An Arginine to Cysteine Amino Acid Substitution at a Critical Thrombin Cleavage Site in a Dysfunctional Factor VIII Molecule

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We have analyzed the factor VIII (FVIII) protein and the nucleotide sequence around two thrombin cleavage sites, at arginine 372 in the FVIII heavy chain and arginine 1689 in the FVIII light chain in a naturally occurring dysfunctional FVIII variant, FVIII Okayama. The patient was a 42-year-old hemophiliac with a FVIII coagulant activity of 0.03 U/mL and a FVIII antigen level of 0.8 U/mL. The patient's FVIII was not thrombin activatable to levels seen in normal plasma. Immunoblotting of partially purified FVIII Okayama and normal FVIII showed that thrombin cleavage of the 92 kilodalton (Kd) heavy chain was impaired in the mutant protein. The patient's genomic DNA was amplified using the polymerase chain reaction with two sets of synthetic oligonucleotide primers spanning amino acid residues 319 to 400 and 1630 to 1720. Sequence analysis of the amplified DNA fragments revealed a cysteine to thymine transition, converting an arginine to a cysteine codon at residue 372. No abnormality was found in the FVIII light chain in association with its activation and inactivation. In most cases, the values for FVIII activity and FVIII antigen correlate well. However, there are some rare patients who have a normal level of FVIII antigen with decreased or absent FVIII activity. Such patients are described as having cross reacting material positive (CRM) hemophilia A. The FVIII gene spans 186 kilobases of DNA and contains 26 exons and 25 introns. FVIII is synthesized as a single chain precursor, with the mature protein containing 2,332 amino acids. FVIII procoagulant activity is associated with proteolytic products of the mature molecule. Based on internal homologies, FVIII is made up of distinct structural domains arranged in the order A1-A2-B-A3-C1-C2 (Fig 1). The A1 and A2 domains are part of the amino-terminal heavy chain, while the A3, C1, and C2 domains are part of the carboxy-terminal light chain. The B domain is a unique domain that is rich in potential asparagine-linked glycosylation sites. FVIII that is lacking the B domain has been produced by recombinant DNA technology and expressed in heterologous systems. This B domainless FVIII still retains coagulant activity.

MATERIALS AND METHODS

Patient. The patient is 42 years old and has moderate hemophilia A. His FVIII coagulant activity was 0.03 U/mL as measured by an activated partial thromboplastin time assay using FVIII deficient plasma from a severe (CRM) hemophilia A patient, his hemophilic brother, and his carrier mother, designated FVIII Okayama. The mutation was found using the polymerase chain reaction and nucleotide sequencing. In addition, the thrombin activation properties of the patient's plasma FVIII and the cleavage properties of partially purified FVIII from this patient were studied.
A factor Xa, proposed; 372 thrombin and factor Xa; 740 to 2332). Numbers indicate arginine residues at the following
sites. (A) Mature FVIII protein showing its domains. Amino acid residues are as follows: A1 (residues 1 to 328); A2 (380 to 711); B
(720 to 1890); A3 (1694 to 2019); C1 (2020 to 2172); and C2 (2173 to 2332). Numbers indicate arginine residues at the following
enzyme cleavage sites: 336, activated protein C, thrombin and factor Xa; proposed: 372, thrombin and factor Xa; 740, thrombin;
1648, unknown enzyme; 1689, thrombin and factor Xa; and 1721, factor Xa. (B) Enlarged section of A showing the acidic region
between the A1 and A2 domains. (C) FVIII amino acid residues 319 to 400 predicted from the DNA fragment that was amplified
by the PCR.

