]Biology Department, Brookhaven National Laboratory, Upton, NY, USA

Keywords

Factor XIII, fibrinogen, fibrin, electron microscopy

Summary

There is an ongoing controversy concerning whether crosslinked γ chains in fibrin are oriented "transversely" between fibril strands or "end-to-end" along fibril strands. From the latter viewpoint, Veklich et al. [Proc Natl Acad Sci (USA) 95: 1438, 1998] observed that fibrinogen fibrils that had been assembled on a fibrin fragment E template, crosslinked with factor XIIIa, and then dissociated in acetic acid solution, were aligned end-to-end. This led to the conclusion that crosslinked γ chains in fibrin under physiological conditions were also aligned end-to-end. To assess its validity we studied the assembly and organization of fibrinogen molecules on a des AB-fibrin fragment E (E-des AB) or a des A-fibrin fragment E (E-des A) template.

We evaluated the roles of E polymerization sites E_A and E_B, and D association sites γ_{XL}, Da, Db, β_C, and αC in this process. E_A:Da interactions caused fibrinogen: E "DED" complexes to form, and markedly enhanced the γ chain crosslinking rates of fibrinogen or des αC-fibrinogen. Fibrinogen crosslinking without added fibrin E was slower, and that of des αC-fibrinogen was still slower. These events showed that although αC domains promote fibrinogen fibril assembly and crosslinking, they contribute little to increasing the E_A:Da-dependent crosslinking rate. Electron microscopic (STEM) images of E-des AB and fibrinogen plus factor XIIIa showed single-, double-, and multi-stranded fibrils with interstrand DED complexes aligned side-to-side. This alignment was due to β_C:β_C contacts resulting from D subdomain rearrangements initiated by the E_B:Db interactions, and also occurred in mixtures of des αC-fibrinogen with E-des AB.

In contrast, a mixture of fibrinogen and E-des A plus XIIIa revealed double-stranded fibrils with interstrand DED complexes in a half-staggered arrangement, an alignment that we attribute to crosslinking of γ_{XL} sites bridging between fibrils strands. These and other features of E-des A-based fibrinogen fibrils, including interstrand γ chain bridges and early and extensive lateral fibril strand associations concomitant with accelerated γ chain crosslinking, indicate that crosslinking of fibrin fibril strands takes place preferentially on transversely positioned γ chains.

Correspondence to: Dr. Michael W. Mosesson, The Blood Research Institute of the Blood Center of Southeastern Wisconsin, PO Box 2178, Milwaukee, WI 53201-2178, USA – Tel.: 00 414 933 5000; Fax: 414 937 6284; E-mail: MWMosesson@bcsew.edu

Abbreviations

Fgn, fibrinogen; Fragment E or E, the proteolytically derived central domain of the fibrinogen molecule; FPA, fibrinopeptide A; FPB, fibrinopeptide B; E_A, polymerization site in the E domain exposed by release of FPA; E_B, polymerization site in the E domain exposed by release of FPB; Da, constitutive association site for the E_A site; D:D, self-association site in the D domain contributing to end-to-end alignment of fibrinogen molecules; Db, constitutive association site for the E_B site; βC, a D subdomain that undergoes conformational rearrangements following E_B:Db interaction. DED, non-covalent complex formed among two fibrinogen D domains and a fibrin E fragment; E-des A, batroxobin-treated fragment E from which FPA has been cleaved; E-des AB, thrombin-treated fragment E from which FPA and FPB have been cleaved; des αC-fibrinogen, fibrinogen molecules from which αC domains have been selectively removed by plasmin treatment; γ_{XL}, the C-terminal region of the γ chain containing the XIIIa crosslinking site; XLNK, an ε-(γ-glutamyl) lysine bond introduced by factor XIIIa; fibril, a single- or double-stranded structure, each strand comprised of end-to-end aligned fibrinogen or fibrin molecules-some investigators refer to these structures as "protofibrils"; STEM, scanning transmission electron microscopy.

Introduction

Thrombin-mediated cleavage of FPA from the central E domain of fibrinogen exposes a polymerization site, E_A (1, 2, 3) which subsequently combines with a complementary site (Da) in the D domain of neighboring molecules (4, 5, 6) resulting in twisting double-stranded fibrin fibrils, with constituent units aligned in a staggered overlapping arrangement (7, 8, 9). Release of FPB by thrombin exposes another polymerization site, E_B (10), that interacts with a Db site in the β chain segment of the D domain (Fig. 1) (6, 10, 11). The E_B:Db interaction, though not absolutely required for lateral fibril and fiber associations, nevertheless contributes to lateral association by inducing rearrangements in the β_C region of the D domain that permit intermolecular β_C:β_C contacts to occur (12). Another domain, termed 'αC', originates at $A\alpha_{220}$ not far from where it emerges from the D domain, and it terminates at $A\alpha_{610}$ (13). Fibrin clots formed from fibrinogen molecules lacking 100 or more C-terminal αC domain residues (such as plasma fractions I-6 or I-9) (14, 15) display a prolonged thrombin time, develop reduced turbidity, and produce thinner fibers (16, 17, 18), the last observations suggesting that αC domains participate in lateral fibril associations (19).

The Da:E_A interaction greatly facilitates anti-parallel C-terminal alignment of intermolecular γ chain pairs, thereby accelerating the rate of XIIIa-mediated crosslinking (20, 21, 22). The location of these γ chain pairs within a crosslinked fibrin polymer has been controversial for more than twenty years. Evidence summarizing this situation was presented in 2000 at the XVI International Fibrinogen Workshop in Leiden and is now in print (23). In brief, we believe that crosslinked γ

