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## The Role of Fibrinogen D Domain Intermolecular Association Sites in the Polymerization of Fibrin and Fibrinogen Tokyo II ( $\gamma$ 275 Arg→Cys)

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### Research Article

Intermolecular end-to-middle domain pairing between a thrombin-exposed 'A' polymerization site in the central 'E' domain of fibrin, and a constitutive complementary 'a' site in each outer 'D' domain ('D:E'), is necessary but not alone sufficient for normal fibrin assembly, as judged from previous studies of a congenital dysfibrinogen, Tokyo II (gamma 275 arg-->cys), which showed defective fibrin clot assembly and a normal D:E interaction (Matsuda, M., M. Baba, K. Morimoto, and C. Nakamikawa, 1983. *J. Clin. Invest.* 72:1034-1041). In addition to the 'a' polymerization site, two other constitutive intermolecular association sites on fibrinogen D domains have been defined: between gamma chain regions containing the carboxy-terminal factor XIIIa crosslinking site ('gamma XL:gamma XL'); and between sites located at the outer ends of each molecule ('D:D') (Mosesson, M. W., K. R. Siebenlist, J. F. Hainfeld, and J. S. Wall, manuscript submitted for publication). We evaluated the function of these sites in Tokyo II fibrinogen, and confirmed that there was a normal fibrin D:E interaction, as determined from a normal fibrin crosslinking rate in the presence of factor XIIIa. We also found a normal gamma XL: gamma XL interaction, as assessed by a normal fibrinogen crosslinking rate. Judging from electron microscopic images, factor XIIIa-crosslinked Tokyo II fibrinogen failed to form elongated double-stranded fibrils like normal fibrinogen. Instead, it formed aggregated disordered collections of [...]

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# The Role of Fibrinogen D Domain Intermolecular Association Sites in the Polymerization of Fibrin and Fibrinogen Tokyo II ( $\gamma 275$ Arg $\rightarrow$ Cys)

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## Abstract

Intermolecular end-to-middle domain pairing between a thrombin-exposed 'A' polymerization site in the central 'E' domain of fibrin, and a constitutive complementary 'a' site in each outer 'D' domain ('D:E'), is necessary but not alone sufficient for normal fibrin assembly, as judged from previous studies of a congenital dysfibrinogen, Tokyo II ( $\gamma 275$  arg $\rightarrow$ cys), which showed defective fibrin clot assembly and a normal D:E interaction (Matsuda, M., M. Baba, K. Morimoto, and C. Nakamikawa, 1983. *J. Clin. Invest.* 72:1034–1041). In addition to the 'a' polymerization site, two other constitutive intermolecular association sites on fibrinogen D domains have been defined: between  $\gamma$  chain regions containing the carboxy-terminal factor XIIIa crosslinking site (' $\gamma_{XL}:\gamma_{XL}$ '); and between sites located at the outer ends of each molecule ('D:D') (Mosesson, M. W., K. R. Siebenlist, J. F. Hainfeld, and J. S. Wall, manuscript submitted for publication). We evaluated the function of these sites in Tokyo II fibrinogen, and confirmed that there was a normal fibrin D:E interaction, as determined from a normal fibrin crosslinking rate in the presence of factor XIIIa. We also found a normal  $\gamma_{XL}:\gamma_{XL}$  interaction, as assessed by a normal fibrinogen crosslinking rate. Judging from electron microscopic images, factor XIIIa-crosslinked Tokyo II fibrinogen failed to form elongated double-stranded fibrils like normal fibrinogen. Instead, it formed aggregated disordered collections of molecules, with occasional short fibrillar segments. In addition, Tokyo II fibrin formed an abnormal, extensively branched clot network containing many tapered terminating fibers. These findings indicate that the Tokyo II fibrinogen defect results in a functionally abnormal D:D self-association site, and that a normal D:D site interaction is required, in addition to D:E, for normal fibrin or fibrinogen assembly. (*J. Clin. Invest.* 1995. 96:1053–1058.) Key words: fibrinogen • dysfibrinogenemia • fibrin • factor XIII • polymerization

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## Introduction

Fibrin polymerization begins after cleavage and release of two amino-terminal A fibrinopeptides from the central 'E' domain of fibrinogen molecules, exposing each of the 'A' polymerization sites they mask in precursor fibrinogen molecules (1–5). Each exposed 'A' site then combines noncovalently with a constitutive complementary 'a' site in the outer 'D' domain of a neighboring fibrin molecule, a necessary interaction ('D:E') for producing the half-staggered, end-to-middle domain pairing that results in twisting (6–10), double-stranded fibrils (6–14) (Fig. 1). Although it is known from many previous studies (1–5, 13, 14 inter alia) that the D:E interaction is required for normal fibrin assembly, it is not clear whether this reaction alone is sufficient for this process to occur.