FVIII as defined by Eaton and Vehar and enzyme cleavage sites. (A) Mature FVIII protein showing its domains. Amino acid residues are as follows: A1 (residues 1 to 328); A2 (380 to 711); B (720 to 1890); A3 (1694 to 2019); C1 (2020 to 2172); and C2 (2173 to 2332). Numbers indicate arginine residues at the following enzyme cleavage sites: 336, activated protein C, thrombin and factor Xa; proposed: 372, thrombin and factor Xa; 740, thrombin; 1648, unknown enzyme; 1689, thrombin and factor Xa; and 1721, factor Xa. (B) Enlarged section of A showing the acidic region between the A1 and A2 domains. (C) FVIII amino acid residues 319 to 400 predicted from the DNA fragment that was amplified by the PCR.

has one younger brother with moderate hemophilia A, an FVIII activity of 0.03 U/mL, and a FVIII antigen level of 0.82 U/mL, with similar clinical features. The patient’s mother had a FVIII activity of 0.61 U/mL and a FVIII antigen level of 1.40 U/mL, which are at the lower and upper end of normal levels, respectively. The patient’s maternal grandfather and a male cousin were also known to have bleeding problems.

Plasma collection and storage. Blood was drawn from the patient 2 weeks after infusion therapy. Plasma samples were prepared using sodium citrate as anticoagulant and stored at −70°C. Plasma samples were shipped frozen on dry ice.

Antibody purification and radiolabeling. Monoclonal antibodies C5 and C2 were purified by protein A-Sepharose chromatography. Human FVIII inhibitor antibody NF was purified by the caprylic acid method and 100 μg was radiolabeled with 1 mCi of 125I using IODO-GEN (Pierce Chemical Co, Rockford, IL). The iodinated IgG was then affinity-purified on a column of FVIII-Sepharose, which contained approximately 500 units of purified FVIII coupled to 1 mL of CL-4B Sepharose. Elution of inhibitor IgG was accomplished using 0.1 mol/L glycine, 0.15 mol/L sodium chloride buffer, pH 2.8, followed by immediate neutralization.

FVIII antigen assay. FVIII antigen was determined by solid phase two-site immunoradiometric assay (IRMA) essentially according to the method of Peake et al. One of three different antibodies was used for coating: a human inhibitor antibody with an epitope(s) in the C2 domain of the FVIII light chain and a titer of 7,000 Bethesda Units/mL (patient NF); a monoclonal antibody to FVIII with an epitope in residues 351 to 362 on the 54 kilodalton (Kd) thrombin fragment of the FVIII heavy chain (antibody C5); and a monoclonal antibody with an epitope in the amino-terminal acidic region of the FVIII light chain (antibody C2) (unpublished data). For the detecting antibody, an affinity-purified 125I-labeled IgG from the human alloantibody was used (see above).

Thrombin activation of FVIII in plasma. Purified human alpha thrombin (specific activity 2,534 U/mg) was added to plasma samples at final concentrations of 0.05, 0.10, and 0.15 U/mL. At the indicated times, samples were taken and immediately diluted 1:80 (for normal plasma) or 1:40 (for severe CRM hemophilia A or FVIII Okayama plasma) into barbital buffer (0.025 mol/L barbital, 0.125 mol/L sodium chloride, and 0.1% sodium azide, pH 7.5) and FVIII activity was measured as described above.

Purification of FVIII from plasma. Five to seven milliliters of citrated plasma containing 10 μmol/L dansyl-L-glutamyl-L-glycyl-L-arginine chloromethyl ketone, 10 μmol/L D-phenylalanyl-L-phenylalanyl-L-arginine chloromethyl ketone, and 10 μmol/L D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (Calbiochem Behring Corp, La Jolla, CA), and 15 μg/mL of leupeptin (Chemicon, El Segundo, CA) were loaded onto a minicolumn containing 1.0 mL of Sepharose CL-2B coupled with 1.3 mg of purified monoclonal antibody to vWF (clone 2.2.9). After washing the column with buffer (0.02 mol/L imidazole, 0.15 mol/L NaCl buffer, pH 7.4) containing the same protease inhibitors, FVIII was eluted with the same buffer containing 0.35 mol/L CaCl₂. The eluate was concentrated by centrifugation through a centricron 30 microconcentrator (Amicon Corporation, Danvers, MA) and dialyzed with multiple changes at 4°C overnight against buffer containing 0.02 mol/L Tris-HCl, 0.15 mol/L sodium chloride, and 0.02% sodium azide, pH 7.4, before thrombin digestion.

