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Purification, identification, and characterization of elastase on erythrocyte membrane as factor IX-activating enzyme☆

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Abstract

In our previous papers, we reported that factor IX (F-IX), when activated by erythrocyte membranes, causes coagulation. We report on purification, identification, and characterization of F-IX-activating enzyme extracted from human erythrocyte membranes. The enzyme whose amino acid sequence is almost in accord with neutrophil elastase was found in normal erythrocyte membrane. The molecular mass was slightly smaller than that of neutrophil elastase. The content of the enzyme in erythrocyte membranes was estimated to be 3.0–3.7 ng per 10^6 erythrocytes. The F-IX sites cleaved by the enzyme were slightly different from those by the ordinary coagulation reaction. The ability of F-IX cleaved by the enzyme to cause coagulation was estimated to be approximately 1/10 as high as that of the F-IX cleaved by activated F-XI. These findings provide evidence that F-IX is activated by erythrocyte membrane, which may serve as a triggering mechanism for blood coagulation.

Keywords: Erythrocyte; Factor IX activation; Amino acid sequence; Elastase; Coagulation

The intrinsic coagulation pathway is triggered by the activation of factor XII (F-XII) or F-XI in the presence of contact surfaces, leading to the activation of F-IX and F-X and, ultimately, to the generation of thrombin [1] and [2]. It is generally thought that this pathway is not critical in the initiation of thrombin generation [2], when compared with the extrinsic coagulation pathway which is associated with vascular wall injury [3] and [4].

Normal human erythrocytes (RBCs) are generally considered to have no procoagulant activity and RBCs must lyse to exhibit clot-promoting activity [5–7]. In our earlier studies [8] and [9], it was shown that only F-IX in coagulation factors is activated by erythrocyte (RBC) membranes in the presence of calcium ions and even if contaminations such as activated coagulation factors including F-VIIa, F-IXa, F-Xa, and F-XIa, tissue factor, platelets, and leukocytes are present, these do not play a role in this process [9]. In addition, it was shown that F-IX, activated by RBCs, was capable of activating F-X in the presence of F-VIIIa and calcium ions and of leading to coagulation [9]. Moreover, it was shown that the rate of F-IX activation by RBCs was enhanced by an elevation in hematocrit [8] and [9]. The initiation of F-IX by RBCs from subjects in hypercoagulable states, including diabetes and normal pregnancy, was much faster than that from normal subjects [9]. Therefore, we supposed that F-IX activation by RBC membranes may serve as a triggering mechanism for blood coagulation.

In this paper, to demonstrate clearly that F-IX is activated by an enzyme on RBC membranes and the activated F-IX has procoagulant activity, we attempted to purify, identify, and characterize the F-IX-activating enzyme in RBC membranes.

☆ *Abbreviations:* F-IX, factor IX; RBC, erythrocyte; RBCs, erythrocytes; EE-IX, F-IX-activating enzyme (erythroelastase-IX); F-IXee, F-IX cleaved by EE-IX; PFP, platelet-free plasma; α_1-PI, α_1-proteinase inhibitor; LDF, logarithmic damping factor.

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Materials and methods

Blood samples. To extract F-IX-activating enzyme from RBC membranes, 1500 ml of blood was collected from healthy human male adult volunteers by venipuncture with 1/10 volume of 3.8% tri-sodium citrate solution. RBCs were obtained by centrifuging whole blood at 1200*g* at 4 °C, and the supernatant and buffy coat were removed. To completely remove leukocytes and platelets from packed RBCs, RBCs were resuspended in Hepes buffer (50 mM Hepes, 115 mM NaCl, 5 mM KCl, and 0.1% (w/v) glucose, pH 7.4) containing 2% dextran 200,000 (MW 180–210 kDa) and the sedimented RBCs were then fully washed with Hepes buffer. In addition, the washed RBCs were suspended in Hepes buffer and then placed in a 60% Percoll solution (Amersham–Pharmacia Biotech AB) with a density of 1.08. The sample was then centrifuged at 200*g* for 15 min and the sedimented RBCs were washed again with Hepes buffer. The absence of platelets and leukocytes in the RBC sample was confirmed with a flowcytometer.

