J. Biochem. 119, 961-969 (1996)

Expression and Release of the *a* and *b* Subunits for Human Coagulation Factor XIII in Baby Hamster Kidney (BHK) Cells[1]

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Received for publication, January 16, 1996

The *a* subunit of coagulation factor XIII lacks a hydrophobic signal sequence for secretion from cells, while the *b* subunit has a typical signal sequence. To determine whether the *a* subunit can be synthesized and released, expression vectors containing the cDNA for either subunit were transfected into baby hamster kidney (BHK) cells. Western blotting analysis and gel filtration chromatography demonstrated that the recombinant *a* and *b* subunits (rXIII*a* and rXIII*b*) had the same molecular weights and subunit structures (a_2, b_2, and a_2b_2) as the native molecules. rXIII*a* was enzymatically active when activated by thrombin. Most rXIII*b* was secreted as measured by ELISA, while most rXIII*a* was detected in the cytosol by subcellular fractionation. Co-expression with rXIII*b* in the same cells did not promote the release of rXIII*a*. Treatment of the cells with brefeldin A, a potent inhibitor of protein transportation, blocked the secretion of rXIII*b*, although it had no effect on the release of rXIII*a*. Several drugs and heat stress induced the release of rXIII*a*, which correlated directly with that of cytoplasmic lactate dehydrogenase. These results suggest that the *a* subunit is released from cells as a consequence of cell injury, which is independent of the classical secretory pathway.

Key words: biosynthesis, blood coagulation, factor XIII, secretory pathway, transglutaminase.

Human coagulation factor XIII (plasma transglutaminase, fibrin stabilizing factor) circulates in blood as a heterotetramer consisting of two catalytic *a* and two noncatalytic *b* subunits (a_2b_2) (*1*). It is a proenzyme which is activated by thrombin that is generated at the final stage of the blood coagulation cascade. Following the release of an activation peptide from the N-terminus of each of the *a* subunits, the activated a_2 dimer dissociates from the b_2 dimer in the presence of calcium ions. Calcium ions also unmask the active sites of the *a* subunits which catalyze the formation of intermolecular ε-(γ-glutamyl)lysine bonds between several proteins, including fibrin, a_2-plasmin inhibitor, fibronectin, *etc.* (for review, see Refs. *2* and *3*). The crosslinking reactions between these proteins lead to an increase in mechanical strength and elasticity of fibrin clots, resistance of the clots to degradation by plasmin, and promotion of wound healing. Thus, a deficiency of factor XIII results in a life-long bleeding tendency, spontaneous abortion, and abnormal wound healing (*2*).

The amino acid sequences of both the *a* and *b* subunits have been determined by a combination of cDNA cloning and amino acid sequence analysis (*4-6*). The *a* subunit consists of 731 amino acids and shares sequence homology with tissue transglutaminase, epidermal transglutaminase, and erythrocyte band 4.2 protein (*3*). The *b* subunit is composed of 641 amino acids and contains 10 tandem repeats called "sushi domains" (*7*) or "GP-I structures" which are found in at least 30 other proteins. The genomic structures for both the *a* and *b* subunits have also been determined (*8, 9*).

Despite extensive searching, no typical hydrophobic leader sequence required for secretion of the *a* subunit from cells was found in its mRNA or its gene (*4, 8*); neither was a possible internal hydrophobic signal detected in the amino acid sequence of the *a* subunit. The *a* subunit begins with an acetylated Ser residue at the N-terminus (*10*), and contains neither carbohydrate nor disulfide bonds; therefore, it displays features of typical cytoplasmic proteins

[1] This study was supported in part by National Institutes of Health (USA) Grant HL 16919 and by research grants from the Mochida Memorial Foundation (Japan), the Ichiro Kanehara Foundation (Japan), and the Japan Research Foundation for Clinical Pharmacology. This paper was presented in part at the 2nd International Conference on Factor XIII in Marburg, Germany (1992) and at the 36th American Society of Hematology meetings in Nashville, Tennessee (1994).

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Abbreviations: BHK, baby hamster kidney; CCCP, carbonyl cyanide *m*-chlorophenyl-hydrazone; DMEM, Dulbecco's modified Eagle's medium; DHFR, dihydrofolate reductase; DNP, 2,4-dinitrophenol; ELISA, enzyme-linked immunosorbent assay; MTX, methotrexate; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; rXIII*a*, recombinant *a* subunit of factor XIII; rXIII*b*, recombinant *b* subunit of factor XIII; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; XIII*a*, the *a* subunit of factor XIII; XIII*b*, the *b* subunit of factor XIII.

(7). Other members of the transglutaminase family not only lack a signal sequence for secretion, but also exist in the cytoplasm of cells, as does the *a* subunit of factor XIII. On the other hand, the *b* subunit has a typical hydrophobic signal sequence for secretion (*5, 9*). Since the *a* subunit of plasma factor XIII exists and functions extracellularly in spite of the lack of the secretory signal, the mechanism(s) for the release of the *a* subunit into the circulation is of interest.

To date, the *a* subunit of factor XIII has been expressed in yeast, but not in mammalian cells. In this paper, we present for the first time the successful expression of both the *a* and *b* subunits of factor XIII in baby hamster kidney (BHK) cells, and provide evidence indicating that the *a* subunit is released from cells as a consequence of cell injury, at least *in vitro*.

MATERIALS AND METHODS

Expression vectors, ZMB3 and 4, and the monoclonal antibody against the *a* subunit of factor XIII were the kind gifts of Ms. C. Sprecher and Dr. C. Hart, respectively (ZymoGenetics, Seattle). Rabbit anti-*a* subunit antisera and anti-*b* subunit antisera were purchased from Calbiochem. Peroxidase-conjugated goat anti-rabbit immunoglobulin and the proteins used as molecular weight standards were obtained from Bio-Rad. Monodansylcadaverine, *N,N'*-dimethylcasein, bovine thrombin, methotrexate (MTX), TPCK-treated trypsin, brefeldin A, 2,4-dinitrophenol (DNP), carbonyl cyanide *m*-chlorophenyl-hydrazone (CCCP), monensin, and calcium ionophore A23187 were purchased from Sigma. Dulbecco's-modified Eagle's medium (DMEM) was purchased from JRH Biosciences. PSN antibiotic mixture (penicillin, streptomycin, and neomycin) and G418 were purchased from GIBCO Life Technologies. Human factor XIII was purified from fresh frozen plasma (*11*). Purified factor XIII was also purchased from American Diagnostica. Factor XIII-deficient plasma was obtained from George King. All other materials used in the experiments were reagent-grade products obtained from commercial sources.

