

# Molecular Basis of Fibrinogen Naples Associated with Defective Thrombin Binding and Thrombophilia

## Homozygous Substitution of B $\beta$ 68 Ala $\rightarrow$ Thr

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### Abstract

In an abnormal fibrinogen (fibrinogen Naples) associated with congenital thrombophilia we have identified a single base substitution (G  $\rightarrow$  A) in the B $\beta$  chain gene that results in an amino acid substitution of alanine by threonine at position 68 in the B $\beta$  chain of fibrinogen. The propositus and two siblings were found to be homozygous for the mutation, whereas the parents and another sibling were found to be heterozygous. Individuals homozygous for the defect had a severe history of both arterial and venous thrombosis; heterozygous individuals had no clinical symptoms. The three homozygotes had a prolonged thrombin clotting time in plasma, whereas the heterozygotes had a normal thrombin clotting time. Fibrinopeptide A and B (FpA and FpB) release from purified fibrinogen by human  $\alpha$ -thrombin was delayed in both the homozygous propositus and a heterozygous family member. Release of FpA from the normal and abnormal amino-terminal disulfide knot (NDSK) corresponded to that found with the intact fibrinogens, indicating a decreased interaction of thrombin with the NDSK part of fibrinogen Naples. Binding studies showed that fibrin from homozygous abnormal fibrinogen bound < 10% of active site inhibited  $\alpha$ -thrombin as compared with normal fibrin, while fibrin formed from heterozygous abnormal fibrinogen bound  $\sim$  50% of  $\alpha$ -thrombin. These results suggest that the mutation of B $\beta$  Ala 68  $\rightarrow$  Thr affects the binding of  $\alpha$ -thrombin to fibrin, and that defective binding results in a decreased release of FpA and FpB in both homozygous and heterozygous abnormal fibrinogens. (*J. Clin. Invest.* 1992. 90:238–244.) Key words: amino-terminal disulfide knot • dysfibrinogenemia • fibrinopeptide release • polymerase chain reaction •  $\alpha$ -thrombin binding

### Introduction

The fibrinogen molecule is involved in the final phase of blood coagulation and consists of pairs of A $\alpha$ , B $\beta$ , and  $\gamma$  chains linked by 29 disulphide bonds (1). The conversion of fibrinogen to fibrin is initiated by thrombin catalyzed cleavage of fibrinopep-

tides A and B (FpA and FpB)<sup>1</sup> from the amino terminus of the A $\alpha$  and B $\beta$  chains, respectively. The interaction of thrombin with fibrin(ogen) involves multiple sites on both thrombin and fibrin(ogen). An important domain of fibrinogen that interacts with the catalytic site of thrombin is located in the A $\alpha$  chain between amino acids 1 and 23 (2–6). The interaction of thrombin with fibrin through a site independent of the catalytic site (anion-binding exosite) (7–12) is located in a cyanogen bromide (CNBr)-derived fragment of the amino-terminal part of the fibrin(ogen) molecule amino-terminal disulfide knot (NDSK) (13–15). Binding of  $\alpha$ -thrombin to fibrin by the anion-binding exosite results in the removal of thrombin from solution (7, 9). It has been proposed that this interaction plays an important role in the regulation of thrombus formation in vivo by limiting the amount of free active thrombin in the circulation (7). Congenitally abnormal fibrinogens are valuable tools for structure–function studies of human fibrinogen. They also provide a basis for correlating the molecular defect with the clinical symptoms of affected individuals. Many abnormal fibrinogens have been described (16), of which a large number show defective release of fibrinopeptides due to a mutation at or near the thrombin cleaved bond (17–20). The structural defect of an abnormal fibrinogen (fibrinogen New York I) with impaired thrombin binding to fibrin (21) has been determined. This fibrinogen lacks amino acids 9–72 in the B $\beta$ -chain (22). The patient, who was shown to be heterozygous, suffered from thrombotic episodes (23). Fibrinogen Naples (Milano II) (24, 25) is another abnormal fibrinogen associated with congenital thrombophilia. Preliminary work on fibrinogen Naples demonstrated a defective interaction between bovine thrombin and fibrin of the propositus (24). In this article we report the structural defect of fibrinogen Naples, inferred from genetic analysis of patient DNA using the polymerase chain reaction (PCR) (26). The presence of the mutation in the family members, the binding of human  $\alpha$ -thrombin to fibrin, and the relationship between the defect and clinical symptoms were also determined.

### Methods

**Patients.** The propositus (II.3) developed postoperative deep-vein thrombosis at the age of 33 yr. His sister (II.1) had a stroke at the age of 25 yr due to thrombotic occlusion of the internal carotid artery and his brother (II.2) had a stroke and thrombosis of the abdominal aorta at the age of 21 yr. Another brother (II.4) of the propositus is asymptomatic, as are his parents (I.1 and I.2) (who are first cousins).

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1. *Abbreviations used in this paper:* CNBr, cyanogen bromide; DFP, difluorophosphate; FpA and FpB, fibrinopeptides A and B; NDSK, amino-terminal disulfide knot; PCR, polymerase chain reaction; t-PA, tissue-type plasminogen activator.

