Characterisation of blood coagulation factor XI T475I

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Summary
PCR-SSCP and DNA sequence analysis of a factor XI (FXI) deficient patient (FXI:C 39 U/dL; FXI:Ag 27 U/dL) identified a C to T transition in exon 12 of the FXI gene (F11 c.1521C>T) that predicts the substitution of Thr475 by Ile (FXI-T475I) within the serine protease domain of FXI. This mutation destroys a consensus sequence for N-linked glycosylation, N473-Y-T475, known to be utilized in vivo. The FXIT475I variant was generated by site-directed mutagenesis, together with other variants that could help explain the phenotype, and recombinant FXI variants were expressed in Chinese hamster ovary cells. FXI:Ag expression was analysed by Western blot analysis, ELISA and immunocytochemical staining. Wild-type FXI:Ag was secreted at high levels, however the mutant (FXI-T475I) was secreted very poorly. Substitution of Thr475 by Ala, Pro, Lys or Arg (all of which abolish the glycosylation consensus sequence) also severely reduced the level of secreted FXI:Ag suggesting that glycosylation at Asn473 is required for folding or secretion. Concordant with this hypothesis the conservative substitution of Thr475 by Ser (which preserves the glycosylation consensus sequence) had no effect on FXI secretion. Thr/Ser475 is highly conserved in serine protease domains but the glycosylation site (Asn473) is not. Surprisingly, substitution of Asn473 by Ala (which removes the N-linked glycosylation site) had no effect on the levels of FXI:Ag secreted. In conclusion, although the FXI-T475I mutation destroys an N-linked glycosylation consensus sequence, the cause of failure to secrete FXI is not the loss of a glycosylation site but rather a direct effect of the substitution of this highly conserved residue.

Keywords
Factor XI, deficiency, mutation, glycosylation

Introduction
Factor (F) XI is a zymogen for a serine protease that participates in blood coagulation by activating FIX through limited proteolysis (1). FXI circulates as a homo-dimer (M, 160kDa) of two identical subunits linked by a disulphide bridge in a non-covalent complex with high molecular weight kinogen (2). Thrombin (3) or FXIIa (4, 5) activate FXI by proteolytic cleavage at Arg369-Ile370 in each monomer, yielding FXIa with two active site serines per dimer. The human FXI gene (F11) has been isolated and completely characterised: it consists of 15 exons spanning 23kb of the long arm of chromosome 4 (4q35) (6, 7).

Hereditary FXI deficiency is a rare autosomal bleeding disorder (OMIM 264900, http://www.ncbi.nlm.nih.gov/omim/) that is not completely recessive since heterozygotes have a mild but definite bleeding tendency. Although the great majority of cases initially reported in the literature were of Ashkenazi Jewish origin, within which population the gene frequency for FXI mutations is as high as 8% (8), there are now an increasing number of mutations described in non-Jewish patients. In unrelated FXI-deficient patients of Ashkenazi Jewish origin, four different mutations have been identified: type I mutation occurs in the 5' splice donor site of intron N; type II mutation in exon 5 is a nonsense mutation (FXI E117X); type III mutation is a missense mutation (FXI F283L) in exon 9; type IV mutation is a 14 base pair deletion at the junction of exon 14 and intron N (9, 10). However, these mutations account for only a small percentage of disease alleles in non-Jewish patients (11). A wide spectrum of mutations has now been described in FXI deficiency, including: insertions, deletions, splice site variants, nonsense and missense mutations. The vast majority of reported mutations are missense mutations and these are found throughout the gene (12).

The majority of identified mutations in the F11 gene associated with deficiency either prevent or greatly reduce protein ex-

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pression. For example, *in vitro* expression studies of the type III mutation, which occurs in the fourth apple domain of the protein, have shown the mutant protein is secreted at reduced levels compared to the wild-type protein due to a failure in the dimerization of the molecule (13). Similarly, expression studies of FXI-D16H, L302P, T304I and E323K showed a marked reduction in the amount of FXI secreted from cells *in vitro* (14).

In the present study, we have identified a novel mutation in a non-Jewish FXI deficient patient, by PCR-SSCP analysis and DNA sequencing. We have characterised the molecular defect by expression studies in Chinese hamster ovary (CHO) cells and show that the mutation greatly reduces secretion of the mutant protein.

