Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 1438–1442, February 1998
Biochemistry

The complementary aggregation sites of fibrin investigated through examination of polymers of fibrinogen with fragment E

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Contributed by Laszlo Lorand, December 22, 1997

ABSTRACT **Fibrin polymerizes through the interaction of sites exposed by the thrombin-mediated cleavage of fibrinopeptides in the central E region of the protein and complementary sites near the ends of the molecules, open in the D regions of both fibrinogen and fibrin. A preparation of fragment E, containing the central domain and part of the coiled-coil regions of fibrin, was used in mixtures with fibrinogen in this electron microscopy study to investigate the formation of fibrillar structures. At short times, linearly ordered oligomers of fibrinogen were observed with an additional mass of E fragments at the end-to-end junctions. At later times, long flexible polymers made up of 30 or more fibrinogen and fragment E units, with a tendency for lateral aggregation and tangle formation, were seen. These single-stranded assemblies could be readily dissociated in dilute acetic acid into their fibrinogen and fragment E components. However, if the aggregates were treated with factor XIIIa so that all γ chains became ligated by $N^{\epsilon}(\gamma$-glutamyl)lysine linkages, the polymers could no longer be taken apart. Because the only γ chains in the preparation are present in the fibrinogen molecules interacting end-to-end, the findings show that the factor XIIIa-induced cross-linking of γ chains in the clotting of fibrinogen or fibrin must occur between molecules that are longitudinal (or end-to-end) rather than transverse (or half-staggered).**

Fibrinogen, which is made up of three pairs of polypeptide chains $(A\alpha B\beta\gamma)_2$, is about 47 nm long and has a molecular mass of 340 kDa (1–6). The amino-terminal ends of all three pairs of chains are joined together by disulfide bonds in the central region of the molecule. The carboxyl-terminal ends of the $B\beta$ chains contain the proximal end regions, and the carboxyl-terminal ends of the γ chain contain the distal end regions (7). Each carboxyl-terminal $A\alpha$ chain consists of a globular αC domain that associates with the central region and a connector to the distal molecular end (8, 9). The central and end regions are connected by a three-chain α-helical coiled-coil. The overall shape of the molecule has been determined by coordinated x-ray crystallography and cryoelectron microscopy (10). Recently, x-ray crystallographic studies have revealed atomic resolution structures of the carboxyl-terminal γ chain and the end regions of the molecule (11, 12).

The particular bonds of fibrin(ogen) cleaved by plasmin and the products of such proteolysis have been characterized (13, 14). The carboxyl-terminal segment of the $A\alpha$ chain and the amino-terminal segment of the $B\beta$ chain are removed first, followed by splitting of all three chains in the middle of the coiled-coil to yield fragment D, which includes the end domains and distal coiled-coil, plus fragment Y, which contains the remainder of the molecule. Fragment Y can then be split to yield another fragment D and fragment E, which contains the central domain and both proximal portions of coiled-coil (Fig. 1*A*).

Because these different fragments contain distinctly different domains of fibrinogen, they have been used for a variety of functional studies. The individual domains involved in interactions of fibrinogen with many other proteins that specifically bind to it have been localized by using these and other fragments. As another example, fragment D has been shown to inhibit polymerization of fibrin (15).

Fibrin monomers produced by cleavage of the A fibrinopeptides from fibrinogen with thrombin interact in a half-staggered manner to yield small oligomers that lengthen to produce two-stranded protofibrils, which consist of fibrin molecules that are half-staggered and bonded end-to-end (1–6). To produce these structures, aggregation sites in the central part of the molecule exposed by removal of the fibrinopeptides interact with complementary sites at each end of the molecule (Fig. 1*B*). The A fibrinopeptides are removed first, exposing what are known as the A sites, which interact with the a sites at the carboxyl-terminal ends of the γ chains. After initiation of polymerization, B fibrinopeptides are cleaved, exposing B sites that interact with b sites at the molecular ends. When protofibrils have grown sufficiently long, they associate with each other laterally to yield fibers that branch to form a three-dimensional network.