**Coagulation studies on plasma.** Blood was collected by venipuncture and anticoagulated with 0.1 vol of 0.11 M trisodium citrate. Plasma was prepared by centrifugation at 2,300 g for 30 min at 4°C. Thrombin clotting times were performed at 37°C with 200  $\mu$ l of plasma and 50  $\mu$ l of human  $\alpha$ -thrombin (1,000 National Institutes of Health (NIH) U/mg; Protogen, Läufelingen, Switzerland), dissolved in 0.15 M NaCl containing 0.025% (wt/vol) gelatin to a concentration of 25 NIH U/ml. Reptilase (Boehringer Mannheim, Mannheim, FRG) clotting time was performed as described (24). Fibrinogen concentration was determined functionally according to Clauss (27) and immunologically according to Mancini et al. (28).

**Purification of fibrinogen and NDSK.** Fibrinogen was purified from plasma as described (29) and dialyzed against PBS for 24 h at 4°C. NDSK was purified after CNBr digestion of purified fibrinogen (30) by a fast protein liquid chromatography (FPLC) system equipped with a Superose 12 column (Pharmacia, Uppsala, Sweden). The column was equilibrated with 10% acetic acid containing 0.1 M NaCl and run at a flow rate of 1.0 ml/min. The CNBr digest, 6 mg of protein in 0.3 ml, was injected and 0.5-ml fractions were collected. Fractions 12–15 (Fig. 1) were pooled and analyzed on SDS-PAGE (31). The purified fibrinogen NDSK showed an  $M_r$   $\sim$  65,000 and a purity of  $\sim$  90% (Fig. 1). Pooled fractions were dialyzed against distilled water, lyophilized, and dissolved in PBS to a concentration of 0.7 mg/ml. The fibrinogen and NDSK concentrations were measured spectrophotometrically at 280 nm (fibrinogen,  $A^{1\%,1\text{cm}} = 15.0$  (32), NDSK,  $A^{1\%,1\text{cm}} = 12.0$  [calculated from amino acid composition]); the yield of this purification was  $\sim$  65%.

**Release of fibrinopeptides from fibrinogen and NDSK.** The rate of FpA and FpB release was determined at 37°C with 100  $\mu$ l of fibrinogen solution (4.0 mg/ml) or NDSK solution (0.7 mg/ml) and 10  $\mu$ l of either 1 NIH U/ml human  $\alpha$ -thrombin or 1:30 diluted Reptilase. The reactions were stopped at various times by placing the samples in a boiling water bath for 2 min. The samples were centrifuged at 12,000 g for 10 min, and 50  $\mu$ l of the supernatant was analyzed by HPLC (LKB Produkter, Bromma, Sweden) on a C-18 reversed-phase column (Chrompack, Middelburg, The Netherlands) (24, 33). The amount of FpA and FpB released was determined by measuring the peak area.

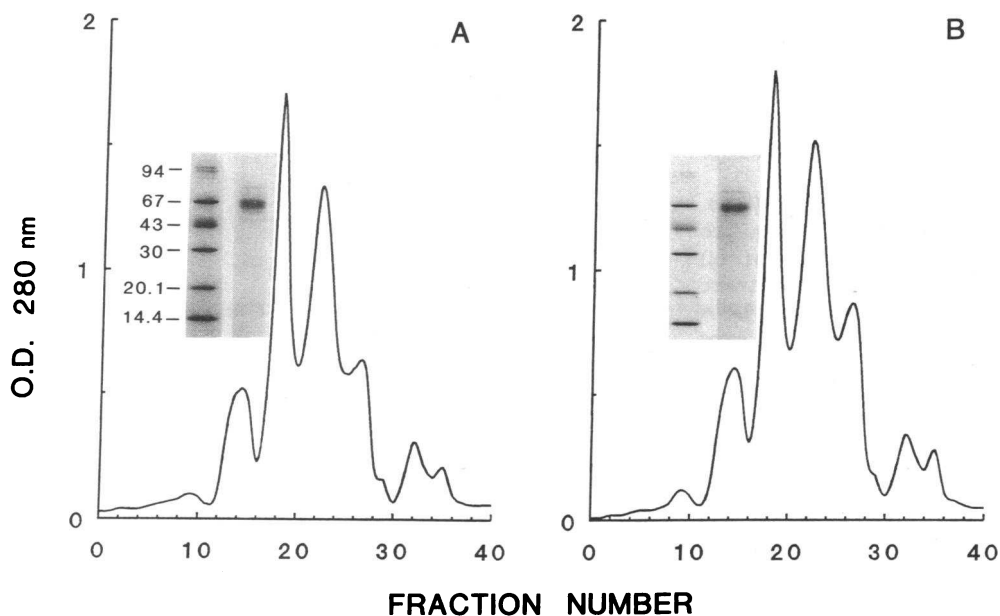
**Fibrin polymerization.** Purified fibrinogen was dissolved in PBS to a concentration of 2.0 mg/ml. 1 ml of this solution was clotted by adding 0.1 ml of human  $\alpha$ -thrombin (25 NIH U/ml) or undiluted Reptilase over a period of 5 h at 37°C. The fibrin was collected by centrifugation, washed four times with PBS, and subsequently dissolved in 20 mM acetic acid to a concentration of 2.0 mg/ml. Polymerization of the

fibrin monomers was obtained by increasing the pH and performed by adding 60  $\mu$ l of the fibrin monomer solution to 740  $\mu$ l of 0.1 M Tris/HCl, pH 7.5. The increase in absorbance at 350 nm was recorded as a function of time on a spectrophotometer (SP 1700; Pye Unicam, Cambridge, UK).

