

Molecular Cloning of Platelet Factor XI, an Alternative Splicing Product of the Plasma Factor XI Gene*

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Platelet factor XI is associated with the platelet plasma membrane and has an apparent M_r (220,000 non-reduced, 55,000 reduced) different from that of plasma factor XI. However, the site of synthesis and the nature of platelet factor XI are not known. Using reverse transcriptase polymerase chain reaction, 12 out of 13 exons (all except exon V) coding for mature plasma factor XI were amplified from human platelet mRNA. The sequence of each of these exons was identical to that of plasma factor XI. *In situ* amplification and hybridization of factor XI mRNA was positive for exon III and negative for exon V in platelets and negative for both exons in other blood cells. By Northern hybridization, a factor XI mRNA transcript of ~1.9 kilobases was detected in megakaryocytic cells, and one of ~2.1 kilobases was detected in liver cells. Factor XI cDNA was cloned from a megakaryocyte library and sequenced. Exon V was absent, and the splicing of exon IV to exon VI maintained the open reading frame without alteration of the amino acid sequence except for the deletion of amino acids Ala⁹¹–Arg¹⁴⁴ within the amino-terminal portion of the Apple 2 domain. Thus, platelet factor XI is an alternative splicing product of the factor XI gene, localized to platelets and megakaryocytes but absent from other blood cells.

Plasma coagulation factor XI is a glycoprotein present in human plasma at a concentration of ~30 nM as a zymogen that, when converted by limited proteolysis to an active serine protease, participates in the contact phase of blood coagulation. This zymogen is a unique plasma coagulation enzyme because it exists as a homodimer (M_r ~143,000) consisting of two identical polypeptide chains linked by disulfide bonds (1, 2).

The sequence of the human factor XI gene has been elucidated by sequencing of the cDNA inserts coding for factor XI from two different λ phage genomic libraries (3, 4). The gene for human factor XI is 23 kilobases (kb)¹ in length and consists of

15 exons (I–XV) and 14 introns. Exon I encodes the 5' untranslated region, and exon II encodes a signal peptide. The next eight exons (III–X) encode four tandem repeat sequences of 90 or 91 amino acids (Apple domains) that are present in the amino-terminal region of the mature protein. The carboxyl-terminal region of the protein, which contains the catalytic domain, is encoded by five exons (XI–XV) that are interrupted by four introns.

Factor XI coagulant activity and antigen are present in well-washed platelet suspensions, and the activity accounts for about 0.5% of the total factor XI activity in blood (5, 6). About half of factor XI-deficient patients have defective hemostasis, whereas the remainder do not experience abnormal bleeding even in the absence of plasma factor XI (7). Subcellular fractionation studies have shown that factor XI activity is enriched in the platelet membrane fraction (8). The washed platelets and isolated platelet membranes obtained from a factor XI-deficient donor without a history of excessive bleeding had normal quantities of factor XI-like activity and normal behavior in the contact phase of coagulation (9). Thus, the functional significance of factor XI associated with platelets is not clear, but it may play a role in maintaining normal hemostasis, possibly complementing plasma factor XI deficiency (9).

Previous studies employing immunofluorescence, immunoelectrophoresis, and immunoprecipitation utilizing monospecific polyclonal anti-factor XI antibodies have demonstrated the presence in and partial purification from human platelets of a molecule with M_r of ~220,000 by SDS-polyacrylamide gel electrophoresis without reducing agents and of ~55,000 upon reduction (9). These results were subsequently confirmed by two separate groups (10, 11). Taken together, these findings suggest that platelet factor XI is either a disulfide-linked tetramer or, alternatively, a M_r 55,000 protein disulfide linked to a platelet membrane protein and that platelet membranes contain an endogenous factor XI-like activity structurally distinct from plasma factor XI. These facts and the observation that platelet factor XI has plasma factor XI activity and antigenic similarity (9) are consistent with the possibility that this protein may be an alternatively spliced product of the gene for circulating plasma factor XI.

Enzymatic amplification (utilizing the polymerase chain reaction) of platelet-specific mRNA after treatment with reverse transcriptase (RT) has been previously reported (12). In this study, we used this method to amplify the vestigial amounts of mRNA corresponding to platelet factor XI. *In situ* amplification and hybridization has been successfully accomplished in peripheral blood mononuclear cells (13). In this method, the polymerase chain reaction (PCR) can be performed *in situ* in fixed cells on specially designed glass slides. After the reaction, the amplified products can be detected by *in situ* hybridization

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AF045649.

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¹ The abbreviations used are: kb, kilobase(s); RT, reverse transcriptase; PCR, polymerase chain reaction; PMN, polymorphonuclear.

TABLE I
Oligonucleotides corresponding to plasma factor XI in PCR amplifications

Exon	5' primer: 5' → 3'	3' primer: 5' → 3'
III	AATGTGTGACTCAGTTGTTGAAAG	CATCGGGTGGGATCCTCAGAT
IV	GTTTACTGTGTCTGAAAGACAG	GCTTATTGGTGTGAGCATTGCTT
V	CTTGCAACAAAGACATTTATGTG	CGATGCTCCAGGCTGGGAAAC
VI	AACATTGTCTACTGAAGCACACC	CAGATTAGAAAGTGCACAGGATTT
VII	CTGTATATAGGGACATTTCCCT	TTTGAGATTCTTTGGGCCATTCCT
VIII	AAATCTTTGTCTCCTTAAACATCTG	TGGGATGCTGTGCCTGCAGCT
IX	TGTTTCTGCCATCTTTCATTTTAC	TCCCTTCGTTGCAGGATGCTTG
X	GGCAAGTGTTACTTAAAGCTTTCTT	ATTATCCATTTTACACACCTTAAATG
XI	AGTGTAACCAACAAATCAAGCC	ATAGAAACAGTGAGCGGCTGATAA
XII	GGTAGAGTCACCTAAGATTTT	TGTGTAATTCAGTGTGGTTTCCAG
XIII	ATTCTCAACGACCCATATGCCT	TCTTAGTTTCTGTACCCCATC
XIV	ACAAAATACAAATACTCTCAGAA	CTTGCAAGCGTCTTCCCTCC
XV	GGAGATTCTGGGAGGCCCTCT	CACTGCTTGAGTTTCTCCAGAAT

