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IN FOCUS

# Megakaryocytes endocytose and subsequently modify human factor V *in vivo* to form the entire pool of a unique platelet-derived cofactor

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**Summary.** Factor Va (FVa), derived from plasma or released from stimulated platelets, is the essential cofactor in thrombin production catalyzed by the prothrombinase complex. Plasma-derived factor V (FV) is synthesized in the liver. The source(s) of the platelet-derived cofactor remains in question. We identified a patient homozygous for the $FV^{Leiden}$ mutation, who received a liver transplant from a homozygous wild-type FV donor. Eighteen days post-transplant, phenotypic analysis of the patient's platelet-derived FV indicated that the platelets were acquiring wild-type FV, consistent with the temporal differentiation of megakaryocytes and subsequent platelet production. Nine months post-transplant, the platelet-derived FV pool consisted entirely of wild-type FV. Consequently, megakaryocyte endocytosis of plasma-derived FV must account for the entire platelet-derived pool, because blood-borne platelets cannot bind or endocytose FV. Subsequent to this endocytic process, the patient's platelet-derived FV was cleaved to a partially active cofactor, and rendered resistant to phosphorylation catalyzed by a platelet-associated kinase, and hence less susceptible to activated protein C-catalyzed inactivation. These data provide the first *in vivo* demonstration of an endocytosed plasma protein undergoing intracellular modifications that alter its function. This process results in the sequestration of active FVa within the platelet compartment, poised for immediate action subsequent to release from platelets at a site of injury.

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## Introduction

Factor Va (FVa), derived from plasma or released from activated platelets, functions as the essential, non-enzymatic cofactor in prothrombinase-catalyzed thrombin formation on the activated platelet surface [1]. FVa profoundly influences the amount of thrombin generated [2], which is clinically underscored by the observation that deficiencies of the procofactor, factor V (FV), can lead to severe hemorrhage and possibly death [3]. The platelet-derived FV/Va pool, which constitutes as much as 25% of the total pool [4], expresses unique physical [5,6] and functional [7–10] characteristics that render it more procoagulant than plasma-derived FVa and more efficient at sustaining procoagulant events on the activated platelet surface. Because, at a site of injury, the concentration of platelet-derived FV/Va can exceed that of its plasma counterpart by more than 100-fold [11], these unique functional properties will impart a distinct hemostatic advantage to the platelet-derived molecule, a concept that is supported by clinical observations [11,12].

The physical and functional differences between human platelet- and plasma-derived FV have prompted great interest in determining the origin of the platelet pool. Prior studies from our laboratory demonstrate that the secretable pool of platelet-derived FV/Va must originate from megakaryocyte endocytosis of the plasma-derived cofactor [13], an observation confirmed recently by Christella and colleagues [14]. Quite recently, however, studies in the murine system indicate that the platelet and plasma pools of FV are biosynthetically distinct [15,16]. In addition, studies conducted more than 20 years ago suggest that human megakaryocytes are capable

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of synthesizing FV [17]. All these observations raise the question that some portion of the platelet-derived FV/Va pool may arise from megakaryocyte synthesis of the protein. Data presented in this report identify, for the first time, the source of the entire pool of human platelet-derived FV/Va *in vivo*. Phenotypic analyses of factor V/Va, present in a platelet lysate, from a patient who genotyped homozygous for $FV^{Leiden}$ and, following thrombosis of the suprahepatic veins, received a liver transplant from a donor expressing only wild-type FV, demonstrate that the entire pool of human platelet-derived FV/Va arises exclusively from endocytosis of the plasma-derived procofactor by megakaryocytes. In addition, these studies identify an unequivocal link to physical modifications occurring subsequent to its endocytosis that lead to some of the functional properties unique to platelet-derived FV/Va.

## Materials and methods

### *Patient information*

The patient was a 43-year-old female with essential thrombocythemia, who developed acquired Budd–Chiari syndrome following thrombosis of the suprahepatic veins. As genotypic analyses demonstrated that she was homozygous for the $FV^{Leiden}$ mutation, she was enrolled in the Padua University Hospital orthotopic liver transplantation (OLTx) program. Low-molecular weight heparin (Enoxaparin) was administered to prevent further thrombotic episodes. Hydroxyurea was used to keep the platelet count within the normal range. Within 1 year, however, the patient's general condition worsened with hepatic imaging revealing an occlusion of the major hepatic veins consistent with parietal thrombosis. The portal vein was totally compressed by a hyperthrophic caudate lobe. The patient underwent OLTx from a normal donor, homozygous for wild-type FV.

