Comparison of Thrombin-Catalyzed Fibrin Polymerization and Factor XIIIa-Catalyzed Cross-Linking of Fibrin Among Three Recombinant Variant Fibrinogens, γ275C, γ275H, and γ275A

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Summary

Background and Objectives: We have reported elsewhere that recombinant γ275Cys fibrinogen exhibits a marked impairment of functions as well as aberrant fibrin clot and bundle structures, as compared with wild-type, γ275Arg, and plasma fibrinogen from a heterozygous proband. Since γArg275His mutations have also been reported in 10 families, we synthesized recombinant γ275His fibrinogen and γ275Ala fibrinogen (as a control), and analyzed and compared them with γ275Cys and γ275Arg.

Methods: A variant γ-chain expression plasmid was transfected into Chinese hamster ovary cells expressing normal human fibrinogen Aα- and Bβ-chains. After purification of the recombinant variant fibrinogens, we performed functional analyses for thrombin-catalyzed fibrin polymerization and Factor XIIIa (FXIIIa)-catalyzed γ-γ dimer formation from fibrin or fibrinogen, and also ultrastructural analysis of fibrin clots and bundles.

Results: By comparison with both γ275His and γ275Ala fibrinogens, recombinant γ275Cys fibrinogen exhibited a more impaired γ-γ dimer formation from fibrin or fibrinogen, a more aberrant fibrin clot structure, and thicker fibers in fibrin bundles. In 1:1 mixtures of γ275Arg and γ275Cys fibrinogens or γ275Arg and γ275His fibrinogens, thrombin-catalyzed fibrin polymerization and both fibrin clot and fiber structures showed some compensation (as compared with γ275Cys or γ275His alone).

Conclusion: These results strongly suggest that an amino acid substitution of γ275Arg alone disrupts D:D interactions in thrombin-catalyzed fibrin polymerization and the formation of fibrin bundles and fibrin clots. Moreover, the existence of a subsequent disulfide-linked Cys in γ275C fibrinogen augments the impairment caused by a His or Ala substitution.

Introduction.

Fibrinogen is converted by thrombin to an insoluble fibrin clot via a two-step process. The first step begins with the release of fibrinopeptide A (FpA). This exposes the "A" site in the E domain, which interacts with the "a" site in the D domain of a neighboring molecule (the so-called D:E association), leading to the formation of half-staggered, double-stranded, twisting protofibrils [1,2]. The second step, the release of fibrinopeptide B (FpB), exposes the "B" site, which most likely interacts with the "b" site in the D domain of another molecule to promote lateral aggregation of the protofibrils [3]. Finally, these fibers assemble into thick, multistranded fibers that branch to form a fibrin network. It is well known that a constitutive self-association site in the γ-chain of each D domain participates in the interfacing of the two abutted D domains in the same protofibril strand (the so-called D:D association), independently of the D:E association [4,5]. In addition, recent high-resolution structural analysis has revealed that the Arg275 in one molecule interacts with γTyr280 in the second molecule, and that the γArg275 in the second molecule interacts with γSer300 in the first molecule [6].

In support of this, γArg275 mutations are the second most common mutations in dysfibrinogenemias, with the Arg being mutated to Cys (15 families), His (10 families), or Ser [7]. Although analysis of dysfibrinogens has improved our understanding of the above-mentioned molecular events, which promote thrombin-catalyzed polymerization [8-10], most of the dysfibrinogens occurred in individuals who are heterozygous for the mutation. This complicates functional analysis because the fibrinogen found in these individuals is a mix of normal and abnormal molecules, consisting, in fact, of three types of molecules (normal homodimer, variant homodimer, and heterodimer) [11,12]. For that reason, protein-engineered recombinant fibrinogens are useful tools for the clarification of certain complications [13,14]. In particular, we have reported

elsewhere that recombinant γ275Cys (γ275C) fibrinogen exhibits a marked impairment of Factor (F) XIIIa-catalyzed γ-γ dimer formation from fibrin or fibrinogen, an aberrant turbidity change during thrombin-catalyzed fibrin polymerization, and thicker fibrin bundles, as compared with wild-type, γ275Arg (γ275R), and heterozygous plasma variant fibrinogens [14].

Here, we describe a comparison and an analysis of another recombinant variant fibrinogen, [namely, γ275His (γ275H), analogous to the dysfibrinogen reported in several families], with γ275Ala (γ275A) used as a control variant. Our data indicate that by comparison with γ275H and γ275A fibrinogens, recombinant γ275C fibrinogen exhibits a more impaired FXIIIa-catalyzed γ-γ dimer formation from fibrin or fibrinogen, a more aberrant fibrin clot structure, and thicker fibrin bundles. This aberrant fibrin clot formation leads to the characteristic turbidity change in thrombin-catalyzed fibrin polymerization. Finally, these results indicate that the existence of a disulfide-linked Cys in γ275C, in addition to the single amino acid substitution at the γ275residue, leads to a marked disruption of the initial alignment of fibrin monomers into protofibrils.

Materials and Methods

Preparation of recombinant variant fibrinogen. Preparation of recombinant variant fibrinogen was performed as described before [13,14]. To change γ275Arg to Cys, His, or Ala, the fibrinogen γ-chain expression vector, pMLP-γ (kindly providing by Dr. Lord ST, University of North Carolina) was altered by oligonucleotide-directed mutagenesis using the mutagenesis primer 5'-TGACAAGTACTGCCTAACATA, 5'-GACAAGTACCACCTAACATAT, or 5'- TGACAAGTACGCCCTAACATAT, respectively (the altered bases are underlined). Each of the variant expression plasmids was cotransfected with a histidinol selection plasmid (pMSVhis) into Chinese hamster ovary (CHO) cells expressing normal human fibrinogen Aα- and Bβ-chains (kindly

providing by Dr. Lord ST, University of North Carolina). A selected and cloned colony was cultured in serum-free medium using 850 cm^2-roller bottles coated with microbeads. Fibrinogen was purified from harvested culture medium by immunoaffinity chromatography, utilizing a calcium-dependent monoclonal antibody (IF-1; Iatron Laboratories, Tokyo) [15]. The fibrinogen concentration was determined from the A_{280} value, assuming that a 1 mg/ml solution has an absorbance of 1.51 [16]. The purity and characterization of the proteins was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) run under either nonreducing (5% polyacrylamide gel) or reducing (10% polyacrylamide gel) conditions.