utilizing specific biotinylated probes. We used this technique to confirm the cell type expression of factor XI mRNA. The length of factor XI mRNA in a megakaryocytic cell line (Meg-01) was 1.9 kb, and in liver cells, 2.1 kb, by Northern hybridization, confirming the results from RT-PCR experiments and *in situ* amplification and hybridization. This paper reports the presence of an alternatively spliced factor XI in human platelets and presents the complete sequence of all exons coding for platelet factor XI (including exons I and II).

EXPERIMENTAL PROCEDURES

Cells and Reagents—Hep G-2 cells (human hepatocellular carcinoma cells) were obtained from American Type Culture Collection (ATCC, Rockville, MD). Human Meg-01 cells (a human megakaryoblastic leukemia cell line) were generously supplied by Dr. Hidehiko Saito (Nagoya, Japan). Plasma factor XI cDNA was kindly provided by Drs. Dominic Chung, Kazuo Fujikawa, and Earl Davie (Department of Biochemistry, University of Washington, Seattle, WA). Proteinase K, DNase, RNase inhibitor, dNTP, Moloney murine leukemia virus-RT, and *Taq* polymerase were purchased from Life Technologies, Inc. Biotinylated probes were obtained from Midland Certified Reagent Co. (Midland, TX). Streptavidin-peroxidase and 3-amino-9-ethyl carbazole were purchased from Pierce. RNazol was obtained from Biotecx (Houston, TX).

Preparation of Platelets, Monocytes, and Polymorphonuclear Leukocytes—Two tubes of 50 ml of blood containing 7 ml of acid citrate dextrose solution (15 g/liter citric acid, 25 g/liter trisodium citrate, 20 g/liter dextrose) were collected from each donor. Prostaglandin E1 was added to a final concentration of 50 ng/ml. The blood samples were centrifuged (1000 rpm for 20 min at room temperature). Platelet-rich plasma, obtained from the upper one-third of the blood sample, was transferred to a new tube for another centrifugation (1000 rpm for 10 min at room temperature) to avoid contamination with leukocytes. The lower part of blood sample was saved for the preparation of monocytes and polymorphonuclear (PMN) leukocytes. The platelet-rich plasma was then transferred to another tube and centrifuged (1800 rpm for 15 min at room temperature). The plasma supernatant was carefully removed from the platelet pellet. The pellet was resuspended in Hepes buffer (consisting of 15 mM Hepes at pH 6.8, 8.6 mM dextrose, 0.376 mM NaH₂PO₄, 0.98 mM MgCl₂, 2.7 mM KCl, 126 mM NaCl, and 1 mg/ml bovine serum albumin), centrifuged (1800 rpm for 10 min at room temperature), and finally resuspended in the same buffer and centrifuged again. For the isolation of human PMN leukocytes and monocytes, Polymorphprep™ (Nycomed Pharma AS, Oslo, Norway) was used. Three to 5 ml of the blood from the above procedure was layered carefully over 3–5 ml of Polymorphprep™ in the tube. The tube was centrifuged at 450 × *g* for 30 min at 20 °C. After centrifugation, there were two visible bands. The upper band, at the sample/medium interface, consisted of monocytes, and the lower band consisted of PMN leukocytes. The cells were diluted by addition of 0.45% NaCl.

Isolation of Platelet RNA—The platelet pellet obtained from the above procedure was resuspended in 3 ml of RNazol (Biotecx), and RNA was isolated as recommended by the manufacturer. Aliquots of the total RNA preparation were frozen at –20 °C until they were further used in experiments employing RT-PCR as described below.

Oligonucleotides—Based upon the published nucleotide sequence of plasma factor XI (3), two oligonucleotide primers were prepared for

each exon using a model 394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA) on a scale of 0.1 μmol per synthesis from the Oligonucleotide Synthesis Laboratory at Temple University School of Medicine. A total of 26 primers were synthesized for the 13 exons of factor XI (Table I).

RT-PCR—Total platelet RNA was analyzed by modifications of the method for RT-PCR originally described by Newman *et al.* (12). The SuperScript™ preamplification system (Life Technologies, Inc.) was used for first strand cDNA synthesis. The RT reaction was carried out in a DNA Thermal Cycler (Perkin-Elmer Corp.) using incubation conditions as follows: room temperature for 10 min, 42 °C for 50 min, 90 °C for 5 min, and 0 °C for 10 min. The cDNA obtained from the RT reaction was used for PCR amplification. Both 5' and 3' primers were added to a final concentration of 0.1 μM. *Taq* DNA polymerase (2.5 units) (Perkin-Elmer Corp.) was then added. Water was used in place of cDNA in negative control experiments. The cDNA from human hepatocellular carcinoma (Hep G-2) cells was also used in place of cDNA from platelets in positive control experiments. The PCR was carried out using the following sequence of cycling conditions: 1) 95 °C for 150 s; 2) 95 °C for 30 s; 3) 58 °C for 45 s; 4) 72 °C for 45 s; 5) repeat of steps 2–4 for 35 cycles; and 6) 72 °C for 7 min.