### *Materials*

Adenosine triphosphate (ATP) was from ICN (Costa Mesa, CA). $^{32}$P-ATP (10 Ci mmol$^{-1}$) was from NEN (Boston, MA, USA). Phospholipid vesicles composed of 75% PC (%wt/wt) and 25% PS (%wt/wt) (PCPS) were prepared [18] and quantified [19] as described previously. Human platelet- [6] and plasma-derived FV [20,21] were purified and quantified as described previously. Human α-thrombin and APC were from Haematologic Technologies (Essex Junction, VT, USA). The α-human FV mouse monoclonal antibodies, αHFV#9 and αHFV#17, were obtained from the Monoclonal Antibody Facility, Biochemistry Department, University of Vermont.

### *Preparation of platelets*

Blood was collected by venipuncture into 5 μmol L$^{-1}$ prostaglandin ($PGE_1$) from the patient or a normal individual in Padua, Italy, subsequent to obtaining their written consent, as described in detail previously [7,8]. Platelet concentrates, containing 5 μmol L$^{-1}$ $PGE_1$, were prepared immediately and shipped to Burlington, VT via air freight (~36 h). Upon receipt, platelets were placed at 37 °C and isolated as described [7,8,13]. Washed platelets were brought to a final concentration of $1 \times 10^9$/mL in 5 mmol L$^{-1}$ HEPES–Tyrode's buffer, pH 7.4.

### *APC-catalyzed cleavage and western blotting analyses of platelet-derived FVa and $FVa^{Leiden}$*

In initial studies, the APC-catalyzed proteolysis of secreted, platelet-derived FVa was analyzed in the presence of PCPS vesicles as described previously [7,8,13]. Platelets ($1 \times 10^9$ platelets mL$^{-1}$) were incubated with thrombin (50 nmol L$^{-1}$, 5 min, ambient temperature) followed by hirudin (75 nmol L$^{-1}$) addition. Platelet releasates containing FVa were prepared by centrifugation (1100 ***g***, 5 min), and treated subsequently with APC (0.25 nmol L$^{-1}$) in the presence of PCPS vesicles (20 μmol L$^{-1}$) for 2 h. Samples of the reaction mixtures were prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting analyses, as described previously [7,13]. The platelet-derived FVa antigen was detected with the $\alpha HFVa_{HC}$#17 MoAb, which recognizes an epitope between amino acids 307–506 in the FVa heavy chain.

In additional studies, the APC-catalyzed proteolysis of FVa in thrombin-activated, platelet lysates was analyzed in order to describe phenotypically the entire platelet-derived FVa pool. Platelets ($1 \times 10^9$ mL$^{-1}$) were lyzed with Triton X-100 (0.25% final concentration) and treated with thrombin (50 nmol L$^{-1}$, 3 min, 37 °C) followed by hirudin (75 nmol L$^{-1}$) addition. APC (0.25 nmol L$^{-1}$) was added and aliquots were removed at timed intervals (0–5 h) and prepared for SDS-PAGE and Western blotting [7,13]. FV present in plasma, as well as a collagen (10 μg mL$^{-1}$, 30 min)-activated platelet releasate were also analyzed by Western blotting analyses [7,13].

### *Analyses of the phosphorylation of platelet- and plasma-derived FVa by a platelet-associated casein kinase II-like enzyme*

Platelets ($2 \times 10^9$ mL$^{-1}$) from the transplant patient and a normal, control individual were activated with thrombin (20 nmol L$^{-1}$) in the presence of ATP (64 μmol L$^{-1}$) supplemented with $^{32}$P-ATP (0.1 Ci μL$^{-1}$) and analyzed as described by Rand and colleagues [5]. In other experiments, purified plasma-derived FV or purified platelet-derived FV/Va (250 nmol L$^{-1}$) was added to the platelet-$^{32}$P reaction mixtures prior to thrombin stimulation. $^{32}$P incorporation into FVa was analyzed by SDS-PAGE according to the method of Laemmli [22] followed by autoradiography. Platelet-derived FVa is the major phosphorylated protein present in a platelet releasate [5], simplifying data interpretation.

