Journal of Thrombosis and Haemostasis, **3**: 541–551

ORIGINAL ARTICLE

Endocytosis of plasma-derived factor V by megakaryocytes occurs via a clathrin-dependent, specific membrane binding event

B. A. BOUCHARD,* J. L. WILLIAMS,† N. T. MEISLER,* M. W. LONG† and P. B. TRACY*‡
*Departments of *Biochemistry and ‡Medicine, University of Vermont College of Medicine, Burlington, VT, USA; and †Department of Pediatrics, University of Michigan, Ann Arbor, MI, USA*

To cite this article: Bouchard BA, Williams JL, Meisler NT, Long MW, Tracy PB. Endocytosis of plasma-derived factor V by megakaryocytes occurs via a clathrin-dependent, specific membrane binding event. *J Thromb Haemost* 2005; **3**: 541–51.

Summary. Megakaryocytes were analyzed for their ability to endocytose factor V to define the cellular mechanisms regulating this process. In contrast to fibrinogen, factor V was endocytosed by megakaryocytes derived from CD34$^+$ cells or megakaryocyte-like cell lines, but not by platelets. CD41$^+$ *ex vivo*-derived megakaryocytes endocytosed factor V, as did subpopulations of the megakaryocyte-like cells MEG-01, and CMK. Similar observations were made for fibrinogen. Phorbol diester-induced megakaryocytic differentiation of the cell lines resulted in a substantial increase in endocytosis of both proteins as compared to untreated cells that did not merely reflect their disparate plasma concentrations. Factor IX, which does not associate with platelets or megakaryocytes, was not endocytosed by any of the cells examined. Endocytosis of factor V by megakaryocytes proceeds through a specific and independent mechanism as CHRF-288 cells endocytosed fibrinogen but not factor V, and the presence of other plasma proteins had no effect on the endocytosis of factor V by MEG-01 cells. Furthermore, as the endocytosis of factor V was also demonstrated to occur through a clathrin-dependent mechanism, these combined data demonstrate that endocytosis of factor V by megakaryocytes occurs via a specific, independent, and most probably receptor-mediated, event.

Keywords: clathrin, endocytosis, factor V, megakaryocyte, platelet, receptor.

Introduction

Platelets contain numerous proteins involved in hemostasis within their α-granules including von Willebrand factor (VWF), plasminogen activator inhibitor-1 (PAI-1), thrombospondin, fibrinogen, vitronectin and factor V, which are released upon platelet activation at sites of vascular injury. As platelets possess little, if any, biosynthetic capability, these proteins are either synthesized by platelet precursors, megakaryocytes, or endocytosed from the plasma. VWF, thrombospondin and PAI-1 are known to be synthesized by megakaryocytes [1–3] and not endocytosed from the plasma [4,5]. Plasma-derived fibrinogen, in contrast, is endocytosed by both megakaryocytes and platelets [6,7], most likely through an integrin-dependent mechanism [7]. No fibrinogen biosynthesis has been detected in megakaryocytes [8] despite the presence of its mRNA in these cells [9]. Vitronectin is also endocytosed by megakaryocytes [4], and possibly platelets as its receptor, $\alpha_v\beta_3$, is found in platelet α-granules [10].

The origin of platelet-derived factor V appears unique in that it appears to be derived from only megakaryocyte endocytosis of the plasma-derived cofactor. This notion is supported by analyses of platelet-derived factor V from individuals heterozygous [11,12] or homozygous [13] for factor V Leiden following transplant of livers from wild-type factor V donors. In these studies, the phenotype of the platelet-derived factor V pool mimicked that of the plasma molecule, and was independent of the megakaryocyte factor V gene. As blood-borne platelets cannot bind [14] or endocytose factor V [11], these combined data support the hypothesis that the origin of platelet-derived factor V is via endocytosis of the plasma molecule by megakaryocytes. Thus, both plasma- and platelet-derived factor V are synthesized by the liver [15]. Subsequent to its endocytosis, factor V is cleaved proteolytically to the active cofactor, and rendered resistant to phosphorylation catalyzed by a platelet-associated kinase [16]. These data are consistent with several observations suggesting that platelet-derived factor V is functionally and physically distinct from its plasma counterpart [17–22]. In addition, several clinical observations suggest that

Correspondence: Paula B. Tracy, Department of Biochemistry, University of Vermont, College of Medicine. 89 Beaumont Avenue, Burlington, VT 05405–0068, USA.
Tel.: (802) 656 1995; fax: (802) 862 8229; e-mail: paula.tracy@uvm.edu

Received 8 October 2004, accepted 29 November 2004

© 2005 International Society on Thrombosis and Haemostasis

once it is converted to factor Va, the essential protein cofactor in thrombin production [23], it may play a more pre-eminent role in maintaining normal hemostasis [23,24]. Taken together these observations represent a new paradigm whereby an essential coagulant protein is endocytosed from plasma and modified intracellularly to yield a functionally distinct molecule.