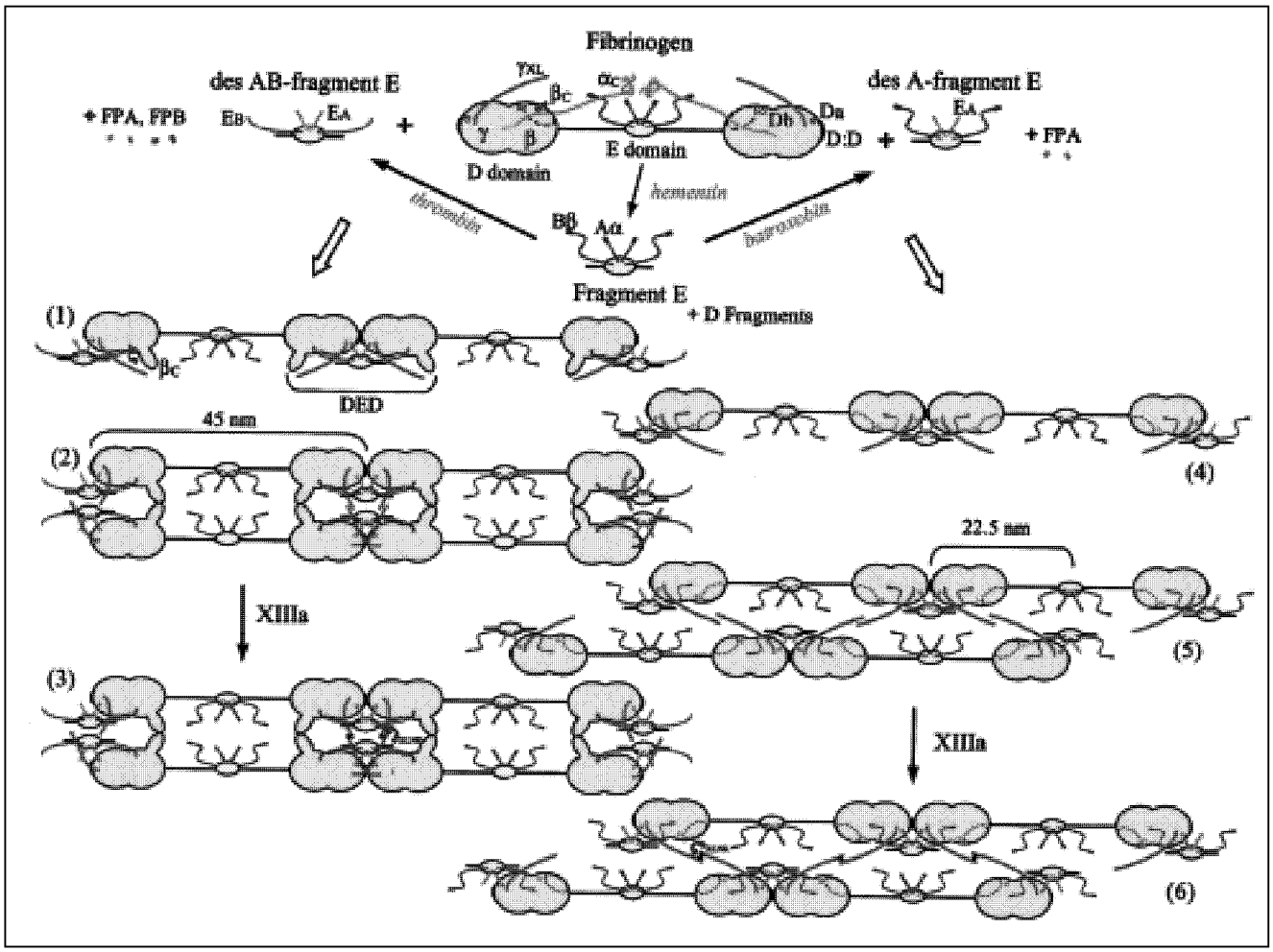
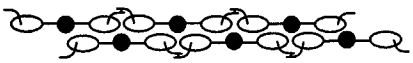


Fig. 1 The assembly and crosslinking of fibrinogen molecules on a fibrin fragment E template. The basis for the structural model of fibrinogen including its major domains, association sites and intermolecular interactions has recently been summarized (23). The recent contribution by Yang et al. based upon crystal structures (12). has clarified the role of the E_B polymerization site in promoting rearrangement of the β_C subdomain, and this information has been incorporated into our model. The preferential orientation of C-terminal regions of γ chains is toward the E domain (proximal), as shown, but this region is known to be flexible and can be found in more distal regions of the fibrinogen molecule (39). We have not attempted to draw the "end-to-end" crosslinking mechanism as an alternative, mainly because it does not fit well with our observations

chain pairs in assembled fibrin or fibrinogen polymers are positioned 'transversely' between D domains on opposing strands of a double-stranded fibrin or fibrinogen fibril as depicted in (1).

(1)

In this scheme each fibrinogen molecule is drawn as two white oval D domains connected to a circular black E domain. C-terminal γ chains protrude inwardly from each fibril strand and their interstrand covalent connections are drawn. The opposite view on the location of cross-linked γ chains involves the belief that crosslinked γ chain regions in fibrin are positioned outwardly, crossing the distal ends of linearly aligned molecules in an end-to-end arrangement as illustrated in (2).

(2) 

The most recent experiments providing evidence for end-to-end crosslinking in fibrin came from electron microscopic images of fibrinogen fibrils that had been assembled as DED complexes on a fibrin fragment E template to form non-covalent DED complexes, and then crosslinked with factor XIIIa (24). Under physiological buffer conditions, single-stranded fibrils were commonly observed and lateral fibril associations were noted as well. In other experiments, crosslinked fibrinogen fibrils that had initially been incubated with fibrin fragment E under physiological conditions, and then dissociated with acetic acid, were examined. Owing to the residual covalent γ chain crosslinks, end-to-end aligned single-stranded fibrils were found. These observations led to the extended conclusion that end-to-end γ chain cross-linking must also exist in native fibrin fibrils. In a companion study, Lorand et al. (25) observed that XIIIa-mediated γ chain crosslinking of fibrinogen was increased in the presence of a bivalent GPRP ligand that mimicked the bilateral valency of the fibrin E fragment. From this result they also inferred that increased crosslinking had resulted from enhanced end-to-end γ chain alignment and crosslinking, no doubt relying upon the conclusions drawn by Veklich et al. (24).

Our present experiments on the assembly and crosslinking of fibrinogen on a fibrin fragment E template were conceived after we had read the Veklich et al. report, and accordingly we designed experi-

ments to address questions that had been raised by their study. We believe that our results provide new and relevant observations on the fibril assembly process, per se, as well as offering a sound basis for modeling fibril assembly, lateral fibril association, and factor XIIIa-mediated crosslinking.

Materials and Methods

Preparation of fibrinogen and factor XIII. Fibrinogen 1 and des αC-fibrinogen: Human fibrinogen fraction I-2 was isolated from normal citrated plasma by glycine precipitation (16) and then separated into fibrinogens 1 and 2 by anion exchange chromatography (26). Des αC-fibrinogen was produced from fibrinogen 1 by limited digestion with plasmin (15) followed by purification of the αC-domainless fibrinogen component, which we now term des αC-fibrinogen (aka, fraction I-9D). Each of these molecules lacked ~390 C-terminal Aα chain residues (27, 28). Factor XIII was purified from human plasma (29) and had a specific crosslinking activity of 2200 Loewy u/mg (30). The XIII level in normal plasma ranges from 85 to 120 Loewy u/ml.