The clot is stabilized by the formation of $N^{\epsilon}(\gamma$-glutamyl)lysine cross-links induced by the transglutaminase factor XIIIa (2, 16, 17). Isopeptide bonds are formed between the carboxyl-terminal γ chains of two fibrin molecules (which are part of fragment D), and several bonds are formed between the carboxyl-terminal α chains. The topology of the γ chain cross-links has been investigated by several different methods, with some apparently conflicting results, giving rise to two distinctly different models of γ–γ chain cross-linking, one linking the γ chains longitudinally (end-to-end) and the other transversely (half-staggered). Studies to differentiate between these two models have used electron microscopy to examine cross-linked fibrin fragments (18). After plasmin digestion of cross-linked fibrin clots, the fragments in the resulting complexes were dissociated from any noncovalently cross-linked species with dilute acetic acid and examined by electron microscopy. Because the fragments observed were single-stranded, the cross-links must be between end-to-end bonded molecules, because they would be double-stranded if the cross-links were between half-staggered molecules. On the other hand, studies of cross-linked fibrinogen and fragment D–fibrin complexes suggested that the cross-links were transverse (19, 20).

In this article, we show electron microscope images of polymers formed by mixing des-AB fragment E with fibrinogen. The polymers reveal aspects of the molecular mechanism of fibrin polymerization, because these studies demonstrate that the A and B sites of fragment E can interact with the a and

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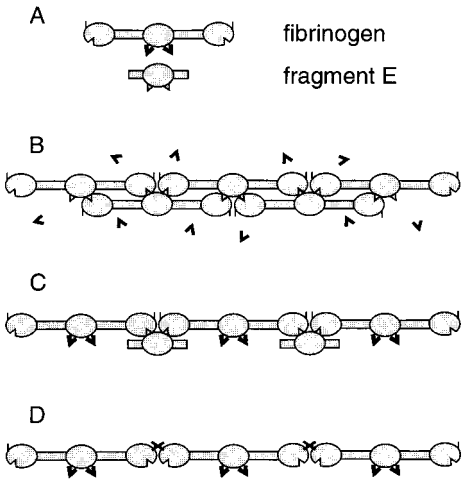

FIG. 1. Schematic diagram of interactions between complementary aggregation sites in fibrin and in fibrinogen–fragment E polymers. Trinodular fibrinogen molecules contain aggregation sites in the middle that are represented as triangular knobs; they are covered by the fibrinopeptides, which are portrayed as dark wedge-shaped structures over the knobs. At each end of the molecules, there are triangular holes that represent the sites complementary to the knobs. The portions of the carboxyl-terminal γ chains that are cross-linked by factor XIIIa are depicted as short strands at the ends of the molecules. (*A*) Individual fibrinogen molecule and des-AB fragment E. (*B*) A two-stranded fibrin protofibril, produced after cleavage of the fibrinopeptides with thrombin and showing interactions between complementary aggregation sites. (*C*) A single-stranded fragment E–fibrinogen polymer, also showing interactions between complementary aggregation sites. (*D*) A single-stranded fragment E–fibrinogen polymer after cross-linking with factor XIIIa (represented as the $\times$ joining carboxyl-terminal γ chains at the ends of molecules) and dissociation of fragment E in acetic acid.

b sites of fibrinogen. In addition, the fact that the fibrinogen molecules in these polymers can be ligated by factor XIIIa indicates that the cross-links in γ–γ dimers occur between molecules that are oriented end-to-end.

MATERIALS AND METHODS

Fibrinogen was obtained from Kabi (A.B. Kabi, Stockholm, Sweden) and prepared as described (21). Recombinant human fibrinogen, prepared as described (22, 23), was a gift from Oleg V. Gorkun and Susan T. Lord (University of North Carolina, Chapel Hill). Fragment E was prepared by methods similar to those used previously (21). Factor XIII was purified as described (24, 25).