Purification of PCR-amplified DNA—PCR amplification products were visualized on 3% agarose gel electrophoresis (ethidium bromide staining). Amplified DNA was purified using the Qiagen (Chatsworth, CA) purification kit.

Sequencing PCR Products—Nucleotide sequences for PCR products were obtained using the dsDNA Cycle Sequencing System (Life Technologies, Inc.). This kit contains a number of modifications based on the sequencing method of Sanger *et al.* (14). Primers used for sequencing were the same as those used for PCR amplification. A modification of the original sequencing electrophoresis method (15), using 40% formamide in the sequencing gel, was used to overcome gel compression caused by the secondary structure of sequencing products during gel electrophoresis.

Preparation of Cells for *in Situ* PCR—Cells and platelets isolated from each individual donor were prepared for *in situ* hybridization and amplification (16) utilizing PCR primers and biotinylated probes for exon III or exon V. Cells (monocytes, PMN leukocytes, erythrocytes, buffy coats, Hep G-2 cells, or platelets) were seeded into the wells of a specially designed slide containing three 8-mm wells/slide. Approximately 1000–10,000 cells in a 10-μl volume were added to each well. Slides were heat-fixed at 105 °C for 90 s and then were incubated in 4% paraformaldehyde for 2 h. Paraformaldehyde was inactivated by incubating slides in 3× phosphate-buffered saline (1× PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7 H₂O, and 1.4 mM KH₂PO₄) for 10 min and then washing slides two times in 1× PBS. The endogenous peroxidase was quenched by incubating slides in 0.3% hydrogen peroxide in PBS overnight. These slides were treated with proteinase K (5 μg/ml) at 55 °C for 10–20 min. During the proteinase K treatment, cells were observed microscopically (× 400 magnification) for appearance of microbubbles on the plasma membranes of the cell surface. Once the microbubbles appeared, the proteinase K treatment was terminated by incubating slides on a heat block at 95 °C for 10 min. The cells were treated with RNase-free DNase, 10 units in DNase buffer (10 mM Tris pH 7.4 and 10 mM MgCl₂ with 10 mM dithiothreitol and 50 units of RNase inhibitor) overnight. DNase was inactivated by exposing cells to 95 °C heat over a heat block. The RT reaction was accomplished by adding to the wells 10 μl of a reaction mixture containing 0.07 M KCl,

0.03 M Tris, pH 8.3, 7.5 mM dithiothreitol, 1.5 mM of all four dNTPs, and 15 mM $MgCl_2$, with Moloney murine leukemia virus-RT and 20 μM downstream primers at 37–42 °C for 1 h. A slide well without RT was used as a negative control to test for DNA contamination. After 1 h incubation, the slides were washed in 1× PBS and then twice in distilled water. The cells were then subjected to *in situ* PCR. The amplification mixture was prepared containing exon III or exon V primers and *Taq* polymerase (50 mM Tris buffered at pH 8.3, 6 mM $MgCl_2$, 40 mM KCl, 1 mM dithiothreitol, 100 pmol of each primer, 2.5 units of *Taq* polymerase, and 200 μM of each deoxyribonucleoside triphosphate). For positive internal controls, we amplified the conserved region of HLA-DQA1 by using primers (HLA-DQ-GH-26/27, Synthetic Genetics). Ten microliters of amplification mixture containing exon III or exon V primers was added to wells 1 and 2, and a mixture containing no primers was added to well 3. Slides were covered with coverslips and sealed with clear nail polish. These slides were placed in the specifically designed heat block of a thermocycler (M. J. Research, Boston, MA). Exon III- or exon V-specific gene sequences were amplified in the individual intact cell structures by adding amplification mixture containing primer pairs representing the conserved regions of these gene segments. Thirty cycles of amplification in an automatic thermal cycler, set at 94, 45, and 72 °C, were employed. Hybridizations were performed with DNA probes biotinylated at the 5' end (Midland Certified Reagent Co.): the exon III probe was 5'-ACATTTATGTGGACCTAGACATGAA-GGGCATAAACTATAACAGCTCAGTGCCAAAGAGTGCTCAAGAATG-CCAAAGAAAGATGCACGGATGACGTCCTCACTG-3' and the exon V probe was 5'-GGACACCTGCTTTGAAGGAGGGGACATTACTACGGT-CTTCACACCAAGCGCCAAGTACTGCCAGGTAGTCTGCACTTACCA-CCCAAGATGTTTACTCTTC-3'. HLA-DQA1 cells were identified by biotinylated probe GH64 (5'-TGGACCTGGAGAGGAAGGAGACTG-3'). Negative controls for each assay were monocytes, erythrocytes, PMN leukocytes, and buffy coats, as well as RT-untreated but DNase-treated cells amplified for exon III or exon V. Positive controls were a mixture of the DNase treated cells with non-DNase-treated monocytes in a 10:1 ratio. Hybridization was carried out in a buffer containing 50% formamide, 10 mM dithiothreitol, 0.3 M NaCl, 0.03 M sodium citrate, 100 $\mu g/ml$ of fragmented salmon sperm DNA, 1 mg/ml of *Escherichia coli* tRNA, and 0.5 pg of probes at 90 °C for 6 min and then at 48 °C for 4 h. These cells were washed to remove unbound probes and incubated with streptavidin-peroxidase for 1 h at 37 °C. Color was developed with 0.3% H_2O_2 , 3-amino-9-ethyl carbazole dissolved in 50 mM acetate buffer. Microscopic examination usually reveals cytoplasmic staining *versus* nuclear staining. Percentage of labeled cells, by morphologic type, were counted on each slide. Multiple areas of the slides were viewed, and the entire slide was surveyed for localized staining or the presence of >1:10⁶ positive cells. Counting was done on blindly labeled slides by at least three observers, including two experienced pathologists.

