Molecular Defect in Coagulation Factor XFriuli Results From a Substitution of Serine for Proline at Position 343

By Harold L. James, Antonio Girolami, and Daryl S. Fair

Our previous findings suggested that coagulation factor XFriuli could be functionally defective owing to a point mutation in the portion of the factor X gene coding for the fully activated heavy chain. To verify the existence of this postulated change, we analyzed all eight exons of the normal and Friuli factor X gene. Each exon was amplified from genomic DNA using the polymerase chain reaction and cloned into the plasmid pUC19. The amplified DNA inserts were subjected to direct sequencing by the dideoxy chain termination method with forward and reverse oligonucleotide sequencing primers. A point mutation (C to T transition at nucleotide position 19,297) that results in coding for serine (TCC) at amino acid position 343 was found. This substitution involves a highly conserved proline residue oriented spatially close to both the cleavage site of the zymogen and the active site of the enzyme and explains the previous observations of discrete biochemical and functional differences between factor XFriuli and normal factor X. The mutation abolished an HgiCl restriction site present in the normal factor X gene, and this change constitutes the basis for a convenient method for screening individuals carrying this molecular defect. Proline343 is in conserved region 5 of the serine protease superfamily to which factor X belongs and is part of a 14-residue LXXXXXXFXXXA motif that occurs in at least 16 other enzymes. Computer analysis suggests that the motif may be an essential aspect of conformational features important to functional properties of factor X as well as other serine proteases.

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ringer Mannheim Biochemicals), and ampicillin (Sigma Chemical, St. Louis, MO). Nusieve GTG and Seakem GTG grades of agarose were purchased from FMC BioProducts (Rockland, ME), and ethidium bromide was obtained from Sigma. All other reagents and chemicals were either molecular biology grade or of the highest available purity.

Genomic DNA. Peripheral blood (PB) samples, approximately 50 mL, were collected using one-tenth volume of 3.8% sodium citrate as anticoagulant using approved institutional protocols. Genomic DNA was isolated according to the method of Bell et al. Each preparation was suspended in 1 mL 10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0 (TE buffer) and dissolved by gentle agitation for 1 to 2 days at 37°C before storage at 4°C.

Polymerase chain reaction (PCR). Known nucleotide sequences11 flanking exons II through VIII of the factor X gene were used to design oligonucleotide primers for use in amplifying the respective exons in the polymerase chain reaction (PCR).12 In addition, published information14 was used to synthesize PCR primers for exon I. Primers were synthesized using a BioSearch model 8750 DNA synthesizer by the phosphoramidite method.15 The primers shown for exon 111 contain HindIII recognition sequences (indicated in lower case). For exons II through V, 5′ “tails” with Sall recognition sequences (bases in lower case), were added to the primers. In the primer sequences for exons VI, VII, and VIII, bases in lower case indicate specified restriction endonuclease recognition sequences created by a single nucleotide substitution, which is italicized in each sequence. The primer sequences are as follows: exon I, 5′ end, GACAAGCAGTCCAGGATGGCGGAC; exon II, 3′ end, CTGGCCGCCCCTCACAGTCTGGCGTGC; exon II, 5′ end, ttatgtcagaGCTCGGGTGAGGTTGACAGAG; exon II, 3′ end, ttatgtcagaAAATCTCCTTTTTTTTTCAG; exon III, 5′ end, ttatgtcagaAACTAGGAGAACAGGAG; exon IV, 5′ end, ttatgtcagaTGAATCCGAAAAACAGCTGAG; exon IV, 3′ end, ttatgtcagaTGCCACTCTTCAGGGCGAT; exon V, 5′ end, ttatgtcagaCCAGCCTCTTTTCTCCAGCTG; exon V, 3′ end, ttatgtcagaTCGACTCTTGTTACCTGCG; exon VI, 5′ end, PstI: TcgcagCTATGGGAGGACCTCT; exon VI, 3′ end, PstI: CAAGCTGGTCTCTCTCrgag; exon VII, 5′ end, AccI: AGTCAGGGATCCTGCTG; exon VIII, 3′ end, AccI: GTAAAACGACGGGCTTCG.

Cloning of factor X gene fragments in pUC19. The unfractionated PCR-amplified DNA products were cleaved with the appropriate restriction endonucleases and were inserted into pUC19 with T4 DNA ligase.18 The insert-containing pUC19 preparations were used to transform DH5α E. coli—competent cells in the presence of ampicillin and X-gal. Verification of isolated colonies carrying the desired DNA inserts was performed using 1.5-mL aliquots of overnight Luria broth cultures in “miniprep” analyses, followed by agarose gel electrophoresis of the restriction enzyme-digested DNA. Plasmid DNA preparations for each insert were then made by growing cultures overnight using 50-mL vol CIRCLEGROW (BIO 101, La Jolla, CA) and a commercial “Midi Preparation” kit (QIAGEN, Studio City, CA), which yielded from 200 to 300 μg highly purified insert-containing plasmid DNA.

