Aberrant Hepatic Processing Causes Removal of Activation Peptide and Primary Polymerrisation Site from Fibrinogen Canterbury (Aα 20 Val → Asp)

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Abstract
A novel mechanism of molecular disease was uncovered in a patient with prolonged thrombin time and a mild bleeding tendency. DNA sequencing of the fibrinogen A α chain indicated heterozygosity for a mutation of 20 Val → Asp. The molar ratio of fibrinopeptide A to B released by thrombin was substantially reduced at 0.64 suggesting either impaired cleavage or that the majority of the variant α-chains lacked the A peptide. The latter novel proposal arises from the observation that the mutation changes the normal "R G P R V V" sequence to R G P R D creating a potential furin cleavage site at Arg 19. Synthetic peptides incorporating both sequences were tested as substrates for both thrombin and furin. There was no substantial difference in the thrombin catalyzed cleavage. However, the variant peptide, but not the normal, was rapidly cleaved at Arg 19 by furin. Predictably intracellular cleavage of the Aα-chain at Arg 19 would remove fibrinopeptide A together with the G P R polymerisation site. This was confirmed by sequence analysis of fibrinogen Aα chains after isolation by SDS-PAGE. The expected normal sequence was detected together with a new sequence (D V E R H Q S A- ) commencing at residue 20. Truncation was further verified by nonreducing SDS-PAGE of the NH2-terminal disulfide bond which indicated the presence of aberrant homo- and heterodimers. (J. Clin Invest. 1995. 96:2854–2858.) Key words: coagulation • furin • proprotein • protein synthesis • dysfibrinogenemia

Introduction
Fibrinogen is a 340-kd dimeric disulfide linked molecule with each half consisting of three polypeptide chains: Aα, Bβ, and γ (1) with molecular weights of 66,062, 54,358, and 48,529, respectively. The final step of the coagulation cascade and the first step of fibrin polymerisation involves the thrombin catalyzed cleavage of the Arg-Gly bond between residues 16 and 17 of the Aα chain of fibrinogen (2). The release of fibrinopeptide A exposes a new Gly Pro Arg NH2-terminal sequence which initiates polymerisation by docking to a preexisting site located in the COOH-terminal domain of the fibrinogen molecule, and centred close to γ 363 Tyr (3). A similar thrombin catalysed reaction results in release of the B peptide from the Bβ chain.

The three fibrinogen chains are synthesized in the liver and assembled into functional fibrinogen molecules before export from the hepatocyte (4). At least two intracellular endoproteases are involved in the export and post translational modification of the molecule. In the endoplasmic reticulum signal peptidase removes the prepeptide from all three chains and a post Golgi cleavage removes a 'pro' peptide from the COOH-terminal of the Aα-chain (5). This fibrinogen convertase has not been identified but an hepatic convertase, first described in 1988, cleaves the propeptide from proalbumin at its Arg Arg (6) site and has been shown to have a preference for cleavage at Arg X Y Arg over X Y Arg Arg sequences (7). This endogenous Ca2+-dependent microsomar serine protease has the same dibasic specificity as recombinant furin (8), a microsomal propeptide expressed at high mRNA levels in the liver. Indeed, furin and the endogenous protease also have the same pH optimum and inhibitory spectrum. Thus the present evidence suggests that they are the same enzyme (9, 10). The Arg Pro Val Arg cleavage sequence at the COOH-terminal of the Aα-chain, conforms to the furin processing motif (5, 9). After cleavage, the newly exposed P1 Arg would be expected to be removed by carboxypeptidase H (11), or a similar circulatory carboxypeptidase to give the mature chain with its COOH-terminal valine.

