To explore the biological and clinical implications of the structure/function relationships in factor XIII, mutations in two patients with type II deficiency were identified and characterized in a mammalian expression system. Nucleotide sequence analysis of the A subunit gene showed that case no. 1 had a deletion of 4 bp (AATT) in exon XI and that, in case no. 2, Gly562 (GGG) had been replaced by Arg(AGG). The deletion in case no. 1 leads to a premature termination at codon 464. Restriction digestion of amplified DNAs confirmed that both cases were homozygous for their respective mutations. Reverse transcription-polymerase chain reaction analysis demonstrated that the level of mRNA was greatly reduced in case no. 1, whereas the level of mutant mRNA in the gene for the B subunit in the former cases, and a single base deletion caused the splicing defect in its mRNA in the latter case. An additional substitution of T for G in the latter case replaced Cys430 with Phe in the seventh Sushi domain and resulted in impaired intracellular transport of the B subunit. Accordingly, it was shown that type I deficiency can be caused by defects in the gene for the B subunit.

Mutations in the gene for the A subunit have also been identified in patients with type II factor XIII deficiency (ie, the A subunit deficiency). These mutations are highly heterogeneous and include a variety of nonsense mutations, small deletions, and insertions with or without out-of-frame shift/premature termination, splicing abnormalities, etc. Various missense mutations in the A subunit will also result in type II deficiency. The mechanisms of defective A subunit biosynthesis caused by the missense mutations have not been clarified, mainly because in vitro expression systems were unavailable using mammalian cells. Recently, we succeeded in synthesizing the A and B subunits in baby hamster kidney (BHK) cells and an abnormal B subunit of type I deficiency in COS cells. However, no abnormal A subunit of type II deficiency has been characterized in a mammalian expression system to date.

It is important to closely examine amino acid substitutions and deletion of peptide regions to understand the structure/function relationships of the factor XIII molecule as well as its clinical implications in factor XIII deficiency. In this study, we identified a 4-bp deletion and a Gly562-Arg mutation in the gene for the A subunit of factor XIII in two patients (cases no. 1 and 2) with A subunit deficiency and predicted altered structures of the mutant A subunits by molecular modeling. In addition, we present for the first time the successful expression of novel mutant A subunits in BHK cells and provide evidence indicating that the mutants are degraded rapidly in the synthesizing cells.

**MATERIALS AND METHODS**

Venous blood was drawn from normal individuals and from two patients with A subunit deficiency and their family members after informed consent had been obtained. Both cases had essentially no enzymatic activity for factor XIII and the A subunit antigen (<5% of normal by Laurell’s method). The A subunit was also absent in the

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patients' platelets. Genomic DNA and RNA samples were prepared from peripheral blood cells by standard techniques, as described previously.

**Southern blotting.** Ten micrograms of genomic DNA was digested with 20 U of EcoRI restriction enzyme at 37°C for 2 hours and applied to a 0.4% Agarose gel. The DNA fragments were transferred to a nylon membrane. The membrane was hybridized overnight at 65°C with a 32P-labeled cDNA encoding the A subunit and exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) at −80°C.

**Polymerase chain reaction (PCR).** One microgram of genomic DNA was amplified using 5.0 U of *Thermus aquaticus* DNA polymerase (Promega, Madison, WI) in a 100 µL reaction mixture. A total of 17 pairs of gene-specific primers were designed from the nucleotide sequence of the normal A subunit (Fig 1, top). After 30 to 35 cycles of amplification, 9 µL of each reaction mixture was applied to a 1.5% Agarose gel.

**Nucleotide sequence analysis.** Amplified DNA samples were digested with restriction enzymes to generate proper ends for ligation into sequencing vectors. The DNA sequence of an insert was obtained using the dideoxynucleotide method with ABI sequence analyzer 373A (Perkin-Elmer, Norwalk, CT). To minimize the possibility of obtaining the DNA sequences with misincorporation by the DNA polymerase, 10 or more samples of each amplified region of the A subunit were examined.