Platelet-free plasma (PFP) was prepared by centrifuging platelet-poor plasma at 16,000*g* for 15 min at 4 °C. To prepare PFP supplemented with RBCs, the packed RBCs were resuspended in PFP. F-IX-deficient PFP was obtained from George King Biomedical.

Other materials. Purified human F-IX (0.2 U/mg) and activated F-IX (F-IXaβ, 0.2 U/mg) were obtained from Enzyme Research Laboratories. Each factor was dissolved in Hepes buffer and stored in a frozen state. Human neutrophil elastase (2.1×10^{-2} U/μg) was obtained from Calbiochem–Nova Biochem and dissolved in Hepes buffer.

Extraction and purification of the enzyme. To extract proteins from the RBC membrane fraction, the membrane preparation was extracted with 50 mM Tris–HCl buffer, pH 7.4 (buffer A), containing 1.0% Tween 20 at 4 °C overnight. Solubilized fractions were subjected to anion-exchange chromatography on a Q-Sepharose column (1.5×20 cm) equilibrated with buffer A containing 0.1% Tween 20 and the absorbed proteins were then eluted with a buffer solution (buffer A + 0.8 M NaCl) at a flow rate of 1.0 ml/min. The active fractions were then subjected to affinity chromatography on a soybean trypsin inhibitor (SBTI)-agarose column (Sigma) equilibrated with 0.1 M sodium acetate buffer (pH 7.4), and the absorbed proteins were then eluted with 0.1 M citric acid solution (pH 2.5). After neutralization, the active fractions were subjected to heparin-affinity chromatography on a heparin Sepharose column (1.5×3 cm). The absorbed fractions were eluted with buffer A using a linear gradient of NaCl from 0.3 to 0.8 M. The active fractions were subjected to reverse-phase HPLC on a CAPCELL PAK Phenyl SG-300 column (1.5×35 mm, Shiseido) equilibrated with 1% solvent B (0.1% trifluoroacetic acid (TFA) in 80% acetonitrile) in solvent A (0.1% TFA in water). Elution was performed using a linear gradient of solvent B (1–99%) at a flow rate of 0.2 ml/min.

The enzymatic activity of each fraction was evaluated by the production of the band corresponding to F-IXa on SDS–PAGE gels (7.5–15% gels). To detect cleaved F-IX, the proteins were subjected to Western blot analysis using rabbit IgG as a factor IX-antibody and peroxidase-conjugated goat anti-rabbit IgG as a secondary antibody. To estimate the degree of purification at each chromatographic step, the protein concentration was determined by a dye-binding method [10].

Fragmentation of the enzyme. Ten microliters of the purified enzyme solution was denatured with 40 μl of 8.0 M guanidine–HCl in 0.8% (v/v) formic acid. The sample was mixed with 50 μl of 2.0 M Tris–HCl (pH 8.5) containing 8 M guanidine–HCl, and 1 mM EDTA, and treated with 1.0% (w/v) of dithiothreitol for 2 h at 37 °C. The reduced enzyme was carboxymethylated by treatment with 2.5% (w/v) iodoacetic acid for 30 min at room temperature in the dark. The sample was subjected to RP-HPLC on a CAPCELL PAK Phenyl SG-300 column equilibrated with 1% solvent B in solvent A. Elution was performed using a linear gradient of solvent B (1–99%) at a flow rate of 0.2 ml/min.

