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The (DD)E Complex Is Maintained by a Composite Fibrin Polymerization Site†

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ABSTRACT: The (DD)E complex is the major cross-linked fibrin degradation fragment. Structural components required for maintenance of the (DD)E complex were examined in order to better understand clot structure and the contribution of specific polypeptide chain segments in the process of polymerization. First, the (DD)E complex was reversibly dissociated by peptides derived from the α-chain NH_2-terminus of fibrin having a minimal sequence of GPR (α 17–19). In addition, the complex was partially dissociated by peptide β 40–54, while β 50–55 and peptides derived from the fibrin β-chain NH_2-terminus had no effect. Second, monoclonal antibody (mAb) 1B6, specific for the α-chain NH_2-terminus of fibrin, reacted rapidly with fragment E_1, but did not recognize the corresponding epitope on the (DD)E complex. On the other hand, mAb 59D8, specific for GHRPL at the β-chain NH_2-terminus of fibrin, reacted with the (DD)E complex in a dose-dependent manner. Third, the (DD)E complex was irreversibly dissociated by proteolytic cleavage of fragment E_1 by either thrombin, which removed GPR from the α-chain NH_2-terminus, or *Crotalus atrox* protease III, which released β 15–42. It has been concluded that fragment E_1 contains a composite polymerization site consisting at least of residues α 17–19 and β 20–49, which together maintain the (DD)E complex. These results illustrate that the complex is kept together by complementary binding sites which form a nucleus of linear fibrin polymerization sites. The (DD)E complex can thus be considered as a soluble model of fibrin clot. The fibrin β-chain NH_2–terminus, GHRPL, appears to be available on the surface of the (DD)E complex and may thus function as a lateral polymerization site.

The (DD)E complex is the major soluble plasmic degradation product of human cross-linked fibrin (Olexa & Budzynski, 1979a). The complex is derived from three separate fibrin monomers and is composed of one molecule each of fragment E_1 or E_2, bound noncovalently to one molecule of fragment DD. Fragment DD contains two COOH-terminal D domains derived from aligned fibrin monomers bound covalently by interchain N^{ϵ}-(γ-glutamyl)lysyl bonds between two γ-chains. Fragments E_1 and E_2 are derived from the NH_2-terminal E domain of fibrin. Fragment E_1 contains intact NH_2-termini of all three polypeptide chains of fibrin, while fragment E_2 is an asymmetric molecule in which one of the two β-chains is cleaved at position 54 (Olexa et al., 1981).

Fragment DD binds to clots (Husain & Budzynski, 1989) and fibrin monomer (Olexa & Budzynski, 1979b, 1980) and inhibits fibrin polymerization (Budzynski et al., 1979), while fragment E_1 interacts weakly with fragment D (Ugarova & Budzynski, 1992), and binds to fibrin clots with high affinity (Husain & Budzynski, 1989; Olexa & Budzynski, 1980a), allowing for its use in humans as a thrombus-imaging agent (Knight et al., 1985). The (DD)E complex, however, is an inert molecule, devoid of anticlotting or fibrin binding properties (Olexa & Budzynski, 1979b, 1980; Budzynski et al., 1979). The existence of complementary binding sites within the (DD)E complex has been postulated, since the complex can be reconstituted from its individually prepared components (Olexa & Budzynski, 1979a). The complex and fibrin accelerate t-PA-catalyzed plasmin formation at equal rates (Weitz et al., 1991; Weitz & Rischke, 1993), demon-

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strating that the (DD)E complex functions in the same manner as fibrin.

Clot formation occurs through the interaction of complementary fibrin polymerization sites. One set of sites resides in the NH_2-terminal E domain and is exposed only after sequential removal of fibrinopeptides A and B by thrombin. Removal of fibrinopeptide A alone induces fine-clot formation by exposing the sequence GPR at the α-chain NH_2-terminus. Subsequent removal of fibrinopeptide B yields thicker clots which are more highly branched.

Synthetic peptide GPRP, analogous to the fibrin α-chain NH_2-terminus, binds to fragment D and inhibits fibrin monomer polymerization (Laudano & Doolittle, 1978, 1980). A complementary polymerization site to GPRP is available in the fibrinogen COOH-terminal D domain and has been localized in the γ 357–373 sequence (Shimizu et al., 1992; Cierniewski & Budzynski, 1993), specifically to Tyr363 (Yamazumi & Doolittle, 1992). However, studies regarding the role of the fibrin β-chain NH_2-terminus (GHRP; β 15–18) have been less clear. GHRP binds to fragment D, and has increased affinity in the presence of Ca^{2+} (Laudano et al., 1983). In separate studies, GHRP was found to inhibit fibrin polymerization (Furlan et al., 1982), and peptide β 15–42 was even more inhibitory (Pandya et al., 1991). Recent evidence, however, suggests that GHRP inhibits polymerization by interacting with α-chain COOH-terminal polar appendages which are not present in fragment D (Hasegawa & Sasaki, 1990).

Fragment DD contains unique polymerization sites which are apparently not available in either fibrinogen or fragment D (Olexa & Budzynski, 1980a). Moreover, since conformational changes in the D domain occur upon the fibrinogen to fibrin conversion (Schielen et al., 1989), the fragment DD structure is certainly more representative of a cross-linked fibrin clot than that of fragment D.

The direct evidence as to the existence of polymerization sites in the (DD)E complex has not been provided. Also, the polypeptide chain sequences involved in keeping the (DD)E complex together have not been studied. Experiments described in this work aimed to demonstrate that the structural integrity of the (DD)E complex is maintained by the interaction of complementary fibrin polymerization sites, to show that the polymerization site in fragment E_1 is dependent upon the collective interaction of segments of the α- and β-chains, and to partially identify the primary sequences involved. In particular, it was attempted to resolve whether the β-chain NH_2-terminus (GHRP) directly participates in the linear polymerization of fibrin. It was also studied whether sequences located deeper in the β-chain may contribute to maintaining the (DD)E complex. The working hypothesis was that the complementary polymerization site present on fragment E is a composite moiety which consists of more than one polypeptide chain segment.