Construction of the Expression Vectors for the a and b Subunits—The *Pst*I fragment of 2.3 kb coding for the *a* subunit (*4*) was digested with *Pvu*II endonuclease to generate 1.8 and 0.5 kb fragments. The 3'-noncoding sequence in the *Pst*I/*Pvu*II fragment of 0.5 kb was then removed as follows. A mutant fragment of 1.6 kb with a *Pst*I site downstream of the stop codon (TGA) was generated by the polymerase chain reaction (PCR) employing the cDNA for the *a* subunit as a template and synthetic oligonucleotides: 5'-side, 5'-ATGATGCTGTGTATCTGGACAATGAGAAAGAA-3' (nucleotide 659-690 as numbered in Ref. *4*); 3'-side, 5'-TCCTCTGCAGTCACATGGAAGGTCGTCTTTGAATCTG-3' (*Pst*I site underlined). The 1.6 kb fragment was digested with *Pst*I and *Pvu*II, and its 3'-part *Pvu*II/*Pst*I fragment of 0.4 kb was ligated into the *Pst*I site of M13 mp19, together with the *Pst*I/*Pvu*II fragment of 1.8 kb. A subclone containing the 2.2 kb insert (*Pst*I/*Pvu*II of 1.8 kb plus *Pvu*II/*Pst*I of 0.4 kb) was selected by *Pst*I digestion, and the DNA sequence of the insert was confirmed to verify the absence of any unexpected mutations. The *Pst*I fragment of 2.2 kb (2,223 bp) was then inserted into a unique *Eco*RI site in a mammalian expression vector, ZMB4 (Fig. 1, top), using an *Eco*RI/*Pst*I adapter:

5'-AATTCGGCAACGAAGGTACCATGGTGCA-3'
3'-GCCGTTGCTTCCATGGTACC-5'.

The resultant expression vector for the *a* subunit was named ZMB4/XIII*a*.

A cDNA of 2.2 kb coding for the *b* subunit (*5*) was employed to construct its expression vector. A codon (ATG) for the initiator Met and two *Eco*RI sites at the 5'-end of this Met codon and at the 3'-end of the stop codon (TAG)

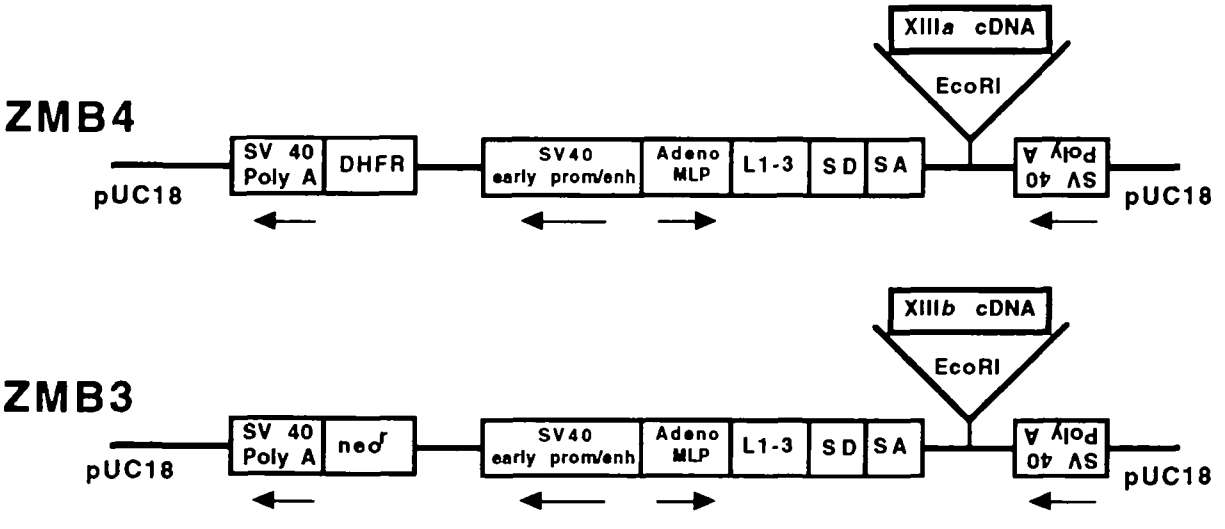

Fig. 1. **Construction of expression vectors for rXIII*a* and rXIII*b*.** A cDNA for the *a* subunit of factor XIII was ligated into a unique *Eco*RI cloning site downstream of the adenovirus promoter in an expression vector, ZMB4. Similarly, a cDNA for the *b* subunit of factor XIII was inserted into an *Eco*RI site in an expression vector, ZMB3 or 4. SV 40 poly A, a polyadenylation signal sequence for SV 40; DHFR, gene coding for dihydrofolate reductase; neo^r, gene coding for aminoglycoside phosphotransferase; SV 40 early prom/enh, early promoter and enhancer of SV 40; Adeno MLP, major late promoter of adenovirus; L1-3, tripartite leader; SD, splice donor; SA, splice acceptor from adenovirus. Arrows indicate direction of transcription.

were created by PCR employing the following synthetic oligonucleotides: 5′-side, 5′-GATCGAATTCATGAGGTT-GAAAAACCTGACTTTTATC-3′; 3′-side, 5′-CATTGAA-TTCTATGTTCTTAAGGGTTCTTGATAA-3′ (*Eco*RI sites underlined). The PCR-generated fragment was then subcloned into the *Eco*RI site of M13 mp18 and sequenced. The *Eco*RI fragment of 1,991 bp encoding the *b* subunit was then inserted into the unique *Eco*RI site of expression vector ZMB4 or ZMB3 (Fig. 1, bottom). The constructed expression vector for the *b* subunit was named ZMB3 or 4/XIII*b*. ZMB4/XIII*a* and ZMB3 or 4/XIII*b* were confirmed to contain the entire coding regions for the *a* and *b* subunits, respectively, in the proper orientation with respect to the promoter by restriction enzyme mapping and DNA sequence analysis.