Preparation of hementin-derived fragment E, des A-fragment E, and des AB-fragment E. Anterior glands from the giant Amazon leech, Haementaria ghilianii were a most generous gift from Dr. Andrei Budzynski of Temple University, Philadelphia, PA. The hementin was obtained by extracting 35 mg dried material twice with cold 20 mM Hepes, 10 mM $CaCl_2$, pH 7.8 buffer, and then centrifuged to remove particulate matter (31). The pooled extracts had an estimated concentration of 2.3 mg/ml (assumed $A^{1\%}_{280\ nm}$, 10). Fibrinogen 1 (8.5 mg/ml) in Hepes buffer, pH 7.8 containing 0.02 % (w/v) sodium azide, was digested by adding 0.25 mg of the extract per 100 mg fibrinogen, and incubating at 37° C for 96 h. As assessed by SDS-PAGE of non-reduced samples on 8-25% Phastgels (Pharmacia Biotechnology, Piscataway, NJ), molecular weight fragments >120 kDa had been degraded by this time, and the enzymatic process was terminated by addition of EDTA (12 mM, final) plus O-phenanthroline (1 mM, final), which inhibits the enzyme. The specimen was then dialyzed against 5 mM PO_4-Tris buffer, pH 8.4, heated at 56° C for 3 min to precipitate D-containing fragments, quickly cooled in an ice bath, and the precipitate removed by centrifugation. The fragment E-enriched supernatant solution was then subjected to ion exchange chromatography on a 2.5 × 20 cm DE-52 (Whatman) column using a programmable FPLC chromatography system (Pharmacia Biotechnology, Piscataway, NJ). Elution buffers were the same as previously described (26), and were: Buffer A, 5 mM PO4-Tris, pH 8.4; Buffer B, 500 mM PO4-Tris, pH 4.1. The DE-52 column was equilibrated with buffer A, and sample then applied in a 95% buffer A, 5% buffer B solution. Following initial wash-off, a 400 ml linear gradient from 5-50% buffer B was imposed. Following this, fragment E was eluted isocratically in 6 ml fractions with a 30% buffer A, 70% buffer B solution. E-containing fractions were pooled on the basis of SDS-PAGE analyses, "vacuum-centrifuged" in a Speed Vac device (Savant, Piscataway, NJ), and then dialyzed against PBS (0.1 M NaCl, 20 mM phosphate, pH 7.4). The final fragment E concentration was 3 mg/ml ($A^{1\%}_{280\ nm}$, 10.2) (32). Confirmation of the intact E fragment fibrinopeptide content was obtained by treating aliquots with batroxobin or thrombin to release FPA or FPB, or both, and evaluating subunit composition by reduced SDS-PAGE on high-density Phastgels. The fragment E molecular weight was estimated to be ~70 kDa based upon its migration in unreduced SDS-PAGE Phastgels. The molecular weight calculated from its amino acid sequence and carbohydrate content (33, 34) was 74 kDa. From this value, the molecular weight of E-des A was a calculated to be 71 kDa, and that of E-des AB, 69 kDa.

Crosslinking conditions for electrophoresis experiments. Factor XIII at a concentration of 1000 u/ml in 0.1 M NaCl, 20 mM Hepes, pH 7 buffer (HBS) was activated by adding human α-thrombin to a concentration of 5 u/ml and then incubating at 37° C for 30 min. Thrombin was then inactivated with a tenfold excess of hirudin (50 u/ml, final concentration). Fibrinogen assembly and crosslinking experiments were carried out in HBS containing 10 mM $CaCl_2$ at a fibrinogen concentration of 1.5 mg/ml (4.4 μM) and E-des A or E-des AB at a ~10% molar excess with respect to fibrinogen. XIIIa was added last at a final concentration of 100 u/ml as timing was initiated. For electrophoretic analyses, reactions were terminated by adding an equal volume of 5% SDS, 10 mM Tris, 1 mM EDTA, 10% β-mercaptoethanol, pH 8 solution. Samples were then subjected to electrophoresis on 8 to 25% gradient gels in a Phast gel apparatus (Pharmacia/LKB). For densitometry, Coomassie blue-stained gels were scanned with an AlphaImager CCD camera and then analyzed with AlphaEase image processing and analysis software (Alpha Innotech Corp., San Leandro, CA). The degree of γ chain crosslinking was determined from the ratio of γ dimers to the total γ chain population, γγ/(γ + γγ). The degree of Aα chain crosslinking to form α polymers was determined from densitometric scans of the Aα chain position, using the Bβ chain band for normalization.

Specimen processing for STEM and mass measurements. Samples for STEM analyses were assembled under the same conditions described for electrophoresis experiments, up to the sample processing step. For STEM measurements, timed samples were rapidly diluted in HBS to a final concentration of 3 to 5 μg/ml, loaded onto ultrathin carbon films by injecting 3 μL into a 3 μL droplet of sample buffer on the grid surface, and allowing an attachment time of 1 min before exchanging the fluid on the grid surface with multiple wicking/washing with 150 mM ammonium acetate, pH 7. The grid was then snap frozen in degassed liquid nitrogen, freeze-dried, and transferred to the STEM microscope stage under vacuum. Specimens were imaged at the Brookhaven STEM Biotechnology Resource Facility by using a 40-kv probe focused at 0.25 nm, usually at a magnification of 125,000 (512 nm full scale width) at an electron dose of ~2.5×10^3 e-/nm^2, and stored digitally as a raster of 512 × 512 pixels. Background filtering of digitized STEM images was optimized for contrast and brightness offset by an image processing program (Adobe Photoshop). Mass measurements on STEM images were based on electron scattering measurements and were performed off-line as described (35, 36, 37).

Results

We used hementin-derived fibrinogen fragment E for fibrinogen assembly experiments. This fragment is intact as far as fibrinopeptide-containing sequences are concerned (Fig. 1), and this unique feature provided us with a useful experimental design advantage over using plasmin-derived E fragments such as were used by Veklich et al. (24), since it permitted us to precisely compare E fragments with both E_A and E_B sites exposed (E-des AB), with E fragments having only E_A sites exposed (E-des A). In contrast, plasmic E fragments are usually obtained as a heterogeneous mixture lacking one, and possibly two, amino-terminal E_B sites, making such comparisons difficult (21).

Crosslinking of fibrinogen or des αC-fibrinogen on a fibrin fragment E template. As assessed by SDS-PAGE analyses of γ chain dimer formation, XIIIa-containing mixtures of fibrinogen or des αC-fibrinogen with E-des AB or E-des A became crosslinked much more rapidly than did fibrinogen alone, or des αC-fibrinogen alone, which was even slower than fibrinogen (Fig. 2). The enhanced E-dependent fibril assembly and crosslinking rates were attributable to the presence of fibrin E fragments, but were nevertheless slower and less complete than that of fibrin, which became 100% dimerized within 5 min. In contrast to the fibrin crosslinking rate, the initial rapid crosslinking rates of fibrinogen-fragment E mixtures slowed and leveled off before reaching 80% completion.* We believe that this effect is related to covalent 'fixation' of fiber network architecture or to formation of single-stranded loops (cf., Fig. 3), or both, which effectively limit fibril-fibril and

* The results shown in Figure 2 were carried out as a single experimental set. Fibrinogen crosslinking experiments on fibrin E templates were repeated 4 to 6 times, and there were no statistically significant differences in the initial crosslinking rates among the various conditions.