Poly(A)⁺ mRNA Preparation and Northern Hybridization—Total RNA from human Meg-01 cells was prepared by suspension and lysis of cells in RNazol. The Meg-01 cell pellet was resuspended in 3 ml of RNazol and aliquoted into three RNase-free Eppendorf tubes. Poly(A)⁺ mRNA was obtained from total RNA of Meg-01 cells by using the Oligotext mRNA kit (Qiagen Inc.). Liver poly(A)⁺ mRNA was purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA). Procedures followed for Northern hybridization were standard protocols as described previously (15).

Screening of a cDNA Library—A cDNA library prepared from CHRF-288–11 cells (17) was kindly donated by Dr. Jerry Ware (Scripps Research Institute, La Jolla, CA). The library was screened using standard procedures (15) using radiolabeled factor XI cDNA as a probe. pBK-CMV was excised from plaque purified recombinant phage by the ExAssit/XLORO system (Stratagene). The cDNA insert was sequenced using Sequenase 2.0 (Amersham Pharmacia Biotech).

RESULTS

Platelet Preparation—It was critical to ensure that the platelet preparation contained no other contaminating cells, such as PMN leukocytes, lymphocytes, monocytes, or erythrocytes. To verify that platelets were the only detectable cells in the suspension, a hemocytometer (American Optical Corp., Buffalo, NY) was used to count the ratio of white cells to platelets. When a series of dilutions of platelet suspension were made (dilutions of 1-, 10-, 200-, and 400-fold), no white cells were found in the hemocytometer chamber. When 7.5×10^5 platelets were present in the hemocytometer chamber and 10 separate chambers were carefully examined, no other contaminating cells were

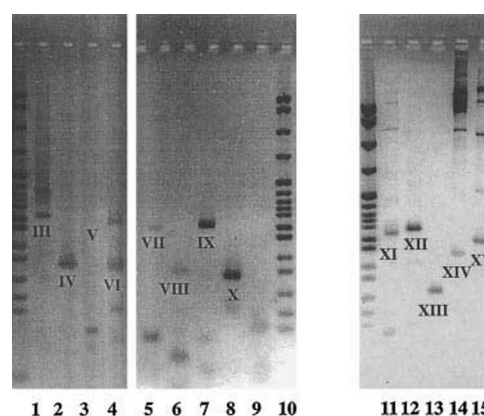


FIG. 1. DNA amplification products of human factor XI by RT-PCR from human platelets. Lanes 1–4, PCR amplification products of factor XI from human platelets (lane 1, exon III; lane 2, exon IV; lane 3, exon V missing (the same position of exon V in Hep G-2 cells, see Fig. 2); lane 4, exon VI); lanes 5–8, PCR amplification products of factor XI from human platelets (lane 5, exon VII; lane 6, exon VIII; lane 7, exon IX; lane 8, exon X). Lane 9, negative control (same components as lane 1, but using water instead of template); lane 10, pBR322 DNA *Msp*-I digest marker (New England Biolabs, Beverly, MA); lanes 11–15, PCR amplification products of factor XI from human platelets (lane 11, exon XI; lane 12, exon XII; lane 13, exon XIII; lane 14, exon XIV; lane 15, exon XV).

observed. Therefore, the platelet preparations were more than 99.9999% pure; *i.e.* there was less than 1 leukocyte per 7.5×10^6 platelets.

Amplification of Platelet Factor XI mRNA—The cDNA from platelet mRNA was prepared as described under “Experimental Procedures.” PCR amplification was carried out by using different pairs of primers for each exon. On the basis of the published nucleotide sequence of plasma factor XI (3), there are 13 exons coding for factor XI. Representative results utilizing platelets from two different donors are presented in Fig. 1 and demonstrate that platelet factor XI consists of 12 exons out of a total of 13 (all except exon V: Fig. 1, lane 3) that encode for plasma factor XI. Similar results were consistently observed in 17 separate experiments utilizing platelets obtained from six separate blood donors, including one (P. N. W.) whose platelets have consistently and repeatedly shown positive results for platelet factor XI activity (5, 8) and antigen (9). The results (Fig. 2) show that all 13 exons coding for plasma factor XI were amplified (including exon V; Fig. 2, lane 4) by PCR from HepG-2 cells.

Amplification across Exon Boundaries of Platelet mRNA Treated with Reverse Transcriptase—Utilizing RT-PCR, exon III and exon IV (which encode the Apple 1 domain) were consistently amplified from platelet mRNA utilizing a 5' primer for exon III and 3' primer for exon IV (Fig. 3, lane 2). This result eliminates the possibility of contamination of platelet RNA with genomic DNA from other cells. It also demonstrates that exons III and IV are present in platelet mRNA in the “correct” orientation, encoding a functional Apple 1 domain (4).

Sequencing of PCR Products—Sequencing of DNA amplification products was accomplished using both the 5'-primer and the 3'-primer for each individual exon of factor XI in a separate sequencing reaction. Full-length sequences were obtained for all 12 exons amplified from platelet mRNA. All of the nucleotide sequences (data not shown) were identical to those reported for plasma factor XI cDNA (3).

