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Research Article

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Biosynthesis of Factor V in Isolated Guinea Pig Megakaryocytes

Hui Chong Chiu, Paul K. Schick, and Robert W. Colman
Thrombosis Research Center and Hematology-Oncology Section, Department of Medicine, Temple University Health Sciences Center, Philadelphia, Pennsylvania 19140

Abstract

Although platelets contain Factor V, localized primarily in the α-granules, the origin of this coagulation cofactor in these cells is not known. We therefore explored whether isolated megakaryocytes could biosynthesize Factor V. Guinea pig plasma Factor V coagulant activity was demonstrated to be neutralized by human monoclonal and rabbit polyclonal antibodies directed monospecifically against human Factor V. These antibodies had been used earlier to purify human Factor V and to quantify Factor V antigen concentration, respectively (1983. Chiu, H. C., E. Whitaker, and R. W. Colman. *J. Clin. Invest.* 72:493–503). As determined by a competitive enzyme-linked immunosorbent assay with guinea pig plasma as a standard, Factor V solubilized from guinea pig megakaryocytes was present at 0.098±0.018 μg/10^5 cells. Each megakaryocyte contained about 500 times as much Factor V as is in a platelet (0.234±0.180 μg/10^8 platelets). The content of Factor V antigen in guinea pig plasma was greater (27.0±3.0 μg/ml) than that of Factor V antigen in human plasma (11.1±0.4 μg/ml). In contrast, human platelets contain ninefold more Factor V antigen (2.01±1.09 μg/10^8 platelets) than do guinea pig platelets. The Factor V coagulant activities in the guinea pig were 2.85±0.30 U/ml plasma, 0.022±0.012 U/10^8 platelets, and 0.032±0.03 U/10^5 megakaryocytes, compared with human values of 0.98±0.02 U/ml plasma and 0.124±0.064 U/10^8 platelets. Isolated megakaryocytes were found to contain Factor V by cytoimmunofluorescence. The megakaryocytes were incubated with [^{35}S]methionine, and radiolabeled intracellular proteins purified were on a human anti-Factor V immunoaffinity column. The purified protein exhibited Factor V coagulant activity and neutralized the inhibitory activity of a rabbit anti-human Factor V antibody, which suggests that megakaryocyte Factor V is functionally and antigenically intact. These results indicate that Factor V is synthesized by guinea pig megakaryocytes. Nonetheless, megakaryocyte Factor V was more slowly activated by thrombin and in the absence of calcium was more stable after activation than was plasma Factor Va. Electrophoresis in sodium dodecyl sulfate and autoradiography of the purified molecule showed a major band of M_r 380,000 and a minor band of M_r 350,000, as compared with guinea pig and human plasma Factor V, where the protein had an M_r of 350,000. Both forms of Factor V were substrates for thrombin. Possible explanations for the higher molecular weight and different thrombin sensitivity and stability observed are that a precursor of Factor V was isolated or that the megakaryocyte Factor V had not been fully processed before isolation.

Introduction

Factor V is a plasma glycoprotein that functions as a nonenzymatic cofactor accelerating the conversion of prothrombin to thrombin by Factor Xa. For blood coagulation or clot formation initiated either by the intrinsic or extrinsic pathway to proceed at a physiologic rate, Factor V is required. The site of prothrombin conversion in vivo is probably the platelet surface membrane. Miletich et al. (1) demonstrated that the binding of Factor Xa to platelets resulted in a 300,000-fold increase in the enzymatic activity of Factor Xa in converting prothrombin to thrombin and required Factor V (2). In support of this concept, patients with congenital Factor V deficiency have decreased Factor Xa binding sites on their platelets. The origin of Factor V associated with platelets has thus occasioned considerable interest. Bovine Factor V in the plasma after thrombin activation binds to bovine platelets with a K_d of 3.4×10^{-10} M (3), and studies in our laboratory (4) demonstrated a requirement for Ca^{++} in the physiological range for binding of bovine Factor V to human platelets. Moreover, Kane and Majerus (5) have observed similar interactions between human Factor V and human platelets. However, Factor V may also arise from an intracellular location in platelets since human platelets separated by gel filtration do not have significant Factor V activity but develop coagulant activity upon repeated freezing and thawing (6). Studies from this laboratory show that both human (7) and bovine (8) platelets contain Factor V in the α-granules where it can be released by collagen. Thrombin can also release Factor V from human platelets (9), where it would be in the form of Factor Va and thus would exhibit high affinity binding to the surface membrane. Since platelets cannot synthesize protein, platelet Factor V might originate from plasma or be synthesized by the precursor of platelets, the megakaryocyte.

In this report, Factor V in isolated guinea pig megakaryocytes was quantified by a competitive enzyme-linked immunosorbent assay (ELISA)[1] assay by use of a cross-reacting rabbit monospecific antibody to human Factor V (10). Factor V was visualized in megakaryocytes by cytoimmunofluorescence. The biosynthesis of Factor V in isolated megakaryocytes was demonstrated by in vitro incubation with radiolabeled amino acids and isolation of biosynthesized radiolabeled Factor V. The purified Factor V had similar coagulant and antigenic activity as compared with plasma Factor V. However, mega-

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1. *Abbreviation used in this paper:* ELISA, enzyme-linked immunosorbent assay.

karyocyte Factor V appeared to be of higher molecular weight and to have different functional properties after exposure to thrombin, which suggests that a precursor or incompletely processed form of Factor V had been synthesized by megakaryocytes.