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## Results

### *Patient treatment and analyses of hemostatic status following OLTx*

During the first 18 days following transplant, several plasma-based assays were performed on a daily basis to monitor various aspects of the patient's hemostatic status. A prothrombin time International Normalized Ratio (PT-INR) was used to assess liver function as defined by the synthesis of normal concentrations of functional coagulation factors. A mildly elevated PT returned to an INR of 1 by day 18 (data not shown). Within 1 week following transplant, the patient's FV cofactor activity rose from 15% to 78% of normal. By day 12, the patient's FV level stabilized at 100% of normal. During this time, an activated protein C sensitivity ratio (APC-SR) was performed to assess the sensitivity of the patient's plasma pool of FV to APC. The patient's APC-SR was within the normal range as early as 2 days following OLTx, indicating a functional switch from $FV^{Leiden}$ to normal, wild-type FV contributed by the transplanted liver. After 18 months of follow-up, the patient was well and the transplanted liver continued to function normally. The patient is currently receiving warfarin and hydroxyurea therapy to control myeloproliferative disease and is exhibiting a high quality of life. The patient never received any platelet transfusions subsequent to the transplant.

### *Phenotypic analyses of platelet-derived FV/Va indicate the entire cofactor pool arises from megakaryocyte endocytosis of FV from plasma*

Transplantation of an individual homozygous for $FV^{Leiden}$ with a liver from an individual expressing only wild-type FV allowed us to determine if megakaryocyte synthesis of FV accounted for even a minor component of the cofactor pool. As $FV^{Leiden}$ can be identified at the protein level due to loss of an APC-catalyzed cleavage site at Arg506 in the FVa heavy chain, treatment of $FVa^{Leiden}$ vs. wild-type FV with APC yields different proteolytic fragments easily distinguishable by Western blotting analyses. Furthermore, such phenotypic analyses are more sensitive than functional assays for identification of the absolute levels of $FV/Va^{Leiden}$ and therefore provide conclusive evidence regarding the extent to which megakaryocyte synthesis of FV contributes to the platelet-derived cofactor pool. In initial studies, platelets were isolated from the transplant patient and normal individuals prior to transplant, 18 days and 61 days post-transplant. Isolated, well-washed platelets were incubated with thrombin to both activate the platelets and to release and activate the platelet-derived FVa. Following the addition of APC (0.25 nmol $L^{-1}$), all reaction mixtures were incubated for 2 h to allow the reaction to proceed to completion in order to compare directly results obtained with the patient between experiments. The APC-catalyzed cleavage of the patient's platelet-derived FVa heavy chain pretransplant demonstrated the presence of only $FV^{Leiden}$