**Preparation of [ $^{14}$ C]difluorophosphate (DFP)  $\alpha$ -thrombin.** 1 ml of human  $\alpha$ -thrombin, dissolved at 1 mg/ml in 10 mM Tris/HCl, pH 8.3, containing 0.75 M NaCl, was incubated with 100  $\mu$ l of [ $^{14}$ C]DFP (108 mCi/mmol, 1.9  $\mu$ M/ml; New England Nuclear, s'Hertogenbosch, The Netherlands) for 90 min at 25°C. Subsequently 10  $\mu$ l of a 0.1 M DFP solution in dry isopropanol was added, and incubated for 30 min. The sample was dialyzed against 50 mM Tris/HCl, pH 7.5, 100 mM NaCl for 24 h at 4°C. Protein concentration was measured spectrophotometrically at 280 nm ( $A^{1\%,1\text{cm}} = 18.0$  [34]). The amidolytic activity of inhibited thrombin, determined by using the synthetic substrate S-2238 (KabiVitrum, Stockholm, Sweden) according to the manufacturer's instructions, was  $< 0.05\%$  of the original activity. The relative  $\alpha$ -,  $\beta$ -, and  $\gamma$ -thrombin content was determined by SDS-PAGE (31) followed by autoradiography. The amount of  $\alpha$ -thrombin was  $> 95\%$  (Fig. 2). To prevent nonspecific adsorption, gelatin (final concentration 0.1% wt/vol) was added to the thrombin solution, and this solution was stored at  $-70^\circ\text{C}$ .

**[ $^{14}$ C]DFP  $\alpha$ -thrombin binding to fibrin.** Fibrinogen was dialyzed against 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, and diluted to 0.5 mg/ml. Part of this fibrinogen solution was radiolabeled with  $^{125}\text{I}$  (Amersham, Buckinghamshire, UK) as described (35). To 0.15 ml of fibrinogen, 50  $\mu$ l of solutions containing different amounts of [ $^{14}$ C]DFP inhibited  $\alpha$ -thrombin diluted in 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 0.1% (wt/vol) gelatin were added. Subsequently, fibrin formation was induced by incubating with 10  $\mu$ l of undiluted Reptilase solution for 30 min. Fibrin was collected by centrifugation for 10 min at 12,000 g. The pellet was washed three times with 0.5 ml 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, and dissolved in 0.2 ml of 50 mM acetic acid. The sample was mixed with 3.0 ml of scintillation fluid (Ultima Gold, Packard Instrument Co., Inc., Downers Grove, IL) and  $^{14}\text{C}$  was counted (Tricarb 1900 CA, Packard Instrument Co., Inc.). The amount of fibrin formed was determined in parallel experiments containing trace amounts of  $^{125}\text{I}$ -fibrinogen. After dissolving the fibrin in acetic acid, the amount of radioactivity was counted in a  $\gamma$  counter (Cobra, Packard Instrument Co., Inc.). Under these conditions normal fibrinogen and fibrinogen Naples II.3 and II.4 were 93–96% clottable.

**Fibrin stimulation of tissue-type plasminogen activator (t-PA).** The stimulatory capacity of Reptilase and thrombin-induced fibrin mono-



**Figure 1.** Elution profiles of FPLC purification of NDSK from CNBr digest of fibrinogen and SDS-PAGE analysis of pooled fractions 12–15. (A) Normal fibrinogen; (B) fibrinogen Naples II.3.

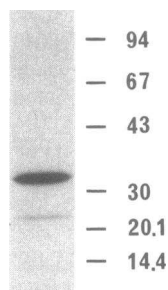


Figure 2. Autoradiogram of [<sup>14</sup>C]DFP  $\alpha$ -thrombin after SDS-PAGE under reducing conditions.

mers, prepared as described for fibrin polymerization, was assayed as described previously (36).