In Situ Amplification and Hybridization—Because RT-PCR determined that exons III–IV and exons VI–XV are present in human platelets, whereas exon V is absent, we next examined both platelets and other blood cells for the presence of factor XI

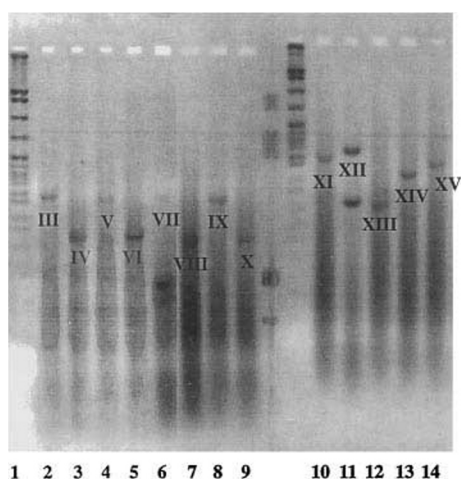


FIG. 2. DNA amplification products of human factor XI by RT-PCR from Hep G-2 cells. Lane 1, pBR322 DNA *Msp*-I digest marker; lanes 2–9, PCR amplification products of factor XI from Hep-G2 cells (lane 2, exon III; lane 3, exon IV; lane 4, exon V; lane 5, exon VI; lane 6, exon VII; lane 7, exon VIII; lane 8, exon IX; lane 9, exon X); lanes 10–14, PCR amplification products of factor XI from Hep G-2 cells (lane 10, exon XI; lane 11, exon XII; lane 12, exon XIII; lane 13, exon XIV; lane 14, exon XV).



FIG. 3. Amplification products across exon boundaries of human platelets mRNA treated with reverse transcriptase. Lane 1, pBR322 DNA *Msp*-I digest marker; lane 2, exons III–IV.

mRNA. *In situ* amplification and hybridization were used to determine the cell type expression of factor XI mRNA using primers and biotinylated probes specific for exons III and V. The experiments were performed three times, each in duplicate, and read blindly by two pathologists and two other observers without knowledge of the primers and probes used for amplification and detection. For all samples, platelets were positive for exon III and negative for exon V, whereas other cells (monocytes, neutrophils, lymphocytes, and erythrocytes) were negative for both exons III and V. Representative results are shown for illustrative purposes in Fig. 4. The results were positive for exon III (Fig. 4A) and negative for exon V (Fig. 4B) in human platelets. Monocytes and PMN leukocytes (DNase-treated) were also used for negative controls to prove that the mRNA that we amplified is not from white cells. The results

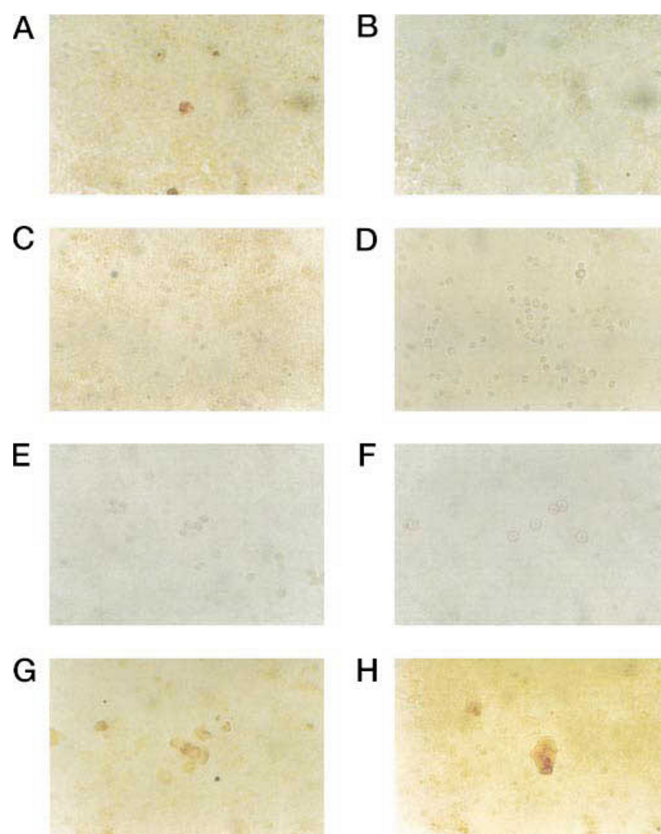


FIG. 4. Platelet factor XI mRNA detected by *in situ* amplification and hybridization. A, human platelets detected by exon III probes (under a $\times 10$ microscope); B, human platelets detected by exon V probes (under a $\times 10$ microscope); C, monocytes detected by exon III probes; D, monocytes detected by exon V probes; E, polymorphonuclear leukocytes detected by exon III probes; F, polymorphonuclear leukocytes detected by exon V probes; G, Hep G-2 cells detected by exon III probes; H, Hep G-2 cells detected by exon V probes.

were negative for both monocytes (Fig. 4, C, exon III and D, exon V), PMN leukocytes (Fig. 4 E, exon III, and F, exon V), erythrocytes, and lymphocytes (data not shown). Hep G-2 cells were also used as positive controls, and the results were positive for both exons III and V (Fig. 4, G and H).

Northern Hybridization—Northern hybridization was used to confirm the presence in platelet progenitors of factor XI mRNA and to determine the length of mRNA in Meg-01 cells and in liver cells by using a full-length “oligolabeled” (18) factor XI cDNA as a probe. The results (Fig. 5) show the presence of an mRNA transcript of ~ 1.9 kb in Meg-01 cells (Fig. 5, lane 1) and a factor XI mRNA of ~ 2.1 kb in liver cells (Fig. 5, lane 2). When a mixture of Meg-01 and liver poly+A mRNA was loaded on the same lane of the gel, two separate bands (~ 1.9 and ~ 2.1 kb) could be visualized using the same factor XI cDNA probe as above (data not shown). These results are consistent with the results from RT-PCR experiments and *in situ* amplification and hybridization: the length of mRNA for factor XI in human platelets is shorter by ~ 200 bases (*i.e.* the approximate size of exon V) than the length of mRNA for factor XI in the human liver cells.

