Articles

Impaired Protein Folding, Dimer Formation, and Heterotetramer Assembly Cause Intra- and Extracellular Instability of a Y283C Mutant of the A Subunit for Coagulation Factor XIII†

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ABSTRACT: Factor XIII (XIII) is a heterotetramer consisting of two catalytic A subunits (XIIIA) and two noncatalytic B subunits (XIIIB). We examined the molecular mechanisms of a Y283C mutation which had previously been identified in a patient with XIIIA deficiency. The recombinant Y283C protein was labile when expressed in MEG-01 cells, which can endogenously synthesize XIIIA. We also included two other mutants, G562R and I464stop, previously characterized in a non-XIIIA-producing cell line. All these mutants exhibited decreased thermostability and resistance against proteolytic digestion when compared with the wild-type. Gel-filtration analysis revealed that the mutants were in monomer form, while the wild-type formed a dimer. These results were consistent with the prediction by molecular modeling that the mutant molecules would be misfolded. Although assembly of a heterotetramer with XIIIB was demonstrated for Y283C, its binding ability was 10% that of the wild-type. No complex formation was observed for the G562R or I464stop mutants. The wild-type was stabilized in plasma by complex formation with XIIIB, resulting in an increased resistance against proteolytic digestion. In contrast, the mutants were unstable in plasma even in the presence of XIIIB. Thus, impaired folding, dimer formation, and heterotetramer assembly of the mutant XIIIAs lead to both intra- and extracellular instability, which must be responsible for XIIIA deficiency in the patient.

Blood coagulation factor XIII (XIII)[1] is a proenzyme of plasma transglutaminase consisting of two catalytic A (XIIIA) and two noncatalytic B subunits (XIIIB) (*1*, *2*). In the final stage of the blood coagulation cascade, XIII is activated by thrombin and promotes clot stability by forming covalent bonds between fibrin molecules and also cross-links fibrin with other proteins including fibronectin, α_2-plasmin inhibitor, and collagen. A XIIIA cDNA encodes a mature protein of 731 amino acids, including an activation peptide of 37 residues at the amino terminus and a catalytic Cys314 residue (*3*). Recently, the three-dimensional structure of XIIIA determined by X-ray crystallography demonstrated that XIIIA is composed of several distinct domains: an activation peptide (residues 1−37), a β-sandwich (38−183), a central core (184−513), and Barrel 1 (514−628) and Barrel 2 (629−731) regions (*4*, *5*). The carboxyl-terminal portion of XIIIA corresponding to the two β-Barrels forms thermostable domains, while three thermolabile domains are formed by the amino-terminal β-sandwich and core domains (*6*). The gene for XIIIA spans more than 160 kb and consists of 15 exons interrupted by 14 introns (*7*). XIIIB is composed of 641 amino acids and contains 10 tandem repeats called "sushi domains" (*3*, *8*). The gene for XIIIB is approximately 28 kb in length and is composed of 12 exons interrupted by 11 introns (*9*).

Deficiency of XIII results in a life-long bleeding tendency and abnormal wound healing in affected patients, and spontaneous abortion in affected females (*1*, *10*). XIII deficiency has been classified into two categories (*11*): type I deficiency, characterized by the lack of both XIIIA and XIIIB; and type II deficiency, characterized by the lack of XIIIA alone. Determination of the genomic organization and sequence for both subunits (*3*, *7*, *9*, *12*) has made it possible to characterize factor XIII deficiency at the DNA level (*13*). Genetic and molecular characterization of XIII deficiency shows that type I and II deficiencies are caused by defects in the genes for XIIIB and XIIIA, respectively. These mutations include a deletion of nucleotide A in the obligatory AG splicing donor sequence at the intron A/exon II boundary, a Cys430-Phe substitution, and a triplet AAC insertion within the codon for Tyr80 in the XIIIB gene; and a 20 bp deletion at the exon I/intron A boundary, an insertion of nucleotide

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[1] Abbreviations: XIII, factor XIII; XIIIA, A subunit of XIII; XIIIB, B subunit of XIII.

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T in the obligatory GT splicing acceptor sequence at the exon IV/intron D boundary, a 4 bp deletion in exon XI, a Gly562-Arg substitution, and an Arg260-Cys change in the XIIIA gene (*13*−*20*). Thus, we have named the two types of this disease XIIIB and XIIIA deficiencies (*21*).

Deficiency of XIIIB in plasma, which is caused by a decreased level of mRNA, intracellular instability, and/or impaired intracellular transport of the translated products, results in a significant decrease in plasma XIIIA, although a normal amount of XIIIA is present in platelets of patients with XIIIB deficiency (*14*−*17*). Therefore, XIIIB is thought to stabilize XIIIA in plasma.