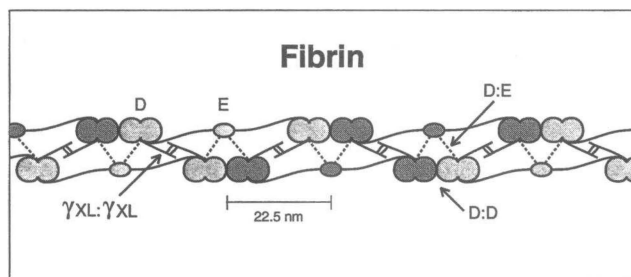
Fibrinogen Tokyo II is an hereditary heterozygotic dysfibrinogenemia associated with defective fibrin assembly (15), and characterized by replacement of  $\gamma 275$  arginine by cysteine in affected molecular D domains (16). The same  $\gamma$  chain substitution and type of abnormal fibrin polymerization have been reported in five other families: fibrinogens Osaka II, Tochigi, Morioka, Baltimore IV and Milano V (16, 17). Six other  $\gamma 275$  mutations (arg $\rightarrow$ his) include fibrinogens Haifa, Bergamo II, Essen, Perugia, Saga, and Osaka III, which also display abnormal fibrin assembly (16).

Defective fibrin assembly of fibrinogen Tokyo II is featured by a markedly prolonged thrombin clotting time that is partially corrected by addition of calcium (15). Tokyo II fibrinogen bound normally to insolubilized normal fibrin, indicating that the D:E interaction was normal. D dimers derived by plasmin digestion of crosslinked fibrin were also normal by SDS-PAGE, but when they were compared functionally with normal, Tokyo II D dimers showed defective binding to fibrin fragment E. This abnormal behavior indicated that there was a unique region in the Tokyo II D domain, distinct from the 'a' polymerization site, that accounted for abnormal fibrin assembly. The defective site was tentatively ascribed to a so-called 'lateral association site' ('bb') (18), that interacts with the E domain 'B' site that becomes exposed after cleavage of fibrinopeptide B (4, 18).

Recently, during the course of evaluating the location of carboxy-terminal  $\gamma$  chain crosslinks in normal fibrin,<sup>1</sup> we identified two constitutive self-association sites on the fibrinogen D domain that were each structurally and functionally distinct from the 'a' site: between  $\gamma$  chain regions containing the carboxy-terminal factor XIIIa crosslinking site (' $\gamma_{XL}:\gamma_{XL}$ '); and between sites located at the ends of each molecule ('D:D') (Fig. 1). The presence of a D:D self-association site in fibrinogen or fibrin had previously been suggested in other studies (19–21).

1. Mosesson, M. W., K. R. Siebenlist, J. F. Hainfeld, and J. S. Wall, manuscript submitted for publication.





**Figure 1.** Diagram showing the “transverse” arrangement of carboxy-terminal  $\gamma$  chain crosslinks in a fibrin fibril based upon recent analyses of crosslinked fibrinogen polymers.<sup>1</sup> Consecutive fibrin molecules comprising each fibril strand are lightly or darkly hatched. The D:E interaction between the D domain ‘a’ site and the E domain ‘A’ site is represented by a dotted line. The locations of the constitutive  $\gamma_{XL}:\gamma_{XL}$  and D:D self-association sites are indicated.

Recognition of the existence of these two self-association sites raised the possibility that the functional abnormality in Tokyo II fibrinogen might be related to a defect in one of them, D:D, rather than to the so-called ‘bb’ site interaction. In this report, we addressed that possibility by investigating the structure of Tokyo II fibrin and factor XIIIa-crosslinked fibrinogen polymers in the context of the relevant functional features of each of the D domain self-association sites.

