Analysis of Aα251 Fibrinogen: The αC Domain Has a Role in Polymerization, Albeit More Subtle Than Anticipated from the Analogous Proteolytic Fragment X†

Oleg V. Gorkun,‡ Agnes H. Henschen-Edman,§ Li Fang Ping,‡ and Susan T. Lord*,‡,||

Department of Pathology and Laboratory Medicine, Curriculum in Genetics and Molecular Biology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7525, and Department of Molecular Biology and Biochemistry, University of California at Irvine, Irvine, California 92697-3900

Received June 30, 1998; Revised Manuscript Received September 1, 1998

ABSTRACT: Numerous experiments have demonstrated that the C-terminal domain of the fibrinogen Aα-chain, the αC domain, has a role in polymerization. To further examine the role of this domain, we synthesized a recombinant fibrinogen, Aα251 fibrinogen, that lacks the αC domain. We examined thrombin-catalyzed fibrinopeptide release and found that the rate of FpB release from Aα251 fibrinogen was 2.5-fold slower than FpB release from normal fibrinogen, while the rate of FpA release was the same for both proteins. We examined thrombin-catalyzed polymerization and found that the rates of protofibril formation and lateral aggregation were similar for both proteins, although discernible differences in lateral aggregation were clear. The rate of protofibril formation for Aα251 fibrinogen was never less than 85% of normal fibrinogen, while the rate of lateral aggregation for Aα251 fibrinogen varied from 64 to 74% of normal. We examined polymerization of fibrin monomers and found that polymerization of Aα251 fibrin was similar to normal fibrin at 0.4 M NaCl, but clearly different from normal at 0.05 M NaCl. These results indicate that the αC domain has a role in lateral aggregation, but this role is more subtle than anticipated from previous experiments, particularly those with fibrinogen fragment X. We interpret this unanticipated finding as indicative of an important contribution from the N-terminus of the β-chain, such that protein heterogeneity that includes small amounts of fibrin lacking that N-terminus of the β-chain leads to markedly altered lateral aggregation.

Fibrinogen is a soluble plasma glycoprotein that has significant roles in hemostasis and wound healing.[1] Fibrinogen is composed of six polypeptides, two copies each of three nonidentical chains called Aα, Bβ, and γ. A model, derived from biochemical and electron microscopy data, shows fibrinogen as a multidomain protein, with a single central domain and a pair of symmetric peripheral domains, linked by rodlike connectors (*1*). The central domain contains the N-termini of all six chains and can be isolated as a plasmin fragment called E. The C-termini of each set of three chains extend in opposite directions from the center, each chain terminating as an independent globular domain. The globular domains of the Bβ- and γ-chains are closely associated (*2*) and can be isolated together from a plasmin digest as the fragment called D. The Aα-chains pass through the peripheral D domains and fold back toward the center of the molecule (*2*, *3*), such that the globular C-terminal domains, called αC, interact with one another and associate with the center of the molecule (*4*−*6*).

During coagulation fibrinogen is converted to fibrin by thrombin-catalyzed cleavage of four peptide bonds, producing two fibrinopeptides A (FpA, Aα 1−16), two fibrinopeptides B (FpB, Bβ1−14), and fibrin monomer (*7*, *8*). Fibrin monomers polymerize to form insoluble fibrin fibers. The assembly of monomers is thought to occur in two stages, the formation of double-stranded protofibrils and the lateral aggregation of these protofibrils into fibers. Both stages are influenced by solution conditions such as pH, salt concentration, and the presence of calcium (*9*−*14*). It is generally accepted that protofibrils form by interactions between the N-terminus of the α-chain of one monomer, exposed upon FpA release, and the C-terminal globular domain of the γ-chain of a second monomer (*15*, *16*). Because fibrin molecules are symmetric, the two N-terminal sites in the central domain of one molecule can interact with C-terminal γ-chain domains on two other fibrin molecules, forming a half-staggered, double-stranded protofibril (*17*). The interactions that promote lateral aggregation of protofibrils are not clearly defined.

Numerous experiments demonstrate that the αC domains have a role in polymerization (*18*−*24*). Experiments with fragment X, a proteolytic fibrinogen derivative that lacks the αC domain, were the first to suggest such a role. Polymerization of fragment X is impaired, and clots formed by fragment X are abnormal. Preparations of fragment X are invariably heterogeneous (*25*−*27*), making it difficult to determine whether changes in polymerization arise from the

† This work was supported by the National Institutes of Health Grants HL 31048 (S.T.L.) and HL 424121 (A.H.H.-E.).

* Corresponding author. Phone: (919) 966-3548. Fax: (919) 966-6718. E-mail: stl@med.unc.edu.

‡ Department of Pathology and Laboratory Medicine.

§ Department of Molecular Biology and Biochemistry.

|| Curriculum in Genetics and Molecular Biology.

[1] Abbreviations: CHO, chinese hamster ovary; FpA, fibrinopeptide A; FpB, fibrinopeptide B; HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction.

10.1021/bi981551t CCC: $15.00
Published on Web 10/16/1998

absence of the αC domain alone. The best studies utilized fragment X monomers prepared by several cycles of polymerization, and even these relatively homogeneous fragment preparations contained up to 15% of degraded β-chains (*23*). Experiments with these fragment X monomers led to the conclusions that αC domains enhance fibrin polymerization, may effect the final clot structure, and probably have a significant role in lateral aggregation. These conclusions are supported by other studies. For example, well-characterized αC domain fragments inhibit polymerization of fibrin monomers (*22*, *23*). Studies with several naturally occurring fibrinogen variants with Aα-chain truncations (*28*−*30*) imply that αC domains are important for polymerization, although the heterogeneity of the variant molecules complicates interpretation of these results. Finally, monoclonal antibodies, which react with epitopes in the αC domain, interfere with fibrinogen polymerization (*21*, *31*).