**Methods**

**Subjects**

An individual with FXI deficiency associated with a mild bleeding tendency was studied. Blood was obtained by venepuncture following informed consent.

**DNA analysis**

Genomic DNA was prepared from peripheral blood leukocytes by established methods (15). Exons 1–15 and the adjacent intronic sequences of the FXI gene were amplified by the polymerase chain reaction (PCR) using the oligonucleotides described previously (16). Single-strand conformational polymorphism (SSCP) analysis was performed according to the method of Hayashi et al. (17) as previously described. PCR fragments were sequenced with an ABI Prism 3700 DNA analyser (Applied Biosystems, Warrington, UK).

**Construction of wild type and variant FXI expression constructs**

cDNAs encoding the FXI variants were generated by site-directed *in vitro* mutagenesis of a 999bp *Hind* III fragment (nucleotides 1088–2087, numbered from the FXI cDNA sequence) (18) by 3 cycles of freeze-thaw lysis. The lysates were centrifuged at 15800g for 15 min at 4°C prior to analysis. Protein levels were measured using a coomassie blue staining assay (BioRad).

**Cell culture, transfection and expression of FXI variants**

CHO K1 cells (ECACC #85050302, Porton Down, UK) were grown in Eagles minimum essential medium (MEM) with 10% heat inactivated foetal bovine serum, in a 5% CO2 atmosphere at 37°C. All culture media were supplemented with 2 mM-glutamine and 100 U/ml of both penicillin G and streptomycin. 10⁷ cells were transfected with approximately 10 µg of linearised plasmid DNA using a Gene Pulser apparatus (BioRad) and stably transfected cells isolated by G418 selection (Geneticin 500 µg/ml; Invitrogen, Paisley, UK).

**Measurement of FXI activity (FXI:C) and FXI antigen (FXI:Ag)**

FXI:C was determined in a one-stage clotting assay using FXI deficient plasma (Diagnostic Reagents, Thame, UK) in a Coag-a-mate X2 coagulometer (Organon Teknika, Cambridge, UK). FXI:Ag was measured by sandwich ELISA using a monoclonal antibody MAb XI-2 (20) as the capture antibody and a polyclonal goat anti-human FXI antibody as the second antibody (GAHXI-PG1, Enzyme Research Laboratories, Swansea, UK) which was detected using a monoclonal anti-goat IgG antibody conjugated to horseradish peroxidase (BioRad). Critated normal pooled plasma (15 normal donors) was used as a standard in both assays, where one unit of coagulation activity and antigen was defined to be equivalent to the amount contained in 1ml of the pooled control plasma and is equivalent to a plasma concentration of approximately 5 µg/ml. Three different dilutions of each sample were assayed in duplicate. FXI:Ag values were calculated using the parallel line bioassay program RANDOM (21). The limit of detection for the antigen assay was 1 mU/ml.

To measure the secreted levels of FXI:Ag in conditioned media CHO cells stably transfected with the FXI variant cDNAs were grown in protein-free medium (HybriMax; Sigma, Poole, UK): after 24–72 h the conditioned media were concentrated by Centricron 30 devices (Amicon, Stonehouse, UK). To measure the levels of intracellular FXI:Ag cell extracts were prepared in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% (w/v) NP-40, 1 mM phenylmethylsulfonyl fluoride) by 3 cycles of freeze-thaw lysis. The lysates were centrifuged at 15800g for 15 min at 4°C prior to analysis. Protein levels were measured using a coomassie blue staining assay (BioRad).

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**Western blot analysis**

Samples of conditioned media with equal protein levels were electrophoresed on 10% precast NuPage gels (Invitrogen, Paisley, UK) with MOPS buffer (50 mM 3-(N-morpholino) propane sulfonic acid, 50 mM Tris-HCl (pH 7.7), 3.5 mM sodium lauryl sulfate, 1 mM EDTA) at constant voltage of 200V according to the manufacturer's instructions. The proteins were transferred from the gel to a nitrocellulose membrane by electrophoretic transfer and blocked with 5% powdered milk. FXI:Ag on the nitrocellulose membrane was detected using a goat anti-human FXI polyclonal antibody (GAHXI-PG1, Enzyme Research Laboratories). The bound antibody was then detected using a horse-