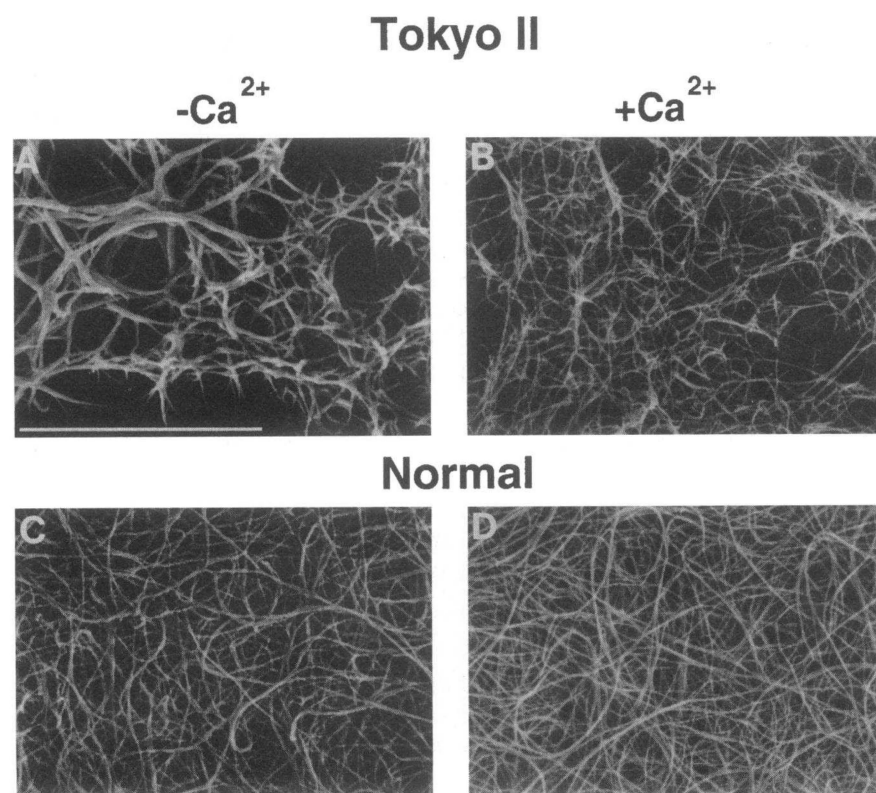
## Methods

**Crosslinked fibrinogen and fibrin.** Normal fibrinogen fraction I-2 was prepared as previously described (22). Tokyo II fibrinogen was prepared

as described by Matsuda et al. (15). Plasma factor XIII (23) at a concentration of 500 Loewy U/ml in 0.1 M NaCl, 20 mM Hepes, pH 7 buffer containing 50 mM DTT, was activated to XIIIa by adding human  $\alpha$ -thrombin (Enzyme Research Laboratories, South Bend, IN; specific activity, 3.4 U/mg) at 5 U/ml, final concentration, and incubating the mixture at 37°C for 30 min. For preparing crosslinked fibrinogen, the thrombin was then inactivated by adding a tenfold excess of hirudin (Sigma Chemical Co, St Louis, MO) (50 U/ml, final concentration). Hirudin-treated factor XIIIa had no measureable residual thrombin activity as assessed by its failure to release detectable FPA from fibrinogen (24) at a final factor XIIIa concentration of 100 U/ml over a 24 hour incubation period, or to cleave the fibrinogen A $\alpha$  chain as assessed by SDS-PAGE of reduced fibrinogen specimens.<sup>1</sup>

For SDS-PAGE analyses of fibrinogen or fibrin crosslinking, factor XIIIa (50 to 100 U/ml, final concentration) containing hirudin-inactivated thrombin or active thrombin (not hirudin treated), respectively, was added to a fibrinogen solution (1 mg/ml final) in 0.1 M NaCl, 20 mM Hepes, pH 7 buffer containing 10 mM CaCl<sub>2</sub> and incubated at room temperature for up to 24 h. At selected time intervals, the crosslinking reaction was terminated by adding an equal volume of 5% SDS, 10 mM Tris, 1 mM EDTA, 10%  $\beta$ -mercaptoethanol, pH 8 solution, and the samples subjected to electrophoresis on 8–25% gradient gels in a Phast gel apparatus (Pharmacia/LKB). Densitometric scanning of Coomassie blue stained gels was carried out at 540 nm in a Gilford Response UV-VIS spectrophotometer.

