

Missense mutations in the human β fibrinogen gene cause congenital afibrinogenemia by impairing fibrinogen secretion

Stefano Duga, Rosanna Asselta, Elena Santagostino, Sirous Zeinali, Tatjana Simoncic, Massimo Malcovati, Pier Mannuccio Mannucci, and Maria Luisa Tenchini

Congenital afibrinogenemia is a rare autosomal recessive disorder characterized by bleeding that varies from mild to severe and by complete absence or extremely low levels of plasma and platelet fibrinogen. Although several mutations in the fibrinogen genes associated with dysfibrinogenemia and hypofibrinogenemia have been described, the genetic defects of congenital afibrinogenemia are largely unknown, except for a recently reported 11-kb deletion of the fibrinogen A α -chain gene. Nevertheless, mutation mechanisms other than the

deletion of a fibrinogen gene are likely to exist because patients with afibrinogenemia showing no gross alteration within the fibrinogen cluster have been reported. We tested this hypothesis by studying the affected members of two families, one Italian and one Iranian, who had no evidence of large deletions in the fibrinogen genes. Sequencing of the fibrinogen genes in the 2 probands detected 2 different homozygous missense mutations in exons 7 and 8 of the B β -chain gene, leading to amino acid substitutions Leu353Arg and Gly400Asp,

respectively. Transient transfection experiments with plasmids expressing wild-type and mutant fibrinogens demonstrated that the presence of either mutation was sufficient to abolish fibrinogen secretion. These findings demonstrated that missense mutations in the B β fibrinogen gene could cause congenital afibrinogenemia by impairing fibrinogen secretion. (Blood. 2000;95:1336-1341)

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Introduction

Fibrinogen is a 340-kd glycoprotein synthesized primarily by hepatocytes and secreted as a hexamer composed of 3 pairs of polypeptide chains (A α , B β , and γ) (for review, see¹). It has a trinodular structure with a central nodule (E-domain) that contains the N-terminus of each chain and 2 lateral globular domains (D-domains) that contain the C-terminus of B β - and γ -chains. The E-domain is linked to the 2 D-domains by a coiled-coil triple helix structure.² The 3 chains are encoded by different genes, clustered in a region of approximately 50 kb on chromosome 4q28.³ Fibrinogen participates in hemostasis by forming the insoluble fibrin clot and mediating platelet aggregation.⁴ Moreover, fibrinogen is a class II protein involved in the acute phase response to injury and stress (for review, see⁵).

Congenital fibrinogen disorders include afibrinogenemia, hypofibrinogenemia, and dysfibrinogenemia, characterized by the complete absence or extremely low levels of plasma fibrinogen, by reduced amounts of plasma fibrinogen, or by the presence of dysfunctional fibrinogen molecules, respectively. Congenital afibrinogenemia (MIM 202 400) is a rare autosomal recessive disorder with a high rate of consanguinity within affected families, and it is characterized by bleeding manifestations that often start at birth with uncontrolled umbilical cord hemorrhages. Bleeding from mucosal membranes, hematomas, hemarthroses, and hemorrhages

after trauma and surgery are relatively frequent.⁶⁻⁸ However, the disease usually can be controlled by fibrinogen replacement therapy.

Although several missense mutations in the 3 fibrinogen genes have been identified as the cause of dysfibrinogenemia, hypofibrinogenemia, or both,⁹ congenital afibrinogenemia, originally described in 1920,¹⁰ has been associated only with a recently reported homozygous 11-kb deletion of the A α -chain gene.¹¹ However, the existence of different genetic alterations in inherited afibrinogenemia was suggested by a previous study on 2 patients with afibrinogenemia who had no gross alterations within the fibrinogen cluster as revealed by Southern blot analysis.¹²

In this study 2 patients with afibrinogenemia, an Italian and an Iranian, each the offspring of a marriage between first cousins, were studied. Both had plasma fibrinogen levels that could not be measured by functional assay, but extremely low levels (between 0.05% and 0.5% of the average normal value) were detected by enzyme immunoassay. Two different homozygous missense mutations (Leu353Arg and Gly400Asp), neither of which has yet been described, were identified in exons 7 and 8 of the fibrinogen B β -chain gene. In vitro expression of the mutant proteins demonstrated that each mutation abolished fibrinogen secretion, thus causing the extremely reduced fibrinogen levels observed in the patients.

