Molecular cloning and biochemical characterization of rabbit factor XI

Dipali SINHA*[1], Mariola MARCINKIEWICZ*, David GAILANI† and Peter N. WALSH*

*The Sol Sherry Thrombosis Research Center, Department of Biochemistry, Temple University School of Medicine, 3400 North Broad Street, Philadelphia, PA 19140, U.S.A., and †Departments of Pathology and Medicine, Vanderbilt University, Nashville, TN 37232, U.S.A.

Human factor XI, a plasma glycoprotein required for normal haemostasis, is a homodimer (160 kDa) formed by a single interchain disulphide bond linking the Cys-321 of each Apple 4 domain. Bovine, porcine and murine factor XI are also disulphide-linked homodimers. Rabbit factor XI, however, is an 80 kDa polypeptide on non-reducing SDS/PAGE, suggesting that rabbit factor XI exists and functions physiologically either as a monomer, as does prekallikrein, a structural homologue to factor XI, or as a non-covalent homodimer. We have investigated the structure and function of rabbit factor XI to gain insight into the relation between homodimeric structure and factor XI function. Characterization of the cDNA sequence of rabbit factor XI and its amino acid translation revealed that in the rabbit protein a His residue replaces the Cys-321 that forms the interchain disulphide linkage in human factor XI, explaining why rabbit factor XI is a monomer in non-reducing SDS/PAGE. On size-exclusion chromatography, however, purified plasma rabbit factor XI, like the human protein and unlike prekallikrein, eluted as a dimer, demonstrating that rabbit factor XI circulates as a non-covalent dimer. In functional assays rabbit factor XI and human factor XI behaved similarly. Both monomeric and dimeric factor XI were detected in extracts of cells expressing rabbit factor XI. We conclude that the failure of rabbit factor XI to form a covalent homodimer due to the replacement of Cys-321 with His does not impair its functional activity because it exists in plasma as a non-covalent homodimer and homodimerization is an intracellular process.

Key words: homodimer, rapid amplification of cDNA ends (RACE), reverse transcription PCR (RT-PCR).

INTRODUCTION

Human factor XI (HFXI) is a glycoprotein that consists of two identical polypeptide chains held together by a single disulphide bond [1]. It is present in plasma as a zymogen at a concentration of $\approx$ 30 nM [2]. Dimeric factor XI (FXI) circulates in plasma in complex with high-molecular-mass kininogen [3]. In human, bovine, porcine and mouse species each subunit of FXI runs as an 80 kDa protein in SDS/PAGE under reducing conditions, whereas the dimer runs as a 160 kDa protein under non-reducing conditions [3–6]. The activated form of FXI (FXIa), a serine protease that activates factor IX (FIX), thereby initiating the intrinsic or consolidation phase of coagulation, consists of an N-terminal heavy chain and a C-terminal trypsin-like catalytic domain [7,8]. The heavy chain of FXI consists of four homologous domains called Apple domains (designated A1, A2, A3 and A4) [1]. The amino acid composition of FXI is 58 % identical with another plasma protein, prekallikrein (PK) [9]. As in FXI, the N-terminal region of PK also has four Apple domains [10]. PK, however, is a monomer. The Cys residue at position 321 in the A4 domain of FXI is involved in the formation of the interchain disulphide bond between the two subunits, whereas the same residue in PK forms an intra-chain disulphide bond with Cys-326. In FXI the residue at position 326 is a Gly.

Unlike HFXI, bovine FXI or mouse FXI, rabbit FXI (RFXI) migrates as a monomer of 80 kDa under both non-reduced and reduced conditions [11], and therefore serves as an appropriate model to examine the functional properties of FXI in relation to its homodimeric structure. We have characterized the complete cDNA sequence of RFXI and studied the physical and functional properties of FXI purified from rabbit plasma. The present manuscript describes a procedure used for characterizing the full-length cDNA sequence of RFXI that did not require a cDNA library, and demonstrates that the rabbit protein is a non-covalently associated homodimer with functional activities very similar to HFXI.

EXPERIMENTAL

Rabbit liver poly(A) RNA was obtained from Clontech (Palo Alto, CA, U.S.A.). Pooled normal plasma and FXI-deficient plasma were from George King Biomedical (Overland Park, KS, U.S.A.). HFXI, HFXIa, activated factor XII (FXIIa), FIX, factor X (FX) and PK were purchased from Enzyme Research Laboratories (South Bend, IN, U.S.A.). Rabbit plasma was from Pel-Freez Biologicals (Rogers, AK, U.S.A.). Recombinant FVIII was from Baxter/Hyland (Glendale, CA, U.S.A.). Expression of recombinant FXI (wtFXI) and its mutant FXI-Ala321 were as described earlier [12,13]. Chromogenic substrates S-2366 (L-pyroglutamyl-L-prolyl-L-arginine-*p*-nitroaniline) and S-2765 (*N*-α-benzoxycarbonyl-D-arginyl-glycyl-L-arginine-*p*-nitroaniline) were from DiaPharma (Westchester, OH, U.S.A.).