Thrombin-catalyzed fibrin polymerization and clottability. Polymerization was followed by a monitoring of turbidity at 350 nm using a UV-110-02 spectrophotometer (Shimadzu Corp., Tokyo, Japan), as described elsewhere [14]. Briefly, fibrinogen (90 μl at 0.1 or 0.5 mg/ml) in 20 mM N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] (HEPES), pH 7.4, 0.12 M NaCl, with added 1 mM $CaCl_2$ was mixed with human α-thrombin (Enzyme Research Laboratories, South Bend, IN) (10 μl at 0.5 unit/ml), and changes in turbidity were monitored at ambient temperature. Three parameters (lag period, maximum slope, and Δabsorbance in 30 min) were obtained from the turbidity curves, as described elsewhere [17]. The reactions were performed in triplicate for each sample.

The clottability of the purified fibrinogens was determined essentially as described before [13], human α-thrombin (final concentration, 0.05 unit/ml) being mixed with fibrinogen (final concentration, 0.45 mg/ml) in the above-mentioned 20 mM HEPES, pH 7.4. Samples were incubated for 3 h at 37 ˚C, followed by an overnight incubation at 4˚C to allow progress to complete gelation. After centrifugation at 15,000 rpm for 15 min, the fibrin(ogen) not incorporated into the fibrin gel was determined from the A_{280}

value of the supernatant, and clottability was calculated as (A_{280} at zero time - A_{280} of the supernatant)÷(A_{280} at zero time) x 100%.

Factor XIIIa-catalyzed cross-linking of fibrin or fibrinogen. FXIIIa-catalyzed cross-linking of fibrin or fibrinogen was performed as described before [14]. Briefly, FXIII (50 unit/ml; Enzyme Research Laboratories, South Bend, IN) was activated with human α-thrombin (1 unit/ml) for 60 min at 37˚C in 20 mM HEPES, pH 7.4, containing 0.12M NaCl and 5 mM $CaCl_2$. For the studies of FXIIIa-catalyzed cross-linking of fibrinogen, thrombin (0.5 units/ml) was inhibited by hirudin (10 units/ml) prior to the addition of fibrinogen. Fibrinogen (final concentration, 0.47 mg/ml) was incubated at 37˚C with FXIIIa (final concentration, 3.3 units/ml) and human α-thrombin (final concentration, 0.07 units/ml) in the presence of 0.67 mM calcium. The reaction was stopped at various times by the addition of an equal volume of SDS-sample buffer with 2-mercaptoethanol and subsequent incubation (5 min) in a boiling-water bath. Samples equivalent to 4.7 μg of fibrinogen were separated on 8 % SDS-PAGE and stained with Coomassie Brilliant Blue R-250.

Scanning electron microscopy (SEM). The SEM preparation was performed as described before [14]. Briefly, 10 μl of thrombin (0.5 unit/ml) was added to 40 μl of fibrinogen solution (0.5 mg/ml). Polymerization proceeded for 10 h at 37˚C, and the clots were then fixed in 2.5% glutaraldehyde overnight, stained with 1% osmium tetroxide, critical-point dried, mounted, osmium plasma-coated at 5-nm thickness in an NL-OPC40 (Nippon Laser and Electronics Lab, Nagoya, Japan), and viewed on a JSM-6000F (Japan Electron Optics Laboratory Co. Ltd., Tokyo, Japan). Images were taken at 3000x or 20000x with a 15.0 kV accelerating voltage. Fiber diameters were measured using a vernier caliper on a 300% enlargement from a photograph of a 20000x observation.

Results

Characterization of recombinant fibrinogens.

We synthesized three recombinant variant fibrinogens each with a single amino acid substitution; namely, γ275C, γ275H, and γ275A. Wild-type recombinant fibrinogen, γ275R, was also produced [14], and served as the normal control. When SDS-PAGE was run under non-reducing conditions with Coomassie staining, all fibrinogens purified from cultured media were pure, and the recombinant variant fibrinogens had the normal Aα-, Bβ-, and γ-chain components (Figure 1A). The mobility of the γ-chain in each of the three recombinant fibrinogens was slightly less than that of the wild type (Figure 1A). Gel resolved under non-reducing conditions revealed that each of the four fibrinogens had two broad bands corresponding to high molecular weight and low molecular weight fractions [18] (Figure 1B).

Thrombin-catalyzed fibrin polymerization and clottability.

Thrombin-catalyzed fibrin polymerization was monitored as the change in turbidity at 350 nm, as described in Materials and Methods. Representative turbidity curves are shown in Figure 2. At a lower concentration of fibrinogen (0.09 mg/ml), we found that polymerization of γ275C, γ275H, and γ275A variant fibrinogens showed no increase in turbidity within 6 h (Figure 2A). When a higher concentration of fibrinogen was used (0.45 mg/ml), γ275C fibrinogen began polymerization after a lag period of 12 min, and its turbidity curve crossed that for γ275R at 30 min. In contrast, γ275H and γ275A fibrinogens started polymerization after lag periods of 23 and 27 min, respectively (Figure 2B), and the values for their Δabsorbance in 60 min were 0.46 and 0.19, respectively (Figure 2B). These results indicate that for all three variant fibrinogens, the turbidity change during fibrin polymerization was markedly decreased by comparison with that for normal fibrinogen, and that the impairment was much greater

for γ275H and γ275A than for γ275C. However, comparison of thrombin-catalyzed fibrin polymerization or fibrin monomer polymerization for plasma variant fibrinogens purified from the heterozygous probands γArg275Cys and γArg275His [19,20] demonstrated that the turbidity change for the heterozygous fibrinogens was greater for γ275Arg and γ275His than for γ275Arg and γ275Cys.