MATERIALS AND METHODS

Reagents. Acrylamide, bis(acrylamide), 10-DG columns, and TEMED were from Bio-Rad, Richmond, CA. The synthetic peptides Gly-Pro-Arg-Pro (GPRP), Gly-His-Arg-Pro (GHRP), and Gly-Pro (GP) were obtained from Bachem California, Torrance, CA. Gly-Pro-Arg (GPR) was from either Novabiochem, La Jolla, CA, or Vega Biotechnologies Inc., Tucson, AZ. Fibrinogen segment α 17–25 Lys was prepared by Peninsula Laboratories, Inc., Belmont, CA. Peptides β 15–26, β 40–54, and β 50–55 were prepared by classical Merrifield solid-phase peptide synthesis (Stewart & Young, 1984). The purity of all peptides was verified by RP-HPLC (Waters Model 600 E) using a standard mobile-phase gradient of CH_3CN in 0.1% TFA. Sepharose CL-6B and CM-50 Sephadex were from Pharmacia, Piscataway, NJ. Hepes was from United States Biochemical, Cleveland, OH. Phosphate-buffered saline (PBS) and bovine serum albumin (BSA) were from Sigma Chemical Co., St. Louis, MO. Human fibrinogen and plasmin were from KabiVitrum, Richfield, OH. Bovine thrombin was obtained from Parke-Davis, Detroit, MI. Human α-thrombin was obtained from Dr. John W. Fenton, New York State Department of Health, Albany, NY. Monoclonal antibodies (mAbs) 1B6, specific for the fibrin α-chain NH_2-terminus, and 59D8, specific for the fibrin β-chain NH_2-terminus (GHRPL), were kindly provided by Dr. Gary Matsueda, Department of Molecular Biology, Princeton University, and Bristol Myers-Squibb Pharmaceuticals (Matsueda & Margolies, 1986). Immobilized mAb C11C1, specific for high molecular weight kininogen, was a generous gift from Cheryl Scott of the Sol Sherry Thrombosis Research Center, Temple University School of Medicine. A reverse-phase C_{18} Delta-Pak column and low binding filters of 5000 molecular weight cutoff were from Waters/Millipore, Bedford, MA. All other reagents were of analytical grade and obtained from Sigma or Fischer Scientific, Pittsburgh, PA.

Protein Purification. Cross-linked fibrin was prepared according to Marder et al. (1976). The (DD)E complex and fragment DD were prepared by digesting 1 g of cross-linked fibrin with 2 and 10 units of plasmin, respectively, at 37 °C for 24 h in 150 mM Tris-HCl buffer, pH 7.8, containing 0.02% NaN_3 (Olexa & Budzynski, 1979a). High molecular weight products were removed from the (DD)E complex by precipitation with $(NH_4)_2SO_4$ at 30% saturation. The (DD)E complex was then precipitated with $(NH_4)_2SO_4$ at 50% saturation and further purified by gel filtration chromatography on a 2 × 100 cm column of Ultrogel AcA 54 equilibrated in 50 mM Tris-HCl, 100 mM NaCl, and 0.02% NaN_3, pH 7.8. Fragments DD and E_3 were separated by cation exchange chromatography on CM-50 Sephadex as previously described for the purification of fragment D (Ugarova & Budzynski, 1992). Fragment E_1 was prepared from the (DD)E complex by dissociation in 3 M urea/50 mM sodium citrate, pH 5.3, at 37 °C for 3 h, followed by column gel filtration chromatography on Sepharose CL-6B (Olexa & Budzynski, 1979a). The 62 kDa NH_2-terminal disulfide knot (NDSK) was prepared by digesting fibrinogen with CNBr in 70% HCOOH as described (Blomback et al., 1968) with minor modifications. Treatment of NDSK with thrombin removed fibrinopeptides A and B to produce tNDSK, having exposed polymerization sites. Briefly, 1.2 mg of NDSK in 1.0 mL of 0.1 M sodium phosphate, pH 7.4, was incubated in a water bath with 0.012 mL of 1000 units/mL human α-thrombin (12 units total) at 23 °C for 3 h. The reaction was stopped by addition of hirudin (5-fold excess over thrombin, unit/unit) and stored at −20 °C. Protease III from *Crotalus atrox* venom was purified exactly as described (Pandya & Budzynski, 1984). A detecting antibody for ELISA, consisting of purified anti-fragment D IgG conjugated to alkaline phosphatase, was prepared as follows: anti-fragment D IgG polyclonal antibodies were obtained in our laboratory by immunization of rabbits with highly purified human fragment D. Anti-fragment D IgG was purified from the rabbit antiserum by affinity chromatography on protein A–Sepharose. The purified IgG was conjugated to alkaline phosphatase using a general glutaraldehyde cross-linking procedure (Voller et al., 1976).

Effect of Synthetic Peptides upon Dissociation of the (DD)E Complex. Synthetic peptide analogues of the fibrin α-chain NH_2-terminus, GP (α 17–18), GPR (α 17–19), GPRP (α 17–19P), GPRVVHDEEK (α 17–24K), and analogues of the β-chain, GHRP (β 15–18), GHRPLDKKREEA (β 15–26), GYRARPAKAAATQKK (β 40–54), and ATQKKV (β 50–55) were dissolved at a concentration of 10 mg/mL in twice-distilled H_2O. A 50 μL aliquot containing 0.4 mg/mL (DD)E complex (1.6 μM) was incubated with a 1000-fold (1.6 mM) or 5000-fold molar excess (8 mM) of each peptide either individually or in combination in 50 mM Hepes/0.02% NaN_3, pH 7.0, at 22 °C for 2 h. The mixture was then diluted with 1 volume of sample buffer (50 mM Tris-HCl, 380 mM glycine, 40% sucrose, and 0.03% bromphenol blue, pH 8.3), and 2 μg of protein was analyzed by nondissociating polyacrylamide electrophoresis (PAGE) on 7% gels (Davis, 1964). In a reconstitution experiment, excess GPRP was removed from the dissociated sample by ultrafiltration on 5000 molecular weight cutoff filters. Buffer was added to the retentate to restore the initial volume, and then the sample was analyzed for (DD)E complex formation by nondissociating PAGE.

