Covalent Crosslinking of Human Coagulation Factor V by Activated Factor XIII
From Guinea Pig Megakaryocytes and Human Plasma

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Coagulation factor V (FV) has been shown to be synthesized in both the liver and megakaryocytes. We now present evidence that FV can be covalently crosslinked by an enzyme originating from megakaryocytes to form polymeric multimers of factor V. The guinea pig megakaryocyte enzyme appears to be factor XIIIa since the FV-crosslinking activity (1) had an absolute requirement for Ca++, (2) was completely inhibited by iodoacetamide, 5,5'-dithiobis-2-nitrobenzoic acid, p-chloromercuribenzenesulfonic acid, and N-ethylmaleimide, all known alkylators of the thiol group at the active site of the factor XIIIa, (3) was blocked by known pseudoamine donor substrates of factor XIII including dansylcadaverine and putrescine, and (4) could be directly demonstrated in the guinea pig megakaryocyte lysate by a specific activity staining procedure. No transglutaminase was detected in guinea pig megakaryocytes in contrast to red cells and liver. A similar pattern of covalent crosslinking of human FV by purified activated human plasma factor XIII was also demonstrated. Analysis of the crosslinked products of FV formed by the guinea pig enzyme by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) indicates the formation of intermediate as well as higher molecular weight polymers, suggesting that the crosslinking is a stepwise polymerization process.

MATERIALS AND METHODS

Materials. Human plasma factor V (FV) was isolated in our laboratory as described below. Purified human plasma factor XIII zymogen was a generous gift of Drs Andrei Budzynski and Joseph Franzi, Thrombosis Research Center, Temple University, and of Drs Sook Il Chung and J. E. Folk, National Institute of Dental Research, NIH. Human plasma from a patient with a fatal hemorrhagic diathesis, which contains a monoclonal anti-human FV antibody and from which the antibody was purified to prepare an immunoadsorbent for purification of human plasma FV was a gift of Dr Helen Glueck, University of Cincinnati Medical School. L-[35S]methionine (specific activity, > 800 Ci/mmol) and [1,4-14C]putrescine (specific activity, 109 mCi/mmol) were purchased from Amersham (Arlington Heights, IL). Cell culture items including Dulbecco's modified eagles medium free of methionine, fetal calf serum, gentamycin, and others were obtained from GIBCO Labs (Grand Island, NY).

Various protease and factor XIIa inhibitors as well as dansylcadaverine...
sine and N, N’ dimethylated casein were purchased from Sigma Chemical Co (St Louis). The reagents required for sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) analysis of FV crosslinking were obtained from Bio-Rad (Richmond, CA). Myosin heavy chain (Mol was 200 kD) used as a molecular weight marker in SDS-PAGE was purchased from BRL (Gaithersburg, MD). Agarose (EE0 0.1-0.15) was obtained from SeaKem (Rockland, ME). α-Thrombin (2,650 U/mg) is a gift from Dr John Fenton, New York State Department of Health, Albany, and hirudin (1,400 U/mg), obtained from Soma. Normal pooled plasma was purchased from George King Biomedical, Inc (Overland Park, KS). CNBr-activated Sepharose 4B was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). YM-100 membrane filters were purchased from Amicon Corp (Lexington, MA). Autoradiography films (Cronex, 20 × 25 cm) were products of Du Pont (Wilmington, DE). Aquasol and Econofluor were obtained from New England Nuclear, Boston. All other common chemicals and biochemicals were of analytical grade and obtained from commercial sources.