To date, more than 30 mutations in the gene for XIIIA have been identified in patients with XIIIA deficiency (*18*−*20, 22*−*43*). These mutations are highly heterogeneous and include a variety of nonsense mutations (Arg171-stop in exon IV, Tyr441-stop in exon XI, Arg661-stop in exon XIV) and missense mutations (Asn60-Lys in exon III, Met242-Thr in exon VI, Arg252-Ile in exon VI, Arg260-His in exon VII, Arg326-Gln in exon VIII, Arg408-Gln in exon X, Val414-Phe in exon X, Leu498-Pro in exon XII, Gly501-Arg in exon XII, Leu660-Pro in exon XIV, Leu667-Pro in exon XIV), deletions and insertions with or without frame-shift/premature termination (20 bp deletion at exon I/intron A boundary, deletion of T in exon II, deletion of AG in exon III, 13 bp deletion in exon III, GG-TCGTCC change in exon III, deletion of A in intron A, 3 bp deletion in exon VIII, 4 bp deletion in exon XI, deletion including exons IV−XI), splicing abnormalities (G-T change in exon III, G-A change in intron C, T-C transition in intron III, G-A change in intron E, G-A change in exon XIV), etc. Five missense mutations of XIIIA (Met242-Thr, Arg252-Ile, Arg326-Gln, Leu498-Pro, Gly562-Arg) have been confirmed to be causative as they resulted in the generation of unstable proteins in mammalian cells (*18, 27*). Most of the missense mutations have been predicted to change the conformations of their translated products; however, experimental evidence for this has not been demonstrated in detail, thus far.

In the present study, we performed detailed biochemical experiments to obtain direct evidence for the molecular abnormalities of a novel Y283C mutant identified in the XIIIA gene of a patient with XIIIA deficiency(*44*), and the two mutants which were previously characterized in BHK cells (*18*). Recombinant XIIIAs were expressed, for the first time, in a megakaryocyte cell line, a cell line especially relevant to the study of the biosynthesis of XIIIA (*2*). In addition, the mutant proteins' complex formation with XIIIB was also examined to compare the extracellular stability of XIIIA with and without XIIIB.

EXPERIMENTAL PROCEDURES

Materials. The human megakaryocytic leukemia cell line MEG-01 was generously provided by Prof. H. Saito (Nagoya University School of Medicine, Nagoya, Japan). The expression vector pCDNA3 was obtained from Invitrogen (San Diego, CA). An Expre^{35}S^{35}S protein labeling mix was purchased from NEN Research Products (Boston, CA). Anti-XIIIA and anti-XIIIB antibodies and Pansorbin cells were obtained from Calbiochem (La Jolla, CA). Bovine pancreas trypsin, bovine thrombin, bovine serum albumin (BSA), and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (St. Louis, MO). Purified human plasma XIII and XIIIB were the kind gifts of Dr. H. Kaetsu (The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan).

Construction of Expression Vectors. An expression vector, pCDNA3, inserted with the XIIIA cDNA for the wild-type (pCDNA3/XIIIA), G562R, or I464stop mutant, and pBluescript II SK(+) inserted with the wild-type XIIIA cDNA (pBSII/XIIIA) were prepared as described previously (*18*). A PCR-amplified fragment of 289 bp for exon VII containing a Y283C mutation was subcloned into pBluescript II SK-(+), and the resultant vector was cleaved with *Bam*HI and *Sac*I. The released fragment of 84 bp was ligated into the XIIIA cDNA in pBSII/XIIIA digested with *Bam*HI−*Sac*I. The generated cDNA of 2.4 kb containing the mutation was excised from pBSII/XIIIA by digestion with *Hin*dIII and *Xba*I and inserted into pCDNA3/XIIIA digested with *Hin*dIII−*Xba*I. Plasmid vectors were prepared using a QIAGEN Plasmid Kit (Qiagen, Valencia, CA) followed by phenol−chloroform extraction and ethanol precipitation. To prepare the Barrel 1 domain-deleted XIIIA (Del-Barrel 1), a sense primer, 5′-TTCCGGAACAACATCCCCTGAGATCATCA-TC-3′ (DB1), which codes for Phe539 to Asn542 and Ile629 to Ile634, was designed. Part of the XIIIA cDNA encoding the entire Barrel 2 domain (Ile629 to 732-stop) was amplified by PCR using a pair of primers, DB1 and M13 reverse primer (GIBCO BRL, Gaithersberg, MD), and pBSII/XIIIA as a template. The PCR product was digested by *Bsp*EI and *Xba*I, and the released fragment was inserted into pBSII/XIIIA digested with *Bsp*EI and *Xba*I. To delete the Barrel 2 domain (Del-Barrel 2), an antisense primer, 5′-TCTAGATCAGGT-TAGCACGGTGGACTTTTG-3′ (DB2), which replaces Ile629 with a stop codon, was designed. A part of the XIIIA cDNA encoding half of the core domain (Asn371 to Asn542) was amplified by PCR using DB2 and a sense primer, 5′-GAACTACCACTGCTGGAATGAAGCATGGATGA-3′, and pBSII/XIIIA as a template. The PCR product was digested by *Stu*I and *Xba*I, and the fragment was inserted into pBSII/XIIIA digested with *Stu*I and *Xba*I.

Transfection and ^{35}S-Labeling. MEG-01 cells were cultured in RPMI 1640 medium containing 10% (v/v) fetal bovine serum (FBS). Twenty micrograms of plasmid DNA was added to 0.25 mL of culture media in a cuvette with 2 $\times$ 10^7 cells, and the cuvette was pulsed at 220 V for 30 ms using an electroporation system (BTX, San Diego, CA). The cells were transferred to a 60 mm dish with 5 mL of culture media and cultured at 37 °C. After 12 h, the medium was changed to 2 mL of methionine-free RPMI 1640 medium containing 5% dialyzed FBS, and the cells were preincubated for 60 min. Fifty microcuries of [^{35}S]methionine was then added, and the cells were incubated at 37 °C for 30 min. For chase experiments, the medium was replaced by 3 mL of the standard medium containing methionine after labeling for 30 min, and the cells were further incubated for an appropriate time.