RFXI cDNA

An overview of the strategy of obtaining the full-length cDNA of RFXI is shown in Figure 1. The procedure consisted of three rounds of reverse transcriptase (RT)-PCR followed by 5′ and 3′ rapid amplification of cDNA ends (RACE) reactions. In all RT reactions described, the Superscript II™ preamplification system (Life Technologies) was used for first-strand synthesis following the protocol described by the manufacturer and using rabbit liver mRNA as the template and oligo(dT) as the primer. Briefly, the RNA/primer mixture was first heated at 70 °C for 10 min followed by cooling in ice. The mixture then had the following

Abbreviations used: FXI, factor XI; HFXI, human FXI; RFXI, rabbit FXI; FXIa, activated FXI; FIX, factor IX; FXIIa, activated factor XII; FX, factor X; PK, prekallikrein; RT, reverse transcriptase; RT-RNA, reverse-transcribed mRNA; RACE, rapid amplification of cDNA ends.

[1] To whom correspondence should be addressed (e-mail dipali@astro.temple.edu).

The nucleotide sequence reported here has been submitted to the GenBank Nucleotide Sequence Database under the accession no. AF395821.

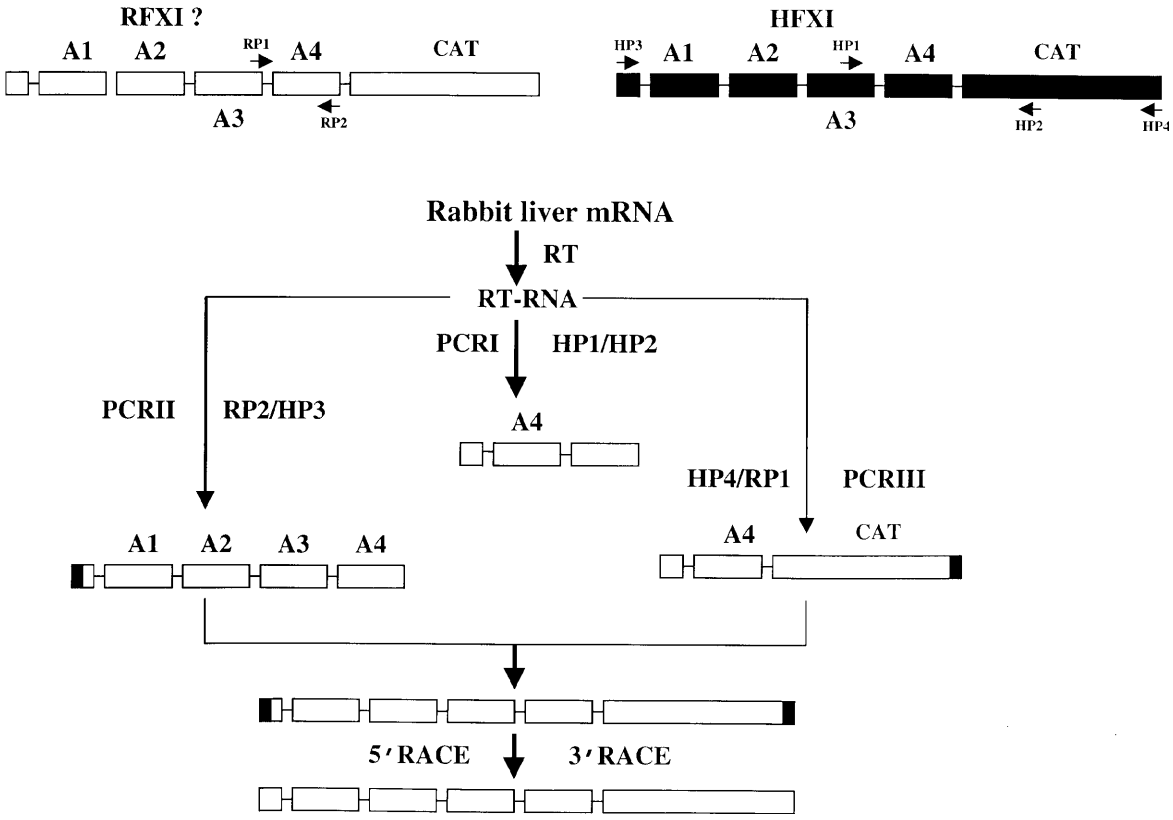

Figure 1 Strategy to obtain the full-length cDNA of RFXI

Rabbit liver mRNA was reverse transcribed using RT and the RT-RNA was used as a template in three separate PCRs. The sequence of the amplified fragment in each PCR is shown as the region of the protein it codes. In PCRI, amplification was done using primers HP1 and HP2 from the human sequence (Table 1). The amplified fragment provided a partial sequence of RFXI that covers part of A3, all of A4 and part of the catalytic domain (CAT). In PCRII the same RT-RNA was amplified using the antisense primer RP2 designed from the partial sequence of RFXI obtained in PCRI and the sense sequence HP3 from the human sequence. The fragment in PCRII provided the complete sequence of the four Apple domains and that of a part of the signal sequence of RFXI. In PCRIII the amplification was done using primers RP1 from the rabbit sequence and HP4 from the human sequence. The fragment thus obtained provided the sequence of part of A3, the entire A4 and almost the entire catalytic domain. 5′-RACE and 3′-RACE reactions provided the sequence of the cDNA ends.