Sandwich ELISA Using mAbs 1B6 and 59D8 as Capture Antibodies. Enzyme-linked immunosorbent assay was performed using standard procedures (Engvall, 1980). One microgram of mAb 1B6 or 59D8 in 50 μL of 100 mM sodium carbonate buffer, pH 9.6, was adsorbed per well on a 96-well microtiter plate (Dynatech Immulon-2) at 4 °C overnight. Nonoccupied sites were then blocked with 1% BSA in PBS containing 0.02% NaN_3 (PBSA). All subsequent dilutions were also performed in this buffer unless otherwise stated. The plate was incubated with 100 μL of (DD)E complex at concentrations ranging between 0.01 and 30.0 μg/mL at room temperature for 1 h. For a negative control, fragments DD and E_3 were used as antigens. Excess antigen was removed by repeated washing with PBSA containing 0.05% Tween-20. Each well was incubated at 22 °C for 1 h with 100 μL of a 500-fold dilution of detecting antibody consisting of purified anti-fragment D IgG conjugated to alkaline phosphatase. The microtiter plate was developed by addition of 100 μL of 1 mg/mL *p*-nitrophenyl phosphate in 1.0 M diethanolamine, 0.5 mM $MgCl_2$, and 0.02% NaN_3, pH 9.8. The reaction was allowed to proceed for 10 min after which the absorbance was monitored at 405 nm on a Bio-Rad Model 4000 plate reader.

Effect of Thrombin upon the Ability of Fragment E_1 To Reconstitute the (DD)E Complex. Fragment E_1 was incubated with varying amounts of human α-thrombin in 20 mM Tris-HCl, pH 8.0, at 37 °C for 1 h. The reaction was terminated with 1 mM phenylmethanesulfonyl fluoride (PMSF). A 10 μL aliquot of thrombin-treated fragment E_1 was removed and mixed at a 1:1 molar ratio with fragment DD in 150 mM Tris-HCl, (pH 7.4)/0.02% NaN_3. Binding was allowed to proceed at room temperature for 1 h; then samples were analyzed for (DD)E complex formation by nondissociating PAGE. The remainder of the sample was passed through a 10 000 molecular weight cutoff low binding filter and analyzed by RP-HPLC. GPR present in the filtrate was quantified by comparison to a synthetic GPR reference standard using a similar method previously developed for measuring fibrinopeptides A and B (Kehl et al., 1981).

Dissociation of (DD)E by Cleavage with Crotalus atrox Protease III. A 100 μL sample of 0.4 mg/mL (DD)E complex was incubated with 1 μg/mL protease III from *Crotalus atrox* venom in 50 mM Tris-HCl, 100 mM NaCl, and 0.02% NaN_3, pH 7.8, at 23 °C for 20 h. Digestion was terminated with 1 mM PMSF, and samples were analyzed by nondissociating PAGE. For preparative studies, 1 mL of 6.7 mg/mL (DD)E complex was digested with 1:1000 (w/w) protease III in 50 mM NH_4HCO_3/2 mM EDTA, pH 7.8, at 37 °C for 24 h. The dissociated products were separated on a 1 × 100 cm column of Ultrogel AcA 34 developed in 50 mM Tris-HCl, 100 mM NaCl, 28 mM sodium citrate, and 0.02% NaN_3, pH 7.4. One milliliter fractions were collected and analyzed by SDS–PAGE.

Polyacrylamide Gel Electrophoresis (PAGE). Two gel systems were used. SDS–PAGE was performed on 11% gels using a 4.5% stacking gel according to Laemmli (1970). Nondissociating electrophoresis was performed on 7% Tris–glycine gels at alkaline pH (Davis, 1964). Gels were stained with Coomassie Brilliant Blue R-250 in both systems.

Particle Concentration Fluorescence Immunoassay (PCFIA). PCFIA is a heterogeneous immunoassay system that utilizes a solid-phase component, either antibody or antigen, which is free to interact with a liquid-phase ligand. Detection is accomplished using a fluorescent-labeled tracer as one of the components (Jolley et al., 1984; MacCrindle et al., 1985). A reaction mixture is incubated on a modified 96-well microtiter plate which has a cellulose acetate membrane with 0.2 μm pores at the bottom of each well, and is enclosed in a vacuum casing which allows for suction. Free components are separated by vacuum filtration of all materials not bound to the beads. After repeated washing, fluorescence is detected in the particle pellet on a Fluorescence Concentration Analyzer, IDEXX Laboratories Inc., Westbrook, ME. A PCFIA protocol was developed to analyze the reactivity of several fibrin degradation products with immobilized monoclonal antibodies of 1B6 and 59D8, specific for the α- and the β-chain NH_2-terminus of fibrin, respectively.

Preparation of Antibody-Coated Particles. The 0.9 μm carboxylate-modified polystyrene beads (Seradyn Corp., Indianapolis, IN) were converted to an active ester derivative by incubation with 20 mM cyanamide and 20 mM *N*-hydroxysuccinimide for 10 min, following a protocol described by Scott and Colman (1992). The activated beads were then coupled with antibody, 60 μg/mL, in 10 mM sodium phosphate buffer, pH 7.0, at room temperature overnight. Excess coupling sites were blocked by the addition of 1% BSA. The final product was washed twice with PBS/Tween-20 containing 0.1% BSA and 0.02% NaN_3. The preparation was suspended to a final particle concentration of 0.25% (w/v) in the same buffer and stored at 4 °C.