From the Department of Biology and Genetics for Medical Sciences, the Institute of Veterinary Physiology and Biochemistry, the Angelo Bianchi Bonomi Hemophilia and Thrombosis Center, and the Fondazione Luigi Villa and the Department of Internal Medicine, University of Milan; the IRCCS Maggiore Hospital, Milan, Italy; and the Department of Biotechnology, Pasteur Institute of Tehran, Iran.

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Reprints: Maria Luisa Tenchini, Dipartimento di Biologia e Genetica per le Scienze Mediche, via Viotti, 3/5-20133 Milano, Italy; e-mail: marialuisa.tenchini@unimi.it.

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Methods

Materials

Full-length α , β and γ cDNA, cloned in the expression vector pRSV-Neo, were kind gifts of Dr C. M. Redman (Lindsley F. Kimball Research Institute, New York Blood Center, New York, NY). Rabbit polyclonal antibodies to human fibrinogen were from DAKO (Copenhagen, Denmark). Oligonucleotides were purchased from Life Technologies (Inchinnan, Paisley, UK).

Coagulation tests

Fibrinogen was measured in plasma by a functional assay based on fibrin polymerization time using a commercial kit (Laboratoire Stago, Asnieres, France), and it was measured in plasma and washed platelets by enzyme immunoassay.¹³ The sensitivity of the functional assay was 5 mg/dL and that of the immunoassay was 0.02 mg/dL.

DNA extraction

All examined subjects signed informed consent. Genomic DNA was extracted from blood samples using the Nucleon BACC1 kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

Linkage analysis

The polymorphic locus used for linkage analysis was the tetranucleotide repeat FGA-i3, located in intron 3 of the fibrinogen α -chain gene.¹⁴ Polymerase chain reaction (PCR) was performed on genomic DNA under standard conditions, and amplified products were separated using an automated 370A DNA sequencer (PE Biosystems, Foster City, CA). Genotyping was performed using the Genescan 3.1 software (PE Biosystems) and the LINKAGE package was used for linkage analysis.¹⁵ The average maximum expected LOD score was calculated using the SIMULATE software.¹⁶

Sequence analysis

DNA sequencing was performed on both strands, either directly on purified-PCR products or on plasmids using the *Taq* dye-deoxy terminator method and an automated 370A DNA sequencer (PE Biosystems). All primers used for sequencing were designed on the basis of known sequences of the fibrinogen genes and intergenic regions (Genbank accession numbers M64 982, M64 983, M10 014, and U36 478). Factura and Sequence Navigator software packages (PE Biosystems) were used for mutation detection.

Allele-specific oligonucleotide hybridization

20 ng PCR products, corresponding to regions in exon 7 or 8 of the fibrinogen β -chain gene, were blotted onto nylon membranes (Amersham Pharmacia Biotech) and hybridized to ³²P-labeled allele-specific oligonucleotides (ASO) according to standard procedures.¹⁷ Wild-type and mutant probes are listed in Table 1.

Site-directed mutagenesis

Leu353Arg and Gly400Asp mutations were introduced in the pRSV-Neo- β plasmid by the Quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer's instructions, using the oligonucleotides L353R-F, L353R-R, and G400D-F, and the oligonucleotide G400D-R, respectively (Table 1).

