Sushi Domains in the B Subunit of Factor XIII Responsible for Oligomer Assembly[†]

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ABSTRACT: Factor XIII (FXIII) is a heterotetramer composed of two catalytic A subunits (FXIII-A) and two B subunits (FXIII-B). FXIII-B has 10 Sushi domains. To explore the structure−function relationship of FXIII-B, we looked for domains in FXIII-B responsible for its homodimer and heterotetramer assembly with FXIII-A. Full-length recombinant human FXIII-B (rFXIII-B) and truncated rFXIII-Bs with various numbers of Sushi domains (rFXIII-B_{x-y}) were expressed in a baculovirus expression system. rFXIII-B was indistinguishable from purified human plasma FXIII-B, in terms of the molecular weight (after being deglycosylated by glycosidases) and the ability to form complexes between the two subunits. rFXIII-B was in dimer form and produced a heterotetramer complex with FXIII-A. Gel-filtration and FXIII-A binding analysis of the various truncated forms of rFXIII-B_{x-y} revealed that the first Sushi domain was responsible for the binding of FXIII-B to FXIII-A and that the fourth and ninth Sushi domains were involved in the FXIII-B homodimer assembly. rFXIII-B and rFXIII-B_{1-9}, which formed a heterotetramer complex with FXIII-A, protected FXIII-A from proteolytic digestion. These findings suggest that only full-length or nearly full-length FXIII-B is large enough to cover the exposed surface of FXIII-A. In conclusion, at least 3 out of the 10 Sushi domains of FXIII-B have the distinct function of forming a homodimer and a heterotetramer, which should be ascribed to the differences in their amino acid sequences. The present studies, however, do not exclude the possibility that additional Sushi domains may also support either or both functions.

Coagulation factor XIII (FXIII)[1]is a proenzyme of plasma transglutaminase that plays a critical role in the generation of a stable hemostatic plug. FXIII circulates in blood as a heterotetramer consisting of two A (FXIII-A) and two B subunits (FXIII-B) (*1, 2*). FXIII-A is a catalytic subunit of FXIII consisting of 731 amino acids and is composed of an amino-terminal activation peptide, β-sandwich, central core containing catalytic residues Cys-314, His-373, and Asp-396, and barrel 1 and 2 domains (*3–5*). FXIII-A also exists as a homodimer inside cells such as monocytes/macrophages (*6–11*) and megakaryocytes/platelets (*11, 12*). FXIII-B, consisting of 641 amino acids, contains 10 tandem repeats called Sushi domains or GP-I structures (*13–15*). Homologous Sushi domains have been found in more than 50 proteins/genes including β_2-glycoprotein I, the α subunit of C4 binding protein, complement receptor type II, complement factor H, etc. (*16*). FXIII-B is synthesized in liver, and half of the synthesized FXIII-B is free of FXIII-A in plasma (*17, 18*). FXIII-A-free FXIII-B is a filamentous and flexible strand; in contrast, cellular FXIII-A consists of two globular particles (*19*).

During coagulation, activation of FXIII is achieved by the cleavage, by thrombin, of an activation peptide in FXIII-A and the subsequent dissociation of FXIII-B from the cleaved FXIII-A (FXIII-A') in the presence of calcium ions (*20*). Congenital FXIII deficiency is caused by a defect in either the FXIII-A or FXIII-B gene. In patients with FXIII-A deficiency, plasma FXIII-A is undetectable and FXIII-A-associated FXIII-B is reduced, while the concentration of free FXIII-B remains constant (*17*). On the other hand, in patients with FXIII-B deficiency, FXIII-B is completely lost and plasma FXIII-A, with its short half-life, is greatly reduced, in spite of the presence of normal levels of FXIII-A in platelets (*21, 22*). Thus, heterotetramer assembly seems to be important for the stability of FXIII in plasma.

Although both the structure and function of FXIII-A have been studied extensively, little is known about the relationship between the structure and function of FXIII-B. In the present study, we explored the function of FXIII-B based on its structure *in vitro* by employing recombinant molecules.

MATERIALS AND METHODS

Materials. The recombinant FXIII-A (rFXIII-A) used in this study was the gift of Dr. P. Bishop of ZymoGenetics

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[1] Abbreviations: FXIII, factor XIII; FXIII-A, A subunits of FXIII; FXIII-B, B subunits of FXIII; rFXIII-A, recombinant FXIII-A; rFXIII-B, recombinant FXIII-B; FXIII-A', activated FXIII-A; PEG, polyethylene glycol; pFXIII-B, plasma-derived FXIII-B.

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Table 1: PCR Primers To Generate Human FXIII-B cDNAs[a]

Signal Sequence
SIG-F, 5′-AGG *AGA TCT* ATG AGG TTG AAA AAC CTG-3′
SIG-R, 5′-ACA *AAG CTT* CTC TTC TGC ATA GAG TTC-3′
Amino-Terminal Truncated cDNAs
S2F, 5′-TTC *AAG CTT* TGC ACT AAG CCT GAC CTG-3′ (for rFXIII-B_{2-10} from Cys71)
S4F, 5′-AAA *AAG CTT* TGC TCT TCT TTA AGA TTA-3′ (for rFXIII-B_{4-10} from Cys193)
S5F, 5′-AGA *AAG CTT* TGT CCT CCT CCA CCT CTG-3′ (for rFXIII-B_{5-10} from Cys254)
S6F, 5′-GAG *AAG CTT* GCC TGT GAG GAA CCA CC-3′ (for rFXIII-B_{6-10} from Ala315)
ER*, 5′-CAT *CTG CAG* TCA TGT TCT TAA GGG TTC TTG-3′
cDNAs Truncated at Carboxyl Terminals
SIG-F (see above)
S4R, TGG *CTG CAG* TCA TCT GTT TCT TCT TCC TTC-3′ (for rFXIII- B_{1-4} to Arg253)
S5R, 5′-TGG *CTG CAG* TCA GGC TAC CTT CTC CTG TCC-3′ (for rFXIII-B_{1-5} to Ala315)
S8R, 5′-AGG *CTG CAG* TCA CAT TCC TTT AGA TTC TTT-3′ (for rFXIII-B_{1-8} to Met503)
S9R, AGA *CTG CAG* TCA YGG CTC TAA ACA CAA TGG-3′ (for rFXIII-B_{1-9} to Pro561)

[a] Italic letters indicate sequences for restriction enzymes: *Bgl*II in SIG-F; *Hin*dIII in SIG-R, S2F, S4F, S5F, and S6F; *Pst*I in ER (*, reverse primer for the 3′-end of FXIII-B cDNA), S4R, S5R, S8R, and S9R.