Preparation of Fluorescein-Labeled tNDSK (F-tNDSK). One milliliter of a 1 mg/mL solution of NDSK in 100 mM sodium phosphate, pH 7.4, was adjusted to pH 9.5 by the addition of 200 μL of 500 mM Na_2CO_3. To this mixture was added 50 μL of 10.7 mg/mL fluorescein isothiocyanate (FITC), freshly prepared in carbonate buffer, and the reaction was allowed to proceed at ambient temperature for 2 h in the dark. FITC-modified NDSK was separated from free FITC on a Bio-Rad 10DG desalting column equilibrated and eluted with 100 mM sodium phosphate, pH 7.4, containing 0.02% NaN_3. The final product had a fluorescein to protein molar ratio of 2.0. FITC-modified NDSK (0.72 mg/mL) was incubated with human α-thrombin (10 units/mL) at 23 °C for 2 h in the dark in order to expose the epitopes masked by fibrinopeptides A and B and to convert FITC-NDSK to FITC-tNDSK (F-tNDSK). The reaction was stopped by addition of hirudin (5-fold excess over thrombin, unit/unit) and stored at 4 °C covered with foil.

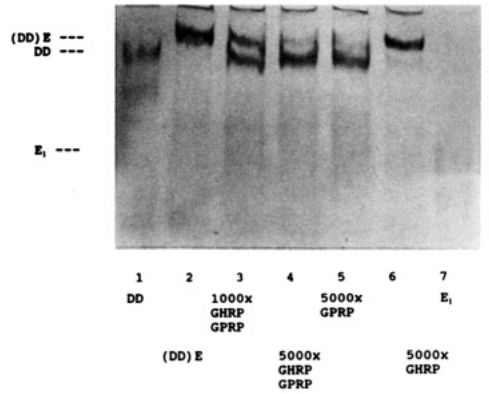

FIGURE 1: Effect of GPRP and GHRP upon dissociation of the (DD)E complex. A 50 μL reaction mixture containing 0.4 mg/mL (DD)E complex was incubated with GPRP, GHRP, or both GPRP and GHRP at 22 °C for 2 h and then analyzed by nondissociating PAGE. Lane 1 contains fragment DD; lane 2, (DD)E complex; lanes 3 and 4, (DD)E complex incubated with either a 1000-fold or a 5000-fold molar excess of both peptides, respectively. Lane 5, (DD)E complex plus a 5000-fold molar excess of GPRP. Lane 6 contains (DD)E complex plus a 5000-fold molar excess of GHRP. Lane 7, fragment E_1.

Competitive PCFIA. Antibody-coated particles were equilibrated in HTNB buffer (20 mM Hepes, pH 7.4, 500 mM NaCl, 0.02% NaN_3, 0.1% BSA, 0.05% Tween-20, and 10 units/mL trasylol) immediately prior to use. A typical assay consisted of 20 μL of 0.25% antibody-coated particles, 20 μL of 7 μg/mL F-tNDSK tracer, and 20 μL of competitor at concentrations ranging between 1 pM and 1 μM in 96-well PCFIA plates. The mixture was incubated at room temperature for 15 min and placed in a Fluorescence Concentration Analyzer where it was washed 3 times with albumin-free buffer. Bound F-tNDSK was detected by fluorescence at an excitation/emission wavelength of 485/535 nm. Nonspecific binding was measured as the relative fluorescence bound to immobilized anti-high molecular weight kininogen mAb C11C1.

RESULTS

Dissociation of the (DD)E Complex with Synthetic Peptides. To address the question whether GPRP and GHRP could dissociate the (DD)E complex, the latter was incubated with various concentrations of either peptide or their mixture and then analyzed for dissociation by nondenaturing PAGE. Lanes 1 and 2 in Figure 1 represent fragment DD and the (DD)E complex, respectively. Lanes 3 and 4 show that the complex incubated with both GPRP and GHRP at 1000- and 5000-fold molar excess dissociated to approximately 50 and 100%, respectively. Fragment E_1 stains poorly with Coomassie Blue and was not present in high enough quantity to be seen clearly on nondissociating gels (lane 7). Lane 5 shows that a 5000-fold molar excess of GPRP completely dissociated the (DD)E complex. In contrast, lane 6 shows that a 5000-fold molar excess of GHRP had no effect. Moreover, addition of 2 mM $CaCl_2$ to the reaction mixtures did not alter dissociation patterns (not shown). Identical results were also observed at physiological ionic strength (0.15 M) and pH (7.4). Functionality of the dissociated components was confirmed by demonstrating that the complex was quantitatively recovered once GPRP was removed by ultrafiltration (not shown).

GPRP dissociated the (DD)E complex, implying that the α-chain, NH_2-terminus of fragment E_1 contained a complementary binding site for fragment DD. On the other hand, GHRP did not dissociate the (DD)E complex, suggesting that the β-chain NH_2-terminus did not mediate formation of the complex. In addition, GHRP did not have any synergistic effect on GPRP-mediated dissociation of (DD)E. However, the fact that such a high molar excess of GPRP was needed to dissociate the complex suggested that the fibrin α-chain NH_2-terminus may contain only a partial binding site. Thus, other synthetic peptides corresponding to the α- and β-chain NH_2-terminal segments of fibrin were tested for their ability to dissociate the (DD)E complex. The results, summarized in Table 1, show that all peptides derived from the α-chain NH_2-terminus having a minimal sequence of GPR dissociated the (DD)E complex. Peptide GP was unable to dissociate the (DD)E complex, and methylation of the NH_2-terminus of GPRP totally abolished its dissociating effect. Of the β-chain peptides tested, β 40–54 significantly dissociated the complex. All other β-chain peptides were completely inert, providing evidence that dissociation by β 40–54 was specific.