In a typical experiment, fibrinogen at a final concentration of 2 μM in 150 mM NaCl/50 mM Tris·HCl, pH 7.5/5 mM $CaCl_2$ was mixed with fragment E at a final concentration of 2 or 4 μM. Samples were taken for electron microscopy at various times from 0.25 hr to 48 hr. Factor XIII was activated with thrombin (21). Factor XIIIa at a final concentration of 9 μg/ml was incubated with the fragment E–fibrinogen polymers for 4 hr at 37°C. Fibrinogen, fragment E, and various cross-linked complexes were analyzed by SDS/PAGE under reducing conditions (26).

Rotary-shadowed samples were prepared by spraying a dilute solution of molecules in a volatile buffer (either 0.05 M ammonium formate at pH 7.4 or 0.125% acetic acid at pH 3.5) containing 30% glycerol onto freshly cleaved mica and shadowing with tungsten in a vacuum evaporator (Denton Vacuum, Cherry Hill, NJ) (7, 8, 27). Samples were prepared by dilution of concentrated protein solution with either ammonium formate or acetic acid and glycerol to a final concentration of about 25 μg/ml. All experiments were repeated several times and many micrographs were taken of randomly selected areas to ensure that the results were reproducible and representative. All of the specimens were examined in a Philips 400 electron microscope (Philips Electronic Instruments, Mahwah, NJ), usually operating at 80 kV.

RESULTS

Electron Microscopy of Fragment E–Fibrinogen Polymers. Fibrinogen was mixed with different ratios of des-AB fragment E in 50 mM Tris·HCl, pH 7.5/150 mM NaCl/5 mM $CaCl_2$. Samples were taken at various times, sprayed onto freshly cleaved mica, shadowed with tungsten in a vacuum evaporator, and examined by transmission electron microscopy. Examination of specimens revealed thin single-stranded chains of globular structures (Fig. 2). The long strands had the general appearance of a string of beads with pairs of nodules alternating with individual nodules. This pattern was very consistent, with the individual nodules having the same size and appearance as those of fibrinogen. In other words, these strands can be described as being made up of a repeating unit that is a trinodular structure with a length of about 45 nm. In some of these strands, additional substructure, such as subdivision of globular domains, could be visualized. Some strands were straight, others were curved, and some had sharp turns or kinks.

At short times, small oligomers that appeared to consist of only a few trinodular fibrinogen molecules were observed joined end-to-end (Fig. 2*A–C*). However, on closer examination, there was additional mass at the end-to-end junction (Fig. 2*A*, arrowheads). There was also some free fragment E in the background of these images (Fig. 2*A*, arrows), depending on the ratios of fragment E to fibrinogen in the starting mixture. At high ratios of fragment E to fibrinogen, there were more of these small structures appearing, whereas there were very few of them at lower ratios of fragment E to fibrinogen. When the structures at the end-to-end junctions in the polymers could be clearly visualized (Fig. 2*A*), they had an appearance similar to that of the free fragment E in the background.

At later times, long polymers made up of 30 or more fibrinogen and fragment E molecules were observed (Fig. 3*B*). The substructure of longer polymers was identical to that of the shorter oligomers. The individual components of fibrinogen and fragment E making up these polymers could be identified. Some of these long polymers were linear, although they often were curved or had a few bends, indicating flexibility (Fig. 3*B*).

Sometimes these polymers associated laterally with each other or formed complex tangles (Figs. 2 and 3). In some cases, two or more polymers were immediately adjacent to each other (Fig. 2*E*). Such lateral aggregation was seen more often for longer polymers than for shorter ones. In these structures, the end-to-end junctions between molecules in one of the polymers were aligned with those of the adjacent polymer or with the single central nodule. Sometimes, long polymers appeared to be folded back so that one end of the structure was interacting with the other end (Fig. 3*B*). Some tangles were too complex to interpret intermolecular interactions unambiguously.