To clarify the discrepancy between the observations relating to the recombinant variant fibrinogens and those to the heterozygous plasma fibrinogens, we performed thrombin-catalyzed fibrin polymerization on 1:1 (0.225mg/ml:0.225mg/ml) mixtures of γ275R and γ275C fibrinogens (γ275R/γ275C), γ275R and γ275H fibrinogens (γ275R/γ275H), and γ275R and γ275A fibrinogens (γ275R/γ275A). Interestingly, the turbidity changes observed for γ275R/γ275H and γ275R/γ275A were similar to each other, and each was significantly larger than that for γ275R/γ275C (Figure 2C). In addition, the turbidity changes observed for all three mixtures of recombinant fibrinogens were larger than that for γ275R (0.225 mg/ml), and no turbidity changes were observed for γ275C, γ275H, or γ275A (0.225 mg/ml) in 1 h (data not shown).

Next, the clottability of fibrinogen was determined, as described in Materials and Methods. The values obtained for the percentage of fibrin(ogen) incorporated into the fibrin gels made from γ275R, γ275C, γ275H, or γ275A were 93 ± 1, 93 ± 2, 91 ± 3, and or 93 ± 3 %, respectively. Thus although the turbidity changes during thrombin-catalyzed fibrin polymerization were quite different among γ275R, γ275C, γ275H, and γ275A, the clottability results indicated that γ275H or γ275A fibrinogen could polymerize into fibrin fibers to a similar extent as the γ275R and γ275C fibrinogens.

FXIIIa-catalyzed cross-linking of fibrin or fibrinogen.

Cross-linking of fibrin $\tilde{\alpha}$ and $\tilde{\gamma}$chains was performed in the presence of FXIII and thrombin, and the reaction products were analyzed by SDS-PAGE, as described in Materials and Methods. After a 1-min reaction with γ275R fibrinogen, a γ-γ dimer

band was weakly evident, while after 5 min, two α-polymer bands were evident (Figure 3; γ275R). During longer incubation periods (up to 60 min), these products increased with time, while the α- and γ-chain monomer bands decreased. Formation of both the γ-γ dimer and α-polymer from γ275H and γ275A fibrinogens was slower than from γ275R, these bands being weakly evident after 3 and 20 min, respectively (Figure 3; γ275H and γ275A). In contrast, in the case of γ275C fibrinogen the γ-γ dimer was faintly evident after 20 min, while α-polymer bands were not evident even at 60 min (Figure 3; γ275C).

To confirm the difference in D:D interactions among the three variant fibrinogens, we examined the FXIIIa-catalyzed cross-linking of fibrinogen. After FXIII had been activated with thrombin, hirudin was added to inhibit thrombin-catalyzed fibrinopeptide release and fibrin polymerization, and then the enzyme mixture was added to fibrinogen solution. After a 0.5 h reaction, the $\tilde{\gamma}\gamma$ dimer was evident clearly for γ275R fibrinogen and weakly evident for both γ275H and γ275A fibrinogens, whereas formation of the α-polymer was faintly evident from 0.5 to 4 or 8 h incubation for all three of these fibrinogens (Figure 4). In contrast, for γ275C fibrinogen neither the γ-γ dimer nor the α-polymer were evident even after 24 h (Figure 4; γ275C). These results indicate that for all three variant fibrinogens, γ-γ dimer formation from fibrin monomers was less than in the case of normal fibrinogen, and that the impairment was much greater for γ275C than for either γ275H or γ275A.

Scanning electron microscopy (SEM) of fibrin clots and fibers.

To clarify the difference in the ultrastructure of fibrin clots among the three recombinant variants and normal fibrinogen, we observed fibrin clots under the SEM. Clots prepared from either γ275H or γ275A fibrinogen with the aid of thrombin differed in ultrastructure from that prepared from γ275R fibrinogen, the density of the bundles of fibrin fibers being less for γ275H and γ275A than for γ275R (Figures 5 and

6) and the fiber diameter being greater for γ275H (279 ± 74 nm) and γ275A (272 ± 79 nm) than for γ275R (137 ± 27 nm) (Figure 6). The clots prepared from γ275C fibrinogen differed markedly in ultrastructure from those prepared not only from γ275R, but also from either γ275H or γ275A fibrinogens. Indeed, in the former there were many aberrant fibrin fibers with tapered ends, and the fiber diameter was much greater (337 ± 50 nm) than in the case of γ275R (Figure 6; γ275C).

To analyze the difference in the ultrastructure of the fibrin clot among normal, variants, and 1:1 mixtures of γ275R and γ275C fibrinogens (γ275R/γ275C) or γ275R and γ275H fibrinogens (γ275R/γ275H), we observed fibrin clots under the SEM. The clot structures for γ275R/γ275C and γ275R/γ275H are shown in Figure 7. The bundles of fibrin clots from γ275R/γ275H were much coarser in density than those from γ275R/γ275C, although both were much finer than those from either γ275C or γ275H alone and similar to those from γ275R (Figure 5). The values obtained for fiber diameter for γ275R/γ275C and γ275R/γ275H were 131 ± 41 nm and 150 ± 42 nm, respectively. These values were larger than that obtained for γ275R (102 ± 17 nm) and smaller than those for γ275C (251 ± 37 nm) or γ275H (208 ± 55 nm). In the case of γ275R/γ275C, aberrant fibrin fibers terminating in tapered ends were much less frequent than in the case of γ275C alone. The difference in the fiber diameter values of corresponding fibrin clot between two sets of SEM observation experiment (Figures 5 and 7) was due to the experimental conditioning difference in the critical-point dried-step of sample preparation.

Discussion

We have reported elsewhere that in recombinant γ275C fibrinogen, there is a marked impairment of FXIIIa-catalyzed γ-γ dimer formation from fibrin or fibrinogen, an aberrant turbidity change during thrombin-catalyzed fibrin polymerization, and a greater fiber diameter in fibrin bundles, as compared with those in the wild-

type, γ275Arg (γ275R) fibrinogen [14]. Since other heterozygous dysfibrinogens with a mutation at the γ275 residue, γArg275His [19,20] and γArg275Ser [20,21], demonstrate impairment of fibrin polymerization similar to that shown by γArg275Cys, we synthesized two other recombinant fibrinogens, γ275H and γ275A, simultaneously with γ275C and the wild type γ275R, and compared all four in terms of the functions of their fibrin and fibrinogen.