To further examine the role of this domain in fibrin polymerization, we synthesized a recombinant fibrinogen, Aα251 fibrinogen, that lacks the αC domain. This protein, which contains normal Bβ- and γ-chains but only the first 251 residues of Aα-chain, is analogous to fragment X; however, unlike fragment X, the six chains in these molecules are homogeneous. Further, internal proteolytic breaks are less likely as the recombinant protein is not subjected to proteolysis, as is required for fragment X preparation. We examined thrombin-catalyzed fibrinopeptide release and fibrin polymerization, as well as fibrin monomer polymerization. We were surprised to find that polymerization of Aα251 fibrinogen was remarkably like that of normal fibrinogen, although differences were seen at low salt concentration. Because changes in salt concentration are associated with changes in lateral aggregation (*9*, *32*), we also examined monomer polymerization as a function of NaCl concentration.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were of reagent grade and, unless specified, were purchased from Sigma. Monoclonal antibody IF-1 (MoAb IF-1) was a generous gift from Dr. Michio Matsuda, Institute of Hematology, Jichi Medical School, Japan. Human α-thrombin was a generous gift from Dr. Frank Church, Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill. The plasmid vectors, CHO cells, and culture medium for normal recombinant fibrinogen have been previously described (*33*).

Vector Construction. The plasmid that encodes the Aα 1−251 fibrinogen chain (pMLP-Aα251) was constructed from the expression vector (pMLPAα1−610), which encodes the Aα1−610 fibrinogen chain, as outlined in Figure 1. The plasmid pMLPAα1−610 was constructed from pMLP-Aα (*34*), which encodes the Aα1−625 fibrinogen chain. To generate pMLPAα1−610, pMLP-Aα was used as a template for PCR with a forward primer containing the unique *Sal*I site and a reverse primer that introduced a TGA stop codon after amino acid 610 and added unique *Sma*I and *Nhe*I sites after the TGA. The PCR product was cleaved with *Sal*I and *Sma*I and cloned into the expression vector p284 (*34*) cleaved with *Sal*I and *Sma*I. Two fragments from pMLPAα1−610 were used to generate pMLP-Aα251. The vector was prepared by cleaving pMLPAα1−610 with *Nhe*I; the single strand ends were filled using vent polymerase and the DNA was then cleaved with *Sal*I. The ∼4230 bp vector fragment was isolated by electroelution. The encoding fragment was prepared by cleaving pMLPAα1−610 with *Xho*I; the single strand ends were removed with mung bean nuclease, and the DNA was then cleaved with *Sal*I. The ∼820 bp fragment was isolated by electroelution. The vector fragment and the encoding fragment were ligated and the products transformed into competent DH5α cells. Plasmid DNAs from individual colonies were screened by restriction enzyme analysis, and the DNA encoding the Aα251 fragment was sequenced as described (*34*). Cloning procedures were as described (*35*) using enzymes from New England Biolabs, Beverly, MA, or Promega, Madison, WI.

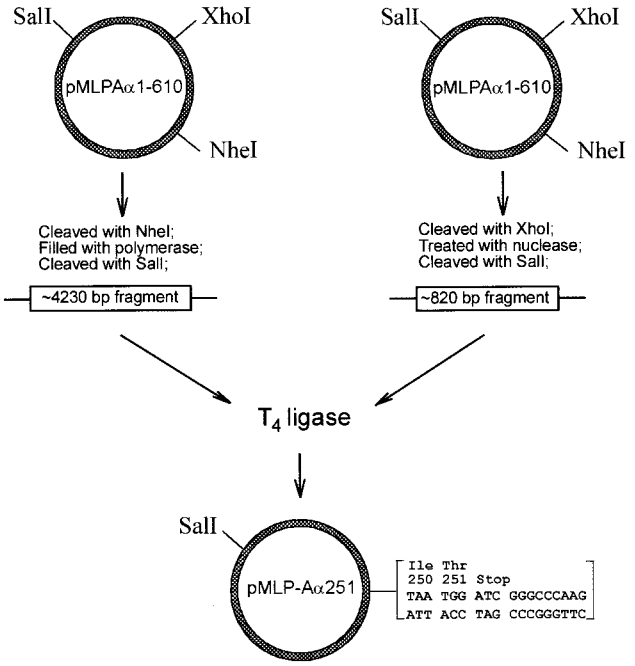

FIGURE 1: Construction of the Aα-chain expression vector pMLP-Aα251. Unique restriction sites in pMLP-Aα610 are *Nhe*I (GCT TGA G\CT AGG), *Xho*I (ATT AC\T CGA GGA). The DNA and amino acid sequences (with corresponding numbers) at the new junction in pMLP-Aα251 are depicted on the right.

Recombinant Fibrinogen Expression. Normal recombinant fibrinogen was synthesized in CHO cell culture as previously described (*33*−*36*). CHO cell lines that express normal human fibrinogen Bβ-chain, CHO-Bβ, were obtained by transfecting the previously described plasmids pMLP-Bβ and pRSVneo into CHO cells, using described procedures (*34*). One cell line that synthesized a high level of Bβ-chain was selected for further transfections. The vectors encoding the Aα 1−251 chain, pMLPAα1−251, and γ 1−411 chain, pMLP-γ, were cotransfected along with the histidinol selection plasmid (pMSVhis) into the CHO-Bβ cells, and histidinol resistant colonies were selected. Selected colonies were expanded, and culture medium was analyzed for fibrinogen concentration by ELISA (*34*). Cell lines producing the highest amounts of fibrinogen were used for large-scale cultures, which were grown in roller bottles supplemented with microcarrier beads (*33*). Serum-free medium containing recombinant fibrinogen was harvested once or twice a week from several roller bottles, the individual harvests were pooled, PMSF was added to 0.15 mM, and pooled samples were stored at −20 °C.

Purification of Fibrinogen. Both recombinant fibrinogens were purified on IF-1 MoAb conjugated Sepharose 4B column as described (*36*, *37*). Purified fibrinogen, eluted from the column, was dialyzed against 20 mM HEPES, pH 7.4, and 0.15 M NaCl at 4° C, centrifuged at 13000*g* for 15 min (4° C), and the supernatant was stored in aliquots at −70° C.