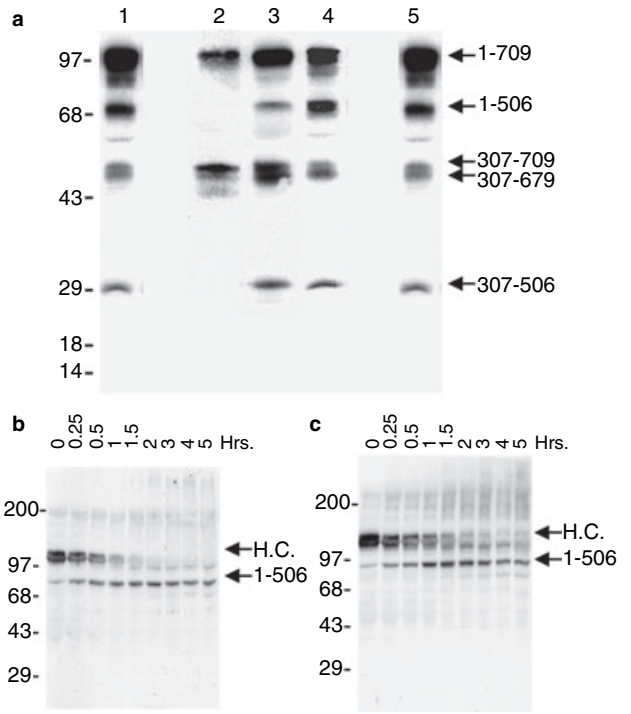


**Fig. 1.** Phenotypic analyses of secreted platelet-derived FVa obtained from a patient homozygous for $FV^{Leiden}$ prior to and following transplant with a liver from an individual homozygous for wild-type FV. (a) Washed platelets ($1 \times 10^9$ $mL^{-1}$) obtained from the patient prior to transplant, and those from a normal individual, were treated with thrombin (50 nmol $L^{-1}$, 5 min). The releasates were subsequently treated with 0.25 nmol $L^{-1}$ APC in the presence of 20 μmol $L^{-1}$ PCPS for 2 h. Samples of the reaction mixtures were then subjected to SDS-PAGE followed by Western blotting analyses using α-human FV #17, a MoAb that distinguishes $FV^{Leiden}$ from wild type [7,8]. Each lane contained ≈50 μg of total platelet protein and the FVa present in the releasate from $5 \times 10^7$ platelets. Molecular weight markers ($\times 10^{-3}$) are indicated on the left. Numbers on the right define the different APC-derived FVa fragments. Lanes 1 and 5, wild-type control; lanes 2–4, patient samples pretransplant and 18 and 61 days post-transplant, respectively. (b) Nine months post-transplant, patient or (c) control platelets ($1 \times 10^9$ $mL^{-1}$) were lyzed by the addition of Triton X-100 (0.25%) and activated with thrombin (50 nmol $L^{-1}$, 5 min) followed by APC addition (0.25 nmol $L^{-1}$). Samples were taken at the indicated times and analyzed by SDS-PAGE and Western blotting. In (b), each lane contains ≈ 50 μg of total platelet protein (the equivalent of $5 \times 10^7$ patient platelets), whereas in (c), each lane contains ≈25 μg of total platelet protein (the equivalent of $2.5 \times 10^7$ platelets).

(Fig. 1a, lane 2). Cleavage at Arg306 was apparent based on the appearance of the 60 kDa fragment and the 54 kDa fragment resulting from further cleavage of this fragment at Arg679. Note the complete absence of 75 and 30 kDa fragments that would result from cleavage at Arg506 followed by cleavage at Arg306, as is observed in wild-type, normal platelet-released FV (lanes 1 and 5). However, these FVa fragments are apparent 18 days post-transplant (lane 3), indicating that platelets containing wild-type FV are beginning to emerge from the patient's bone marrow. The presence of $FV^{Leiden}$ is still apparent, but not surprising, and would be consistent with the temporal differentiation of megakaryocytes in the bone marrow and their subsequent platelet production.

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In contrast, by 61 days post-transplant (lane 4) only wild-type, normal FV is present based on comparison to a normal wild-type control. Fragments corresponding to FVa cleavage initially at Arg306 appear to a small extent. A similar amount can be seen in normal, wild-type FVa. This cleavage event has been documented to occur consistently to varying degrees in individuals expressing wild-type FV and therefore is not unique to $FV^{Leiden}$ [7]. These studies suggest that megakaryocyte endocytosis of plasma FV, rather than endogenous synthesis, defines a substantial fraction, if not all, of the platelet's secretable pool of FV.

To confirm that the entire pool of platelet-derived FV was derived from endocytosis of the plasma molecule, additional blood samples were drawn from the patient and a control individual, 9 months post-transplant, and the entire platelet-derived FVa pool was analyzed using thrombin-activated, Triton X-100-solubilized platelet lysates. To direct APC-catalyzed cleavage to Arg506, experiments were performed in the absence of a membrane surface, which reduces the cleavage at Arg306 to a negligible rate [23,24]. Consequently, if any $FV^{Leiden}$ was present in the patient's platelets, an intact heavy chain would remain subsequent to APC addition as detected by Western blotting analyses. The heavy chain doublet observed in the patient's FVa initially was no longer detectable after 2 h of APC cleavage (Fig. 1b). Over-exposure of the Western blot revealed no remaining intact heavy chain, indicating that the entire pool of platelet-derived FVa was wild-type. Furthermore, the FVa cleavage pattern and rate of cleavage was nearly indistinguishable from that observed with platelet-derived FVa from a wild-type control (Fig. 1c). As resting platelets are unable to bind and endocytose FV [13,25], the human platelet-derived FV/Va pool must result from megakaryocyte endocytosis of FV from plasma.