<table>
<thead>
<tr>
<th>Table 1: Oligonucleotides used for <em>in vitro</em> mutagenesis</th>
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<tbody>
<tr>
<td><strong>Amino acid sequence</strong></td>
</tr>
<tr>
<td>N-XI</td>
</tr>
<tr>
<td>N-YS</td>
</tr>
<tr>
<td>N-YA</td>
</tr>
<tr>
<td>A-Y-T</td>
</tr>
<tr>
<td>A-XI</td>
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</tbody>
</table>

The mutated bases are shown in lower case, the codon is shown in bold.
radish peroxidase conjugated anti-goat IgG monoclonal antibody (BioRad) and chemiluminescence detection reagents (ECL; Amersham Biosciences, Little Chalfont, UK) according to the manufacturer’s instructions. The nitrocellulose membrane was then exposed to Kodak XAR-5 film.

**Northern blot analysis**

Polyadenylated total RNA was isolated from transfected cells using a commercially available kit (Amersham Bioscience). 1 µg of polyadenylated RNA was loaded per lane, electrophoresed in a denaturing 1.1% agarose/formaldehyde gel, blotted and probed with the human FXI cDNA and murine α-actin cDNA as previously described (14). The blot was either exposed to Kodak XAR-5 film at –70°C with an intensifying screen, or for quantification it was exposed to a phosphor screen, detected using a phosphorimager (Phosphorimager 445 SI; Molecular Dynamics, Kent, UK) and analysed using Image QuaNT software (Molecular Dynamics).

**Immunocytochemical detection of FXI**

For indirect immunofluorescence staining, cells were grown on 8 well culture chamber slides (Nunc, Paisley, UK) and fixed with 4% formalin in phosphate buffered saline (PBS) for 10 minutes. After three PBS washes, the slides were incubated with 5% normal rabbit serum in 1% bovine serum albumin, 1 mM EGTA, 0.1% saponin in PBS for 20 minutes. The primary antibody (MAb XI-2), diluted 1:250 in the same buffer was pipetted onto the culture slides and incubated for 2 hours. After three washes with PBS, the slides were further incubated in similar conditions with a biotinylated rabbit polyclonal anti-mouse IgG antibody (Dako, High Wycombe, UK) diluted 1:300. After a further three washes with PBS, the slides were incubated with streptavidin Texas Red (Amersham Bioscience). The slides were rinsed again three times with PBS and mounted in Vectashield (Vector Laboratories, Peterborough, UK). All incubations were carried out at room temperature. The immunofluorescence was visualised using a Zeiss Axioshot (Zeiss, Welwyn Garden City, UK) microscope with Metamorph imaging software (Universal Imaging Corp, Pennsylvania, USA).

**Molecular modelling**

Insight II (Accelrys, Cambridge, UK) was used with the crystal coordinates of the catalytic domain of FXIa in complex with EcotinM84R (1XX9) available in the Protein Data Bank (http://www.rcsb.org/pdb/).

**Results**

**Case history and coagulation studies**

The index case was identified as a result of investigations for asymptomatic microscopic haematuria identified at a medical examination at age 14. Despite extensive investigation the cause of the haematuria was not identified. Prior to diagnosis he had bled for 2 days following tooth extraction and he may also have bled excessively after an earlier tonsillectomy in childhood. The FXI:C and FXI:Ag levels of the index case were 39 U/dl and 27 U/dl respectively. The patient has a brother with no bleeding history whose FXI:C level is 84 U/dl. His father, who died from cancer 11 years prior to diagnosis of FXI deficiency in the index case, was reported to have bled for 3 days after dental extractions. His mother who has no bleeding history (she did not bleed during child birth or after dental extractions, and does not suffer heavy periods) has a FXI:C level of 96 U/dl.

**Identification of the mutation in the FXI gene**

Exons 1 to 15 and adjacent intron-exon junctions of the *F11* genes of the propositus with FXI deficiency were amplified by PCR. The PCR products showed one distinct band of appropriate size on agarose gel electrophoresis, and no differences were observed between the subject and the normal controls. SSCP analysis identified only a single PCR product with aberrant mobility relative to the normal control, an extra band with slower mobility in the analysis of exon 12. DNA sequence analysis identified
a C to T substitution at position 1521 (c.1521C>T; numbered from the ATG of the FXI cDNA sequence, accession number M13142); the ACA codon for Thr475 is therefore replaced with the codon ATA for Ile (numbered +1 from the Glu that forms the mature N-terminus of the protein, according to Fujikawa [18]). Both SSCP analysis and DNA sequence analysis indicated this individual is heterozygous for the (FXI T475I) mutation.