Clottability. Fibrinogen (0.3 mg/mL) was incubated with thrombin (1 units/mL) for 2 h as described (*36*). Fibrinogen clottability was determined based on the residual absorption of the supernatant, at 280 nm, after the fibrin clot was removed by centrifugation. The clottabilities of both recombinant fibrinogens were about 97%.

Fibrin Monomer Preparation. Fibrin monomer was prepared by clotting fibrinogen with thrombin and dissolving the clot in 0.125% acetic acid as described (*38*) with modifications (*36*). We monitored cleavage of fibrinopeptides by HPLC to ensure that 100% of fibrinopeptide A and B was cleaved from both normal and Aα251 recombinant fibrin monomers. Both fibrin monomers were prepared and treated in the same way. Fibrin monomer was stored at 4° C and used within 1 month of preparation.

Release of Fibrinopeptides. Aliquots of purified fibrinogens were thawed at 37 °C and dialyzed against 20 mM HEPES, pH 7.4, 0.15 M NaCl, 5 mM ϵACA, and 1 mM $CaCl_2$ at 4 °C. Human α-thrombin (4300 units/mL) was diluted to 0.1 units/mL with the same buffer immediately prior to the reaction and was kept on ice. Fibrinogen of particular concentration was prepared, assuming that $\epsilon_{280} = 15.06$ for a 10 mg/mL solution of normal ($M_r = 340$ kDa) recombinant fibrinogen (*39*), and $\epsilon_{280} = 16.0$ for a 10 mg/mL solution of Aα251 ($M_r = 260$ kDa) recombinant fibrinogen. The final concentrations of fibrinogen and thrombin in the reaction mixture were 0.3 μM and 0.11 nM (0.01 units/mL), respectively. The reactions were performed as described (*33*). Fibrinopeptides were detected by reversed-phase HPLC on an ISCO chromatography system directed by ISCO Chemresearch 2.4 software, using a Vydak C-18 column (4.6 × 250 mm) with an acetonitrile gradient as previously described (*40*). The quantity of fibrinopeptide was calculated from the areas under the HPLC peak, and the data were fit to first-order equations, using the software ENZFITTER (Biosoft), as described (*33*). All experiments were performed four times.

Polymerization Turbidity Curves. Polymerization was measured by monitoring turbidity changes with time at 350 nm using a Shimadzu BioSpec 1601 UV−vis spectrophotometer equipped with a thermostatic cuvette holder. Prior to polymerization, both fibrinogens were dialyzed against 20 mM HEPES, pH 7.4, 5 mM ϵACA, and 1 mM $CaCl_2$ buffer. To 90 μL of fibrinogen in a 100 μL microcuvette (Starna Cells, Inc, Alscadero, CA) was added 10 μL of thrombin at the zero time point. The final concentrations were 0.7, 0.3, and 0.07 μM for fibrinogen and 0.1 unit/mL for thrombin. Fibrin monomer polymerization was initiated by adding 10 μL of 0.59 μM fibrin monomer to 90 μL of 20 mM HEPES, pH 7.4, 5 mM ϵACA, and 1 mM $CaCl_2$ buffer, with NaCl concentration as indicated. All experiments were performed at 25 °C. Polymerization curves were characterized by two parameters—the lag period, which is the time required for protofibrils to form, and the maximum rate of assembly (V_{max}) which is the rate of the lateral aggregation of protofibrils (*41*). The polymerization experiments were done three times for each NaCl concentration used, and standard deviation was calculated for the parameters.

SDS−PAGE and Western Blot. Reduced samples of both fibrinogens were run on 10% gels and nonreduced samples on 6% gels and stained with Coomassie Blue G-250. In addition, reduced samples of fibrinogen separated by 10% SDS−PAGE were transferred onto 0.45 μm nitrocellulose (Bio-Rad, Hercules, CA) for Western blotting. The blots were developed as described (*34*) with either an anti-fibrinogen polyclonal antibody (DAKO, Carpinteria, CA), monoclonal antibody Y18, which recognizes the N terminus of the Aα-chain of human fibrinogen (*42*), or a monoclonal antibody against residues 15−21 of the Bβ-chain (*43*). Immunoreactive bands were visualized using alkaline phosphatase conjugated goat anti-rabbit (Pierce, Rockford, IL) or goat anti-mouse antiserum (Promega, Madison, WI).

Protein Characterization. The two recombinant fibrinogens and plasma fibrinogen (KabiVitrum, Stockholm, Sweden) were analyzed in parallel. About 0.3 mg of each protein was dissolved in 0.7 mL of a 10% solution of cyanogen bromide (w/v) in 80% formic acid (v/v) and left for 2 h at room temperature. Fragments in the sample were fractionated using the Sephadex G-50SF (Pharmacia, Uppsala, Sweden) column (1 × 100 cm) at the flow rate of 6 mL/h. The elution buffer was 0.2% trifluoroacetic acid in water (v/v). Fractions from each chromatogram were pooled and subjected to N-terminal amino acid sequence analysis for 5−12 cycles in a Hewlett-Packard model G1005A Protein Sequencer (Palo Alto, CA). The fractions, known to contain the N-terminal disulfide knot, were also treated with thrombin and sequenced. The pool containing the C-terminal fragment of the Aα251 chain was analyzed by matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Voyager-DE, PerSeptive Biosystems, Framingham, MA).