In contrast to observations in the human system, the murine plasma- and platelet-derived factor V pools appear to be biosynthetically distinct [25,26], an observation that is consistent with earlier studies demonstrating factor V synthesis by guinea pig megakaryocytes [27]. However, it should be noted that fundamentally important cross-species differences have been demonstrated in mammalian coagulation systems [28–31], suggesting that some animal studies may not model the human system appropriately.

The purpose of this study was to demonstrate directly that human megakaryocytes endocytose factor V from plasma and to begin to define the cellular processes regulating the endocytic process. Our observations strongly support the concept that megakaryocytes endocytose factor V by a specific, clathrin-dependent and, most probably, receptor-mediated mechanism.

Materials and methods

Proteins

Thrombopoietin (TPO), human transferrin and human factor VIII were generous gifts of Drs Dan Eaton (Genentech, San Francisco, CA, USA), Anne Mason (University of Vermont), and Kenneth Mann (University of Vermont), respectively. Stem cell factor was from R & D Systems (Minneapolis, MN, USA) and human factor IX was from Haematologic Technologies, Inc. (Essex Junction, VT, USA) Human factor V [32] and fibrinogen [33] were purified from fresh-frozen plasma and characterized as described. Factor V was iodinated with ^{125}I and characterized as described previously [18]. Factor V and transferrin were fluorescently labeled with AlexaFluor dyes using commercially available kits (Molecular Probes, Inc., Eugene, OR, USA) at fluorescence to protein (F : P) ratios of 1.5–11.7 mol fluorophore per mol protein. AlexaFluor488-labeled fibrinogen was also from Molecular Probes, Inc. (F : P ratio = 8.1–15). Endocytosis was not affected by protein modification as different preparations of modified proteins yielded similar, if not identical, results, and the data obtained mirrored those using unlabeled proteins followed by detection with fluorescently labeled monoclonal antibodies (mAbs).

Purified mouse mAbs against human factor V, fibrinogen and factor IX were obtained from the Core Antibody Facility, at the University of Vermont, and labeled with AlexaFluor488 at F : P ratios ranging from 4 to 6. All other antibodies were obtained commercially.

Megkaryocyte production ex vivo

Bone marrow cells were obtained from vertebral bodies acquired from the National Disease Resource Interchange, Philadelphia, PA. CD34^{+} hematopoietic progenitor cells were isolated and subjected to *ex vivo* expansion and differentiation into megakaryocytes as described [34].

Megakaryocyte-like cell lines

MEG-01 cells were from the American Type Culture Collection (Manassas, VA, USA) and were cultured following the supplier's directions. CMK [35] and CHRF-288 [36] cells, generous gifts of Dr Hava Avraham (Harvard Medical School) and Dr Michael Lieberman (University of Cincinnati College of Medicine), respectively, were cultured as described. Quiescent cells were used for all experiments.

Where indicated, MEG-01 and CMK cells were stimulated with phorbol myristate acetate PMA [1×10^{-8} mol L^{-1} in dimethyl sulfoxide at 2×10^{5} cells mL^{-1}] for 3 days in media containing 10% fetal bovine serum [37]. Following removal of nonadherent MEG-01 cells, the adherent cells were removed with phosphate-buffered saline containing 0.53 mM EDTA (10 min, 37 °C), and used in subsequent assays. CHRF-288 cells were stimulated with PMA (1×10^{-8} mol L^{-1} at 2×10^{5} cells mL^{-1}) for 2–5 days in media containing 20% horse serum.

Analyses of factor V endocytosed by ex vivo*-derived megakaryocytes*

CD34^{+} bone marrow cells were subjected to *ex vivo* expansion and differentiation into megakaryocytes for 7 or 10 days. On days 4 or 7, factors V (30 nmol L^{-1}) or IX (90 nmol L^{-1}) were added for 72 h. For immunocytochemical localization of endocytosed protein, cells were adhered to slides, air-dried, then fixed and permeabilized with methanol. Cells, adhered to slides, were incubated with 1% horse serum and anti-factor V (66.7 nmol L^{-1}) or anti-CD41 (66.7 nmol L^{-1}, Dako Corporation, Carpinteria, CA, USA) mAbs (45 min). Unless otherwise indicated all incubations were performed at ambient temperature. Anti-factor V or anti-CD41 immunostaining was detected using Immunopure ABC-Horseradish Peroxidase or -Phosphatase staining kits (Pierce, Rockford, IL, USA), respectively. Double antibody staining reactions were performed by sequential immunostaining for factor V and then CD41. Non-specific binding interactions were identified using isotyped-matched, non-immune antibody or secondary antibody alone. The cell nuclei were counterstained using Evans Blue.