**PCR.** Genomic DNA was isolated from blood cells as described (37). Oligonucleotides were synthesized on a model 380A DNA synthesizer (Applied Biosystems, Inc., Foster City, CA). Oligonucleotides  $\alpha$ 1a(5'GCGATAGTGGTGAAGGTGAC3') and  $\alpha$ 1b(5'GTTATTGCTGAGGAAAAATCGCC3') were used to amplify the part of the  $\alpha$ -chain gene, coding for amino acids 1–95. Oligonucleotides  $\beta$ 1a(5'GGTGTGGAAATAGTTACATTCC3') and  $\beta$ 1b(5'GGTGTGTGAGTTCCTCTGGA3') were used to amplify the part of the  $\beta$ -chain gene, coding for amino acids 9–209.  $\beta$ 2a(5'GCCTTAAGGTTGTAGGAATTCTTCAG3') and  $\beta$ 2b(5'ATCAGTGCACCCACCAAGTCTGGG3') were used to amplify the  $\beta$ -gene segment coding for amino acids 9–72. Oligonucleotides  $\gamma$ 1a(5'GCTCTTCACAAAACGTTGTTTTAAAAATGGAATTCTGG3') and  $\gamma$ 1b(5'CAGTCTTGCAGAGCAAATTTAAAAACAAAATCCTTAC3') were used to amplify the part of the  $\gamma$ -chain gene coding for amino acids 1–108. Amplification by PCR (26) was performed in a 100- $\mu$ l reaction volume containing 1  $\mu$ g of genomic DNA, 0.2 mM of each dNTP (Pharmacia), 0.2  $\mu$ M of each primer in 1 $\times$  reaction buffer (10 mM Tris/HCl, pH 8.3 at 25°C, 50 mM KCl, 3.0 mM MgCl<sub>2</sub> and 0.001% (wt/vol) gelatin). The DNA was denatured at 95°C for 8 min and 2.0 U Taq DNA polymerase (Perkin-Elmer Cetus, Emeryville, CA) were added. Cycles consisted of a 1-min 95°C, 0.5-min 60°C, and 3-min 70°C incubation. After 30 cycles 5  $\mu$ l of the sample was analyzed on a 1.0% agarose gel (A-6013, Sigma Chemical Co., St. Louis, MO).

**Direct sequencing PCR fragments.** PCR samples were run on a 1% (wt/vol) ultralow gelling agarose gel (A-5030, Sigma Chemical Co.). The band with the appropriate size, as predicted by the genomic sequence of the  $\alpha$ A (16),  $\beta$ B (16, 38), and  $\gamma$  chain genes (36), was cut out of the gel and heated to 65°C. 1  $\mu$ l of the melted agarose, containing ~ 10 ng of DNA, was mixed with 1  $\mu$ l of the appropriate PCR primer (60 ng), and 2  $\mu$ l of 5 $\times$  sequence buffer (T7 sequence kit, Promega Corp., Madison, WI), the volume was brought up to 10  $\mu$ l with distilled water. The mixture was heated to 95°C for 3 min and immediately put on ice. Labeling and termination reactions were performed using the T7 DNA sequence kit (Promega Corp.) according to the manufacturer's instruction.

**Southern blot analysis of family members and normal population.** Amplified fragments of the  $\beta$ B chain gene containing the sequence coding for amino acids 9–72 (~ 50 ng) were run on a 2% (wt/vol) agarose gel (A-6013, Sigma Chemical Co.). The gel was washed (twice for 15 min) with 0.5 M NaOH, 1.5 M NaCl, and the denatured DNA was transferred to a nylon membrane (Hybond N, Amersham) using the Vacugene blotting system (LKB Produkter). 100 ng of sequence-specific oligonucleotides ( $\beta$ -normal: 5'TGTCTTCACGCTGACC-CAG3' and  $\beta$ -Naples: 5'TGTCTTCACACTGACCCAG3') were radiolabeled with T4 polynucleotide kinase (Gibco BRL, Breda, The Netherlands) according to the manufacturer's instructions, using [ $\gamma$ -<sup>32</sup>P]ATP (3,000 Ci/mmol, Amersham). The blots were washed with 2 $\times$  SSC (1 $\times$  SSC contains 0.15 M NaCl and 0.015 M Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>) and hybridized with the labeled oligonucleotides in 40 ml of 7% SDS, 0.36 M Na<sub>2</sub>HPO<sub>4</sub>, 0.14 M NaH<sub>2</sub>PO<sub>4</sub>, and 10 mM EDTA for 4 h at 42°C. Blots were washed three times for 30 min at 58°C with 6 $\times$  SSC contain-

ing 0.5% (wt/vol) SDS, and exposed to x-ray film (X-AR, Eastman Kodak Co., Rochester, NY) for 16 h.

## Results

**Coagulation studies on plasma.** Table I shows the results of the thrombin and Reptilase clotting time assays and of the immunological and functional fibrinogen assays performed on plasma of normal individuals, the propositus (II.3), and his family members. The propositus and two of his siblings (II.1, II.2) showed a strongly delayed thrombin clotting time, but a normal Reptilase time. They also showed a discrepancy between the immunologically and the functionally determined fibrinogen concentration. Three other family members (father, mother, and one other sibling: I.1, I.2, and II.4) showed normal thrombin and Reptilase clotting times and no significant difference between the immunologically and functionally determined fibrinogen concentration.