Methods

Materials. Albumin, Triton X-100, bovine serum albumin (BSA), soybean trypsin inhibitor, benzamidine hydrochloride, phenylmethylsulfonyl fluoride, ε-aminocaproic acid, and ethylene glycol were purchased from Sigma Chemical Co. (St. Louis, MO). Reagents for sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories (Richmond, CA). Medium and chemicals for tissue culture were obtained from Gibco Laboratories (Grand Island, NY). ^{35}S-methionine was from Amersham Corp. (Arlington Heights, IL). Factor V deficient plasma and normal plasma were obtained from George King Biomedical Inc. (Overland Park, KS). Plasma containing human anti-human Factor V antibody (11) was a kind gift of Dr. Helen Glueck, University of Cincinnati Medical School (Cincinnati, OH). Rabbit anti-human Factor V antibodies, a human anti-Factor V immunoaffinity column, and purified human Factor V were prepared in our laboratory as previously described (10).

Factor V coagulant assay. Factor V coagulant activity was monitored by a one-stage Factor V coagulation assay of Ware et al. (12) using plasma in which Factor V has been inactivated (13) or congenitally deficient plasma. 1 U of Factor V is defined as the amount in 1 ml of pooled normal plasma. A standard curve was constructed with dilutions of human pooled normal plasma, with the measured clotting time plotted against the concentration of Factor V present in the clotting assay.

Neutralization of guinea pig plasma Factor V coagulant activity by anti-human Factor V antibodies. A human anti-human Factor V antibody (11) and a rabbit polyclonal anti-human Factor V antibody (10) were assayed for their ability to inhibit the guinea pig plasma Factor V coagulant activity. In the inhibition assay twofold serial dilutions of antiserum were incubated with equal volumes of guinea pig plasma for 18 h at 4°C. Residual Factor V activities after incubation were determined by a coagulant assay and expressed as percentages of the original activity.

Preparation and solubilization of guinea pig megakaryocytes, platelets, and plasma. Megakaryocytes were isolated from bone marrow obtained from humeri, femurs, and tibiae of Detrick Dunkin Hartley guinea pigs, 300 to 400 g. The procedure used an albumin density gradient centrifugation followed by two velocity gradients (14, 15). Blood was obtained by cardiac puncture and platelet-rich plasma was prepared as previously described (14, 15). Megakaryocytes were isolated to 80% purity by cell number and up to 97% purity based on cell volume. Platelets were counted using a MK/H4 platelet counter (Baker Instruments Corp., Bethlehem, PA) and megakaryocytes were counted with a hemocytometer. Purity and viability of the megakaryocytes suspensions were determined as previously described (14, 15).

The isolated megakaryocytes were then washed three times by centrifugation in 0.1 M sodium phosphate, pH 7.4, containing 0.15 M NaCl, and the final pellet was resuspended in 0.5 ml of phosphate-buffered saline containing, in addition, 0.1% BSA, 0.2% Triton-X 100, and soybean trypsin inhibitor (2.4 M), benzamidine (1.0 mM), phenylmethyl sulfonyl fluoride (1.0 mM), ε-aminocaproic acid (1.0 mM), and D-phenylalanine-propyl-arginyl-chloromethyl ketone (1.0 mM) to prevent hydrolysis by proteases. In some experiments, we also added leupeptin (50 M). Protease inhibitors also were added to platelets and plasma before extraction of Factor V. After sonication at maximum speed (Sonicator model W-220 F; Heat systems-Ultrasonics, Inc., Aberdeen, MD) for 7 min, the disrupted cell suspension were further subjected to freezing at −80°C and thawing at 25°C for three cycles. After centrifugation at 10,000 *g* at 4°C an aliquot of the supernatant was monitored for the presence of Factor V activity by coagulant assay.

Quantification of Factor V in solubilized megakaryocytes. A competitive ELISA (10) was used to assay the Factor V antigen levels in the megakaryocyte solubilized as described above. For clarity, the principles of the assay procedure are briefly described. Purified human Factor V was coated on the surface of a polystyrene cuvette. A predetermined limiting amount of the rabbit anti-Factor V antibody solution that will bind 70–90% of the coated Factor V was incubated in the presence of different amounts of Factor V from pooled normal plasmas or the test samples. A goat antibody directed against rabbit gamma-globulin conjugated to alkaline phosphatase in a concentration calculated to be in excess of the rabbit IgG was reacted with the rabbit anti-human Factor V antibody on the surface. A chromogenic substrate solution containing 1 mg/ml of *p*-nitrophenylphosphate was added and the color developed was measured at 405 nm after a standard incubation. In each step, all reactions were carried out by incubation at 23°C, the unbound reagents were removed by aspiration, and the cuvettes were thoroughly washed before the addition of each new reagent. The results obtained with the dilutions of pooled normal human or guinea pig plasma were used to construct a standard curve and calculate a Factor V antigen concentration in the samples from the respective species. The control with anti-Factor V antibody alone without any added free Factor V to compete was included and used as a maximum binding reference. Negative controls with buffer alone or normal rabbit serum were also employed and the values were subtracted from the experimental values. Each experiment was carried out in triplicate.