DNA sequencing. For direct sequencing, each plasmid DNA was denatured in 0.1 mol/L NaOH and incubated at 37°C for 20 minutes in the presence of forward (GTAAACAGGAGGC- CAGT) and reverse (AACACTTGAGGACAT) M13 sequencing primers (U.S. Biochemicals), using 30 ng of each primer for each 2 μg denatured plasmid DNA.19 Because the insert-containing exon VIII was almost 900 base pairs (bp) long, three forward and three reverse internal sequencing primers were synthesized for use in obtaining the total sequence of exon VIII. The three forward internal sequencing primers were: ATGACTTCGACATCCGGCAGT, ACCGGCAACACGTCCAGCAGT, and CCAAGGTACCCGGCTTCCTC. The three reverse internal sequencing primers were: AATCGAGAGCAAGAAACCAGGC, GAAGTAGTGGTCTCTTGAAGC, and TGCCCGCCAGCGCGTCACAAATG.

Results

The complete nucleotide sequences (sense and antisense directions) of all eight exons for the factor X derived gene were sequenced in two separate experiments, beginning with amplification of each exon by the PCR. The exon sequences for the DNA isolated from an individual with normal factor X antigen and activity agreed with those reported previously.20 A single transition from a C to T at nucleotide 19,297 in exon VIII of the variant gene was observed (Fig 1). Sequence analysis of exon VIII derived from a related individual with the Friuli defect gave the same result as shown in Fig 1. This point mutation is consistent with the coding for serine (TCC) in place of
proline (CCC) at amino acid position 343 in factor X\textsubscript{Friuli}. The consistent substitution of C by T confirms that the deficiency is homozygous. The proline\textsuperscript{30} was part of a L\textsubscript{******}P\textsubscript{******}C motif that occurs in as many as 16 other serine proteases as well as haptoglobin (Table 1). Computer-analyzed structural homology of the motif in three serine proteases is shown in Fig 2. The motif occurs in conserved region 5 of factor X and other serine proteases.\textsuperscript{21}

The point mutation in factor X\textsubscript{Friuli} results in abolishment of recognition sites for at least three different restriction endonucleases: HgiCl, NlaIV, and SduI. Because the PCR-amplified exon VIII-containing fragment of the normal factor X gene contains only two HgiCl recognition sites, loss of one of the two sites in factor X\textsubscript{Friuli} gene could be demonstrated easily by agarose electrophoresis (Fig 3). The

### Table 1. Sequence Alignment of an Invariant Leucine, Proline, and Cysteine Motif Among the Serine Proteases

<table>
<thead>
<tr>
<th>Protein</th>
<th>Amino Acid Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor X</td>
<td>LKMLEPYPVRDNSC</td>
<td>9,11</td>
</tr>
<tr>
<td>Prothrombin</td>
<td>LQVVLPLPVRPS</td>
<td>25</td>
</tr>
<tr>
<td>Factor VII</td>
<td>LMVLNLPLMTDQC</td>
<td>26</td>
</tr>
<tr>
<td>Factor X</td>
<td>LQYLRPLVDATC</td>
<td>27</td>
</tr>
<tr>
<td>Protein C</td>
<td>LNFLNIPVPHNEC</td>
<td>28</td>
</tr>
<tr>
<td>Trypsinogen\textsuperscript{t}</td>
<td>LKCLKAPILSNSSC</td>
<td>29</td>
</tr>
<tr>
<td>Chymotrypsinogen\textsuperscript{t}</td>
<td>LQASLPFLSNTNC</td>
<td>29</td>
</tr>
<tr>
<td>Prekallikrein</td>
<td>LQKVNLPLTVEEC</td>
<td>30</td>
</tr>
<tr>
<td>Factor XI</td>
<td>LGKEKGPLVNEEC</td>
<td>31</td>
</tr>
<tr>
<td>Factor XII</td>
<td>LQEAQWPFLSNERC</td>
<td>32</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>LKEAQLLPILNKVC</td>
<td>33</td>
</tr>
<tr>
<td>Factor D</td>
<td>LHVVLPLVDRAKC</td>
<td>34</td>
</tr>
<tr>
<td>C1r</td>
<td>LRFVRPLPVAPQAC</td>
<td>35</td>
</tr>
<tr>
<td>C1s</td>
<td>LKAARLPVAPRLKC</td>
<td>36</td>
</tr>
<tr>
<td>Hepsin</td>
<td>LEARVPILSNVCE</td>
<td>37</td>
</tr>
<tr>
<td>Elastase\textsuperscript{t}</td>
<td>LQAYLPSVDSYIC</td>
<td>38</td>
</tr>
<tr>
<td>Elastase\textsuperscript{s}</td>
<td>LQATLPTVDYAIC</td>
<td>39</td>
</tr>
</tbody>
</table>

*Invariant residues among human proteins within the serine protease family.
†Bovine.
‡Murine rat.
§Porcine.