For flow cytometric analyses of endocytosed protein, the cells were labeled with anti CD41-phycoerythrin (PE) (45 min), then washed, fixed, permeabilized and counterstained with AlexaFluor488-labeled anti-factor V (66.7 nmol L^{-1}) or anti-factor IX (66.7 nmol L^{-1}) mAbs (45 min). Fluorescence from 10 000 cells was analyzed on a FACscan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Two-color analyses were accompanied by single-antibody staining to allow adequate compensation of each fluorescence detector.

© 2005 International Society on Thrombosis and Haemostasis

The positive gate was set such that ≥97% of the cells stained with isotyped-matched, non-immune antibodies were negative. Megakaryocyte ploidy analyses were performed as described [34].

Analyses of factor V endocytosed by megakaryocyte-like cells

Untreated or PMA-treated cells (1×10^6 mL^{-1}) were cultured in serum-free media, containing 0.1% endotoxin-free bovine serum albumin, in the presence or absence of plasma concentrations of unlabeled or AlexaFluor-labeled factor V (30 nmol L^{-1}), fibrinogen (10 μmol L^{-1}), factor IX (90 nmol L^{-1}), or transferrin (0.3 μmol L^{-1}) for 1, 4, or 16 h (37 °C). Where necessary, washed cells were fixed with 2% paraformaldehyde (30 min), followed by permeabilization with 0.15% Triton X-100 (15 min). Endocytosed protein was detected by incubation (30 min) with immunostaining reaction mixtures containing human immunoglobulin G (IgG) Fc fragment (66.7 nmol L^{-1}, Calbiochem, La Jolla, CA, USA) and 0.1 μmol L^{-1} of the appropriate AlexaFluor488-labeled mAb. Fluorescence from 10 000 cells was analyzed on a Coulter EPICS Elite Flow Cytometer.

Following incubation with AlexaFluor-labeled proteins, 10 000 washed and fixed cells were adhered to poly L-lysine (2 mg mL^{-1}) coated slides, and visualized using an Olympus BX50 Upright Fluorescence Microscope. The AlexaFluor488 and AlexaFluor568 fluorophores were excited at 488 and 568 nm, respectively, with a krypton/argon laser. The individual fluorescent images were merged using Bio-Rad LASER PIX image analysis software. Colocalization was quantified using the MetaMorph Imaging System (Universal Imaging Corporation, Dauningtown, PA, USA).

Immunostaining for clathrin and α-adaptin

PMA-treated CMK cells were cultured with AlexaFluor488-labeled factor V (30 nMOL L^{-1}) or transferrin (0.3 μmol L^{-1}) for 4 h (37 °C). The cells were fixed and permeabilized as described above and incubated (30 min) with human Fc (66.7 nmol L^{-1}), and mouse anti-clathrin or mouse anti-α-adaptin mAbs (66.7 nmol L^{-1}, BD Biosciences, San Jose, CA, USA). Reactivity with the mAbs was detected with goat anti-mouse IgG-AlexaFluor568 (66.7 nmol L^{-1}, Molecular Probes) (30 min). The cells were analyzed by confocal microscopy as described above.

Disruption of clathrin-dependent, receptor-mediated endocytosis by cell culture in hypertonic tissue culture media

PMA-stimulated CMK cells were washed twice with serum-free medium, cultured in normal tissue culture medium or tissue culture medium made hypertonic by the addition of 0.45 mol L^{-1} sucrose (30 min), and incubated with AlexaFluor488-labeled factor V (30 nmol L^{-1}), fibrinogen (0.83 μmol L^{-1}), or transferrin (0.3 μmol L^{-1}) for 60 min. The cells were analyzed by confocal microscopy or flow cytometry as described above. The viability of the cells was unaffected by their culture in hypertonic tissue culture media.

Quantification of MEG-01 or CMK cell binding and/or endocytosis of ^{125}I-labeled-factor V

MEG-01 or CMK cells (± PMA stimulation) were cultured in serum-free media containing 30 nmol L^{-1} factor V plus trace ^{125}I-labeled factor V (1×10^7 c.p.m. mL^{-1}). Aliquots of the cell suspension were removed at timed intervals (0–21 h), layered over an Apiezon A oil : *n*-butylpthalate mixture (1 : 9, v/v) in a microcentrifuge tube, and centrifuged (12 000 × *g*, 30 s). The radioactivity associated with the cell pellets, which represented both cell-bound and internalized ^{125}I-labeled factor V, was determined using a γ-counter.

The specificity of the binding interaction regulating factor V endocytosis was determined using CMK cells cultured with 30 nmol L^{-1} factor V plus ^{125}I-labeled factor V$^{\pm}$plasma concentrations of fibrinogen (10 μmol L^{-1}), transferrin (38 μmol L^{-1}), factor VIII (0.3 nmol L^{-1}), and IgG (666 μmol L^{-1}) for 1 h in serum-free medium (37 °C). The cells were washed extensively to ensure the removal of membrane-bound radioactivity to quantify the amount of ^{125}I-labeled factor V endocytosed.