Cell Culture, DNA Transfection, and Selection—BHK cells were grown, transfected, and selected as described in Refs. *11* and *12*; approximately 3×10^6 BHK cells were transfected with 10 μg of the expression plasmids. In order to obtain stable clones, a selective agent was added to the culture medium: 10 μM MTX for ZMB4/XIII*a* or XIII*b*, or 5 mg/ml G418 for ZMB3/XIII*b*. Positive clones for production of either the recombinant *a* subunit or *b* subunit (rXIII*a* or rXIII*b*) were selected (*11, 13*) and were grown individually for analysis. For co-expression of rXIII*a* and rXIII*b*, the stable clones expressing rXIII*b* through ZMB3/XIII*b* were transfected a second time with ZMB4/XIII*a*, and transfectants were selected in culture media containing both MTX and G418. Resistant colonies were screened for rXIII*a* production and cultured individually for analysis.

SDS-PAGE and Western Blotting Analysis—The cells expressing rXIII*a* and/or rXIII*b* were cultured in 100-mm Falcon dishes. After washing with phosphate-buffered saline (PBS), the cells were cultured further in 3 ml of the culture medium described above without fetal bovine serum (serum-free medium). After 24 h, the cells and media were separated. Samples of the culture media or cell lysates were prepared as described (*12*) and 20 μl aliquots were subjected to electrophoresis on 8% Laemmli gels; all SDS-PAGE analyses were performed under non-reducing conditions except for a crosslinking experiment of fibrin. For Western blotting analysis, proteins were transferred from a gel onto a nitrocellulose membrane (0.45 μm, Schleicher & Schuell) using the Fast-Blot semi-dry electroblotter (Biometra). Protein bands were developed by employing either 1/1,000 dilution of rabbit anti-*a* subunit or anti-*b* subunit antiserum, and 1/1,000 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin and peroxidase-4-chloro-1-naphthol.

Assay of Factor XIII Activity—Culture media containing rXIII*a* or both rXIII*a* and rXIII*b* were concentrated 10-fold by a filtration system (Amicon), then the factor XIII activity was measured in terms of the incorporation of monodansylcadaverine into dimethylcasein. A solution was prepared by mixing 20 μl of 50 mM Tris-HCl, pH 7.5, 20 μl of 100 mM $CaCl_2$ in 50 mM Tris-HCl, pH 7.5, 40 μl of sample, and 100 μl of 2 mM monodansylcadaverine in 50 mM Tris-HCl, pH 7.5. The solution was mixed with 2.5 μl of 500 units/ml thrombin in 0.15 M NaCl, 0.05 M sodium citrate, and 25% glycerol, pH 6.5, and incubated at 37°C for 20 min to activate factor XIII in the sample. A 20 μl aliquot of 0.1 M dithiothreitol was then added and after 5 min 200 μl of 0.4% *N,N′*-dimethylcasein in 50 mM Tris-HCl, and 10% glycerol, pH 7.5, was mixed into the solution. After 30 min of incubation at 37°C, 0.4 ml of 10% trichloroacetic acid was added to terminate the reaction and the precipitate was washed three times with 0.5 ml of ethanol-ether (1 : 1, v/v). The air-dried precipitate was dissolved in 3 ml of 50 mM Tris-HCl, pH 8.0, containing 8 M urea and 0.5% SDS. Fluorescence intensities were then measured with excitation at 355 nm and emission at 525 nm. Heat-denatured normal human plasma (factor XIII: 1 unit/ml) was used as a standard for factor XIII activity in samples.

Factor XIII-deficient plasma (0.5 ml) was mixed with 0.5 ml of the concentrated culture media of the BHK cells transfected with ZMB4/XIII*a*; 50 μl of 20 units/ml thrombin and 20 μl of 1 M $CaCl_2$ or 250 mM EDTA were then added. After incubation for 1.5 h at room temperature, the formed fibrin clot was separated from the serum and solubilized in 2% SDS, 8 M urea, and 2% β-mercaptoethanol, kept at 100°C for 5 min, then subjected to SDS-PAGE.

Gel Filtration Chromatography of rXIIIa and rXIIIb—Culture media containing rXIII*a* and/or rXIII*b* were concentrated 10-fold in the Amicon filtration system. One ml of the concentrated medium was dialyzed against 50 mM Tris-HCl, 1 mM EDTA, and 0.3 M NaCl, pH 7.5, and was applied to a gel filtration column (2.5 × 90 cm) of Sepharose CL-6B (Pharmacia, Sweden). The chromatography was performed at 4°C, and fractions of approximately 5.2 ml were collected. The column was first calibrated using standard proteins ranging from $M_r = 1{,}350$ to 670,000, including bovine γ-globulin ($M_r = 158{,}000$) as well as plasma factor XIII ($M_r = 320{,}000$). In the experiment on complex formation between the two subunits, the culture medium of rXIII*a* was mixed with that of rXIII*b* at a ratio of three to one after dialysis, and kept at 4°C overnight. The mixture was then applied to a Sepharose CL-6B gel filtration column. Both rXIII*a* and rXIII*b* in the fractions were detected by ELISA.

ELISA for rXIIIa and rXIIIb—Concentrations of rXIII*a* and rXIII*b* were measured as follows (*12*): 96-well ELISA plates (Corning, NY) were first coated with 50 μl of culture media or cell lysates containing rXIII*a* and/or rXIII*b* by incubation at 4°C overnight. The wells were incubated with 1/1,000 dilution of primary antibody, 1/1,000 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin, and a substrate solution (tetramethylbenzidine plus peroxidase, Microwell peroxidase substrate system, Kirkegaard & Perry Lab.). Purified *a* and *b* subunits from normal plasma were used as standards to estimate the concentrations of target proteins. When a monoclonal antibody against the *a* subunit of factor XIII was employed in the sandwich method, wells were first coated with the monoclonal antibody before samples were incubated.

Subcellular Fractionation and Trypsin Digestion—Cells transfected with ZMB4/XIII*a* and/or ZMB3/XIII*b* were cultured in five 150-mm plates and harvested by trypsin treatment. After having been washed twice in PBS, the cells were resuspended in 5 ml of 50 mM Tris-HCl, pH 7.5, kept for 5 min in ice, disrupted by 20 strokes with a glass pestle in a homogenizer (Wheaton, NJ), and supplemented with 5 ml of 0.66 M sucrose in the same buffer. Nuclei and undisrupted cells were eliminated by centrifuging for 10 min at 200 × *g*, and the post-nuclear supernatant was centrifuged at 100,000 × *g* for 1 h at 4°C. After centrifuga-

tion, supernatants were collected and kept as cell cytosol, and the pellet (particulate fraction) was resuspended in 0.5 ml of 50 mM Tris-HCl, pH 7.5. The cytosol and particulate fractions were incubated for 3 h in ice in the presence or absence of 100 μg/ml of trypsin. Samples were then analyzed by Western blotting after SDS-PAGE.