(Seattle, WA). The FXIII-B was purified from pooled human plasma (*13*). An anti-human FXIII-A antibody was yielded by the immunization of purified human FXIII-A to rabbit (*3*) and was affinity-purified using rFXIII-A immobilized to cyanogen bromide-activated Sepharose 4B (Amersham Bioscience AB, Uppsala, Sweden). An anti-human FXIII-B antibody was purchased from Calbiochem (San Diego, CA). Bovine pancreas trypsin and bovine plasma thrombin (436 units/mg of protein) were purchased from Sigma (St. Louis, MO), and proteinase K (endopeptidase K) was from Wako Pure Chemical Ind. Ltd. (Tokyo, Japan).

Vector Construction. A human FXIII-B cDNA was inserted into transfer vector pBlueBacIII (Invitrogen, Carlsbad, CA; pBBIII-rFXIII-B). To generate amino-terminal truncation of FXIII-B, its signal sequence was amplified by PCR using specific primers (Table 1), in which a *Bgl*II site was introduced at the 5′-end for insertion into a vector and a *Hin*dIII site at the 3′-end to connect with Sushi domains. A PCR-amplified signal sequence was inserted into the *Bam*HI−*Hin*dIII sites of the pBlueBacIII vector (pBBIII-FXIII-B/SIG). Amino-terminal truncated cDNAs were generated by PCR using primers listed in Table 1. The amplified cDNAs were inserted into the *Hin*dIII−*Pst*I sites of pBBIII-FXIII-B/SIG. cDNAs truncated at carboxyl terminals were also generated by PCR, and the amplified cDNA was inserted into the *Bam*HI−*Pst*I sites of the pBlueBacIII vector.

Baculovirus Expression. A transfer vector was cotransfected to *Spodoptera frugiperda* (Sf) 21 cells with the Autographa californica nuclear polyhedrosis virus (AcNPV) DNA cut with *Sau*36I. The generated recombinant virus was isolated by a plaque assay. Sf21 cells were infected with a recombinant virus at a multiple of infection (moi) ≥ 1 in Sf900II serum-free medium (Invitrogen) for 4 days.

Purification of rFXIII-Bs. For purification of rFXIII-B and rFXIII-B_{2-10}, 30 g of polyethylene glycol (PEG) 6000 was added to 300 mL of the medium from Sf21 cells that were infected with recombinant baculovirus (rbv)FXIII-B or rbvFXIII-B_{2-10}. The sample was left on ice for 1 h. After centrifugation at 10000*g* for 20 min, the precipitate was dissolved with 15 mL of 20 mM Tris-HCl buffer of pH 6.8 (TB) and subjected to a DEAE-Sephadex A-50 column (10 mL of gel) preequilibrated with TB. The column was washed with 20 mL of TB, and proteins were eluted with a linear gradient of 0−0.5 M NaCl in TB. Fractions containing rFXIII-B were pooled and concentrated in a dialysis tube by 50% PEG 20000 in TB. The concentrate was subjected to a Sepharose CL6B column (10 mm i.d. × 45 cm) and eluted with TB. Fractions containing rFXIII-B were applied to a DEAE-Sephadex A-50 column (0.5 mL gel), and rFXIII-B was eluted with 1 mL of 0.3 M NaCl in TB.

For purification of rFXIII-B_{1-5}, proteins in 400 mL of medium from Sf21 cells infected with rbvFXIII-B_{1-5} were precipitated by the addition of 156 g of ammonium sulfate and dissolved with 10 mL of TB. The sample was desalted by a Sephadex G-25 column and applied to a phosphocellulose P-11 column (5 mL of gel) preequilibrated with TB. The column was washed with 10 mL of TB, and the proteins were eluted with a linear gradient of 0−0.5 M NaCl in TB. Fractions containing rFXIII-B_{1-5} were desalted and applied to a DEAE-Sephadex column (2 mL of gel) preequilibrated with TB. The column was washed with 10 mL of TB, and the proteins were eluted with a linear gradient of 0−0.5 M NaCl in TB. The rFXIII-B_{1-5} fractions were desalted and applied to a DEAE-Sephadex A-50 column (0.5 mL of gel), and rFXIII-B_{1-5} was eluted with 1 mL of 0.3 M NaCl in TB.

For partial purification of rFXIII-B_{1-9}, a 700 mL cultured medium containing Sf21 cells infected with rbvFXIII-B_{1-9} was dialyzed against 20 mM sodium phosphate (pH 6.4) and mixed with 200 mL of calcium phosphate gel (approximately 50% wet gel) at 4 °C for 2 h. After centrifugation, the gel pellet was washed twice with 200 mL of 20 mM sodium phosphate (pH 6.4), and the absorbed proteins were eluted with 100 mL of 0.2 M sodium phosphate (pH 7.2). The eluate was diluted 5-fold with ice-cold water and applied to a DEAE-Sephadex column (20 mL of gel). The column was washed with 40 mL of TB, and the proteins were eluted with a linear gradient of 0−0.5 M NaCl in TB. Fractions containing rFXIII-B_{1-9} were desalted and applied to a DEAE-Sephadex A-50 column (0.5 mL of gel), and rFXIII-B_{1-9} was eluted with 1 mL of 0.3 M NaCl in TB.

All purified rFXIII-B proteins were quantified as samples by densitometry after sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis (PAGE), followed by Coomassie brilliant blue (CBB) staining. Estimated yields of various rFXIII-B preparations were as follows: rFXIII-B, 0.5 mg; rFXIII-B_{2-10}, 0.3 mg; rFXIII-B_{1-9} and rFXIII-B_{1-5}, 0.2 mg. The purity of all preparations was estimated to be 50−60%. Other truncated rFXIII-Bs could not be purified because the yields of these mutants were too low in the medium.