To assess thrombin-induced secretion, CMK cells cultured in serum-free media for 4 h with 30 nmol L^{-1} factor V containing trace ^{125}I-labeled factor V, were washed extensively to ensure the removal of membrane-bound radioactivity, resuspended at 1×10^7 mL^{-1}, and treated ± 2 U mL^{-1} thrombin (5 min, 37 °C) [38]. Bound and internalized ^{125}I-labeled factor V was separated from free ^{125}I-labeled factor V by centrifugation through oil, and the radioactivity associated with the cell supernatants and pellets was determined.

Analyses of platelet endocytosis of fibrinogen and factor V in whole blood

Blood was drawn by phlebotomy from consenting adults into heparin (50 U mL^{-1}). Aliquots of blood were incubated with equal volumes of plasma concentrations of AlexaFluor488-labeled fibrinogen (10 μmol L^{-1}) or factor V (30 nmol L^{-1}) (60 min). Washed platelets were incubated (15 min) with anti-CD41-PE (100 nmol L^{-1}) or an equivalent concentration of control mouse IgG-PE. The platelets (10 000) were analyzed on a Coulter EPICS Elite Flow Cytometer.

Results

Endocytosis of plasma factor V by CD34$^+$ cell-derived megakaryocytes

Human CD34$^+$ bone marrow cells, subjected to *ex vivo* expansion and differentiation into megakaryocytes under serum-free conditions in the presence of stem cell factor and thrombopoietin for 10 days, expressed no detectable,

© 2005 International Society on Thrombosis and Haemostasis

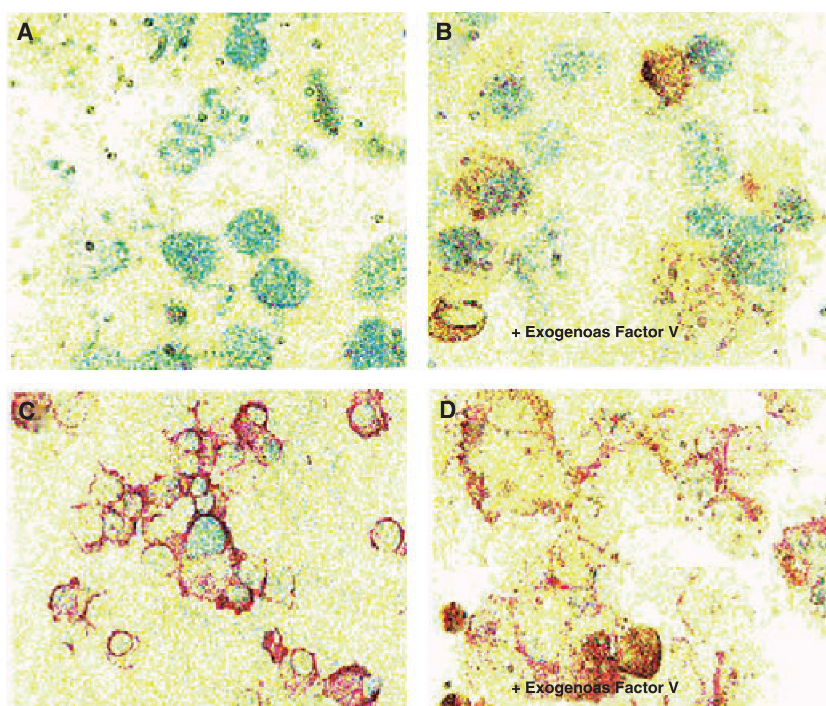

Fig. 1. Immunocytochemical localization of factor V endocytosed by *ex vivo*-derived megakaryocytes. *Ex vivo*-derived megakaryocytes were cultured in the presence (B, D) or absence (A, C) of plasma concentrations of factor V for 72 h. On day 10, adherent cell preparations were subjected to immunocytochemical localization of factor V using anti-factor V (A, D) and/or anti-CD41 (C, D) monoclonal antibodies subsequent to cell permeabilization. Controls using non-specific, isotyped-matched antibody or secondary antibody alone were uniformly negative (data not shown). Immunostaining for factor V appears brown and immunostaining for CD41 appears pink. The cell nuclei are counterstained using Evans Blue.

endogenous factor V subsequent to cell permeabilization and immunocytochemical staining (Fig. 1A,C). In contrast, addition of a plasma concentration of factor V to the megakaryocyte cultures on day 7 followed by identical analyses on day 10 demonstrated its intracellular localization (Fig. 1B) in those cells immunostaining positively for CD41 (Fig. 1D).