The reduced and carboxymethylated enzyme was digested with modified trypsin (Promega) at an enzyme/substrate ratio of 1/10 (w/w) in 0.1 M Tris–HCl, pH 8.5, for 18 h at 37 °C. For tryptophanyl bond cleavage, the reduced and carboxymethylated enzyme was treated with 0.1% (w/v) 3-bromo-3-methyl-2-(2-nitrophenylmercapto)-3H-indole (BNPS-skatole) in 55% (v/v) acetic acid for 18 h. The sample was then washed with chlorobutane three times. Cleaved peptides were fractionated by HPLC on a Mightysil RP-18 column (Kanto-kagaku) equilibrated with 1% solvent B in solvent A. Elution was performed using a linear gradient of solvent B (1–99%) over 80 min under a flow rate of 20 μl/min. Isolated peptides were subjected to mass spectrometry and amino acid sequence analysis.

Mass spectrometry and amino acid sequence analysis. Mass spectra of the cleaved peptide fragments were obtained by matrix-associated laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry with a REFLEX mass spectrometer (Bruker-Franzen Analytik) using 2,5-dihydroxybenzoic acid, α-cyano-4-hydroxycinnamic acid or 3,5-dimethoxy-4-hydroxycinnamic acid as the matrix. The mass signals were in accord with the predicted molecular masses of the corresponding peptides. To determine the N-terminal amino acid sequence, peptides were subjected to Edman degradation using a Procise 494cLC protein sequencer (Applied Biosystems).

Estimation of the concentration of the enzyme. The content of F-IX-activating enzyme in RBC membrane was estimated from a comparison of fluorescence intensity of a mixture of a fluorogenic synthetic substrate (Suc(OMe)-Ala-Ala-Pro-Val-MCA, Calbiochem–Nova Biochem), specific for elastase [11] with elastase and with RBCs. To measure changes in the fluorescence intensity of a mixture of elastase and fluorogenic synthetic substrate, 3 μl of 1.5 μM fluorogenic synthetic substrate and 10 μl of a fixed concentration of neutrophil elastase dissolved in Hepes buffer (pH 7.4) were added to 190 μl Hepes buffer. After incubating the mixture for a predetermined time at 37 °C, 100 μl of the mixture was added to 2 ml Hepes buffer and the time course of fluorescence intensity was measured using a fluorescence spectrophotometer (F-1200, Hitachi) at $\lambda_{ex} = 380$ nm and $\lambda_{em} = 460$ nm. To measure changes in the fluorescence intensity of a mixture of RBCs and the synthetic substrate, 3 μl of 1.5 μM synthetic substrate and 3.76 μl of packed RBCs were added to 196 μl of Hepes buffer and incubated for a predetermined time at 37 °C. After centrifuging the mixture at 700*g* for 3 min, 100 μl of the supernatant was added to 2 ml Hepes buffer.

Determination of cleavage sites of F-IX by the enzyme. A mixture of purified F-IX (final concentration: 100 U/ml) and the enzyme (approximately 0.2 μg/ml) in Hepes buffer was incubated for 60 min at 37 °C. The cleaved F-IX was reduced with dithiothreitol and carboxylated with iodoacetic acid. The heavy and light chains were then separated by reverse-phase HPLC on a CAPCELL PAK Phenyl SG300 column and the light chain was digested with endoproteinase Asp-N (Asp-N) for 20 h. The digest was then subjected to MALDI-TOF mass spectrometry using 2-mercaptobenzothiazole as a matrix and the enzyme cleavage sites were identified from the measured molecular mass. To determine the enzyme cleavage sites further, the activated F-IX was subjected to SDS–PAGE and transferred to a PVDF membrane. The band corresponding to F-IXaβ was subjected to protein sequence analysis.

Rheological measurement of coagulation. The progress of coagulation of a blood sample in a polypropylene tube (diameter, 0.7 cm; length 2.5 cm) was monitored using a damped oscillation rheometer [8] and [12]. The change in logarithmic damping factor (LDF) during the coagulation of blood sample was measured at 37 °C. The LDF is closely related to the fluidity of blood sample in the tube. The time of onset of coagulation was determined from the initial change in LDF, which corresponds to the initiation of clot formation.