### *Platelet-derived FV/Va is proteolytically processed subsequent to megakaryocyte endocytosis*

The concept that platelet-derived FV/Va originates from FV circulating in plasma contradicts the observations that platelet- and plasma-derived FVa display disparate functional and physical properties [5–10]. Consequently, additional analyses were performed to determine if characteristics unique to platelet-derived FV/Va were expressed in the patient's platelet-derived cofactor pool, and hence arose from modifications subsequent to its endocytosis from plasma. Western blotting analyses of the patient's plasma-derived FV 9 months post-transplant demonstrated that it circulated as a single chain procofactor of 330 kDa similar to that of a normal individual (Fig. 2a, lanes 1 and 2, respectively). In contrast, platelet-derived FV/Va, present in collagen releasates from the patient and normal individual, had undergone substantial proteolysis (Fig. 2b, lanes 1 and 2, respectively). This pattern of peptides represents a partially activated platelet-derived FV/Va pool that typically expresses 30–50% the activity of thrombin-activated, plasma-derived FVa [10]. Activation of both patient and control platelets with thrombin yielded a fully activated

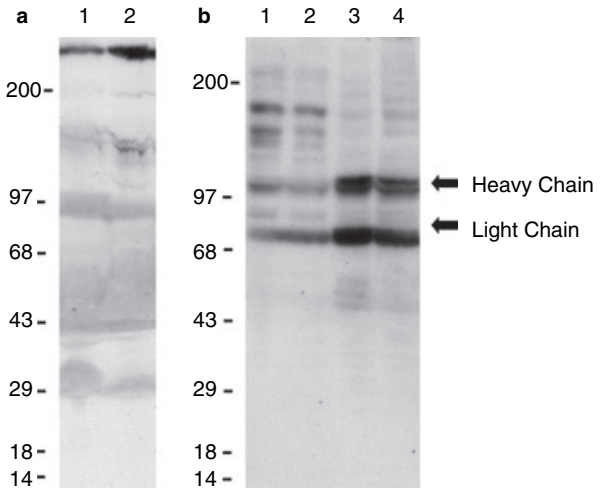


**Fig. 2.** Platelet-derived FV/Va is proteolytically processed subsequent to megakaryocyte endocytosis of plasma-derived FV. (a) Patient plasma obtained 9 months post-transplant (lane 1) or control plasma (lane 2) was analyzed by SDS-PAGE and Western blotting techniques using a combination of α-human FV#9 and #17. Each lane contained the equivalent of 10 μL of citrated plasma in attempts to achieve a FV antigen concentration of 40–140 ng $lane^{-1}$. Densitometric analyses of the autoradiograph indicated that patient's plasma-derived FV concentration was ≈35% of the control. (b) Washed platelets ($1 \times 10^9$ $mL^{-1}$) from the patient (lanes 2 and 4) or a control individual (lanes 1 and 3) were activated with collagen (10 μg $mL^{-1}$, 30 min, lanes 1 and 2) or thrombin (50 nmol $L^{-1}$, 37 °C, 15 min, lanes 3 and 4). FV/Va present in the samples was visualized by SDS-PAGE and Western blotting. Each lane contained ≈60 μg of total platelet protein and the FV/Va present in the releasate from $5.6 \times 10^7$ platelets. Densitometric analyses of the the FVa heavy and light chains indicated that the patient's platelet-derived FVa concentration was ≈60% of the control.

platelet-derived cofactor indicated by the presence of only intact heavy and light chains of platelet-derived FVa (Fig. 2b, lanes 3 and 4, respectively). These data represent the first *in vivo* evidence demonstrating a direct link from endocytosis of FV by megakaryocytes to proteolytic processing of the procofactor to yield the partially activated cofactor stored within platelet α-granules.