Glycosidase Treatment. Five micrograms of FXIII-B, that had been obtained from human plasma and rFXIII-B, was treated overnight with 0.2 unit of *N*-glycosidase F and/or 1 milliunit of *O*-glycosidase (Roche, Mannheim, Germany) in a 10 μL mixture of 50 mM sodium phosphate (pH 7.2), 0.05% SDS, and 0.5% Triton X-100 at 37 °C. The reaction was terminated by boiling with an equal volume of 125 mM Tris-HCl (pH 6.8), 2% SDS, 15% glycerol, 0.02% bromphe-

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REGION   NUMBER    SEQUENCE (ONE LETTER CODE)
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SIGNAL    -19     MRLKNLTFIIILIISGELYA
                    ↓rFXIII-BN, B1-9N, B1-8N, B1-5N, B1-4N
SUSHI 1     1       EEKP CGFPHVENGRIAQYYYTFKSFYFPMSIDKKLSFF CLAGYTTESGRQEEQTT    CTTEGWSPEPRC
                         ↓rFXIII-B2-10N
SUSHI 2    68         FKK CTKPDLSNGYISDVKLLYKIQENMRYG        CASGYKTTGGKDEEVVQ    CLSDGWSSQPTC

SUSHI 3   127      RKEHET CLAPELYNGNYSTTQKTFKVKDKVQYE        CATGYYTAGGKKTEEVE    CLTYGWSLTPKC
                          ↓rFXIII-B4-10N
SUSHI 4   189        TKLK CSSLRLIENGYFHPVKQTYEEGDVVQFF        CHENYYLSGSDLIQ       CYNFGWYPESPVC
                           ↓rFXIII-B5-10N
SUSHI 5   248      EGRRNR CPPPPLPINSKIQTHSTTYRHGEIVHIE        CELNFEIHGSAEIR       CEDGKWTEPPKC
          rFXIII-B1-4C↑
                           ↓rFXIII-B6-10N
SUSHI 6   308    IEGQEKVA CEEPPFIENGAANLHSKIYYNGDKVTYA        CKSGYLLHGSNEIT       CNRGKWTLPPEC
          rFXIII-B1-5C↑
SUSHI 7   370      VENNEN CKHPPVVMNGAVADGILASYATGSSVEYR        CNEYYLLRGSKISR       CEQGKWSSPPVC

SUSHI 8   431         LEP CTVMVDYMNRNNIEMKMKYEGKVLHGDLIDFV     CKQGYDLSPLTPLSELSVQ   CNRGEVKYPLC

SUSHI 9   496    TRKESKGM CTSPPLIKHGVIISSTVDTYENGSSVEYR      CFDHHFLEGSREAY        CLDGMWTTPPLC
          rFXIII-B1-8C↑
SUSHI 10  559         LEP CTLSFTEMEKNNLLLKMDFDNRPHILHGEYIEFI CRGDTYPAELYITGSILRMQ CDRGQLKYPRC
          rFXIII-B1-9C↑
C-TERM    627    IPRQSTLSYQEPLRT-641
                              ↑rFXIII-BC, B6-10C, B5-10C, B4-10C, B2-10C
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FIGURE 1: N- and C-termini of truncated recombinant FXIII-Bs (rFXIII-Bs). Amino acid sequences of 10 Sushi domains as well as the signal peptide and the C-terminal tail of FXIII-B are shown in one-letter code. Downward and upward arrows indicate the N- and C-termini of truncated rXIII-Bs, respectively. Letters N and C at the end of each rFXIII-B_{x-y} stand for its N- and C-termini, respectively. The number prior to each Sushi domain identifies the residue number of the first amino acid residue in each row.

nol blue, and 5% β-mercaptoethanol (sample solution). A sample was subjected to SDS−PAGE and stained with CBB.

Gel-Filtration Analysis. A 2 mL medium of Sf21 cells infected with the recombinant virus was concentrated to approximately 0.5 mL in a dialysis tube by 50% PEG 20000 in 50 mM Tris-HCl (pH 7.5) and 150 mM NaCl (TBS) and was dialyzed against TBS. Two hundred microliters of the concentrated medium was mixed with 0.1% blue dextran, 0.5 mg of thyroglobulin, 10 μg of lactate dehydrogenase, 0.2 mg of bovine serum albumin, and 0.5 mg of ovalbumin, with or without 10 μg of rFXIII-A, and subjected to a Sepharose CL6B column (10 mm i.d. × 45 cm). Elution was performed using TBS at a flow rate of 8 mL/h, and 0.8 mL fractions were collected. Ten microliters of the fractions was analyzed for FXIII-B by an enzyme-linked immunosorbent assay (ELISA) using an anti-FXIII-B antibody. The fractions were also analyzed by Western blotting using an anti-human FXIII-B antibody. Elution of the protein standards was monitored by densitometry of SDS−PAGE gels, followed by CBB staining, and then by an enzyme assay for lactate dehydrogenase.

Native PAGE. One microgram of rFXIII-B, rFXIII-B_{1-9}, or rFXIII-B_{2-10} or 0.5 μg of FXIII-B_{1-5} was mixed with 0.5, 1, or 2 μg of rFXIII-A in a 10 μL mixture and left on ice for 20 min. One microgram of rFXIII-A was also treated with 0.1 μg of thrombin in TBS at 37 °C for 15 min and mixed with 1 μg of rFXIII-B or rFXIII-B_{1-9} or 0.5 μg of rFXIII-B_{1-5} in TBS with or without 5 mM $CaCl_2$. Two microliters of 50% glycerol and 0.1% bromphenol blue was added, and a sample was electrophoresed using a 6% polyacrylamide gel containing 45 mM Tris-HCl (pH 8.8) and a running buffer of 25 mM Tris and 192 mM glycine. The gel was stained with CBB.