Screening the cDNA Library and Sequencing—A CHRF-288–11 cDNA library (a generous gift from Dr. Jerry Ware) was obtained from a human megakaryoblastic cell line (19), which was treated with phorbol 12-myristate 13-acetate to induce maturation and differentiation into the megakaryocyte/platelet lineage. The fact that these cells express platelet peroxidase, platelet factor 4, and glycoprotein IIb/IIIa (19) pro-

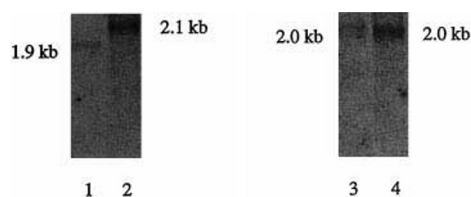


FIG. 5. **Northern hybridization.** Using human factor XI cDNA (~2.1 kb) as a probe: lane 1, poly(A)+ mRNA preparation of Meg-01 cells; lane 2, poly(A)+ mRNA preparation of human liver cells. Using human β -actin cDNA (~2.0 kb) as a probe: lane 3, poly(A)+ mRNA preparation of Meg-01 cells; lane 4, poly(A)+ mRNA preparation of human liver cells. Because very small quantities of poly(A)+ mRNA were recovered from Meg-01 cells, the amount of Meg-01 cell mRNA applied to the gel (~1 μ g) was ~35% of the liver cell poly(A)+ mRNA applied (~2.5 μ g). The same membrane after washing to remove the factor XI probe was employed for another Northern hybridization using human β -actin cDNA (2.0 kb) as a probe for the positive control.

vides evidence that the CHRF-288-11 cell line is a megakaryocytic cell line. The CHRF-288-11 cDNA library has been reported to contain $\sim 1 \times 10^6$ recombinant phage, with an average insert size of ~0.8 to ~4.0 kb (17). The titer of this library was 1×10^6 plaque-forming units/ μ l before screening.

During primary screening of the CHRF-288-11 cDNA library, 16 positive clones were initially identified. Twelve of these 16 clones remained positive after secondary and tertiary screening. One (λ XI3-2-3-1-1) (Fig. 6) contained a cDNA insert of 361 base pairs (nucleotides 1727–2087), and the other one (λ XI12-1-1-1) (Fig. 6) contained a cDNA insert of 1069 base pairs (nucleotides 1019–2087). However, these two sequences represent only a partial sequence for factor XI.

To avoid selecting the same clones on subsequent screening, a probe was constructed representing the 5' 43% of total factor XI cDNA (nucleotides 1–908). Eight positive clones were identified on the primary screening; four of those eight clones remained positive after secondary and tertiary screening. The DNA inserts of each of those four clones were sequenced. One clone (λ XI10) (Fig. 6) contained an insert of 1567 base pairs, with a nucleotide sequence identical to that for factor XI cDNA encompassing nucleotides 359–2087, which excludes exon V (nucleotides 367–528). The splicing of exon IV to exon VI within this clone maintains the open reading frame without alteration of the amino acid sequence (Fig. 7) except for the deletion of amino acids Ala⁹¹–Arg¹⁴⁴ within the amino-terminal protein of the Apple 2 domain.

Subsequently, a probe was constructed from nucleotide 1 to nucleotide 528. This probe represents only the 5' ~25% of the full length of plasma factor XI cDNA. Twelve positive clones were identified on the primary screening. Two clones (λ XI5-1 and λ XI5-2) have identical sequences (Fig. 6). Two clones contained an insert of 1916 base pairs with a nucleotide sequence identical to that for factor XI cDNA, encompassing nucleotides 10–2087, with deletion of exon V (nucleotides 367–528).² Again, the splicing of exon IV to exon VI maintains the open reading frame without alteration of the amino acid sequence, as described above. Moreover, these two clones contained the signal peptide (exon II) and the 5' untranslated region (exon I) of factor XI.

DISCUSSION

The functional significance of platelet factor XI is presently uncertain, but platelet factor XI may play an important role

Clones of Platelet Factor XI from CHRF288-11 cDNA Libraries

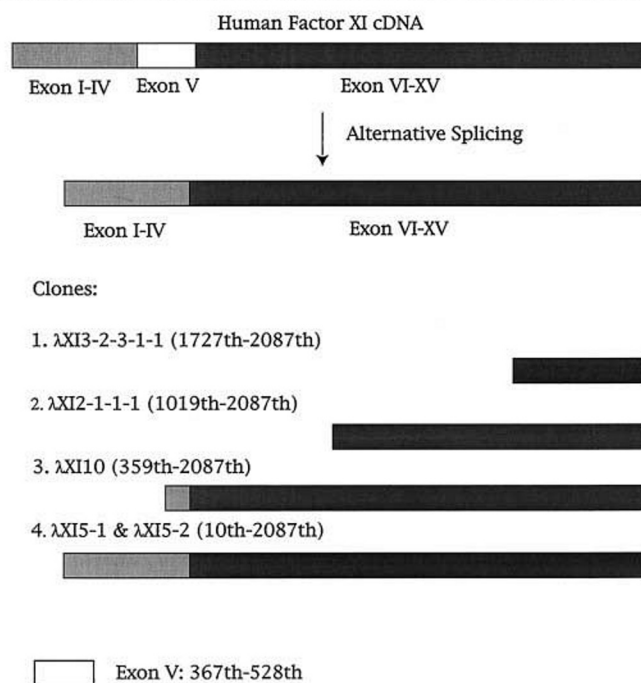


FIG. 6. **Clones of platelet factor XI from CHRF288-11 cDNA libraries.** Shown are λ XI3-2-3-1-1, λ XI2-1-1-1, λ XI10, λ XI5-1, and λ XI5-2.