Drug Treatment (Brefeldin A, DNP, CCCP, Monensin, and A23187)—The BHK cells transfected with either ZMB4/XIII*a* and/or ZMB3/XIII*b* on 100-mm dishes were preincubated at 37°C for 1 h with or without drugs in DMEM (serum-free medium). The culture media of the preincubation period were removed and the cells were washed with PBS. The cells were then continuously cultured in 7 ml of the DMEM (serum-free medium) including the indicated concentrations of drugs. At intervals, aliquots (500 μl) of the culture media were taken and used for ELISA to measure the antigen levels of rXIII*a* or rXIII*b*.

Cell viability at the end of the incubation period (24 h) was evaluated by measuring LDH activity in the culture media of cells using a colorimetric kit (340-UV, Sigma). The BHK cells were lysed by three cycles of freezing and thawing, and LDH activity of all the cells used in the experiment was determined.

RESULTS

*Detection of rXIII*a *and rXIII*b *in Culture Media and Cell Lysates*—Amounts of intracellular and released rXIII*a* and rXIII*b* were determined by ELISA. Two typical experiments yielded averages of 4.9 μg of rXIII*a* and 4.7 μg of rXIII*b*/1×10^6 cells/24 h in the culture media of BHK cells transfected with ZMB4/XIII*a* and ZMB3/XIII*b*, respectively. On the other hand, 66 μg of rXIII*a* and 1.4 μg of rXIII*b*/1×10^6 cells were found in the cell lysates of the BHK cells. These results indicated that most rXIII*a* remained in the cells, while most rXIII*b* was secreted into the culture medium. Similar results were obtained when human 293 cells were employed.

*Molecular Weights of rXIII*a *and rXIII*b—To characterize rXIII*a* and rXIII*b*, both the culture media and cell lysates of the BHK cells were examined by Western blotting. rXIII*a* in the cell lysates had the same molecular weight as the *a* subunit of plasma factor XIII (Fig. 2A). rXIII*a* in the culture media showed a very weak band since the sensitivity of Western blotting (and ELISA) for the *a* subunit was about 50 times lower than that for the *b* subunit. With considerably longer staining, or after having been concentrated 10-fold, rXIII*a* in the culture media was confirmed to have the same molecular weight as that in the cell lysates (Fig. 2A, inset). These results indicate that rXIII*a* was synthesized by the BHK cells and accumulated inside the cells, and that only a small amount of the rXIII*a* was released from the cells.

rXIII*b* in the culture media and cell lysates also had the same molecular weight as the *b* subunit of plasma factor XIII (Fig. 2B). The ratio of rXIII*b* in the cell lysates and

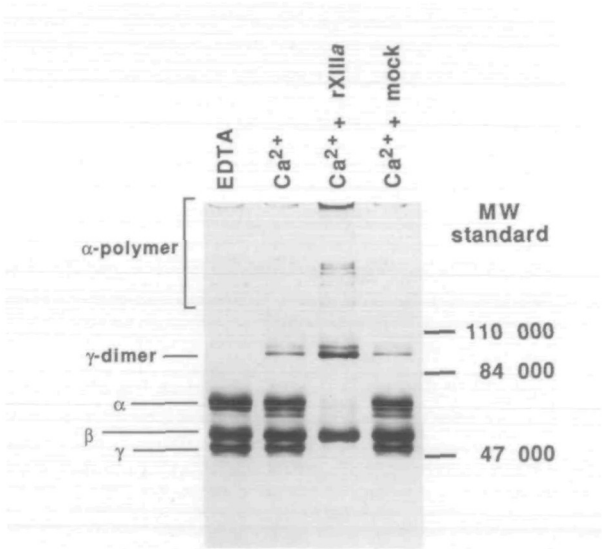

Fig. 3. **Crosslinking of fibrin by rXIII*a*.** A mixture of factor XIII-deficient plasma plus concentrated culture medium containing rXIII*a* was clotted by thrombin in the presence of calcium ions or EDTA, and incubated for 1.5 h. The fibrin clot was collected and subjected to SDS-PAGE under reducing conditions after being solubilized in SDS-urea solution.

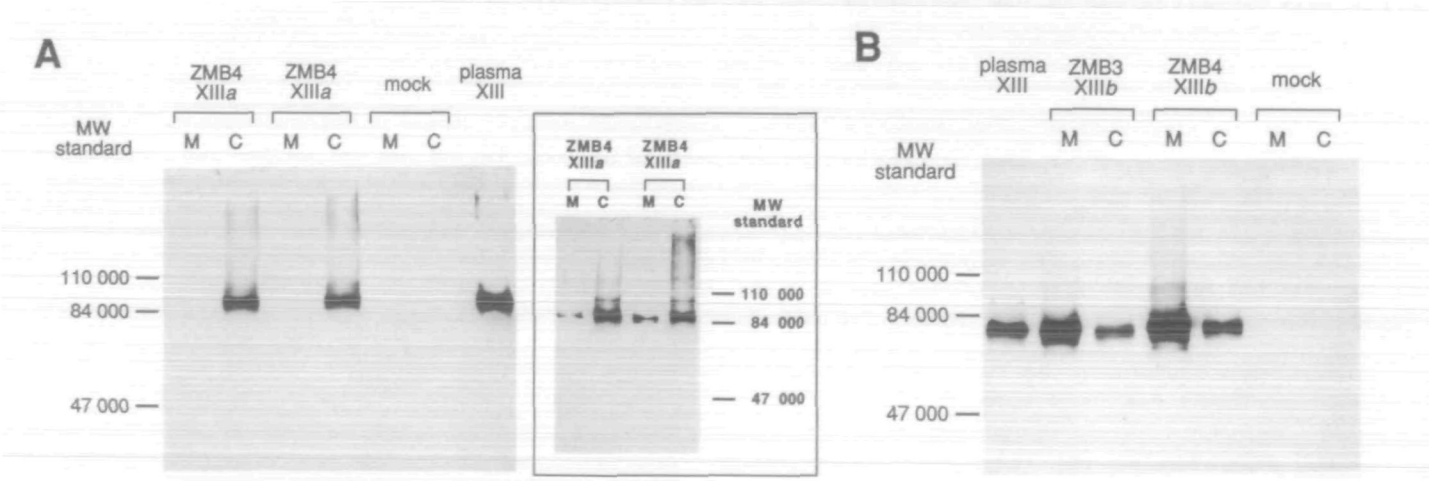

Fig. 2. **Western blotting of rXIII*a* and rXIII*b* in culture media and cell lysates.** Culture media and cell lysates were prepared as described under "MATERIALS AND METHODS" and subjected to SDS-PAGE and Western blotting. Purified factor XIII from plasma was used for comparison. Panel A demonstrates rXIII*a* synthesized by two separate clones of ZMB4/XIII*a*. Inset: the 10-fold-concentrated culture media. Panel B presents rXIII*b* produced by two clones of ZMB3 and 4/XIII*b*. M, culture media; C, cell lysates.