Table 1: Dissociation of (DD)E by Fibrin-Derived Synthetic Peptides[a]

peptide	sequence	dissociation of (DD)E
α-chain		
α 17–18	GP	–
α 17–19	GPR	++
α 17–19 Pro	GPRP	++++
Me_2–α 17–19 Pro	$(CH_3)_2$-GPRP	–
α 17–24 Lys	GPRVVERHK	++
β-chain		
β 15–18	GHRP	–
β 15–26	GHRPLDKKREEA	–
β 40–54	GYRARPAKAAATQKK	++
β 50–55	ATQKKV	–

[a] Synthetic peptide analogues of the fibrin α- or β-chain NH_2-terminus were tested for their ability to dissociate the (DD)E complex exactly as described in Figure 1. Each peptide was used at a 5000-fold molar excess over (DD)E complex in 0.05 M Hepes–NaOH, pH 7.4. One plus sign (+) represents approximately 25% dissociation.

Availability of Epitopes on the (DD)E Complex. The exposure of epitopes in the α- and β-chain NH_2-termini was analyzed using ELISA. In this assay, the (DD)E complex was in the liquid phase in order to preserve conformation, which would have been altered upon adsorption to polystyrene. A detecting antibody, consisting of polyclonal anti-fragment D IgG cross-linked to alkaline phosphatase, was used to ensure that the (DD)E complex, not simply fragment E_1, would be recognized. The results in Figure 2 show that (DD)E complex bound to mAb 59D8, but not to mAb 1B6. Antibody 1B6 was highly reactive with fragment E_1 and tNDSK, confirming it recognized the fibrin α-chain NH_2-terminus (not shown). A lack of the (DD)E complex reaction with mAb 1B6 indicated that the NH_2-terminus of the α-chain in fragment E_1, bound within the (DD)E complex, was not accessible since it may have been engaged in binding to a complementary site on fragment DD.

Since GHRP did not dissociate the (DD)E complex (Figure 1), and the β-chain NH_2-terminus appeared to be exposed, it was considered that the free β-chain NH_2-terminus of fragment E_1 may not be required for maintenance of the (DD)E complex. This hypothesis was further treated using monoclonal antibody 59D8 in a competitive binding assay between F-tNDSK and a variety of fibrin degradation fragments and synthetic peptides structurally related to the fibrin E domain. The following species were used as negative controls: GPRP, GHRP, fibrinogen, and fragment E_3. The most relevant fragments were the (DD)E complex, fragment E_1, and tNDSK. The assay system, PCFIA, measured competition in a liquid phase between unlabeled fibrin fragments and F-tNDSK tracer for

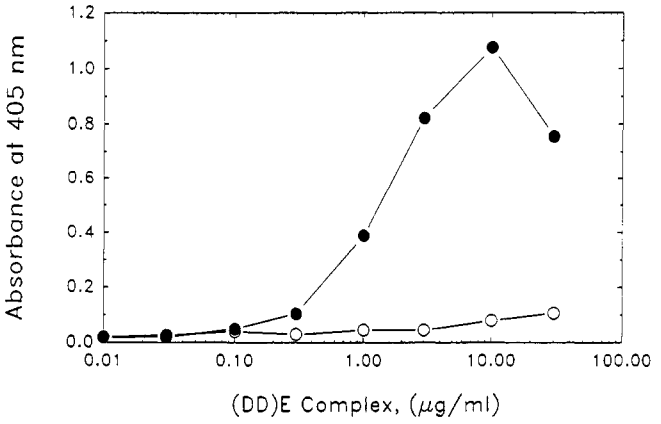

FIGURE 2: Reactivity of the (DD)E complex with antibodies specific for the α- or β-chain NH_2-terminus of fibrin. Monoclonal antibodies specific for the α-chain (mAb 1B6, open circles) or the β-chain (mAb 59D8, filled circles) NH_2-terminus of fibrin were obtained from Dr. Gary Matsueda, Princeton University. Each antibody was used as a capture antibody for the (DD)E complex in a solid-phase ELISA experiment. Bound (DD)E complex was detected using an alkaline phosphatase–anti-fragment D IgG conjugate.

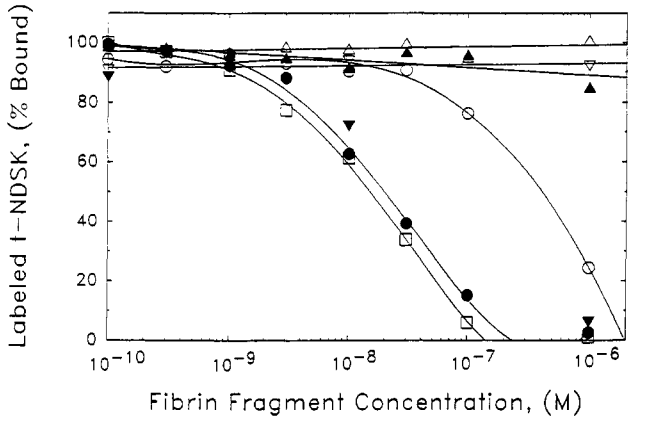

FIGURE 3: Competition of fibrin derivatives for antibody 59D8 (anti-β-chain NH_2-terminus of fibrin). Antibody 59D8 coated particles were incubated simultaneously with F-tNDSK tracer and competitors derived from fibrin fragment E_1 as described under Materials and Methods. Bound F-tNDSK was detected by fluorescence at an excitation/emission wavelength of 485/535 nm. (DD)E complex, open circles; fragment E_1, filled circles; fragment E_3, open triangles; tNDSK, open squares; fibrinogen, filled triangles; GHRP (β 15–18), open inverse triangles; β 15–26, filled inverse triangles.