Our results confirm that substitution of the γ-chain residue Arg275 by Cys leads to a much greater impairment of FXIIIa-catalyzed γ-γ dimer formation from fibrin or fibrinogen than substitution of residue Arg275 by His or Ala. Moreover, turbidity changes during thrombin-catalyzed fibrin polymerization were much smaller for γ275H and γ275A fibrinogens than for γ275C or for γ275R. Surprisingly, fibrin clot structures made from γ275H or γ275A fibrinogens differed markedly not only from those made from γ275R, but also from those made from γ275C. In addition, the fiber diameter in both γ275H and γ275A fibrin bundles was smaller than in the case of γ275C, but much larger than in γ275R. We speculate that the above-mentioned discrepancy between the turbidity changes during thrombin-catalyzed fibrin polymerization and clot structure was caused by the existence in the case of γ275C of many aberrant fibrin fibers with short and tapered ends. However, we cannot explain the mechanism by which fibrin monomers derived from γ275C form thicker fibers in fibrin bundles.

Comparison of either thrombin-catalyzed fibrin polymerization or fibrin monomer polymerization between the plasma variant fibrinogens purified from the heterozygous probands γArg275Cys and γArg275His [19,20] demonstrated that the heterozygous fibrinogen for γArg275His showed a greater turbidity change than that for γArg275Cys. To clarify the discrepancy between the observations made for recombinant variant fibrinogens (variant homodimer) and heterozygous plasma fibrinogens (containing three types of molecules; normal homodimer, variant homodimer, and heterodimer),

we performed thrombin-catalyzed fibrin polymerization and also observed clot structure for 1:1 mixtures of γ275R and γ275C fibrinogens or γ275R and γ275H fibrinogens. Interestingly, the turbidity change was larger for the latter than for the former mixture, while fiber diameter was greater for the latter than for the former, both diameters being greater than for γ275R alone, but less than for either γ275C or γ275H alone. The plot of fibrin diameter (shown in Figure 7) against maximum turbidity change (shown in Figure 2B and C) is found to be almost oppositely linear among γ275R, 1:1 mixtures of γ275R and γ275C, 1:1 mixtures of γ275R and γ275H, and γ275H fibrinogen, while only γ275C fibrinogen is completely different from the linear interaction (data not shown). These results indicate that fibrin fiber and clot structure derived from γ275C fibrinogen was markedly different from those derived from other fibrinogens. The mixture experiment results indicate that γ275H and γ275C fibrinogens may interact with γ275R fibrinogen, resulting in the formation of thinner fibers than in the case of γ275C or γ275H alone, and a marked shape change in the thrombin-catalyzed fibrin polymerization turbidity curves as compared with those for γ275C or γ275H alone. Because the interaction among the three types of molecules in plasma fibrinogens from heterozygous probands might differ between γArg275His and γArg275Cys, we speculated that the fibrinogen mixture γ275R plus γ275H is associated with a larger absorbance change and the formation of thicker fibers than a mixture of γ275R plus γ275C.

A fairly recent high-resolution structural analysis showed that the γ-chain residue Arg275 is located at the so-called ‘D:D interface’ and plays a central role in the initial alignment of fibrin monomers into protofibrils (that is, γArg275 in one molecule interacts with γTyr280 in the second molecule, while γArg275 in the second molecule interacts with γSer300 in the first molecule) [6]. The present results indicate that all three variants of γ275Arg exhibited an impaired thrombin-catalyzed fibrin polymerization curve and impaired FXIIIa-catalyzed γ-γ dimer formation from fibrin

or fibrinogen, an aberrant fibrin clot structure, and thicker fibers in the fibrin bundles, as compared with γ275R. In addition, γ275C fibrinogen showed the greatest impairment of FXIIIa-catalyzed γ-γ dimer formation from fibrin or fibrinogen, the most aberrant fibrin clot structure, the thickest fibers in the fibrin bundles, and the greatest thrombin-catalyzed fibrin polymerization turbidity change among the four recombinant fibrinogens. Thus, the degree of impairment in several functions was markedly different for γ275C fibrinogen than for γ275H or γ275A fibrinogen. Finally, our results strongly suggest that a single amino acid substitution at γ275Arg disrupts D:D interactions in thrombin-catalyzed fibrin polymerization, and leads to an aberrant formation of fibrin bundles and an aberrant fibrin clot structure. Moreover, the existence of a subsequent disulfide-linked Cys in γ275C fibrinogen [14] appears to augment the impairment due to His or Ala substitution.

In conclusion, recombinant γ275C fibrinogen exhibits a more impaired FXIIIa-catalyzed γ-γ dimer formation from fibrin or fibrinogen, a more aberrant fibrin clot structure, and thicker fibers in fibrin bundles than either γ275H or γ275A fibrinogen. These results indicate that the presence of a disulfide-linked Cys in γ275C, in addition to the single amino acid substitution at the γ275residue, augments the disruption of the initial alignment of fibrin monomers into protofibrils. Furthermore, they raise the interesting question as to how the markedly affected D: D interaction in γ275C leads to the formation of aberrant and much thicker fibers in fibrin bundles.

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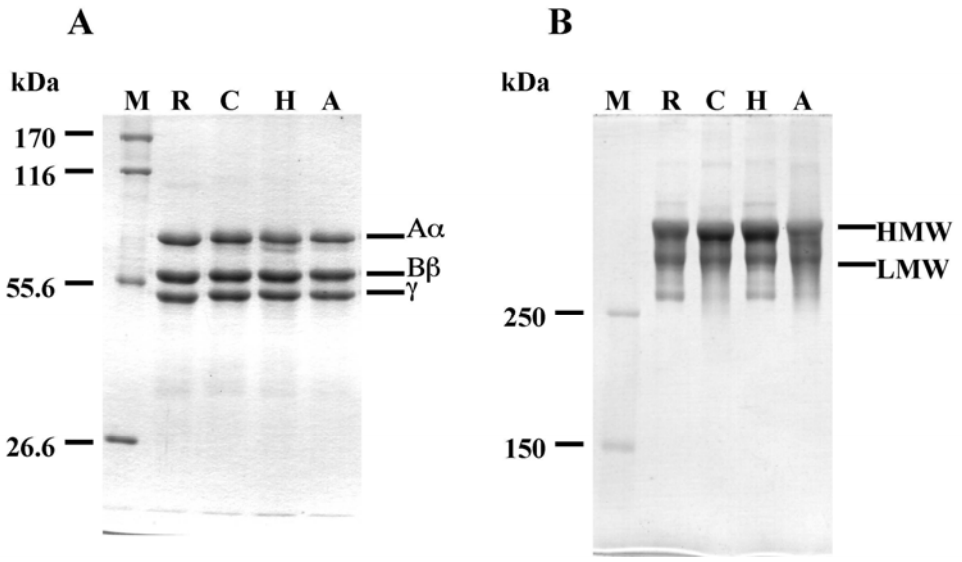