**Fibrin for scanning electron microscopy (SEM).** Fibrin was formed directly on carbon-formvar coated gold grids by adding 1  $\mu$ l thrombin (0.5 U/ml) to a 4  $\mu$ l droplet of fibrinogen at 500  $\mu$ g/ml in 10 mM Tris, 0.15 M NaCl, pH 7.4 buffer, and incubating the clotting mixture overnight at room temperature. CaCl<sub>2</sub>, 10 mM final concentration, was added to some clotting mixtures. The clots were then fixed with 2.5% glutaraldehyde in 0.1 M Hepes pH 7 buffer, containing 0.2% tannic acid, and washed several times with Hepes buffer. These specimens were dehydrated in graded ethanol solutions (20% to 100%), CO<sub>2</sub> critical point dried using a Tousimis model 780A critical point drying apparatus,



**Figure 2.** SEM images of Tokyo II fibrin (A and B) and normal fibrin (C and D). Fibrin formed in Hepes pH 7 buffer are at the left (A and C), and fibrin formed in the same buffer containing 10 mM CaCl<sub>2</sub> are at the right (B and D). Bar, 10  $\mu$ m.

Table I. Assessment of Constitutive D Domain Polymerization or Association Sites on Tokyo II Fibrinogen

D domain site	Fibrin assembly (thrombin time)	Binding to fibrin sepharose	Fibrin crosslinking rate	Fibrinogen crosslinking rate	Crosslinked fibrinogen fibrils
D:E ('a' site)	—	Normal	Normal	—	—
$\gamma$ XL: $\gamma$ XL	—	—	—	Normal	—
D:D	Abnormal (prolonged)	Normal	Normal	Normal	Disordered

The experimental results that define the constitutive polymerization or intermolecular association sites on fibrinogen Tokyo II, are indicated.

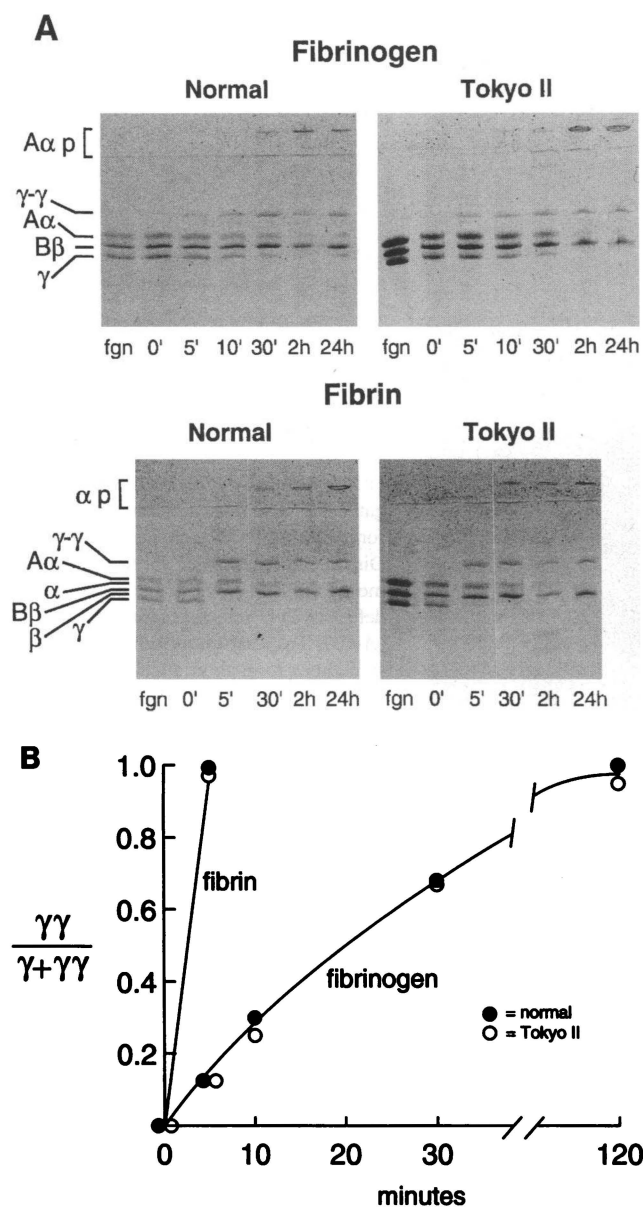


Figure 3. Factor XIIIa crosslinking of Tokyo II fibrinogen or fibrin assessed by SDS-PAGE. A, hirudin-treated factor XIIIa (100 U/ml, final) was added to normal or Tokyo II fibrinogen to produce fibrinogen crosslinking mixtures (*upper panels*). Non-hirudin treated factor XIIIa was added to produce fibrin crosslinking mixtures (*lower panels*). Sampling times are indicated beneath the gels; (B) a plot of the densitometric scan of the  $\gamma$  and  $\gamma\gamma$  band positions expressed as the ratio of  $\gamma\gamma$  to  $\gamma + \gamma\gamma$ . (●) Normal fibrinogen or fibrin; (○) Tokyo II fibrinogen or fibrin.