**Fibrinopeptide release from fibrinogen.** Fig. 3 shows the thrombin-catalyzed release of the FpA and FpB from normal fibrinogen, fibrinogen Naples II.3 (propositus with prolonged thrombin clotting time), and Naples II.4 (sibling with normal thrombin clotting time). FpA release (Fig. 3 A) from both fibrinogens Naples II.3 and II.4 is strongly delayed as compared with normal fibrinogen. There is a large difference in the amount of FpA released after 60 min from normal fibrinogen (100%) and from fibrinogens Naples II.4 (63%) and Naples II.3 (24%). Fibrinogen Naples II.3 also showed a lag of ~ 10 min before any FpA could be detected; fibrinogen Naples II.4 and normal fibrinogen did not show this lag period. The release of FpB (Fig. 3 B) from fibrinogens Naples II.3 and II.4 was also strongly delayed as compared with normal fibrinogen. After 60 min, normal fibrinogen released 92% of FpB, fibrinogen Naples II.4 released 30% of FpB, and Naples II.3 only released 10% of FpB. With normal fibrinogen and fibrinogen Naples II.4, FpB was detected after a lag period of 2.5 min; with fibrinogen Naples II.3 this lag period was 25 min. Reptilase-catalyzed release of FpA was the same for all three fibrinogens (data not shown).

**Fibrinopeptide release of NDSK.** Fig. 4 shows the release of FpA and FpB by thrombin from NDSK purified from normal fibrinogen and fibrinogen Naples II.3. As was found with intact fibrinogens, the release of FpA from NDSK Naples II.3 was strongly delayed as compared with that of normal NDSK. The release of FpA from both normal and Naples II.3 NDSK was

Table I. Plasma Coagulation Studies

	Plasma clotting time		Plasma fibrinogen	
	Human $\alpha$ -thrombin	Reptilase	Functional	Immunological
	s		mg/ml	
Normal	19.8	22.1	2–4	2–4
I.1	21.3	22.3	2.1	2.5
I.2	20.7	22.6	2.6	2.8
II.1	> 180	21.9	1.6	2.7
II.2	> 180	22.4	1.4	2.4
II.3	> 180	21.8	1.7	3.0
II.4	21.0	22.9	2.3	2.5

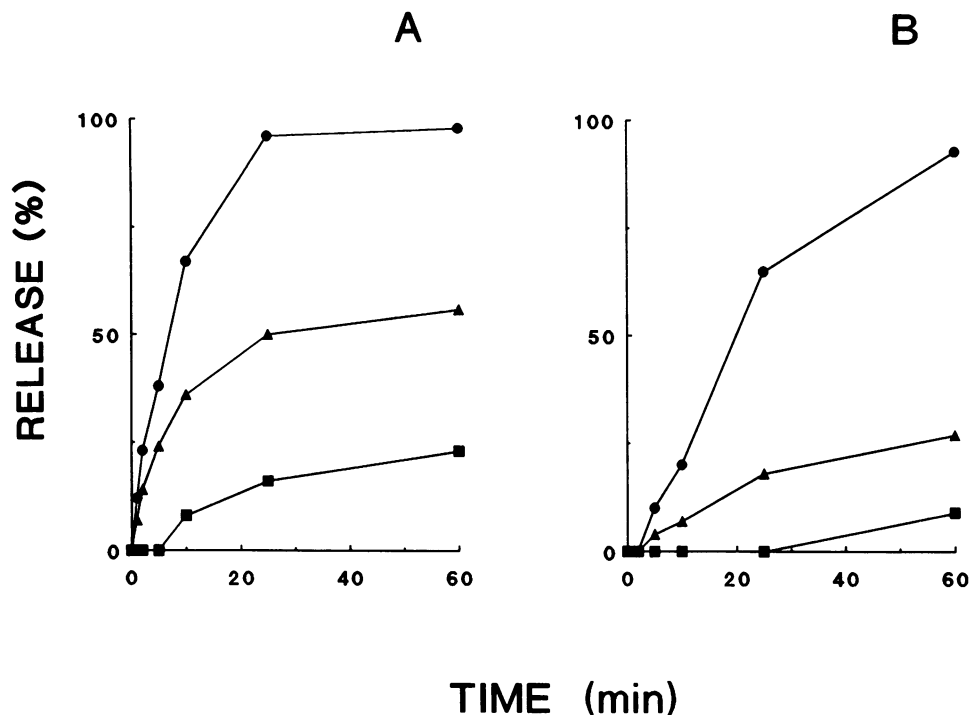


Figure 3. Release of fibrinopeptides from intact fibrinogen by human  $\alpha$ -thrombin, determined by HPLC. (A) FpA and (B) FpB release from (●) normal fibrinogen (▲) fibrinogen Naples II.4, and (■) fibrinogen Naples II.3.

identical to the release from intact normal and Naples II.3 fibrinogen, respectively (compare Fig. 4 with Fig. 3 A).

The release of FpB from NDSK Naples II.3 was delayed as compared with normal NDSK. In contrast to the FpA release, the FpB release from normal NDSK is slower than the FpB release from intact normal fibrinogen (compare Fig. 4 with Fig. 3 B). However, the FpB release of NDSK Naples II.3 was approximately the same as the FpB release of intact fibrinogen Naples II.3 (compare Fig. 4 with Fig. 3 B).