Cell cultures, transfections, and metabolic labeling

The human hepatoma cell line HepG2 was cultured in Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 media (1:1, vol/vol), supplemented with 10% fetal calf serum. The African green monkey kidney cell line COS-1 was cultured in DMEM containing 10% fetal calf serum. Antibiotics (100 IU/mL penicillin and 100 μ g/mL streptomycin) and

Table 1. Oligonucleotide primers used in ASO hybridizations and in site-directed mutagenesis

Primer	Sequence	Position*	Location (FGB exon)
FGB-L353†	5' TAATGCCCTCATGGATG 3'	7148-7164	7
FGB-R353†	5' TAATGCCCGCATGGATG 3'	7148-7164	7
FGB-G400†	5' AGACGGTGGTGGATGGT 3'	7907-7923	8
FGB-D400†	5' AGACGGTGGTGGATGGT 3'	7907-7923	8
L353R-F‡	5' CCGTAATGCCCGCATGGATGGAGC 3'	7144-7168	7
L353R-R‡	5' GCTCCATCCATGCGGGCATTACCGG 3'	7168-7144	7
G400D-F‡	5' AAGAAGACGGTGGATGGTGGTA 3'	7903-7927	8
G400D-R‡	5' TACCACCATCCATCACCCTCTTCTT 3'	7927-7903	8

*Numbering according to Genbank accession number M64983.

†Probes used for ASO hybridization.

‡Oligonucleotides used for site-directed mutagenesis.

FGB, fibrinogen β -chain gene.

glutamine (1%) were added to the media. Cells were grown at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Using CELL FECTIN (Life Technologies), semiconfluent COS-1 cells were transfected with equimolar amounts (5 μ g each) pRSV-Neo- α , pRSV-Neo- γ , and pRSV-Neo- β plasmids, the latter either wild-type or mutant. Thirty-six hours after transfection, the cells were labeled by incubating each 100-mm dish for 3 hours with 5 mL methionine- and cysteine-free DMEM (ICN Biomedicals, High Wycombe, Berks, UK) supplemented with 150 μ Ci L-[³⁵S] methionine and 60 μ Ci L-[³⁵S] cysteine (Amersham Pharmacia Biotech), with 10% dialyzed fetal calf serum, 0.1 mg/mL heparin, and 1% glutamine. In pulse-chase experiments, cells were pulse labeled for 3 hours as described above and then chase incubated for various periods of time (30, 90, 180 minutes) with fresh medium containing 10 mmol/L methionine and 10 mmol/L cysteine.

Immunoprecipitation and sodium dodecyl sulphate–polyacrylamide gel electrophoresis

After metabolic labeling, culture media were collected and a protease inhibitor mixture was added (Complete; Roche, Basel, Switzerland). Five milliliters medium from each plate was centrifuged to remove cell debris and concentrated using a Centricon Plus-20 column (Millipore, Bedford, MA). Cells were washed 3 times with prechilled phosphate-buffered saline (PBS) and lysed for 1 hour on ice with lysis buffer containing 1 \times PBS, 1% Triton X-100, and the protease inhibitor mixture. Cell lysates were centrifuged to remove cell debris. Rabbit antihuman fibrinogen antibodies, preadsorbed with protein G-Sepharose (Sigma Chemical, St. Louis, MO) at room temperature for 3 hours, were added to cell lysates and media and incubated for 4 hours on ice. Pellets were collected by centrifugation for 3 minutes and washed 3 times with lysis buffer. The immunoprecipitated proteins were released from protein G-Sepharose by boiling for 5 minutes in sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Samples were analyzed by SDS-PAGE according to Laemmli¹⁸ and were detected by autoradiography or by a STORM PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Results

Patient data

The proband of the Italian family was a 17-year old boy whose parents were first cousins (Figure 1A). The diagnosis of afibrinogenemia was made at birth because the patient had life-threatening bleeding from the umbilical cord, which rendered necessary transfusion with whole blood and fibrinogen concentrates. After that he had relatively mild symptoms, such as epistaxis and posttraumatic muscle hematomas. His parents, sister, and other