Cross-Linking of Fragment E–Fibrinogen Polymers with Factor XIIIa. After formation of these fragment E–fibrinogen polymers, they were incubated for 4 hr with factor XIIIa. Electrophoresis on SDS/PAGE gels revealed that cross-linking occurred to such an extent that there were no individual γ chains remaining, with γ dimers and some α multimers being prominent features of these gels. In other words, all γ chains were ligated to form γ dimers and there were α polymers. There was no difference in the effects of 4 μM fragment E, in comparison with 2 μM fragment E. However, in the fragment E–fibrinogen polymers the rate of cross-linking of γ chains was

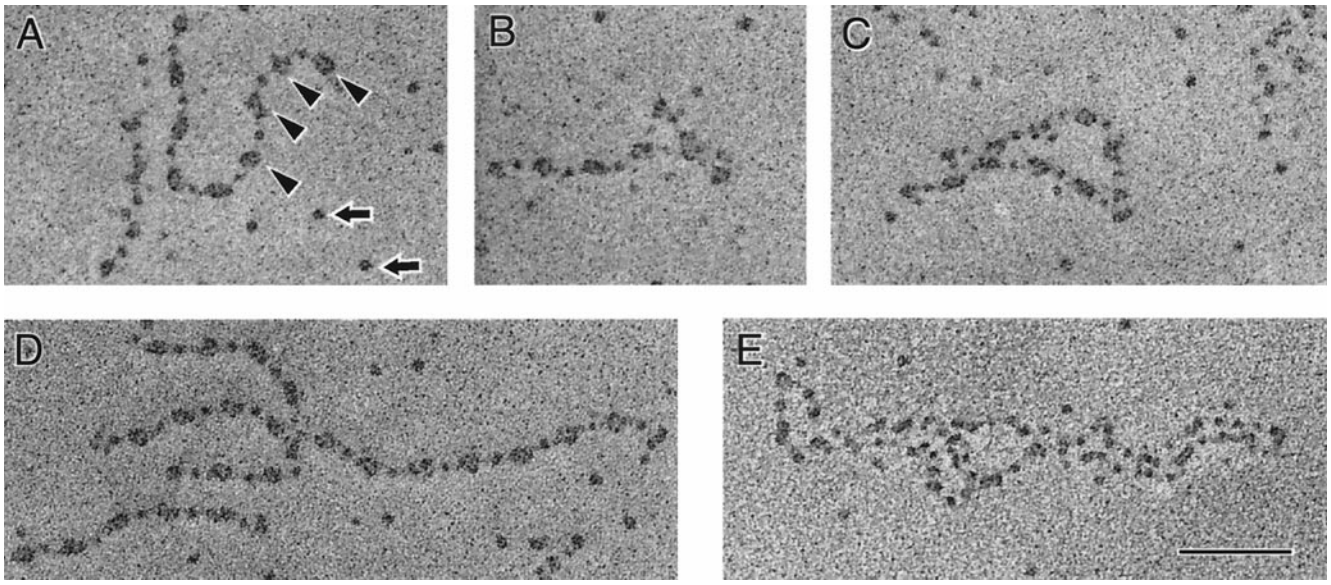

FIG. 2. Short polymers formed from des-AB fragment E and fibrinogen. Fragment E was mixed with fibrinogen at 4°C and incubated for 12–24 hr before rotary shadowing for electron microscopy. It is important to note that these experiments were carried out with recombinant fibrinogen, so that these preparations had no possible contamination of thrombin, which could otherwise be present in low amounts and cause slow conversion of fibrinogen to fibrin and subsequent polymerization. (*A*) Two single-stranded fragment E–fibrinogen polymers. The aggregate on the left is made up of three fibrinogen molecules and the curved one on the right is made up of eight fibrinogen molecules. Both of these polymers are held together by fragment E at the end-to-end junctions (arrowheads), and there is also some fragment E in the background (arrows). (*B*) Two short oligomers of des-AB fragment E and fibrinogen interacting with each other. (*C*) Another example of two short oligomers of des-AB fragment E and fibrinogen interacting with each other. (*D*) Longer polymers of des-AB fragment E and fibrinogen. (*E*) Polymers showing more extensive lateral aggregation. (Bar = 100 nm.)

greater than that of α chains, whereas the reverse was true for fibrinogen alone.