Results

Extraction and purification of the enzyme

To purify F-IX-activating enzyme, the active portion of extracts was subjected to a series of chromato-

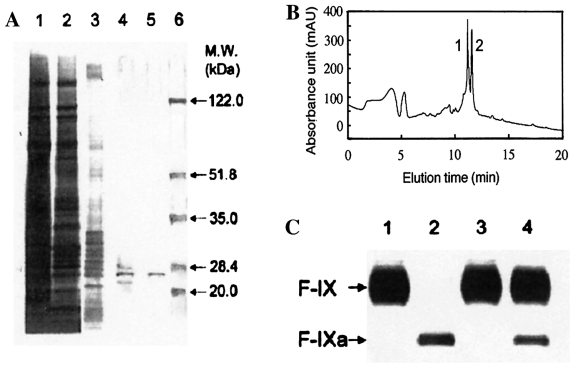

Fig. 1. Extraction and purification of F-IX-activating enzyme. (A) SDS–PAGE profiles of each purification step. A crude extract obtained from the RBC membrane preparation was subjected to a series of chromatographies. Lane 1, the crude extract; lane 2, after Q-Sepharose column; lane 3, after SBTI affinity column; lane 4, after heparin Sepharose column; lane 5, after RP-HPLC; and lane 6, molecular weight standards. (B) Elution profile of the RP-HPLC on a CAPCELL PAK phenyl SG-300 column. (C) Western blot assay of each fraction: lane 1, F-IX standard; lane 2, activated F-IX standard; and lanes 3 and 4, F-IX mixed with RP-HPLC fractions 1 and 2, respectively.

graphic fractionations on different columns (Fig. 1A). F-IX activation by the anion-exchange chromatography fractions was inhibited by α_1-proteinase inhibitor (α_1-PI) or soybean trypsin inhibitor (SBTI) (data not shown), suggesting that the enzyme formed a complex with the inhibitors. Therefore, to perform further purification, the active fractions were subjected to chromatography on an SBTI-agarose column, followed by heparin-affinity chromatography. The elusion profile for the reverse-phase HPLC following the heparin-affinity chromatography is shown in Fig. 1B. Western blotting of the final two peak fractions with F-IX suggested that the fraction 2 was enzymatically active (Fig. 1C, lane 4).

The specific activity of the purified enzyme corresponded to approximately a 25,800-fold purification of the crude extract with an overall yield of 10.5%. The fraction showed only one band by SDS–PAGE (Fig. 1A, lane 5), indicating that the preparation was pure. The amino acid sequence of the active protein was then determined.

Amino acid sequence determination

Masses of cleaved fragments of the purified enzyme were determined (Table 1). Although most masses of minor peaks were identified, but these are not listed. The enzyme consisted of 219 amino acid residues and the molecular mass was 25.7 kDa. Sugar residues were estimated from a comparison of the measured masses of sugar-linked fragment and the theoretically predicted mass. The difference was approximately 880 Da for fragments 75–115 and 135–154 which suggests that the sugar chains are likely GlcNac(Fuc)-GlcNac-Man-Man. Mass of the whole protein differed to the calculated mass by about 1760, which is in accord with two N-linked carbohydrate chains. We designated the F-IX-activating enzyme as erythroelastase-IX (EE-IX).