Thrombin digestion. Purified human alpha thrombin (specific activity 2,534 U/mg) was added to the dialyzed, partially purified FVIII at a final concentration of 10 U/mL (0.25 units of thrombin per unit of FVIII antigen) and the mixture was incubated for one hour at 37°C. This was followed by preparation for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (see below).

Immunoblotting. The FVIII and thrombin-treated FVIII samples were mixed with sample buffer and contained a final concentration of 3% SDS, 7.4% glycerol, 0.02% bromphenol blue, and 10 mmol/L dithiothreitol. After heating for 30 minutes at 56°C, samples containing approximately 1.0 unit of FVIII antigen were loaded onto a 7.5% SDS polyacrylamide gel and electrophoresed. After electrophoresis, the samples in the gel were transferred onto a nitrocellulose membrane. For detection of reactive FVIII protein bands, a combination of two monoclonal antibodies to FVIII, C2 and C5 (see above), were used at a final concentration of 10 μg/mL, followed by 125I affinity-purified rabbit anti-mouse IgG and autoradiography.

Oligonucleotides. Oligonucleotide primers were synthesized with an Applied Biosystems 380 DNA synthesizer (Applied Biosystems Inc, Forest City, CA). The synthetic primers were purified by reverse-phase high performance liquid chromatography using an Oligo Column 4.2 mm × 8 cm (Du Pont, Wilmington, DE). The iodinated oligonucleotides used were as follows: MS-5, 5′GTTTTGTCGTACCTCGAGATG′ containing a portion of exon 8, preceded by flanking intron sequence; MS-4, 5′TGTCTTACCTGTACCTCGAGGG′ from exon 8 and adjoining intron sequence; MS-1, 5′CCGAAATCGGTAGGACTGAAAGGCTGTGC′ from exon 14; and MS-6, 5′CGCATTCCTACTGACTCG′ from exon 14 and one nucleotide from the flanking intron.

DNA amplification and sequencing. The genomic DNA of the patient was isolated and amplified using the polymerase chain reaction (PCR). The reaction was done in a 100 μL mixture containing 2 μg of the genomic DNA, 1 μg of each synthetic oligonucleotide primer, and a buffer consisting of 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L MgCl₂, 0.01% gelatin, and 200 μmol/L of each dNTP (Boehringer-Mannheim, Indianapolis). After heating at 92°C for two minutes, 2.5 units of the thermostable Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) was added to the mixture. After overlaying the sample with mineral oil (Sigma, St Louis), PCR was performed automatically with a DNA thermal cycler (Perkin Elmer Cetus). Amplification was performed using 35 cycles of 30 seconds at 91°C, 25 seconds at 55°C, and 30 seconds at 72°C. The amplified DNA was purified by electrophoresis through a 2% agarose gel. The gel-purified DNA was

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kinased and cloned into M13mp18 for sequence analysis using Sequenase (US Biochemical, Cleveland) and dideoxy chain termination reactions with [*H]dATP.\textsuperscript{24}

**RESULTS**

The relative level of FVIII antigen in this CRM\textsuperscript{+} hemophilia A patient was investigated by two-site immunoradiometric assay using either a human inhibitor antiserum or a monoclonal antibody to the 54 Kd thrombin fragment of the FVIII heavy chain, or a monoclonal antibody to the amino-terminus of the FVIII light chain as solid phase antibodies. The same human inhibitor antibody was radiolabeled and used for detection (see Materials and Methods). The level of FVIII antigen measured using the three different coating antibodies was 0.80 U/mL, 0.82 U/mL, and 0.75 U/mL, respectively, where the FVIII antigen level in normal pooled plasma is assigned a value of 1.0 U/mL. These data are in general agreement and are within the range for FVIII antigen in normal individuals,\textsuperscript{2} suggesting that an intact FVIII molecule was present in this patient.