The polymers were diluted into 0.125% acetic acid (pH 3.5), sprayed, and shadowed with tungsten. Electron microscopy of these preparations demonstrated that polymers were as prevalent as in preparations at neutral pH, and they could not be distinguished in appearance from nonligated polymers, except that there was no extra mass (from fragment E) at the end-to-end junctions. A typical example of these cross-linked polymers is shown in Fig. 3*A*. In other words, these cross-linked polymers could not be dissociated at pH 3.5. In contrast, polymers that were not ligated with factor XIIIa could be completely dissociated into individual fragment E and fibrinogen components in 0.125% acetic acid (pH 3.5; Fig. 3*C*).

DISCUSSION

Fibrinogen is normally soluble in saline, whereas fibrin is not. In fact, fibrin can only be dissolved at acid pH (28, 29) or by the addition of solutes such as urea (30, 31) or NaBr (32). The event that is responsible for this dramatic change in solubility is cleavage of the fibrinopeptides, exposing aggregation sites in the central part of the molecule. These aggregation sites are complementary to sites at the ends of the molecule. Various fragments of fibrinogen have been used to explore these interactions. For example, fragment D, which contains the sites at the molecular ends, can inhibit polymerization of fibrin by associating with the aggregation sites in fibrin's central region and blocking their interactions with the equivalent sites on other fibrin molecules (15).

Fragment E consists of the central disulfide knot of fibrinogen plus the proximal part of each of the coiled-coils. If the fibrinopeptides are cleaved from fragment E, the aggregation sites at that location will be exposed. Because the complementary sites should be available on fibrinogen without cleavage of any fibrinopeptides, fragment E missing its fibrinopeptides should be able to interact with fibrinogen, causing it to polymerize. However, such polymers have been difficult to demonstrate, suggesting that the distal polymerization sites may not in fact normally be exposed in fibrinogen but may be exposed only after the cleavage of the fibrinopeptides. After a negative report (33), the slow formation of aggregates between fibrinogen and the amino-terminal disulfide knot of fibrin (which is the near equivalent of fragment E) has been published (34–39). Moreover, recently it was shown that fragment E definitely bound to fibrinogen strongly in solution and substantially enhanced the rate of γ chain ligation by factor XIIIa (21).

The results presented herein demonstrate that des-AB fragment E preparations can interact with fibrinogen to produce polymers. In other words, the aggregation sites that are exposed in fragment E (without the fibrinopeptides) can interact with complementary sites that are always exposed at the ends of fibrinogen molecules still containing their fibrinopeptides. These interactions occur in the same way as they normally do in fibrin, such that single-stranded polymers are formed (Fig. 1*C*).

In a fibrin protofibril, each molecule interacts with an adjacent half-staggered molecule by at least two pairs of complementary aggregation sites. For example, with cleavage of the A fibrinopeptides only, in each molecule of a dimer, there will be an A–a interaction in the middle and an a–A interaction at one molecular end (Fig. 1*B*). Cleavage of the B fibrinopeptides then exposes B sites. Fragment E contains only A and B sites. Thus, the fragment E–fibrinogen polymers are held together only by a single set of A–a and B–b interactions (Fig. 1*C*). Therefore, reciprocal interactions between ends and middle of each molecule are not necessary for polymerization.

The fragment E–fibrinogen polymers sometimes bend back on themselves with a 180° turn or form complex tangles, which also incorporate sharp bends. In contrast, fibrin protofibrils are often gently curved but do not generally display such sharp bends (40–42). Thus, although the basic interactions are similar in both structures, the additional sets of reciprocal interactions that occur in the protofibril serve to give them more rigidity and mechanical stability.