Purification of human plasma FV. Human plasma FV was isolated by a modified procedure of our previously described immunoaffinity method.13 Briefly, blood (eg, 445 mL) was drawn from normal donors by venepuncture into a bottle containing 1 vol (50 mL) of 0.13 mol/L trisodium citrate and 0.01 vol (5 mL) of a protease inhibitor cocktail that consisted of 100 mol/L benzamidine, 100 mol/L L-aminocaproic acid, 100 mol/L phenylmethylsulfonylfluoride, 240 μmol/L soybean trypsin inhibitor, 10 μmol/L leupeptin, 1 mmol/L pepstatin and 10,000 KIU/mL aprotinin. Platelet-free plasma was obtained by centrifugation of the anticoagulated blood at 2,000 g at 4°C for 15 minutes. The plasma was subjected to fractionation with BaCl2 and 4% to 10% PEG 600 in a manner identical with the procedure described by Nesheim et al.17 The resulting precipitates were dissolved in 1/10 vol (50 mL) of Buffer A (20 mmol/L Tris-HCl, pH 7.2, containing 150 mmol/L NaCl and 0.02% NaN3) and the FV solution applied to a column (5 × 5 cm) of the immunoadsorbent Sepharose 4B coupled with a naturally occurring human monoclonal antibody directed against human FV.14 The column was then washed successively with 5 × bed volume each of Buffer A, Buffer B (20 mmol/L Tris HCl, pH 7.2, 370 mmol/L NaCl and 0.02% NaN3), and Buffer C (20 mmol/L Tris-HCl, pH 7.2, 150 mmol/L NaCl, 5 mmol/L CaCl2, 5% gycerol, and 0.02% NaN3). The specifically bound FV was eluted with Buffer D (20 mmol/L Tris-HCl, pH 7.2, 150 mmol/L NaCl, 5 mmol/L CaCl2, 0.02 Na2HPO4, 20% gycerol and 50% ethylene glycol). The calcium was used to stabilize factor V activity. Fractions containing the highest FV coagulant activity were pooled and concentrated by ultrafiltration using Amicon YM-100 membrane.

The purified factor was characterized to be authentic FV by three criteria: (1) a single major band on SDS-PAGE analysis of FV crosslinking was contained in a final volume of 0.05 mL 50 mmol/L Tris-HCl, pH 7.4; 5 mmol/L CaCl2 unless otherwise stated; 5 to 10 mmol/L crosslinking inhibitors, Ca++ chelators, or pseudo amine donor competitive substrates where indicated; 2 μg of purified human plasma FV as crosslinking substrate; and 0.1 to 0.5 μg of FV crosslinking enzyme derived from guinea pig megakaryocytes (see Results). The crosslinking enzyme preparation was the material eluted with buffer D (Fig 1A). This material containing factor XIII was found to be activated to FXIIIa presumably by calpain19 on storage. Reactions were initiated by addition of the labeled guinea pig megakaryocyte FV as well as for FV-crosslinking guinea pig megakaryocyte enzyme (vide infra).

FV crosslinking reaction. Crosslinking reaction mixtures contained in a final volume of 0.05 mL 50 mmol/L Tris-HCl, pH 7.4; 5 mmol/L CaCl2 unless otherwise stated; 5 to 10 mmol/L crosslinking inhibitors, Ca++ chelators, or pseudo amine donor competitive substrates where indicated; 2 μg of purified human plasma FV as crosslinking substrate; and 0.1 to 0.5 μg of FV crosslinking enzyme derived from guinea pig megakaryocytes (see Results). The crosslinking enzyme preparation was the material eluted with buffer D (Fig 1). This material containing factor XIII was found to be activated to FXIIIa presumably by calpain19 on storage. Reactions were initiated by addition of the labeled guinea pig crosslinking enzyme. After incubation at 37°C for 20 minutes, reactions were stopped by addition of 0.025 mL of Laemmli’s electrophoresis sample buffer (250 mmol/L Tris-HCl, pH 6.8, containing 20% 2-mercaptoethanol, 8% SDS, 40% gycerol, and 0.004% bromophenol blue). After the final volume of the reaction mixtures was made to 0.1 mL with distilled water, the reaction mixtures were processed for SDS-PAGE analysis as described below.

SDS-PAGE analysis of crosslinked products. Crosslinking reaction mixtures, prepared as described above, were analyzed for crosslinked products, on a 4% linear gel without a stacking gel essentially as described by Laemmli,20 using a 16 × 18 × 0.15 cm slab in a Bio-Rad apparatus (Model 360). Electrophoresis was at 70 mA for a 2-slab gel run for four hours with cooling at 20°C. At the end of the run, the gel was stained for one hour with shaking in a staining solution (0.1% Coomassie Blue R-250 in 10% acetic acid and 25% isopropyl alcohol), destained overnight in a destaining solution (10% acetic acid and 5% methanol), dried on a filter paper as described,21 and photographed. In experiments where [35S]-