binding to each immobilized specific antibody. Figure 3 shows competitive binding of fibrinogen or fibrin derivatives to mAb 59D8, which recognizes epitope of sequence 15–19 (GHRPL) in the β-chain NH_2–terminus of fibrin (Matsueda & Margolies, 1986). The best competitors were tNDSK and fragment E_1, inhibiting effectively F-tNDSK binding to mAb 59D8. The concentrations required to displace 50% of F-tNDSK were 13 nM for tNDSK and 20 nM for fragment E_1. The epitope was recognizable on the (DD)E complex, albeit to a lesser extent (IC_{50} = 450 nM). The approximately 25-fold difference in the reactivity of 59D8 to the (DD)E complex as compared to tNDSK or fragment E_1 may be caused by steric hindrance of the epitope by fragment DD, which is larger than the antibody itself. In addition, peptide β 15–26, but not GHRP (β 15–18), was a competitor for mAb 59D8, supporting the conclusion that β L19 is required as the structural determinant of the 59D8 epitope (Matsueda & Margolies, 1986). Moreover, since the β 15–21 epitope is readily available on cross-linked fibrin clots (Chen et al., 1992), the finding that the same epitope is available on the (DD)E complex supports a conclusion that (DD)E may be considered a soluble model of fibrin.

Role of the NH_2-Terminus of the α-Chain in (DD)E Complex Formation. It has been reported by Blomback that high thrombin concentration produced secondary cleavage in the α-chain of NDSK, in which the R19–V20 bond in the sequence GPRV was also hydrolyzed (Blomback et al., 1967). In addition, thrombin cleaves GPR in fibrinogen partially reduced in the absence of Ca^{2+} (Procyk & Blomback, 1990). The results of experiments described in Figures 1 and 2 and Table 1 suggest that GPR is a necessary component of the complementary binding site on fragment E_1 involved in maintaining the (DD)E complex. Since tNDSK and fragment E_1 are structurally equivalent, we postulated that removal of GPR from the α-chain NH_2-terminus of fragment E_1 would abolish binding to fragment DD. Thus, fragment E_1 was incubated with thrombin, the enzyme inactivated by PMSF, and the digest mixed with fragment DD in a 1:1 molar ratio and analyzed by nondissociating PAGE for formation of the (DD)E complex (Figure 4, left panel). Lane 1 contains fragment DD alone. Lanes 2, 3, 4, and 5 show fragment E_1 incubated with 0, 10, 100, and 1000 units of thrombin per milligram of fragment E_1, respectively, prior to the addition of fragment DD. Lanes 4 and 5 show that (DD)E complex formation is abolished at thrombin concentrations greater than 10 units/mg of E_1. Quantitative removal of GPR from fragment E_1 was monitored by RP-HPLC using a synthetic GPR standard (Figure 4, right panel). These results show that specific and quantitative removal of GPR by thrombin is accompanied by destruction of a complementary binding site on fragment E_1 for DD. This result is in accord with results obtained in Figures 1 and 2, confirming that the α-chain NH_2-terminus of fragment E_1, GPR, contains a functional complementary binding site involved in maintenance of the (DD)E complex.

In contrast, fragment E_1, that was in complex with fragment DD, was resistant to thrombin-catalyzed removal of GPR (not shown). This observation provided evidence that the (DD)E complex was not appreciably dissociated in aqueous solution. If the equilibrium constant would have favored dissociated fragments, GPR on free fragment E_1 would have been cleaved, and the (DD)E complex would have been readily dissociated by thrombin.

Cleavage of (DD)E Complex with Crotalus atrox Protease III. Since the β-chain NH_2-terminus was accessible to mAb 59D8 on the (DD)E complex, we asked if other regions of the fibrin β-chain involved in maintaining the (DD)E complex could be localized using a specific protease. A des(Bβ 1–42) fibrinogen, having imparied thrombin-coagulability, can be prepared using protease III from *Crotalus atrox venom*, which specifically cleaves at the β R42–A43 bond, leaving the rest of the molecule intact (Pandya & Budzynski, 1984; Pandya et al., 1985). It was tested whether cleavage of the same bond in the (DD)E complex would result in its dissociation by releasing the β 15–42 peptide. Thus, the (DD)E complex was incubated with protease III, and the digests were analyzed by nondissociating PAGE (Figure 5). Lanes 1 and 2 represent, respectively, (DD)E complex and (DD)E complex after incubation with the enzyme. The appearance of fragment DD in lane 2 provided evidence that cleavage of the β 42–43 peptide bond resulted in dissociation of the (DD)E complex. The result of this experiment suggested that β 15–42 contains a critical component of a complementary binding site on fragment E_1 required for maintenance of the (DD)E complex. In addition, since the (DD)E complex was cleaved by protease