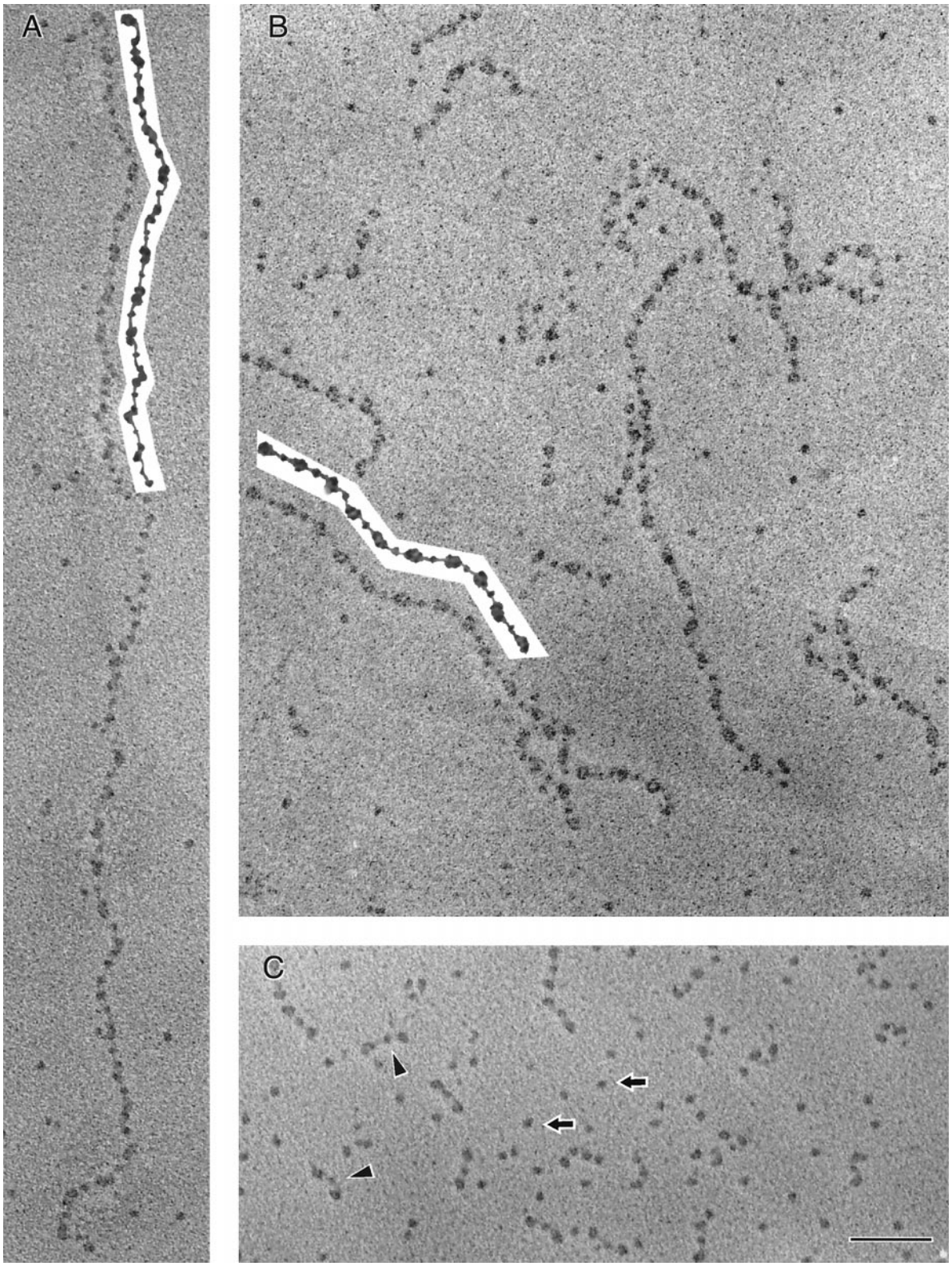

FIG. 3. Long polymers formed from des-AB fragment E and fibrinogen and cross-linked polymers. Fragment E was mixed with fibrinogen at 4°C and incubated for 24–48 hr before prior rotary shadowing for electron microscopy. (*A*) Long single-stranded polymer that was produced by mixing des-AB fragment E with fibrinogen, cross-linking with factor XIIIa, and dissociation of any complexes not held together by covalent bonds with dilute acetic acid. This polymer appears thinner than those in *B* and Fig. 2 because the fragment E has been dissociated. To facilitate interpretation of this image of the polymer, a part of the image of this structure was filtered and enhanced using Adobe PHOTOSHOP and is displayed next to the original image. (*B*) A field containing a variety of short oligomers and longer polymers of des-AB fragment E and fibrinogen. There are bends in some polymers and some are interacting with each other. To facilitate interpretation of these images, a portion of one polymer image was filtered and enhanced by using Adobe PHOTOSHOP and is displayed next to the original image. (*C*) Preparation of des-AB fragment E–fibrinogen polymers put into dilute acetic acid without ligation by factor XIIIa. In this case, the aggregates of fibrinogen and fragment E have dissociated. Two fibrinogen molecules are indicated by arrowheads, and two fragment E moieties are indicated by arrows. (Bar = 100 nm.)

It has been proposed that polymers formed from the N-terminal disulfide knot and fibrinogen consist of a 2:1 ratio of these components (34–37). In examining the mass located at the end-to-end junctions in our images, it appears that there is only a single fragment E at each junction, but the resolution of these images is not sufficient to settle this point definitively. However, higher ratios of fragment E to fibrinogen beyond 1:1 did not promote polymerization to any noticeably greater extent and did not lead to increased cross-linking by factor XIIIa, also suggesting that there probably was a 1:1 ratio of fragment E to fibrinogen.