### In vitro expression of wild type FXI and FXI T475I in CHO cells

In order to investigate the functional or structural significance of this mutation we used site-directed mutagenesis to generate a FXI cDNA encoding the FXI T475I variant. The wild-type and variant FXI cDNA sequences were cloned into a high level expression vector and stably transfected CHO cell lines were established. This expression system had previously been shown to express recombinant FXI that was indistinguishable from the plasma derived-protein in molecular mass, activation site or N-terminal sequence (19). Conditioned protein-free growth media collected from cells stably transfected with the pNeoG502 expression vector alone, the wild-type FXI cDNA expression vector and the FXI T475I cDNA expression vector were concentrated and analyzed by western blot using a polyclonal anti-human FXI antibody (Fig. 1A). A major band of about 160 Kd was observed under non-reducing conditions and 80 Kd under reducing conditions in conditioned medium from cells transfected with wild-type FXI cDNA, indicating that the secreted FXI was a disulphide-linked dimer. In contrast, no immuno-detectable material was observed in the conditioned medium from cells transfected with the FXI-T475I cDNA.

To determine whether the cells transfected with the FXI expression constructs express comparable levels of FXI mRNA Northern blot analysis was performed. The blots were probed for both FXI and α-actin mRNAs (Fig. 1B). FXI mRNA was detected in cells transfected with both the wild type and variant FXI T475 expression constructs. Quantification of the amount of FXI mRNA relative to the α-actin control by phosphorimager analysis revealed equivalent amounts of FXI mRNA in cell lines stably transfected with these FXI expression constructs.

The T475I mutation occurs in a consensus sequence for N-linked glycosylation (Asn-X-Thr or Ser), where the Asn residue has been shown to be glycosylated in plasma-derived human FXI (22). Since the substitution of Thr475 by Ile abolishes this consensus sequence we tested whether maintenance of this glycosylation was required for secretion by generating additional variants in both the Asn (AYT, AYI) and the Thr amino acid residues (NYS, NYA). CHO cells stably transfected with these constructs were isolated and studied by Western blot analysis (Fig. 2). The FXI-NYS and FXI-AYT expression constructs directed expression and secretion of immuno-reactive FXI that was indistinguishable from the wild-type. In contrast, no immuno-reactive material was observed in the conditioned media from cells transfected with either the FXI-AYI or the FXI-NYA expression constructs. Additional substitutions of Thr475 by Pro, Lys or Arg, all of which abolish the glycosylation consensus sequence also severely reduced the level of immuno-reactive FXI detected in conditioned media transfected with appropriate expression constructs (data not shown).

**Figure 2:** Western blot analysis of wild-type FXI and variants at N473 and T475 secreted from transfected CHO cells. CHO cells stably transfected with the wild type and variant FXI cDNAs in the expression vector were grown in protein-free medium, and after 72 hours conditioned media were removed and concentrated using Centricon 30 devices. 5 µg of protein were analysed by SDS-PAGE under non-reducing conditions. The proteins were electroblotted and the membrane probed with a goat anti-human FXI polyclonal antibody. Vector only, CHO cells transfected with the expression vector containing no FXI sequences. The amino acid sequence of each variant at N473-Y-T475 is indicated.

**Figure 3:** Immunocytochemical localization of wild-type and variant FXI in CHO cells. CHO cells stably transfected with the wild-type and variant FXI cDNAs were fixed with 4% formalin and FXI Ag was detected using an anti-human FXI monoclonal antibody. The amino acid sequence of each variant at N473-Y-T475 is indicated.
To determine whether FXI protein accumulated abnormally in cells that failed to secrete detectable FXI:Ag we performed immunofluorescence staining of the stably transfected CHO cells using a polyclonal anti-FXI antibody (Fig. 3). All cells transfected with a FXI expression construct stained positive whereas cells transfected with the expression vector alone were negative (data not shown). Both cells secreting and cells not secreting FXI protein into the culture media had a similar punctate cytoplasmic staining pattern; there was no evidence in non-secreting variants of intracellular accumulation of variant FXI in a particular subcellular compartment.