Cytoimmunofluorescence detection of Factor V in megakaryocytes. Isolated megakaryocytes were cytocentrifuged at 700 *g* for 4 min on glass slides. The cells were fixed and made permeable with acetone/methanol 10:1. An indirect cytoimmunofluorescence technique was used as previously described (15). In this method, anti-human Factor V raised in rabbits was used. Fluorescein isothiocyanate-labeled goat anti-rabbit IgG (Sigma Chemical Co.) was used as the second antibody. Pre-immune serum served as a control. Megakaryocytes were examined by a microscope equipped for phase contrast and fluorescence (Zeiss No. 16; Carl Zeiss, Inc., New York, NY).

Preparation and solubilization of [^{35}S]L-methionine-labeled megakaryocytes. Megakaryocytes were isolated from guinea pig bone marrow as described above. The isolated megakaryocytes were then placed into Dulbelcco's Modified Eagles Medium, free of methionine and containing 10% fetal calf serum, penicillin (50 IU/ml), and streptomycin (50 μg/ml) in a glass vial. 250 μCi of ^{35}S-methionine was then added to the culture and incubated for 18 h at 37°C in a 5% CO_2 incubator. The cells were then washed and lysed as described above by the combined sonication and freezing and thawing of the cells in the presence of nonionic detergent. Aliquots of the supernatant were taken and proteins were precipitated with 10% TCA. The radioactivities of the precipitable proteins and supernatant were counted in Scintiverse II (Fischer Scientific Co., Allied Corp., Pittsburgh, PA) in a liquid scintillation radioactive counter.

Purification of [^{35}S]L-methionine-labeled Factor V from megakaryocytes by immunoaffinity chromatography. The solubilized solutions of [^{35}S]methionine-labeled megakaryocytes were subjected to immunoaffinity chromatography with immobilized anti-human Factor V antibody (10). The immunoaffinity column was first washed and equilibrated with a 0.02 M Tris buffer, pH 7.2 containing 0.1% BSA. The solubilized solution of [^{35}S]methionine-labeled megakaryocyte was two times diluted with the buffer used to equilibrate the column, and mixed with the anti Factor V immunoaffinity gel in a 10-ml syringe. The reaction was allowed to proceed overnight at 4°C on a shaker at 20 vibrations/min. The unbound solution was then eluted from the column with 0.02 M Tris buffer, pH 7.2. The nonspecifically bound proteins were eluted with the same buffer containing 0.65 M NaCl and 0.1% BSA. Finally, the specifically bound proteins were eluted with the same buffer containing, in addition, 50% ethylene glycol. The

radioactivities of the three pooled eluted fractions were counted in a liquid scintillation counter in Scintiverse II.

Immunoreactivity of [^{35}S]L-methionine-labeled megakaryocyte Factor V. Neutralization of the inhibitory activity of anti Factor V antibody was used as an indication of the antigenic properties of the purified molecules. In the neutralization assay, equal volumes of pooled normal plasma and monospecific rabbit anti-human Factor V antibody solutions were incubated and the residual Factor V activities were measured by coagulant assay. The minimum amount of antibody that will inhibit >90% of the Factor V activity was then used in the antibody neutralization assay, in which equal volumes of the defined antibody solution, test sample, and pooled normal plasma were incubated, in that order, for 18 h at 4°C. The clotting time of the above mixture was then measured and the presence of antigenically active Factor V in the test sample was indicated by comparison of residual coagulant activity with that obtained with pooled normal plasma and antibody without the test samples.

Biological activity of the purified megakaryocyte [^{35}S]methionine-labeled Factor V. The immunoaffinity purified [^{35}S]labeled protein was analyzed for Factor V coagulant activity and the changes in activity after exposure to thrombin. Aliquots of purified megakaryocyte Factor V were incubated with α-thrombin at 25°C and assayed at various times for Factor V coagulant activity. The degree of activation was expressed as the ratio of coagulant activity before and after exposure to thrombin. Stability was assessed by the percentage of the maximum activity obtained after 18 h at 25°C.

Molecular weight of purified megakaryocytes [^{35}S]methionine-labeled Factor V. SDS slab gel electrophoresis (16) was performed to characterize the molecular weight of the immunoaffinity purified [^{35}S]methionine-labeled protein. The concentration of the polyacrylamide gel used was 3% and purified human Factor V and fibrinogen were included as references. After electrophoresis, the gel was dried and stained with Coomassie Brilliant Blue R250. The dried gel was subjected to autoradiography with Kodak X-Omat AR X-ray film (Eastman Kodak Co., Rochester, NY) for 18 h at −80°C.

Results

Inhibition of guinea pig plasma Factor V coagulant activity by the anti-human Factor V antibodies. The guinea pig plasma Factor V coagulant activity was found to be 2.85 times higher than that of the human plasma, as determined by reference to a standard curve constructed with different amounts of human Factor V in a coagulant assay. In contrast, guinea pig plasma contains more antigen (27.0 μg/ml) than does human plasma (11.1 μg/ml), as calculated from the specific activity of purified guinea pig plasma Factor V (see below). Thus, the specific activity of Factor V in the guinea pig plasma is similar to that in human plasma (Table I). Inhibition of the guinea pig plasma Factor V and human plasma Factor V coagulant activity by two kinds of anti-human Factor V antibodies is contrasted in Fig. 1, *A* and *B*. At higher dilutions of both antibodies, less neutralization of guinea pig plasma Factor V than of Factor V was observed, but at higher antibody concentration, the extent of inhibition is the same. These differences in reactivity may be partially due to the higher content of Factor V in guinea pig plasma (2.85 U/ml) than in human plasma (1.0 U/ml), but some of the decreased reactivity may be due to species specificity. Both human anti-Factor V antibody and monospecific rabbit anti human-Factor V antibody showed considerable cross-reactivity against the guinea pig plasma Factor V.