To prepare the biosynthetically labeled FV, isolated megakaryocytes (eg, approximately 1 × 10^9 cells) were incubated at 37°C in a humidified chamber with circulating 5% CO2 and 95% air, with [35S]methionine (200 μCi/mL) in a volume of 5 mL of suspension medium, which consisted of modified Dulbecco’s eagles medium medium free of methionine, supplemented with 10% fetal calf serum and an antibiotic (eg, gentamycin). After incubation for 18 hours, cells were harvested by centrifugation at 205 g for ten minutes, washed three times with 0.01 mol/L sodium phosphate, pH 7.4, containing 140 mmol/L NaCl and 2.7 mmol/L KCl (PBS), and collected by centrifugation. The cell pellets were then suspended in 0.2 mL of a lysis buffer (PBS containing 0.1% BSA, 0.2% Triton X-100, 1 mmol/L benzamidine, 1 mmol/L L-aminocaproic acid, 1 mmol/L phenylmethylsulfonylfluoride, 2.4 μmol/L soybean trypsin inhibitor, 50 μmol/L leupeptin, 10 μmol/L pepstatin, and 10 KIU/mL aprotinin) and lysed by sonication and centrifuged as described by Chi et al.13

Preparation and purification of [35S]methionine-labeled guinea pig megakaryocyte FV. Megakaryocyte lysate (eg, 0.2 mL of either 10,000 g [S-10] or 105,000 g supernatant [S-105]), prepared as described above, was applied to a column of anti-human FV-coupled Sepharose 4B (a total bed volume of 3 mL in a 5-mL plastic syringe), which had been previously equilibrated with buffer A (vide supra). The column was then washed successively with 5 × bed volume of buffer A and 50 × bed volume of buffer B until no radioactive material emerged from the column. Two-milliliter fractions were collected. The bound FV was then eluted with 20 mL of buffer D free of CaCl2 (Fig 1A). Aliquots of each fraction were assayed for FV coagulant activity and for radioactivity. The FV peak fractions (tubes no. 51 through 54) were then pooled, dialyzed against 2 L of 20 mmol/L Tris-HCl (pH 6.8) containing 0.1 mmol/L EDTA to minimize proteolysis by calcium activated proteases, and concentrated by applying PEG 15,000 to 20,000 to the surface of the dialysis bag at 4°C. The concentrated fraction was used as a source for the labeled guinea pig megakaryocyte FV as well as for FV-crosslinking guinea pig megakaryocyte enzyme (vide infra).
methionine-labeled FV crosslinked products were analyzed, the stained gel was treated with Enhance (NEN, Boston), dried, and subjected to autoradiography using Cronex x-ray films with exposure at ~70°C.

**FVIII assay.** FVIII was assayed by the procedure essentially as described by Folk and Chung,21 which is based on the ability of activated FVIII to incorporate an amine donor such as [3H]putrescine into a protein acceptor, dimethylated casein, with subsequent determination of TCA-precipitable radioactivity.

**Thrombin activation of FVIII.** Purified human plasma FVIII was activated by thrombin in a reaction mixture which contained, in a final volume of 0.1 mL: 10 mmol/L Tris-HCl, pH 7.5; 70 µg FVIII; and 0.1 NIH unit of thrombin. The reaction was initiated by addition of thrombin and, after incubation at 37°C for 30 minutes, stopped by addition of hirudin at a final concentration of 20 U/mL. Activation was monitored by the FVIII assay as described above.

**Cell lysates and tissue extract for FVIII and transglutaminase analyses.** Guinea pig megakaryocyte lysate was prepared as follows: Cells (1 x 10⁸ cells) were suspended in 0.1 mL of a lysis buffer (20 mmol/L Tris-HCl, pH 7.4, containing 0.2% Triton X-100, 2 mmol/L EDTA, 2.4 µmol/L soybean trypsin inhibitor, and 50 µmol/L leupeptin) and sonicated at maximum speed six times for ten seconds at 4°C followed by freezing and thawing six times. After centrifugation at 10,000 g for ten minutes, the supernatant was collected. Guinea pig RBCs and platelets and plasma were prepared according to a standard procedure24 using sodium citrated blood (1 vol of 3.8% trisodium citrate + 9 vol of blood) as a starting material. Guinea pig RBCs (2 x 10⁹ cells) and platelets (5 x 10⁹ cells) suspended in 0.1 mL of the lysis buffer were lysed in a manner similar to that for megakaryocytes. Guinea pig liver extract was prepared as described.24 Human RBCs, platelets, and plasma were prepared in a similar manner.25 Human RBCs (2 x 10⁹ cells) and platelets (5 x 10⁹ cells) suspended in 0.1 mL of the lysis buffer were lysed as described above.