Western Blotting. The cells at 48 h after transfection were lysed with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 1 mM PMSF. One microgram of protein of the lysate was electrophoresed on an 8% polyacrylamide gel containing 0.1% SDS, and then transferred to a nitrocellulose membrane. The membrane was incubated with phosphate-buffered saline containing an anti-human XIIIA antibody. Protein detection was achieved using

an ECL Western blotting analysis kit (Amersham, Buckinghamshire, U.K.).

Thermostability. ^{35}S-labeled cells were sonicated in a lysate buffer of 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1 mM EDTA, and 1 mM PMSF, and the suspension was centrifuged at 10000*g* for 10 min. The supernatant adjusted the protein concentration to 5 mg/mL with the same buffer, and the solution was heated at 42, 45, 48, 51, 54, 57, 60, or 63 °C for 2 min and then immediately cooled with ice/water. After centrifugation at 10000*g* for 20 min, an anti-XIIIA antiserum and Pansorbin cells were added to the supernatant and incubated at 4 °C for 60 min. The immunoprecipitate was collected by centrifugation, washed twice with a solution of 40 mM Tris-HCl (pH 7.5), 0.3 M NaCl, and 0.2% Tween 20, and then boiled in 20 μL of an SDS-sample buffer containing 60 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, and 7% glycerol. The samples were electrophoresed on an 8% polyacrylamide gel containing 0.1% SDS. The gel was dried, and the radioactive band for XIIIA was quantified by fluorography using a FLA-2000 Fluoroimage Analyzer (Fuji Film, Tokyo, Japan).

Tryptic Digestion. Thirty micrograms of protein of the cell lysate from ^{35}S-labeled cells was treated with 3, 15, or 75 ng of trypsin at 37 °C for 30 min. For the tryptic treatment in the presence or absence of XIIIB, 1 μg of XIIIB or BSA was added to 30 μg of protein of the lysate, and the sample was left on ice for 30 min and then treated with 0.15, 0.3, or 0.6 μg of trypsin at 37 °C for 30 min. The reaction was terminated by adding PMSF (a final concentration of 5 mM), and the samples were subjected to immunoprecipitation followed by SDS−PAGE using a 10% polyacrylamide gel and fluorography.

Gel-Filtration. ^{35}S-labeled cells were sonicated in the lysis buffer, and the suspension was centrifuged at 10000*g* for 10 min. The supernatant was then filtrated by a 0.45 μm filter using Artkiss (Advantec, Tokyo, Japan), and the filtrate was applied onto a TSK-G3000SW column (7.5 mm i.d. × 60 cm; Tosoh, Tokyo, Japan) equilibrated with 50 mM Tris-HCl (pH 7.5), 0.2 M NaCl, and 1 mM EDTA. The elution was performed with the same buffer at a flow rate of 0.5 mL/min, and fractions of 0.5 mL/tube were collected. Fractions were subjected to immunoprecipitation, SDS−PAGE, and fluorography, as described above. For a study of the complex formation of XIIIA with XIIIB, 5 μg of XIIIB was added to the lysate (approximately 200 μg of protein) after filtration by the Artkiss filter and left on ice for 30 min; then the sample was subjected to the gel-filtration analysis. For calibration of the column, plasma XIII, γ-globulin, and BSA were used as molecular weight markers.

RESULTS

Expression of Y283C Mutant Protein. To examine whether the Y283C mutation is causative for XIIIA deficiency, both the wild-type and Y283C XIIIAs were expressed using the XIIIA cDNAs and MEG-01 cells. Although native MEG-01 cells expressed the XIIIA mRNA and protein weakly (Souri and Ichinose, unpublished observation), an endogenous XIIIA protein was not detected by Western blotting when 1 μg of cell lysate protein from MEG-01 with mock-transfection was used (Figure 1). In the MEG-01 cells transfected with the wild-type cDNA, XIIIA was readily detected by an anti-XIIIA antibody at the position corresponding to purified human plasma XIIIA, indicating definitely that the XIIIA protein was expressed. The mutant XIIIA protein was also found in the cells transfected with the Y283C cDNA at the same position as the wild-type, and its amount was 10% that of the wild-type, when determined by densitometry. The mRNA levels for XIIIA determined by Northern blot analysis were similar among the cells transfected with the wild-type and Y283C cDNAs (data not shown).

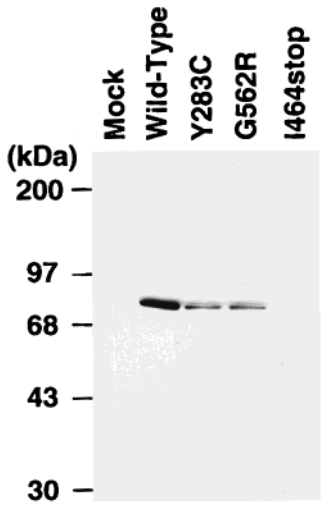

FIGURE 1: Western blotting analysis of recombinant XIIIAs expressed in MEG-01 cells. Expression of the wild-type and mutant XIIIAs. One microgram of each cell lysate protein was subjected to SDS−PAGE and Western blotting. The bands for XIIIA were detected by employing an anti-human XIIIA antibody.

Pulse−chase experiments were carried out to examine the intracellular stability of the Y283C protein. ^{35}S-labeled wild-type XIIIA was stable in the MEG-01 cells, since 75% of the labeled protein remained after 24 h of the chase phase (Figure 2). On the other hand, the ^{35}S-labeled Y283C protein decreased to 60% after 2 h, and gradually decreased to 10% at 24 h. In both cases, the release of XIIIA to the cultured medium was not observed during the entire incubation period of the chase phase (data not shown). Thus, the Y283C protein was confirmed to be labile, for the first time, inside human cells that endogenously synthesize XIIIA.