Although the patient’s FVIII antigen measurement revealed a level of FVIII protein within the normal range, his FVIII coagulant activity was only 0.03 U/mL. This is in contrast to a value of 1.0 U/mL assigned to the FVIII activity in normal pooled plasma and is far below the normal range.\textsuperscript{2} We have named this dysfunctional protein, FVIII\textsuperscript{Okayama}.

Given a normal level of FVIII antigen and abnormally low level of FVIII activity, a missense point mutation was hypothesized to account for this variant. Two areas in the FVIII gene, each known to code for amino acids spanning a thrombin cleavage site involved in FVIII activation were selected for nucleotide sequence analysis. The critical thrombin cleavage sites are located at arginine 372 in the FVIII heavy chain and arginine 1689 in the FVIII light chain\textsuperscript{14} (Fig 1). These sites are adjacent to the two acidic regions in the FVIII amino acid sequence and this analysis was simplified by the fact that both acidic regions are encoded by single exons within the normal FVIII gene.\textsuperscript{25} The first acidic region, which separates the A1 and A2 domains of the heavy chain, is encoded by exon 8 of the FVIII gene and includes amino acids 337 to 372, while the second acidic region, at the amino-terminus of the light chain, is encoded by exon 14 and contains amino acids 1630 to 1720.

For this analysis, two sets of oligonucleotide primers were synthesized (see Materials and Methods). The first set of primers, MS-5 and MS-4, direct the amplification of a 261 base pair segment of the FVIII gene using the PCR. The amplified fragment included nucleotides 1010 to 1271 of the FVIII cDNA sequence.\textsuperscript{17} These oligonucleotides were used to amplify and subsequently determine the DNA sequence of the portion of the FVIII\textsuperscript{Okayama} gene that codes for amino acid residues 319 to 400 in the heavy chain of mature FVIII (Fig 1C). Another set of oligonucleotides, termed MS-1 and MS-6, allow a similar analysis by amplifying a 274 base pair fragment that encodes amino acid residues 1630 to 1720 in the light chain of mature FVIII.

Sequence analysis of the amplified DNA produced by MS-4 and MS-5 revealed a missense mutation within the coding sequence of the FVIII\textsuperscript{Okayama} gene. A cytosine to thymine transition causes the arginine 372 codon (CGC) to be replaced with a cysteine 372 codon (TGC) (Fig 2). The observed mutation was confirmed by independent PCRs and cloning experiments to ensure that the defect was not the result of a DNA replication error during the PCR amplification, or the subsequent M13 cloning experiment. No additional mutations were found in the amplified fragments produced by MS-4 and MS-5. The DNA sequence of the amplified product of MS-1 and MS-6 was also identical to the published sequence for the FVIII cDNA,\textsuperscript{7} thereby predicting a normal peptide sequence within the FVIII light chain acidic region. A similar analysis performed on the patient’s hemophilic brother yielded identical results. In addition, multiple templates from the patient’s mother were sequenced and both normal and mutant sequences were found, confirming her carrier status.

In order to evaluate the possibility that the substitution at residue 372 is the pathogenetic factor in this dysfunctional FVIII protein, FVIII\textsuperscript{Okayama} was compared with normal FVIII in its ability to be activated by thrombin, as manifested by an increase in FVIII activity when small amounts of thrombin are added to plasma (in amounts low enough to allow FVIII activity measurement without causing immediate clot formation). The results of this experiment are shown in Fig 3. Despite its high antigen level, FVIII\textsuperscript{Okayama} was impaired in its ability to be activated by thrombin. Although a consistent 2.3 to 4.8-fold increase in activity was seen in this mutant protein using three different concentrations of thrombin, which was temporally similar to normal, the maximum activation achieved for FVIII\textsuperscript{Okayama} at each concentration of thrombin was over tenfold lower than that achieved for normal pooled plasma. The control plasma from a CRM\textsuperscript{+} severe hemophilia A patient showed no activation.