Detection and quantitation of Factor V in guinea pig megakaryocytes platelets and plasma and in human platelets.

Table I. Factor V Coagulant Activities and Antigen Levels of Guinea Pig (GP) Megakaryocytes, Platelets, and Plasma

	Coagulant activity*	Antigen‡	Specific activity
	u	*µg*	*U/mg*
GP megakaryocytes (10^5)	0.032±0.030	0.098±0.018‖	326
GP platelets (10^8 cells)	0.022±0.012	0.234±0.18‖	85
GP plasma (1 ml)	2.85±0.30	27.0±3.0‖	—
Human platelets (10^8)	0.124±0.064	2.01±1.09	60
Human plasma§ (1 ml)	0.98±0.02	11.1±0.4	90

* Coagulant activity was calculated by reference to a standard line constructed with dilutions of human pooled normal plasma. The coagulant units are defined based on 1 U/ml of normal human plasma.
‡ Factor V antigen level was obtained by reference to a standard line constructed with dilutions of human pooled normal plasma for human samples and guinea pig pooled plasma for guinea pig samples by a competitive ELISA method. All samples represent a mean±SD of four or five separate samples assayed in triplicate at three dilutions, except for human plasma that was determined from 14 normal individuals (10).
§ Data from reference 10.
‖ The specific activity of purified guinea pig Factor V (105 U/mg) was used in combination with coagulant activity of pooled guinea pig plasma to calculate the concentration of Factor V antigen in plasma. The absolute antigen levels are only estimates since they are based on the specific activity of purified Factor V, which could be influenced by small amounts of Factor Va. The plasma value was used with the results of the ELISA and a plasma standard curve in order to calculate platelet and megakaryocyte antigenic levels.

Factor V coagulant activity was observed in isolated megakaryocytes after disruption and lysis by sonication, freezing, and thawing of the cells in the presence of nonionic detergent. The clotting time was substantially shortened when the resulting solution was added to the Factor V deficient plasma, and a mean of 0.032 U of Factor V coagulant activity was obtained per 10^5 megakaryocytes, as calculated by reference to a coag-

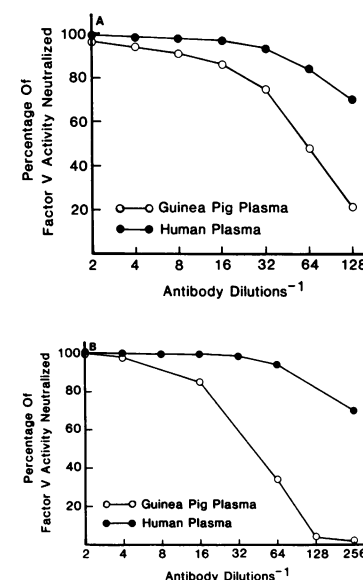

Figure 1. Neutralization of Factor V clotting activity by human (*A*) and rabbit (*B*) anti Factor V antibodies. Serial twofold dilutions of human or rabbit anti-Factor V antiserum were incubated with equal volumes of pooled human plasma or guinea pig plasma at 4°C for 18 h. The clotting times of the mixtures were measured nd the residual Factor V coagulant activities were obtained by reference to a standard curve and ex pressed as a percentage of the original Factor V activity.

ulant assay standard curve. A mean value of 0.022 U of Factor V coagulant activity was obtained per 10^8 guinea pig platelets that were solubilized by the same method as described for guinea pig megakaryocytes (Table I). The corresponding mean guinea pig Factor V antigen levels determined by use of guinea pig plasma as a standard curve determined by the competitive ELISA method were 0.234 $\mu g/10^8$ platelets and 0.098 $\mu g/10^5$ megakaryocytes, respectively (Table I). Since preparation by albumin density gradient had been used for cells, velocity sedimentation for megakaryocytes, and gel filtration for platelets, contamination of Factor V from plasma was minimized. Therefore, the measured Factor V coagulant activity and antigen levels appear to arise from the cells themselves. For comparison, human platelets had a mean coagulant activity of 0.124 U and mean antigen of 2.01 $\mu g/10^8$ platelets.

Cytoimmunofluorescence detection of Factor V in megakaryocytes. Isolated megakaryocytes were shown to contain Factor V by cytoimmunofluorescence (Fig. 2). Most megakaryocytes reacted with the rabbit anti-human Factor V antibody. No fluorescence was detected in megakaryocyte nuclei or when pre-immune rabbit serum was substituted for antiserum. Except for the presence of weak fluorescence in a few mononuclear cells, there was no evidence of immunofluorescence in other bone marrow cells. Thus anti-human Factor V IgG can identify Factor V in guinea pig megakaryocytes.