Structural Defects of Mutant XIIIAs. We previously reported two other mutant XIIIAs (G562R and I464stop), which were identified in two separate Italian families with XIIIA deficiency and were found to be unstable when they were expressed in baby hamster kidney (BHK) cells (*18*), which do not produce XIIIA endogenously. When these mutants were expressed in MEG-01 in the present study, the band for the G562R mutant was faint, and that for I464stop was barely detected (Figure 1), results which are consistent with those of the previous study carried out in BHK cells.

Since the Tyr283 residue of XIIIA is completely conserved among members of the transglutaminase family, it may be important for the structure of XIIIA. To explore the molecular defects in the Y283C mutant protein, biochemical studies were carried out. The G562R and I464stop mutants were also included in the following experiments. Figure 3 shows the thermostability of the wild-type and mutant XIIIAs. Amounts of XIIIA proteins in the cell lysate remaining in a

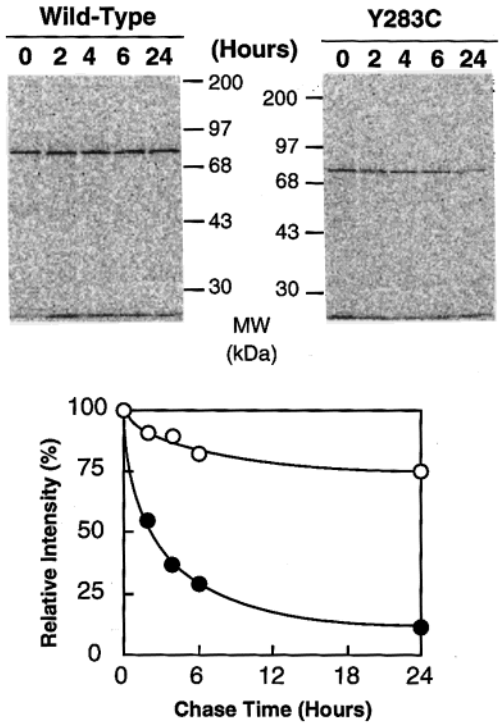

FIGURE 2: Pulse−chase experiment of wild-type and Y283C XIIIAs. MEG-01 cells transfected with the expression vectors were incubated with 50 μCi of [^{35}S]methionine for 30 min and then chased for the indicated time periods. The cell lysate was subjected to immunoprecipitation using an anti-XIIIA antibody, followed by SDS−PAGE and fluorography. Results of quantification by fluorography are shown in the panel at the bottom. Open circles, wild-type; closed circles, Y283C.

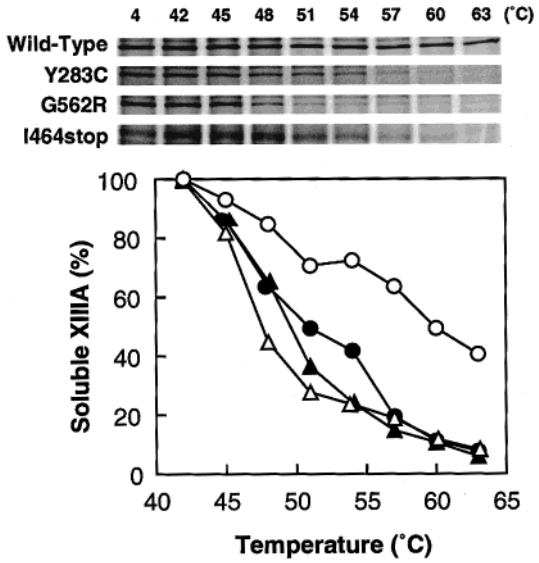

FIGURE 3: Thermostability of wild-type and mutant XIIIAs. A cell lysate from ^{35}S-labeled cells was heated at the indicated temperature for 2 min, and then centrifuged at 10000*g* for 20 min. The supernatant was subjected to immunoprecipitation using an anti-XIIIA antibody, followed by SDS−PAGE and fluorography. The amount of soluble XIIIA in each sample after heat treatment was compared to that in an untreated sample (4 °C). Open circles, wild-type; closed circles, Y283C; open triangles, G562R; closed triangles, I464stop.

soluble state after heat treatment were analyzed by fluorography (Figure 3, top). Soluble wild-type XIIIA gradually decreased as temperature increased, to 40% at 63 °C. For Y283C, half of the soluble protein disappeared after 2 min at 51 °C, and the Y283C protein was not detectable over 60 °C (Figure 3, bottom). Soluble G562R and I464stop XIIIAs also decreased to half their starting amounts by the heat treatment at 51 °C.