The release of FpA by Reptilase from NDSK Naples II.3 was the same as that from normal NDSK (data not shown).

**Fibrin polymerization.** The polymerization profiles of fibrin monomers obtained from fibrinogen Naples II.3, by Reptilase and thrombin, were similar to the profiles obtained with the corresponding monomers prepared from normal fibrinogen (data not shown). These results are consistent with the normal Reptilase clotting time and prothrombin-staphylococulase clotting time of purified fibrinogen Naples II.3, re-

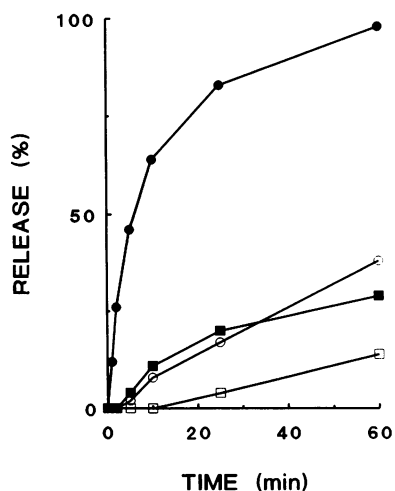


Figure 4. Release of fibrinopeptides from purified NDSK by human  $\alpha$ -thrombin determined by HPLC. (●) FpA and (○) FpB from normal NDSK, and (■) FpA and (□) FpB from NDSK Naples II.3.

ported previously (24), and indicate that the mutation in fibrinogen Naples does not affect the fibrin polymerization.

**Binding of active-site inhibited human  $\alpha$ -thrombin to fibrin.** Fig. 5 shows that the binding of [ $^{14}$ C]DFP inhibited  $\alpha$ -thrombin to Reptilase-induced fibrin clots from normal fibrinogen and fibrinogens Naples II.3 and II.4. With normal fibrin, thrombin binding increased with increasing thrombin concentration, reaching saturation at  $\sim 1$  mol of inhibited  $\alpha$ -thrombin per mol of fibrin. With fibrin Naples II.4, the shape of the binding curve was similar, but saturation was achieved at  $\sim 0.5$  mol of thrombin per mol of fibrin. Thrombin bound poorly to fibrin Naples II.3 with  $< 0.1$  mol of thrombin bound/mol of fibrin at the highest thrombin concentration tested. Scatchard analysis of the binding data for normal fibrinogen and Naples II.4 (inset, Fig. 5) indicated one class of binding sites. The maximal molar binding ratio (thrombin/fibrin) for normal fibrin was  $1.1 \pm 0.25$  with  $K_a = 12.7 \pm 3.0 \times 10^5 \text{ M}^{-1}$ , for fibrin Naples II.4 this ratio was  $0.70 \pm 0.15$  with  $K_a = 13.8 \pm 3.1 \times 10^5 \text{ M}^{-1}$ . No Scatchard plot could be constructed for the binding data obtained with fibrin Naples II.3, because of low binding under these conditions.

**Fibrin stimulation of t-PA-induced plasminogen activation.** The stimulatory effect of fibrin Naples II.3 on t-PA-induced plasminogen activation was normal, as compared with fibrin prepared from normal fibrinogen, by both Reptilase and thrombin (data not shown). These results indicate that the interaction among fibrin Naples, t-PA, and plasminogen in a purified system is not affected by the mutation in fibrinogen Naples. This concurs with the previously described normal interaction between fibrin Naples and several components of the fibrinolytic system in plasma (24). It also indicates that the observed thrombophilia in the affected individuals is not related to defective fibrinolysis.

**Amplification and direct sequencing of genomic DNA fragments.** Based on the evidence of fibrinopeptide release by thrombin, which showed that the defect was located in the

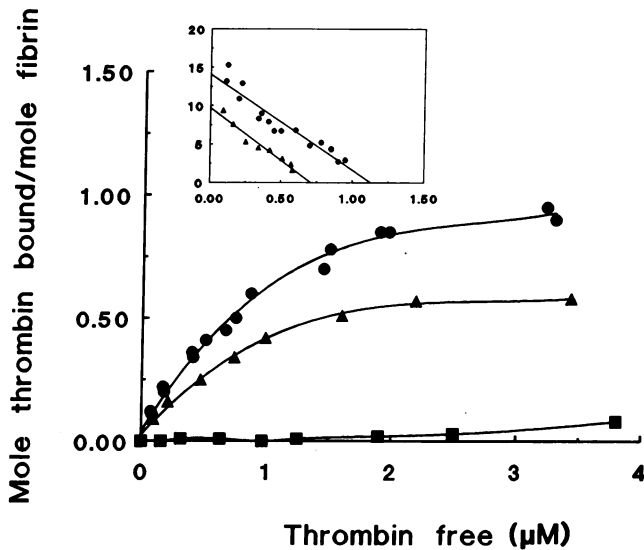


Figure 5. Binding of [<sup>14</sup>C]DFP  $\alpha$ -thrombin to Reptilase-induced fibrin clots. (●) Normal fibrinogens; (▲) fibrinogen Naples II.4; and (■) fibrinogen Naples II.3. *Inset*: Scatchard plot of binding data (normal fibrinogen and fibrinogen Naples II.4).