and coated with 5 nm platinum in a VCR IBS/TM200S Ion Beam Sputter System. SEM was carried out in a JOEL JSM6300F Field Emission Scanning Electron Microscope.

*Scanning transmission electron microscopic (STEM) analysis of crosslinked fibrinogen polymers.* To prepare electron microscope grids of fresh, partially crosslinked fibrinogen for STEM analyses, factor XIIIa/fibrinogen crosslinking mixtures, prepared as described above, were deposited as 50  $\mu$ l drops on a parafilm surface and incubated at room temperature for 10–20 min. At the selected time interval, a specimen was mixed by drawing the solution back and forth several times into a shortened plastic pipette tip, and diluted to 5–10  $\mu$ g/ml in the Hepes pH 7 buffer. 3  $\mu$ l was then injected into a 3- $\mu$ l droplet of the

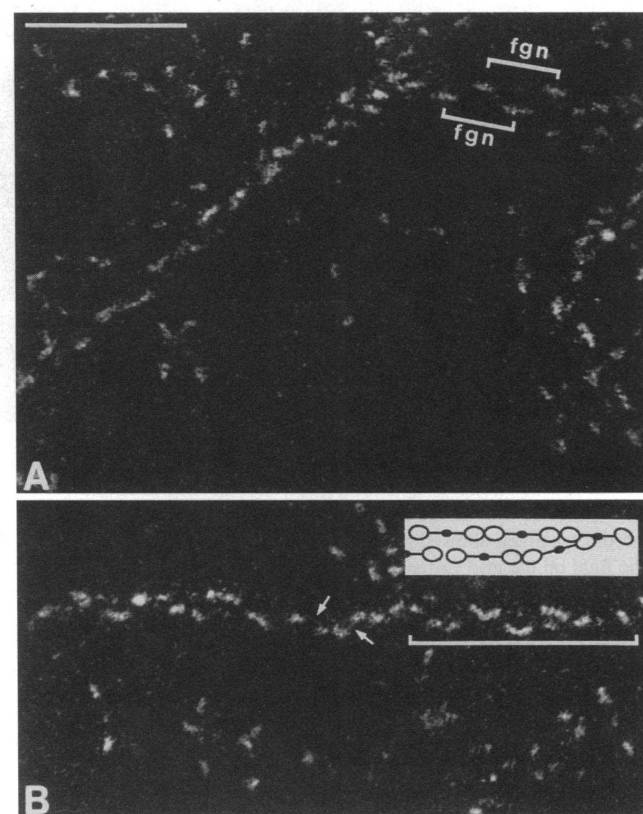
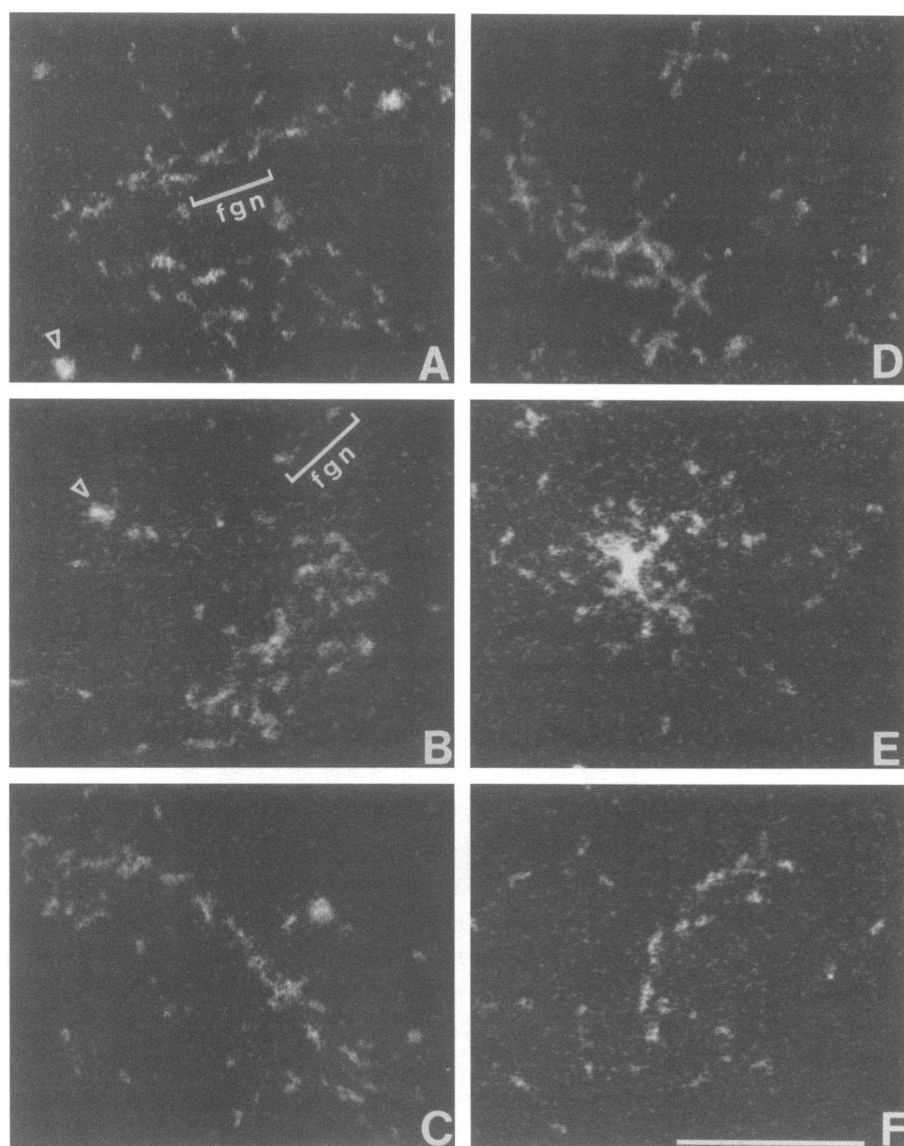