FVIII\textsuperscript{Okayama} protein was also partially purified and compared with partially purified normal FVIII before and after extensive thrombin digestion. This was done by immunoblotting of the partially purified proteins using a mixture of two monoclonal antibodies, one to the FVIII heavy chain (C5) and one to the FVIII light chain (C2) (see Materials and Methods). The immunoblot in Fig 4 shows that both antibodies bound to FVIII\textsuperscript{Okayama} and to normal FVIII. In Fig 4A and C, FVIII purified from normal plasma and from the patient plasma show the characteristic multiple polypeptide bands ranging from 80 Kd to >200 Kd. Normal FVIII also shows a band at approximately 54 Kd, which is presumably a proteolytic fragment of the 92 Kd FVIII heavy chain that arose either before or during purification and contained the C5 epitope. A fragment of similar molecular weight was only faintly present in FVIII\textsuperscript{Okayama}.

After thrombin treatment, however, (Fig 4B and D) FVIII\textsuperscript{Okayama} showed a pattern different from normal FVIII. In normal FVIII (Fig 4B), thrombin cleavage of the 80 Kd light chain to its 72 Kd product occurred, although the 72 Kd product is not visible due to loss of the C2 epitope.\textsuperscript{20} Cleavage of all but a trace of the 92 Kd heavy chain and its higher molecular weight precursors also occurred, as evidenced by their greatly decreased intensity. The 54 Kd thrombin cleav-
Fig 2. (A) Nucleotide sequence around the thrombin cleavage site at residue 372 in the heavy chain of normal FVIII and FVIII Okayama, showing the arginine to cysteine mutation. The asterisk shows the substituted nucleotide. (B) Autoradiograph of the nucleotide sequencing gel showing the G to A base pair transition mutation within the FVIII Okayama noncoding strand.

...age product of the FVIII heavy chain, which contains the C5 epitope, was generated as evidenced by its increase in intensity. A less intense band just below the 54 Kd fragment is also seen and may represent either microheterogeneity or further proteolysis. In Fig 4D, after thrombin treatment FVIII Okayama also showed a loss of the higher molecular weight bands, but unlike normal FVIII, an apparent accumulation of the 92 Kd heavy chain (derived from the higher molecular weight precursors) occurred. There was also no apparent 54 Kd thrombin cleavage product of the 92 Kd heavy chain resulting from this thrombin treatment. Several immunoreactive bands below 92 Kd did appear, however, including peptide material at the tracking dye. The identity of these bands is not known, but they are presumed to be derived from the heavy chain (due to the known loss of light chain reactivity with the C2 antibody after thrombin cleavage) and may represent thrombin cleavage of the heavy chain at new sites, not used in normal FVIII. These results suggest that FVIII Okayama was greatly (although perhaps not entirely) impaired in its ability to undergo thrombin cleavage at arginine 372 in the 92 Kd heavy chain, which produces the 54 Kd and 44 Kd cleavage products.

FIG 3. (A) Thrombin activation of FVIII in normal pooled plasma. The letters a to c indicate thrombin concentrations of 0.15 U/mL, 0.10 U/mL, and 0.05 U/mL, respectively. (B) Thrombin activation of FVIII in FVIII Okayama plasma. The letters a to c represent the same thrombin concentrations as in A. The letter d indicates a severe CRM hemophilia A plasma treated with 0.15 U/mL of thrombin. The interruption in the ordinate indicates that this activity was below the sensitivity limit of the assay.

Fig 4. (A) Nucleotide sequence around the thrombin cleavage site at residue 372 in the heavy chain of normal FVIII and FVIII Okayama, showing the arginine to cysteine mutation. The asterisk shows the substituted nucleotide. (B) Autoradiograph of the nucleotide sequencing gel showing the G to A base pair transition mutation within the FVIII Okayama noncoding strand.