Table 1
Determination of cleaved fragments of F-IX-activating enzyme

Fragment type	Fragment	Peak number	Observed mass	Calculated mass	Assigned sequence
Tryptic fragments	1–6	1	659.08	657.79	IVGGRR
	7–21	6	1825.18	1810.18 (1826.18)	ARPHAWPFMVSLQLR (methionine sulfoxide)
	22–49	5	2990.28	2974.42 (2990.42)	GGHFCGATLI...AHCVANVNVR (methionine sulfoxide)
	53–62	2	1065.39	1066.25	VVLGAHNLSR
	63–74	3	1486.64	1487.70	REPTRQVFAVQR
	68–74	2	847.25	847.99	QVFAVQR
	75–115	10	5331.75	4450.04 (5327.86)	IFENGYDPVN...QVAQLPAQGRR (+N-linked glycan)
	116–132	7	1822.28	1804.15 (1820.15)	LGNGVQCLAMGWGLLGR (methionine sulfoxide)
	135–154	9	3094.36	2217.41 (3094.41)	GIASVLQELNVTVVTSLCRR (+N-linked glycan)
	154–162	4	1105.54	1106.29	RSNVCTLVR
	165–191	8	2821.98	2822.19	QAGVCFGDSG...GLIHGIASFVR
	192–219	11	3059.34	3055.44	GGCASGLYPD...VNWIDSIIQR
BNPS-skatole fragments	1–12	12	1376.61	1375.79	IVGGRRARPHAW
	13–127			13486.41	PFMVSLQLRG...NGVQCLAMGW
	128–212			10019.26	GLLGRNRGIA...FAPVAQFVNW
	213–219	13	844.58	844.99	IDSIIQR
Whole protein	1–219		25673.27	23915.56 (25669.19)	IVGGRRARPH...VNWIDSIIQR (+N-linked glycans)

Each fragment except for 13–127 and 128–212 was confirmed by amino acid sequence analysis.

Content of the enzyme

From the measurements of the time course of fluorescence intensity of a mixture of fluorogenic substrate and elastase (Fig. 2A), a standard curve of the increment of fluorescence intensity per one minute ($\Delta I/\Delta t$) plotted against the concentration of elastase was obtained (Fig. 2B). Assuming that the relation between the value of $\Delta I/\Delta t$ and the concentration of EE-IX on RBC membranes is expressed by the same equation as that for elastase, the concentration of EE-IX was estimated from the measurement of $\Delta I/\Delta t$ for RBCs (Fig. 2A, line 4). The concentration was in the range of 3.0–3.7 ng per 10^6 RBCs ($n = 24$).

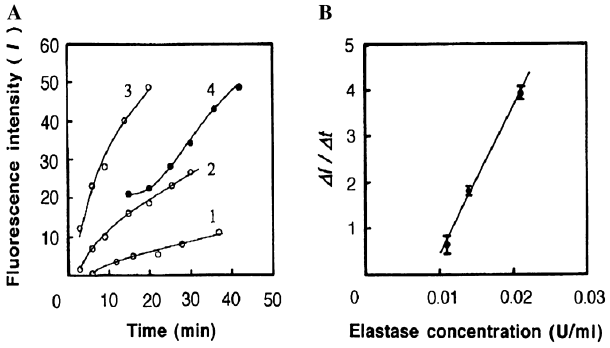

Fig. 2. Estimation of the concentration of F-IX-activating enzyme (EE-IX). (A) Examples of the time course of fluorescence intensity (I) of an incubation mixture of fluorogenic synthetic substrate with elastase and with RBCs. Lines 1, 2, and 3 for elastase (elastase concentration: line 1, 0.011 U/ml; line 2, 0.014; line 3, 0.021); and line 4 for RBCs (RBC count: approximately $17 \times 10^4/\mu l$). (B) A standard curve of the increment of fluorescence intensity per 1 min ($\Delta I/\Delta t$) plotted against the concentration of elastase, where the value of $\Delta I/\Delta t$ was obtained from the initial slope shown in (A). The relation between the value of $\Delta I/\Delta t$ and elastase concentration (C) is expressed by $\Delta I/\Delta t = 321.4C - 2.8$ at the concentration range between 0.01 and 0.021 U/ml.