culture medium was about 1:3 as estimated from the intensities of the rXIII*b* bands in serially diluted culture media and that of the cell lysate (data not shown). Thus, rXIII*b* was synthesized in the BHK cells and effectively secreted into the culture medium.

*Enzymatic Properties of rXIII*a—In order to see whether rXIII*a* is biologically functional, the 10-fold-concentrated culture medium of the BHK cells transfected with ZMB4/XIII*a* was treated with thrombin; its transglutaminase activity was then measured by a method based on amine incorporation. The activity of rXIII*a* was 1.7 and 0.2 units/ml with and without the thrombin treatment, respectively. In contrast, the concentrated culture medium of mock cells had no activity (0 unit/ml) with or without the thrombin treatment. Western blotting analysis after SDS-PAGE demonstrated that the molecular weight of rXIII*a* was lower by about $M_r = 4{,}000$ after the thrombin treatment (data not shown). These results indicate that rXIII*a* retains a transglutaminase activity which can be induced proteolytically by thrombin.

The culture medium containing rXIII*a* was then added to factor XIII-deficient plasma and treated with thrombin in the presence of calcium ions. After incubation for 1.5 h, both the γ and α chains of fibrin disappeared and the γ-dimer and α-polymer were formed (Fig. 3). When the factor XIII-deficient plasma was mixed with the culture medium of the mock cells, both the monomeric γ and α chains remained and only a trace amount of the γ-dimer was formed. Thus, rXIII*a* was found to be capable of crosslinking its natural macromolecular substrate, fibrin.

*Subunit Structure of rXIII*a *and rXIII*b—Gel filtration chromatography of rXIII*a* and rXIII*b* was carried out in order to examine whether rXIII*a* and rXIII*b* exist in the culture media in a monomeric or dimeric form. rXIII*a* was eluted as a single peak at fraction 75 (Fig. 4A), which corresponds to the elution peak of bovine γ-globulin ($M_r =$

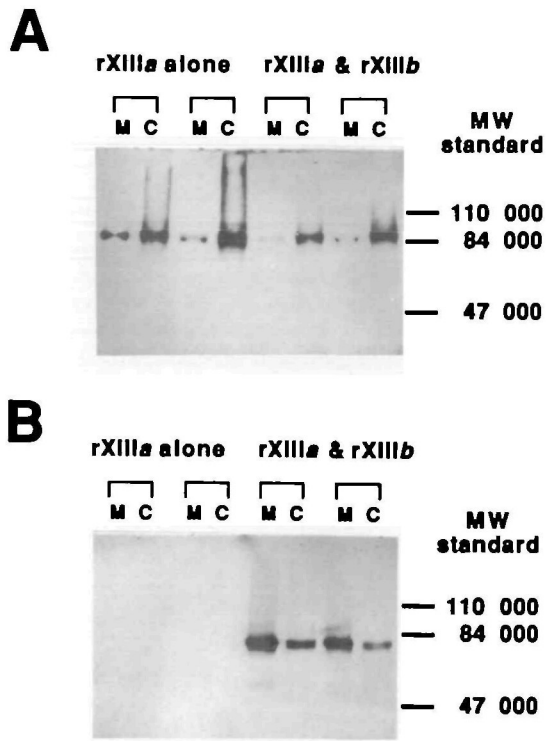

Fig. 5. **Western blotting of co-expressed rXIII*a* and rXIII*b*.** BHK cells (1×10^7 cells/dish) transfected with ZMB4/XIII*a* alone or both ZMB4/XIII*a* and ZMB3/XIII*b* were cultured in 3 ml of DMEM (serum-free medium) for 24 h. The culture media were then collected, and the cells were lysed in 3 ml of the detergent solution. Two clones producing rXIII*a* alone and two of those co-expressing rXIII*a* and rXIII*b* were selected for detection of rXIII*a* by anti-*a* subunit antibody (panel A), and for detection of rXIII*b* by anti-*b* subunit antibody (panel B). M, culture media; C, cell lysates.