Figure 4. STEM images obtained from a ten minute sample of a crosslinking mixture of normal fibrinogen in pH 7 Hepes buffer, showing examples of the double-stranded fibrils that form as a result of  $\gamma$  chain crosslinking. Fibrinogen molecules become aligned within a fibril strand, and form end-to-end DD complexes between neighboring molecules (*brackets*), as a result of the D:D self-association reaction. A schematic diagram illustrating the molecular arrangement is shown in panel B. Some of the thin filaments (representing crosslinked  $\gamma$  chains) that connect the D domains of fibril strands are indicated by arrows. Bar, 100 nm.



**Figure 5.** STEM images obtained from a ten minute sample of a crosslinking mixture of Tokyo II fibrinogen in pH 7 Hepes buffer, showing examples of the aggregates and short double-stranded fibrils that form as a result of  $\gamma$  chain crosslinking. Disordered crosslinked fibrinogen polymers form as a result of the presence of defective D:D self-association sites (A–E). Tokyo II fibrinogen molecules sometimes form short double-stranded fibrils, probably resulting from competent D:D self-association reactions (A, C, and F). Factor XIIIa molecules are indicated by open arrowheads. Bar, 100 nm.

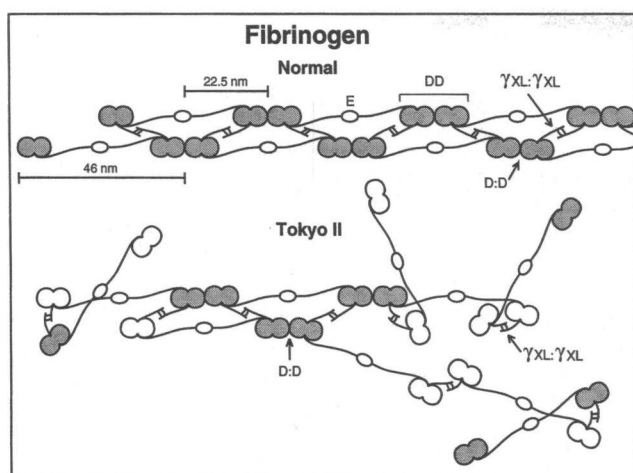
same buffer on the surface of a carbon-coated electron microscope grid, and the specimen allowed to attach to the grid surface for 1 min (25). The fluid on the grid surface was then exchanged 8–10 times with 150 mM ammonium acetate solution, frozen in liquid nitrogen, freeze-dried, transferred under vacuum to the microscope stage, and imaged at the Brookhaven STEM facility using a 40 kv probe focused at 0.25 nm. Background filtering of digitized STEM images was optimized for contrast and brightness offset by an image processing program (Adobe Photoshop), and the adjusted images downloaded to a GCC Technologies ColorFast film recorder.