Cleavage sites of F-IX by EE-IX

We determined the cleavage sites of F-IX by EE-IX from the masses of the Asp-N fragments derived from the light chain of EE-IX cleaved F-IX (F-IXee) and from the amino acid sequences of the C-terminal light chain fragment (Fig. 3A) and the N-terminal heavy chain fragments of F-IXee (Fig. 3B). The identification of the cleavage sites of F-IX indicated that EE-IX activates F-IX by cleaving at Thr_{140}–Ser_{141}, Val_{181}–Val_{182}, and/or Val_{182}–Gly_{183} bonds (Fig. 3C). On the other hand, the neutrophil elastase cleavage sites in F-IX were Thr_{140}–Ser_{141}, Thr_{144}–Arg_{145}, and Val_{181}–Val_{182}, which sites were in accord with those reported by other worker [13].

Coagulation ability of F-IX cleaved by EE-IX (F-IXee)

The issue of whether F-IXee is capable of initiating coagulation was examined. Coagulation of PFP and PFP with added purified EE-IX did not occur (Fig. 4A, lines 1 and 2), whereas coagulation of PFP supplemented with RBCs occurred (line 5). When F-IXee was added to PFP, coagulation occurred, but the prolonged preincubation of F-IX with EE-IX caused a delay in the initiation of coagulation (Fig. 4A, lines 3 and 4). This result may be expected from the result obtained by SDS–PAGE analysis which shows that an incubation of F-IX with EE-IX produced F-IXee with almost the same molecular weight as that of F-IXa, but further

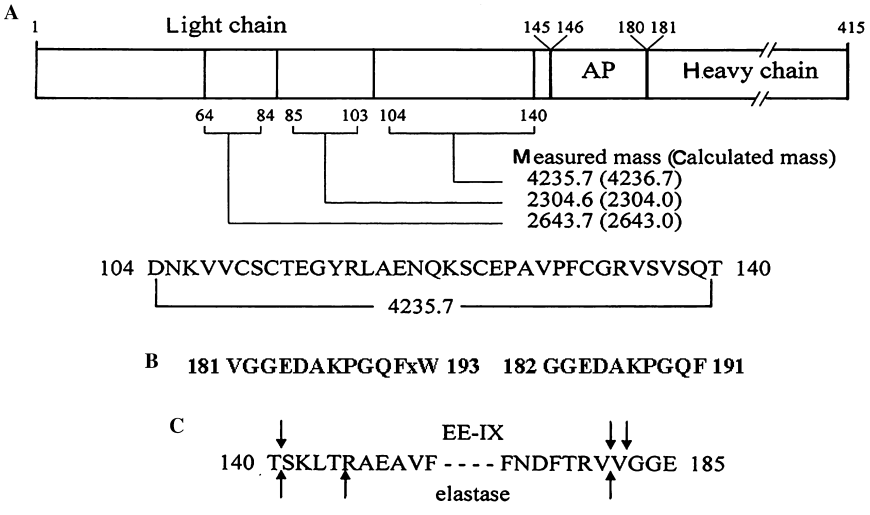

Fig. 3. Determination of cleavage sites of F-IX by EE-IX. (A) The mass of the light chain fragments of F-IXee and the amino acid sequence of the C-terminal light chain fragment. The light chain, activation peptide (AP), and heavy chain signify those of F-IX cleaved by F-XIa. The amino acid sequence of the C-terminal fragment (104–140) was determined by amino acid sequence analysis. (B) The amino acid sequence of the N-terminal heavy chain fragments of F-IXee, which was identified by protein sequence analysis of the SDS–PAGE band corresponding to F-IXaβ. (C) Sites of F-IX cleavage by EE-IX and neutrophil elastase.