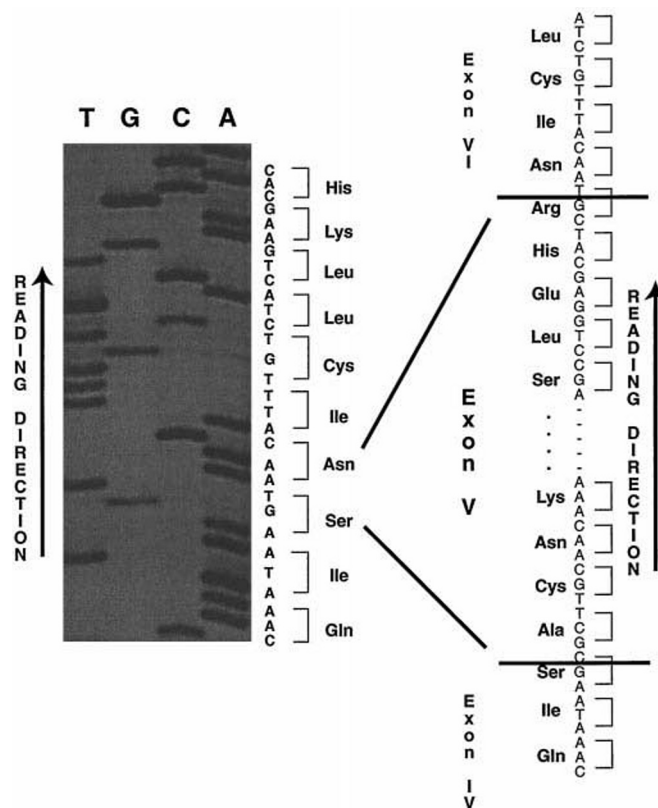


FIG. 7. **Amino acid sequence comparison of platelet factor XI without exon V (sequencing gel) and plasma factor XI with exon V.**

² This sequence was also scanned against both EMBL FASTA and BLAST data bases and is 100% identical to human plasma factor XI (accession no. M13142) and 62% identical to human plasma prekallikrein (accession no. M13143).

both in the maintenance of normal hemostasis and as a substitute for plasma factor XI (6, 8, 20). In fact, many patients, even among those with severe factor XI deficiency, have no evidence of abnormal bleeding, even after surgical or traumatic

challenge (7–9), and their platelets and isolated platelet membranes possess normal levels of factor XI activity and antigen (9). In contrast, platelets from hemostatically abnormal factor XI-deficient donors were reported to contain no detectable factor XI activity (21). These facts suggest that platelet factor XI may play a major role in the hemostatic process.

The experiments reported in this paper demonstrate the presence of an alternative splicing product of the plasma factor XI gene in human platelets. The gene for human plasma factor XI is 23 kb in length and consists of 15 exons and 14 introns (3). Our present studies using RT-PCR demonstrated the presence in a liver cell line (Hep G-2) of exons III–XV, encoding a mature plasma factor XI protein: four Apple domains (exons III–X) and the catalytic domain (exons XI–XV) were all amplified. In contrast, platelets were shown to contain mRNA sequences that are products of exons III, IV, and VI–XV, representing the full-length sequence of factor XI with the exception of the first (exon V) of two exons encoding the second Apple domain.

The cell type expression of factor XI mRNA was also determined. Using *in situ* amplification and hybridization, factor XI mRNA was amplified from human platelets but not from other peripheral blood cells, such as monocytes, lymphocytes, erythrocytes, and polymorphonuclear leukocytes. The results of these studies were consistent with those presented here using RT-PCR: exon III could be detected in human platelets but exon V could not, whereas both exon III and exon V were present in a liver cell line (Hep G-2). Moreover, the results from *in situ* amplification and hybridization confirm the conclusion that the products amplified by RT-PCR do not originate in DNA or mRNA present in peripheral blood leukocytes nor do they arise from illegitimate transcription (22) of DNA from cells other than platelets.

To test the hypothesis that exon V is missing from platelet factor XI, we utilized Northern blot analysis of platelet mRNA with plasma factor XI cDNA as a probe. The observations from Northern blot analysis show that the length of mRNA in Meg-01 cells (~1.9 kb) is shorter than that in the liver cells (~2.1 kb), a difference almost identical to that expected from the absence of exon V (162 bases), as demonstrated by the results of the RT-PCR experiments and the *in situ* amplification and hybridization experiments.

Finally, the screening and sequencing of three separate cDNAs isolated from the CHRF-288 library confirm that platelet factor XI cDNA lacks exon V, whereas the splicing of exon IV to exon VI (Figs. 6 and 7) maintains the open reading frame. The amino acid sequence, except for the deletion of amino acids Ala⁹¹–Arg¹⁴⁴ within the amino-terminal portion of the Apple 2 domain, is identical to the previously identified factor XI cDNA. Platelet factor XI, identified in and partially purified from human platelets, has an apparent subunit M_r by reduced SDS-polyacrylamide gel electrophoresis of ~55,000 compared with ~80,000 for plasma factor XI (9–11). Because the carbohydrate content of plasma factor XI has been reported to be ~7% of its mass, it seemed unlikely that differences in glycosylation between platelet factor XI and plasma factor XI alone can explain the difference in apparent subunit mass between the platelet and plasma proteins. However, observations in our laboratory³ have demonstrated that when purified factor XI is treated with *N*-glycosidase to remove carbohydrate, its apparent subunit M_r by gel electrophoresis is decreased from ~80,000 to ~64,000, an apparent 20% decrease in weight. It is not known whether or not platelet factor XI contains carbohydrate, but if it does not, the further decrease in M_r (*i.e.* ~6050) due to the absence of exon V would result in a protein with an apparent M_r on