The enzyme BanI (isoschizomer of HgiCl) produced, as expected, three fragments (537, 212, and 129 bp long) from the amplified normal DNA. Only two fragments, 666 and 212 bp long, were noted in the amplified Friuli DNA. When the genomic DNA preparation from a second individual (described above) exhibiting the point mutation associated

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Computer-generated projections of the L\textsubscript{******}P\textsubscript{******}C motif in three different serine proteases. The invariant leucine, proline, and cysteine residues are indicated. Two additional amino acids are shown, to illustrate continuity in the homology of the backbones, on both ends of the three sequences. The bovine \alpha-chymotrypsinogen numbering system was used.}
\end{figure}
Fig 3. Ethidium bromide staining patterns following agarose electrophoresis (3% Nusieve GTG/1% Seakem GTG) of PCR-amplified DNA fragments containing exon VIII of the normal and Friuli factor X gene before and after cleavage with the Banl restriction enzyme. The lanes contained the following: 1-kb ladder size standard, 1 µg (A,F); exon VIII of the normal factor X gene, before (B) and after (C) digestion with Banl (isoschizomer of HgiCI); and exon VIII of the Friuli factor X gene, before (D) and after (E) cleavage with Banl. Both amplified fragments of normal and Friuli exon VIII migrated with a size of 878 bp. Fragments consistent with the calculated sizes of 537, 212, and 129 bp were generated from the normal DNA, whereas fragments with sizes of 666 and 212 bp were produced from the Friuli DNA after restriction enzyme digestion.

with the Friuli defect was analyzed by the same methods, the same two fragments of DNA were observed (Fig 3), providing further substantiation, owing to the well-defined patterns, that the patients from whom the materials were derived are homozygous for the defect.

DISCUSSION

We identified the existence of a point mutation in factor X

Friuli, as previously suspected on the basis of peptide mapping, active site analysis, and differential activities in reaction systems using purified components. On amplification and comparison of sequences of exon VIII from normal and two Friuli variant DNA isolates, a single nucleotide difference was observed that produces a coding change from that for proline to serine at amino acid position 343. This finding is consistent with the report from one of our laboratories that the factor X

Friuli gene has an apparently normal basic structure, as evidenced by the Southern blot hybridization technique for a variety of restriction fragment length polymorphisms.

When we aligned the amino acid sequences around proline in factor X with corresponding sequences from other serine proteases, we observed a highly conserved leucine, proline, cysteine motif (Table 1). The extent of sequence homology (ie, identity of amino acid residues in the motif) with factor X varies from 21% for chymotrypsinogen to 57% for factor IX. This motif is composed of 14 residues, defined by leucine, five variant amino acids, proline, six variant amino acids, and cysteine, or L+++++P++++++C, and is located within conserved region 5 of blood coagulation proteases and in the corresponding region in other serine proteases. This unique amino acid sequence is present in several subfamilies of serine proteases, including those involved in blood coagulation, protein digestion, contact activation, fibrinolysis, complement, and others. The total sequence of the motif has a remarkably similar backbone structure among the enzymes shown in Fig 2, and great variation exists in the residues between the leucine, proline, and cysteine, except for the predominant occurrence of hydrophobic residues in the one preceding position and two subsequent positions relative to the central proline of the motif (Table 1). Based on studies with bovine trypsin, the motif represents slightly less than the first half of conserved region 5, the second half of which is a disulfide loop. In chymotrypsin, this disulfide loop lies on the surface, whereas the portion of the motif near its amino terminus, from leucine through proline, lies "behind" the substrate binding pocket. An extra disulfide bond in trypsin also binds the amino terminus of the motif at residue 157,
another feature that indicates proximity of the motif to the activation cleavage site.\textsuperscript{29} Although this motif may be important to maintain the conformational integrity of many serine proteases, it is not a strict requirement of the enzyme superfamily. Urokinase,\textsuperscript{30} tissue plasminogen activator,\textsuperscript{31} cathepsin G,\textsuperscript{32} human neutrophil elastase,\textsuperscript{33} and rat elastase II\textsuperscript{34} lack the central proline; complement factors B and I,\textsuperscript{35,36} as well as rat pancreatic kallikrein,\textsuperscript{37} lack this motif altogether. On the other hand, variation of residues within the motif may confer properties allowing specificity of interaction of individual enzymes with their activators, cofactors, or substrates.