Table 1 Primers used in reverse transcription and RT-PCR

Primer name	Type	Nucleotide sequence from	At position	Oligonucleotide sequence (5′ → 3′)
HP1	5′	HFXI	863	GCTCTTTCTGGTTTCAGTCTACAA
HP2	3′	HFXI	1589	CACTGACCTACCCCCATGTCT
RP1	5′	RFXI	908	GGTAGAGTGTTCTGCCATTCTTCATTT
RP2	3′	RFXI	1160	TTACACATTATCCATTTTACATAA
HP3	5′	HFXI	44	ATGATTTTCTTATATCAAGTG
HP4	3′	HFXI	1901	TCACACTGCTTGAGTTTTCTC
3′GSP1	3′	RFXI	464	AAAGTGACAGTGGGCATCATTCGT
3′GSP2	3′	RFXI	389	ACCTTGCATGTCTAGATCCAC
3′GSP3	3′	RFXI	329	TCTGGGTATTCTTTCAAGCAATGC
5′GSP1	5′	RFXI	1435	GATACGGCTTTCTTTGGGGTACAA
5′GSP2	5′	RFXI	1592	ACTGGATGGGGGTACAGGAAA
AAP				GGCCACGCGTCGACTAGTACGGGIIGGIIGGGIIG
AUAP				GGCCACGCGTCGACTAGTAC
AP				GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT

ingredients added: 20 mM Tris/HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM dithiothreitol and 0.5 mM of dATP, dGTP, dCTP and dTTP, followed by incubation for 5 min at 42°C. Superscript II (1 μl; 200 units) was added followed by incubation at 42 °C for 50 min. The reaction was terminated by incubation at 70 °C for 10 min. The RNA from the RNA–DNA duplex was degraded by incubation with RNaseH at 37 °C for 30 min. The reverse-transcribed mRNA (RT-RNA), thus obtained, served as a template for amplification by PCR using PrecisionTaq polymerase (Stratagene, La Jolla, CA, U.S.A.). Three separate pairs of primers as shown in Figure 1 and described below were used in PCRI, PCRII and PCRIII. Amplification was performed in a DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT, U.S.A.) using the following sequence of cycling conditions: 1 cycle at 95 °C for 2 min, followed by 35 cycles of 95 °C for 2 min, 57 °C for 1 min and 72 °C for 3 min. The reaction was completed by incubation at 72 °C for 7 min followed by a final incubation at 4 °C.

In PCRI a partial cDNA sequence of RFXI was obtained by amplification of the RT-RNA using primers HP1 and HP2 (Table 1 and Figure 1) designed from known cDNA sequence of HFXI. Since there is a considerable homology between FXI and PK, primer sequences were chosen from regions that are highly conserved in HFXI and mouse FXI and have minimal homology with human PK. The amplified fragment thus obtained was $\approx$ 700 bp long as determined by agarose gel electrophoresis and ethidium bromide staining. It was gel purified using the Qiaquick gel purification kit (Qiagen, Valencia, CA, U.S.A.) and sequenced in both directions using the same pair of primers HP1 and HP2. The sequence of this fragment was 84 % identical when compared

with the sequence of HFXI in the same region. Thus a partial sequence of RFXI was obtained.

In PCRII the same RT-RNA obtained as above was amplified using one antisense primer (RP2; Table 1) designed from the partial sequence of RFXI and a sense primer (HP3; Table 1) corresponding to the N-terminal of the signal sequence of HFXI. The amplified product showed a major fragment of $\approx$ 1100 bp.

In PCRIII the RT-RNA from rabbit liver was amplified using sense primer RP1 from the partial sequence of RFXI and antisense primer HP4 (Table 1) corresponding to the seven amino acids at the C-terminal end of HFXI. The amplified fragment in this case was $\approx$ 1000 bp long.

The sequence of RFXI cDNA ends was obtained by the procedure called RACE using kits from Life Technologies. In 5′-RACE the RT reaction was primed with the antisense gene-specific primer 3′GSP1 (Table 1) designed from the partial sequence of RFXI obtained above. Homopolymeric tail was added to the 3′ end of the cDNA using terminal transferase. Tailed cDNA was then amplified using a nested gene-specific primer 3′GSP2 and a complementary homopolymer-containing anchor primer AAP (abridged anchor primer; see Table 1). This amplified reaction (0.5 μl) was reamplified using a second nested gene-specific primer, 3′GSP3 (Table 1), from the RFXI sequence, and AUAP (abridged universal anchor primer; see Table 1) that contains the same adapter sequence as AAP. The amplified fragment from this step was a single band of $\approx$ 500 bp.