DISCUSSION

The amplified genomic DNA of FVIII Okayama revealed a predicted arginine to cysteine substitution at the arginine 372 thrombin cleavage site in the FVIII heavy chain. Although this mutation is probably responsible for the functional defect in this protein, until the entire sequence of FVIII Okayama is determined, the possibility of another mutation(s) affecting function cannot be ruled out. Our finding of an arginine 372 to cysteine mutation is in accordance with the prediction of Gitschier et al that the
Fig 4. Autoradiograph of an immunoblot of partially purified FVIII samples detected by a mixture of a monoclonal antibody to the FVIII heavy chain (C5) and a monoclonal antibody to the FVIII light chain (C2) (see Materials and Methods). (A) FVIII purified from normal pooled plasma. (B) The same sample as in A after thrombin treatment. (C) FVIII purified from FVIII Okayama plasma. (D) The same sample as in C after thrombin treatment. Molecular weights in kilodaltons are shown for the heavy chain (92), light chain (80), and amino-terminal thrombin fragment of the heavy chain (54).

arginine residues at positions 336, 372, 1648, and 1689 in the FVIII sequence are mutational hot spots, due to the presence of a CpG dinucleotide in these codons. The presence of a CpG dinucleotide has been correlated with a high frequency of DNA polymorphisms in humans and evidence has been presented by Youssoufian et al. for its role in FVIII mutations, which cause hemophilia A. The mechanism is thought to be based on the occurrence of 5-methylcytosine in a CpG dinucleotide, the deamination of which leads to a thymine transition mutation (discussed in reference 35).

It is interesting to note that all but one of the FVIII molecules with a mutation at a thrombin cleavage site involved in activation described to date, still retain a low level of procoagulant activity. The site-directed mutagenesis studies of Pittman and Kaufman showed that substitution of an isoleucine for the arginine residue at either position 372 in the heavy chain, or 1689 in the light chain, resulted in a molecule that was resistant to thrombin cleavage at the mutated site and was not susceptible to thrombin activation. However, these molecules had a low level of residual FVIII activity. Similarly, a naturally occurring variant FVIII molecule with an arginine to histidine mutation at residue 372 in the heavy chain has recently been reported by Arai et al. This mutation resulted in mild hemophilia A, with a residual FVIII activity of 5% and a higher than normal FVIII antigen level. The partially purified FVIII from this patient had a slower rate of thrombin proteolysis at this site, as compared with normal FVIII. In this report we describe a moderate hemophilia A patient with 3% FVIII activity, a normal FVIII antigen level, and a heavy chain arginine 372 to cysteine mutation. This mutant protein showed a four to fivefold activation when plasma was treated with 0.15 U/mL of thrombin, although the maximum level of activity reached was much less than that seen in similarly treated normal plasma. The partially purified molecule also appeared to be resistant to thrombin cleavage after prolonged digestion. In contrast, however, Gitschier et al. described a light chain arginine 1689 to cysteine mutation in a severe hemophilia A patient with no detectable FVIII activity and a normal FVIII antigen level. The basis of the variable level of FVIII activity in these mutant molecules has not yet been explained. However, it seems possible that cleavage at arginine 1689 alone could support a low level of activity, depending on the amino acid substitution; other enzymes in plasma or cell culture medium might generate fragments with low activity; or thrombin might cleave at a very low rate at the mutated site, again depending on the amino acid substitution. Further characterization of these and other FVIII mutations in CRM hemophilia A patients should provide further insights into the structure and function of FVIII.

ACKNOWLEDGMENT

We thank Dr Carol K. Kasper for the gift of human FVIII inhibitor plasma from patient NF. We also thank Dr John W. Fenton II for the gift of purified human alpha thrombin. The support and encouragement of the late Dr Theodore S. Zimmerman is also gratefully acknowledged.

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