Proteolytic Degradation of rFXIII-A in the Presence of rFXIIIBs. One microgram of rFXIII-A was mixed with 1 μg of rFXIII-B, rFXIII-B_{1-9}, or rFXIII-B_{2-10}, 0.5 μg of rFXIII-B_{1-5}, or 1 μg of BSA in a total volume of 50 μL of TBS and kept on ice for 20 min. Ten microliters of the mixture was divided into four tubes and reacted with 10 μL of 0, 1, 3, or 10 μg/mL trypsin or 0, 1, 2, or 4 μg/mL proteinase K at 37 °C for 60 min. The reaction was terminated by boiling with 80 μL of the sample solution. A sample was subjected to SDS−PAGE followed by Western blotting using an anti-FXIII-A antibody.

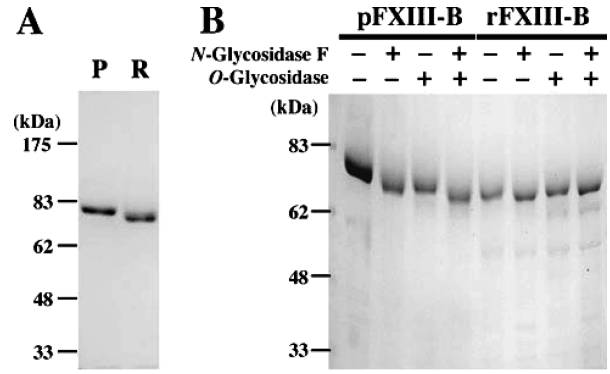

FIGURE 2: Expression of FXIII-B. rFXIII-B was expressed by a baculovirus expression system and partially purified from a culture medium of recombinant virus-infected Sf21 cells. (A) rFXIII-B (R) and human plasma FXIII-B (P) were subjected to SDS−PAGE under reducing conditions. The gel was stained with CBB. (B) rFXIII-B and pFXIII-B were incubated at 37 °C overnight in the presence (+) or absence (−) of *N*-glycosidase F and/or *O*-glycosidase; for example, the presence of both glycosidases is depicted by +, +. Sample proteins were subjected to SDS−PAGE under reducing conditions and stained with CBB.

RESULTS

Dimer Assembly of FXIII-B. In order to produce the amount of recombinant proteins including deletion mutants necessary for the present study (Figure 1), FXIII-B was expressed by a baculovirus expression system and partially purified from a culture medium of recombinant virus-infected cells. rFXIII-B (R in Figure 2A) migrated on SDS−PAGE faster than human plasma FXIII-B (pFXIII-B; P in Figure 2A), suggesting a difference in carbohydrate moieties in rFXIII-B, which would be consistent with its heterologous expression in the baculovirus system. Since the migration of rFXIII-B was slightly altered by *N*-glycosidase treatment but not by *O*-glycosidase treatment, it was concluded that

rFXIII-B was slightly *N*-glycosylated but not *O*-glycosylated (Figure 2B, right). Removal of sugar chains from pFXIII-B by treatment with both *N*-glycosidase F and *O*-glycosidase resulted in its migrating faster than untreated pFXIII-B (Figure 2B, left) up to the same position as rFXIII-B treated with *N*-glycosidase F.

Gel-filtration analysis was performed to confirm whether or not rFXIII-B was capable of forming a homodimer. The rFXIII-B elution peak was observed by ELISA in a fraction corresponding to the molecular mass of 150 kDa, estimated by calibration using standard proteins, although the elution pattern was relatively broad (Figure 3, Table 2). The results of this procedure suggested that rFXIII-B was in dimer form.

rFXIII-Bs truncated by the deletion of varying numbers of Sushi domains were constructed to determine which Sushi domain(s) was (were) responsible for the homodimer assembly of FXIII-B (Figure 1). A medium for the cells infected with the recombinant virus was concentrated and subjected to gel-filtration analysis. All rFXIII-Bs lacking the ninth Sushi domain (rFXIII-B_{1-4}, rFXIII-B_{1-5}, rFXIII-B_{1-6}, and rFXIII-B_{1-8}) were in monomer form, while rFXIII-B_{1-9} that lacked the 10th Sushi domain alone could form a homodimer (Figure 3, Table 2). By contrast, amino-terminal truncations from the first to third Sushi domains (rFXIII-B_{2-10} and rFXIII-B_{4-10}) did not affect the FXIII-Bs' capacity for dimer formation, and additional removal of the fourth Sushi domain (rFXIII-B_{5-10} and rFXIII-B_{6-10}) resulted in the disappearance of the dimer form in spite of the presence of the ninth Sushi domain. Accordingly, it was found that both the fourth and ninth Sushi domains were essential for dimer formation of FXIII-B.

Heterotetramer Formation with FXIII-A. To examine whether rFXIII-B could bind to FXIII-A, rFXIII-B was mixed with rFXIII-A and subjected to gel-filtration chromatography. In the presence of rFXIII-A, rFXIII-B was eluted into fractions corresponding to the estimated molecular mass of 380 kDa (Figure 3A, Table 2), the elution of which occurred much sooner than that in the absence of rFXIII-A.

Native PAGE was also carried out to confirm the binding of rFXIII-B to rFXIII-A after mixing at various molar ratios. In native PAGE, rFXIII-B migrated to a position similar to that of pFXIII-B (Figure 4A). When a small amount of rFXIII-A was added, a new band appeared and migrated more slowly than either rFXIII-B or rFXIII-A, indicating a complex formation between rFXIII-A and either pFXIII-B or rFXIII-B. The complex formation was not affected by the treatment of FXIII-Bs with *N*-glycosidase, an observation that would seem to exclude any possible involvement of FXIII-Bs' sugar chains in the binding to rFXIII-A. These results were consistent with the fact that the removal of sugar chains from FXIII-B did not affect its ability to form a complex with rFXIII-A, which is similar to a previous report (*23*). This rFXIII-A/rFXIII-B complex increased with each increment of added rFXIII-A, while the free rFXIII-B diminished at the same time (Figure 4B, top). When an amount of rFXIII-A in excess of rFXIII-B was added, the free rFXIII-A also appeared, indicating that the stoichiometric 1:1 molar ratio was the best condition under which a complex could form between rFXIII-B and rFXIII-A. These results are consistent with the formation of an A_2B_2 ensemble (in tetramer form) based on the molecular mass determined by gel-filtration analysis (Figure 3A, Table 2).