**Detection of mutations by restriction digestion.** For restriction enzyme analysis to detect the presence of the 4-bp deletion in exon XI and the replacement of G by A in exon XII (Fig 1, top), 15 µL of the amplified DNA samples was incubated with Tru9I and HpaII endonucleases (Boehringer Mannheim GmbH, Mannheim, Germany), respectively. After digestion for 2 hours, half of each sample was applied to a 2% Agarose gel.

**Reverse transcription-PCR (RT-PCR) assay of mRNA for the A subunit.** RT of the total RNA (1.0 µg) was performed using an oligo dT (dT18) primer and Superscript II RNase H+ (GIBCO-BRL, Grand Island, NY), as described previously. One fiftieth of the synthesized first-strand cDNA was used for PCR in a reaction mixture of 50 µL by employing two pairs of primers for exons II-IV of the A subunit (Fig 1, bottom), 5′-ATGTCAGAAACTTCCAGGACC-3′ (sense) and 5′-CGTGTCTGTTTCTGGGTGTTGTTTCC-3′ (antisense); for human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control, 5′-CATCACCAGTTTCCAGGACC-3′ (sense) and 5′-TAAGCAGTTGGTGGTGCAGG-3′ (antisense). After 25 cycles, the reaction mixtures were electrophoresed in a 2% Agarose gel. The gel was subjected to quantitation of the amplified products by Densitograph 3.01 Imaging Analyzer (Atto Inc, Tokyo, Japan). The fluorescence intensity of the PCR product for the A subunit was normalized to that for GAPDH.

One fiftieth of the synthesized first-strand cDNA was also used for

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![Fig 1.](https://example.com/fig1.png)

**Fig 1.** (Top) Gene for the A subunit and mutations identified. Exons are indicated by wide vertical bars and Roman numerals, and introns are indicated by capital letters. Each exon and its boundaries and the 5′-flanking regions were amplified one by one using 17 pairs of primers (arrows under exons). Solid and open circles with sequences represent homozygous causative mutations and changes known as common polymorphisms, respectively. Normal sequences are followed by those corresponding to substitutions found in the probands' DNAs. For analysis of the two mutations identified, two amplified fragments were digested with appropriate endonucleases as indicated by their names and arrows. (Bottom) mRNAs for the A subunit in the probands. Three regions of exons II-IV, X-XIII, and XI-XIII were amplified by RT-PCR. A solid line represents the expressed mRNA, whereas a broken line indicates a possible mRNA that was not detected.
PCR in a reaction mixture of 50 µL to amplify two regions (Fig 1, bottom) by employing two pairs of primers: for exons X-XIII, 5′-CATCAAGCCAGGCAATGTCTGC-3′ (sense) and 5′-CTTGAATGATGATCCTAGG-3′ (antisense); for exons XI-XIII, 5′-TCCGGAAACACGCCACACCCG-3′ (sense) and 5′-CTTGAATGATGATCCTAGG-3′ (antisense). To detect extremely small amounts of cDNAs, prolonged amplification was performed using 35 to 40 cycles. Amplified fragments were subjected to digestion by either Tru9I or Hpa II to detect expressed mutations.

Molecular modeling. To predict the tertiary structure of the mutant molecule, molecular modeling was performed by using the coordinates of the A subunit defined by X-ray crystallography.30 To discuss the molecule, molecular modeling was performed by using the coordinates detected expressed mutations.

bovine serum (FBS) and antibiotics (penicillin, streptomycin, and (Invitrogen, San Diego, CA).

pBluescriptII vector and inserted into expression vector pcDNA3 wild-type and the mutants were released by

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and 5

-GGAAACAGCTATGACCA TG-3

[35 S]Met in Met-free DMEM with 10% dialyzed FBS for 30 minutes for 1 hour at 37°C. The cells were then labeled with 50 µCi/well of

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using oligonucleotide primers: for 4-bp deletion, 5