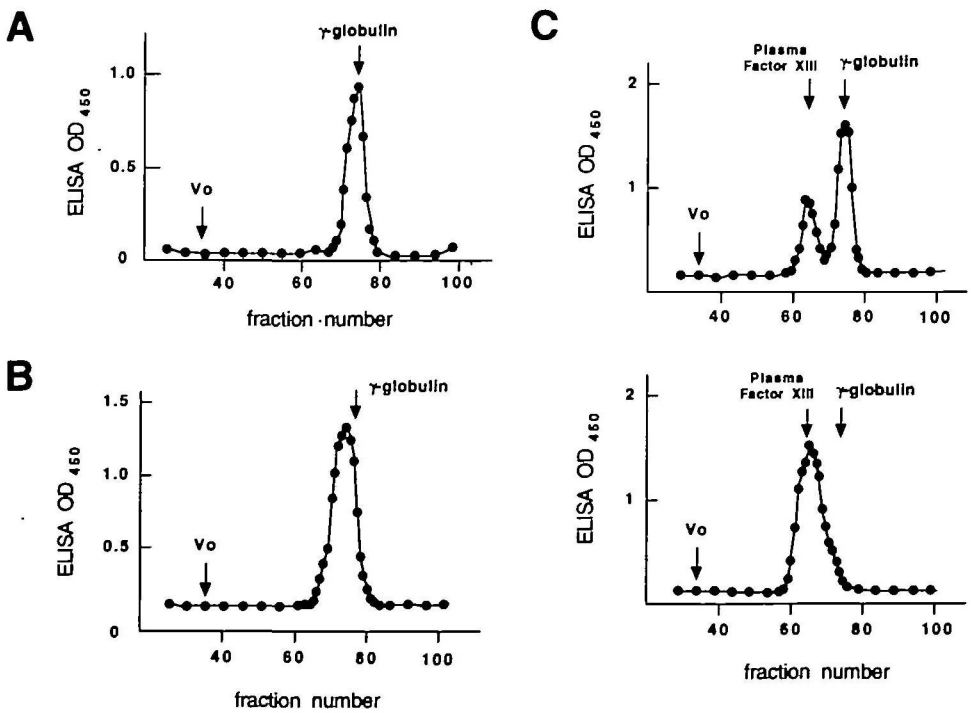

Fig. 4. **Gel filtration profiles of rXIII*a* and rXIII*b*, and complex formation of rXIII*a* and rXIII*b*.** Culture media containing rXIII*a* and rXIII*b* were concentrated 10-fold, and rXIII*a* (panel A), rXIII*b* (panel B), and a mixture of rXIII*a* and rXIII*b* (panel C) were applied to a column (2.5 × 90 cm) of Sepharose CL-6B. Fractions of approximately 5.2 ml/tubes were collected and rXIII*a* and/or rXIII*b* in these fractions were detected by ELISA (panel A and panel C (top), detection of rXIII*a*; panel B and panel C (bottom), detection of rXIII*b*). Arrows with Vo indicate the void volume of the column, and arrows with γ-globulin show the elution peak of bovine γ-globulin. Arrows with Plasma Factor XIII indicate the elution peak of human plasma factor XIII.

158,000). This result suggests that rXIII*a* exists as a dimer, because the molecular weight of each of the *a* subunits is 75,000. rXIII*b* was eluted as a single peak at fraction 73 (Fig. 4B), which is two fractions prior to the elution peak of bovine γ-globulin. This result indicates that rXIII*b* also exists as a dimer, since the molecular weight of each of the *b* subunits is 80,000.

To see whether rXIII*a* and rXIII*b* in the culture media retain the binding capability of forming a heterotetramer, rXIII*a* was mixed with rXIII*b* at a ratio of 3:1, and the mixture was subjected to gel filtration chromatography. rXIII*a* was eluted as double peaks at fractions 65 and 76 (top of Fig. 4C, ELISA for the *a* subunit), which correspond to the elution peaks of human plasma factor XIII (a_2b_2 tetramer) and rXIII*a* itself (a_2 dimer), respectively. When the amount of rXIII*b* added to rXIII*a* was doubled, the peak at fraction 76 was lowered and that at fraction 65 was elevated (data not shown). rXIII*b* was eluted as a single peak at fraction 65 (bottom of Fig. 4C, ELISA for the *b* subunit), which corresponds to the elution peak of human plasma factor XIII (a_2b_2 tetramer). When the amount of rXIII*b* was doubled, the height of the peak at fraction 65 was increased (data not shown). These results indicate that rXIII*a* and rXIII*b* both have the same binding capability of forming a a_2b_2 heterotetramer as plasma factor XIII.

Co-expression of rXIIIa and rXIIIb—Co-expression of rXIII*a* and rXIII*b* in the same BHK cells was carried out to test for possible interaction of the *b* subunits in the release of factor XIII*a*. Samples of the co-transfected clones certainly showed the band corresponding to rXIII*b* (Fig. 5B); however, there was no clear promoting effect of rXIII*b* on the release of rXIII*a* from the cells (Fig. 5A). The distribution of rXIII*a* in the culture media and cell lysates was also examined by ELISA, and the rates of released rXIII*a* *versus* total (intracellular plus released) rXIII*a* were calculated. The cells producing rXIII*a* alone released 6.7% of the total rXIII*a* into the culture media in 24 h, while the co-transfected cells released 7.3%. Therefore, the difference in the rates of released rXIII*a* between the cells producing rXIII*a* alone and those producing both subunits was not significant. Thus, the co-expression of rXIII*a* and rXIII*b* does not promote the release of rXIII*a*.

Effect of Brefeldin A on Release of rXIIIa and Secretion of rXIIIb—In order to test whether the release of rXIII*a* can be blocked, brefeldin A, a potent inhibitor of the classical secretory pathway, was added to the culture medium of BHK cells transfected with ZMB4/XIII*a*. Brefeldin A showed no inhibitory effect on the release of rXIII*a* up to 5 μg/ml (Fig. 6A). In contrast, the secretion of

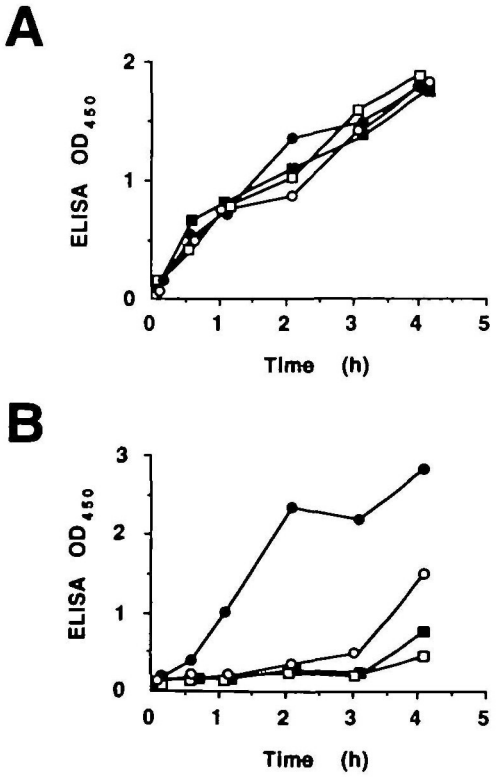

Fig. 6. **Effect of brefeldin A on release of rXIII*a* and secretion of rXIII*b* from the cells.** After preincubation at 37°C for 1 h, the BHK cells (1×10^7 cells/dish) transfected with ZMB4/XIII*a* (Panel A) or ZMB3/XIII*b* (Panel B) were incubated in the absence (closed circles) or presence of brefeldin A at 0.1 (open circles), 1 (closed squares), or 5 μg/ml (open squares). At the indicated times, aliquots were collected from the culture media and used for ELISA to measure the antigen levels of rXIII*a* or rXIII*b*.