RESULTS

Characterization of Aα251 Fibrinogen Structure. We characterized the purified recombinant fibrinogens by SDS−PAGE (Figure 2, panels A and B) and immunoblot analysis (Figure 2C). As shown in Figure 2A, under nonreducing conditions, each fibrinogen appeared as a single band demonstrating that these proteins are pure and that the polypeptide chains are assembled into a single molecule. Further, the mobility of each band is consistent with the anticipated molecular mass of 340 kDa for normal fibrinogen and 265 kDa for Aα251 fibrinogen. Under reducing conditions, each fibrinogen appeared as three bands, two of which were the same for both proteins and ran with the mobility expected for the normal Bβ- and γ-chains (Figure 2B, lanes 1 and 2). For normal fibrinogen, the third band ran as expected for the Aα-chain, while for Aα251 fibrinogen the third band ran alongside the 30 kDa marker protein, consistent with the calculated mass, 28.95 kDa, for this truncated chain. To confirm the chain identities, we examined immunoblots of reduced SDS−PAGE developed with several antibodies. All the polypeptides seen by SDS−PAGE reacted with a polyclonal antibody to human fibrinogen (Figure 2C, lanes 1 and 2). Immunoblots developed with a monoclonal antibody to the Bβ-chain (Figure 2C, lanes 3

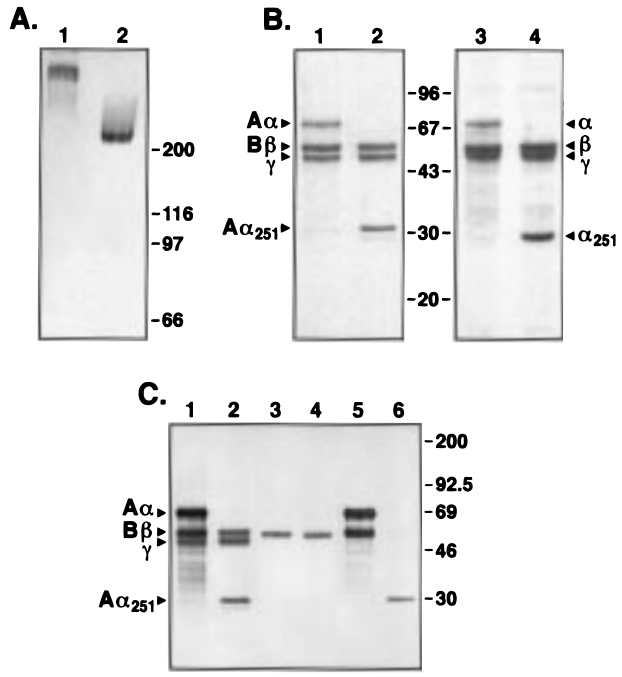

FIGURE 2: (A) Coomassie stained 6% SDS−PAGE run under nonreducing conditions; lane 1, normal fibrinogen; lane 2, Aα251 fibrinogen. Size markers are indicated on the right. (B) Coomassie stained 10% SDS−PAGE run under reducing conditions; lane 1, normal fibrinogen; lane 2, Aα251 fibrinogen; lane 3, normal fibrin; lane 4 Aα251 fibrin. Size markers are indicated between the panels; Fibrinogen Aα-, Bβ-, γ-, and Aα251-chains are indicated on the left and fibrin α-, β-, γ-, and α251-chains are indicated on the right. (C) Western blot of 10% SDS−PAGE of normal (lanes 1, 3, and 5) and Aα251 fibrinogen (lanes 2, 4, and 6) under reducing conditions. The primary antibodies for lane 1 and 2 was polyclonal antiserum raised against human fibrinogen (DAKO, Carpinteria, CA), for lanes 3 and 4, a monoclonal antiserum specific for the N-terminus of the Bβ-chain (*43*), and for lanes 5 and 6 a monoclonal antiserum specific for the N-terminus of Aα-chain (*42*). The position of Aα-, Bβ-, γ-, and Aα251-chains are indicated on the left and the size markers are indicated on the right.

and 4) or the Aα-chain (Figure 2C, lanes 5 and 6) showed that the Bβ-chain of Aα251 fibrinogen was indistinguishable from normal, while the Aα-chain from Aα251 fibrinogen differed from normal and was, as expected, the band with the 30 kDa relative mobility. The immunoblots further demonstrated that the individual chains in Aα251 fibrinogen were homogeneous. In particular, while normal fibrinogen displayed the usual heterogeneity in Aα (two bands which run close to 67 kDa and one at 54 kDa), the Aα-chain from Aα251 fibrinogen was a single band. The homogeneity of all three chains in recombinant Aα251 fibrinogen is in a marked contrast to fragments X prepared from plasma fibrinogen, where heterogeneity is present in both the Aα- and the Bβ-chains (*23*). We also examined fibrin monomers, prepared by treating fibrinogen with thrombin and dissolving the resulting clot in the 0.125% acetic acid. As expected, the mobility of α, α_{251}, and β-chains increased after fibrinopeptide cleavage (Figure 2B, lanes 3 and 4).

To confirm that the individual chains of the two recombinant fibrinogens were those predicted from the transfected cDNAs, we examined by amino acid sequence analysis all amino-terminal and carboxyl-terminal fragments of all chains as well as a large number of internal fragments obtained after cyanogen bromide cleavage. Before sequencing, the fragments were partially purified by gel-filtration chromatography. Both plasma and normal recombinant fibrinogens displayed the same pattern of elution during chromatography while the pattern of Aα251 fibrinogen was different. With both recombinant proteins, thrombin digestion exposed the same sequences of the corresponding fibrin chains. Sequences from 25 fragments were identified in normal recombinant fibrinogen, and sequences from 31 fragments were identified in the Aα251 fibrinogen. All data were in agreement with the anticipated sequences at the expected ratios. Mass-spectrometric analysis of the fractions confirmed the presence of the fragments with the predicted mass. The data summarized in Table 3 demonstrate that the correct reading frames have been maintained throughout all peptide chains of both recombinant proteins.

Table 1: Specificity Constants, k_{cat}/K_m, for FpA and FpB Release by Thrombin[a]

substrate	FpA	FpB
normal fibrinogen	8.1 ± 0.8	5.8 ± 0.5
Aα251 fibrinogen	6.2 ± 0.8	2.3 ± 0.9

[a] Values are × 10^6 M^{-1} s^{-1} ± standard deviation (*33*).