In order to quantify the relative amounts of intracellular and secreted FXI:Ag ELISAs were performed on cell lysates and conditioned media from cells stably transfected with the expression constructs (Table 2). Low but detectable levels of FXI:Ag were measured in the cell lysates of all cells transfected with FXI expression constructs. The amount of FXI:Ag detected in conditioned media from cells transfected with the FXI-NY1, NYA and AYI expression constructs was extremely low in comparison with that detected in the media from cells transfected with the wild-type (FXI-NYT), FXI-NYS or FXI-AYT expression constructs, consistent with the Western blot analysis. Since the polyclonal cell lines all express variable amounts of FXI it is difficult to directly compare expression levels. However, the ratio of secreted to intracellular FXI:Ag enables a comparison of the effect of each mutation on the relative secretion of FXI variants. Mutations that replace the Thr475 with Ile (NYI and AYI) or Ala (NYA) lead to a ratio of secreted to intracellular antigen of 4–20 fold less than that observed for mutations that either replace Thr475 with Ser (NYS) or maintain Thr475 but abolish the glycosylation consensus sequence by mutation of Asn473 to Ala (AYT).

Figure 4: Sequence alignments of 22 human serine proteases. The sequences were aligned using ClustalW, following a phi-blast search (http://www.ncbi.nlm.nih.gov/BLAST) with the human FXI amino acid sequence (D462 to W501) to identify human serine proteases with sequence identity. D462 is the Asp residue in the active site catalytic triad Ser-His-Asp found in all mammalian serine proteases. The figure was generated using Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). White on black indicates identity with the consensus; black on grey indicates similarity with the consensus.

Table 2: FXI:Ag measured by ELISA in cell lysates and conditioned media of CHO cells transfected with wild-type and FXI variants.

<table>
<thead>
<tr>
<th>Amino acid sequence N473-Y475</th>
<th>Predicted Glycosylation disruption</th>
<th>Western blot analysis FXI:Ag in conditioned media</th>
<th>FXI:Ag in conditioned media†</th>
<th>FXI:Ag in cell lysates¶</th>
<th>Ratio of secreted to intracellular FXI:Ag %</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Y-T (WT)</td>
<td>No</td>
<td>15.4 (14.1–17.0)</td>
<td>7.6 (7.1–8.2)</td>
<td>100</td>
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<tr>
<td>N-YI</td>
<td>Yes</td>
<td>0.31 (0.29–0.31)</td>
<td>2.9 (2.7–3)</td>
<td>5</td>
<td></td>
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<tr>
<td>N-YA</td>
<td>Yes</td>
<td>30.4 (48–54.1)</td>
<td>17.2 (16.4–18.4)</td>
<td>145</td>
<td></td>
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<tr>
<td>N-Y-S</td>
<td>No</td>
<td>5.3 (5.1–5.7)</td>
<td>10.8 (10.4–11.6)</td>
<td>25</td>
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<tr>
<td>A-Y-I</td>
<td>Yes</td>
<td>1.4 (1.2–1.5)</td>
<td>7.7 (7.1–8.5)</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>A-Y-T</td>
<td>Yes</td>
<td>169.6 (158.1–187)</td>
<td>35.4 (31.8–40.7)</td>
<td>150</td>
<td></td>
</tr>
</tbody>
</table>

FXI:Ag values are the geometric means with 95% confidence limits given in parentheses. †, mU FXI:Ag/mg lysate protein (normalised to 24 h expression; ¶mU FXI:Ag/mg lysate protein; #, where WT = 100%.

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Discussion

We investigated the molecular defect responsible for FXI deficiency in a non-Jewish individual with a mild bleeding diathesis. PCR-SSCP and DNA sequence analysis revealed the patient was heterozygous for a novel C to T transition, which results in Thr475 in the protease domain being replaced by Ile. To confirm that this substitution was responsible for the FXI deficiency we stably transfected CHO cells with an expression vector containing FXI cDNA encoding either the wild-type or T475I mutation and showed that the secretion of the mutant protein was severely reduced (Table 2, Fig. 1). The expression and mutation data were consistent with the FXI:C value measured in the patient’s plasma if the variant allele is not expressed and/or secreted. However, the FXI:Ag value measured in the patients plasma (27U/dl) are lower than expected and are suggestive of a dominant negative effect of the mutation, where only homodimers of wild-type FXI are secreted (23).