In 3′-RACE, the RT reaction was primed with an oligo(dT) adapter primer (AP; see Table 1) that targets the poly(A) tail region. Specific cDNA was then directly amplified by PCR using the gene-specific primer 5′GSP1 that anneals near the 5′ end of the cDNA and adapter primer AUAP. Reamplification using nested primer 5′GSP2 and the adapter primer generated a fragment that was $\approx$ 900 bp long.

Amplification was performed in three separate tubes for each set of RT-PCRs, and each amplified product was gel purified and sequenced in both directions with the same primer pair used in the respective RT-PCR.

Purification of RFXI from plasma

Citrated rabbit plasma (2.5 litres) was dialysed against 40 mM Tris/HCl, pH 8.3, containing 10 mM succinic acid, 1 mM benzamidine, 1 mM EDTA and 50 μg/ml polybrene, and then passed over an 8 litre DEAE–Sephadex A50 column equilibrated in the same buffer. The flow-through fraction containing FXI was dialysed against 50 mM sodium acetate, pH 5.2, 1 mM EDTA and 1 mM benzamidine and applied to a 200 ml S-Sepharose fast-flow column. Protein was eluted with a 3 litre NaCl gradient (0–700 mM). FXI-containing fractions were dialysed against 50 mM sodium acetate, pH 5.2, with 1 mM EDTA, and applied to a mono-S 5/5 column. Protein was eluted with an 85 ml NaCl gradient (0–700 mM), dialysed against 150 mM sodium acetate, pH 5.2, applied to a 3 ml heparin sepharose column and eluted with a 100 ml NaCl gradient (1–1000 mM). RFXI was concentrated in an ultra-filtration concentrator (Amicon, Beverly, MA, U.S.A.), dialysed against Tris-buffered saline (TBS) and stored at -80 °C. The protein appeared homogeneous on Gelcode Blue (Pierce, Rockford, IL, U.S.A.)-stained gels and had a specific activity of 370 units/mg as determined by activated partial thromboplastin time assay using FXI-deficient human plasma as a substrate and pooled normal human plasma as a standard.

Size-exclusion chromatography

Protein (10–20 μg) in 100–200 μl of TBS underwent size fractionation on a Superose-12 gel-filtration column (Amersham Biosciences, Piscataway, NJ, U.S.A.) fitted to a BioLogic FPLC workstation (Bio-Rad, Richmond, CA, U.S.A.). The column was equilibrated with 50 mM sodium phosphate, pH 7.3, containing 150 mM NaCl. Fractions of eluate (500 μl) were collected. Retention times of proteins were compared with a series of protein standards. The identity of the protein was confirmed by performing Western immunoblots on column fractions (results not shown).

Activation of RFXI and HFXI by FXIIa

Purified RFXI or HFXI (100–200 μg/ml) in 25 mM Tris/HCl, pH 7.4, containing 100 mM NaCl was supplemented with human FXIIa (2 μg/ml) and incubated at 37 °C. Activation was confirmed by demonstrating complete conversion of the 80 kDa subunit into the 45 and 35 kDa chains on Coomassie Blue-stained SDS/PAGE run under reducing conditions (results not shown).

Cleavage of S-2366 by HFXIa and RFXIa

Activated proteins were diluted to 0.5 μg/ml in TBS with 0.1 % BSA (TBSA) containing 50–1000 μM S-2366, and change in absorbance at 405 nm was followed on a microtitre plate reader. Michaelis–Menten constants (K_m and V_{max}) were determined by standard methods using the average of duplicate measurements of initial rates at varying substrate concentrations. Two separate experiments were carried out with similar results. Values for V_{max} were converted into nM *p*-nitroanilide/s using an molar absorption coefficient of 9800 at 405 nm/mol of *p*-nitroanilide. Turnover number (k_{cat}) was calculated from the ratio of V_{max} to enzyme concentration.

Activation of HFIX by HFXIa and RFXIa

Activation of FIX by FXIa was assessed by a modification of the methods of Wagenvoord et al. [14] and Sun et al. [15]. All reagents were diluted in TBSA. HFIX (0.5–10 μM) was incubated with 5 mM $CaCl_2$ and 1.0 nM HFXIa or RFXIa for 60 s at 37 °C in a 50 μl volume. Adding EDTA to 25 mM and chilling on ice stopped the reactions. The reactions were diluted 1:100 or 1:10, and 10 μl of each dilution was added to 50 μl of a mixture of human FVIII (8 units/ml), $CaCl_2$ (10 mM) and rabbit brain cephalin (1:5 dilution of the stock). Human thrombin (10 μl, 0.6 units/ml) was added, and the sample was incubated at 37 °C for 60 s to allow the thrombin to activate FVIII. Subsequently, 30 μl of human FX (450 nM) was added, and incubation was continued for 10 min. Adding EDTA to 25 mM and placing on ice stopped the activation of FX by FIXa. The final concentrations of reagents were 135 nM FX, 4 units/ml FVIII, 5 mM $CaCl_2$ and 1:10 dilution of rabbit brain cephalin. Each reaction (50 μl) was mixed with 50 μl of 1 mM S-2765, and the change in absorbance at 405 nm was followed on the ThermoMax microtitre plate reader. Results were compared with a control curve constructed with known amounts of purified FIXa. There was a linear correlation between the results of the assay and FIXa concentrations between 20 and 300 pM. Michaelis–Menten constants were determined using averages from three separate experiments. Rabbit brain cephalin was made from rabbit brain acetone extract (Sigma, St. Louis, MO, U.S.A.) by the method of Bell and Alton [16].