**Fluorescent activity staining procedure for identification of FVIII and transglutaminase.** A rapid method for detection of and distinguishing between factor XII and transglutaminase (TGase) in crude cell extracts was used essentially as described by Steinberg and Stenberg.26 The procedure involved the separation of the two enzymes by agarose gel electrophoresis under nondenaturing conditions27 followed by the detection of the enzyme activities by their ability to incorporate a fluorescent amine, dansylcadaverine, into an acceptor dimethylated casein with subsequent visualization under a UV lamp of the fluorescent spot on the gel. Briefly, cell lysates (50 µL) were subjected to agarose gel electrophoresis in a horizontal apparatus28 at 4°C at 40 mA in 2-mm thick gels (14.7 x 12.6 cm) containing 0.8% agarose using 0.1 mol/L barbitral, pH 8.6, containing 2 mmol/L EDTA as electrode and gel buffers. To detect FVIII, the gel was incubated at 37°C for 90 minutes in staining solution A (50 mmol/L Tris-HCl, pH 7.5, containing 1% dimethylated casein, 0.7 mmol/L monodansylcadaverine, 35 mmol/L CaCl₂, 2 mmol/L DTT, and 10 NIH U/mL α-thrombin), after which the gel was fixed by immersing in 10% TCA for 20 minutes and the excess stain was removed by repeated washing with 10% acetic acid followed by neutralization with 50 mmol/L Tris-HCl (pH 7.5). To detect TGase, a duplicate gel was processed in an identical manner except that the gel was treated in staining solution B, which consisted of the same constituents as staining solution A except that α-thrombin was omitted. Both gels were then dried on filter papers as described,22 the fluorescent bands visualized under UV light, and they were then photographed through a Wratten No. 15 filter using Polaroid Land Camera (Model CU-5).

**Protein determination.** Protein contents were measured by the procedure of Lowry et al.29 using bovine serum albumin as a standard.

**RESULTS**

**Preparation and purification of FV-crosslinking guinea pig megakaryocyte enzymatic factor.** FV-crosslinking enzymatic activity was prepared in a manner identical with that for purification of guinea pig megakaryocyte FV (Fig 1A) since it was found that a small percentage of the FV-crosslinking copurifies with FV after the application of buffer D. The chromatographic behavior of human plasma FV and FVIII were similar as were FV and FVIII from guinea pig plasma (Fig 1C). In both cases plasma FVIII required thrombin for conversion to FXIIIα as measured by ability to catalyze putrescine incorporation into dimethyl casein. In the case of guinea pig platelet lysate (Fig 1D), FXIIIα activity coeluted at the same region as plasma human FXIII and FV (Fig 1B) and plasma guinea pig FXIII and FV (Fig 1C). Although the majority of FXIII activity did not bind to the affinity gel in each case, in all experiments some FXIII activity coeluted with FV. The crosslinking activity that emerged at the void volume of the column required thrombin to express its transaminidase activity.
However, in stored preparations both guinea pig megakaryocyte and platelet lysates showed crosslinking activity in the absence of thrombin. However, when freshly prepared fractions were assayed no FXIIIa activity was evident until after thrombin and calcium were added. This latter data indicates that FXIII is present in the zymogen form in platelets and megakaryocytes but may be activated by endogenous proteases such as calpain in addition to thrombin.