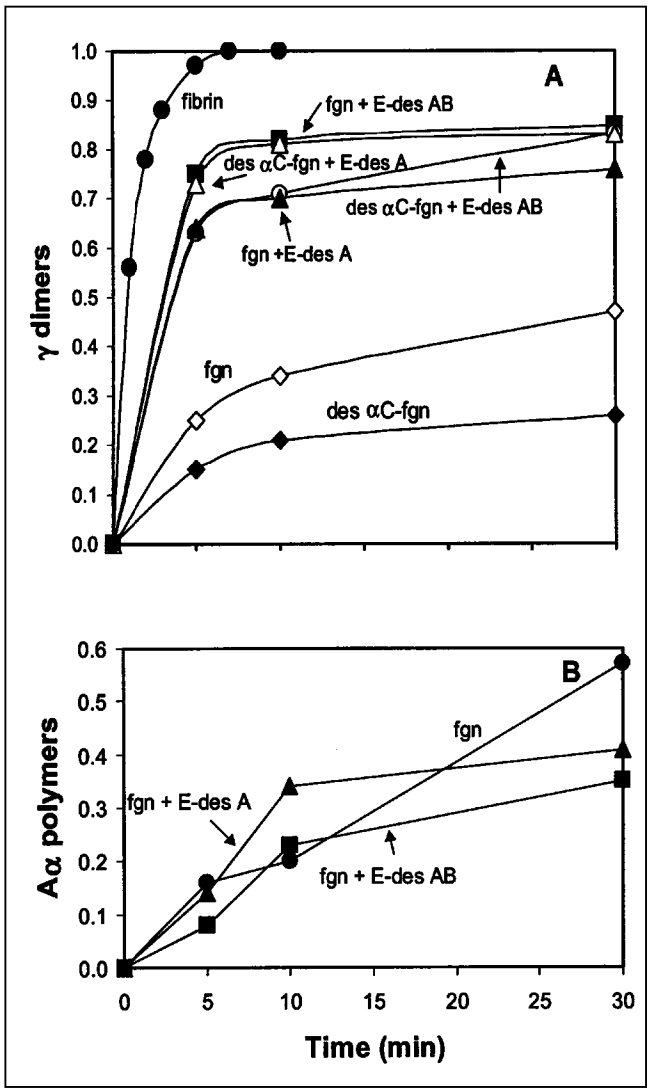


Fig. 2 The rate of factor XIIIa-mediated γ and Aα chain crosslinking of various mixtures of fibrinogen, des αC-fibrinogen, E-des AB, and E-des A or fibrin. Panel A, γ dimers; panel B, Aα polymers

strand-strand interactions. The relatively rapid initial crosslinking rate of fibrinogen compared with des αC-fibrinogen suggests that αC domains enhance lateral association of fibril strands, and thereby the γ chain crosslinking rate. Such observations fit well with other data indicating that fibrinogen αC domains participate in lateral fibrin fibril associations (16, 17, 18, 19), thus enhancing fibrinogen γ chain crosslinking (38).

The initial Aα chain crosslinking rate was about the same under all conditions (panel B) and always was less than that of the γ chains. In this respect, we did not observe the reversal in the sequence of initial chain crosslinking (i.e., Aα > γ) reported by Veklich et al. (24). However, in the "fibrinogen-alone" system, the level of Aα chain crosslinking eventually exceeded that of the γ chains.

We also compared the fibrinogen/E-des AB and the fibrinogen/E-des A crosslinking systems by SDS-PAGE analyses of timed samples on non-reduced as well as disulfide-reduced gels, with respect to their rates of γ chain crosslinking and the size of the crosslinked fibrinogen polymers (data not shown). The crosslinking rates and the sizes of the crosslinked fibrinogen polymers formed were indistinguishable from one another during the entire period of sampling and included rapid and progressive formation of very large crosslinked fibrinogen polymers. In other words, there were no unusual or unexpected γ chain pairings (e.g., "closed" crosslinked fibrinogen tetramers** in these systems other than the concatenary crosslinking arrangement that is expected to take place during assembly of crosslinked fibrinogen fibrils (20). This observation was useful for correctly modeling the γ chain crosslinking pattern in these systems (vide infra).

STEM images of mixtures of fibrinogen and fibrin fragment E. STEM images of mixtures of fibrinogen, E-des AB, plus factor XIIIa sampled at 3 min. (Fig. 3, panel A) or 10 min (Fig. 3, panel B) showed numerous single-stranded fibrils which commonly overlapped one another (panel B-fields 1 and 2) or formed overlapping loops (panel A-field 2). The oblong-shaped structures disposed regularly along the fibril strands were "DED" complexes that were comprised of a hemen-

** This type of crosslinking arrangement might be expected to occur if γ chains became crosslinked directly across side-to-side oriented DED complexe, rather than in the expected concatenated pattern that is illustrated in figure 1 (3).

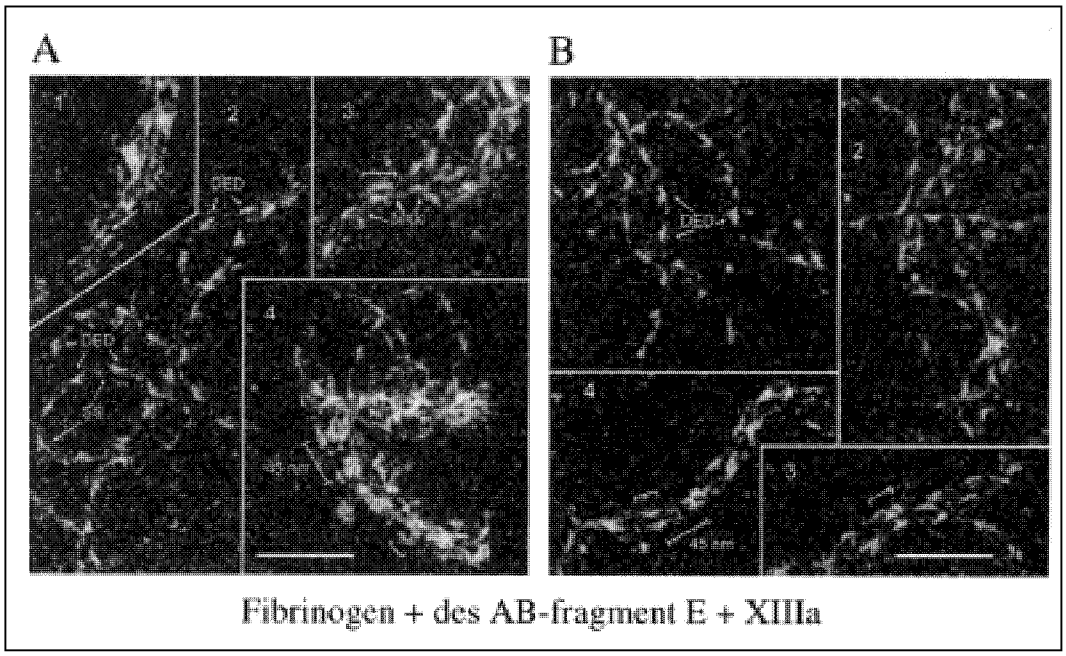


Fig. 3 STEM images of fibrinogen assembly and crosslinking on a des AB-fragment E template. Panel A, 3 min sample; Panel B, 10 min sample. "γ:γ" indicates intermolecular bridging between D domains. DED complexes in single-stranded ("SS") arrangements are indicated. Side-to-side oriented DED complexes are indicated by unidirectional brackets; 45 nm spacing by bi-directional brackets. Probable intermolecular β_C:β_C contacts are indicated. Horizontal bars, 100 nm