Biosynthesis of Factor V by the isolated guinea pig megakaryocytes and their isolation by immunoaffinity chromatography. Although megakaryocytes contain Factor V as quantified in the solubilized solution of coagulant activity and antigen levels by ELISA, these Factor V molecules could result from adsorption from plasma or biosynthesis by megakaryocytes. Therefore, experiments to confirm whether or not megakaryocytes could synthesize Factor V were carried out by incubation of the isolated megakaryocytes with radiolabeled amino acid in an in vitro culture medium. Incorporation of the $[^{35}S]$methionine (or $[^{14}C]$isoleucine in one experiment) into protein molecules was observed in the solubilized solution. Before solubilization the cells were washed to remove the free $[^{35}S]$methionine from the labeled megakaryocytes. In four experiments 52±31% (SD) of the incorporated radioactivity was precipitable with 10% TCA. In order to isolate the biosynthesized radiolabeled Factor V, the lysate was passed through an anti-Factor V IgG-Sepharose immunoaffinity column.

The conditions for elution of Factor V from the immunoaffinity column were determined by prior purification of Factor V from guinea pig plasma by use of the same immunoaffinity column. Tris buffer (0.02 M), pH 7.2, containing 0.65 M NaCl and 50% ethylene glycol was able to elute guinea pig plasma Factor V from the column efficiently, as shown in Fig. 3. The resulting guinea pig Factor V was a single band on sodium dodecyl sulfate electrophoresis with M_r = 350,000 (Fig. 3, inset). On this 4% gel proteolytic breakdown products as low as M_r = 50,000 could have been visualized; none was seen. The specific activity in the column fraction was 61 U/mg, which, in combination with the single band on the gel, suggests that the isolated protein is Factor V rather than Factor Va.

Similar conditions used to isolate guinea pig plasma Factor V were used to purify the biosynthesized megakaryocyte Factor V antigen. The solution derived from labeled guinea pig megakaryocytes was applied directly to the column. Before

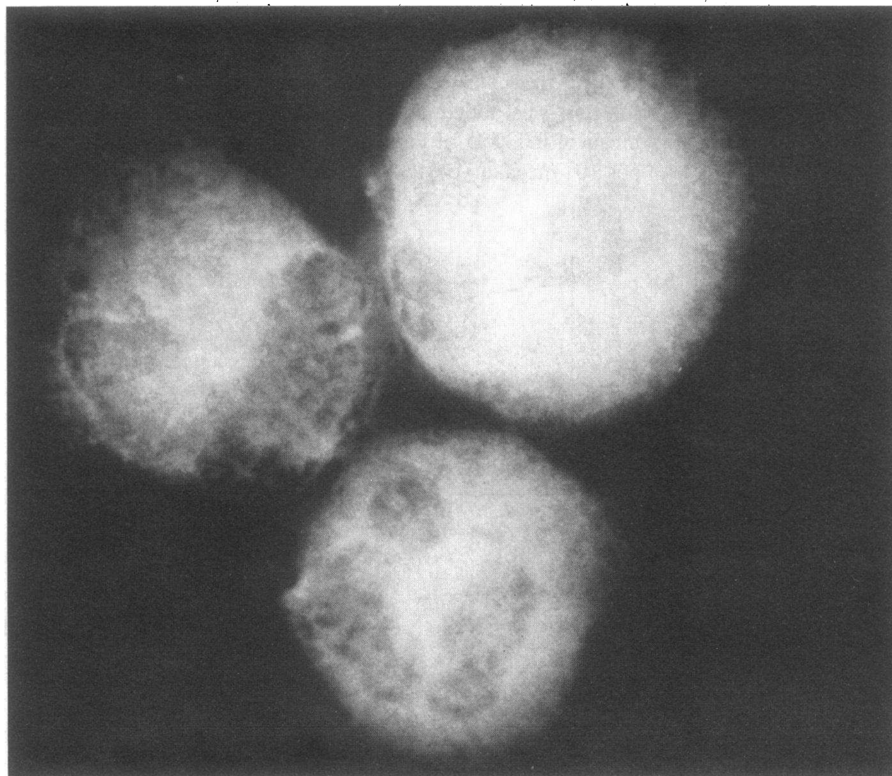

Figure 2. Cytoimmunofluorescent demonstration of Factor V in megakaryocyte. An indirect cytoimmunofluorescence technique was used to demonstrate Factor V in cytocentrifuged isolated megakaryocytes. Anti-human Factor V antibody raised in rabbits and fluorescein isothiocyanate-labeled goat anti-rabbit IgG were used as a second antibody. Megakaryocytes were examined under a microscope (Carl Zeiss Inc.). The bar represents 50 μm.