Since the two carboxyl-terminal domains of XIIIA (Barrels 1 and 2) are resistant to proteolytic cleavage, treatment of XIIIA with several enzymes generates terminal 24 and 12 kDa fragments; the former corresponds to both Barrels 1 and 2, and the latter to Barrel 2 (*6*). When the cell lysate containing the wild-type XIIIA was treated with varying amounts of trypsin, 71, 24, and 12 kDa bands were produced (Figure 4). The 71 kDa fragment migrated to the same position as the thrombin-cleaved XIIIA and remained even after treatment with the highest amount of trypsin. Three mutant proteins were much more sensitive to tryptic digestion: the 71 kDa band was not detectable in these three samples. In Y283C XIIIA, the 24 and 12 kDa fragments were observed as in the wild-type. Only the 12 kDa band was detectable in G562R XIIIA, indicating that Barrel 2 was intact but that Barrel 1 was disrupted by the mutation. The I464stop protein was small in size because of truncation at the carboxyl-terminal portion by the premature termination codon. The I464stop XIIIA disappeared rapidly, and no fragment was detected after a 15 min tryptic digestion. These results indicate that the carboxyl-terminal domains are fairly intact in the Y283C mutant, but that the conformations of its β-sandwich and core domains are drastically altered by the mutation.

Gel-filtration analysis was then performed to examine the subunit structure of the mutant XIIIAs, such as dimer assembly. A peak of the wild-type XIIIA was observed at an elution time of 26 min, the molecular mass of which was estimated to be 150 kDa (Figure 5). On the other hand, elution of the Y283C and G562R XIIIAs was delayed two fractions (2 min) from that of the wild-type, and their molecular masses were estimated to be 75 kDa. The I464stop XIIIA was eluted at a fraction of 29 min, the molecular mass of which was calculated to be 50 kDa. These results clearly indicate that none of these mutant proteins can assemble to form a dimer.

Complex Formation with XIIIB. To test whether the mutant XIIIAs can form a complex with XIIIB, a co-immunoprecipitation study was performed using purified human plasma XIIIB and an anti-XIIIB antibody. Although a minimal amount of the wild-type XIIIA was precipitated by an anti-XIIIB antibody in the absence of XIIIB (<5% that by an anti-XIIIA antibody), 60% of the labeled XIIIA was precipitated by the anti-XIIIB antibody after the addition of 0.5 μg of XIIIB (Figure 6A). All the wild-type XIIIA was precipitated by the anti-XIIIB antibody using 1 μg of XIIIB/30 μg of the lysate protein (Figure 6B). The precipitation of labeled XIIIA was abolished by the addition of an excess amount (10 μg) of unlabeled XIIIA (Figure 6A), indicating the specific binding of XIIIA to XIIIB. An increasing amount of Y283C XIIIA was also precipitated by the anti-XIIIB antibody as the amount of added XIIIB increased, but its precipitation ratio, even after the addition of 1 μg of XIIIB, was only 10% that of the anti-XIIIA antibody. On the other hand, both the G562R and I464stop XIIIAs were not co-immunoprecipitated with XIIIB, indicating that these mutants could not bind to XIIIB. Two mutant proteins lacking either

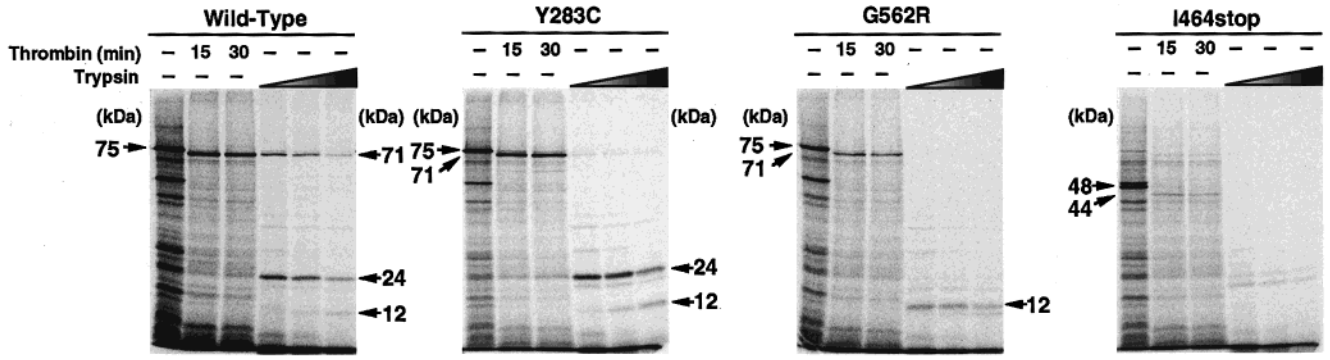

FIGURE 4: Proteolytic digestion of wild-type and mutant XIIIAs. Thirty micrograms of the lysate protein from ^{35}S-labeled cells was treated with 2.5 μg of thrombin for 15 or 30 min, or with 3, 15, or 75 ng of trypsin for 30 min at 37 °C. The reaction was terminated by the addition of PMSF; the samples were then subjected to immunoprecipitation, SDS−PAGE, and fluorography.

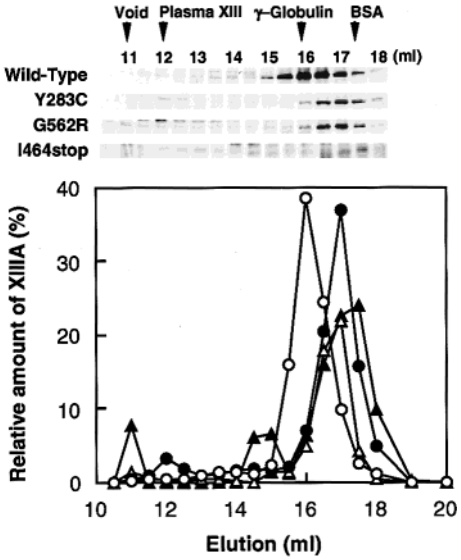

FIGURE 5: Gel-filtration analysis of wild-type and mutant XIIIAs. A cell lysate from ^{35}S-labeled cells was centrifuged, and the suspension was filtered and then subjected to gel-filtration on a TSK-G3000SW column. Fractions of 0.5 mL/tube were collected. Plasma XIII (320 kDa), γ-globulin (160 kDa), and BSA (68 kDa) were used as molecular mass markers. Open circles, wild-type; closed circles, Y283C; open triangles, G562R; closed triangles, I464stop.

the Barrel 1 or the Barrel 2 domain (Del-Barrel 1 or Del-Barrel 2) also demonstrated no binding to XIIIB (Figure 6B), suggesting that the carboxyl-terminal domains are important for the complex formation of XIIIA with XIIIB.