Table 2: Polymerization Parameters[a]

	normal fibrinogen		Aα251 fibrinogen	
concn (mg/mL)	lag-period (s)	maximum rate (× 10^{-5} s^{-1})	lag-period (s)	maximum rate (× 10^{-5} s^{-1})
0.2	165	78	186	51
0.1	192	35	194	26
0.02	270	3.6	300	2.3

[a] Data are calculated as described in the Experimental Procedures from the curves shown in Figure 4.

Table 3: Comparison of Polypeptide Chain Compositions

	Aα251 fibrinogen	X_1 monomer[a]	X_2 monomer[a]
Aα-chain and derivatives	1−251	17−609	17−219
		17−262	
Bβ-chain and derivatives	1−461	15−461	15−461
		43−461[b]	43−461[c]
γ-chain and derivatives	1−411	1−411	1−411
		1−419	1−419

[a] From Gorkun et al. (*23*). [b] This form is 4% of the total Bβ-chain. [c] This form is 15% of the total Bβ-chain.

Thrombin-Catalyzed Fibrinopeptide Cleavage. The thrombin-catalyzed release of fibrinopeptides was monitored by HPLC, as described (*33*, *40*), and % peptide released was plotted as a function of time. The average data from four experiments were fitted assuming that the release of both fibrinopeptides is first order and that the release of FpB follows the release of FpA (*33*, *44*). As shown in Figure 3, the release of FpA was similar for both fibrinogens as expected from previous experiments. In contrast, the release of FpB from Aα251 fibrinogen was slower than FpB release from normal fibrinogen. We determined the specificity constants for each reaction as described in the Experimental Procedures; the data are presented in Table 1. The data showed that the rate of FpB release from Aα251 fibrinogen was 2.5-fold slower than FpB release from normal fibrinogen, while the rates of FpA release were essentially the same for both proteins.

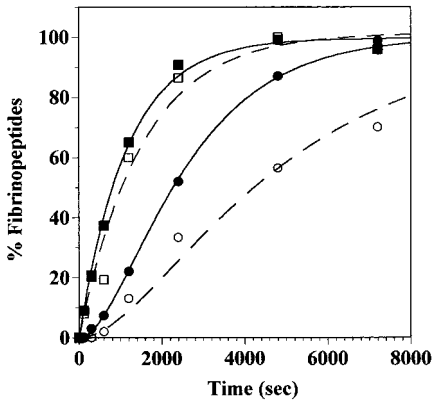

FIGURE 3: Progress curves for fibrinopeptide release. Thrombin catalyzed fibrinopeptide release of normal fibrinogen, solid lines, FpA (■) and FpB (●), and of Aα251 fibrinogen, dashed lines, FpA (□) and FpB (○). Curves represent the best fit of average data ($n = 4$) to first-order rate equations, assuming FpA release before FpB release, as described (*34*, *44*).

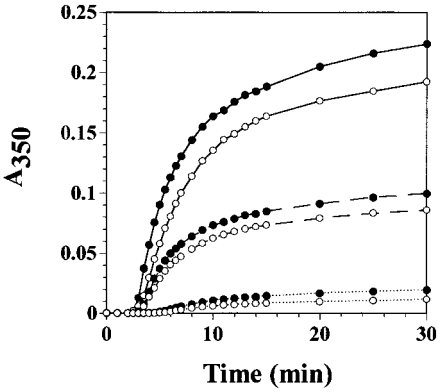

FIGURE 4: Thrombin-catalyzed polymerization. Representative polymerization curves of normal (●) and Aα251 (○) fibrinogen at concentrations: 0.2 mg/mL (solid line), 0.1 mg/mL (dashed line), and 0.02 mg/mL (dotted line). Polymerization was initiated by the addition of thrombin at time 0 (0.1 units/mL) and polymer formation was measured as change in turbidity at 350 nm with time.

Thrombin-Catalyzed Fibrin Polymerization. Polymerization was monitored as the change in turbidity at 350 nm (*41*). Representative data obtained at three concentrations of fibrinogen are shown in Figure 4. We were surprised that the curves for Aα251 fibrinogen were very similar to the curves for normal fibrinogen, as polymerization of fibrinogen fragment X differed markedly from normal fibrinogen (*23*). From the curves in Figure 4, we determined the lag time, which represents the time required to form protofibrils, and the maximum slope, which represents the rate of protofibril assembly into fibers (the rate of lateral aggregation). The results, presented in Table 2, demonstrated that the lag period for Aα251 fibrinogen was essentially the same, and the maximum rate was slightly smaller than for normal fibrinogen. The rates of protofibril formation and protofibril assembly decreased significantly with lower fibrinogen concentration, but the magnitude of these changes with Aα251 fibrinogen mirrored those seen with normal fibrinogen. At all concentrations tested, the lag period for Aα251 fibrinogen was minimally different, less than 15% longer than normal fibrinogen. Thus, the rate of protofibril formation was basically the same for both proteins. The rates of fibril assembly for Aα251 fibrinogen were slower than those for normal fibrinogen; Aα251 fibrinogen rates varied from 64 to 74% of normal. These results indicated that the rate of lateral aggregation was slower with Aα251 fibrinogen compared to normal fibrinogen.

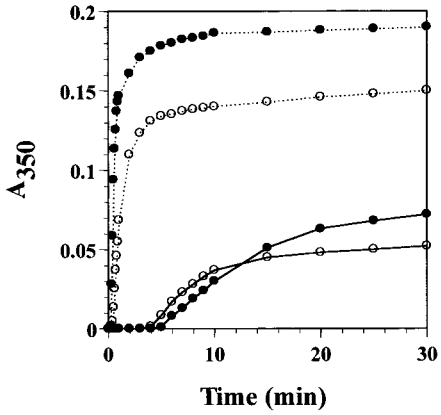

FIGURE 5: Fibrin monomer polymerization. Polymerization of normal (●) and Aα251 (○) fibrin monomers at 0.4 M (solid line) and 0.05 M (dotted line) concentration of NaCl. Fibrin monomers (0.59 μM) were diluted 10-fold into neutral buffer at time 0 and the changes in turbidity monitored at 350 nm with time. Representative curves are shown.