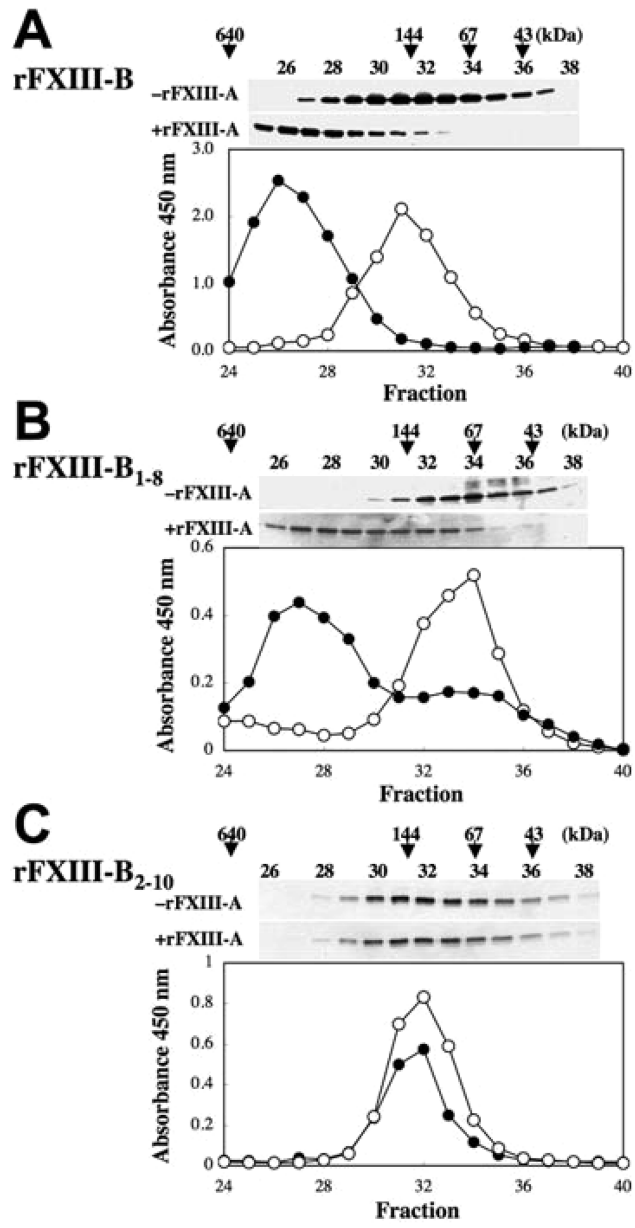

FIGURE 3: Gel-filtration analysis of rFXIII-Bs with or without rFXIII-A. Fractions obtained from gel-filtration chromotograpy using a Sepharose CL6B column (10 mm i.d. and 45 cm in length) were analyzed by SDS−PAGE followed by ELISA (bottom) and/or Western blotting (top) using an anti-FXIII-B antibody. Peaks of standard proteins (thyroglobulin, 640 kDa; lactate dehydrogenase, 144 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa) are shown by arrows on the top. Analyzed were 14 consecutive fractions for rFXIII-B (A) and rFXIII-B_{1-8} (B) and 12 fractions for rFXIII-B_{2-10} (C). rFXIII-Bs were detected by ELISA at 450 nm. Open circles, without rFXIII-A; closed circles, with 10 μg of rFXIII-A.

To determine which Sushi domain(s) of FXIII-B contribute(s) to its binding to FXIII-A, rFXIII-A was added to a concentrated medium containing various truncated rFXIII-Bs and subjected to gel-filtration chromatography. In the presence of rFXIII-A, rFXIII-Bs having the first Sushi domain (rFXIII-B_{1-9}, rFXIII-B_{1-8}, rFXIII-B_{1-6}, rFXIII-B_{1-5}, and rFXIII-B_{1-4}) were eluted in fractions of higher molecular mass than in the absence of rFXIII-A (Figure 3, Table 2). In contrast, rFXIII-Bs without the first Sushi domain (rFXIII-B_{6-10}, rFXIII-B_{5-10}, rFXIII-B_{4-10}, rFXIII-B_{2-10}) demonstrated no change in their elution profiles by the addition of rFXIII-

Table 2: Molecular Sizes of Truncated rFXIII-Bs Estimated by Gel-Filtration Chromatography

	molecular size (kDa)				
construct	−rFXIIIA	predicted[a]	M/D[b]	+rFXIIIA	complex
rFXIII-A				150	
rFXIII-B	150	73.6	D	380	+
rFXIII-B_{1-9}	120	63.7	D	280	+
rFXIII-B_{1-8}	78	57.6	M	260	+
rFXIII-B_{1-5}	44	36.6	M	250	+
rFXIII-B_{1-4}	41	29.6	M	210	+
rFXIII-B_{6-10}	47	37.1	M	52	−
rFXIII-B_{5-10}	49	44.0	M	49	−
rFXIII-B_{4-10}	100	51.8	D	110	−
rFXIII-B_{2-10}	130	65.0	D	130	−

[a] Based on the amino acid sequence of monomeric FXIII-B. [b] Monomer (M)/dimer (D).

A. Thus, it was evident that the first Sushi domain in FXIII-B was responsible for its binding to FXIII-A.

Partially purified rFXIII-B_{1-9}, rFXIII-B_{1-5}, and rFXIII-B_{2-10} were also subjected to native PAGE analysis after mixing with rFXIII-A in varying molar ratios. rFXIII-B_{1-9} and rFXIII-A formed a complex at the molar ratio of 1:1, as did rFXIII-B (Figure 4B). Although rFXIII-B_{1-5} was in monomer form, it could make a complex with rFXIII-A at the molar ratio of 1:1, indicating that the rFXIII-A/rFXIII-B_{1-5} complex was in an A_2B_2 heterotetramer form. Again, no complex formation between rFXIII-B_{2-10} and rFXIII-A was observed, as shown by gel-filtration analysis (Figure 3C).