For both the wild-type and Y283C XIIIAs, a heterotetramer assembly with XIIIB was demonstrated by gel-filtration chromatography, although most Y283C XIIIA eluted in fractions as a monomer (Figure 7). All wild-type XIIIA transformed to a heterotetramer with XIIIB. These results suggest that two Y283C monomers bind to a XIIIB dimer.

The stability of the wild-type and mutant XIIIAs in plasma was then investigated in the presence and absence of XIIIB. In this experiment, plasma obtained from a patient with complete XIIIB deficiency (*17*) was used, because it contains neither XIIIA nor XIIIB. In the absence of XIIIB, the wild-type XIIIA decreased to 60% after a 2 h incubation at 37 °C, but 40% still remained after 24 h, while most Y283C protein disappeared within 2 h (Figure 8A). The addition of XIIIB to the reaction mixture stabilized both the wild-type

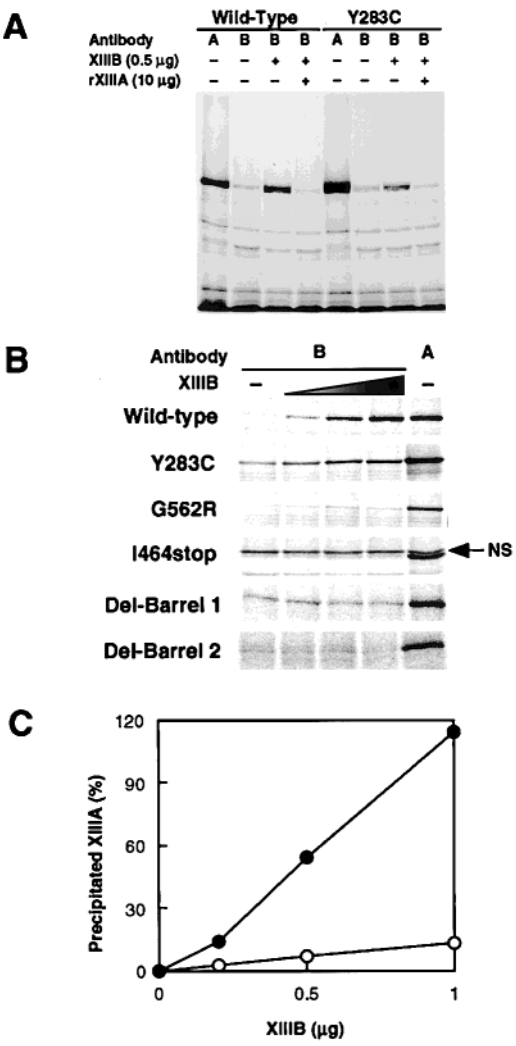

FIGURE 6: Co-immunoprecipitation of XIIIA with XIIIB using an anti-XIIIB antibody. (A) 0.5 μg of XIIIB was added to 30 μg of the lysate protein from ^{35}S-labeled cells with or without 10 μg of purified recombinant XIIIA, and left on ice for 30 min. Immunoprecipitation was then performed using an anti-XIIIB antibody, and the precipitates were subjected to SDS−PAGE and fluorography. NS indicates a nonspecific band. (B and C) Co-immunoprecipitation of wild-type and mutant XIIIAs with various amounts of XIIIB using an anti-XIIIB antibody. 0.2, 0.5, or 1 μg of XIIIB was added. The amounts of XIIIA precipitated with an anti-XIIIB antibody were compared with those obtained by an anti-XIIIA antibody. Del-Barrel 1 and 2 stand for the deletion mutants without Barrel 1 or 2 domains, respectively. Open circles, Y283C; closed circles, wild-type.