### *Platelet-derived FVa is resistant to phosphorylation catalyzed by a platelet-associated casein kinase II-like enzyme subsequent to megakaryocyte endocytosis*

Another characteristic unique to platelet-derived FVa is its inability to be phosphorylated on the heavy chain by a platelet-associated casein kinase II-like enzyme [5,6]. Under identical conditions, plasma-derived FVa is readily phosphorylated in this region [5,6,26]. Therefore, experiments were performed to determine if the patient's plasma-derived FV, subsequent to its endocytosis by megakaryocytes and packaging into platelets, was altered in a manner that prevented the phosphorylation of its heavy chain by a casein kinase II-like enzyme. Analyses of FVa present in releasates (Fig. 3), prepared subsequent to the thrombin-catalyzed activation of equal numbers of patient and control platelets in the presence of $^{32}$P-ATP, demonstrate that

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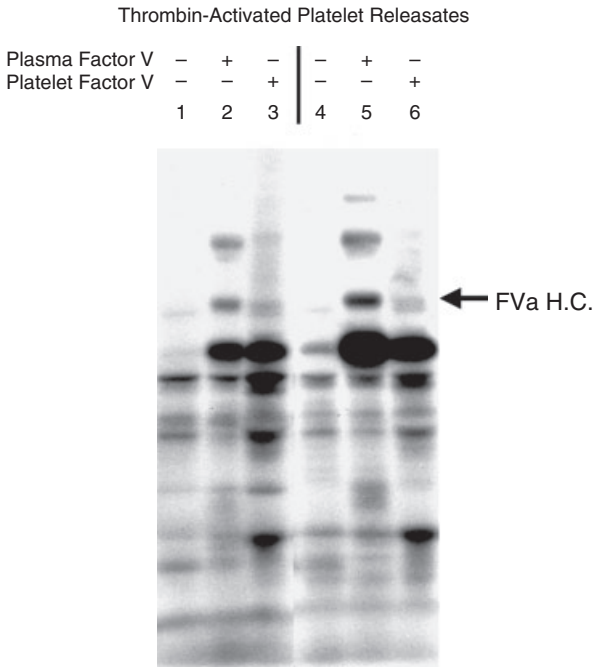


**Fig. 3.** Platelet-associated casein kinase II catalyzes the phosphorylation of plasma- rather than platelet-derived FVa heavy chain.Washed platelets ($2 \times 10^9$ $mL^{-1}$) obtained from the patient 9 months post-transplant (lanes 1–3) or a control individual (lanes 4–6) were activated with thrombin (20 nmol $L^{-1}$, 15 min, 37 °C) in a reaction mixture containing ATP (64 μmol $L^{-1}$) supplemented with trace $^{32}$P-ATP. Purified plasma-derived FV (lanes 2 and 5) or purified platelet-derived FV (lanes 4 and 6) was added before thrombin stimulation. $^{32}$P-labeled FVa peptides were visualized subsequently by autoradiography. Each lane contained ≈120 μg of total platelet protein. Lanes 1 and 4 contain only the FVa present in a releasate prepared from $1.2 \times 10^8$ thrombin-activated patient or control platelets, respectively.

no phosphate was incorporated into the heavy chain of the intrinsic platelet-derived FVa from either patient or control (lanes 1 and 4, respectively). Furthermore, no phosphate incorporation was observed in the heavy chain of added purified, platelet-derived FV added prior to thrombin-catalyzed activation of the platelets (lanes 3 and 6, respectively). As expected, however, the addition of purified plasma-derived FV to the patient or control platelet reaction mixtures, prior to thrombin addition, resulted in significant $^{32}$P incorporation into both heavy chains (lanes 3 and 7, respectively). In addition, a platelet-associated protein kinase C isoform catalyzed the incorporation of $^{32}$P into the light chain [5,27] of both cofactors (lanes 1 and 4), although densitometric analyses of the autoradiograph indicate that the $^{32}$P incorporated into the patient's platelet-derived FVa light chain (lane 1) was only 40% of that incorporated into FVa light chain of the control individual (lane 4). This observation is due to the reduced amount of platelet-derived FVa present in the patient's platelets (approximately 60%) when compared to the control (Fig. 2) as determined by densitometric analyses of the autoradiographs. Because in these experiments the concentration of FVa light chain (low nmol $L^{-1}$ [7]), is substantially below the $K_m$ for protein kinase-C catalyzed reactions (μmol $L^{-1}$ [28]), even small differences in the concentration of FVa light chain would have a significant impact on the amount of $^{32}$P incorporated. These combined data indicate that, subsequent to its endocytosis from plasma by megakaryocytes, the factor V/Va stored in and released from the patient's platelets mimics that obtained from a normal individual and is modified such that its heavy chain region can no longer be phosphorylated.