**Evidence for enzymatic covalent crosslinking of human FV.** In the course of studies showing that FV is biosynthesized de novo in isolated guinea pig megakaryocytes in culture, we noted that incubation of purified human plasma FV with an enzyme fraction derived from guinea pig megakaryocytes resulted in the conversion of the FV to high molecular weight derivatives as monitored by SDS-PAGE. Purified plasma FV, analyzed by SDS-PAGE under reducing conditions on a 4% gel, exhibited homogeneous behavior as evidenced by a single protein band with an estimated molecular weight of about 330 kd (Fig 2, lane 2). No higher molecular weight components were noted. However, preincubation of the human FV with an enzyme from guinea pig megakaryocyte lysate resulted in the disappearance of the monomeric FV band with a concomitant appearance of intermediate molecular weight polymeric forms together with high molecular weight polymers (Fig 2, lane 3). The existence of these forms after incubation in 5% 2-mercaptoethanol and 2% SDS before electrophoresis indicated that the high molecular weight forms are held together by covalent bonds. One possibility is the crosslinking of human FV by an enzyme derived from guinea pig megakaryocytes. The FV-crosslinking activity of the purified guinea pig megakaryocyte enzyme was not abolished by dialysis of the enzyme (lane 4), suggesting that the crosslinking of FV was not effected nonenzymatically by low molecular weight compounds. Incubation of the human FV with enzyme elution buffer (buffer D) alone (without the guinea pig megakaryocyte component) did not produce high molecular weight derivatives of FV (Fig 2, lane 10), again indicating that buffer components were not responsible. Inclusion in the reaction mixture of increasing amounts of the megakaryocyte component (0.1, 0.2, and 0.3 μg) resulted in the formation of high molecular weight crosslinked products at the expense of intermediate molecular weight polymers (lanes 7, 8, and 9, respectively). These results suggest that the process is covalent crosslinking of FV by a guinea pig megakaryocyte enzyme and that the reaction is a stepwise polymerization process. The substrate is plasma FV since the proteins from the megakaryocyte lysate are too low to be detected by protein staining. Since the higher molecular weight crosslinked products barely entered a 4% polyacrylamide gel, it seems to suggest that the crosslinked multimers possess molecular weights of more than a million. Purified human plasma FV crosslinked under these conditions had a specific activity of 90.0 ± 1.5 U/mg while the unmodified FV had an

![Figure 2](https://example.com/figure2.png)

**Fig 2.** Evidence for covalent crosslinking of human plasma FV by an enzyme derived from guinea pig megakaryocytes. Purified human plasma FV (2 μg) was incubated at 37°C for 20 minutes with or without purified guinea pig megakaryocyte lysate enzyme (0.1 μg) in crosslinking reaction mixture (0.05 mL) containing 50 mmol/L Tris-HCl, pH 7.4. The reaction was then terminated by the addition of Laemmli’s sample buffer containing 2-mercaptoethanol and SDS, at final concentrations of 5% and 2%, respectively. After heating the mixtures in a boiling water bath for five minutes, they were subjected to SDS-PAGE on a 4% gel, after which the gel was stained with Coomassie Blue and dried as described under Materials and Methods. Lane 1 contained myosin heavy chain (2 μg); lane 2, human plasma FV (hp FV); lane 3, human plasma FV incubated with guinea pig megakaryocyte lysate enzyme; lane 4, human plasma FV incubated with dialyzed guinea pig megakaryocyte lysate enzyme; lane 5, myosin H chain (2 μg) plus human plasma FV; lane 6, human plasma FV incubated without guinea pig megakaryocyte lysate enzyme; lane 7, human plasma FV incubated with guinea pig megakaryocyte lysate enzyme (0.1 μg); lane 8, human plasma FV incubated with guinea pig megakaryocyte lysate enzyme (0.2 μg); lane 9, human plasma FV incubated with guinea pig megakaryocyte lysate enzyme (0.3 μg); and lane 10, human plasma FV incubated with elution buffer (buffer D).
activity of 90.6 ± 1.2 U/mg (mean ± SD, n = 5). Thus, crosslinking does not affect FV activity.

**Requirement of Ca**²⁺ for FV-crosslinking activity. FV-crosslinking activity of the purified enzyme from guinea pig megakaryocyte lysate required Ca**²⁺** for its activity. In the absence of the megakaryocyte enzyme no crosslinking is seen (Fig 3, lane 3). Preincubation of FV with the crosslinking enzyme and CaCl₂ (5 mmol/L) resulted in the conversion of a substantial portion of the monomeric FV to higher molecular weight polymers (lane 4), indicating an effect of Ca**²⁺**. This conclusion is supported by the observation that omission of CaCl₂ and addition of EDTA at a concentration of 10 mmol/L to the crosslinking reaction mixture almost completely abolished the FV-crosslinking activity of the guinea pig enzyme (lane 5). Since all known FXIII and transglutaminases are absolutely dependent on Ca**²⁺** for their activity, these results are consistent with the possibility that the FV-crosslinking activity of guinea pig megakaryocytes is a function of FXIII and/or transglutaminase present in the megakaryocyte.