Furthermore, like fibrin protofibrils, these single-stranded polymers can associate with each other laterally. Thus, the interactions responsible for lateral aggregation of protofibrils are also present in these single-stranded fibrils. It is not necessary to have a complete two-stranded protofibril for lateral aggregation to occur. Because generally only longer fibrils interact, these results imply that there are many weak interactions that add up over the length of the fibril. This conclusion is also similar to what has been observed for normal fibrin protofibrils (43–46).

There has been some controversy over whether cross-links between γ chains in fibrin occur between molecules that are end-to-end (longitudinal) or half-staggered (transverse). Because the only γ chains present in these polymers are those of the fibrinogen molecules interacting end-to-end, these results demonstrate that the factor XIIIa-induced γ–γ cross-links in fibrin(ogen) must occur between molecules that are longitudinal (or end-to-end) rather than transverse (or half-staggered) (Fig. 1*D*). This conclusion is consistent with a recent structure determination of fragment D dimer, which shows the end-to-end relationship of the carboxyl-terminal γ chains (11, 12).

The results of these experiments reveal the exquisite control that is possible with such a system of complementary aggregation sites. Fibrinogen is a large complex molecule, but it is only necessary to remove very small peptides for the exposure of aggregation sites, promoting rapid and precise intermolecular interactions. A temporary plug consisting of a three-dimensional network of fibers is rapidly and efficiently constructed to stop bleeding. The strength of the bonding is increased by the recruitment of multiple sets of these aggregation sites. Finally, the polymers are further stabilized with covalent linkages catalyzed by factor XIIIa. The ordered assembly of fibrin was shown to play a unique role in regulating the rate of factor XIIIa-catalyzed end-to-end ligation of the γ chains (21, 47).

We are grateful to Drs. Oleg V. Gorkun and Susan T. Lord (University of North Carolina, Chapel Hill) for their generous gift of recombinant fibrinogen. We acknowledge the support of National Institutes of Health Grants HL30954 to J.W.W. and HL16346 and HL02212 to L.L.

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