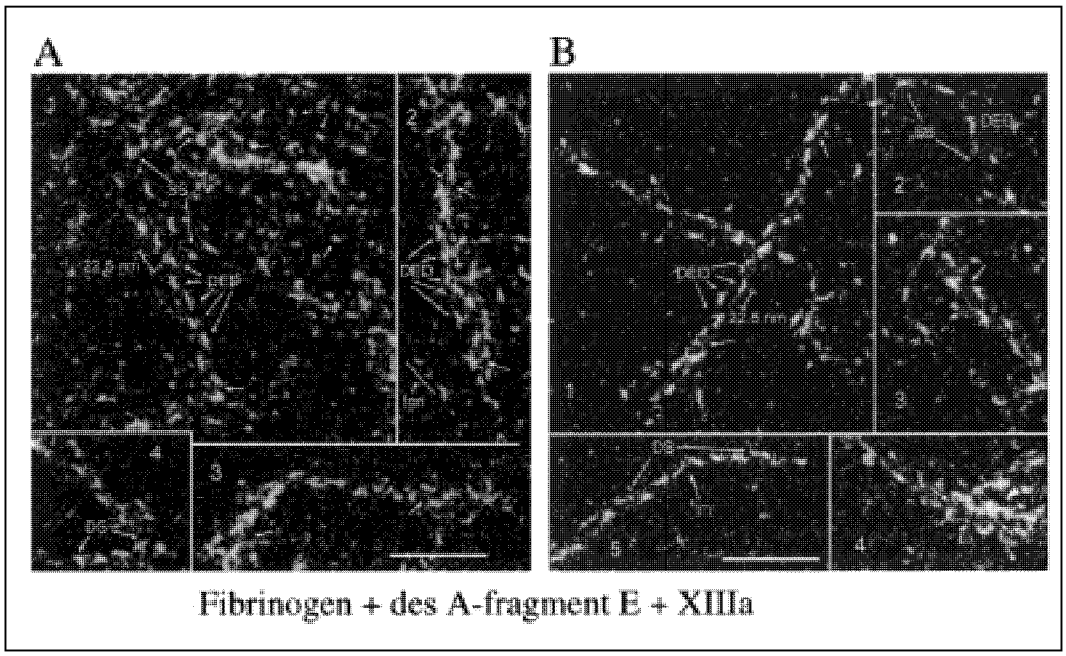


Fig. 4 STEM images of fibrinogen assembly and crosslinking on a des A-fragment E template. Panel A, 5 min sample; Panel B, 10 min sample. Some DED complexes and hementin E fragments ("E") are labeled. Single- ("SS") and double-stranded ("DS") fibrils possessing 22.5 nm spacing are indicated. "γ:γ" indicates intermolecular bridging between D domains. The arrowheads point toward molecular aggregates. Horizontal bars, 100 nm.

tin-derived fibrin E fragment that was non-covalently complexed with two fibrinogen D domains (DED), as diagrammed in Fig. 1. The molecular composition of DED was verified by STEM mass measurements under each condition, and indicated a mass of 271 ± 15 kDa (n, 31) for E-des AB-containing DED complexes (computed mass, 269 kDa), and 267 ± 22 kDa (n, 23) for E-des A-containing DED complexes (computed mass, 271 kDa). Double- or multi-stranded fibrils were found commonly (panel A-fields 1, 3, 4; panel B-fields 3, 4) and DED complexes between fibril strands were usually aligned side-to-side (brackets), thus retaining the major 45 nm periodicity conferred by the constituent fibrinogen molecules***. Side-to-side alignment of DED complexes is attributable to E_B:Db interactions triggering β_C subdomain rearrangements that in turn promote intermolecular contacts between β_C domains [see Fig. 1, (2) or (3)]. The putative β_C:β_C contacts were often identifiable as proteinaceous or filamentous bridges at the proximal ends of DED complexes ("β_C:β_C"). Filamentous bridges between the middle portions of DED complexes most likely reflect γ chain interactions ("γ:γ")****.

Images of crosslinking fibrinogen/E-des A mixtures revealed relatively fewer single-stranded fibrils (Fig. 4, panels A and B) compared to the fibrinogen/E-des AB system, but in their place were double-stranded fibrils (some marked "DS"), multi-stranded fibrils or fibrillar aggregates (Panel A-fields 1, 2; panel B-field 4). Fibrillar structures seemed to be better organized in the 10 min sample (panel B) compared to the 5 min sample (panel A), consistent with the degree of crosslinking that had taken place. Filaments bridging between strands of these fibrils were commonly observed and in this crosslinking system reflected inter-strand γ chain bridging ("γ:γ" and unlabeled arrows). The inter-strand alignment of laterally associated E-des A-based fibrils differed markedly from that observed in the E-des AB system, in that DED complexes in double-stranded fibrils were in a half-staggered arrangement, yielding a major period of 22.5 nm as diagrammed in Figs. 1, (5) or (6). Our interpretation of the half-staggered double-stranded arrangement is when only E_A sites are available on E-des A for interaction with Da sites, fibrinogen γ_{XL} sites bridge between fibril strands and preferentially orient opposing strands in a half-staggered arrangement. These interacting sites become crosslinked by factor XIIIa as depicted in Fig. 1 (6). The same intermolecular arrangement and 22.5 nm periodicity occurs with crosslinked fibrinogen fibrils (20).

STEM images of crosslinking des αC-fibrinogen/E-des AB mixtures with factor XIIIa revealed single-stranded fibrils ("SS") that were indistinguishable from the single-stranded structures formed with intact fibrinogen (Fig. 5, panel A). Double-stranded polymers tended to be shorter and less-well organized than those formed from mixtures containing intact fibrinogen, and often formed difficult-to-interpret aggregates (panel A, fields 1, 3). Nevertheless, DED complexes comprising these fibrils were often associated side-to-side (brackets).

To assess the contribution of factor XIIIa-mediated crosslinking to fibrinogen fibril assembly, we studied fibrin E-fibrinogen mixtures without added factor XIIIa (Fig. 5, panel B; Fig. 6). Images of fibrinogen/E-des AB mixtures revealed the same DED-based fibrillar structures as had been found in the presence of XIIIa (Fig. 6, panel A). Single-stranded fibrinogen fibrils (SS) were found commonly, as well as poorly defined aggregates (arrowheads) and multi-stranded fibrillar aggregates (double arrowhead, field 2). Double-stranded structures, per se, were uncommon, suggesting that γ chain crosslinking by XIIIa (cf., Fig. 3) helps to "maintain" double-strandedness over multi-stranded fibrils or aggregates. Nevertheless, there were recognizable side-to-side DED alignments (brackets). Occasional filamentous structures ("γ:γ") bridging between DED domains were observed, and probably represented

*** C-terminal regions of γ chains are known to be flexible (39). Although their preferred molecular orientation is proximal, as depicted in figure 1 (1) or (4), it is easy to visualize how they might become repositioned as a result of β_C:β_C contacts, and thus come to lie in the positions depicted in (2) or (3).

****The ability of STEM to resolve filamentous structures extending from or bridging between globular protein domains has previously been demonstrated in studies of porcine factor VIII (40) as well as in studies on crosslinked fibrn or fibrinogen (20, 41).