reduced SDS gels of ~58,000, which is very close to the apparent M_r of ~55,000 reported previously (9, 10). Furthermore, one of the five potential *N*-glycosylation sites in plasma factor XI is encoded within the amino-terminal portion of the A2 domain of factor XI by exon V, which is missing from platelet factor XI. Moreover, the specific coagulant activity of factor XI was unaffected by removal of *N*-linked carbohydrate (data not shown). Therefore, the function of platelet factor XI in coagulation reactions would not be expected to be influenced by either the presence or absence of carbohydrate. Studies discussed above support the hypothesis that the molecular mass differences between platelet factor XI and plasma factor XI are a consequence of alternative splicing of the factor XI gene in addition to the possible absence or deficiency of carbohydrate in platelet factor XI. Alternatively, posttranslational processing (*e.g.* proteolytic cleavage) of the platelet factor XI protein could account for the mass difference between platelet factor XI and plasma factor XI.

We conclude from these results that platelets, but not other peripheral blood cells, contain a truncated form of factor XI mRNA lacking exon V that represents an alternatively spliced product of the factor XI gene, possibly expressed in megakaryocytes. Similarly, alternative splicing of transcripts of other proteins in platelets, including glycoprotein IIb (23, 24) and the granule membrane protein GMP-140 (25), have been reported. For example, alternative splicing of platelet glycoprotein IIb with a deletion of exon 28 has been demonstrated in mRNA isolated from a human erythroleukemia cell cDNA library (23, 24).

What might be the functional consequences of deletion of amino acids Ala⁹¹–Arg¹⁴⁴ (exon V) from platelet factor XI? Exon V encodes the amino-terminal portion of the Apple 2 (A2) domain of plasma factor XI (3, 4). Previous studies from our laboratory (26) have identified a sequence of amino acids (Ala¹³⁴–Leu¹⁷²) within the carboxyl-terminal portion of the A2 domain (encoded by exon VI) as a substrate-binding site for factor IX that is essential for promoting optimal rates of factor XIa-catalyzed factor IX activation. In contrast, recent observations by Sun and Gailani (27) are consistent with the presence of a substrate-binding site for factor IX within the A3 domain of factor XIa. Because the Apple domains are stabilized by three internal disulfide bonds, they are likely to fold independently, and therefore it is possible that the conformation of the putative A2 substrate-binding site for factor IX could be affected by the absence of the amino-terminal portion of the A2 domain. This may adversely affect the affinity of platelet factor XIa (relative to plasma factor XIa) for factor IX. Therefore, it will be important to determine the substrate specificity of platelet factor XIa. The major plasma substrates of plasma factor XIa are factor IX (28, 29) and factor XI (30, 31). Recent data from our laboratory⁴ indicate that platelets can promote the proteolytic activation of plasma factor XI by thrombin at rates comparable to or greater than those achieved in the presence of dextran sulfate. Even in the absence of added thrombin, platelets activated by the thrombin receptor activation peptide (SFLLRN amide) can activate factor XI, albeit at rates lower than those observed in the presence of thrombin. These observations lead us to postulate that the preferred substrate for platelet factor XIa is plasma factor XI, not factor IX. Future studies will be required to refute or confirm this hypothesis.

The platelets of several unrelated patients with severe (<0.05 unit/ml) plasma factor XI deficiency contain platelet factor XI antigen and/or activity (8–11), suggesting that the platelet-specific expression of functional platelet factor XI is

³ T.-C. Hsu and P. N. Walsh, unpublished observations.

⁴ F. A. Baglia and P. N. Walsh, unpublished observations.

independent of plasma factor XI expression. This raises interesting questions about the molecular genetics of deficiencies of plasma factor XI and platelet factor XI. Severe factor XI deficiency, especially common among Ashkenazi Jewish families, arise from three specific DNA mutations (32). Among the most common of these is a nonsense mutation that replaces Glu¹¹⁷ (GAA) with a stop codon (TAA) (32). This mutation causes premature termination of translation of the plasma factor XI gene. However, because this so-called type II mutation is located in exon V, which is spliced out in platelet factor XI, patients with the type II mutation would be expected to lack plasma factor XI but to produce normal platelet factor XI. Therefore, it will be important to define the genotype(s) that results in deficiencies of plasma factor XI with either normal quantities or with deficiencies of platelet factor XI.

The observation that platelet factor XI has an M_r of ~220,000 by SDS-polyacrylamide gel electrophoresis without reducing agents and ~55,000 on reduction (9–11) suggests that platelet factor XI may be a disulfide-linked tetramer or, alternatively, a protein with M_r 55,000 disulfide-linked to a membrane protein of M_r 165,000 (9). One possible candidate for this putative factor XI binding membrane protein on the platelet surface is glycoprotein Ib (M_r ~170,000). Thus, platelet factor XI may consist of a M_r 55,000 protein disulfide-linked to glycoprotein Ib. In support of this possibility are the results of previous studies from our laboratory (33) demonstrating that platelets from two related patients with the hereditary giant platelet (Bernard-Soulier) syndrome contained no detectable factor XI activity in washed platelet suspensions. Furthermore, these platelets lacked the capacity to support factor X activation after incubation with collagen (33). A reasonable interpretation of these results is that platelet factor XI is activated when platelets are stimulated by collagen, leading to sequential activation of factor IX to factor IXa, which in turn activates factor X on the platelet surface in the presence of factor VIII (34, 35). Because platelet factor XI is absent in Bernard-Soulier platelets, the failure of these platelets to promote factor X activation when stimulated by collagen suggests that platelet factor XI is required for the events leading to factor X activation on the platelet surface.

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