It is known that FXIII-B dissociates from activated FXIII-A by thrombin (FXIII-A') in the presence of calcium ions. In the present study, calcium ions did not affect the ability of rFXIII-Bs to form a complex with rFXIII-A (Figure 4C). When rFXIII-A was pretreated with thrombin before mixing with rFXIII-Bs, the resulting A_2B_2 complexes clearly decreased, in the absence of calcium ions, and almost completely disappeared, in the presence of calcium ions, regardless of which of the three forms of rFXIII-B was employed. These results indicate that the binding capacity of FXIII-B to FXIII-A' is much lower in the presence of calcium ions than in their absence.

Protective Effect of FXIII-B on Proteolytic Degradation of FXIII-A. We previously showed that FXIII-B could stabilize wild-type FXIII-A but not mutant FXIII-A, which demonstrated impaired complex formation with FXIII-B *in vitro* (*24*). To examine whether FXIII-B could protect FXIII-A from proteolytic degradation *in vitro*, rFXIII-A was mixed with various types of rFXIII-Bs and treated with trypsin or proteinase K. rFXIII-A that had been mixed with BSA was nearly completely digested with a minimal amount of trypsin (Figure 5A). In the presence of rFXIII-B, however, the digestion of rFXIII-A with trypsin yielded a 71 kDa fragment corresponding to that generated by thrombin treatment (*24*). This 71 kDa fragment remained even after treatment with the highest amount of trypsin. A similar protective pattern of tryptic digestion of rFXIII-A was observed in the presence of either rFXIII-B_{1-9} or rFXIII-B_{1-5}. In contrast, the total amount of rFXIII-A rapidly decreased, as was seen in the case of BSA when rFXIII-A was treated with trypsin in the presence of rFXIII-B_{2-10}.

rFXIII-B and rFXIII-B_{1-9} also protected rFXIII-A from digestion with proteinase K treatment, although only small amounts of the 71 kDa fragment were observed (Figure 5B). On the other hand, rFXIII-A in the mixture with rFXIII-B_{2-10} was as sensitive to proteinase K digestion as it was in the mixture with BSA. rFXIII-A in the mixture with rFXIII-B_{1-5} was also sensitive to proteinase K digestion. It is of interest, however, that the 71 kDa fragment was produced in the sample of rFXIII-A treated with proteinase K in the presence of rFXIII-B_{1-5} but not in the presence of either BSA or rFXIII-B_{2-10}, both of which lacked the ability to specifically bind FXIII-A. These findings suggest the existence of differential substrate specificities between the trypsin and subtilisin subfamilies of serine proteases: Trypsin prefers to cleave the C-terminal peptide bonds of Arg and Lys residues, whereas proteinase K is rather unspecific and hydrolyzes mainly at the C-terminal ends of aromatic or aliphatic amino acids.

DISCUSSION

In this study we describe the relationship between the oligomer assembly of FXIII-B and its function *in vitro*. Seelig and Folk (*25*) first determined by gel-filtration chromatography and electrophoresis that plasma-derived FXIII-B (pFXIII-B) existed as a "dimer". By contrast, Carrell et al. (*19*) demonstrated by sedimentation analysis that pFXIII-B was "monomeric". There is no good explanation for this discrepancy. The pFXIII-B specimens used in their studies, however, were treated with thrombin in the presence of calcium ions to separate FXIII-B from FXIII-A, and thus the specimens might have been modified by proteolytic action: During purification from plasma or after separation from pFXIII-A, dimeric pFXIII-B might have become a monomer, or *vice versa*. Alternatively, some polymorphisms in FXIII-B may be related to the differences in the monomer/dimer conformations.

We had previously shown by gel-filtration chromatography that rFXIII-B expressed in mammalian BHK cells was in dimer form (*26*). In the present study, rFXIII-B synthesized by a baculovirus expression system also formed a homodimer. Accordingly, it is evident that native rFXIII-B forms a homodimer at least when it is synthesized under FXIII-A-free conditions. The present studies also revealed that rFXIII-B is capable of forming a heterotetramer complex with FXIII-A. Moreover, both the native free pFXIII-B and rFXIII-Bs stoichiometrically formed an indistinguishable A_2B_2 heterotetramer with rFXIII-A. It is of note that "monomeric" mutant rFXIII-A formed an A_2B_2 heterotetramer with native free pFXIII-B purified from plasma without thrombin treatment (*24*). This fact also supports our conclusion that at least native human pFXIII-B is in dimer form.

Analysis of various truncated rFXIII-Bs clearly indicated that both the fourth and ninth Sushi domains were required for homodimer assembly and that the first Sushi domain was the site for binding to FXIII-A. FXIII-B bound to FXIII-A at a molar ratio of 1:1, consistent with the well-established theory. It is of interest that even "monomeric" rFXIII-Bs (rFXIII-B_{1-4}, rFXIII-B_{1-5}, and rFXIII-B_{1-8}) formed a heterotetramer with a FXIII-A dimer. Since a "monomeric" mutant FXIII-A also formed a heterotetramer with FXIII-B (*24*), a stable heterotetramer could be generated when either one of the two subunits was in dimer form. In theory, it may also be possible that a hetero "trimer", such as A_2B and AB_2,