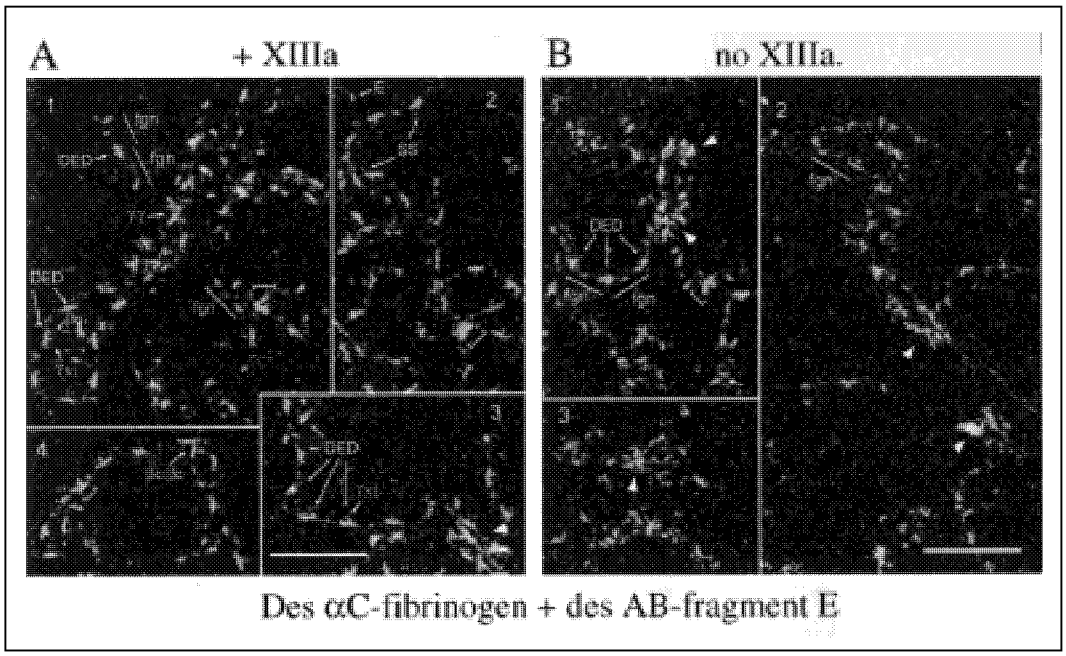


Fig. 5 STEM images of des αC-fibrinogen assembly on a des AB-fragment E template. Panel A, plus factor XIIIa sampled at 12 min; panel B, without factor XIIIa sampled at 12 min. "γ:γ" indicates filaments that are bridging between D domains; "γ_{XL}" indicates a probable γ chain extension from a DED complex. Proposed intermolecular β_C:β_C contacts or putative "β_C" subdomains are indicated. Arrowheads point toward molecular aggregates. Some DED complexes and fibrinogen molecules are highlighted. Side-to-side oriented DED complexes are indicated by unidirectional brackets. Horizontal bars, 100 nm

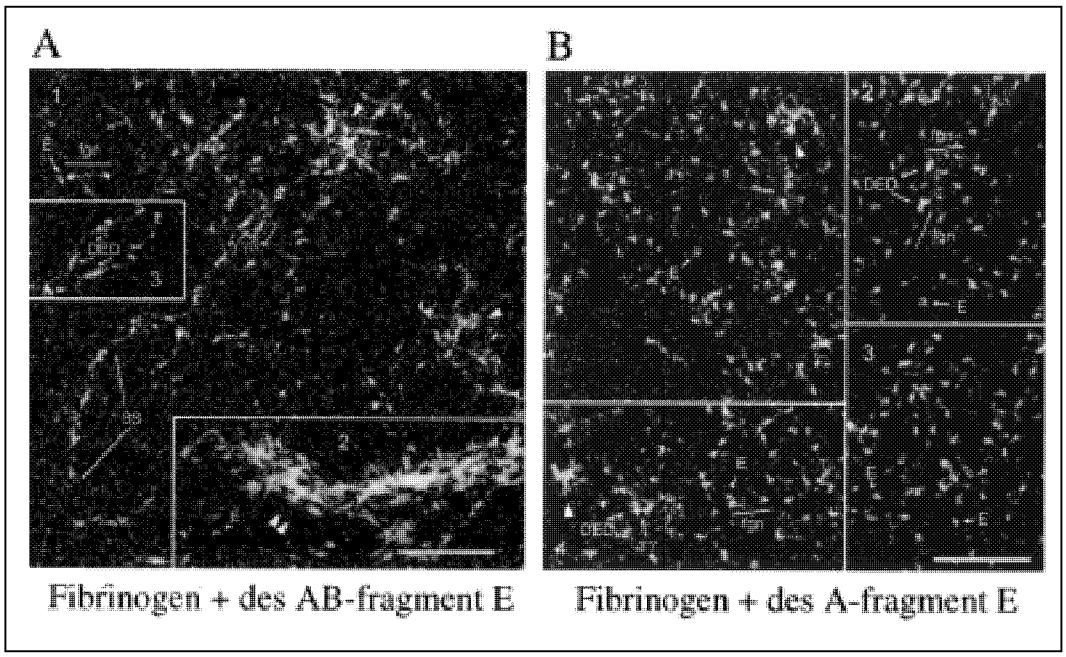


Fig. 6 STEM images of assembly of fibrinogen on a des AB-fragment E template (Panel A, 10 min sample) or on a des A-Fragment E template (Panel B, 10 min sample) without added factor XIIIa. Arrowheads point toward molecular aggregates. Double arrowheads point to multistranded linear aggregates. Some DED complexes, E fragments, and fibrinogen molecules are highlighted. Side-to-side oriented DED complexes are indicated by unidirectional brackets. Horizontal bars, 100 nm

interacting γ chains. Unassociated fibrinogen molecules and E fragments were also found, as in the other systems.

Images of fibrinogen/E-des A mixtures without XIIIa (Fig. 6, panel B) formed DED-based structures that tended to be shorter than in other assembly systems (e.g., panel B, field 2), plus numerous ill-defined aggregates (arrowheads) and occasional filamentous bridges between DED complexes ("γ:γ"). Double-stranded fibrils, per se, in these mixtures were not well delineated, suggesting that γ_{XL} site crosslinking plays a prominent role in forming double-stranded fibrils in this system.

Des αC-fibrinogen-E-des AB mixtures lacking XIIIa (Fig. 5, panel B), formed similar structures to those observed in the crosslinking mixture (Fig. 5, panel A) including single-stranded DED-based fibrils plus loosely organized and ill-defined aggregates (arrowheads). Side-to-side oriented DED complexes were difficult to find. This suggests that although DED assembly involving des αC-fibrinogen takes place readily on the fibrin E template, αC domains play a constructive role in orienting the fibril strands, even when E_B sites are available to promote lateral association.