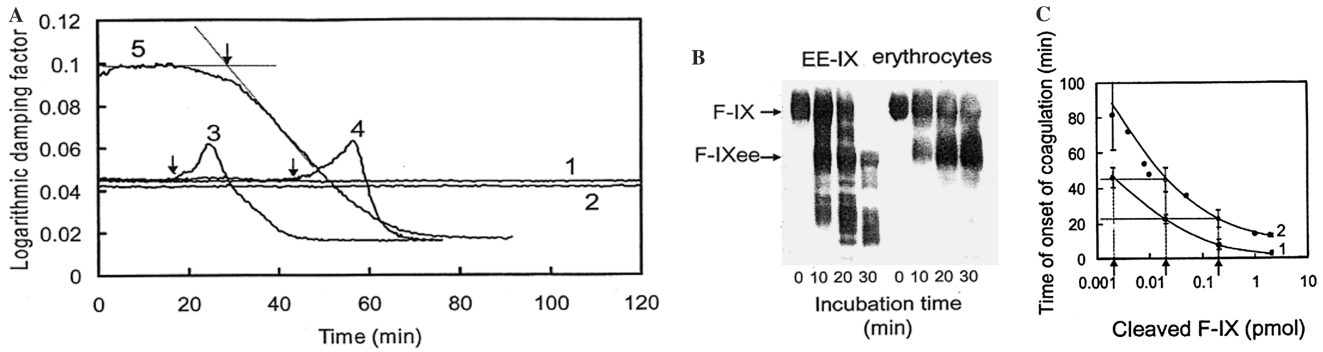

Fig. 4. Coagulant ability of F-IXee. (A) Changes in LDF during the coagulation of PFP and PFP supplemented with RBCs. For PFP with added F-IXee, F-IX dissolved in Hepes at a concentration of 2 U/ml was preincubated with EE-IX for a predetermined time at 37 °C, and 1 μl of the mixture was added to the PFP. Line 1, without EE-IX; line 2, with added EE-IX (10 ng/ml); lines 3 and 4, with added F-IX pretreated with EE-IX, the estimated concentration of F-IXee: line 3, 0.2 pmol; (F-IX: 2 U/ml; EE-IX/F-IX: 1/1000 (w/w); preincubation: 4 min); line 4, 0.02 pmol (F-IX: 2 U/ml; EE-IX/F-IX: 1/1000; preincubation: 30 min); and line 5, PFP supplemented with RBCs (hematocrit: 40%). To initiate coagulation, 85 μl of a 0.25 M $CaCl_2$ solution was added to 1.0 ml of the blood sample. The arrow indicates the time of onset of coagulation. (B) The effect of incubation time on F-IX with EE-IX and with RBCs. Purified F-IX was incubated with EE-IX or RBCs for a predetermined time. The supernatant was subjected to Western blot or SDS–PAGE analysis. The concentration of F-IX was 20 U/ml with the ratio of EE-IX/F-IX = 1/100. Left four lanes, SDS–PAGE for a mixture of F-IX with EE-IX; right four lanes, Western blot for that with RBCs (hematocrit: 40%). (C) The time of onset of coagulation of PFP plotted against the concentration of cleaved F-IX. Curve 1, F-IX-deficient PFP with added F-IXa; curve 2, F-IX-deficient PFP with added F-IXee (EE-IX/F-IX: 1/100). The concentration of F-IXee was estimated from a quantitation comparison of the SDS–PAGE band of F-IXee and F-IXa.

incubation produced fragments of F-IX with lower molecular masses (Fig. 4B, left).

The ability of F-IXee to cause coagulation was obtained from the ratio of the concentration of F-IXa to that of the cleaved F-IX at the same value of the time of onset of coagulation (Fig. 4C). The ability was estimated to be approximately 1/10 as high as that of the F-IXa,

Discussion

The amino acid sequence of EE-IX is in accord with that of neutrophil elastase although the neutrophil elastase consists of 218 amino acid residues [14] and [15] and the molecular weight (29.5 kDa) [16] was slightly larger than that of EE-IX. The difference in the molecular weight is assumed to be due to the difference in the number of asparaginyl N-linked oligosaccharide chains. EE-IX contained N-linked glycans at Asn_{95} and Asn_{144}, while elastase is glycosylated at Asn_{59}, Asn_{95}, and Asn_{144}.