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(antisense); for exons XI-XIII, 5′-GCCACCCACACATTGGGAAAATTGTAGCAACAAAACATTG-3′ (sense), and 5′-CTTGAATGATGATCCTAGG-3′ (antisense); for Gly562-Arg mutation, 5

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-(CA TCAAGCACGGCCA TGTCTGC)-3

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and 5

8

polyacrylamide gel. The gel was dried and the radioactive band for the

RESULTS

Genomic Southern blotting analysis and in vitro amplification of the A subunit gene. To test for the presence of large deletion(s), insertion(s), and recombination(s), genomic Southern blotting analysis was performed first. EcoRI-digested genomic DNAs of two Italian cases and normal individuals showed an identical pattern (data not shown). To search for possible smaller mutation(s) in and around the exons by PCR, 17 pairs of amplification primers were used, and these produced 17 different DNA fragments, respectively (data not shown). The sizes of these fragments were the same as predicted from the normal genomic sequence.7 These results confirmed that there was no gross change(s) in the probands’ genes.

Identification of the 4-bp deletion and Gly562-Arg mutation. Nucleotide sequences of the 17 amplified fragments were then examined to search for differences from the normal sequence.7 When exon XI of case no. 1 was sequenced, all of 15 ssDNA samples showed AAATTGTGTA (Fig 2, top), whereas the normal sequence is AAATTAATGTGTA. One obvious result of this 4-bp deletion is the creation of a new termination codon at amino acid position 464 (Fig 2, top).

Three additional changes in the nucleotide sequence were found in case no. 1 (Fig 1, top), but all of these were known genetic polymorphisms in the coding regions: Val34-Leu(GTG-TTG) in exon II, Pro331-Pro(CCA-CCC) in exon VIII, and Pro564-Leu(CCG-CTG) in exon XII.18 No other mutations were detected in and around the 15 exons or in a section of the 5′-flanking region. This region of about 300 bp, 1.3 kb upstream from the 5′-end of exon I, contains several TATA and CAAT boxes (A. Ichinose, unpublished data). Taken together, these results suggest that the 4-bp deletion is very likely causative for the A subunit deficiency in case no. 1.

When exon XII of case no. 2 was examined, all of 12 ssDNA samples demonstrated AGG for Arg in place of the normal sequence of GGG coding for Gly562 (Fig 2, bottom). Another nucleotide substitution, Val34-Leu (GTG-TTG), was identified in exon II; this is the same known polymorphism as found in case no. 1. No other mutation was detected in and around all exons or in a section of the 5′-flanking region. Accordingly, it was concluded that the Gly562-Arg mutation was responsible for the A subunit deficiency in case no. 2.

Detection of mutations by restriction enzyme digestion. Because the deletion of 4-bp (AATT) in the DNA of case no. 1 destroys an intrinsic Tru9I site (TTAA), a fragment spanning exon XI was amplified and digested with Tru9I endonuclease. Digestion of the 291-bp fragment from a normal individual yielded two bands of 165 bp and 126 bp (Fig 3, left), whereas the 287-bp fragment of the proband remained unchanged. The proband’s mother showed both cleaved and uncleaved bands. These results indicated that case no. 1 was a homozygote and that the mother was a heterozygote of this mutant allele. This same mutant allele was not found in 54 normal Italian individuals (data not shown).
Because the G-A substitution in case no. 2 destroyed an intrinsic Hpa II site (CCGG), a fragment spanning exon XII was amplified and digested with Hpa II endonuclease. Digestion of the 389-bp fragments from a normal individual and the proband’s brother (Fig 3, right) yielded three fragments of 208, 116, and 65 bp. In contrast, the digest of the 389-bp fragment from the proband yielded two fragments of 208 and 181 bp. Thus, the brother was normal, and case no. 1 was homozygous for the Gly562-Arg mutation. This mutant allele also was not found in 53 normal Italian individuals (data not shown).