Similar dibasic sequences also mark the intracellular proprotein processing sites in peptide hormones, growth factors, plasma proteins, receptors, bacterial toxins, viral envelope glycoproteins, and other liver-derived coagulation factors, including prothrombin, profactors IX, X, and XII, and proproteins C, S, and Z (7, 9, 12). Human mutations that abolish these dibasic processing motifs result in diseases with pathologies dependent on the product of the mature protein. For example, mutations in the Arg Pro Lys Arg processing site of profactor IX, at –1Arg → Ser (13) or –4Arg → Gln (14), result in hemophilia since the uncleaved precursors are unable to be activated by factor Xa. The mutation of –1Arg → Ser in the insulin proreceptor results in noninsulin dependent diabetes (15), while mutations of either the –1 or –2Arg of proalbumin (10) result in circulating proalbumin with no associated pathology. Most importantly, the mutation of +1Asp → Val in proalbumin Blenheim changes the processing sequence from Val Phe Arg Arg Asp to Val Phe Arg Arg Val and this prevents processing of the proalbumin precursor (10, 16).

Here we report a new disease mechanism in a patient with a prolonged thrombin time. The mutation introduces an inappropriate proprotein cleavage sequence at residue 19 of the Aα-chain of fibrinogen and is the first report of a disease being caused by this mechanism. Here the mutation results in removal of fibrinopeptide A and the primary Gly Pro Arg polymerisation sequence before the fibrinogen molecule enters the circulation.

Methods
Materials. The protease inhibitors, E-64 (thiol), o-phenanthroline (metal) and pepstatin (aspartyl) were obtained from Boehringer Mannheim

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Biochemicals (Indianapolis, IN). Bothrops atrox venom (atroxin) was obtained from Sigma Chemical Co. (St. Louis, MO) and stored at 5 mg/mL in water at −20°C. Human α-globin was prepared by chromatography of hemoglobin A globin on CM-cellulose in 6 M urea.

**Plasma samples** Plasma was prepared from trisodium citrate anticoagulated whole blood and thrombin and reptilase clotting times were performed by standard protocols. Fibrinogen functional concentrations were determined using the Clauss method and molar concentrations were determined by quantification of the amount of fibrinopeptide B released from plasma on treatment with thrombin. For fibrinopeptide release assays 50 μg of plasma and 50 μL of 50 mM Tris HCl, 50 mM NaCl, 10 mM CaCl2, were incubated with 5 μL of 10 mM phenanthroline and 1 μL of bovine thrombin (Parke Davis Thrombostat, Ann Arbor, MI). After 5 min 100 μL of 49 mM phosphate buffer pH 2.9 was added, the solution boiled for 5 min, microfuged, and 50 μL of the supernatant injected on to a Nova Pac C-18 column. The column was eluted with a linear gradient from 25% to 50% solvent B where the initial solvent, A, was 49 mM phosphate buffer pH 2.9 and solvent B was an equal mixture of A and acetonitrile (8). Fibrinogen was purified by precipitation with 20% saturated ammonium sulphate (20°C), after standing (4°C) for 30 min the precipitate was collected by centrifugation and washed twice with 20% saturated ammonium sulphate before being dissolved in water at 37°C.

**DNA amplification.** Genomic DNA was isolated from white blood cells and oligonucleotide primers were synthesized on an DNA synthesizer (390; Applied Biosystems, Inc., CA). The Aα primer pair targeted nucleotides 1111–1130 (5′ ATT GCT TGT CCT GTC TTT TTG 3′) and 1290–1309 (5′ AAT CTC CTT CCC CCG CT 3′), and the Bβ primer pair target2ed nucleotides 3187–3206 (5′ GGG TGT TGG AAT AGT TAC AT 3′) and 3495–3515 (5′ CGT CCA TGA CTA CAG GCT TT 3′). These primers flank the second exon of each chain.

After a hot start (98°C, 10 min) Taq polymerase (Boehringer Mannheim Biochemicals) was added to the amplification mixture which contained 100 ng of DNA, 50 pmol primers, and 200 μM dNTPs. 30 amplification cycles were performed of denaturation (94°C, 30 s), annealing (60°C, 30 s), and extension (72°C, 30 s), with a final extension of 7 min. The PCR product was gel purified and sequenced directly by the dyeoxy chain termination method using [35S]ATP and Taq DNA polymerase (18).