**Effects of –SH blocking agents on FV-crosslinking activity.** To further test the hypothesis that megakaryocyte FXIII was responsible for the crosslinking of FV we used the knowledge that these enzymes contain a sulphydryl group at their active site. Known –SH group inhibitors of FXIII and transglutaminases were therefore examined for their effect on the FV-crosslinking catalyzed by the guinea pig megakaryocyte enzyme and their crosslinked products analyzed by SDS-PAGE. In the presence of Ca**²⁺** (5 mmol/L), addition to the reaction mixture of –SH blocking agents such as iodoacetamide, 5,5'-dithiobis-(2-nitrobenzoic acid), p-chloromercuribenzenesulfonate, and N-ethylmaleimide at concentrations of 10 mmol/L, 5 mmol/L, 5 mmol/L, and 5 mmol/L, respectively, completely inhibited the FV-crosslinking activity of the guinea pig enzyme (Fig 3, lanes 6, 7, 8, and 9, respectively). The more rapid migration of the FV polypeptide is due to the absence of reducing agent. If 2-mercaptoethanol is added at the end of the crosslinking reaction together with SDS, the migration is the same as in lanes 2, 3, and 5 (results not shown). These results are consistent with the hypothesis that the FV-crosslinking activity of guinea pig megakaryocyte is FXIIIa and/or transglutaminase.

**Effect of known amine donor substrates of FXIIIa on FV-crosslinking activity.** In order to further evaluate the possibility that FV-crosslinking enzyme of guinea pig megakaryocytes is FXIIIa, we used dansylcadaverine and putrescine, which are known to competitively inhibit the

*Fig 3. Requirement of the FV-crosslinking activity of guinea pig megakaryocyte for Ca**²⁺** and inhibition of the activity by EDTA, SH group blocking reagents, and pseudoamine donors. For conditions of incubations and SDS-PAGE, see Fig 2 legend. In lanes 6 through 9 where the effect of –SH blocking agents were evaluated, 2-mercaptoethanol was omitted from the reaction mixture. Lane 1 contained myosin heavy chain (2 µg); lane 2, human plasma FV; lane 3, human plasma FV incubated without guinea pig megakaryocyte lysate enzyme; lane 4, human plasma FV incubated with guinea pig megakaryocyte lysate enzyme in the presence of 5 mmol/L CaCl₂; lane 5, human plasma FV incubated with guinea pig megakaryocyte lysate enzyme in the presence of 10 mmol/L EDTA; lane 6, human plasma FV incubated with guinea pig megakaryocyte lysate enzyme in the presence of 10 mmol/L iodoacetamide; lane 7, human plasma FV incubated with guinea pig megakaryocyte lysate enzyme in the presence of 5 mmol/L 5,5'-dithiobis-(2-nitrobenzoic acid); lane 8, human plasma FV incubated with guinea pig megakaryocyte lysate enzyme in the presence of 5 mmol/L p-chloromercuribenzenesulfonate; and lane 9, human plasma FV incubated with guinea pig megakaryocyte lysate enzyme in the presence of 5 mmol/L N-ethylmaleimide; lane 10, human plasma FV incubated with guinea pig megakaryocyte lysate enzyme in the presence of 2 mmol/L dansylcadaverine; and lane 11, human plasma FV incubated with guinea pig megakaryocyte lysate enzyme in the presence of 10 mmol/L putrescine.*
The presence of dansylcadaverine (2 mmol/L) and putrescine (10 mmol/L) in the preincubation mixture completely inhibited the crosslinking of FV (Fig 3, lanes 10 and 11). The results, once again, strongly support the postulate that the FV-crosslinking enzyme of guinea pig megakaryocyte is FXIII and/or transglutaminase.

**Evidence that guinea pig megakaryocyte FV can also form crosslinked polymers.** To see if guinea pig megakaryocyte FV was also a substrate for FXIII or transglutaminase, we incubated $[^{35}S]$methionine-labeled affinity-purified guinea pig MK FV in the presence of Ca$^{++}$ and purified human plasma FV, and analyzed the reaction products by SDS-PAGE followed by autoradiography. As can be seen in Fig 4 inclusion of radiolabeled guinea pig FV (containing 10,000 or 20,000 cpm) in such reaction mixtures resulted in the crosslinking of the radiolabeled guinea pig FV as visualized by autoradiography (Fig 4B, lanes 3 and 4, respectively), as well as in that of the nonlabeled human FV as visualized by protein staining (Fig 4A, lanes 3 and 4, respectively). The labeled crosslinked products of guinea pig FV was associated with those of human FV at regions precisely corresponding to high and intermediate molecular weight polymers (lane 4), suggesting the copolymerization of human and guinea pig FV and/or the homologous crosslinking of the FV of the two species.