Semiquantitation of mRNA for the A subunit. Because neither factor XIII activity in plasma nor the A subunit antigen in platelets was detected in either patient, the mRNA for the A subunit was examined. An RT-PCR assay was performed to estimate the amount of mRNA for the A subunit in peripheral blood cells, where the A subunit may be aberrantly expressed, although the site of biosynthesis for the plasma A subunit is still unknown. A 543-bp amplified fragment for the A subunit and a 253-bp fragment for GAPDH were obtained by RT-PCR from a normal individual (Fig 4). Under experimental conditions, a linear relationship was observed between the amounts of total RNA used and the fluorescence intensity of these bands (data not shown). A very faint band for the A subunit was detected in case no. 1, whereas a band was clearly observed in case no. 2 (Fig 4). The ratio of the A subunit mRNA/GAPDH mRNA was 1.0, 0.04, and 1.0 for a normal subject, case no. 1, and case no. 2, respectively. Thus, the amount of the mRNA in case no. 1 was markedly reduced, strongly suggesting that the mutant mRNA is not synthesized as stably as normal mRNA. In contrast, the amount of the mRNA of case no. 2 was comparable to that of the normal individual.

Detection of the mutant transcript for the A subunit. A band of normal size was obtained in the amplified sample from case

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**Figure 2.** (Top) Nucleotide sequence of a part of exon XI for normal and case no. 1. The 4-bp deletion is boxed. Codon 464 becomes a termination signal because of a reading frame-shift. (Bottom) Nucleotide sequence of a part of exon XII for normal and case no. 2. Mutated nucleotide A is indicated in a box, and a normal Gly562 residue is substituted with an abnormal Arg sequence.
no. 2 by using primers for exons XI-XIII. Digestion with Hpa II of the 290-bp fragment from case no. 2 yielded three bands of 155, 133, and 2 bp (the last fragment was unresolved), whereas a normal fragment produced four bands of 133, 90, 65, and 2 bp (Fig 5, right), indicating that the mRNA from case no. 2 contained the Gly562-Arg mutation. These results confirmed that the mRNA with the Gly562-Arg mutation was the sole transcript expressed in case no. 2.

No discrete band was obtained when the mRNA sample from case no. 1 was amplified, even by prolonged RT-PCR for 35 to 40 cycles using a pair of primers for exons X-XIII. Accordingly, PCR-restriction fragment length polymorphism (RFLP) analysis could not be performed for case no. 1.

Molecular modeling. To calculate the effects of the Gly562-Arg mutation on the structure of the mutant A subunit, molecular modeling was performed. The solvent accessibility of the Arg562 side chain was 47% for domain IV alone and 6% for domains II, III, and IV, indicating that the Arg562 residue of the mutant was present on the surface of domain IV and on the interdomain surface between domains IV, II, and III of the molecule (refer to the coordinates for the mutant molecule presented at http://prtds.pharm.kitasato-u.ac). Domain IV interacts with each of domains II and III, and Arg562 in domain IV interacts with several amino acid residues on the surface of domains II and III. Although the Gly562 residue is completely conserved among transglutaminases, the structure of the A subunit was partly compatible with that of the elongated side chain of the mutated Arg residue. However, there were atomic pairs that had distances shorter than van der Waals contacts between the Arg and other residues (Fig 6, top right); these included Phe559, Tyr560, Thr561, Val563, His605, and Gln622...
in domain III, which were about 2.0 Å distant from the Arg residue; for domain IV, Ser368 and Val369 were at about 2.6 Å.

The molecular structure of the 464Stop mutant is truncated to 463 residues by the 4-bp deletion. In the native form, the first half of domain III (residues 320-463) makes contact with domain II and the second half of domain III (residues 464-513) over a wide area (Fig 6, bottom left). When the second half of domain III is absent, as in the deletion mutant of case no. 1, the surfaces of domain II and the first half of domain III become exposed extensively to solvents. Thus, it may be said that the surfaces of the mutant molecule will be susceptible to proteolytic degradation. The dimer formation of the mutant A subunit must also be impaired by the truncation of residues 464-731, because, in the native A subunit, this second part of domain III in one monomer contacts residues Pro383 and Asp384 directly in the counterpart monomer (Fig 6, bottom right).
Pulse-chase experiments in mammalian expression system.