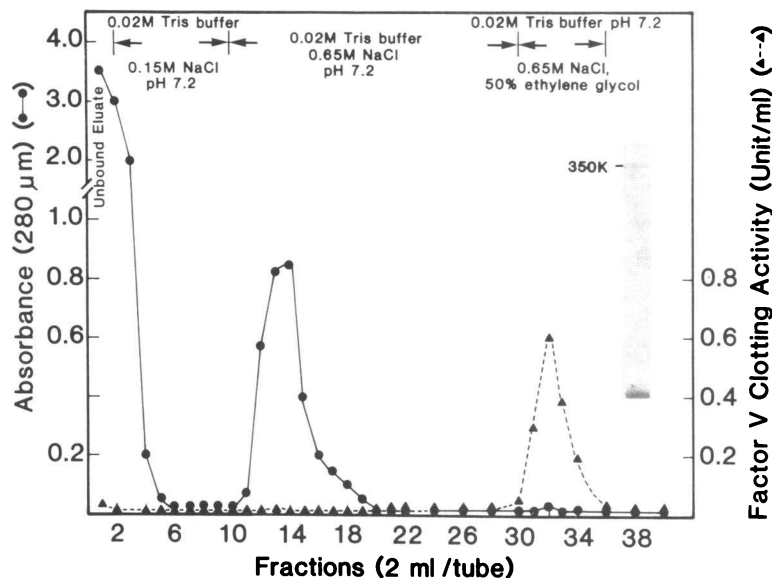

Figure 3. Purification of guinea pig plasma Factor V. Guinea pig plasma Factor V was isolated by one-step immunoaffinity chromatography with immobilized human anti-Factor V IgG. 5 ml of guinea pig plasma was passed through an anti-Factor V immunoaffinity column. After washing was done with a 0.02 M Tris buffer, pH 7.2, nonspecifically bound proteins were eluted with the same buffer containing 0.65 M NaCl. Finally, the specifically bound Factor V was eluted with the same buffer containing 0.65 NaCl and 50% ethylene glycol. The inset shows the purified plasma Factor V electrophoresed on a 4% SDS-polyacrylamide gel.

elution of Factor V, the column was washed and the nonspecifically bound protein was eluted with the same buffer containing 0.65 M NaCl. Thus, contamination by nonspecific proteins was eliminated or negligible in the isolated Factor V preparation. Factor V coagulant activity was observed neither in the effluent that was unbound by the anti-Factor V antibody column nor in the high salt containing buffer solution, although >94% of the radioactivity was recovered in these combined fractions. The Factor V clotting activity was observed only in the final elution solution in four separate experiments which contained 0.23±0.17% of the total radioactivity.

Characterization of the immunoaffinity purified [^{35}S]methionine-labeled Factor V. To study the antigenic properties of the biosynthesized Factor V, neutralization of the inhibitory activity of anti-Factor V antibody was performed with the immunoaffinity purified [^{35}S]methionine-labeled megakaryocyte Factor V (Table II). After incubation of pooled normal plasma (1.0 U/ml) with an equal volume of 1:64 diluted rabbit anti-Factor V antibody (final dilution, 1:128) only 0.06 U/ml of the Factor V coagulant activity remained (94% neutralization), as seen in experiment B. In experiment D, incubation of the same amount of the antiserum with an equal volume of the purified [^{35}S]labeled megakaryocyte Factor V (0.15 U/ml) left only 0.01 U/ml residual activity (94% neutralization). In experiment C, when megakaryocyte Factor V was pre-incubated with the same concentration of antibody and followed by incubation of the mixture with the pooled normal plasma, 0.25 U/ml of the residual activity remained (78% neutralization). The difference (C − B) was 0.19 U/ml (16% residual), which agrees well with the 0.15 U/ml of megakaryocyte Factor V used in the pre-incubation mixture. Thus, megakaryocyte Factor V combined with the antibody to reduce its neutralizing activity to the extent predicted by its biological activity. We conclude that the purified megakaryocyte Factor V was functionally and antigenically intact.

The anti-Factor V antibody immunoaffinity purified [^{35}S]methionine-labeled molecules that showed Factor V clotting activity were studied for their ability to be activated by thrombin. As shown in Fig. 4, the purified Factor V from guinea pig megakaryocytes was activated by thrombin at a slower rate and to a lesser extent than was the purified Factor V from either human and guinea plasma. After the increase of Factor V coagulant activity, the clotting activity of the purified Factor V from human and guinea pig plasma completely disappeared after 24 h at 25°C in the absence of added calcium. In contrast, the coagulant activity of the purified Factor V from guinea pig megakaryocytes studied without the addition of calcium retained 55% of its activity.

The molecular weight of the [^{35}S]labeled guinea pig Factor V was determined by a 3% SDS-polyacrylamide gel electro-

Table II. Neutralization of Inhibitory Activity of Anti-Factor V Antibody

Experiment	Antibody volume	GP megakaryocyte lysate volume	Factor V activity	Human plasma volume	Factor V activity	Factor V observed	Percent neutralized
	μl	*μl*	*U/ml*	*μl*	*U/ml*	*U/ml*	
A	0	0	0	100	1.0	1.00	0
B	100	0	0	100	1.0	0.06	94
C	100	100	0.15	100	1.0	0.25	78
D	100	100	0.15	0	0	0.01	94

The antibody (initial concentration, 1/64) was pre-incubated by itself, or with megakaryocyte Factor V for 18 h at 4°C. Normal human plasma was then added in some cases. The assay volume was adjusted to represent a constant percentage (10%) of the total volume. The experiment is representative of three similar studies. GP, guinea pig.

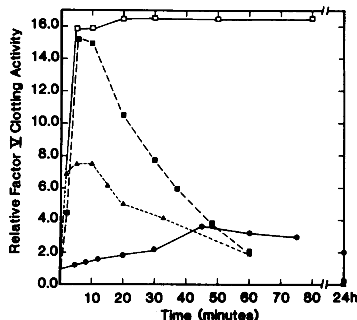

Figure 4. Time-dependent Factor V activation by a thrombin. Activation of Factor V clotting activity by thrombin was carried by incubation of purified guinea pig plasma (triangles), human plasma (squares), or guinea pig megakaryocytes (circles) Factor V (0.36 U/ml) with 0.1 U/ml of α-thrombin in 0.02 M sodium barbital buffer, pH 7.2, containing 0.15 M NaCl. Purified guinea pig plasma Factor V was also activated in the presence of 5 mM $CaCl_2$ (triangles). Aliquots were taken at specific times before and after the addition of thrombin. After appropriate dilution, Factor V coagulant activity was measured. The degree of activation was expressed as a ratio of Factor V activity after and before thrombin addition, and the percentage of maximum activity was determined after 18 h at 25°C.

phoresis and compared with a standard protein myosin and purified human Factor V (Fig. 5). Autoradiography of the dried gel showed a major band of molecular weight 380,000 and two minor bands, one of them at a migration position the same as that of the purified Factor V from human plasma, corresponding to a molecular weight of 350,000 determined previously.