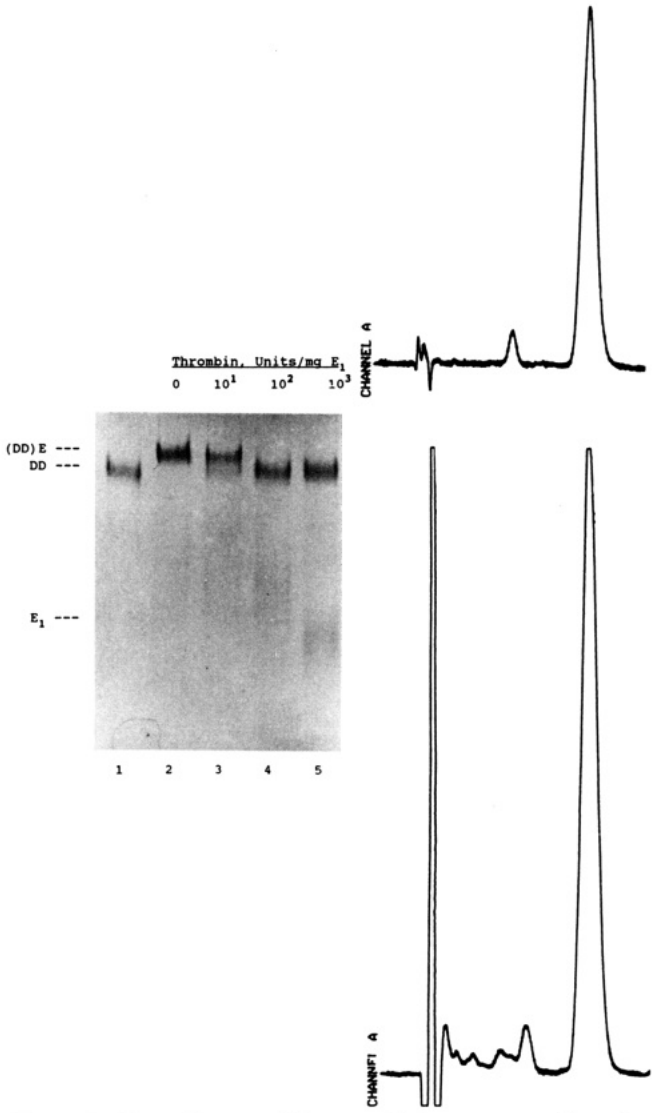

FIGURE 4: Thrombin-treated fragment E_1 does not reconstitute the (DD)E complex. Fragment E_1 was incubated with varying amounts of human α-thrombin at 37 °C for 1 h. The reaction was terminated with 1 mM PMSF. An aliquot was removed and added at a 1:1 molar ratio to native fragment DD. Binding was allowed to proceed at room temperature for 1 h; then samples were analyzed for (DD)E complex formation by nondissociating PAGE. The remainder of the sample was centrifuged through low binding filters, exclusion limit 5 kDa. The amount of GPR present in the filtrate was analyzed by RP-HPLC using synthetic GPR as a reference standard. (Left panel) PAGE. Lane 1, fragment DD; lanes 2–5, fragment E_1 incubated with 0, 10, 100, and 1000 units of thrombin/mg of E_1, respectively, prior to the addition of fragment DD. (Right panel) RP-HPLC. Top: 4 μg of synthetic GPR standard. Bottom: 100 μL of filtrate from the 1000 unit/mg digest.

III, the β R42–A43 bond was apparently accessible on the (DD)E complex surface.

In order to assure that the enzyme did not affect fragment DD, the (DD)E complex was digested with protease III, and the generated fragments DD and E were separated by column gel filtration. The appearance of two separate peaks provided further evidence that the complex was dissociated by protease III. The isolated fragment DD was completely able to reconstitute the (DD)E complex when incubated with native fragment E_1 (not shown).

DISCUSSION

Dissociation of the (DD)E complex by GPR, GPRP, and GRPVVERHK (Table 1, Figure 1) provided original evidence

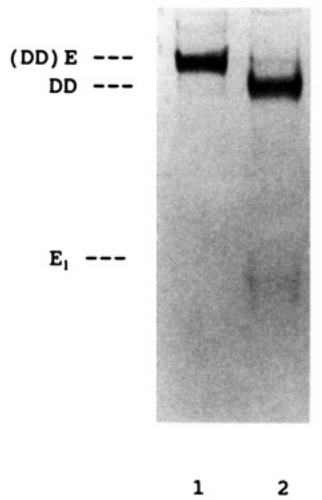

FIGURE 5: Dissociation of the (DD)E complex by cleavage with *Crotalus atrox* protease III. (DD)E complex was incubated with a 1:1000 (w/w) ratio of protease III from *Crotalus atrox* venom at 37 °C for 24 h. Digestion was terminated with PMSF, and samples were analyzed by nondissociating PAGE. Lane 1, (DD)E complex. Lane 2, (DD)E complex after incubation with protease III.

that the complex is bound by fibrin polymerization sites. This conclusion stemmed from previous observations showing that the fibrin α-chain NH_2-terminus is involved in a polymerization site. Synthetic peptides GPR, GPRV, and GPRP bound to fragment D_1 and inhibited polymerization of fibrin monomers, GPRP being the most active (Laudano & Doolittle, 1978, 1980, 1981; Hsieh et al., 1981; Furlan et al., 1982; Laudano et al., 1983). The same tetrapeptide altered the mechanical properties of fibrin (Shimizu & Ferry, 1988; Bale et al., 1985) and has been shown to partially liquefy non-cross-linked fine clots (Shimizu et al., 1988). Substitution of Gly, Pro, or Arg or alkylation of the NH_2-terminal glycine abolished the anticlotting effect of all GPR analogues (Hsieh et al., 1981), demonstrating the importance of each residue. The finding that reductive methylation of GPRP destroyed its dissociating activity (Table 1) demonstrated that the free α-chain NH_2-terminus is required as part of a complementary binding site maintaining the (DD)E complex. Further evidence suggesting the importance of each residue of GPR was derived from abnormal fibrinogens Kyoto II, Aα P18→L (Yoshida et al., 1991a), Detroit, Aα R19→S, and Munchen, Aα R19→N (Henschen et al., 1980), which all had delayed thrombin coagulability despite normal fibrinopeptide release. Also, dipeptidylpeptidase IV inhibited the maximal rate of fibrin monomer polymerization by cleaving GP dipeptide from the α-chain NH_2-terminus (Mentlein & Heymann, 1982). Most notably, removal of GPR rendered fibrinogen incoagulable after thrombin cleavage of the Aα R19–V20 bond in partially reduced fibrinogen (Procyk & Blomback, 1990). This is consistent with the observation that removal of GPR from fragment E_1 prevented formation of the (DD)E complex (Figure 4). Further evidence supporting the involvement of the α-chain NH_2-terminus in binding the components of the (DD)E complex was obtained using monoclonal antibody 1B6, a fibrin-specific antibody directed against a synthetic octapeptide corresponding to the α-chain NH_2-terminus of fibrin (Hui et al., 1983). The finding that mAb 1B6 did not react with the (DD)E complex suggested that the α-chain NH_2-terminus of fragment E is bound to a complementary polymerization site, therefore unavailable for reaction (Figure 2).