Characterization of intracellular FXI

RFXI cDNA comprising the amino acid sequence of the mature protein was introduced into the *Hin*dIII and *Xho*I sites of the pSecTag2 expression vector (Invitrogen). Human fetal kidney

Figure 2 Comparison of the amino acid sequences of HFXI and RFXI

Numbering is according to the human sequence [8]. The alignment required a deletion in the rabbit sequence after amino acid 47 in the A1 domain, a deletion after amino acid 532 and an insertion after amino acid 531 in the catalytic domain. The FXIIa cleavage site is shown by the arrow and the catalytic triad by solid circles. Identical amino acids are boxed.

fibroblasts (HEK-293 cells) were transfected with the above construct using lipofectamine (Life Technologies, Gaithersburg, MD, U.S.A.) according to the protocol provided by the supplier. Preliminary experiments were performed to ascertain the time at which a maximum level of the protein was expressed, as judged by ELISA on the conditioned media. Based on these results, after 40 h of transfection, HEK-293 cells were processed for characterization of the intracellular FXI. For this purpose transfected cells in 100 mm dishes were washed three times with PBS followed by lysis using a lysis buffer (Pierce, Rockville, IL, U.S.A.) supplemented with inhibitor cocktail (Calbiochem, San Diego, CA, U.S.A.). The lysates were centrifuged at 16000 ***g*** in a microcentrifuge at 4 °C for 1 h and the supernatant was immediately size fractionated over Superose 12 as described in the Size-exclusion chromatography section (above). The eluted fractions were assayed for the presence of FXI using an FXI ELISA kit from Enzyme Research Laboratories. Purified plasma FXI and plasma PK were fractionated in the same column under identical conditions.

RESULTS

RFXI cDNA sequence

The conventional procedure for characterizing the cDNA sequence of a gene is to screen a cDNA library. The protocol for obtaining the full-length cDNA of RFXI described in this manuscript did not require a cDNA library. Our approach consists of a number of steps of RT-PCR. The procedure is rapid and simple and should be useful for obtaining complete cDNA sequences of other proteins. The primers and their sequences used for RT and PCR in our sequencing strategy are listed in Table 1. The nucleotide sequence of the 700 bp fragment obtained in PCRI shown in Figure 1 was found to have 86 % identity