**Proteolytic cleavage of synthetic fibrinogen.** Synthetic peptides of the sequence D F L A E G G V G R P R V E R H W and D F L A E G G G V G R G P R V E R H W, corresponding to fibrinogen Aα-residues 7–25, were obtained from Chiron Mimotopes (Vic, Australia) and contained blocked NH2 (acetyl) and COOH (amide) terminals. Trp rather than the normal Gln was incorporated at residue 25 (numbering based on the NH2-terminus of the Aα-chain rather than that of the peptide) to facilitate the rapid identification of the NH2- and COOH-terminal fragments. The purity and sequence fidelity of the peptides were determined/verified by the manufacturer using ion spray triple quadruple mass spectrometry. Their measured masses (MH+) were 2178.4 and 2194.3 D, respectively.

For thrombin digests, 1 μL bovine thrombin (0.02 U) was added to 0.7 nmol of peptide in 5 μL of 50 mM Tris/HCl pH 7.4, 50 mM NaCl. Separate tubes were incubated for 2.5, 5, 10, 15, 20, and 30 min and diluted with 50 μL of 49 mM phosphate buffer pH 2.9 before analysis by reverse phase HPLC (8). The amount of peptide cleaved at each time point was determined as a percentage based on the appearance of the COOH-terminal product and disappearance of the parent peptide. For furin digests, the purified Δ704 mutant soluble form of the mouse enzyme was used (10). The furin, 0.3 μL (4 μU), was added to 0.7 nmol of peptide in 2.6 μL 50 mM MES, 1 mM CaCl2, pH 5.5 containing 50 μM, phenanthroline, 20 μM pepstatin, 0.1 mg/mL E-64, and 1 mg/mL α-globin. Individual tubes were incubated at 30°C for up to 6 h before being diluted with 50 μL of 49 mM phosphate buffer, pH 2.9 and analyzed by reverse phase HPLC (8). As for thrombin digests the effluent was monitored at 254 as well as 215 nm.

**Protein sequence analysis.** Fibrinogen (20 μg) was separated into its three constituent chains by SDS-PAGE in a 7.5% gel under reducing conditions and electroblotted onto a polyvinylidene difluoride membrane. Direct sequencing was carried out on an Applied Biosystems 471 instrument using a Problot cartridge.

**CNBr digestion.** 1 mg of fibrinogen and 2 mg of CNBr were incubated overnight in 75 μL of 70% formic acid. After drying (over NaOH), peptides were redissolved in 100 μL of 1% SDS, 6 μL urea and analyzed directly by nonreducing SDS-PAGE. For cleavage with Bothrops atrox venom (atroxin) 20 μg of CNBr digest was diluted into 50 μL 25 mM Tris HCl pH 7.4, 25 mM NaCl, and 25 μg of venom was added. Samples were incubated at 20°C for 20 min and again analyzed by nonreducing SDS-PAGE.

**Results**

**Case history.** The propositus, a 45-y male vegan with Type III hyperlipidemia, was initially seen at a specialized lipid clinic. Fibrinogen was measured as part of a routine panel of cardiovascular risk factors. On specific questioning, he admitted a tendency to prolonged bleeding, lasting up to 15 min, from even minor cuts. He also noted that these cuts would readily resume bleeding after minor bumps. He had no history of major bleeds despite several episodes of surgery including an amputation of one arm after trauma sustained in a motor vehicle accident. He denied any easy bruising although soft tissue injuries such as a sprained ankle often resulted in large hematomas. He has two siblings and two children, who were not available for testing, but was not aware of any familial bleeding or thrombotic tendency.