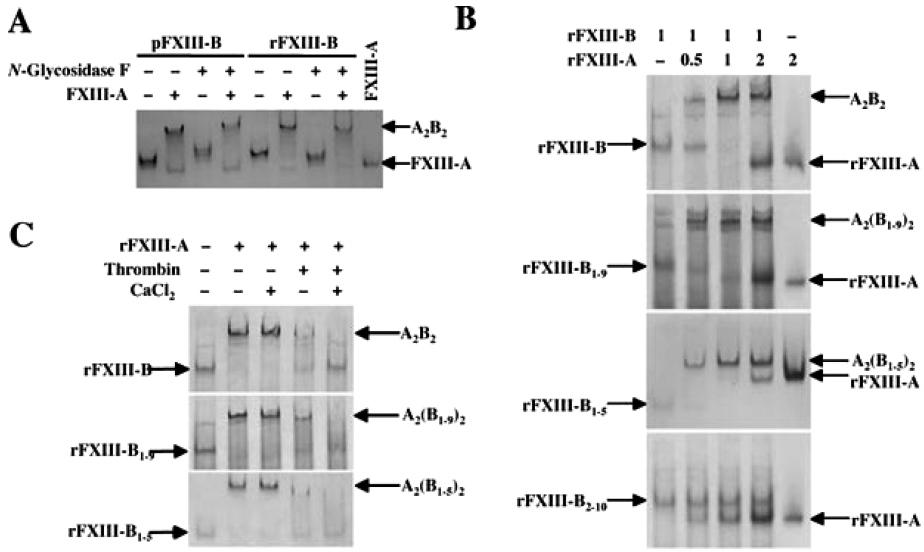

FIGURE 4: Complex formation of rFXIII-Bs with rFXIII-A. (A)1 μg of pFXIII-B or rFXIII-B was mixed with 2 μg of rFXIII-A and loaded onto native PAGE. The gel was stained with CBB. (B) Partially purified rFXIII-Bs were loaded onto native PAGE after mixing with rFXIII-A at varying molar ratios (indicated by numbers on the top). (C) Binding of rFXIII-Bs to thrombin-treated rFXIII-A in the absence or the presence of 5 mM $CaCl_2$.

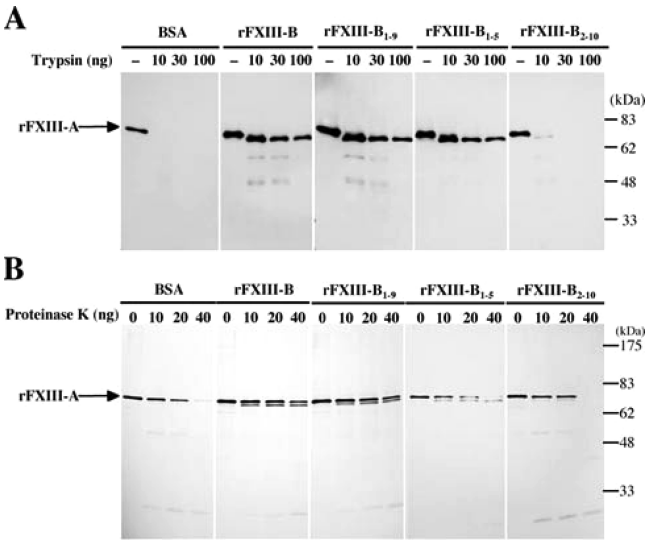

FIGURE 5: Proteolytic digestion of rFXIII-A in the presence of rFXIII-Bs. rFXIII-A was mixed with rFXIII-Bs at a molar ratio of 1:1 and treated with varying amounts of trypsin [(A) 0, 1, 3, or 10 μg/mL] or proteinase K [(B) 0, 1, 2, or 4 μg/mL] at 37 °C for 60 min. Samples were analyzed by Western blotting using an anti-FXIII-A antibody.

can form under certain conditions where either FXIII-A or FXIII-B "dimers" exist in excess of the other subunit monomers.

Radek et al. reported that pFXIII-B, treated with neuraminidase or with *N*-glycosidase F to remove sialic acid and carbohydrate moieties, formed an A_2B_2 complex with rFXIII-A (*23*). The present studies also demonstrated that insect cell-derived rFXIII-B is capable of forming an indistinguishable stoichiometric A_2B_2 heterotetramer with rFXIII-A. This occurs independent of sugar chains, since rXIII-B is synthesized with different carbohydrate attachments by insect (in the present study) and mammalian (in ref *26*) cells. These findings suggest that the heterologous binding sites of FXIII-B to FXIII-A reside in the protein part and not in the carbohydrate attachments of the FXIII-B molecule. In contrast to our results, higher ordered oligomers

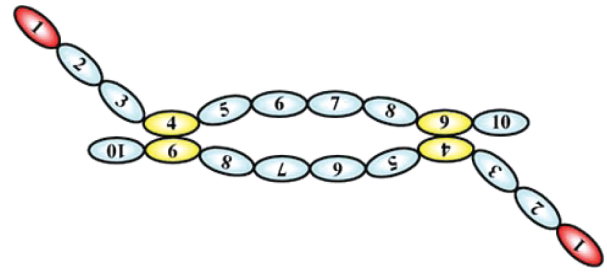

FIGURE 6: Proposed model for the FXIII-B homodimer. How two molecules of FXIII-B may form a homodimer in symmetric configuration due to the interactions between the fourth and ninth Sushi domains (yellow). FXIII-B should be a filamentous and flexible strand as shown by electron microscopy (ref *19*).

(A_2B_n, where $n > 2$) also formed in the presence of excess FXIII-B in Radek's work, even though the stoichiometric A_2B_2 heterotetramer remained the main product of assembly (*23*). The reason of these contrasting findings is unknown. Radek's SDS−PAGE system may have been of higher resolution than ours. The higher ordered oligomers, however, may be artifacts, because such products are not observed in human plasma despite the presence of FXIII-B in excess amounts of about 2-fold of FXIII-A.

Since the crystal structure of FXIII-B is not available yet, it is only possible to theorize that the fourth Sushi domain of one FXIII-B molecule binds to the ninth Sushi domain of the other half of the dimer, likely resulting in a symmetric orientation of both molecules (Figure 6). The possible symmetric orientation of FXIII-B is plausible because the FXIII-A dimer itself is symmetrical (*5, 27*). The first to third and the tenth Sushi domains may be free, and the FXIII-B molecule is flexible as shown by electron microscopy (*19*); in so being, FXIII-B may bind to FXIII-A via the first Sushi domain. It is possible that the first Sushi domain may bind to barrels 1 and/or 2 of FXIII-A, because FXIII-A lacking either barrel 1 or barrel 2, or both, demonstrated no binding to FXIII-B (*24*). Alternatively, the overall structure of barrels 1 and/or 2-less FXIII-A may be quite different from the intact molecule, which may not allow FXIII-B to bind.