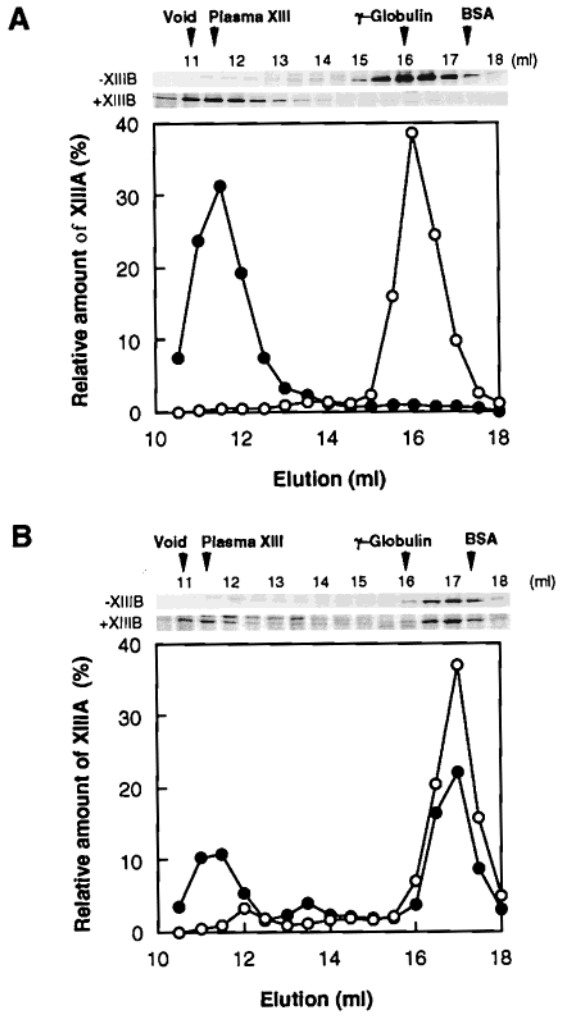

FIGURE 7: Gel-filtration analysis of wild-type (A) and Y283C (B) after incubation with XIIIB. Experiments were carried out following exactly the same procedures as in Figure 5. Open circles, without XIIIB; closed circles, with 5 μg of XIIIB.

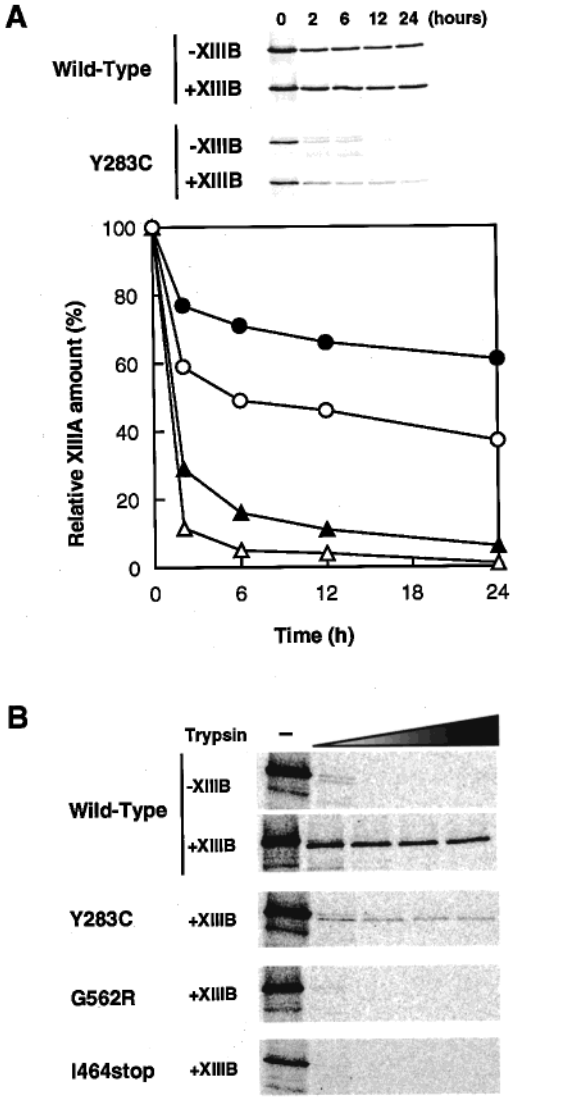

FIGURE 8: Effect of XIIIB on the stability of XIIIA in plasma. (A) Cell lysate was incubated with 10 volumes of XIIIB-deficient plasma at 37 °C, with or without XIIIB. The samples were subjected to immunoprecipitation using an anti-XIIIA antibody, SDS−PAGE, and fluorography. Open circles, wild-type without XIIIB; closed circles, wild-type with XIIIB; open triangles, Y283C without XIIIB; closed triangles, Y283C with XIIIB. (B) Tryptic digestion of the wild-type and mutant XIIIAs in the presence and absence of XIIIB, as described in Figure 4, except for the amount of trypsin added.

and Y283C XIIIAs in plasma, although the Y283C protein decreased to 5% after 24 h.

Tryptic digestion of mutant XIIIA was also carried out in the presence or absence of XIIIB (Figure 8B). Because a larger amount of trypsin was used in this experiment than in those shown in Figure 4, the 71 kDa band was not detected even in the wild-type in the absence of XIIIB. The addition of XIIIB clearly increased the resistance of the wild-type XIIIA to tryptic digestion: the 71 kDa fragment remained even after treatment with the highest amount of trypsin. A faint band of 71 kDa was observed after tryptic digestion of the Y283C protein in the presence of XIIIB. On the other hand, no 71 kDa fragment was detected for either the G562R or the I464stop XIIIAs.

DISCUSSION

Many mutations in the XIIIA gene have been detected by in vitro amplification of DNA samples obtained from patients with A subunit deficiency. More than one-third of the patients have point mutations that cause amino acid substitutions (*45*). The effects of these natural mutations on A subunit biosynthesis have been confirmed in several cases by expressing mutant proteins in yeast (*24*, *38*, *46*), COS cells (*27*), or BHK cells (*18*). However, to date none of the XIIIA mutants has been expressed in the cells that *endogenously* synthesize XIIIA. It has been known that placenta, macrophages, and megakaryocytes do synthesize the A subunit (*2*). In the present study we, for the first time, successfully expressed both the wild-type and three mutant XIIIAs in MEG-01 cells, a human megakaryocytic cell line. It is important to examine the effects of mutations on protein biosynthesis in such a cell line, since the quality-control and proteolytic degradation mechanisms for abnormal molecules in mammalian cells may differ from those in other systems, like yeast (*18*), and may depend on the cell type employed. A shortcoming of expression in MEG-01 cells is noted as well: The low efficiency of transfection into the cells leads to production of only a small amount of recombinant protein. However, it was estimated by Northern blotting, Western blotting, and pulse-labeling analyses that the expression level of the