## Results

**Tokyo II fibrin.** The Tokyo II fibrin clot formed an extensively branched fiber network that had a lower fiber density than normal fibrin, and was featured by the presence of numerous tapered terminating fibers (Fig. 2 A). Including calcium ions in the Tokyo II clotting mixture resulted in somewhat greater fiber density and thinner fibers, but the extensive branching and tapering terminal fibers remained (Fig. 2 B). Morphological differences in normal fibrin matrix structure in the presence or ab-

sence of calcium ions were minimal. The greater widths of Tokyo II fibers in buffer compared with normal fibrin or Tokyo II fibrin formed in the presence of calcium, is probably related to the slow kinetics of Tokyo II fibrin assembly in buffer, since slowly polymerizing clots develop thicker fiber bundles than more rapidly formed clots (8, 26–28).

**The crosslinking rates of Tokyo II fibrinogen and fibrin.** A summary of the various evaluations of Tokyo II fibrinogen/fibrin function, that result in determination of the competency of its polymerization or self-association sites is given in Table I. The crosslinking rate of Tokyo II fibrinogen  $\gamma$  and  $\alpha\alpha$  chains was the same as for normal fibrinogen, as assessed by visual inspection (Fig. 3 A) and by densitometric gel scanning (Fig. 3 B). Complete  $\gamma$  chain crosslinking was observed for both normal and Tokyo II fibrin at the earliest time point (5 min), suggesting that their crosslinking rates were the same. When the fibrin crosslinking experiment was repeated under the same conditions with earlier sampling, complete  $\gamma$  chain crosslinking occurred at the earliest sampling time (1 min; data not shown), indicating that fibrin  $\gamma$  chain crosslinking is at least 40-fold faster than that of each respective fibrinogen. These observa-



**Figure 6.** Schematic diagram showing the molecular arrangement of factor XIIIa-crosslinked normal and Tokyo II fibrinogen. The end-to-end association between normal D domains of fibrinogen at the functional D:D self-association site is indicated, as is the location of the  $\gamma_{XL}:\gamma_{XL}$  self-association site within which the  $\gamma$  chain crosslinking site is situated. Gamma chain crosslinking between strands results in parallel fibrils with  $\sim 22.5$  nm periodicity.<sup>1</sup> In the bottom part of the diagram, disordered assembly of crosslinked fibrinogen molecules derived from the heterozygotic Tokyo II subject, is depicted. The D domains of Tokyo II fibrinogen molecules containing a defective D:D polymerization site are unfilled; normal D:D sites are hatched.

tions are consistent with our recent findings on the differences between the crosslinking rates of normal fibrinogen and fibrin,<sup>1</sup> and confirms the conclusion that the fibrin D:E association accounts for the higher crosslinking rate of fibrin over fibrinogen (29, 30). The fact that Tokyo II fibrinogen manifests the same degree of accelerated crosslinking as normal fibrin, provides confirmation for the previous finding based on fibrin affinity chromatography experiments that the D:E interaction in Tokyo II is normal (15).

**Crosslinked Tokyo II fibrinogen polymer structure.** Normal fibrinogen that had been crosslinked in the presence of factor XIIIa formed elongated double-stranded fibrils (Fig. 4), whereas Tokyo II fibrinogen did not (Fig. 5). The constituent fibrinogen molecules within each normal fibrinogen fibril strand were aligned end-to-end to form the intermolecular DD complexes that resulted from the 'D:D' association reaction, and the fibril strands became aligned in parallel because of the  $\gamma$  chain crosslinks that bridge the fibril strands. The half-staggered arrangement of molecules constituting the fibrinogen fibrils causes them to have 22.5 nm periodicity.<sup>1</sup>

Crosslinked Tokyo II fibrinogen, although it had become crosslinked at the same rate and to the same extent as normal fibrinogen (Fig. 3), formed polymeric structures that were not as well ordered as normal crosslinked fibrinogen fibrils (Fig. 5). Most commonly, Tokyo II fibrinogen formed aggregated collections of crosslinked molecules, with occasional short double-stranded fibrillar segments with  $\sim 22.5$  nm periodicity (Fig. 5 A, C, and F). These 'normal' fibril segments probably reflect a subpopulation of Tokyo II fibrinogen molecules having two functionally normal D domains (i.e., "normal homodimers"), that are present in the heterozygous fibrinogen population (Fig. 6).