Along this line, it is of particular interest that the active form of human tissue TGase is in an extended "open" conformation with the two C-terminal barrel domains displaced by 120 Å (*28*), while the inactive tissue TGase is in a "closed" form. Similarly, the activation of FXIII-A may occur sequentially in concert: (1) the activation peptide of FXIII-A is cleaved by thrombin, (2) FXIII-B dissociates from FXIII-A' in the presence of calcium ions, (3) the two C-terminal barrel domains extend, (4) the "closed" conformation of FXIII-A' converts to its "open" form, and (5) the buried active site becomes exposed to its large substrates. The removal of FXIII-B from FXIII-A' may occur in conjunction with the extension of the two barrel domains, resulting in the unmasking of the active site.

In this study, rFXIII-B was shown to protect most of FXIII-A from proteolytic degradation, indicating that binding with FXIII-B masks various protease cleavage sites in the FXIII-A molecule. The protective effect was similar in rFXIII-B_{1-9} but seemed to be less in rFXIII-B_{1-5}. rFXIII-B_{1-5} was probably too small to cover the FXIII-A molecule, and/or the binding of rFXIII-B_{1-5} to FXIII-A might be inconsistent and unstable because rFXIII-B_{1-5} itself was "monomeric". A cleavage site at the C-terminal end of the activation peptide of FXIII-A protrudes from the FXIII-A structure (*27*) and is therefore likely to be exposed on the surface of the heterotetramer complex, which must allow the activation of FXIII by thrombin and the cleavage by trypsin even in the presence of bound FXIII-B.

It is also important to recognize that the thrombin-catalyzed removal of the activation peptide from FXIII-A causes a significant reduction in the strength of binding to FXIII-B (ref *23*). This is consistent with our results that the thrombin-treated rFXIII-A bound various forms of rFXIII-Bs much less efficiently, indicating an increased tendency of the A_2B_2 ensemble to release a FXIII-B dimer after activation by thrombin, especially in the presence of calcium ions even at a concentration as low as 5 mM.

It is very likely that in three Sushi domains of FXIII-B the distinct functions of forming a homodimer and a heterotetramer are ascribed to the differences in their amino acid sequences. However, the present studies do not exclude the possibility that additional Sushi domains may also be required for both functions. In addition, the position of each functional Sushi domain may be important if it turned out to physically constrain the region of FXIII-A with which it would bind to FXIII-B.

A naturally occurring Cys430Phe (in the seventh Sushi domain) mutant of FXIII-B cannot be secreted from synthesizing cells (*29*), hepatocytes *in vivo*, and thus unlikely contacts with FXIII-A in circulation. In contrast, a His95Arg variant, a polymorphic allele of the FXIII-B gene, is in fact present in plasma and forms a complex with FXIII-A (*30*). Since His95Arg exists in the second Sushi domain, adjacent to the first Sushi domain that binds to FXIII-A, it is possible that the amino acid substitution of His95 by Arg may lead to a drastic conformational change(s) in the first Sushi domain as well as in the second Sushi domain through unknown interactions between these two Sushi domains. However, purified His95 and Arg95 proteins did not show significant differences in the dissociation constant of FXIII-A:FXIII-B interaction, although increased subunit dissociation was found in plasma obtained from subjects possessing the Arg95 allele (*30*). Accordingly, other plasma components and/or common polymorphisms of FXIII-A may be responsible for the differential dissociated fractions between His95 and Arg95.

Sushi domains have been found in more than 50 proteins/genes including β_2-glycoprotein I, the α subunit of C4 binding protein, complement receptor type II, complement factor H, etc. (*16*). When we carried out an homology search of the amino acid sequence of FXIII-B, the third to seventh Sushi domains showed sequence similarity with other proteins such as complement factor H, complement receptor types I and II, CD46, C4-binding protein, etc., while the first, second, eighth, and tenth Sushi domains revealed low degrees of homology with other proteins (data not shown). In the present study, we found that the relatively unique first Sushi domain of FXIII-B contributed to its specific binding to FXIII-A. Since other Sushi domains in FXIII-B have sequence homology with several proteins in the complement system, it is fair to say that FXIII could also be involved in such a system.

In our previous work, we analyzed in detail a Tyr283Cys mutant of rFXIII-A, using a [^{35}S]methionine-metabolic labeling procedure (*24*). The Tyr283 residue is close to the active site Cys314 on a large surface loop of the core domain; its interactions seem to be critical for securing the loop to the rest of the core domain as well as securing the barrel 1 domain to the core domain (*31*). The Tyr283Cys mutation hindered the formation of not only the FXIII-A homodimer completely but also the A_2B_2 heterotetramer significantly, implying that two Tyr283 residues on the surface of the large "concave" of a FXIII-A dimer may be involved to some extent in the heterotetramerization with a FXIII-B dimer. In addition, both mutants Ile464Stop (truncated at the C-terminal third of the core domain) and Gly562Arg (located in the first barrel domain) did not form a complex with FXIII-B at all, suggesting that the C-terminal barrel domains of FXIII-A are important for the A_2B_2 assembly.

Finally, the demonstrated ability of rFXIII-B to bind to FXIII-A in a combination analogous to that of a plasma FXIII ensemble is encouraging for the possible use of rFXIII-B in clinical situations, especially for the treatment of acute bleeding events in patients with both congenital and acquired FXIII-B deficiency. This assumption is consistent with the fact that the injection of only a small amount of rFXIII-B was seen to increase FXIII-A levels in FXIII-B knockout mice (*28*). Thus, heterotetramer assembly seems to be important for the stability of FXIII in blood circulation. Replenishing the missing FXIII-B together with administering recombinant FXIII-A seems to be the most rational therapy when, in coming years, plasma-derived FXIII concentrates are abandoned because of possible contamination with infectious pathogens such as prions and viruses. In fact, conventional plasma-derived concentrates of factors VIII and IX are already being replaced with recombinant products in most countries in the world.

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performed research. A.I. designed the research, analyzed data, and wrote the paper.

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