Factor V endocytosis appeared to be developmentally regulated (Fig. 2A). Addition of factor V to the cytokine-stimulated megakaryocyte cultures on day 4 followed by flow cytometric analyses on day 7 demonstrated that only a small subpopulation of $CD41^+$ megakaryocytes (5.9 $\pm$ 1.2%) endocytosed factor V. In marked contrast, addition of factor V on day 7 followed by flow cytometric analyses on day 10 resulted in substantial factor V uptake by 34 $\pm$ 3.8% of the $CD41^+$ megakaryocytes. Ploidy analyses of this same cell population demonstrated that all of the $CD41^+$ cells with $>$ 4C DNA content (the more mature cell population) had endocytosed factor V (Fig. 2B, bottom right) whereas a much smaller percentage of the 2C/4C $CD41^+$ cells was positive for factor V uptake. In this series of experiments, culture of the megakaryocytes in the presence of a plasma concentration of factor IX did not result in any factor IX uptake, consistent with its absence from platelets and megakaryocytes, and confirming the specificity of factor V endocytosis.

In these experiments, and those described subsequently, factor V endocytosis was not a result of its proteolysis over time in culture. Western blots of supernatants, from cells incubated with unmodified factor V using a mixture of mAbs against the factor V light chain and heavy chain, or autoradiography of supernatants from cells incubated with ^{125}I-labeled factor V, demonstrated that single-chain factor V remained intact throughout the course of the experiments. However, subsequent to its endocytosis, substantial proteolysis occurred to yield factor V fragments that appeared similar in size to those stored in and released from platelets [39] (Bouchard *et al.*, manuscript in preparation).

Endocytosis of plasma factor V by megakaryocyte-like cell lines

Similar protocols were used to determine if MEG-01 and CMK cells endocytose factor V. Analyses of factor IX and fibrinogen endocytosis served as negative and positive controls, respectively. Cells cultured in the absence of exogenous factor V showed no reactivity with the anti-factor V antibody following cell permeabilization, confirming that neither cell line synthesizes factor V (data not shown). Identical analyses performed subsequent to cell culture in the presence of plasma concentrations of factor V or fibrinogen demonstrated that subpopulations of CMK and MEG-01 cells endocytose these proteins (Table 1, top). A significantly greater proportion of both the MEG-01 and CMK cell populations endocytosed fibrinogen when compared to factor V; therefore, not every cell that endocytoses fibrinogen, endocytoses factor V. Since treatment