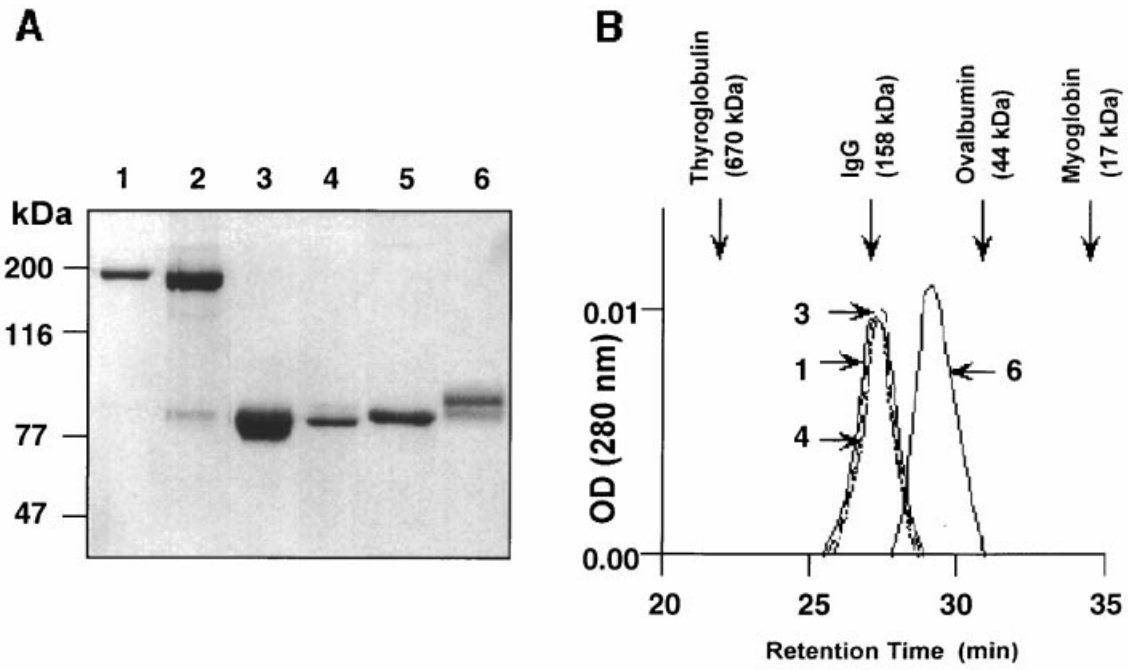

Figure 3 SDS/PAGE and size-exclusion chromatography

(**A**) SDS/PAGE of proteins stained with Gelcode Blue. All lanes contain unreduced protein except for lane 5. Lane 1, human plasma FXI; lane 2, human recombinant FXI; lane 3, recombinant FXI-Ala321; lane 4, rabbit plasma FXI; lane 5, human plasma FXI (reduced); lane 6, human plasma PK. (**B**) Retention times of proteins shown in (**A**) size fractionated on a Superose-12 gel-filtration column. The numbers next to the protein peaks correspond to the lane numbers in (**A**). The peak for human plasma FXI (lane 1) and PK (lane 6) are drawn as solid lines, FXI-Ala321 (lane 3) as a dashed line and RFXI (lane 4) as a dotted line.

when aligned with the corresponding region of the human sequence. This fragment consisted of the region that coded for part of the A3 domain, the entire A4 domain and a portion of the catalytic domain. From this partial sequence of RFXI a pair of primers RP1 and RP2 were designed and two more PCR amplifications were performed. The generated fragment in PCRII was $\approx$ 1100 bp long and represented the sequence of the entire heavy chain and a part of the signal sequence of RFXI. The 21 bp sequence at the 5′ end of the fragment represented the primer sequence of HP3 corresponding to the N-terminal seven amino acids of the signal peptide of HFXI. The amplified fragment obtained in PCRIII contained the sequence of RFXI corresponding to the entire A4 domain, part of A3 and the entire catalytic domain except for the 21 bp sequence at the 3′ end. The 21 bp at the C-terminal end of the fragment represented the primer sequence of HP4 corresponding to the C-terminal seven amino acids of HFXI used in the amplification.

Thus, from the above three RT-PCRs the cDNA sequence of a 1872 bp open reading frame coding for 624 amino acids was obtained. Except for the 21 bp sequence at the 5′ end and 21 bp sequence at the 3′ end, the entire 1872 bp sequence, represented the sequence of RFXI.

The 5′- and 3′-RACE reactions were then performed to obtain the cDNA ends of RFXI. The 500 bp fragment obtained in 5′-RACE provided a 96 bp sequence of the 5′ untranslated region and a 354 bp open reading frame coding for 118 amino acids at the N-terminal end including the signal sequence of 18 amino acids. Amino acid sequence Leu (-8) to Gly (103) obtained by the 5′-RACE fragment matched perfectly with that obtained from the fragment in PCRII in Figure 1. Thus the sequence of the entire A1 domain, signal peptide and that of 96 bp of the untranslated region were obtained from the 5′-RACE reaction.

The 900 bp fragment obtained from 3′-RACE provided a 222 bp open reading frame coding for 74 amino acids at the C-terminal end followed by a stop codon, and 306 bp of the 3′ untranslated region.

Sequence analysis of products from PCRII, PCRIII, 5′-RACE and 3′-RACE (Figure 1) provided the complete cDNA sequence of RFXI. The sequence thus obtained consists of a 1872 bp open reading frame coding for 624 amino acids, including an 18 amino acid leader peptide. Alignment with the human amino acid sequence required a gap in the rabbit sequence after amino acid 47 in the A1 domain, a gap after amino acid 532 and an insertion after amino acid 531 in the catalytic domain (Figure 2). The overall identity of the mature proteins from the two species is 87%. When individual Apple domains of the two proteins are compared the percentage identities are 75, 86, 87 and 84 respectively for A1, A2, A3 and A4, whereas the catalytic domains are 91% identical. The Cys-321 that is involved in disulphide-bond formation in HFXI is replaced by a His residue in the rabbit sequence. Cys-11 in the human forms a disulphide bond with a free cysteine and its function is unknown. In rabbit a Tyr replaces the Cys-11.

Comparison of RFXI and HFXI by SDS/PAGE and size-exclusion chromatography

HFXI is a homodimer and runs as a 160 kDa protein on SDS/PAGE under non-reducing conditions (Figure 3A, lane 1); recombinant HFXI was run for comparison (Figure 3A, lane 2). Human recombinant FXI-Ala321, in which Cys-321 was replaced by Ala, runs as an 80 kDa moiety under the same conditions (Figure 3A, lane 3). The Cys-321 in PK forms an intrachain disulphide bond with Cys-326 and non-reduced PK also runs as

Table 2 Comparison of RFXI and HFXI in functional assays

Specific activity was measured by activated partial thromboplastin time assay (see the Experimental section).

		Kinetic parameters			
		Chromogenic substrate		Human FIX	
Protein	Specific activity (units/mg)	K_m (μM)	k_{cat} (s^{-1})	K_m (nM)	k_{cat} (min^{-1})
HFXI	230	510	200	100	10.0
RFXI	370	690	150	110	9.3

a monomer (Figure 3A, lane 6). Non-reduced RFXI, like the mutant FXI-Ala321, also runs as an 80 kDa moiety (Figure 3A, lane 4). This was expected since the cDNA sequence revealed that the Cys at position 321 in HFXI is replaced by a His residue in RFXI. HFXI has the same size only when reduced (Figure 3A, lane 5). Size-exclusion chromatography, however, demonstrated that RFXI, as well as HFXI-Ala321, elutes with the same retention time as plasma FXI at physiological pH and salt concentration (Figure 3B). Thus RFXI, like FXI-Ala321, exists as a non-covalent dimer. In contrast, PK, a protein with homologous domain structure to FXI [1,10], has a higher retention time corresponding to a molecular mass of $\approx$90 kDa. This confirms that PK is a monomer.