Fibrinogen levels measured functionally by thrombin clotting gave values of 0.8 and 0.7 mg/mL on two occasions while quantitation based on the amount of fibrinopeptide B release was 1.3 mg/mL for both these samples. The prothrombin time (INR) and APP time were normal at 1.2 and 32 s, respectively (normal ranges 0.8–1.2 and 26–37 s). The prolonged thrombin and reptilase time of 32 s (normal 18–22 s) and 29 s (normal 18–22 s) however, suggested an impairment of fibrinopeptide release and/or fibrin polymerisation.

**Fibrinopeptide release.** Reverse phase thrombin catalysed fibrinopeptide release assays (Fig. 1) were performed on whole plasma and incorporated 10 mM phenanthroline to inhibit the carboxypeptidase B mediated conversion of peptide B to its des-Arg form. Analysis of 10 different normal plasmas indicated that the average fibrinopeptide A to B ratio, based on peak areas, was 0.92. Analysis of three separate cases (Brennan, S. O., B. Hammond and P. M. George unpublished data) with the fibrinogen Christchurch mutation (Bf14 Arg → Cys) gave average A to B ratios of 2.1 indicating, as expected, that the B peptide was not being removed from ~50% of the Bβ chains.

In contrast, repeated analysis of plasma from the present case (Fig. 1 b) showed a decrease in fibrinopeptide A release with an A/B ratio of 0.58. This result explains the observed prolonged thrombin and reptilase times and suggested that either the propositus is heterozygous for a mutation that prevents the thrombin catalysed release of the A peptide, or that the A peptide was missing from approximately half of the Aα chains in the circulating fibrinogen.

**DNA sequence analysis.** Genomic DNA encoding residues 1–41 of the Aα chain and 9 to 72 of the Bβ chain, spanning the two thrombin cleavage sites (18), was amplified by the polymerase chain reaction and the products subjected to direct DNA sequence analysis as described earlier. The Bβ DNA had an entirely normal sequence, however, two bands, A and T, were detected at nucleotide 1215 of the Aα gene (Fig. 2). This
indicates that the propositus is heterozygous for a mutation of A20 Val → Asp (GTT→GAT). This result was confirmed by sequencing the complementary strand and represents a new mutation; fibrinogen Canterbury.

**Cleavage of synthetic fibrinopeptides.** The prolonged thrombin time and decreased yield of fibrinopeptide A could be readily explained if this mutation inhibited thrombin cleavage as is reported to occur in other fibrinogens with mutations at or near the cleavage site (19, 20); e.g. fibrinogens Ledyard 16 Aα Arg → Cys and Aarhus 19 Aα Arg → Gly (21, 22). To test this possibility, synthetic peptides spanning residues 7–25 of the normal and fibrinogen Canterbury sequence were incubated with thrombin and the released peptides (A and B) separated by reverse phase HPLC. Gradient 25–50% B over 14 min.

**Figure 1.** Reverse phase HPLC analysis of fibrinopeptide release. (a) normal control; (b) propositus with fibrinogen Canterbury; and (c) control patient with fibrinogen Christchurch B314 Arg → Cys. 50 µl of plasma was incubated with thrombin and the released peptides (A and B) separated by reverse phase HPLC. Gradient 25-50% B over 14 min.

**Figure 2.** DNA sequencing of the fibrinogen Aα gene, exon 2. Normal control (right) and a heterozygote for fibrinogen Canterbury (left), showing that the affected individual is heterozygous for A and T at the second base of the codon for amino acid 20. The abnormal sequence encodes a mutation of A20 Val to Asp.