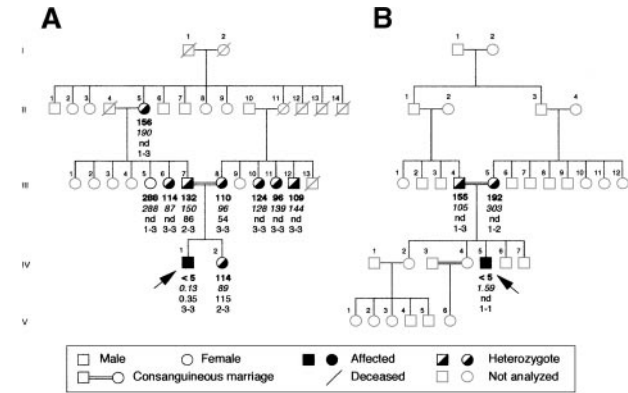


Figure 1. Family pedigrees of the 2 probands with congenital afibrinogenemia analyzed in this study. (A) Pedigree of the Italian and (B) of the Iranian family. Plasma functional fibrinogen levels (mg/dL), immunoreactive fibrinogen levels (mg/dL), intraplatelet levels ($\mu\text{g}/10^9$ platelets) and haplotypes of the FGA-i3 tetranucleotide repeat marker (alleles numbered from the largest to the smallest) are indicated in this order below each symbol. Arrows indicate the probands. nd, not done.

family members available for study were asymptomatic. In the proband, plasma fibrinogen levels could not be measured by functional assay (less than 5 mg/dL; normal range, 160-400 mg/dL), and they were found to be very low by enzyme immunoassay (0.13 mg/dL; normal range, 160-400 mg/dL). Platelet levels of immunoreactive fibrinogen were also very low (0.35×10^9 platelets; normal range, 60-190 $\mu\text{g}/10^9$ platelets). His parents and sister had approximately half the normal levels of plasma fibrinogen and normal intraplatelet levels.

The proband of the Iranian family (Figure 1B) was a 24-year-old man, also born of a consanguineous marriage. He bled at birth from the umbilical cord and later during circumcision, and he was treated with whole blood and fresh-frozen plasma on both occasions. Subsequently, he suffered repeatedly from muscle hematomas and hemarthroses that occurred spontaneously or after minor trauma. His parents, 2 brothers, and 2 sisters were asymptomatic, and there was no history of bleeding in the other family members not investigated. In the proband, fibrinogen could not be measured by functional assay, and very low values (1.6 mg/dL) were detected by the more sensitive immunoassay. His father's plasma fibrinogen level was half the normal level, and his mother had a low borderline level of functional plasma fibrinogen and a normal level of immunoreactive fibrinogen. Platelet samples could not be obtained.

Mutational analysis

The absence of gross deletions in the fibrinogen cluster has been verified by Southern blot analysis and by long PCR amplification of each fibrinogen gene (data not shown). To verify whether the fibrinogen cluster was still a candidate for mutation screening, linkage analysis using the tetranucleotide repeat FGA-i3 marker located within the α -chain gene was performed in the Italian family. Evaluation of plasma levels of functional fibrinogen allowed us to classify phenotypically the 9 available members of this family as homozygous normal (more than 160 mg/dL) or heterozygous (less than 160 mg/dL) (Figure 1A). Linkage analysis resulted in an LOD score of 2.02 at $\theta = 0$. This LOD score, close to the average maximum expected LOD score of 2.37 calculated for this pedigree, suggested the existence of linkage to the fibrinogen cluster. Because of the small number of available members, the Iranian pedigree could not be considered for this analysis. The proband received the same FGA-i3 allele from both parents, as expected for the affected offspring of a consanguineous marriage (Figure 1B).

On the basis of these results in each proband, the entire coding region was sequenced, including exon-intron boundaries and approximately 300 bp of the promoter region of each fibrinogen gene. In the Italian proband, a single homozygous T-G transversion was found in exon 7 of the fibrinogen β -chain gene at position 7156 (numbering according to Genbank accession number M64983) (Figure 2A). This resulted in a missense mutation leading to a Leu-Arg substitution at position 353 (L353R). The proband's parents were heterozygous for the mutation (Figure 2A). All available family members were tested for the mutation by dot blot hybridization with ASO probes. Besides the proband's parents, the mutation was present in the heterozygous state in the proband's grandmother and in phenotypically heterozygous relatives (Figure 2B). Two hundred aploid genomes from unrelated persons in a northern Italian control population were also analyzed, and the mutation was absent. In the Iranian proband, a single G-A homozygous transition at position 7915 was identified in exon 8 of the fibrinogen β -chain gene, replacing glycine with aspartate at position 400 (G400D) (Figure 2C). This mutation was also present in the heterozygous state in the proband's parents (Figure 2C), but not in 200 unrelated Iranian aploid control genomes. Both amino acid substitutions are nonconservative and reside in the C-terminal globular portion of the β -chain.