## Discussion

The data presented in this study are the first to demonstrate that the vast majority, if not the entire pool, of platelet-derived FV/Va originates from megakaryocyte endocytosis of plasma-derived FV *in vivo*. Furthermore, subsequent to endocytosis, events occur that, at a minimum, partially proteolytically activate the intracellular procofactor and modify it such that it is no longer a substrate for an associated casein kinase II-like enzyme. Thus, the combined data provide a direct link between megakaryocyte endocytosis of plasma-derived FV and its subsequent acquisition of characteristics unique to the released and purified protein, which cannot be attributed to either megakaryocyte synthesis of the protein or alterations acquired during the purification process.

These studies extend previous studies reported independently by our laboratories [13] and more recently by those of Christella and colleagues [14], as only the secretable pool of platelet-derived FV was analyzed phenotypically using either Western blotting analyses or prothrombinase-based functional assays in the earlier studies. The current study evaluated the entire platelet-derived cofactor pool and demonstrated that its phenotype was identical to that of the plasma-derived cofactor and was independent of the $FV^{Leiden}$ alleles present in the megakaryocyte genome.

Our inability to demonstrate the presence of any $FV^{Leiden}$ in the patient's platelets 9 months post-transplant using Western blotting techniques is due to the inability of the megakaryocyte to synthesize the protein, rather than a lack of assay sensitivity for the following reasons. Based on Western blotting analyses and densitometric analyses of the resulting autoradiographs, the amount of FVa present in the patient's platelet lysate (Fig. 1b) or thrombin-activated releasate (Fig. 2b) is approximately 60% of the normal control, indicating that her platelets should contain from 2700 to 9000 molecules of FV/platelet [4]. Therefore, under the conditions of the study shown in Fig. 1c, each lane of the Western blot could contain from 75 ng to 250 ng of FVa. Because the antibodies used in our Western blotting analyses allow detection of as little as 2 ng FV $lane^{-1}$ under normal conditions, we would be able to detect synthesized protein even if it represented as little as 1% of the total pool [28]. In addition, in analyses of the Western blot shown in Fig. 1c, the blot was over-exposed to ensure that no $FVa^{Leiden}$ heavy chain could be observed.

Our inability to demonstrate the presence of any $FV^{Leiden}$ in the patient's platelets nine months post-transplant also does not appear to be due to the antirejection pharmaceutical regimen she is receiving or to the possible engraftment of

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hematopoietic cells derived from the transplanted liver. The apparent concentration of FV in the patient's platelets (60% of normal) reflects her reduced plasma-derived FV concentration (35% of normal) (Fig. 2a). A direct relationship is apparent between her plasma- and platelet-derived FV/Va concentrations, mimicking the relationship established previously in normal individuals. These data indicate that the megakaryocyte endocytosis mechanism has not been altered significantly, if at all.

In addition, subsequent to endocytosis of the wild-type FV, the patient's megakaryocytes appear to process proteolytically (Fig. 2b) and alter phenotypically (Fig. 3) the endocytosed protein identically to a normal control individual. Finally, PCR (36 cycles) of DNA extracted from the peripheral blood cells of the patient at 9 months post-transplant failed to reveal the presence of a wild-type FV allele (data not shown), which could be due to engraftment of liver-derived hematopoietic cells.

Studies have not been conducted to demonstrate that the patient's megakaryocytes are capable of normal protein synthesis, therefore our inability to demonstrate the presence of any $FV^{Leiden}$ in the patient's platelets might reflect a condition-related abnormality of her megakaryocytes. However, the concept that megakaryocyte synthesis of FV accounts for little, if any, of the platelet-derived cofactor pool is also consistent with recent reports in which no FV protein or mRNA could be detected in $CD34^+$ cell-derived megakaryocytes derived from either peripheral blood or bone marrow and grown in serum-free media containing thrombopoietin to induce megakaryocyte differentiation and α-granule production [25,29–31]. Work in our laboratory has shown that megakaryocyte endocytosis of FV is dependent upon the stage of megakaryocyte differentiation [31] (Bouchard, submitted), and more recent studies indicate that it is regulated by a clathrin-dependent, receptor-mediated event (Bouchard, submitted).