**Figure 3.** Reverse phase separation of thrombin (a) and furin (b) digests of synthetic peptides incorporating the normal, A F L A E G G G V R16 G P R V 20 V E R H W sequence (peptide A) and the variant peptide with the 20 Val → Asp substitution (peptide R). The thrombin digests shown are for a 5-min incubation and the gradient is from 14 to 55% B. The furin digests shown were for 2 h and gradient was from 14 to 62% B. N and C peptides are derived from cleavage at Arg 16 and N’ and C’ from cleavage at Arg 19, respectively.
erved (Fig. 3 b) and sequence/composition analysis established that N’ was D F L A E G G G V R G P R and that C’ was D V E R H W confirming that cleavage was occurring at Arg 19. The rate of this reaction, 0.6 nmol/min per unit of furin, was comparable to that observed for the cleavage of a peptide RGVFRRDANKSEVAV (0.5 nmol/min per unit) which incorporates the processing site of proalbumin (8). There was, however, no detectable cleavage of the normal fibrinogen peptide even after prolonged incubations of 6 h.

Analysis of fibrinogen. Fibrinogen was purified by ammonium sulphate precipitation and analyzed by agarose gel electrophoresis, nonreducing SDS-PAGE in 4% gels and reducing 7.5% SDS-PAGE gels. Normal patterns were observed in all cases, specifically the expected 340- and 305-kD forms were observed in nonreducing SDS gels (23), and on reducing gels the individual Aα, Bβ, and γ chains comigrated with the corresponding chains from normal individuals. However, protein sequence analysis of the individual chains, after transfer to a polyvinylidene difluoride membrane, showed two major sequences in the Aα band of the propositus. One was the expected NH₂-terminal sequence of Ala Asp Ser Gly Glu Gly Asp Phe Leu- and the second was Asp Val Glu Arg His Gin (Ser) Ala- . Two PTH amino acids were clearly detectable at each cycle except for residue seven where only Asp was detected and the other residue was presumed to be Ser. The aberrant sequence commences at the Aα 20 Val → Asp mutation site and proceeds with a normal primary structure beyond residue 21. The two products were present in the ratio 60:40, normal to truncated variant.

The six NH₂ termini of the pairs of Aα, Bβ and γ chains that make up the central domain of the fibrinogen molecule, are held rigidly together by a series of disulfide bonds. This ‘NH₂-terminal disulfide knot’ can be liberated as a unit by CNBr cleavage. It consists of residues (Aα 1-51, Bβ 1-118, and γ 1-78) × 2 and, containing 494 amino acids, it is the largest expected CNBr fragment (2) apart from higher Mr forms which result from incomplete cleavage, possibly resulting from partial denaturation of the methionines. Fig. 4 A shows the SDS-PAGE pattern of CNBr digests of nonreduced fibrinogen. The propositus (lanes 1 and 4) showed the expected normal 494 residue fragment (a) together with two lower molecular weight forms (b and c) which were not present in controls (lanes 2, 3, and 5). As expected the (a) band runs above the albumin marker (lane 6) which, because of its intense disulfide bonding, has an apparent Mr of 50 kD in nonreducing gels (24, 25). Incubation with Bothrops atrox venom confirmed that band a was the 494 residue NH₂-terminal disulfide knot since removal of the two A peptides resulted in a decrease in Mr (Fig. 4 B, lanes 1 and 2). Similar incubation of the CNBr digest from the propositus resulted in normalisation of the band pattern (lanes 4 and 3). The atroxin cleavage products all migrated below band b and in the position of the minor c band. These data confirm the amino acid sequence data which indicated truncation of the Aα chain and further show that the circulating fibrinogen contains both homo- and heterodimers of the abnormal Aα chains. Similarly homo- and heterodimers have been observed with fibrinogen Birmingham (26).

Discussion

DNA sequence analysis readily established that the propositus was heterozygous for a mutation of Aα 20 Val → Asp. Since this substitution is close to the thrombin cleavage site at Arg 16, it initially seemed plausible that the prolonged thrombin time might be explained by defective thrombin binding and cleavage. This has been reported for other variants (19–22) and is consistent with the observation that fibrinopeptide A was not released from some 40% of the Aα chains after treatment with excess thrombin.