Polymerization of Fibrin Monomers. Because the ordered release of fibrinopeptides influences polymerization, the thrombin-catalyzed polymerization of fibrinogen differs from polymerization of fibrin monomer (*45*). To investigate polymerization apart from catalysis, we prepared fibrin monomers from normal and Aα251 fibrinogens. We examined the polymerization of fibrin monomers at high and low salt concentrations; representative curves are shown in Figure 5. As others have found with normal plasma fibrinogen (*12*, *32*), we found that fibrin monomer polymerization was slower at high salt (solid lines) than at low salt (dotted lines) for both recombinant fibrinogens. Significantly, monomer polymerization of Aα251 fibrinogen was similar to normal fibrinogen at 0.4 M NaCl, but clearly different from normal fibrinogen at 0.05 M NaCl.

To characterize the difference observed at low salt, we monitored fibrin monomer polymerization at several NaCl concentrations. We determined the lag times and the maximum polymerization rates from these curves, and plotted these data as a function of NaCl concentration. As shown in Figure 6A, the lag period increased with higher NaCl concentration, and the results with Aα251 fibrin resemble those with normal fibrin. In contrast, as shown in Figure 6B, the maximum rate of polymerization increased with decreasing of NaCl concentration, and more importantly, obvious differences between Aα251 fibrin and normal fibrin were detected at the lowest NaCl concentrations. These experiments support the previous finding that fibril assembly was slower with Aα251 fibrinogen in comparison with normal fibrinogen.

DISCUSSION

Earlier experiments showed that the αC domains of fibrinogen have a significant role in fibrin polymerization. This conclusion is based largely on studies with fibrinogen fragments, in particular, fragments X isolated from plasmin digests. In the experiments presented here, we synthesized a recombinant fibrinogen molecule similar to fragment X, Aα251 fibrinogen, and examined the biochemical properties

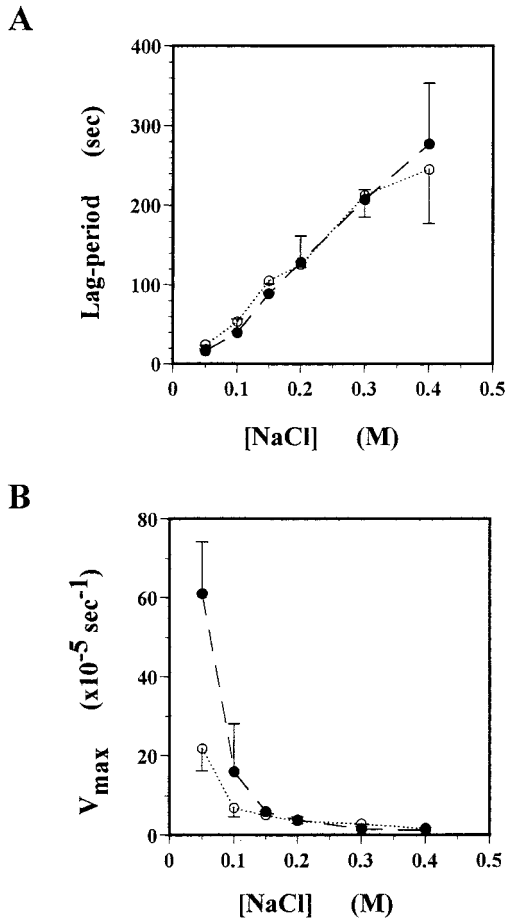

FIGURE 6: Effect of NaCl concentration on the lag-period (A) and maximum rate of polymerization, V_{max}, (B) during polymerization of normal fibrin monomer (dashed line, ●) and Aα251 fibrin monomer (dotted line, ○). Each data point is an average of three determinations and bars represent standard deviation.

of this molecule. Surprisingly, we found that Aα251 fibrinogen differed substantially from fibrinogen fragment X. Nevertheless, our data suggest that the αC domains of fibrinogen have a role in fibrin polymerization, particularly in promoting lateral aggregation.

With Aα251 fibrinogen, we found that the rate of FpB release was reduced 2−3-fold relative to normal fibrinogen. Because several previous studies indicate that FpB is preferentially released from fibrin polymers, the delayed FpB release suggested that we should expect a significant delay in polymerization (*7*, *8*, *26*). However, this delay in FpB release occurred with only a slight delay in polymerization. The lag periods were essentially the same and the rate of fibril assembly for thrombin-catalyzed polymerization of Aα251 fibrinogen was about 70% of that of normal fibrinogen. Taken together, these results suggest that the C-terminal domain of the Aα-chain has a role in regulating both the release of FpB and the lateral aggregation of protofibrils.

Our data are consistent with a model where the C-terminal domains of the Aα-chain facilitate the release of FpB and thereby participate in the initiation of normal lateral aggregation. This model is also consistent with published results that have demonstrated a correlation between FpB release and lateral aggregation (*7*, *8*, *26*, *45*). This model, however, does not explain the results from our experiments with fibrin monomer. Because FpB release is not relevant to monomer polymerization, the model would predict that polymerization of Aα251 monomers would be the same as of normal fibrin monomers, yet at low salt concentration, the polymerization profiles were significantly different. This result indicates that the αC domains of fibrinogen not only facilitate the release of FpB, but also participate directly in lateral aggregation.

We found that polymerization of fibrin monomers prepared from Aα251 fibrinogen was comparable to polymerization of fibrin monomers prepared from normal fibrinogen, when measured at physiologic salt concentrations. When we examined polymerization at lower salt concentrations, we saw minor differences between the lag phases, but significant differences in the rates of turbidity increase. Because the maximum rate for Aα251 monomers was less sensitive to changes in NaCl concentration, it follows that the loss of the αC domain alters electrostatic interactions that participate in lateral aggregation. We concluded that the αC domain has a more significant role in lateral aggregation than in protofibril formation.