Discussion

Our studies provide new observations on the assembly and crosslinking of fibrinogen molecules on a fibrin E template, including information on the roles played by E_A and E_B sites, γ chains, αC domains, β_C domains, and factor XIIIa in this process. The overall results provide strong evidence for "transverse" γ chain positioning and crosslinking between fibrinogen fibril strands, adding to an existing body of experiments that seem to us to be persuasive in their own right (23). The present observations and considerations that contribute to this conclusion are the following: 1) Fibrin fragment E is required for enhancing XIIIa-mediated crosslinking of fibrinogen or des αC-fibrinogen, and γ chain crosslinking is increased to virtually the same extent in either case, thus indicating that αC domains, per se, contribute little to this process. However, since crosslinking of des αC-fibrinogen alone is much slower than that of fibrinogen itself, αC domains do indeed promote fibrinogen fibril assembly and crosslinking, thus confirming observations made in previous studies on fibrinogen crosslinking (20, 38). 2) Fibrinogen or des αC-fibrinogen assembly and crosslinking on a fibrin E template is accompanied by accelerated crosslinking and concomitant lateral strand and fibril associations. Even without benefit of further details, this type of event favors transverse γ chain crosslinking, though not to the exclusion of at least some degree of end-to-end crosslinking. 3) The arrangement of laterally associated fibril strands depends upon whether E_B sites are available in addition to E_A sites, and when both sites are available, opposing DED complexes become oriented side-to-side. This is evidently due to β_C:β_C contacts that are promoted as a result of E_B:Db interactions (12). In this system, filamentous bridging across fibril strands is attributable either to β_C:β_C contacts or to bridging by γ chain γ_{XL} sites, and a morphological distinction between them can often be made. Bridges due to β_C:β_C contacts are situated in the more N-terminal regions (i.e., proximal) of side-to-side oriented DED complexes, whereas filamentous bridging in the middle portion of side-to-side oriented DED complexes is most likely attributable to γ chain bridging. The fact that in this system there exist two contact mechanisms for forming laterally associated fibril strands, seems to be consistent with either an end-to-end or transverse crosslinking mechanism. 4) γ_{XL} sites comprise an independent self-association and singular crosslinking site that contributes importantly to the lateral strand association and half-staggered alignment of crosslinked fibrinogen fibril strands (20, 39). By that same token, the half-staggered alignment of fibril strands that we observed in the fibrinogen/E-des A system almost certainly results from "transverse" interstrand γ_{XL} interactions and crosslinking. Filamentous connections that bridge between the DED complexes of fibril strands in this system provide additional evidence for the location of γ_{XL} sites between fibril strands. Taken together, these findings constitute a persuasive argument for transverse positioning of γ_{XL} sites between fibrinogen fibril strands.

Our conclusions regarding the location of crosslinked γ chains in fibrinogen fibrils are distinctly different from those drawn by our colleagues in Pennsylvania (24). We think it is valuable to examine the details behind their argument that crosslinked γ chains in fibrin are situated in end-to-end positions along fibril strands. This interpretation was mainly based upon the appearance of factor XIIIa-crosslinked fibrinogen fibrils that had been dissociated in solutions containing 30% glycerol under non-physiological conditions, i.e., in acetic acid solutions. The fibrinogen molecules in these single-stranded fibrils were aligned end-to-end, as were the crosslinked γ chains that connected them. Although this much is indisputable, the further extrapolation that the alignment in acetic acid solutions predicts the arrangement in native fibrin fibrils is inconsistent with existing data (23). For one example, in related studies on the structure of crosslinked D-fibrin-D complexes we showed that under physiological conditions, crosslinked γ chains in D-fibrin-D complexes were transversely oriented with respect to the arrangement of D domains on the fibrin template (41). This folded arrangement had resulted from non-covalent Da:E_A interactions. However, in the presence of acetic acid, D-fibrin-D complexes became unfolded, and the covalently linked D fragments came to reside at the ends of fibrin molecules in end-to-end alignment with respect to their crosslinked γ chains.

Secondly, in our current experiments we've shown that DED-driven assembly and crosslinking of fibrinogen fibrils formed on E-des AB or E-des A is always accompanied by extensive lateral strand and fibril associations. Since DED assembly takes place without crosslinking (Figs. 5 and 6), the mere demonstration that fibrinogen molecules become assembled on a fibrin E template, cannot alone predict where the γ chains are situated. In our view, a valid argument for end-to-end crosslinking of γ chains in the fibrin fragment E assembly system under physiological buffer conditions would require that all DED-based fibrils remain single-stranded throughout the period of factor XIIIa crosslinking. Simply stated, this does not occur.

In this connection, it is worth noting that Veklich et al. reported a tendency for lateral fibril aggregation, but they apparently did not apply this observation in rendering their interpretations. Since they sampled their mixtures for microscopy well after γ chain crosslinking had been completed (12 to 24 h), we speculate that they would not have observed the extensive lateral fibril association and aggregation that is evident at much earlier times. At their late sampling time, the crosslinking mixture would probably have become largely depleted of observable multi-stranded fibrils and aggregates due to extensive self-association or precipitation, or both.

In summary, when E_B and E_A sites are available in the fibrin E fragment (E-des AB), DED complexes in fibrinogen fibril strands become aligned side-to-side. This observation is consistent with transverse crosslinking, but does not exclude end-to-end crosslinking. However, when only E_A sites are available in fibrin fragment E (E-des A), DED complexes become aligned in a staggered overlapping arrangement owing to preferential orientation by γ_{XL} sites [Fig. 1, (5), (6)]. In this case, only transverse γ chain crosslinking can account for the formation of such structures. We further believe that it is valid to extend this logic as a general model for fibrin fibril γ chain crosslinking.

Acknowledgements

This study was supported by NIH Grant HL-59407 and RR-01777, and by the U.S. Dept of Energy, Office of Health and Environmental Research. The recent report by Yang et al. (12) was helpful for modeling the assembly of fibrinogen molecules on a des AB-fragment E template.

References

1. Scheraga HA, Laskowski MJr. The fibrinogen-fibrin conversion. Adv Prot Chem 1957; 12: 1-131.
2. Blombäck B. Studies on the action of thrombotic enzymes on bovine fibrinogen as measured by N-terminal analysis. Arkiv Kemi 1958; 12: 321-35.
3. Blombäck B, Hessel B, Hogg D, Therkildsen L. A two-step fibrinogen-fibrin transition in blood coagulation. Nature 1978; 275: 501-5.
4. Shimizu A, Nagel GM, Doolittle RF. Photoaffinity labeling of the primary fibrin polymerization site: Isolation of a CNBr fragment corresponding to γ 337-379. Proc Natl Acad Sci USA 1992; 89: 2888-92.