Figure 1. **Characterization of recombinant fibrinogens.** Coomassie-stained SDS-PAGE: 10% SDS-PAGE run under reducing conditions (A) or 5% SDS-PAGE run under nonreducing conditions (B). Recombinant wild-type γ275R (lane R), variant γ275C (lane C), γ275H (lane H), and γ275A (lane A). The molecular markers were run in lane M. HMW and LMW are abbreviations for high molecular weight and low molecular weight fibrinogen, respectively.

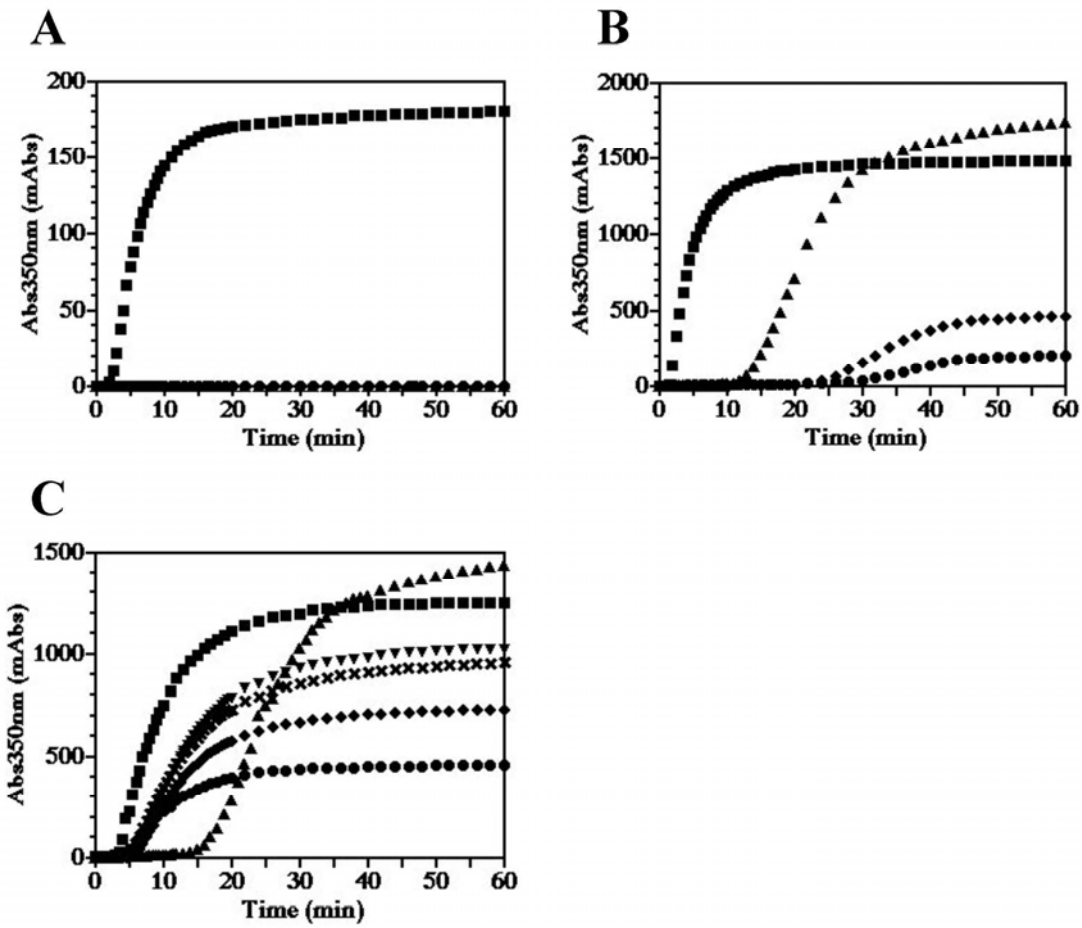

Figure 2. Thrombin-catalyzed fibrin polymerization. Polymerization of fibrinogen (0.09 mg/ml, A ; 0.45 mg/ml, B) was initiated with thrombin (0.05 U/ml), and the change in turbidity with time was followed at 350 nm for recombinant γ275R (■), γ275C (▲), γ275A (●) or γ275H (◆) fibrinogens in 20 mM HEPES, pH 7.4, 0.12 M NaCl. Other thrombin-catalyzed fibrin polymerizations were also performed as

described above (C): 0.225 mg/ml of γ275R (●); 0.45 mg/ml of γ275R (■); 0.45 mg/ml of γ275C (▲); a 0.225 mg/ml : 0.225 mg/ml mixture of γ275R and γ275C (◆), γ275R and γ275H (▼), or γ275R and γ275A (×).