© 2005 International Society on Thrombosis and Haemostasis

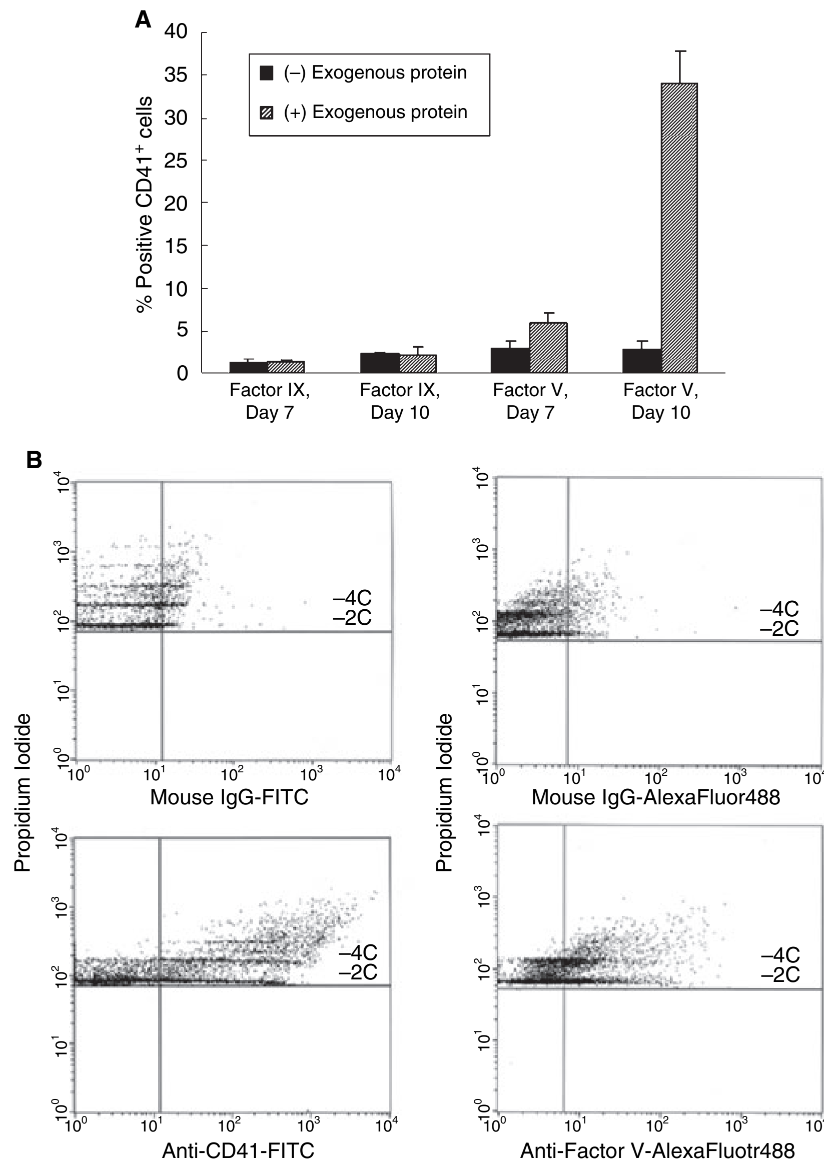

Fig. 2. Endocytosis of factor V by *ex vivo*-derived megakaryocytes is dependent upon the stage of megakaryocyte differentiation. (A) $CD34^+$ bone marrow-derived cells were cytokine stimulated for 7 or 10 days. Plasma concentrations of factor V or factor IX were added at days 4 or 7. Endocytosis was analyzed using multiparameter flow cytometry subsequent to cell permeabilization using anti-CD41-PE, and AlexaFluor488-labeled anti-factor V or anti-factor IX mAbs. The data represent the mean ± SEM ($n = 12$). (B) $CD34^+$ bone marrow-derived cells were cytokine stimulated for 10 days. At day 7, factor V was added. At day 10, the cells were permeabilized, and stained with fluorophore-labeled antibodies and propidium iodide. Cells were analyzed by multiparameter flow cytometry. Cells that were stained with propidium iodide and non-immune mAbs were used as controls.

of MEG-01 and CMK cells with phorbol diesters induces further differentiation along the megakaryocyte lineage [36,40,41], a 2–3-fold increase in the number of CMK and MEG-01 cells endocytosing factor V and fibrinogen was observed as a result of PMA treatment (Table 1, top). Uptake of factor IX could not be demonstrated under any of the conditions examined.

In these experiments, the detection of cellular factor V and fibrinogen with fluorescently labeled mAbs was absolutely dependent upon cell permeabilization with Triton X-100, indicating that the antigens were intracellular. To confirm these observations directly, endocytosis was verified by confocal microscopy of CMK cells, cultured in the presence of AlexaFluor488-labeled factor V or fibrinogen, and extensively washed to remove membrane-bound protein (Fig. 3). A vertical (XZ) section through the region of the cells where the majority of the fluorescent factor V or fibrinogen was observed in the XY plane demonstrated that the visualized proteins were located within the cell, and were absent from the cell membrane surface (Fig. 3B).

Endocytosis of factor V and fibrinogen was independent of the protein concentration to which the cells were exposed. Even though the fibrinogen concentration used in these experiments was 300 times greater than that of factor V (10 μmol L^{-1} vs. 30 nmol L^{-1}), comparison of the relative amounts of endocytosed protein revealed that fibrinogen endocytosis, at best

© 2005 International Society on Thrombosis and Haemostasis

Table 1 Quantitative analyses of the endocytosis of factor V and fibrinogen by megakaryocyte-like cells

	Factor IX	Factor V	Fibrinogen
% positive cells*			
CMK	2.9 ± 2.0	9.1 ± 3.4	13.2 ± 5.3
(+) PMA	0	16.2 ± 4.6	26.5 ± 7.6
MEG-01	0	7.7 ± 0.6	18.5 ± 1.1
(+) PMA	2.2 ± 2.2	20.2 ± 2.9	32.6 ± 7.9
Fluorescence Index†			
CMK	50 ± 50	300 ± 180	620 ± 280
(+) PMA	0	540 ± 260	920 ± 190
MEG-01	0	170 ± 40	470 ± 60
(+) PMA	50 ± 50	370 ± 160	850 ± 230

Megakaryocyte-like cells were cultured in serum-free medium in the presence or absence of plasma concentrations of factor IX, factor V, or fibrinogen for 16 h. Endocytosis was analyzed by immunostaining of permeabilized cells using AlexaFluor488-labeled anti-factor IX, factor V, or fibrinogen mAbs, labeled with the same F : P ratios, followed by flow cytometry.

*The analyses regions were set such that ~97% of the cells stained with isotype-matched, non-immune antibodies were negative. The data are presented as the mean percentage (± SD) of cells that immunostained positively for each antibody ($n = 8$).

†The mean fluorescence index is defined as the product of the percent positive cells and the mean cell fluorescence of that positive cell population. As such, it is a measure of antigen density.