We were surprised that our data differed from published data for fragment X (*6*, *23*, *46*, *47*). The differences may be explained by the proteins per se, as our experiments used proteins with the human sequence and the previous data were obtained with fragments of bovine fibrinogen. This rationale seems unlikely because fibrinogen is a highly conserved molecule. Further, both bovine and human fibrinogens have been extensively studied, and no significant differences in polymerization have been noted. Therefore, we reexamined the published data for fragment X in light of our data with Aα251 fibrinogen. The proteolytic derivative of fibrinogen has been extensively studied, and as reviewed in Gorkun et al. (*23*), the data from these studies have been inconsistent. Because both the plasma fibrinogen substrate and the proteolytic cleavage sites are heterogeneous, it is likely that no two fragment X preparations are exactly alike. Assuming that the published inconsistencies arose from this molecular heterogeneity, the authors (*23*) performed detailed analyses on two carefully characterized fragments, X_1 and X_2, whose purification included the selection of functionally active molecules. Despite this selective purification, neither fragment was homogeneous. The chain compositions for fragments X_1 and X_2 are presented in Table 3. Fragment X_1 was a mixture of six different chains, and X_2 was a mixture of five, and the α-chains of X_1 were entirely different from those of X_2. Polymerization of the two fragments differed from one another, likely reflecting the differences in chain composition. Nevertheless, the differences between X_1 and X_2 were small relative to the differences between either X_1 or X_2 and normal monomers. Comparing intact fibrinogen to X_1 or X_2, more significant differences in polymerization were seen at low (0.09 mg/mL) compared to high (0.5 mg/mL) concentrations of fibrin monomers. In contrast to these results, we did not see significant differences in thrombin-catalyzed polymerization when comparing normal and Aα251 fibrinogens. The polymerization profiles were similar at both high (0.2 mg/mL) and low (0.02 mg/mL) concentrations. We conclude that the apparent contradiction between our data and that reported by Gorkun et al. (*23*) arises from the differences in the chain compositions of the proteolytic fragments X and Aα251 fibrinogen.

It is reasonable to conclude that any or all of the chain heterogeneities contribute to these differences. We favor an important contribution from the N-terminal residues of the β-chain (*48*−*50*). It is known that β15−18 participates in fibrin polymerization, so loss of these residues in X_1 and X_2 could have a dramatic effect. Further, polymerization of fibrinogen lacking Bβ1−42 is impaired. For example, with 100 mM NaCl and no added calcium, polymerization of des-(Bβ1−42)-fibrin monomers was 20-fold slower than normal fibrin monomers (*51*, *52*). Thus, the N-terminus of the β-chain appears to have a significant role in the lateral aggregation of fibrin monomers. Although the N-terminal shortened chain is present in small amounts in X_1 (4%) and X_2 (15%), previous experiments have demonstrated that small amounts of nonclottable material can dramatically influence clot structure (*20*, *23*, *47*). Specifically, fragment X_1 treated with thrombin forms a defective clot, but X_1 monomer obtained after cycles of dissolution and polymerization of the fragment X_1 forms a normal gel (*47*).

We should also emphasize the differences between the C-termini of the α-chain of fragments X_1 and X_2, as compared to Aα251. X_1 has one complete α-chain, and one that ends at reside 268. This mix of αC domains with different degrees of degradation complicates interpretation of the data obtained with fragment X_1. X_2 has more homogeneous α-chains that end at residue 219, so the α-chains in fragment X_2 are shorter than those in the recombinant protein. Because the α-chain C-terminus for X_2 is homogeneous, Gorkun et al. (*23*) concluded that the αC domains enhance polymerization, probably most significantly during lateral aggregation. We found, however, that polymerization of Aα251 was remarkably similar to polymerization of normal fibrinogen. We concluded that either the loss of 15% of β15−42, the loss of α219−251, or both is the basis for the significantly abnormal polymerization of fragment X_2. Alternate interpretations of our data are reasonable. For example, the loss of the αC domains may have two simultaneous, but opposite, effects on polymerization, such that the turbidity profiles were more normal than expected. Currently, we favor our more direct interpretation.

In conclusion, the results from our experiments with Aα251 fibrinogen suggest that the αC domains have a role in lateral aggregation, but this role is more subtle than that anticipated from the published data obtained with fibrinogen fragment X.

ACKNOWLEDGMENT

We thank Dr. Michio Matsuda for providing the monoclonal antibody IF-1 and Dr. Frank Church for providing human α-thrombin. We are also grateful to Michael Rooney, Alyssa Gulledge, Jennifer Mullin, and Kelly Lee for their assistance in preparing this manuscript.