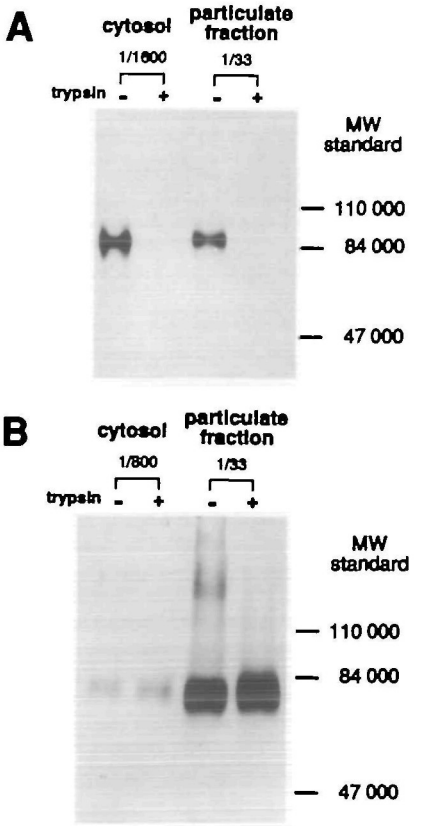

Fig. 7. **Subcellular fractionation of rXIII*a* and rXIII*b* in the BHK cells.** Cytosol and particulate fractions were prepared as described under "MATERIALS AND METHODS." Aliquots of the samples were treated with (+) or without (−) 100 μg/ml of trypsin for 3 h on ice before solubilization by a detergent solution. Panel A, 1/1,600 of the cytosol or 1/33 of the particulate fraction was applied to each lane and detected by Western blotting employing anti-*a* subunit antibody. Panel B, 1/800 of the cytosol or 1/33 of the particulate fraction was analyzed by anti-*b* subunit antibody.

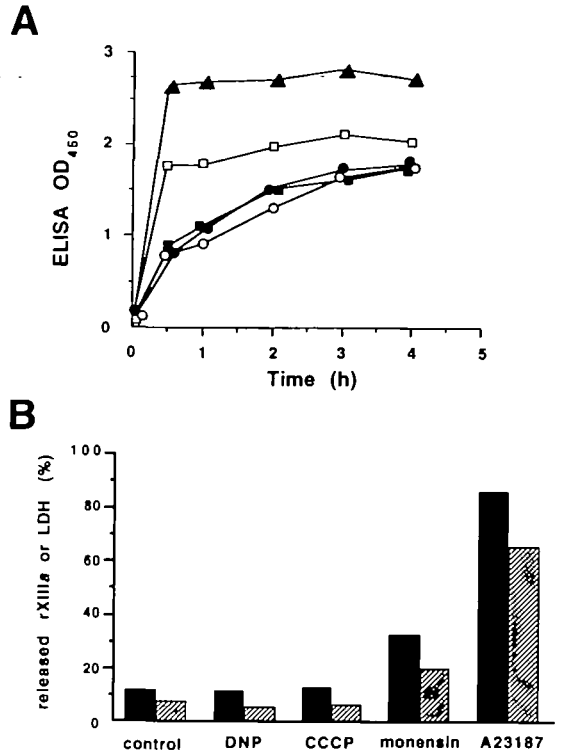

Fig. 8. **Effects of DNP, CCCP, monensin, and A23187 on the release of rXIII*a*.** Panel A, after preincubation at 37°C for 1 h, the BHK cells (1×10^7 cells/dish) were incubated at 37°C in the absence of drugs (closed circles), or in the presence of 1 mM DNP (open circles), 5 μM CCCP (closed squares), 10 μM monensin (open squares), or 5 μM A23187 (closed triangles). At the indicated times, aliquots of media were taken and subjected to ELISA to measure the antigen levels of rXIII*a* in the culture media. Panel B, after 24 h incubation, the media and cell lysates were tested for the antigen levels of rXIII*a* (solid bars) and for LDH activity (hatched bars). The percentages of rXIII*a* released and LDH released equaled the amount released divided by the amount released plus the amount present in the cell lysates.

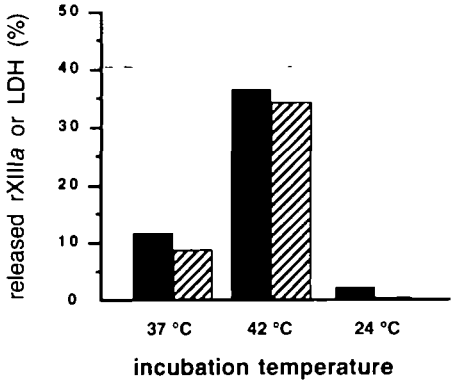

Fig. 9. **Effects of heat shock and low temperature on the release of rXIII*a*.** After 24 h incubation at 37°C (control), 42°C, or 24°C, media and cell lysates were tested for the antigen levels of rXIII*a* (solid bars) and for LDH activity (hatched bars).

rXIII*b* from the cells transfected with ZMB3/XIII*b* was inhibited in a dose-dependent manner (Fig. 6B) by brefeldin A. Exactly the same results were obtained when the BHK cells co-transfected with both ZMB4/XIII*a* and ZMB3/XIII*b* were treated with brefeldin A (data not shown). The brefeldin A-induced accumulation of rXIII*b* inside the cells was confirmed by Western blotting analysis of cell lysates after 4 h incubation (data not shown). These results suggest that the release of rXIII*a* from the BHK cells does not depend on the classical secretory pathway, which rXIII*b* follows.

*Subcellular Fractionation of rXIII*a *and rXIII*b—In order to localize rXIII*a* in the BHK cells, subcellular fractionation was performed. rXIII*a* was found exclusively in the soluble cytosol fraction and little, if any, was found in the particulate fraction (Fig. 7A). ELISA data showed that rXIII*a* in the particulate fraction amounted to only 0.3% of total rXIII*a* and that 99.7% was in the cytosol. Treatment of these two fractions with trypsin demonstrated that rXIII*a* both in the cytosol and particulate fractions was sensitive to digestion, indicating that no rXIII*a* is contained within membrane-surrounded vesicles. In contrast, rXIII*b* was found exclusively in the particulate fraction, and was resistant to trypsin digestion (Fig. 7B). Therefore, it is concluded that rXIII*b* is localized inside the membrane vesicles.