NDSK part of fibrinogen Naples, we amplified the genomic DNA which codes for NDSK, specifically amino acids A $\alpha$  1–51, B $\beta$  1–118, and  $\gamma$  1–79 of the fibrinogen molecule. After amplification, fragments with the sizes predicted from the genomic DNA sequences for the human fibrinogen A $\alpha$ , B $\beta$ , and  $\gamma$  chain genes (16, 38, 39) were sequenced. The fragments containing the A $\alpha$  or  $\gamma$  gene sequence were completely normal, whereas the B $\beta$  fragment of Naples II.3 had a single base substitution (Fig. 6) in the codon normally coding for alanine at position 68. This mutation changed the codon GCT (alanine) to ACT which codes for threonine. The normal sequence was completely missing in Naples II.3, indicating that the propositus was homozygous for this mutation.

*Detection of mutation in family members and normal individuals.* The amplified B $\beta$  fragments of the family members were hybridized with two synthetic oligonucleotides, one with the normal sequence ( $\beta$ -normal) and the other with the sequence found in fibrinogen Naples ( $\beta$ -Naples). Fig. 7 shows that the amplified B $\beta$  fragments of Naples I.1, I.2, and II.4

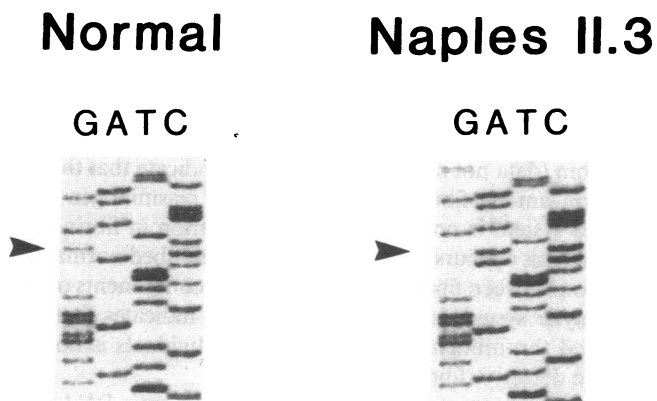


Figure 6. DNA sequence of B $\beta$ -chain gene fragment after amplification coding for amino acids 9–209 (arrows indicate the mutation).

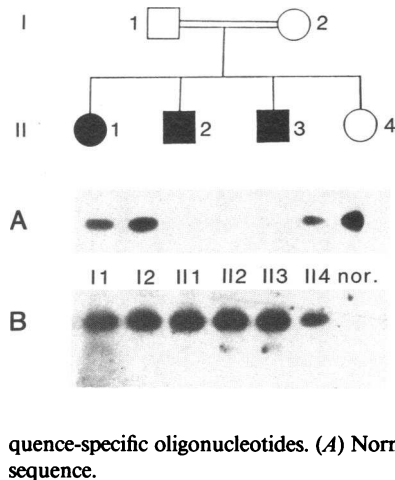


Figure 7. (Top) Pedigree of the family. The propositus (II.3) has symptoms of venous thromboembolism, and his sister (II.1) and brother (II.2) have symptoms of arterial thrombosis, whereas the parents I.1 and I.2 (first cousins) and the brother II.4 are asymptomatic. (Bottom) Southern blot analysis of the B $\beta$  chain gene fragment of normal and Naples family using sequence-specific oligonucleotides. (A) Normal sequence; (B) Naples sequence.

hybridize with both oligonucleotides, indicating that the asymptomatic family members are heterozygous for the mutation found in the B $\beta$  fragment. Amplified B $\beta$  fragments of Naples II.1, II.2, and II.3 hybridized only with the  $\beta$ -Naples oligonucleotide, showing that the symptomatic family members were homozygous for the mutation. The corresponding fragment of 120 normal individuals hybridized only with the  $\beta$ -normal oligonucleotide (data not shown), indicating that the mutation was not a common polymorphism.

## Discussion

The mutation in fibrinogen Naples is associated with a defective release of FpA and FpB by thrombin. Because this defect was present in the purified NDSK fragment of fibrinogen Naples, we amplified and sequenced the genomic DNA segments encoding the NDSK fragments A $\alpha$ , B $\beta$ , and  $\gamma$  chains. Sequence analysis of the amplified products demonstrated that the propositus (II.3) was homozygous for a single base substitution in the codon for B $\beta$  Ala 68 (GCT) resulting in a Thr (ACT) at this position. Southern blot analysis using sequence specific oligonucleotides showed that all three asymptomatic family members were heterozygous for the mutation while the three symptomatic members (including the propositus) were homozygous. This indicates that the homozygous mutation (B $\beta$  68 Ala $\rightarrow$ Thr) is associated with thrombophilia, and that this type of dysfibrinogenemia is clinically recessive.