To determine if the high molecular bands seen in the megakaryocyte Factor V were due to a limited proteolysis by platelet calcium-activated protease, leupeptin was included in the buffer used to lyse and solubilize the megakaryocytes. No change in the pattern of the components over M_r 300,000 was seen. Despite these precautions, some labeled protein was observed with M_r 105,000, which could represent proteolysis. The specific activity was 326, somewhat higher than that of the purified guinea pig plasma Factor V. However, this represents only a threefold increase, as compared with the eightfold increase observed in Fig. 4 for purified plasma Factor V, indicating that mostly unactivated Factor V was present. Moreover, the Factor V's of both M_r 380,000 and 350,000 were cleaved by thrombin. When the radiolabeled guinea pig megakaryocyte Factor V was incubated with thrombin, both bands disappeared and a new component of M_r 150,000 appeared, corresponding to one of the Factor V activation peptides (Fig. 6). The difference in the pattern of thrombin proteolysis is consistent with the slower rate and decreased extent of activation seen in Fig. 4.

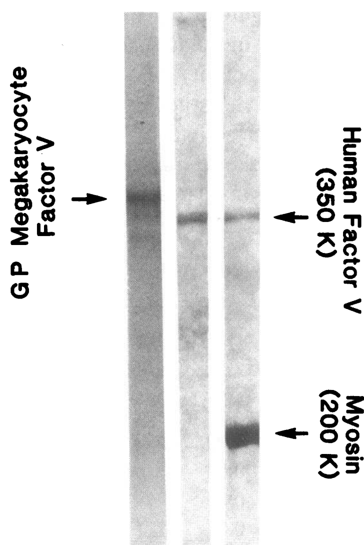

Figure 5. Determination of the molecular weight of purified Factor V biosynthetically [^{35}S]methionine-labeled guinea pig megakaryocyte Factor V. Guinea pig (GP) megakaryocytes were incubated with [^{35}S]methionine for 18 h and solubilized, and Factor V was isolated on an anti-Factor V immunoadsorbent column. The protein was dialyzed against the SDS electrophoresis buffer (see Methods) and a SDS-3–3.5% polyacrylamide gel electrophoresis was performed. (*Left*) Autoradiography of guinea pig megakaryocyte Factor V. (*Middle*) Purified human plasma Factor V stained with Coomassie Blue. (*Right*) Purified human plasma Factor V and myosin stained with Coomassie Blue. Numbers are molecular weights; K, thousands.

Discussion

This study is the first to quantify Factor V in isolated megakaryocytes by both coagulant and immunochemical assays and

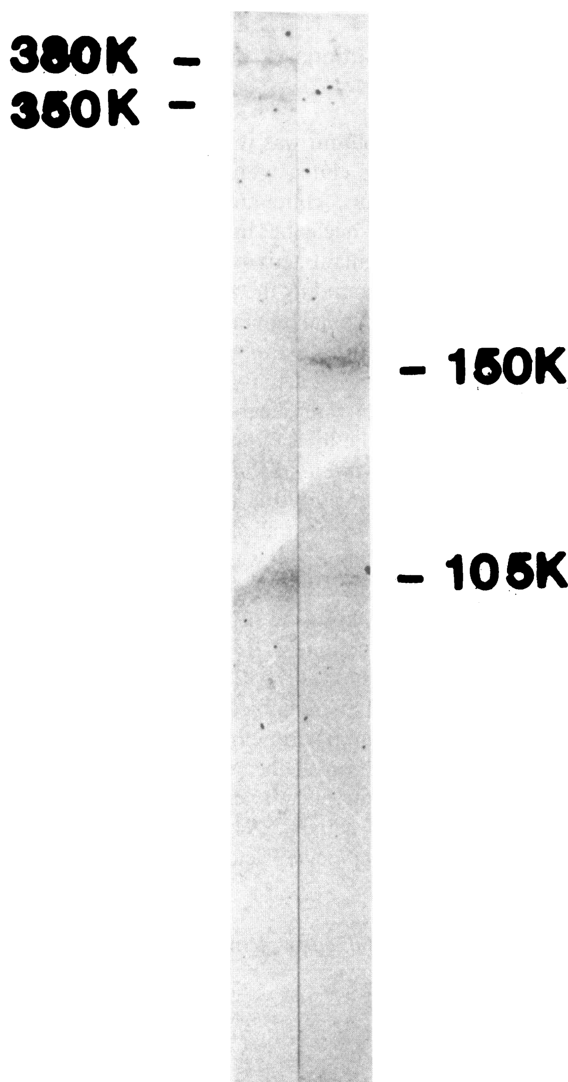

Figure 6. Cleavage of [^{35}S]methionine guinea pig megakaryocyte Factor V by α-thrombin. Megakaryocyte Factor V was prepared and purified as in Fig. 5 except that leupeptin (50 μM) was added to the solubilizing buffer in addition to the other inhibitors. The protein was analyzed by 4% SDS-polyacrylamide gel electrophoresis (*left*). An aliquot was incubated with α-thrombin (1 U/ml) at 37°C for 10 min and similarly analyzed (*right*). Numbers are molecular weights; K, thousands.

to demonstrate visually Factor V antigen by cytoimmunofluorescence by use of a monospecific polyclonal rabbit anti-human Factor V IgG, which cross-reacted and neutralized guinea pig Factor V. Beside megakaryocytes and platelets, only a few mononuclear cells showed fluorescence, in agreement with Tracy et al. (17). Thus, anti-human Factor VIII related antigen (14) and anti-human Factor V cross-react with guinea pig proteins.