Cleavage of the chromogenic substrate S-2366 by RFXIa and HFXIa

The chromogenic substrate S-2366 is commonly used for active-site titration of HFXIa. We compared the kinetic parameters of S-2366 cleavage by HFXIa and RFXIa (Table 2). The calculated catalytic efficiency (k_{cat}/K_m) was very similar for the two proteins, whereas that of murine FXIa was shown to be 2–3-fold lower [6].

Activation of human FIX by RFXIa and HFXIa

The kinetic parameters for the activation of human FIX by HFXIa and RFXIa are shown in Table 2. Since a chromogenic substrate sensitive enough to assay the generation of FIXa was unavailable a two-stage assay was performed. FIX was activated by FXIa in the first step and the resulting FIXa was then used to activate FX in the presence of FVIII and phospholipid. The activated FX thus generated was assayed by cleavage of S-2765. The results shown in Table 2 demonstrate that both HFXIa and RFXIa activate FIX similarly. The values of the parameters obtained in this report for HFXIa are similar to the values reported earlier [15].

Characterization of intracellular FXI

Supernatants from RFXI-expressing cells were tested for the presence of FXI antigen using an FXI ELISA kit and for FXI clotting activity by activated partial thromboplastin time assay as described for plasma FXI. The level of FXI secreted was found to be around 1–1.5 μg/ml and the clotting activity was estimated to be in the range of 250–300 units/mg. In order to characterize the intracellular FXI in the RFXI-expressing cells, elution profiles of purified plasma FXI and plasma PK on Superose 12 gel-filtration columns were first established as described above. Plasma FXI was found to elute with a peak maximum at 11.33 ml whereas PK eluted at 12.48 ml (Figure 4). ELISA assay on the fractions obtained after size-exclusion chromatography of the RFXI-expressing cell extract showed two peaks with retention volumes identical to those obtained when a mixture of FXI and PK was size fractionated in the same column. No FXI antigen was detected on fractions obtained

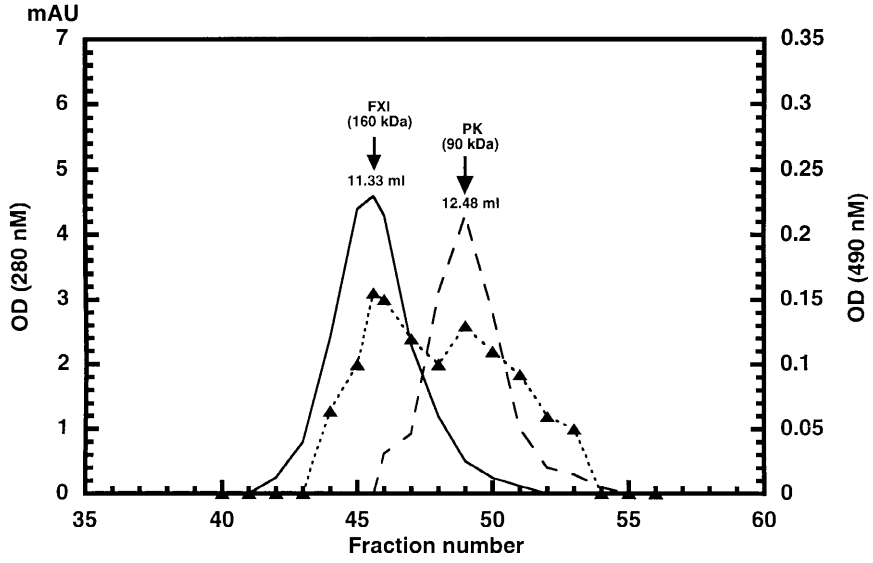

Figure 4 Characterization of intracellular FXI

Fractions obtained from size-exclusion chromatography of the RFXI-expressing cell extract were assayed colorimetrically (A_{490}) (OD) using an ELISA kit for detection of FXI antigen and the results are shown by ▲. Each point is an average of duplicate readings. Similar elution profiles were obtained in two separate experiments. Elution profiles (A_{280}) of purified plasma FXI (160 kDa) and plasma PK (90 kDa) are shown by solid and broken lines respectively. mAU, milli-absorbance units.

after size-exclusion chromatography of mock-transfected cell extract. The results demonstrated that both monomeric and dimeric forms of FXI are present in the cell extract.