REFERENCES

1. Weisel, J. W., Stauffacher, C. V., Bullitt, E., and Cohen, C. (1985) *Science 230* (4732), 1388−91.
2. Spraggon, G., Everse, S. J., and Doolittle, R. F. (1997) *Nature 389* (6650), 455−62.
3. Mosesson, M. W., Hainfeld, J., Wall, J., and Haschemeyer, R. H. (1981) *J. Mol. Biol. 153* (3), 695−718.
4. Medved, L. V., Gorkun, O. V., and Privalov, P. L. (1983) *FEBS Lett. 160* (1−2), 291−5.
5. Cierniewski, C. S., Plow, E. F., and Edgington, T. S. (1984) *Eur. J. Biochem. 141* (3), 489−96.
6. Veklich, Y. I., Gorkun, O. V., Medved, L. V., Nieuwenhuizen, W., and Weisel, J. W. (1993) *J. Biol. Chem. 268* (18), 13577−85.
7. Blomback, B., Hessel, B., Hogg, D., and Therkildsen, L. (1978) *Nature 275* (5680), 501−5.
8. Higgins, D. L., Lewis, S. D., and Shafer, J. A. (1983) *J. Biol. Chem. 258* (15), 9276−82.
9. Ferry, J. D., and Morrison, P. R. (1947) *J. Am. Chem. Soc. 69*, 388−400.
10. Hantgan, R. R., and Hermans, J. (1979) *J. Biol. Chem. 254* (22), 11272−81.
11. Weisel, J. W. (1986) *Biophys. J. 50* (6), 1079−93.
12. Nair, C. H., Shah, G. A., and Dhall, D. P. (1986) *Thromb. Res. 42* (6), 809−16.
13. Dang, C. V., Shin, C. K., Bell, W. R., Nagaswami, C., and Weisel, J. W. (1989) *J. Biol. Chem. 264* (25), 15104−8.
14. Carr, M. E., and Powers, P. L. (1991) *Blood Coagulat. Fibrinol. 2* (6), 741−7.
15. Olexa, S. A., and Budzynski, A. Z. (1981) *J. Biol. Chem. 256* (7), 3544−9.
16. Laudano, A. P., Cottrell, B. A., and Doolittle, R. F. (1983) *Ann. N.Y. Acad. Sci. 408*, 315−29.
17. Erickson, H. P., and Fowler, W. E. (1983) *Ann. N.Y. Acad. Sci. 408*, 146−63.
18. Marder, V. J., Shulman, N. R., and Carroll, W. R. (1969) *J. Biol. Chem. 244* (8), 2111−9.
19. Hasegawa, N., and Sasaki, S. (1990) *Thromb. Res. 57* (2), 183−95.
20. Sato, H., and Weisel, J. W. (1990) *Thromb. Res. 58* (3), 205−12.
21. Cierniewski, C. S., and Budzynski, A. Z. (1992) *Biochemistry 31* (17), 4248−53.
22. Lau, H. K. (1993) *Blood 81* (12), 3277−84.
23. Gorkun, O. V., Veklich, Y. I., Medved, L. V., Henschen, A. H., and Weisel, J. W. (1994) *Biochemistry 33* (22), 6986−97.
24. Wu, J. H., and Diamond, S. L. (1995) *Anal. Biochem. 224* (1), 83−91.
25. Shen, L. L., McDonagh, R. P., McDonagh, J., and Hermans, J. (1977) *J. Biol. Chem. 252* (17), 6184−9.
26. Nossel, H. L., Hurlet-Jensen, A., Liu, C. Y., Koehn, J. A., and Canfield, R. E. (1983) *Ann. N.Y. Acad. Sci. 408*, 269−78.
27. Mosesson, M. W. (1983) *Ann. N.Y. Acad. Sci. 408*, 97−113.
28. Sobel, J. H., Trakht, I., Wu, H. Q., Rudchenko, S., and Egbring, R. (1995) *Blood 86* (3), 989−1000.
29. Ridgway, H. J., Brennan, S. O., Gibbons, S., and George, P. M. (1996) *Br. J. Haematol. 93* (1), 177−84.
30. Ridgway, H. J., Brennan, S. O., Faed, J. M., and George, P. M. (1997) *Br. J. Haematol. 98* (3), 632−639.
31. Ehrlich, P. H., Sobel, J. H., Moustafa, Z. A., and Canfield, R. E. (1983) *Biochemistry 22* (18), 4184−92.
32. Weisel, J. W., and Nagaswami, C. (1992) *Biophys. J. 63* (1), 111−28.
33. Lord, S. T., Strickland, E., and Jayjock, E. (1996) *Biochemistry 35* (7), 2342−8.
34. Binnie, C. G., Hettasch, J. M., Strickland, E., and Lord, S. T. (1993) *Biochemistry 32* (1), 107−13.
35. Lord, S. T., Binnie, C. G., Hettasch, J. M., and Strickland, E. (1993) *Blood Coagulat. Fibrinol. 4* (1), 55−9.
36. Gorkun, O. V., Veklich, Y. I., Weisel, J. W., and Lord, S. T. (1997) *Blood 89* (12), 4407−14.
37. Okumura, N., Gorkun, O. V., and Lord, S. T. (1997) *J. Biol. Chem. 272* (47), 29596−29601.
38. Belitser, V. A., Varetskaja, T. V., and Malneva, G. V. (1968) *Biochim. Biophys. Acta 154* (2), 367−75.
39. Mihalyi, E. (1968) *Biochemistry 7* (1), 208−23.
40. Haverkate, F., Koopman, J., Kluft, C., A, D. A., Cattaneo, M., and Mannucci, P. M. (1986) *Thromb. Haemost. 55* (1), 131−5.
41. Furlan, M., Rupp, C., and Beck, E. A. (1983) *Biochim. Biophys. Acta 742* (1), 25−32.

42. Koppert, P. W., Huijsmans, C. M., and Nieuwenhuizen, W. (1985) *Blood 66* (3), 503−7.
43. Valenzuela, R., Shainoff, J. R., DiBello, P. M., Urbanic, D. A., Anderson, J. M., Matsueda, G. R., and Kudryk, B. J. (1992) *Am. J. Pathol. 141* (4), 861−80.
44. Ng, A. S., Lewis, S. D., and Shafer, J. A. (1993) *Methods Enzymol. 222*, 341−58.
45. Weisel, J. W., Veklich, Y., and Gorkun, O. (1993) *J. Mol. Biol. 232* (1), 285−97.
46. Medved, L. V., Gorkun, O. V., Manyakov, V. F., and Belitser, V. A. (1985) *FEBS Lett. 181* (1), 109−12.
47. Medved, L. V., Gorkun, O. V., Maniakov, V. F., and Belitser, V. A. (1986) *Mol. Biol. 20* (2), 461−70.
48. Shimizu, A., Saito, Y., and Inada, Y. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83* (3), 591−3.
49. Pandya, B. V., Gabriel, J. L., J, O. B., and Budzynski, A. Z. (1991) *Biochemistry 30* (1), 162−8.
50. Hsieh, K. H. (1997) *Biochemistry 36* (31), 9381−9387.
51. Mosesson, M. W., DiOrio, J. P., Muller, M. F., Shainoff, J. R., Siebenlist, K. R., Amrani, D. L., Homandberg, G. A., Soria, J., Soria, C., and Samama, M. (1987) *Blood 69* (4), 1073−81.
52. Siebenlist, K. R., DiOrio, J. P., Budzynski, A. Z., and Mosesson, M. W. (1990) *J. Biol. Chem. 265* (30), 18650−5.

BI981551T