No values are available for human megakaryocytes, but a direct comparison of Factor V concentration in platelets from guinea pigs with Factor V concentration in human and bovine platelets can be made. Guinea pig platelets contain less Factor V antigen (0.234 $\mu g/10^8$ platelets) than do human platelets (2.01 $\mu g/10^8$ platelets). The human platelet data are somewhat higher than those reported by radioimmunoassay for human platelets (0.25–0.77 $\mu g/10^8$ platelets) (18); similarly, somewhat higher antigen levels (11.4 μg/ml) in human plasma are determined by ELISA (10), as compared with radioimmunoassay (7.0 μg/ml) (17). Bovine platelets (9) assayed by a coagulant assay contain only 0.0098 $U/10^8$, about half as much as guinea pig platelets (0.022 $U/10^8$ platelets), and human platelets contain much more Factor V coagulant activity (0.124 $U/10^8$ platelets). A 1000-fold more platelets than megakaryocytes were compared for their respective content since this ratio equalized total protein. This ratio has been useful for the comparison of lipid content and ability to accumulate serotonin in these cells (14, 15). A ratio of ~1: 1,000 for the content of intracellular components of platelets to megakaryocytes has been suggested (14, 15) on the basis of lipid content and serotonin accumulation. This ratio appears to be similar to that determined for guinea pig platelets and megakaryocytes (1:687). The specific coagulant activity is similar in a given species for platelets or megakaryocytes and plasma. Factor V in guinea pig megakaryocytes appears to be immunologically similar to that in guinea pig plasma and functionally resembles Factor V in that it exhibits coagulant activity. Furthermore, megakaryocyte Factor V can be activated by thrombin, albeit at a lower rate and to a lesser extent than plasma Factor V, and can be cleaved by thrombin to a lower molecular weight species.

This study is the first to show that megakaryocytes can synthesize Factor V. This demonstration was facilitated by the use of a human monoclonal antibody which neutralized and bound guinea pig Factor V and allowed its purification on a immunoaffinity column. Platelets are anucleate "cells" which cannot synthesize protein; therefore, protein synthesis probably occurs exclusively in the megakaryocyte. Megakaryocytes are nucleated cells that are known to contain Factor VIII-related antigen (19), platelet membrane glycoproteins, and platelet Factor 4 (20, 21), actin (22), fibrinogen (22) and Factor XIII (23). Thus far, megakaryocytes have been shown to be able to synthesize Factor VIII-related antigen (19), platelet Factor 4 (24), fibrinogen (25), and actin (22). The observation that guinea pig megakaryocytes can synthesize Factor V suggests that granule development occurs early in megakaryocyte development since Factor V's, at least in the case of platelets, are located in α-granules (7).

Megakaryocytes have a considerably greater capacity to synthesize lipids than do platelets (14, 26). Megakaryocytes, but not platelets, can synthesize cholesterol (27) and desaturate eicosatrienoic acid for the synthesis of arachidonic acid (28). It appears that synthesis of lipids and proteins are critical for the production and assembly of megakaryocyte and platelet membranes. Coagulant proteins and lipids probably are synthesized in later stages in the course of megakaryocyte development when platelets are formed and packaged.

The biosynthesized megakaryocyte Factor V was shown to be immunochemically similar to plasma Factor V. Adsorption of a monospecific antibody to Factor V with [^{35}S]L-methionine-labeled Factor V decreased the ability of the antibody to inhibit Factor V coagulant activity in plasma. Moreover, the biosynthesized Factor V contained coagulant activity. We conclude that the isolated guinea pig megakaryocytes synthesize Factor V that is functionally active and antigenically intact. Despite these similarities to plasma Factor V, certain unique functional and physical differences should be noted. Kinetic study of activation of the isolated [^{35}S]labeled megakaryocyte Factor V by purified human thrombin indicated a lesser extent and slower rate of activation. Moreover, in the absence of added calcium, the coagulant activity of the purified megakaryocyte Factor V was more stable after thrombin activation than was coagulant activity of that purified from human and guinea pig plasma. Furthermore, the purified protein from guinea pig megakaryocytes has a major component of molecular weight 380,000, greater than that of guinea pig plasma Factor V, and a minor component with molecular weight of 350,000, equivalent to that of the purified Factor V from human plasma (Fig. 5) and guinea pig plasma (Fig. 3, inset). Both components were cleaved by thrombin and therefore represented intact Factor V. A logical hypothesis is that the larger molecular weight form represents a precursor of Factor V or an incompletely processed form of the molecule synthesized by the isolated megakaryocytes in vitro. Studies are underway in our laboratory to test the validity of this explanation.

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