**Demonstration of the presence of FXIII in guinea pig megakaryocytes by a specific activity staining procedure.** To attempt to determine whether FXIIIa or transglutaminase is involved in the crosslinking of FV, we separated FXIII and transglutaminase by agarose gel electrophoresis on the basis of their different molecular weights followed by the localization of the two enzymes by activity staining for the fluorescent amine incorporation into dimethylated casein. In the absence of thrombin (Fig 5B) only the TGase present in guinea pig and human RBCs and guinea pig liver were visualized (lanes 4, 8, and 5, respectively). They migrated most rapidly consistent with their monomeric structure with a molecular weight of approximately 80 kD. In contrast, FXIII present in human or guinea pig plasma (Fig 5A, lanes 1 and 6) required thrombin and migrated most slowly consistent with a hetero-tetrameric structure ($a_2b_2$) and a 320 kD mol wt. Guinea pig and human platelets (Fig 5A, lanes 2 and 7) required thrombin and migrated in an intermediate position consistent with its homodimeric structure ($a_3$). The trace of more slowly migrating enzyme seen in human platelet lysate (Fig 5A, lane 7) may be due to a small degree of human plasma contamination. Guinea pig megakaryocytes contain predominantly homodimeric ($a_3$) FXIII similar to platelets (Fig 5A, lane 3). No TGase was detected in the guinea pig megakaryocytes (Fig 5B, lane 3) in contrast to liver and RBCs.

**Evidence for the formation of polymers involving guinea pig megakaryocyte FV.** Human plasma FV (2 $\mu$g) and $[^{35}S]$methionine-labeled guinea pig megakaryocyte FV were incubated with guinea pig megakaryocyte enzyme (0.1 $\mu$g) at 37°C for 20 minutes, in a reaction mixture containing 50 mmol/L Tris-HCl (pH 7.4) and 4 mmol/L CaCl$_2$. Reaction was stopped by addition of Laemmli’s sample buffer, containing 2-mercaptoethanol and SDS at final concentrations of 5% and 2%, respectively. The mixtures were then boiled for five minutes and subjected to SDS-PAGE and the gel then stained with Coomassie Blue, dried, and subjected to autoradiography as described under Materials and Methods. Lane 1 contained myosin heavy chain (2 $\mu$g); lane 2, human plasma FV (2 $\mu$g); lane 3, human plasma FV (2 $\mu$g) incubated with labeled guinea pig megakaryocyte FV (containing approximately 10,000 cpm) in the presence of purified guinea pig megakaryocyte lysate enzyme; and lane 4, human plasma FV incubated with labeled guinea pig megakaryocyte FV (containing approximately 20,000 cpm) in the presence of guinea pig megakaryocyte lysate enzyme.
Fig 5. Demonstration of the presence of FXIII in guinea pig megakaryocytes by a specific activity staining procedure for the fluorescent amine incorporation into dimethylcasein. Cell lysates and tissue enzyme fractions were prepared and subjected in duplicate to 0.8% agarose gel electrophoresis under nondenaturing conditions, after which the gel was incubated in activity staining solution containing dansylcadaverine, dimethylcasein, and in case of gel 1, thrombin. Gel 2 was identically treated except for omission of thrombin. After destaining, both gels were dried. The fluorescent spots were visualized under UV light and photographed as described under Materials and Methods. Lane 1 contained guinea pig plasma (30 μL); lane 2, guinea pig platelet lysate (derived from 2 × 10⁶ cells); lane 3, guinea pig megakaryocyte lysate (derived from 5 × 10⁶ cells); lane 4, guinea pig RBC lysate (derived from 2 × 10⁶ cells); lane 5, guinea pig liver extract (20 μg protein); lane 6, human plasma (30 μL); lane 7, human platelet lysate (derived from 6 × 10⁶ cells); and lane 8, human RBC lysate (derived from 2 × 10⁶ cells).

The FV-crosslinking activity of guinea pig megakaryocytes is a function of FXIIIa.