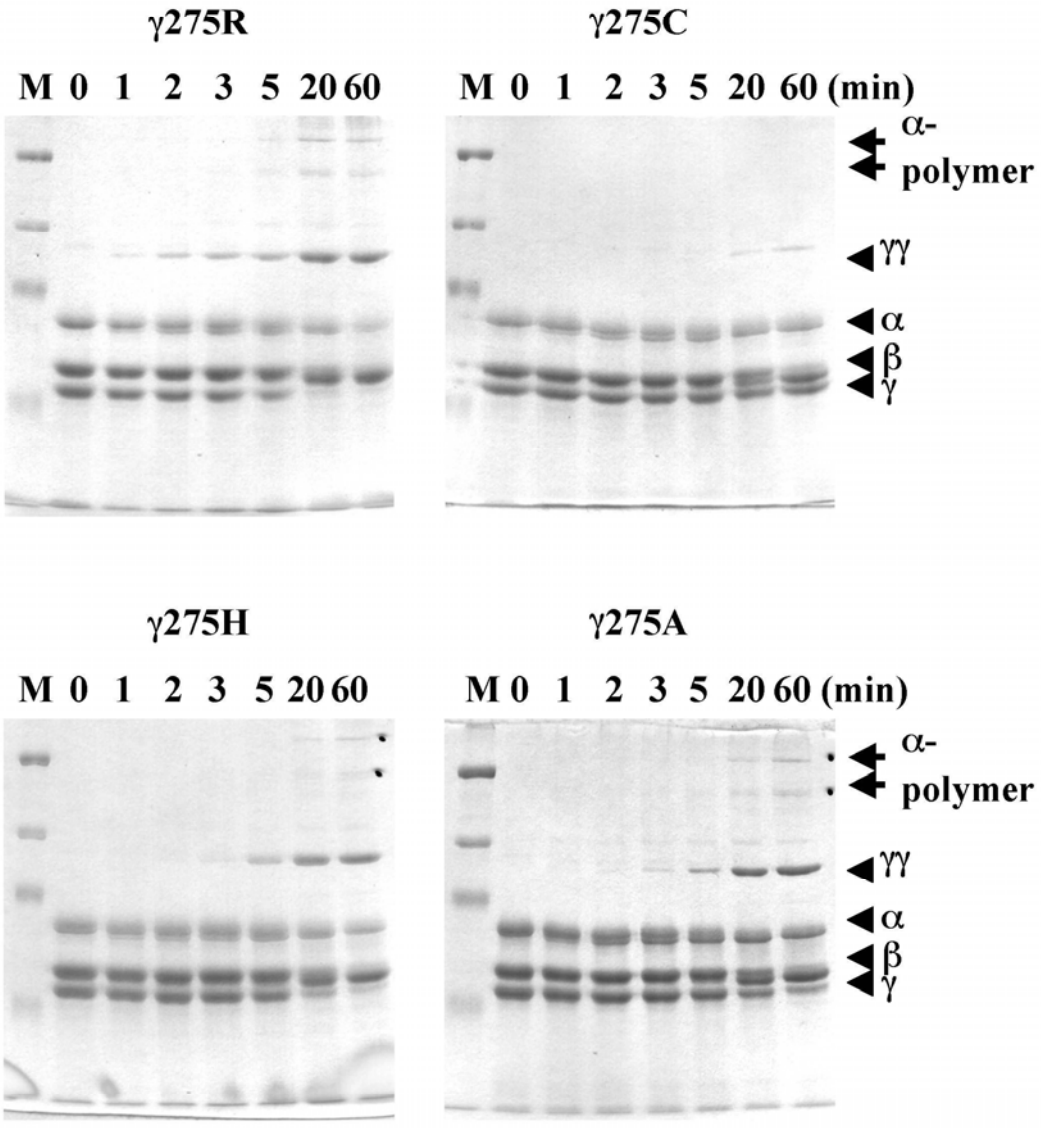

Figure 3. FXIIIa-catalyzed cross-linking of fibrin. Time-dependent FXIIIa-catalyzed cross-linking of fibrin was examined, with 8% SDS-PAGE being run under reducing conditions with Coomassie Brilliant Blue staining, as described in Materials and Methods. The individual fibrinogen chains (α, β, γ, cross-linked γ-γ dimer, and cross-linked α-chain polymers) are indicated on the right side of the gels. The molecular markers (213, 120, 76, and 47 kDa; from top to bottom) were run in lane M.