The replacement of Thr475 by Ile is relatively conservative, substituting a moderately small polar side chain by a slightly larger hydrophobic residue. The mutation also disrupts an N-linked glycosylation consensus sequence in FXI (22). N-linked glycosylation of proteins is an essential process in eukaryotic cells (24). Addition of N-linked oligosaccharides to many glycoproteins is an obligatory event for the folding and assembly of many (but not all) newly synthesized glycoproteins. Furthermore, the presence of oligosaccharides is often required for the efficient transport of individual glycoproteins through the secretory pathway (25). In addition, N-linked glycosylation affects the plasma half-life (26–28) and biological activity of glycoproteins. Human FXI contains 5% carbohydrate and there are 5 potential N-linked glycosylation sites, 2 of which occur in the protease domain (Asn432 and Asn473) and are conserved between FXI and the homologous protein plasma prekallikrein (18). Indirect evidence from protein sequence analysis suggests that Asn473 in human FXI is glycosylated. To further investigate the functional significance of the mutation at position 475 of FXI and to support the hypothesis that the reduced levels of protein secretion were a result of the loss of the carbohydrate side chain at Asn473, we generated a series of additional mutants which either maintained or destroyed the consensus glycosylation sequence and performed expression studies in stably transfected CHO cells. The mutations replaced the small polar residue (Thr) with a very small polar residue (Ser) or a very small hydrophobic residue (Ala). A substitution that maintained the consensus (NYT to NYS) had no significant effect on FXI:Ag secretion; consistent with the primary defect being the loss of a glycosylation site. Substitutions which destroyed the consensus sequence severely reduced the amount of FXI:Ag secreted (Table 2). To confirm that the causative defect was loss of glycosylation we mutated Asn473 to Ala, removing the glycosylation target. However, unexpectedly the Asn to Ala substitution had no significant effect on FXI:Ag secretion (Fig. 2, Table 2). This data suggested the primary defect was the substitution of Thr475, although we could not exclude the possibility that substitution of a glycosylated-Asn residue by a non-glycosylated Asn residue (as a result of mutation at Thr475) disrupted protein folding, and that the N473A mutation corrected or ‘rescued’ this defect. To differentiate between these two possibilities we studied a double variant with mutations at both Asn473 and Thr475 (AYT; Fig. 2, Table 2). Expression data strongly suggests that the molecular defect in FXI T474I is the substitution of Thr by Ile rather than loss of N-linked glycosylation at Asn473.

To study the intracellular processing of the mutant proteins we performed immunocytochemical staining of the stably transfected CHO cells expressing FXI N473 and T475 variants (Fig. 3). Our results showed that the proteins did not accumulate abnormally within the cell in spite of a major secretion defect.

The mutation T475I occurs in the protease domain, which is homologous to members of the trypsin family of serine proteases. The alignment of known serine protease structures by Greer (29) indicated that FXI Thr475 occurs in a structurally conserved region, however this region displays limited sequence identity within the family of serine proteases. An alignment of 22 human serine protease sequences including FXI (Fig. 4) shows a Ser or Thr at the equivalent position in 18 of the sequences aligned, suggesting this is a structurally conserved residue. The residue only forms part of a N-linked glycosylation consensus in 4 out of 22, implying the presence of a glycosylation signal is secondary to its structural role. Examination of the crystal structure of catalytic domain of FXI in complex with EctoinM84R (1XX9; Protein Data Bank, http://www.rcsb.org/pdb/) shows this structurally conserved region forms a loop that lies on the surface of the molecule. It is predicted that the replacement of Thr475 by Ile will be accommodated without any steric clash.

In conclusion, we have identified a novel heterozygous mutation, T475I, in the gene encoding blood coagulation FXI in a non-Jewish FXI deficient patient. We have characterised the molecular defect by expression studies in CHO cells. The mutation greatly reduces the secretion of the mutant protein. Furthermore, we show that although the mutation destroys a N-linked glycosylation site the primary molecular defect lies in the substitution of Thr475 and not the loss of a carbohydrate side-chain at Asn473.

References