DISCUSSION

The observations in this report indicate that human coagulation FV is covalently crosslinked by an enzyme derived from guinea pig megakaryocytes to form polymeric derivatives, and that this transamidase is likely to be FXIIIa derived from factor XIII present in the megakaryocytes. FXIII and tissue TGase represent a group of transamidating enzymes that catalyze the covalent crosslinking of fibrin and many other proteins through a γ-glutamyl-ε-lysine isopeptide bond. The enzymes require Ca²⁺ for activity, are inhibited by reagents reacting with the free sulfhydryl group essential for catalytic activity, and are inhibited by dansylcadaverine and putrescine, which compete with lysyl sites of protein
An interesting observation was the copurification of FV and FXIII present in guinea pig plasma and platelet lysate. This observation may reflect a substrate-enzyme interaction of a noncovalent type. Data indicating that the crosslinking of FV does not occur in the absence of the crosslinking enzyme rules out the possibility of a nonenzymatic crosslinking of an internal thioester of cysteine with a lysyl residue, a reaction observed within α2-macroglobulin or in the C4 and C5 components of complement. The crosslinking of guinea pig radiolabeled FV requires the presence of enough unlabeled human FV to exceed the Km for the enzyme.

FXIII and tissue TGases are distinguished from each other by molecular weight, ability to be activated by thrombin, lack of immunological crossreactivity, and by patterns of crosslinking. FXIII but not TGase activity, could be unequivocally demonstrated in guinea pig megakaryocytes by a specific activity staining procedure. The ability of the megakaryocyte enzyme preparation after storage of the lysate to catalyze the crosslinking of FV without thrombin activation can be best explained by the fact that FXIII activation may occur readily during cell lysis and lysate storage by thrombin-independent pathway(s). Recently platelet FXIII has been shown to be converted to an active enzyme by calpain in the presence of CaCl2. Since the megakaryocytes were cultured in a medium containing 1.8 mmol/L CaCl2, and the purified enzyme was treated with 2-mercaptoethanol before SDS-PAGE analysis, it is quite possible that the megakaryocyte FXIII was activated by calpain under these conditions. Multiple intracellular tissue TGase occur in different cell types both as membrane-bound enzymes and cytosolic enzymes. The protocols used would not have detected a particulate enzyme.

The finding that FXIII can crosslink FV raises an intriguing question of its possible pathophysiologic significance in hemostasis. Whether FV is covalently incorporated into fibrin and plasma clots is not known and, if so, whether the FXIII incorporation into the clots would play a role analogous to those of fibronectin, thrombospondin, and α2-plasmin inhibitor remains to be determined. Our data on the FXIII-catalyzed crosslinking of FV monomer to form homopolymeric multimers indicates that FV possesses both amine acceptor (acyl donor) glutaminyl and acyl acceptor lysyl crosslinking sites. The actual glutaminyl and lysyl residues involved in the crosslinking reaction in the primary sequence of FV is not yet known. Thus, it is highly likely that FV can covalently crosslink to other known protein substrates of FXIII to form heteropolymers as is known for fibronectin.

Crosslinking of FV mediated by FXIIIa may have a role in cytoketoskeleton-mediated secretion of platelet α-granular FV, receptor-mediated endocytosis of surface membrane-bound FV, or assembly of the prothrombinase complex on the platelet surface. Tuszynski et al demonstrated that on stimulation of human platelets with the physiologically important activator, thrombin α-granular FV (as much as 38% of the total platelet FV) becomes irreversibly bound to the cytoketoskeleton and the bound FV can promote FXa conversion of prothrombin to thrombin in the presence of Ca2+ and phospholipids. In contrast, the cytoketoskeleton prepared from resting platelets (not exposed to thrombin) contained only
0.08% of the total FV activity. This reaction may be mediated by FXIII, present in the platelet cytosol. Since the cytoskeletal proteins actin, myosin, and vinculin have been demonstrated to be substrates of plasma FXIII, and to undergo heterologous crosslinking to other proteins to form heteropolymers, such heterologous crosslinking could occur between FV and these cytoskeletal proteins during platelet activation when intracellular Ca²⁺ concentration increases. Data from our laboratory indicates that human FV can form crosslinked heteropolymers with cytoskeletal actin in the presence of human FXIII, from plasma or thrombin-activated platelets. An additional possible role of the FXIII-mediated crosslinking of FV is that crosslinked polymers of FV might be rendered insensitive to degradation by activated protein C (APC). Since APC degrades FV, more efficiently than FV, the resistance of crosslinked FV, multimers to degradation by APC would provide an economical means to conserve FV. The present studies should stimulate further investigation of the role of crosslinked FV or FV multimers in hemostasis.

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Covalent crosslinking of human coagulation factor V by activated factor XIII from guinea pig megakaryocytes and human plasma

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