By using fibrinogen purified from the homozygous propositus,  $\alpha$ -thrombin-catalyzed FpA release was delayed relative to normal fibrinogen. The rate of FpA release from fibrinogen isolated from a heterozygous family member (II.4) was approximately half that of normal, indicating that heterozygous individuals have both normal and abnormal molecules. Human  $\alpha$ -thrombin-catalyzed release of FpA from the purified NDSK fragment of normal fibrinogen was identical to that of intact fibrinogen. As previously reported (2, 3), this provides evidence that the NDSK fragment contains all the essential information for an effective interaction of thrombin with fibrinogen. Analogously, NDSK from the homozygous fibrinogen Naples II.3 had the same decreased rate of FpA release by  $\alpha$ -thrombin as intact fibrinogen Naples II.3, indicating that the defective domain is located in the NDSK part of the fibrinogen Naples molecule. The release of FpA by Reptilase from fibrinogen

Naples and NDSK Naples II.3 was normal, indicating that the mutation did not affect the substrate cleavage site of FpA in the A $\alpha$ -chain.

The rate of FpB release from homozygous fibrinogen Naples II.3 was also strongly delayed as compared to normal fibrinogen. The FpB release from NDSK Naples II.3 was approximately the same as from intact fibrinogen Naples II.3, which is in contrast with the slower release of FpB from normal NDSK as compared with normal fibrinogen. The reduced release of FpB from normal NDSK as compared with normal intact fibrinogen, can be explained by the accelerating effect of fibrin polymerization on FpB release (40–43), which is absent when using NDSK. The similar release of FpB from NDSK Naples II.3 and intact fibrinogen Naples II.3 suggests that this accelerating effect of fibrin polymerization is absent in fibrin Naples.

Active site-inhibited thrombin binds to fibrin by a site independent of the catalytic site designated the anion-binding exosite (7–12). Active site-inhibited human  $\alpha$ -thrombin bound to normal fibrin formed by Reptilase. In contrast, thrombin binding to fibrin Naples II.3 was very low whereas thrombin binding to fibrin Naples II.4 was about half that of normal fibrin. Scatchard analysis of the binding data indicated a single class of binding sites with maximal binding of 1.1 mol of thrombin per mole of fibrin and  $K_a = 1.3 \times 10^6 \text{ M}^{-1}$ . This approximates the strongest binding ( $K_a = 5.8 \times 10^5 \text{ M}^{-1}$ ) determined by Liu et al. (7), who reported that fibrin contains two classes of binding sites. Our data determined under different experimental conditions indicate only one class of binding sites. Irrespective of these differences, many studies indicate that  $\alpha$ -thrombin binds to fibrin by a site distinct from its catalytic centre and that the binding site on fibrin is located in the NDSK part of the fibrinogen molecule (13–15). The results with fibrinogen Naples indicate that the B $\beta$  Ala 68  $\rightarrow$  Thr substitution fully disrupts thrombin binding at this site.

The loss of the anion-binding exosite from  $\alpha$ -thrombin in the formation of  $\beta$ - and  $\gamma$ -thrombins, results in a reduced clotting activity of  $\beta$ - and  $\gamma$ -thrombins for fibrinogen (9, 13). This suggests that the reduced fibrinopeptide release observed in fibrinogen Naples could be fully accounted for by the decreased noncatalytic binding of  $\alpha$ -thrombin to fibrinogen Naples. However, the possibility of influence by amino acid substitution in fibrinogen Naples on the catalytic binding of  $\alpha$ -thrombin cannot be ruled out.

Fibrinogen New York I (23) also showed a decreased release of FpA and FpB, and a defective binding of thrombin (24). In contrast to fibrinogen Naples, the defect in fibrinogen New York I is extensive, in that amino acids B $\beta$  9–72 are deleted. This large deletion is likely to have multiple effects on the defective fibrinogen structure, particularly because B $\beta$  Cys 65, which normally forms a disulphide bond with A $\alpha$  Cys 36, is missing. Without information about the three-dimensional structure of NDSK, it is difficult to determine the exact influence of the B $\beta$  68 Ala  $\rightarrow$  Thr substitution on the binding to thrombin. One possible explanation is that B $\beta$  68 alanine participates in a nonpolar interaction with thrombin and that this is disrupted by threonine. Alternatively, the slightly larger side chain of threonine could lead to incorrect folding or disulfide bond formation of this part of the fibrinogen molecule, preventing thrombin binding.

It has been suggested that thrombin binding to fibrin is a mechanism that prevents active thrombin from existing free in the circulation (7). In the absence of this binding, free active

thrombin in the circulation results in excessive coagulation and/or platelet aggregation which in its turn can lead to thrombosis. From the dramatic history of thrombophilia in the family with fibrinogen Naples and the genetic analysis of the family, it is clear that the occurrence of thrombosis is related to the defect in fibrinogen Naples only in homozygous family members. The mutation in fibrinogen Naples (B $\beta$  68 Ala  $\rightarrow$  Thr) prevents thrombin binding to fibrin and is correlated with thrombophilia. These results demonstrate that thrombin binding to fibrin is an important in vivo mechanism to limit the presence of free active thrombin in circulation and to prevent excessive coagulation and/or platelet activation leading to thrombosis.

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