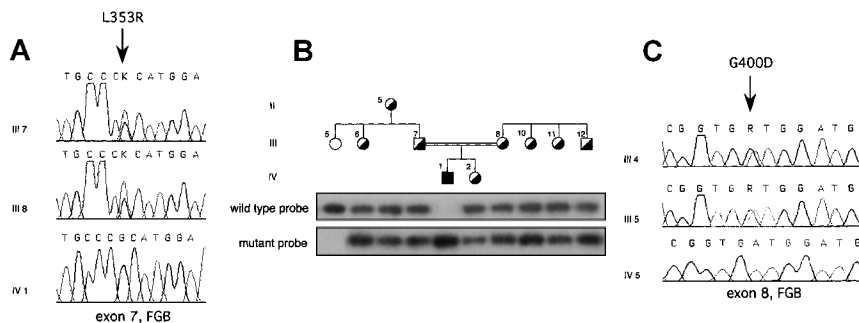


Figure 2. L353R and G400D mutations. (A) Electropherograms showing the mutation identified in the Italian proband with afibrinogenemia. The T-G transversion leading to the L353R mutation, indicated by an arrow, was present in the heterozygous state in both parents. (B) ASO hybridization showing cosegregation of the L353R mutation and clinical phenotype in the Italian kindred. Family members were phenotypically classified as homozygous normal or heterozygote on the basis of plasma fibrinogen levels. Individuals within the pedigree are positioned above the corresponding lanes. Wild-type (FGB-L353) and mutant (FGB-R353) probes are listed in Table 1. (C) Electropherograms showing the mutation identified in the Iranian proband with afibrinogenemia. The G-A transition leading to the G400D mutation, indicated by an arrow, was present in the heterozygous state in both parents. Family members are labeled as in Figure 1. FGB, fibrinogen β -chain gene; K, G or T nucleotide; R, A or G nucleotide.

In vitro protein expression

To determine whether the L353R and G400D mutations affect fibrinogen assembly or secretion, mutant proteins were transiently expressed in COS-1 cells that do not express fibrinogen. The 2 point mutations were independently introduced by site-directed mutagenesis in a B β -chain expression plasmid (pRSV-Neo-B β)¹⁹ and checked by sequencing. Each mutated plasmid was transiently cotransfected in COS-1 cells, together with plasmids pRSV-Neo-A α and pRSV-Neo- γ expressing the wild-type A α - and γ -chains, respectively.²⁰ Transfections with equal amounts of wild-type and each of the mutated B β -chain cDNA were also performed, to mimic the heterozygous state. Transfected cells were incubated with L-[³⁵S] methionine and L-[³⁵S] cysteine. Fibrinogen expression was analyzed by immunoprecipitation using polyclonal antihuman fibrinogen antibodies and protein G-Sepharose and then by SDS-PAGE.

After SDS-PAGE under nonreducing conditions, a specific fibrinogen band was observed in cell lysates and culture media of COS-1 cells transfected with plasmids containing wild-type fibrinogen cDNA (Figures 3A, 3B). The same band was also observed in untransfected control fibrinogen-expressing HepG2 cells (Figures 3A, 3B). By contrast, in cells transfected with plasmids expressing L353R or G400D B β -chains, hexameric fibrinogen was detectable only in cell lysates (Figure 3A) but was undetectable in the corresponding culture media (Figure 3B). Cotransfection of wild-type and each of the mutated B β -chain cDNA restored fibrinogen secretion to the medium (Figure 3B). Mock-transfected (pUC18) COS-1 cells showed only background bands in cell lysate and medium (Figures 3A, 3B). Immunoprecipitated proteins from the same culture media as in Figure 3B were also separated on SDS-PAGE under reducing conditions. The expected pattern of A α , B β and γ -chains was observed in fibrinogen-containing media (Figure 3C).