DISCUSSION

FXI is unique among coagulation proteins by virtue of its homodimeric structure, the functional significance of which is not clearly understood. RFXI, unlike its human, bovine, porcine and murine counterparts runs as a monomer on non-reduced SDS/PAGE. To investigate whether this biochemical behaviour reflects a physiological monomeric structure resulting from an intra-chain disulphide bond that involves Cys-321 as in PK, we have characterized the cDNA sequence of RFXI and compared the predicted amino acid sequence with that of HFXI. Furthermore, we have examined how the protein exists and functions in plasma in comparison with HFXI, which is a disulphide-linked homodimer.

Alignment of the translated amino acid sequence of RFXI with that of HFXI (Figure 2) suggests a domain organization highly similar to those for human and murine FXI. The Cys residue at position 321 that forms the interchain disulphide bond in the human protein is replaced by a His residue in the rabbit protein and thus explains why the rabbit protein runs as an 80 kDa protein under non-reducing conditions (Figure 3A).

This discovery still does not reveal whether the rabbit protein exists in plasma as a monomer or homodimer, since multimeric structure can result from intermolecular forces other than covalent disulphide linkage. Work by Wiggins et al. [11] on RFXI suggested that it might exist as a monomer, or as a mixture of monomers and dimers. In contrast, our study using Superose-12 size-exclusion chromatography demonstrated that HFXI and RFXI are similar in size at physiological pH and salt concentration. In comparison, PK had a longer retention time corresponding to a molecular mass of ≈ 90 kDa, which is consistent with its monomeric structure. The data clearly indicate that RFXI is dimeric despite lacking an interchain disulphide bond. Therefore, in RFXI the two subunits form a homodimer through non-covalent interactions. Do the functional properties of FXI benefit from the presence or absence of the interchain disulphide linkage? We have demonstrated that RFXI and HFXI behave very similarly in functional assays (Table 2). We also found that FXIIa and thrombin activate both RFXI and HFXI in a comparable fashion (results not shown). The sequence Lys-Pro-Arg immediately N-terminal to the FXIIa cleavage site is identical in HFXI, mouse FXI and RFXI (Figure 2) and is a typical sequence for a thrombin cleavage site [17]; RFXI also undergoes autoactivation in the presence of dextran sulphate, as does the human molecule (results not shown). Thus the failure of RFXI to form an interchain disulphide bond does not impair its functional activities. The comparable functional properties of RFXI and HFXI suggest that the two proteins are structurally similar.

What is the physiological significance of FXI being a dimer? One hypothesis not yet rigorously proven is that FXI needs to be dimeric for proper secretion. Meijers et al. [18] studied the mutant FXI-Leu283 (type III mutation) that causes FXI deficiency in humans, and hypothesized that FXI needs to be dimeric for proper secretion. In this study [18] they demonstrated that there was poor secretion of the protein from BHK cells transfected with an FXI cDNA containing the type III mutation and that there was accumulation of the monomeric form within the cells. The hypothesis that dimerization is needed for proper secretion is further supported by the fact that FXI deficiency is almost always caused by a lack of the protein in the plasma, and not by a dysfunctional protein that is present at normal levels [19,20]. Other mutations (Asp-16 → His [21], Leu-302 → Pro [21], Arg-308 → Cys [22], Thr-304 → Ile [21], Glu-323 → Lys [21], Gly-350 → Glu [23], Phe-442 → Val [24] and Thr-475 → Ile [24]), besides FXI-Leu283, have been reported to cause a reduction in the amount of FXI secreted from cells. It has been hypothesized that, as in the case of FXI-Leu283, the reduction in FXI associated with these mutations is the result of inadequate dimer formation.

Gailani et al. [12,13] recently hypothesized that the dimeric conformation of FXIa is essential for optimal activation of FIX on the platelet surface. A recombinant monomeric FXI molecule, FXI/PKA4, was constructed by replacing the A4 domain of FXI with that of PK. The functional properties of this protein were compared with those of a dimeric version of FXI/PKA4 (FXI/PKA4-Gly326) and wild-type FXI. The monomeric and dimeric proteins were shown to have similar functional properties in solution. However, in the presence of activated platelets, monomeric FXI/PKA4 was a poor activator of FIX, while its dimeric counterpart FXI/PKA4-Gly326 and wild-type FXI activated FIX well. The authors interpreted the findings as supporting a model in which one chain of the FXIa dimer binds to the platelet surface, while the other binds to FIX. An alternative hypothesis is that the monomeric protein may be inhibited more rapidly by protease inhibitors, such a protease nexin II, released from the platelets [25]. Nonetheless, it can be unequivocally concluded that in the presence of activated platelets the homodimeric structure of FXI is required for normal function.

Our discovery that RFXI, despite lacking the interchain disulphide bond, nevertheless exists as a dimer supports the concept that dimerization of FXI is required for its optimal expression and/or function. We have, therefore, examined the intracellular protein to assess whether dimerization of FXI occurs within the cell. Our results demonstrated the presence of both forms of FXI within the cell. The presence of approximately equal proportions of FXI monomer and dimer clearly indicate that dimerization is an intracellular process. It also suggests that the kinetic conversion of monomeric into dimeric protein is not an instantaneous process, but rather represents a dynamic equilibrium in which monomer is converted to dimer in a process in which chaperones could be involved.

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