To evaluate the intracellular stability of the newly synthesized A subunit, the wild-type and two mutants were examined by pulse-chase studies. RT-PCR analysis, using mRNA samples obtained from cells transfected with the expression vectors, showed that there were comparable amounts of the transcripts for both the wild-type and two mutants (data not shown).

Nascent wild-type A subunit seen at 0 hours gradually decreased in the cells but did not appear in the culture medium at all (Fig 7, top right), as described previously.27 At 24 hours, the band of the wild-type A subunit was still intense (64% of 0 hours). In contrast, the mutant A subunits in the cell lysates rapidly decreased with time but, again, did not appear in the culture medium (Fig 7, bottom). The disappearance of a truncated 48-kD band in the 4-bp deletion mutant was much faster than that for the Gly562-Arg mutant (0% within 2 hours v 8 hours, Figs 7 and 8). The complete loss of both mutants in BHK cells indicated that these molecules degraded rapidly in an intracellular compartment(s).

These results strongly suggest that the mutant A subunits are unstable. This is in good agreement with the results obtained by molecular modeling as described above.

DISCUSSION

Factor XIII deficiency is caused by defects in either the A or B subunit of the gene.14 These can include a variety of missense and nonsense mutations, small deletions and insertions with or without out-of-frame shift/premature termination, splicing abnormalities, etc.11-26 Therefore, mutations causing factor XIII deficiency are highly heterogeneous. In the present study, nucleotide sequence analysis of amplified DNAs showed that cases no. 1 and 2 with severe factor XIII deficiency28 had a deletion of 4 bp in exon XI and a mutation of Gly562-Arg in exon XII of the gene for the A subunit, respectively.

The premature termination newly created by the 4-bp deletion leads to a marked reduction in the mRNA level of the A subunit in case no. 1. However, the mechanism by which a new in-frame termination codon results in a decrease in the concentration of steady-state cytoplasmic mRNA has not been understood to date. As discussed by Cooper,36 nonsense mutations would halt the pulling process of the pre-mRNA through the splicing apparatus and the nuclear pores, leaving the RNA molecule vulnerable to RNase digestion. Another model is proposed whereby detection of an in-frame termination codon by putative nuclear scanning machinery would result in a slowing down of mRNA splicing/translocation. Despite the fact that the steady-state mRNA of the mutant allele was not detectable in peripheral blood cells from case no. 1, the level of mutant mRNA was comparable to that of the wild-type in the transfected mammalian cells, strongly suggesting that this mutation impairs some steps involved in pre-mRNA processing before the completion of the mature mRNA. The marked reduction in the level of A subunit mRNA in case no. 1 would also result in a marked decrease in production of the A subunit protein.

The premature termination at codon 464, in turn, results in the deletion of the C-terminal third of the A subunit molecule (268 amino acids, residues 464-731) and in the creation of a C-terminal truncated protein. Because domains I and II (residues 1-319) are structurally independent of domains III, IV, and V,36 domains I and II of the mutant will fold like that of the native A subunit. It is generally accepted that a hydrophobic
core in a domain plays essential roles in protein folding. Because residues 320-463 nearly completely the hydrophobic core in domain III, it is likely that the 464Stop mutant folds in a manner similar to the native A subunit. Because the missing C-terminal portion is essential for interdomain interactions and dimer formation of the A subunit, the truncated protein of 463 residues is predicted to be unstable. This hypothesis has been confirmed by pulse-chase experiments in a mammalian expression system in which the truncated protein of 48 kD was degraded rapidly inside BHK cells. The disappearance of the truncated mutant was even faster than that of the Gly562-Arg mutant. Accordingly, it may be said that the A subunit deficiency in case no. 1 was caused by two steps: first, by a severe reduction in its mRNA level; and, second, by the instability of its truncated molecule.