*Effect of DNP, CCCP, Monensin, and A23187 on the Release of rXIII*a—Possible effects of other drugs on the release of rXIII*a* were examined, since these drugs are known to affect different intracellular compartments (Fig. 8A). It was found that 1 mM DNP and 5 μM CCCP had no effect on the release of rXIII*a*, while 10 μM monensin and 5 μM A23187 increased the release of rXIII*a*. Microscopic examination of culture dishes revealed a significant change in shape and a decrease in the number of BHK cells after treatment with monensin or A23187. Thus, the media and cell lysates after 24 h incubation were tested by an activity assay for lactate dehydrogenase (LDH), which is a cytosolic enzyme commonly used as an indicator of cell lysis (Fig. 8B). The release of rXIII*a* was found to be parallel to that of the LDH activity, suggesting that rXIII*a* was released from the cytosol by cell lysis. The percentage of the released LDH was slightly lower than that of rXIII*a*, probably because of a mild decline in LDH activity during the 24 h incubation in the culture media.

*Effects of Heat Stress on the Release of rXIII*a—Following the method of Rubartelli *et al.* (*14*), possible effects of the incubation temperature on the release of rXIII*a* were examined after 24 h of incubation. When the media and cell lysates were tested for the antigen level of rXIII*a* by ELISA and for LDH activity (Fig. 9), a significant increase in the release of both rXIII*a* and LDH was obtained by raising the culture temperature from 37°C (control) to 42°C. Thirty-seven percent of total rXIII*a* was found in the culture media, whereas only 2.3% was released by incubation at 24°C. The release of rXIII*a* was well correlated with that of LDH under both conditions. These results also support the idea that rXIII*a* is released from cells as a consequence of cellular injury.

DISCUSSION

Recombinant *a* and *b* subunits of factor XIII (rXIII*a* and rXIII*b*) have been successfully expressed in BHK cells by employing their cDNAs (*4, 5*) and mammalian expression vectors ZMB3 and 4. The molecular weights of rXIII*a* and

rXIII*b* estimated by Western blotting analysis after SDS-PAGE were consistent with those of the *a* and *b* subunits of plasma factor XIII (Fig. 2, A and B). Gel filtration chromatography confirmed that both rXIII*a* and rXIII*b* in the culture media exist as dimers (Fig. 4, A and B), and retain the same binding capability of forming a heterotetramer as plasma factor XIII (Fig. 4C). rXIII*a* and rXIII*b* bind each other and also exist as a heterotetramer in the culture media of co-transfected cells (data not shown). rXIII*a* is able to be activated by thrombin, and shows transglutaminase activity as judged from an amine incorporation assay. The activated rXIII*a* is also capable of catalyzing crosslinking reactions of its natural substrate (γ-dimerization and α-polymerization of fibrin in Fig. 3). Thus, all properties of rXIII*a* and rXIII*b* examined here are consistent with those of native human plasma factor XIII.

As demonstrated by ELISA and Western blotting analysis, most rXIII*a* is not released into the culture medium, but is accumulated in the cytosol of BHK cells, as confirmed by subcellular fractionation. Intracellular accumulation of rXIII*a* has also been confirmed by pulse-chase experiments (Izumi and Ichinose, unpublished observation). This is consistent with the fact that the *a* subunits have been found diffusely in the cytoplasm of megakaryocytes (*15*), platelets (*16*), and macrophages/monocytes (*17-19*) by immunohistochemical methods, and localized in the cytosol of platelets by subcellular fractionation (*20, 21*). These intracellular *a* subunits are not released either from platelets (*21, 22*) or from macrophages/monocytes (*17*). Thus, both the *a* subunit *in vivo* and rXIII*a* *in vitro* are synthesized as cytosol proteins, as predicted from the structural features (*7*). In contrast, most rXIII*b* is secreted into the culture medium (*12*) and is localized in the particulate fraction by subcellular fractionation. The release of rXIII*a* is not promoted by its co-expression with rXIII*b* in the same cells. Treatment of BHK cells with brefeldin A—a potent inhibitor of protein transportation from the endoplasmic reticulum to the Golgi apparatus—blocks the secretion of rXIII*b*, whereas it has no effect on the release of rXIII*a*. Therefore, rXIII*b* is probably synthesized in the endoplasmic reticulum and secreted through the Golgi pathway (the classical secretory pathway) as shown by an immunohistochemical method (*12*). It is also clear that the *b* subunit does not assist the release of the *a* subunit, and that the release of rXIII*a* from BHK cells is not dependent on the classical secretory pathway.

The release of rXIII*a* is increased by raising the culture temperature to 42°C or by the presence of monensin or A23187. These treatments induce cell injury, and the release of rXIII*a* correlates with the release of cellular LDH; on the other hand, the release of rXIII*a* is decreased by low temperature (24°C). In this case, the release of LDH is also reduced and the BHK cells remain intact. These results suggest that rXIII*a* is released from BHK cells as a consequence of cellular injury. It has been demonstrated that the release of interleukin-1β, which also lacks a signal sequence, despite its extracellular function, was efficiently induced by cell injury (*23*). Similar to the release of rXIII*a*, the release of interleukin-1β by activated monocytes was blocked by low temperature, and was increased by raising the culture temperature to 42°C or by the presence of A23187 or monensin (*14*). These data suggest that cell injury results in the release from cells of the mature interleukin-1β, as well as the biologically active *a* subunit of factor XIII. Cell injury may also induce the release of other proteins which have extracellular functions but lack a signal sequence, such as basic fibroblast growth factor, platelet-derived endothelial cell growth factor, plasminogen activator inhibitor-2, *etc.* Although the existence of other mechanisms for the release of the *a* subunit of plasma factor XIII *in vivo* cannot be excluded completely, cell injury can account for the release of the *a* subunit observed at least *in vitro*. Regardless of the release mechanism(s) *in vivo*, there must be a large population(s) of cells producing the *a* subunit to supply the relatively high levels of extracellular *a* subunit. At present, the site of synthesis of the *a* subunit of plasma factor XIII has not been elucidated; most researchers in this field believe that it is of hematopoietic origin (*24, 25*), whereas a single group claims that it is synthesized in and secreted from the liver (*26*). Studies on the site of synthesis of the *a* subunit of plasma factor XIII are now under way.

rXIII*a* and rXIII*b* are indistinguishable from the native *a* and *b* subunits of plasma factor XIII in terms of their physical and functional properties. Thus, a mammalian expression system is being applied to the expression of abnormal molecules of either subunit found in defective genes of patients with congenital deficiencies (*12*). In addition, this system will be suitable for expression of various artificial mutants in order to study structure/function relationships of factor XIII.

The authors thank Prof. E.W. Davie for his support throughout this study, Dr. T. Izumi for helpful discussions, and Ms. L. Boba for her assistance in the preparation of the manuscript.

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