It is difficult to rationalize the discrepancy between the collection of recent studies indicating that megakaryocytes do not synthesize FV with those reported several years ago, which demonstrated *in vitro* incorporation of $^{35}$S-Met-labeled amino acids into the procofactor, FV [17]. One could argue that if megakaryocytes do synthesize FV, it accounts for an insignificant fraction of the total pool (< 1%). The presence of $FV^{Leiden}$ in the patient's platelets 18 days following liver transplant does not represent endogenous synthesis but, rather, most probably reflects platelet production by differentiating megakaryocytes which had endocytosed plasma-derived $FV^{Leiden}$ prior to transplant. The bone marrow transit time (i.e. 'maturation time') for postmitotic human megakaryocytes is 6–10 days and a reservoir of immature megakaryocytes exists in the marrow that feeds into the mature cell compartment [32]. Considering that the half-life of human platelets is approximately 2.5 days [32], it is reasonable to speculate that exhaustion of the pretransplantation megakaryocyte pool (and, hence, $FV^{Leiden}$-positive platelets) would have followed an asymptotic, negative temporal slope. Consequently, to observe a mixture of wild-type FV and $FV^{Leiden}$ on day 18 post-transplant would be within the expected variability of megakaryocyte differentiation.

The origin of platelet-derived FV in the human vs. murine system is markedly different. Yang *et al.* [16] demonstrated that liver-specific FV expression prevented neonatal hemorrhage observed previously in FV-deficient mice, even though FV antigen could not be detected in their platelets. Platelet-specific expression of FV similarly prevented neonatal hemorrhage observed with FV deficiency and confirmed that in the murine system, megakaryocyte synthesis of FV accounts for the platelet-derived cofactor pool and is biosynthetically distinct from the plasma-derived pool [15]. Despite these observations, it should be noted that platelets from different species display several disparate properties [33–37], several related to FV expression [35–37] and that the extension of results in animal models to the human system should be made with caution.

Although we have established clearly that platelet- and plasma-derived FV originate from the same source, the platelet-derived cofactor is unique in many respects. Platelet-derived FVa is a different substrate for proteases including APC [8] and plasmin [9], when compared to its plasma counterpart. Platelet-derived FVa is stored in α-granules in a partially activated state and as such appears to be activated more efficiently by factor Xa than by thrombin, even though a cofactor expressing the same functional activity is expressed [10]. The current study establishes that this phenotypic and functional difference occurs subsequent to FV endocytosis by megakaryocytes. Platelet-derived FVa is also resistant to phosphorylation catalyzed by a platelet-associated casein kinase II-like enzyme. The current study demonstrates that the mechanism, which confers this resistance to phosphorylation, also occurs subsequent to the endocytosis of plasma-derived FV by megakaryocytes. It has been proposed that phosphorylation at Ser692 may lead to an increase in the rate of cofactor inactivation [26], and thus the resistance to phosphorylation observed in platelet-derived FVa [5,6] may explain its resistance to APC inactivation [8]. Other physical differences have been observed between purified platelet- and plasma-derived FV/Va [6], such as the addition of a single O-liked glycoform to the heavy chain of platelet-derived FV at Thr402, and a unique, activating cleavage at $Tyr^{1543}$ producing a functional FVa light chain. It is reasonable to hypothesize that these modifications also occur subsequent to megakaryocyte endocytosis of plasma-derived FV.

In conclusion, we have demonstrated unequivocally that the *in vivo* production of the human platelet-derived FV/Va pool occurs through endocytosis of plasma-derived FV by megakaryocytes. Subsequent to this event, the single chain procofactor is physically modified and is proteolytically processed forming a pool of a partially activated, mature platelet-derived cofactor. These events provide the first evidence for the modification of an endocytosed plasma protein that produces an alteration in its functional characteristics. This process results in the sequestration of an essential and unique blood coagulation protein within the platelet compartment, in an active form and poised for immediate and sustained procoagulant function subsequent to release at a site of injury.

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