Alternatively, without residues 464-513, domain III may not be able to fold like the native domain III, in which case the result would also be the premature degradation of the entire mutant protein. However, this hypothesis cannot be substantiated at present.

Although this 4-bp deletion was previously identified at the DNA level in a compound heterozygote of French origin, it was not characterized further. A founder effect for this mutation is not likely, because none of the three polymorphisms found in our Italian homozygote was detected in the heterozygote mentioned above. This deletion mutation may be a recurring one, because the direct repeat of the AA TT sequence at the deletion site in exon XI seems to be a hotspot for slippage of DNA polymerases.

The mRNA level of the Gly562-Arg mutation was not reduced at all in case no. 2. Gly562 is highly conserved throughout all 15 members of the transglutaminase family; therefore, it may play an important role(s) in the enzymatic function and/or structural integration of transglutaminases. This amino acid is located on the interface of domains IV, II, and III, based on the three-dimensional structure of the molecule. It may be said that the Gly562-Arg mutant protein cannot fold into exactly the same structure as the wild-type, especially in the formation of the domain complex of II, III, and IV. Even if the gross structure consisting of domains II, III, and IV for the mutant were close to the native x-ray structure, molecular modeling showed a space somewhat smaller than required for the elongated side chain of Arg562. Furthermore, molecular mechanics predicted that the Gly562-Arg mutation increased the total potential energy of the A subunit molecule by +73.4 kcal/mole (Tsukamoto et al, unpublished data), suggesting that the mutation from Gly to Arg makes the molecule less stable than the native form. Therefore, the Gly562-Arg mutation is not adaptable for the A subunit molecule. This hypothesis is consistent with the results obtained by pulse-chase experiments that showed that the Gly562-Arg mutant was synthesized normally, but degraded rapidly in cells. Accordingly, the Gly562-Arg mutation leads to deficiency of the A subunit not only in plasma but in platelets as well.

It may be that transport and secretion of the mutant A subunit are impaired in addition to its folding capabilities; however, these questions must remain unanswered until the mechanisms of its normal biosynthesis are better understood.

Eight additional missense mutations have been reported, including Asn60-Lys, Met242-Thr, Arg252-Ile, Arg326-Gln, Arg408-Gln, Leu498-Pro, Gly501-Arg, and Leu667-Pro. To our knowledge, two of these mutants, Asn60-Lys and Gly501-Arg, have been expressed in yeast to date, but not in mammalian cells. It was concluded by Coggan et al that the Asn60-Lys mutant was very unstable and/or the subject of increased proteolytic degradation. However, this conclusion may not always be valid in vivo, because the quality control and proteolytic degradation mechanisms for abnormal molecules in mammalian cells may differ from those in other systems. For example, C-terminal truncated mutants (including one composed of 462 residues) were expressed in Escherichia coli at levels comparable to those of the wild-type, whereas our mutant of 463 residues was lost within 2 hours in mammalian cells, indicating that the effects of mutations on protein biosynthesis depend on the type of expression systems used. We have recently developed mammalian expression systems both for the A and B subunits and have confirmed that both recombinant A and B subunits were indistinguishable from the native A and B subunits of plasma factor XIII in terms of their physical and functional properties. Thus, in this study, we present for the first time the successful expression of novel mutant A subunits in mammalian cells and provide evidence indicating that rapid degradation of the A subunit mutants in the synthesizing cells leads to type II factor XIII deficiency.

Finally, this study has shown that molecular modeling is, at least to some extent, useful in predicting possible effects of missense mutations on the structure and stability of the A subunit, because an expression analysis of each mutant individually would require a great outlay of time and resources.

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Molecular Mechanisms of Type II Factor XIII Deficiency: Novel Gly562-Arg Mutation and C-Terminal Truncation of the A Subunit Cause Factor XIII Deficiency as Characterized in a Mammalian Expression System

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