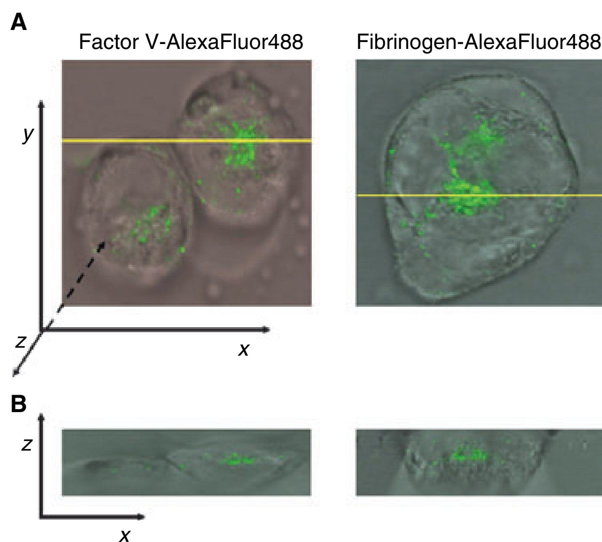

Fig. 3. Fluorescence micrograph of factor V or fibrinogen endocytosed by megakaryocyte-like cell lines. (A) CMK cells were cultured in the presence of AlexaFluor488-labeled factor V or fibrinogen for 4 h. Cells were visualized using an Olympus BX50 Upright Fluorescence Microscope. In these micrographs, the fluorescence images were overlayed on the phase contrast images (original magnification ×600). (B) The laser was scanned repeatedly across the regions of the cells indicated by the yellow lines in (A) as the focus motor was moved vertically through the images at different *z*-axis positions to produce vertical sections of the images.

exceeded factor V endocytosis 3-fold (Table 1, bottom), indicating that endocytosis of each of these proteins was specific and differentially regulated. The amount of antigen present in the cells was determined by calculation of a fluorescence index – the product of the percentage of positive cells and the mean fluorescence of that positive cell population. This is a valid measure of the relative density of the proteins associated with the cells as the AlexaFluor488-labeled mAbs used had almost identical F : P ratios.

Consistent with this notion, the percentage of ^{125}I-labeled factor V endocytosed by cells (100%) was not significantly affected by the presence of plasma concentrations of transferrin (94.9%), IgG (95.4%), fibrinogen (87.4%), or factor VIII (98.0%), whereas, a 50-fold molar excess of unmodified factor V inhibited ^{125}I-labeled factor V endocytosis by > 98%. In addition, another megakaryocyte-like cell line, CHRF-288, endocytosed fibrinogen, but not factor V. In six different experiments approximately the same proportion of CHRF-288 cells as CMK and MEG-01 cells endocytosed fibrinogen, while virtually no endocytosed factor V could be detected. Although PMA-treatment of CHRF-288 cells increased their expression of CD41, factor V endocytosis was not induced. These combined results are consistent with the hypothesis that factor V and fibrinogen are endocytosed by megakaryocytes via specific, membrane-binding events.

Endocytosis of ^{125}I-labeled factor V by MEG-01 and CMK cells was time-dependent. The amount of ^{125}I-labeled factor V associated with MEG-01 cells was maximal at 8 h (Fig. 4). The subsequent gradual decrease in cell-associated factor V was not the result of cell death, but rather appeared more consistent with an increased rate of constitutive secretion vs. endocytosis as MEG-01 cells do not possess α-granule-like structures [41]. In contrast, the amount of ^{125}I-labeled factor V associated with both unstimulated and PMA-stimulated CMK cells was maximal at 6 h and could be sustained (Fig. 4, inset), perhaps more consistent with the presence of α-granule-like structures in these cells [38,40]. PMA stimulation resulted in a ~3-fold increase in factor V endocytosis, as observed previously (Table 1).

Subsequent to a 4-h incubation with ^{125}I-labeled factor V, thrombin treatment of CMK cells [38] induced the release of ~25% of the endocytosed ^{125}I-labeled factor V consistent with its partial localization to secretory granules. Furthermore, a significant fraction of endocytosed, fluorescently labeled factor V and fibrinogen colocalized within CMK cells (Fig. 5). METAMORPH analysis of the 'boxed' cell indicated that 73% of the endocytosed factor V colocalized with endocytosed fibrinogen, and 70% of the fibrinogen colocalized with factor V. Since factor V and fibrinogen are stored in megakaryocyte α-granules [42], one would anticipate that subsequent to their specific and independent endocytosis, factor V and fibrinogen would be sorted together to α-granule-like structures. It should also be noted that some cells will only endocytose fibrinogen (see Fig. 5), which is consistent with the flow cytometric analyses demonstrating that a larger number of cells endocytose fibrinogen when compared to factor V (see Table 1).