5. Pratt KP, Côté HCF, Chung DW, Stenkamp RE, Davie EW. The primary fibrin polymerization pocket: three-dimensional structure of a 30-kDa C-terminal γ chain fragment complexed with the peptide gly-pro-arg-pro. Proc Natl Acad Sci USA 1997; 94: 7176-81.
6. Everse SJ, Spraggon G, Veerapandian L, Riley M, Doolittle RF. Crystal structure of fragment double-D from human fibrin with two different bound ligands. Biochemistry 1998; 37: 8637-42.
7. Ferry JD. The mechanism of polymerization of fibrinogen. Proc Natl Acad Sci USA 1952; 38: 566-9.
8. Krakow W, Endres GF, Siegel BM, Scheraga HA. An electron microscopic investigation of the polymerization of bovine fibrin monomer. J Mol Biol 1972; 71: 95-103.
9. Fowler WE, Hantgan RR, Hermans J, et al. Structure of the fibrin protofibril. Proc Natl Acad Sci USA 1981; 78: 4872-6.
10. Shainoff JR, Dardik BN. Fibrinopeptide B in fibrin assembly and metabolism: physiologic significance in delayed release of the peptide. Ann NY Acad Sci 1983; 408: 254-67.
11. Medved LV, Litvinovich SV, Ugarova TP, Lukinova NI, Kilikhevich VN, Ardemasova ZA. Localization of a fibrin polymerization site complimentary to Gly-His-Arg sequence. FEBS Letters 1993; 320: 239-42.
12. Yang Z, Mochalkin I, Doolittle RF. A model of fibrin formation based on crystal structures of fibrinogen and fibrin fragments complexed with synthetic peptides. Proc Natl Acad Sci USA 2000; 97: 14156-61.
13. Weisel JW, Medved L. The structure and function of the αC domain of fibrinogen. Ann NY Acad Sci 2001; 936: 312-27.
14. Mosesson MW, Finlayson JS, Umfleet RA, Galanakis DK. Human fibrinogen heterogeneities. I. Structural and related studies of plasma fibrinogens which are high solubility catabolic intermediates. J Biol Chem 1972; 247: 5210-9.
15. Mosesson MW, Galanakis DK, Finlayson JS. Comparison of human plasma fibrinogen subfractions and early plasmic fibrinogen derivatives. J Biol Chem 1974; 249: 4656-64.
16. Mosesson MW, Sherry S. The preparation and properties of human fibrinogen of relatively high solubility. Biochemistry 1966; 5: 2829-35.
17. Mosesson MW. Fibrinogen Heterogeneity. Ann NY Acad Sci 1983; 408: 97-113.
18. Hasegawa N, Sasaki S. Location of the binding site "b" for lateral polymerization of fibrin. Thromb Res 1990; 57: 183-95.
19. Medved LV, Gorkun OV, Manyakov VF, Belitser VA. The role of fibrinogen αC-domains in the fibrin assembly process. FEBS Letter 1985; 181: 109-12.
20. Mosesson MW, Siebenlist KR, Hainfeld JF, Wall JS. The covalent structure of factor XIIIa crosslinked fibrinogen fibrils. J Struct Biol 1995; 115: 88-101.
21. Samokhin GP, Lorand L. Contact with the N termini in the central E domain enhances the reactivities of the distal D domains of fibrin to factor XIIIa. J Biol Chem 1995; 270: 21827-32.
22. Kanaide H, Shainoff JR. Cross-linking of fibrinogen and fibrin by fibrin-stabilizing factor (factor XIIIa). J Lab Clin Med 1975; 85: 574-97.
23. Mosesson MW, Siebenlist KR, Meh DA. The structure and biological features of fibrinogen and fibrin. Ann NY Acad Sci 2001; 936: 11-30.
24. Veklich YI, Ang EK, Lorand L, Weisel JW. The complementary aggregation sites of fibrin investigated through examination of polymers of fibrinogen with fragment E. Proc Natl Acad Sci USA 1998; 95: 1438-42.
25. Lorand L, Parameswaren KN, Murthy SNP. A double-headed Gly-Pro-Arg-Pro ligand mimics the functions of the E domain of fibrin for promoting the end-to-end crosslinking of γ chains by factor XIII_{α}. Proc Natl Acad Sci USA 1998; 95: 537-41.
26. Siebenlist KR, Meh DA, Mosesson MW. Plasma factor XIII binds specifically to fibrinogen molecules containing γ' chains. Biochemistry 1996; 35: 10448-53.
27. Henschen A, Lottspeich F, Kehl M, Southan C, Lucas J. Structure-function-evolution relationship in fibrinogen. In: Fibrinogen. Recent biochemical and medical aspects. Henschen A, Graeff H, Lottspeich F, eds. Berlin: W de Gruyter; 1982: 67-82.
28. Veklich YI, Gorkun OV, Medved LV, Niewenhuizen W, Weisel JW. Carboxyl-terminal portions of the a chains of fibrinogen and fibrin. J Biol Chem 1993; 268: 13577-85.
29. Lorand L, Gotoh T. Fibrinoligase. The fibrin stabilizing factor. Methods Enzymol 1970; 19: 770-82.
30. Loewy AG, Dunathan K, Kriel R, Wolfinger HL. Fibrinase I. Purification of substrate and enzyme. J Biol Chem 1961; 236: 2625-33.
31. Malinconico SM, Katz JB, Budzynski AZ. Hementin: anticoagulant protease from the salivary gland of the leech Haementeria ghilianii. J Lab Clin Med 1984; 103: 44-58.
32. Marder VJ, Budzynski AZ, James HL. High molecular weight derivatives of human fibrinogen produced by plasmin: (III) Their NH2-terminal amino acids and comparison with the NH2-terminal disulfide knot. J Biol Chem 1972; 247: 4775-81.
33. Townsend RR, Hilliker E, Li YT, Laine RA, Bell WR, Lee YC. Carbohydrate structure of human fibrinogen. J Biol Chem 1982; 257: 9704.
34. Kirschbaum NE, Budzynski AZ. A unique proteolytic fragment of human fibrinogen containing the Aα COOH-terminal domain of the native molecule. J Biol Chem 1990; 265: 13669-76.
35. Mosesson MW, Hainfeld JF, Haschemeyer RH, Wall JS. Identification and mass analysis of human fibrinogen molecules and their domains by scanning transmission electron microscopy. J Mol Biol 1981; 153: 695-718.
36. Hainfeld JF, Wall JS, Desmond EJ. A small computer system for micrograph analysis. Ultramicroscopy 1982; 8: 263-70.
37. Wall JS, Hainfeld JF. Mass mapping with the scanning transmission microscope. Ann Rev Biophys Biophys Chem 1986; 15: 355-76.
38. Mosesson MW, Siebenlist KR, Hainfeld JF, et al. The relationship between the fibrinogen D domain self-association/crosslinking site (γXL) and the fibrinogen Dusart abnormality (Aα R554C-albumin). J Clin Invest 1996; 97: 2342-50.
39. Mosesson MW, Siebenlist KR, Meh DA, Wall JS, Hainfeld JF. The location of the carboxy-terminal region of γ chains in fibrinogen and fibrin D domains. Proc Natl Acad Sci USA 1998; 95: 10511-6.
40. Mosesson MW, Fass DN, Lollar P, et al. Structural model of porcine factor VIII and factor VIIIa molecules based on scanning transmission electron microscope (STEM) images and STEM mass analysis. J Clin Invest 1990; 85: 1983-90.
41. Siebenlist KR, Meh DA, Wall JS, Hainfeld JF, Mosesson MW. Orientation of the carboxy-terminal regions of fibrin γ chain dimers determined from the crosslinked products formed in mixtures of fibrin, fragment D, and factor XIIIa. Thromb Haemost 1995; 74: 1113-9.

Received September 28, 2001 Accepted after revision December 14, 2001