## Discussion

Our recent studies on fibrinogen crosslinking<sup>1</sup> led to recognition of the existence and general location of two constitutive intermolecular association sites on fibrinogen D domains, that are distinct from the D domain 'a' polymerization site that interacts with an exposed 'A' site in the fibrin E domain (D:E). These sites evidently participate in fibrin assembly, and have the following defining characteristics:  $\gamma_{XL}:\gamma_{XL}$ —this site is in the carboxy terminal region of the  $\gamma$  chain and overlaps the  $\gamma$  chain crosslinking site. The site is recognizable from ultrastructural analyses of crosslinked or uncrosslinked fibrinogen, and from kinetic studies of fibrinogen and fibrin crosslinking;<sup>1</sup> and D:D—this site is located at the outer end of each fibrinogen D domain and was identified from ultrastructural analyses of native fibrinogen solutions showing frequent noncovalent D-to-D associations<sup>1</sup> (Table I). The existence of a D:D self-association site has also been suggested from other studies on fibrinogen or fibrin assembly (19–21).

Previous studies had indicated that the fibrin D:E interaction was normal in Tokyo II fibrin, and that factor XIIIa-mediated crosslinking of Tokyo II fibrin was also normal (15). Our present studies extend those observations to show that the rate as well as the extent of crosslinking of fibrin Tokyo II is the same as that of its normal counterpart, strongly reinforcing the notion of a normal D:E interaction. The fact that the crosslinking rate of Tokyo II fibrinogen is also the same as its normal counterpart indicates that  $\gamma_{XL}:\gamma_{XL}$  self-association sites are normal. Despite this, assembly of crosslinked Tokyo II fibrinogen is grossly abnormal, thus pointing directly to a defective D:D self-association site interaction. This heterozygous deficiency, which is caused by  $\gamma 275 \text{ arg} \rightarrow \text{cys}$  mutations involving half the fibrinogen D domains, results in abnormal end-to-end associations during factor XIIIa-catalyzed fibrinogen polymer assembly and also, as assessed by morphological appearances, during fibrin assembly. Thus, these studies on Tokyo II fibrin and crosslinked fibrinogen polymer structure, advance our understanding of the specific self-association sites that participate in the fibrin assembly process. They indicate that the D:E interaction is necessary but not alone sufficient for fibril assembly, and that the D:D interaction is also required.

Our findings also indicate that the D:D interaction is an important determinant of the ultimate network structure of the fibrin clot. The Tokyo II fibrin network is more highly branched than its normal counterpart (Fig. 2), an observation that is very similar to those we have made on fibrinogens having a mutation at the same site—Haifa ( $\gamma 275 \text{ arg} \rightarrow \text{his}$ ) (31) and Morioka ( $\gamma 275 \text{ arg} \rightarrow \text{cys}$ ) (32). The likely explanation for increased branching is that impairment of intermolecular D:D interactions permits fibrin molecules to extend outward from linearly propagating fibril strands more frequently, thus favoring formation of greater numbers of branch points (25).

The abnormality in Tokyo II D dimer binding to the fibrin E domain had tentatively been attributed to a defect in a region expressed in the linearly aligned dimeric D domain ('bb') that participates in lateral fibril association (18) via interaction with an exposed 'B' site in the fibrin E domain. Based upon our present observations on crosslinked fibrinogen polymers, in which the fibrinogen E domain plays no obvious role in the assembly process, this surmise does not seem to be the case. Our findings indicate instead that the primary defect in the Tokyo II molecule, as formerly reflected in its defective D

dimer function, is due to an abnormality in self-association of its constituent D domains that results in defective fibril assembly. Lateral association of thrombin-cleaved fibrin fibrils due to the 'bb' site interaction may prove to be related to the D:D interaction, requiring DD association for full expression, or it may be related less directly to DD assembly. These possibilities remain to be investigated.

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