Results of the present work suggest that sequences located distal to the NH_2-terminus of the β-chain of fragment E_1 also

participate in a binding site for fragment DD. This conclusion is based primarily on the following observations. First, the (DD)E complex was dissociated after removal of β 15–42 by cleavage with *Crotalus atrox* protease III (Figure 5). Therefore, the β 15–42 segment likely contains a partial complementary binding site on fragment E. Involvement of β 15–42 in fibrin assembly has been reported (Pandya et al., 1985, 1991; Chen et al., 1988; Siebenlist et al., 1990). Second, the (DD)E complex reacted with mAb 59D8 (Figures 2 and 3), whose epitope is GHRPL, the β-chain NH_2 terminus of fibrin (Matsueda & Margulies, 1986). Therefore, the sequence GHRPL must be accessible on the (DD)E complex surface. Third, β 15–18, β 15–26, and β 50–55 did not dissociate the (DD)E complex, while β 40–54 did (Table 1). Since only one β-chain peptide was active, nonspecific binding was unlikely, especially since β 50–55, containing one-third the β 40–54 sequence, was inert. Thus, the sequence β 20–49 appears to be part of a binding site which keeps the (DD)E complex together. The rationale is as follows: Removal of β 15–42 dissociated the (DD)E complex; therefore, β 15–42 contains at least a partial complementary binding site required for maintenance of the (DD)E complex. The sequence GHRPL (β 15–19) was available on the (DD)E complex surface, so β 15–19 cannot be directly bound to fragment DD within the complex. This conclusion is supported by the inability of GHRP to dissociate the (DD)E complex. Peptide β 40–54, encompassing the R42–A43 bond hydrolyzed by cleavage of (DD)E with protease III, partially dissociated the (DD)E complex, while β 50–55 was inert. Since the two peptides overlap, residues 40–49 are apparently the active portion of β 40–54. Taken together, the sequence β 20–49 appears to be the likely contact site on fragment E which interacts with fragment DD in forming the (DD)E complex.

The finding that the fibrin β-chain NH_2-terminus is not directly bound to fragment DD in the (DD)E complex has indirect support. Photooxidation of β H16 in tNSDK abolished its binding to immobilized fibrinogen (Shimizu et al., 1983), but the same fragment retained affinity for fragment DD (Shimizu et al., 1986). This residue appeared to be required for binding to fibrinogen, but not fragment DD. Since GHRPL (β 15–19) is located on the (DD)E complex surface (Figures 2 and 3), β H16 predictably would not be involved in maintaining a (DD)E or (DD)–tNDSK complex. Furthermore, synthetic peptide β 19–42 bound to fibrinogen, presumably via a complementary polymerization site, and prevented the binding of fibrinogen to activated platelets (Chen et al., 1988), although β 15–42 was a more effective competitor. Interestingly, GHRP had no effect on platelet aggregation or fibrinogen binding to activated platelets, clearly demonstrating that β 19–42, which lacks the GHRP sequence, can bind to fibrinogen, further supporting our model. In addition, abnormal fibrinogen Ise, Bβ G15→C (Yoshida et al., 1991b) is characterized by polymerization profiles consistent with our observations.

However, GHRP may stabilize a secondary structure within the NH_2-terminus of the β-chain. For example, retention of fibrinopeptide B on α-fibrin, prepared by selective cleavage of fibrinopeptide A only, altered the early plasmic degradation products from (DD)E to fragments DD and E_3, suggesting that removal of fibrinopeptide B stabilized the (DD)E complex against rapid plasminolysis. Molecular dynamics predictions of the β 1–42 and β 15–42 peptides suggested that a conformational change occurred upon removal of fibrinopeptide B, since residues located near GHRP were bought into contact with the β 40–55 segment (Pandya et al., 1991). Immunological methods have lead to a proposal that long-range interactions stabilized the structure of the bovine fibrinogen β-chain (Nagy et al., 1985).

Recent evidence suggests that GHRP may bind to the fibrinogen α-chain COOH-terminal domains (Hasegawa & Sasaki, 1990). The involvement of the α-chain COOH-terminal domains in fibrin polymerization has recently gained considerable support (Medved et al., 1983, 1985; Weisel & Papsun, 1987; Cierniewski & Budzynski, 1992; Velkich et al., 1993). Since GHRP appears exposed on the (DD)E surface (Figures 2 and 3), one can envision this region could function as a lateral polymerization site, originally bound to cross-linked α-chains in fibrin clots. Interestingly, we consistently observed that the cross-linked α-chain remnants, M_r 24 000–29 000, cofractionated with the (DD)E complex during precipitation of the latter with 2.1 M ammonium sulfate (unpublished observations).

It has been suggested that the polymerization site in the fibrin E domain is dependent upon the intact conformation of all three chains (Budzynski et al., 1983). In the present paper, cleavage of fragment E_1 with thrombin (Figure 4) or protease III (Figure 5) produced novel fragment E species having intact fibrin β- or α-chains, respectively. Since neither novel fragment could reconstitute the (DD)E complex, the polymerization site in fragment E_1 must be dependent upon the collective interaction of both polypeptide chain segments.

In conclusion, the results of this work support a hypothesis that the (DD)E complex is maintained by complementary binding sites which form a nucleus of linear fibrin polymerization sites. Experimental data imply that fragment E_1 contains a composite polymerization site consisting at least of residues α 17–19 (GPR) and β 20–49 (DKKREEAPSLRPAPPPISGGGYRARPAKAA), required for maintenance of the (DD)E complex, and presumably involved in linear polymerization during protofibril formation. Both sequences likely combine to form a composite linear polymerization site, since disruption of either the α-chain or β-chain binding site on fragment E_1 resulted in dissociation of the (DD)E complex. The sequence β 15–19 (GHRPL), located at the β-chain NH_2-terminus of fibrin, does not seem to directly participate in the linear polymerization site, is exposed to solvent on the (DD)E complex surface, and thus may function as a lateral polymerization site.

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