To evaluate whether the observed absence of mutant fibrinogens in culture media resulted from an impairment of fibrinogen secretion or from increased intracellular or extracellular degradation, a pulse-chase experiment was performed. Transfected COS-1 cells were labeled for 3 hours with L-[³⁵S] methionine and L-[³⁵S] cysteine and were chase incubated with an excess of the corresponding unlabeled amino acids for various periods up to 3 hours. As

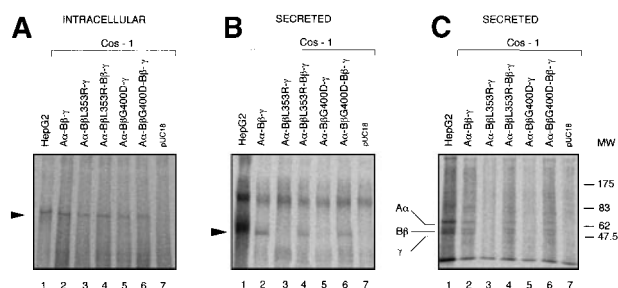


Figure 3. Expression of wild-type and mutant fibrinogen chains in COS-1 cells. Intracellular and secreted fibrinogen from COS-1 cells transfected with wild-type A α and γ -chains together with wild-type and/or L353R or G400D B β chains have been analyzed. Metabolic labeling, immunoprecipitation, and SDS-PAGE were carried out as described in "Materials and Methods." (A) Immunoprecipitable proteins in cell lysates separated on 4% SDS-PAGE under nonreducing conditions. The arrowhead indicates the 340-kd fibrinogen complex. (B) Immunoprecipitable proteins in the culture media separated on 4% SDS-PAGE under nonreducing conditions. The arrowhead indicates the 340-kd fibrinogen complex. (C) Immunoprecipitable proteins in the culture media separated on 10% SDS-PAGE under reducing conditions. The positions of A α , B β , and γ chains are indicated. The position of molecular weight markers (MW) in kd is denoted at the right-hand side of the figure.

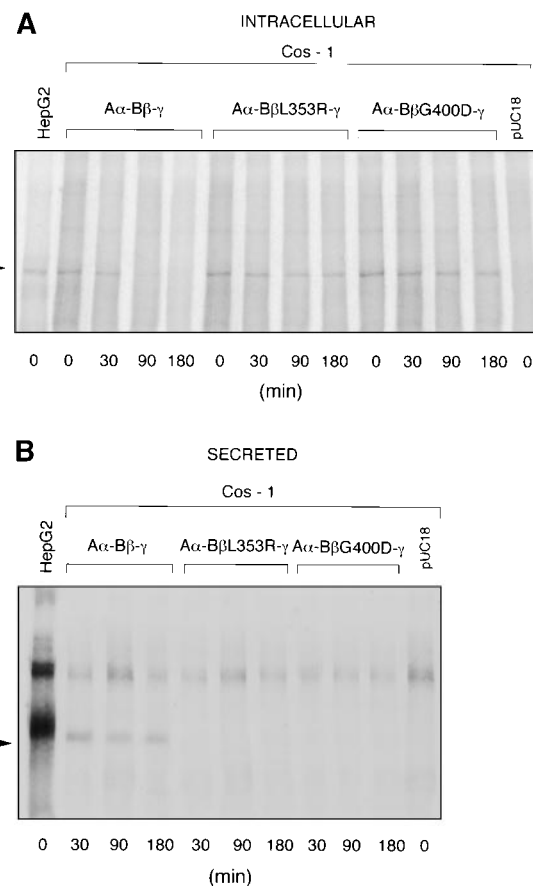


Figure 4. Intracellular retention of L353R and G400D mutant fibrinogens. COS-1 cells were incubated with L-[³⁵S] methionine and L-[³⁵S] cysteine for 3 hours and then chase incubated with 10 mmol/L L-methionine and L-cysteine for various periods of time up to 3 hours. At the specified chase periods (0, 30, 90, and 180 minutes), radioactive fibrinogen was immunoprecipitated from cell lysates and from the corresponding culture media, as described in "Materials and Methods." Intracellular (A) and secreted (B) immunoprecipitable proteins were separated by 4% SDS-PAGE under nonreducing conditions. The arrowheads indicate the 340-kd fibrinogen complex. Intracellular and secreted fibrinogen at the end of the pulse period from control HepG2 cells was also analyzed. In both panels, pUC18 lane contains immunoprecipitable proteins at the end of the pulse period from COS-1 cells transfected with the unrelated pUC18 plasmid.