Recent findings in our laboratory indicated that a common recognition site(s) on factor X is recognized by the enzymes that normally activate factor X.\textsuperscript{49} Activation of factor X by both the extrinsic and intrinsic activation pathways requires membrane-associated nonproteolytic protein cofactors, and the ternary complex of membrane-cofactor-enzyme may be assumed to be in a fixed organization. Activation of factor X by RVV requires only calcium ions, and the activating enzyme is therefore considered relatively free to move within the aqueous environment. Analysis of the bovine chymotrypsinogen structure deduced from x-ray crystallography showed that the homologous invariant proline\textsuperscript{8} of the L****P*A-****C motif is located within 8 to 9 Å of the cleavage site of the zymogen, near residues leucine\textsuperscript{16}-isoleucine\textsuperscript{17,18} A change in the secondary structure caused by a serine substitution of proline might influence the spatial orientation of the potential cleavage site. With analogy to factor X\textsubscript{RVV}, the arginine\textsuperscript{194}-isoleucine\textsuperscript{195} site could be disturbed sufficiently by the substituted serine to inhibit cleavage of the variant protein by the extrinsic and intrinsic activation complexes significantly. Because the enzyme in RVV is not bound to a membrane surface, it would have less imposed binding constraints for recognition of the cleavage site on factor X\textsubscript{RVV}; thus, it could activate the zymogen at a near-normal rate.

Differences associated with the catalytic site of this variant enzyme relative to normal factor Xa include lack of cleavage of small chromogenic substrates, altered orientation of a bound dansylated inhibitor within the active site, and a reduction in the catalytic efficiency toward the substrate prothrombin.\textsuperscript{6} Substitution of proline\textsuperscript{36} by the polar residue serine could be responsible for these multiple effects, although the observed changes owing to the mutation are relative because some degree of activation and catalytic potential remains. It is unfortunate that without availability of the three-dimensional structure of factor Xa, it is difficult to assign specific changes within the conformation attributable to this mutation. Extensive perturbations in the conformation of lysozyme were observed, however, when a specific proline was substituted by several amino acids, including serine, and these changes could be propagated as far distal as 20 Å,\textsuperscript{51} suggesting that conformational disturbances associated with proline substitution can be quite dramatic. In this regard, two factor IX variants with point mutations in the corresponding general vicinity of the factor X\textsubscript{RVV} mutation confer quite different aberrations. A patient with severe hemophilia B was shown to possess factor IX, in which valine is substituted at position 309 in place of glycine.\textsuperscript{52} Activation of the variant factor IX is normal, but the active site is completely blocked, presumably by prevention of an ion pair formation. In a patient with hemophilia B Oxford h5, valine is replaced at position 328 by phenylalanine.\textsuperscript{53} In this variant, by analogy with the chymotrypsin structure,\textsuperscript{51} residue 328 is probably surface-oriented and susceptible to proteolytic attack by se- rine proteases such as thrombin. Furthermore, by analogy with properties of other thrombin substrates, including fibrinogen, the presence of phenylalanine at position 328 was postulated to enhance thrombin cleavage of the variant protein at the arginine\textsuperscript{308}-serine\textsuperscript{309} site.\textsuperscript{54} Reduced clotting and antigen levels (both 4% of normal) suggested that the variant factor IX protein is fully active but unstable.

Loss of an HgiCI restriction site in exon VII of the factor X\textsubscript{RVV} gene provides the basis for a relatively easy screening procedure, shown in Fig 3, for the variant gene within this population. The analysis requires only about 1 μg genomic DNA, which can be easily derived from the cellular DNA in 1 ml blood.\textsuperscript{12}

The goal of elucidating the relationships between structural and functional aspects of factor X by determining point mutations within the genes from individuals expressing a variant molecule has been limited by the small number of reported factor X abnormalities. Before the present study was performed, investigation of the normal and abnormal variations in the factor X gene had been limited to elucidation of restriction fragment length polymorphism,\textsuperscript{23-25,29-31} some preliminary studies showing mutations involving the signal peptide,\textsuperscript{27} and Gla domain,\textsuperscript{38} and the recent report of a point mutation in factor X\textsubscript{San Antonio} gene\textsuperscript{44} resulting in substitution of cysteine for arginine at amino acid position 366. Because of the structural homology observed for the vitamin K-dependent serine protease zymogens, however, naturally occurring mutations in the factor IX and prothrombin genes should complement our understanding of the structure-function relationships of factor X specifically and of serine proteases in general.

ACKNOWLEDGMENT

We thank Drs Sujoy Dasgupta and Tushar Chakraborty for many helpful suggestions and discussions during the course of this work. We also thank Drs Maitreeyee Dasgupta and Donald K. Blumenthal for computer analyses involving sequence and structural homologies of blood coagulation and other serine proteases.

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