endogenous XIIIA is less than 5% of that of the exogenous recombinant XIIIA (data not shown). It is not likely that the coexistence of the mutant recombinant protein with the endogenous XIIIA molecule significantly influenced the results. A low concentration of recombinant XIIIA may affect some experiments, such as its stability test in solution; however, both the wild-type and mutant XIIIAs were examined and compared under identical conditions. Thus, we believe that the low expression levels of recombinant proteins do not invalidate our conclusions.

A novel mutation, Y283C, was previously identified in the XIIIA gene of a proband of complete XIIIA deficiency and his brother. Tyr283 of XIIIA is highly conserved among all transglutaminases (*47*, *48*). To examine the effects of this mutation on XIIIA biosynthesis, the mutant protein was expressed and characterized in detail at the cellular and protein levels. Western blotting detected lesser amounts of the Y283C mutant than of a wild-type XIIIA, and pulse−chase experiments revealed that the disappearance rate of the Y283C mutant in expressing cells was faster than that of the wild-type. In addition, the Y283C protein exhibited lower thermostability and was more sensitive against proteolytic digestion than the wild-type, suggesting that the conformation of the Y283C protein was significantly altered by the mutation. Rapid intracellular degradation of other mutant proteins has also been reported in COS cells (*27*) and in BHK cells (*18*). Decreased thermostability was previously demonstrated only in a G501R mutation by Coggan et al. (*24*); however, it is difficult to compare their results with our current study, since the G501R mutant was tested only at 55 °C for 15 min.

To explore the effects of this mutation on the structure/function of XIIIA, its capacity to form a homodimer and a heterotetramer and its stability were examined for the first time. Gel-filtration analysis revealed that the Y283C mutant was a monomer, while the wild-type formed a native homodimer. Dimer formation must be very important for the stability of the XIIIA molecule, since 2280 Å^2 of its solvent-accessible surface area and 10.3−14.4 kcal/mol of its free energy decrease upon dimerization. The Y283C protein could bind to XIIIB, although the amount of its complex was much less than that of the wild-type. The decrease in the binding ability of the Y283C protein to XIIIB must be caused by the altered conformation of the mutant XIIIA molecule, which results in impaired interaction between domains. This is exactly what was predicted by molecular modeling (*44*). It is of interest that the complex formed between the Y283C protein and XIIIB was a heterotetramer like a native XIII, despite the fact that Y283C itself was a monomer. Accordingly, one Y283C monomer binds to one XIIIB molecule of a XIIIB dimer, and another Y283C monomer binds to the remaining XIIIB molecule of the XIIIB dimer.

In the present study, we also examined the G562R and I464stop mutations, which have previously been identified and found to be labile in BHK cells (*18*). These mutants have changes in the primary structure of their carboxyl termini: Gly562 locates in Barrel 1, and I464stop deletes the carboxyl-terminal third of the XIIIA molecule. Both of these mutant proteins were shown to have lower thermostability and to be more sensitive to proteolytic digestion than the wild-type, and to be monomers as determined by gel-filtration chromatography. These results indicate that the mutations truly impaired the folding and dimer assembly of the XIIIA molecule. Furthermore, both the G562R and I464stop mutants were incapable of forming a heterotetramer with XIIIB.

The complex formation of XIIIA with XIIIB must be dependent on a binding site(s) in the XIIIA molecule. Two carboxyl-terminal domains of XIIIA (Barrels 1 and 2) may, at least to some extent, contribute to its binding to XIIIB. This assumption is supported by the following facts: (1) The Y283C protein did partially bind to XIIIB. It is likely that intradomain structures of Barrels 1 and 2 in the Y283C protein are similar to those in the wild-type, since the carboxyl terminus of Y283C was as resistant to tryptic digestion as that of the wild-type. (2) The G562R and I464stop mutants did not bind to XIIIB at all. The Barrel 1 domain of the G562R protein is structurally altered by the mutation, and it was readily digested by trypsin. Both the Barrel 1 and Barrel 2 domains are completely lost in the I464stop mutant. (3) Two additional mutants with a deletion of either the Barrel 1 or the Barrel 2 domain did not bind to XIIIB at all.

The complex formation of XIIIA with XIIIB must be critical for its stability in plasma, because the wild-type XIIIA is more stable in plasma containing XIIIB than in that lacking XIIIB. In addition, the wild-type XIIIA is protected by XIIIB against proteolytic attack. Accordingly, all three mutant XIIIA proteins are unstable even in the plasma containing XIIIB, since they have no or limited ability to bind XIIIB. The protection of XIIIA by XIIIB may be more prominent in circulation in vivo, because there are many cellular components, such as blood cells, vascular endothelial cells, reticuloendothelial system, etc., that may interact with XIIIA. This hypothesis is consistent with the fact that the XIIIA antigen level is significantly low in plasma of patients with XIIIB deficiency, although their platelets contain normal amounts of XIIIA (*16*).

In conclusion, the Y283C mutation is causative for XIIIA deficiency, because its translational product is unstable both inside cells and in plasma. The mutant XIIIA protein would thus be deficient in the patients' blood.

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