Failure of thrombin cleavage was, however, not verified in experiments with synthetic peptides which showed that both the variant and normal peptide were cleaved with similar efficiencies (0.5 and 0.9 nmol/min per unit, respectively). The predicted change in amino acid sequence from Arg Gly Pro Arg Val to Arg Gly Pro Arg Asp suggested an alternative explanation for the prolonged thrombin time; that the variant Aα chain was being cleaved, within the intracellular export pathway, by the hepatic proprotein convertase (7). This novel suggestion was substantiated in three ways: (a) by sequence analysis of the Aα chains from circulating fibrinogen which showed that 40% of the molecules commenced at the new aspartic acid at residue 20; (b) by the demonstration of truncation in the NH₂-terminal disulfide knot after CNBr cleavage; and (c) by the demonstration that peptides incorporating the new sequence were rapidly cleaved by purified recombinant furin the proposed in situ hepatic convertase.

The sequence requirements for cleavage by the endogenous KEX2-like hepatic convertase (7) and furin (8, 10) are identical, with absolute requirements for paired basic residues in either an X Y Arg Arg or preferably an Arg X Y Arg sequence. For processing to occur however the P₁₁ residue immediately after the cleavage site must not be valine. The clearest demonstration of this precise requirement comes from the observed failure of proalbumin processing in individuals with the proalbumin Blenheim (+1 Asp → Val) mutation. Here the existing Val Phe Arg Arg Asp site is replaced by an ineffective Val Phe Arg Arg Val sequence (16). In fibrinogen Canterbury the converse mutation occurs and thus the new Aα sequence of Arg Gly Pro Arg Asp would be expected to permit the intracellular removal of the A peptide together with the Gly Pro Arg polymerization site. It would also be necessary for Arg 19 to be on
the surface of the molecule and thus accessible to proteases. However, this appears to be the case since in prolonged diges-
tions with thrombin normal fibrinogen can be cleaved at this site.

It also appears that fibrinogen is exposed to furin in the hepatic export pathway since the cDNA for the Aα chain en-
codes an additional 15 residue propedeptide not found in the circu-
lating molecule (5). In this instance, cleavage occurs at an Arg
Pro Val Gly sequence and is followed by carboxypeptidase
H exoproteolytic removal of the exposed Arg to generate the
observed mature COOH-terminal sequence of Arg Pro Val.
When fibrinogen is expressed in kidney cells (5) which, like the
hepatocyte, contain high levels of furin (27), the secreted
protein contains two species of Aα chain: one with the extension
and one with the mature circulating sequence. As would be
expected for furin mediated cleavage, mutation of the P1, Arg →
Gly totally prevented fibrinogen cleavage in the baby hamster
kidney expression system. Further investigation showed that
this extended molecule underwent normal assembly and secre-
tion, and that there was no alteration in its ability to clot in
the presence of thrombin or crosslink in the presence of factor
XIIIa. Similarly intracleavage of the new propedeptide
sequence at the NH2-terminal of fibrinogen Canterbury does not appear to
have a major effect on assembly or secretion since the truncated
molecule constitutes 40% of the circulating α chains and homo-
dimers (α*βγ)y2 as well as heterodimers were detected after
CNBr cleavage. However, removal of the NH2-terminal twenty
residues has a predictably major impact on function since the
intracellular cleavage removes the Gly Pro Arg sequence which
is the primary initiator of polymerisation.

This mutation reveals a new disease mechanism but also
implies a general constraint on the sequence of secreted pro-
teins. Specifically valine residues that follow exposed dibasic
sequences might be important in preventing unwanted cleavage
of any protein that transits the constitutive export pathway.
Similarly other large alkyl P1 residues, noticeably leucine and
isoleucine, also inhibit proalbumin cleavage (28) and histidine
is not allowed at the P2 position (8). Thus, in the Bβ chain,
histidine 19, which occurs in a sequence of Arg Gly His Arg,
might have the specific role of preventing similar intracellular
cleavage, and mutations at this residue might allow similar intra-
cellular cleavage of the Bβ chain.

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