shown in Figure 4A, in lysates from cells transfected with the 3 wild-type cDNA, immunoprecipitated radioactive fibrinogen markedly declined at 90 minutes and disappeared at 180 minutes. By contrast, in lysates from cells transfected with plasmids expressing L353R or G400D B β -chains, hexameric fibrinogen persisted intracellularly up to 3 hours (Figure 4A). Secreted fibrinogen was present, at the analyzed chase-periods, in culture media from COS-1 cells cotransfected with A α , B β , and γ wild-type cDNA, whereas it was absent in culture media from cells cotransfected with A α and γ wild-type chains and either B β mutant chain expressing plasmids (Figure 4B).

Discussion

Inherited afibrinogenemia is a rare recessive bleeding disease characterized by fibrinogen deficiency in plasma and platelets. Even though, strictly speaking, the term should refer to the complete absence of fibrinogen, minute amounts of fibrinogen can sometimes be detected through sensitive immunoassays. Asymptom-

fibrinogen secretion because mutant fibrinogen molecules were synthesized and assembled intracellularly but were undetectable in culture media. The observation that mutant fibrinogen molecules are assembled intracellularly might explain the minute amounts of protein measured in a patient's plasma using a sensitive immunoassay. This finding is consistent with the concept that missense mutations do not necessarily block protein production completely. The hypothesis that the main defect caused by these 2 mutations was at the secretion level was confirmed by the results of pulse-chase experiments showing the lack of cellular depletion of L353R and G400D mutant fibrinogens.

In conclusion, 2 different homozygous missense mutations were identified in the same region of the B β -chain in 2 patients with afibrinogenemia. Transfection experiments in COS-1 cells with plasmids expressing the mutant proteins demonstrated, for the first time, that these mutations can abolish fibrinogen secretion without

affecting the protein synthesis. These results demonstrated that congenital afibrinogenemia can be caused by point mutations that impair the normal fibrinogen secretion pathway.