The enzymatic activity of reticulocyte-enriched erythrocyte, which was estimated from the fluorescence intensity of a mixture of reticulocyte-enriched erythrocytes and the fluorogenic substrate, was approximately 20 times higher than those of normal RBCs (unpublished data). Incubation of RBCs with leukocyte elastase failed to cause any adhesion of the elastase to RBC membranes (data not shown). The results, together with the fact that EE-IX was extracted from RBC membrane, suggest that the gene for this enzyme may be expressed in bone marrow precursors prior to RBC maturation and the enzyme is not derived from leukocytes. However, the result of RT-PCR for total RNA extracted from reticulocyte-enriched erythrocytes indicated that elastase cDNA was not amplified from reticulocyte-derived cDNA and the number of copy for elastase mRNA was negligible (unpublished data). Therefore, it is assumed that elastase gene is not expressed in reticulocytes. In neutrophil elastase, it is reported that the gene is expressed in bone marrow precursors, but not in leukocytes that carry elastase [15]. As it is reported that the sequence of neutrophil elastase precursor has R after the last amino acid Q [17], it would be reasonable to assume that both enzymes may be products from the same gene.

As the amount of elastase in polymorphonuclear neutrophils is reported to be 1.1×10^3 ng per 10^6 cells [18], the content of EE-IX in RBC membranes is approximately 1/300 as high as that of neutrophil elastase. An estimate is that 100 ml of blood will contain approximately 1.5–1.8 mg of this enzyme. As the content of reticulocytes is 1–2% of the total erythrocytes, most of the enzyme may be contained in normal (old) RBC membrane.

The cleavage sites of F-IX by EE-IX were different from Arg_{145}–Ala_{146} and Arg_{180}–Val_{181}, cleaved by F-XIa [19] and [20] although the respective sites were close to each other. Our data suggest that the sites of F-IX cleaved by EE-IX may serve a previously unrecognized role in the formation of an active species [21] and [22] since EE-IX catalyzed a slow but definite activation of F-IX. What was unclear in the current experiments was that either cleavage at Val_{181}–Val_{182} or at Val_{182}–Gly_{183} had higher ability to activate F-X.

It is known that when neutrophil elastase is added to PFP, coagulation does not occur because of complex formation between elastase and plasma α_1-PI [16]. We were able to confirm that when the isolated EE-IX is

added to PFP, no coagulation occurred. Moreover, it has been reported that an incubation of F-IX with leukocyte elastase generated F-IX fragments with lower molecular masses, leading to the loss of coagulant activity of F-IX [13] and [23]. Our data indicated that F-IXee was capable of initiating coagulation, but the coagulant activity of F-IXee lowered with the increase in the preincubation time. Therefore, we conclude that F-IXee possesses procoagulant activity when the degradation does not proceed to completion. This may not be contradictory to other reported results on leukocyte elastase, in which 30–40% of the initial coagulant activity remained after a 10 min incubation [13] and [23].

The amount of F-IX cleaved by RBC membranes increased with increasing incubation time (Fig. 4B, right). Furthermore, the coagulation of PFP supplemented with RBCs occurred at about 30 min (Fig. 4A, line 5). This suggests that EE-IX in RBC membranes does not form a complex with α_1-PI. The location of EE-IX on RBC membranes may be critical for the effective activation of F-IX to cause coagulation although the molecular mechanism of F-IX activation on RBC membranes remains unclear. Although the ability of F-IXee to cause coagulation was lower than that of F-IXa, the enzymatic activity may be sufficiently large to activate F-X, thus leading to thrombin.

In conclusion, our study clearly demonstrated that the F-IX-activating enzyme, erythroelastase-IX (EE-IX), is located in the RBC membrane. F-IX activated by EE-IX in RBC membranes is capable of initiating blood coagulation. Our findings may provide a framework for further defining the roles in clot formation.

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