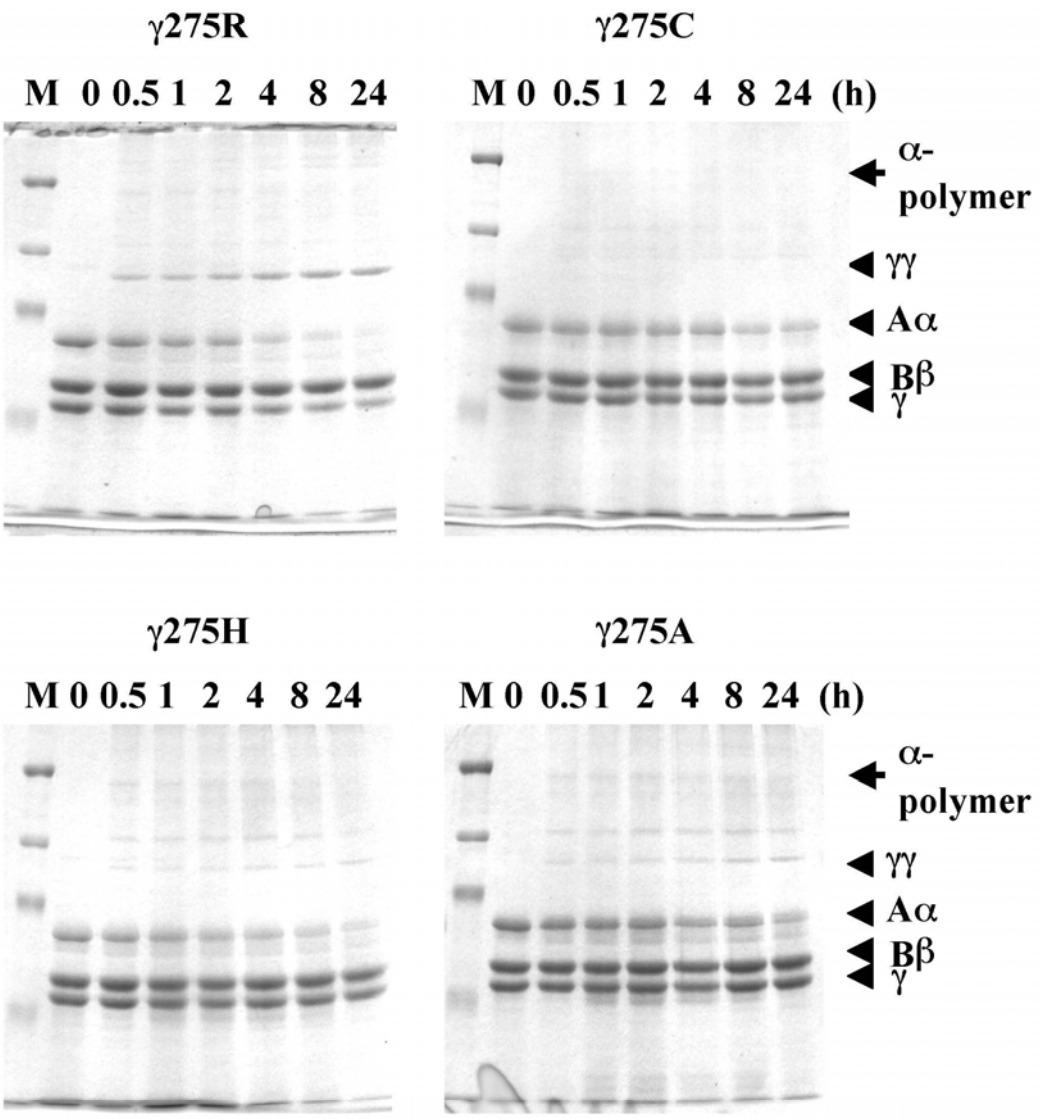

Figure 4. FXIIIa-catalyzed cross-linking of fibrinogen. Time-dependent FXIIIa-catalyzed cross-linking of fibrinogen was examined as described in Materials and Methods and in Figure 3 legend. The individual fibrinogen chains (Aα, Bβ, γ, cross-linked γ-γ dimer, and cross-linked α-chain polymer) are indicated on the right side of the gels.

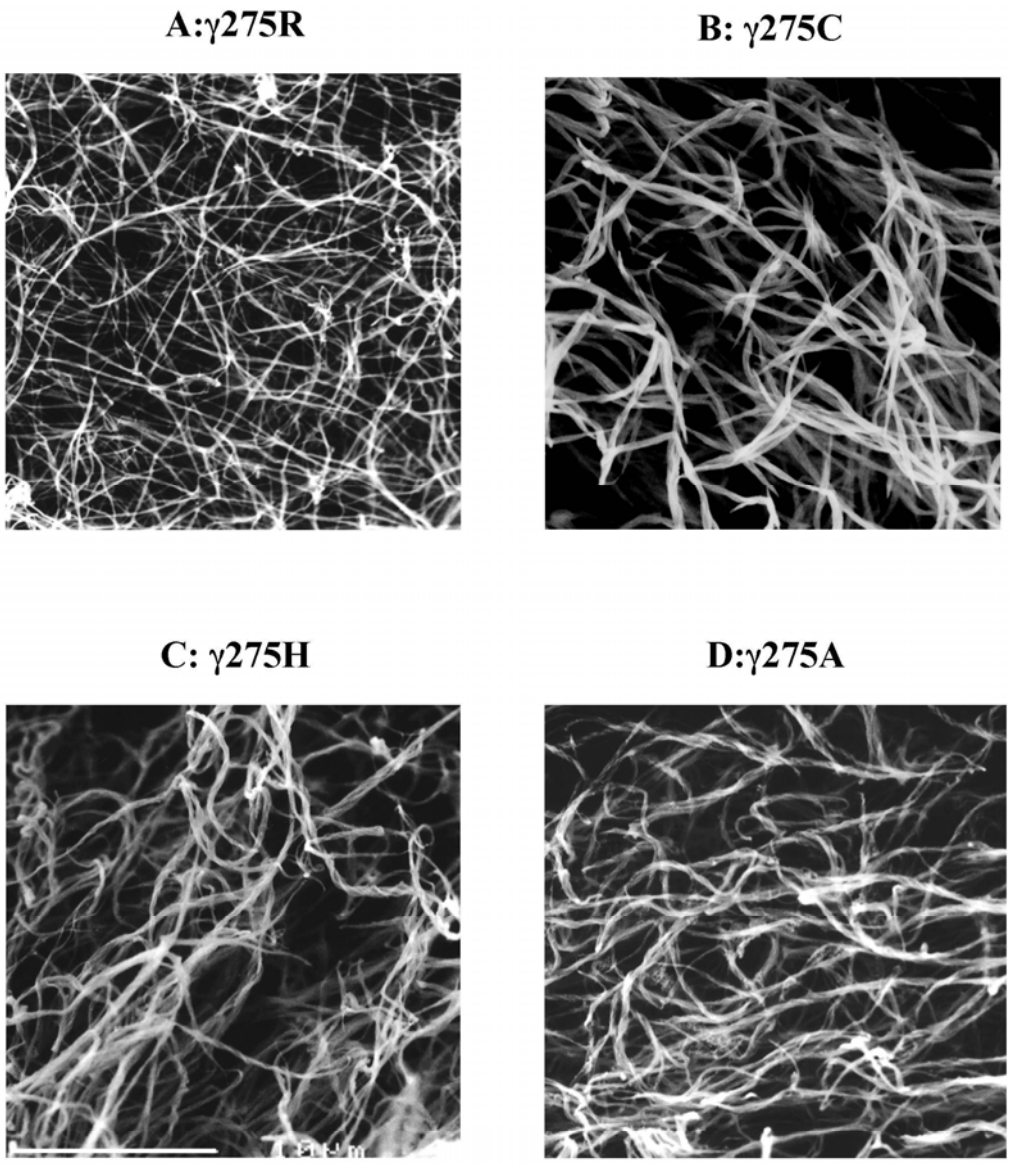

Figure 5. Scanning electron microscopy of fibrin clots. All samples were prepared as described in Materials and Methods. Micrographs were taken at 3000×. Bar**,** 10 μm.