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References

- Doolittle RF. Fibrinogen and fibrin. *Ann Rev Biochem.* 1984;53:195.
- Spraggon G, Everse SJ, Doolittle RF. Crystal structures of fragment D from human fibrinogen and its crosslinked counterpart from fibrin. *Nature.* 1997;389:655.
- Marino MW, Fuller GM, Elder FF. Chromosomal localization of human and rat A alpha, B beta, and gamma fibrinogen genes by in situ hybridization. *Cytogenet Cell Gene.* 1986;422:36.
- Mosesson MW. Fibrinogen structure and fibrin clot assembly. *Semin Thromb Hemost.* 1998;24:169.
- Kushner I. The acute phase response: an overview. *Methods Enzymol.* 1988;163:373.
- Mammen EF. Fibrinogen abnormalities. *Semin Thromb Hemost.* 1983;9:1.
- Al-Mondhiry H, Ehmann WC. Congenital afibrinogenemia. *Am J Hematol.* 1994;46:343.
- Lak M, Keihani M, Elahi P, Peyvandi F, Mannucci PM. Bleeding and thrombosis in 55 patients with inherited afibrinogenemia. *Br J Haematol.* 1997;107:204.
- Martinez J. Congenital dysfibrinogenemia. *Curr Opin Hematol.* 1997;4:357.
- Rabe F, Salomon E. Über-faserstoffmangel im Blute bei einem Falle von Hämophilie. *Arch Int Med.* 1920;95:2.
- Neerman-Arbez M, Honsberger A, Antonarakis SE, Morris MA. Deletion of the fibrinogen alpha-chain gene (FGA) causes congenital afibrinogenemia. *J Clin Invest.* 1999;103:215.
- Uzan G, Courtois G, Besmond C, et al. Analysis of fibrinogen genes in patients with congenital afibrinogenemia. *Biochem Biophys Res Commun.* 1984;120:376.
- Cattaneo M, Bettega D, Lombardi R, Lecchi A, Mannucci PM. Sustained correction of the bleeding time in an afibrinogenemic patient after infusion of fresh frozen plasma. *Br J Haematol.* 1992;82:388.
- Mills KA, Even D, Murray JC. Tetranucleotide repeat polymorphism at the human alpha fibrinogen locus (FGA). *Hum Mol Genet.* 1992;1:779.
- Lathrop GM, Lalouel JM. Easy calculations of LOD scores and genetic risks on small computers. *Am J Hum Genet.* 1984;36:460.
- Weeks DE, Ott J, Lathrop GM. Detection of genetic interference: simulation studies and mouse data. *Genetics.* 1994;136:1217.
- Sambrook J, Fritsch EF, Maniatis T. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory; 1989.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 1970;227:680.
- Roy SN, Mukhopadhyay G, Redman CM. Regulation of fibrinogen assembly. Transfection of Hep G2 cells with B beta cDNA specifically enhances synthesis of the three component chains of fibrinogen. *J Biol Chem.* 1990;265:6389.
- Roy SN, Procyk R, Kudryk BJ, Redman CM. Assembly and secretion of recombinant human fibrinogen. *J Biol Chem.* 1991;266:4758.
- Handagama P, Scarborough RM, Shuman MA, Bainton DF. Endocytosis of fibrinogen into megakaryocyte and platelet alpha-granules is mediated by alpha IIb beta 3 (glycoprotein IIb-IIIa). *Blood.* 1993;82:135.
- Koopman J, Haverkate F, Grimbergen J, Egbring R, Lord ST. Fibrinogen Marburg: a homozygous case of dysfibrinogenemia, lacking amino acids A alpha 461-610 (Lys 461 AAA—>stop TAA). *Blood.* 1992;80:1972.
- Ridgway HJ, Brennan SO, Faed JM, George PM. Fibrinogen Otago: a major alpha chain truncation associated with severe hypofibrinogenemia and recurrent miscarriage. *Br J Haematol.* 1997;98:632.
- Furlan M, Steinmann C, Jungo M, et al. A frame-shift mutation in Exon V of the A alpha-chain gene leading to truncated A alpha-chains in the homozygous dysfibrinogen Milano III. *J Biol Chem.* 1994;269:33,129.
- Zhang Z, Redman CM. Identification of B beta chain domains involved in human fibrinogen assembly. *J Biol Chem.* 1992;267:21,727.
- Kim PS, Hossain SA, Park YN, Lee I, Yoo SE, Arvan P. A single amino acid change in the acetylcholinesterase-like domain of thyroglobulin causes congenital goiter with hypothyroidism in the cog/cog mouse: a model of human endoplasmic reticulum storage diseases. *Proc Natl Acad Sci U S A.* 1998;95:9909.
- Ward CL, Omura S, Kopito RR. Degradation of CFTR by the ubiquitin-proteasome pathway. *Cell.* 1995;83:121.
- Sifers RN, Finegold MJ, Woo SL. Molecular biology and genetics of alpha 1-antitrypsin deficiency. *Semin Liver Dis.* 1992;12:301.
- Prockop DJ, Kivirikko KI. Collagens: molecular biology, diseases, and potentials for therapy. *Annu Rev Biochem.* 1995;64:403.
- Ito M, Jameson JL, Ito M. Molecular basis of autosomal dominant neurohypophyseal diabetes insipidus: cellular toxicity caused by the accumulation of mutant vasopressin precursors within the endoplasmic reticulum. *J Clin Invest.* 1997;99:1897.
- Pathak RK, Merkle RK, Cummings RD, Goldstein JL, Brown MS, Anderson RG. Immunocytochemical localization of mutant low density lipoprotein receptors that fail to reach the Golgi complex. *J Cell Biol.* 1988;106:1831.