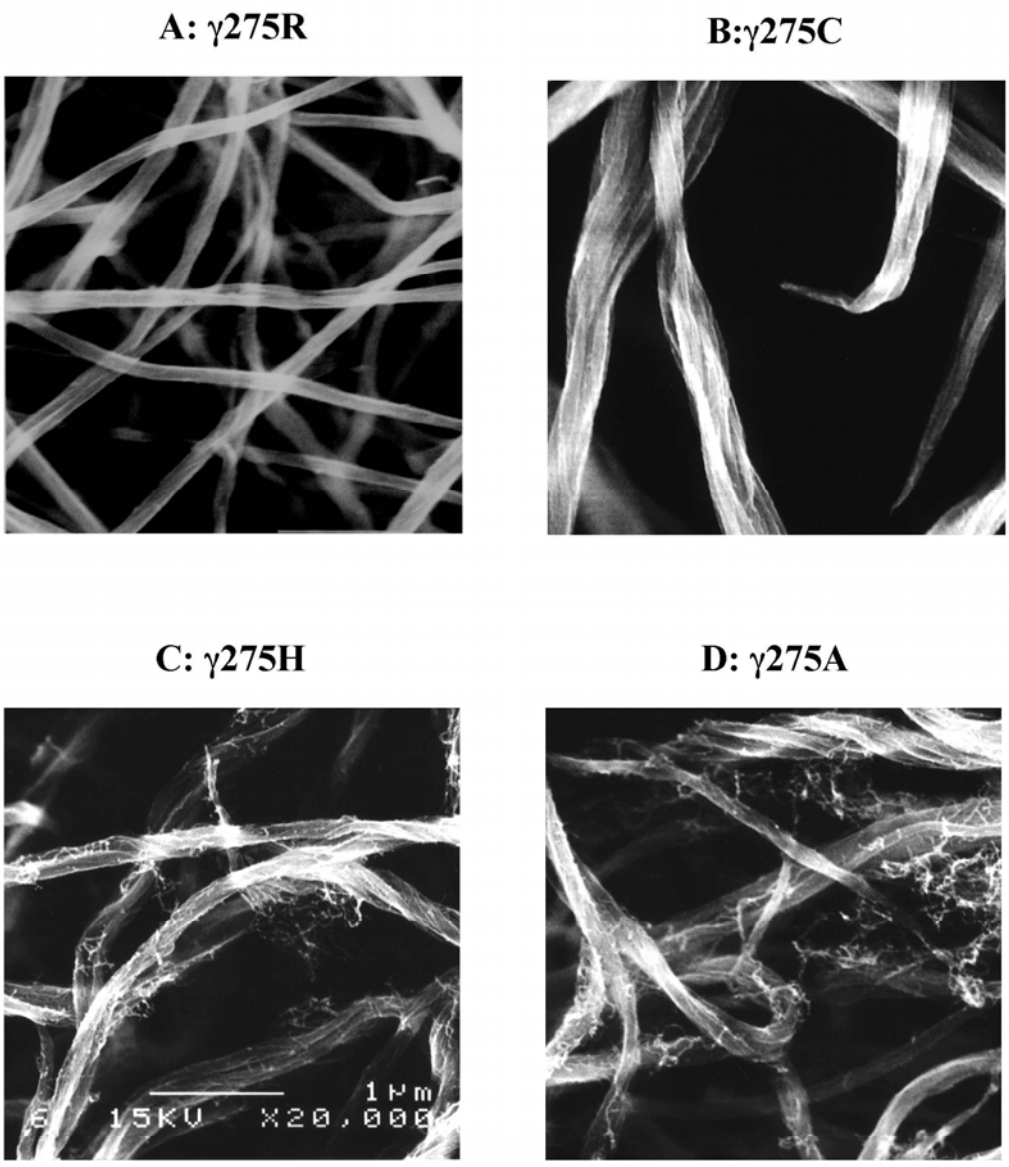

Figure 6. Scanning electron microscopy of fibrin clots. All samples were prepared as described in Materials and Methods. Micrographs were taken at 20000×. Bar**,** 1 μm.

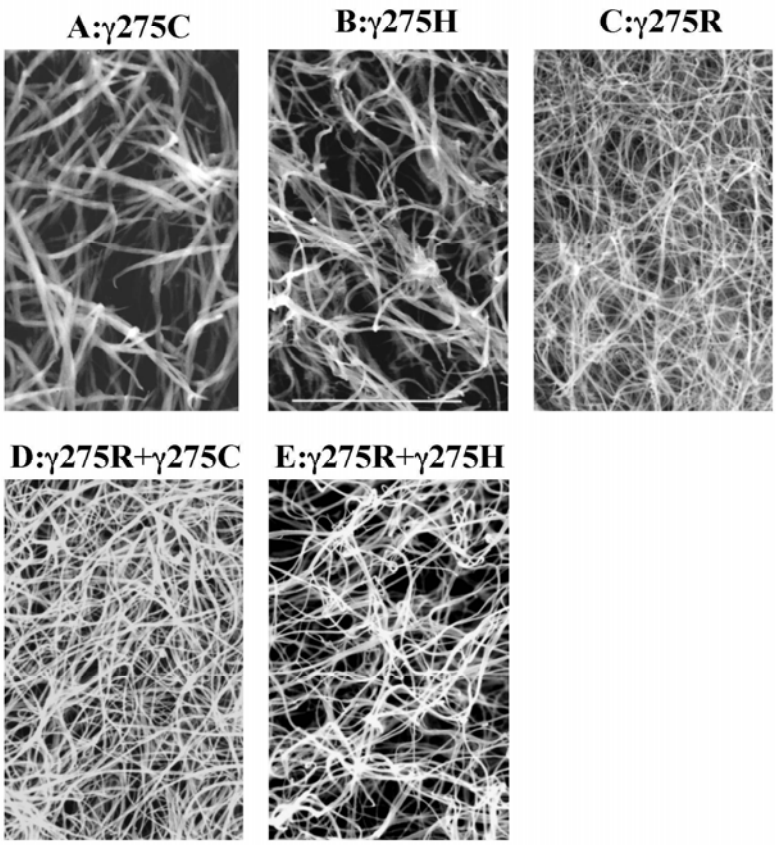

Figure 7. Scanning electron microscopy of fibrin clots formed using a 1:1 mixture of two recombinant fibrinogens. All samples were prepared as described in Materials and Methods. (D) and (E) represent 1:1 fibrinogen mixtures □□ γ275R plus γ275C and γ275R plus γ275H, respectively. Micrographs were taken at 3000×. White bar**,** 10 μm.