Factor V endocytosis appears specific to megakaryocytes. Flow cytometric analyses of platelets in whole blood diluted with plasma concentrations of fluorophore-labeled factor V

© 2005 International Society on Thrombosis and Haemostasis

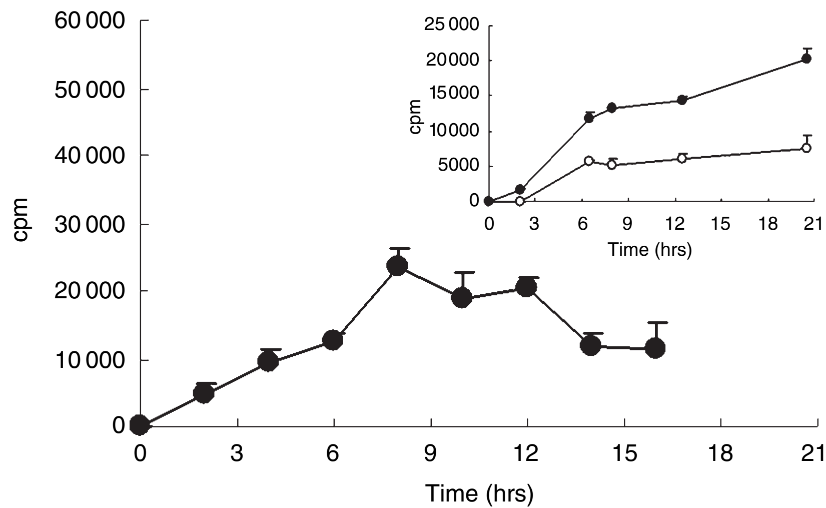

Fig. 4. Time dependence of ^{125}I-labeled factor V endocytosis by MEG-01 and CMK cells. MEG-01 cells were cultured in the presence of factor V containing trace ^{125}I-labeled factor V for 1–21 h. Cell-associated ^{125}I-labeled factor V was determined at each time point by subtracting the radioactivity at time = 0 from the radioactivity associated with each aliquot. The data represent the mean ± SD of three determinations. Inset: Endocytosis of ^{125}I-labeled factor V by untreated (○) or PMA-treated (●) CMK cells over time.

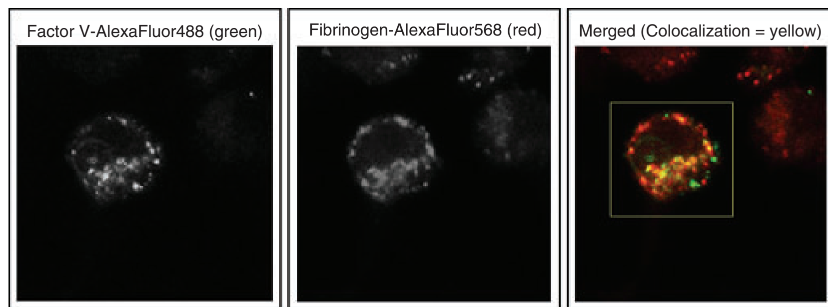

Fig. 5. Factor V colocalizes with fibrinogen subsequent to their endocytosis by megakaryocyte-like cells. PMA-stimulated CMK cells were cultured in the presence of AlexaFluor488-labeled factor V (green fluorescence), or AlexaFluor568-labeled fibrinogen (red fluorescence) for 4 h. The individual images were merged (yellow fluorescence) using Bio-Rad LASERPIX image analysis software. METAMORPH analyses were performed on the 'boxed' cell, which endocytosed both proteins.

or fibrinogen demonstrated that > 80% of the platelets endocytosed fibrinogen. Less than 1% of the platelets were positive for factor V, which is consistent with the observation that factor V does not bind to the unactivated, human platelet surface [14]. Thus, endocytosis of these two proteins by megakaryocytes must be regulated by independent and specific mechanisms.

Endocytosis of factor V by megakaryocytes occurs via a clathrin-dependent, receptor-mediated event

The colocalization of factor V with transferrin was quantified, because the cellular accumulation of transferrin is regulated by clathrin-dependent, receptor-mediated endocytosis, and therefore is used as a tool to distinguish clathrin-dependent from clathrin-independent endocytosis [43]. Subsequent to their endocytosis by PMA-stimulated CMK cells, AlexaFluor488-factor V accumulated in the same endocytic vesicles as AlexaFluor568-transferrin in a time-dependent manner (Fig. 6A). At 1 and 4 h, respectively, 66% and 75% of the endocytosed factor V colocalized with endocytosed transferrin, whereas ≈ 83% of the endocytosed transferrin was colocalized with factor V. Incomplete colocalization was not surprising because factor V, a storage protein, would probably only follow transferrin to the early endosome.

Endocytosed, AlexaFluor488-factor V was also partially colocalized with clathrin and α-adaptin, components of clathrin-coated vesicles. AlexaFluor488-factor V was observed within some of the same endocytic vesicles that stained positively with anticlathrin and anti-α-adaptin antibodies (Fig. 6B), following a 4-h incubation. METAMORPH analyses of the merged images demonstrated that 84% of the endocytosed factor V colocalized with clathrin, while 68% of the clathrin was colocalized with factor V. Similarly, 61% of the factor V colocalized with α-adaptin and 42% of the α-adaptin colocalized with factor V. In separate experiments, similar percentages of endocytosed transferrin colocalized with clathrin and α-adaptin (~ 50%). These combined data support the

© 2005 International Society on Thrombosis and Haemostasis

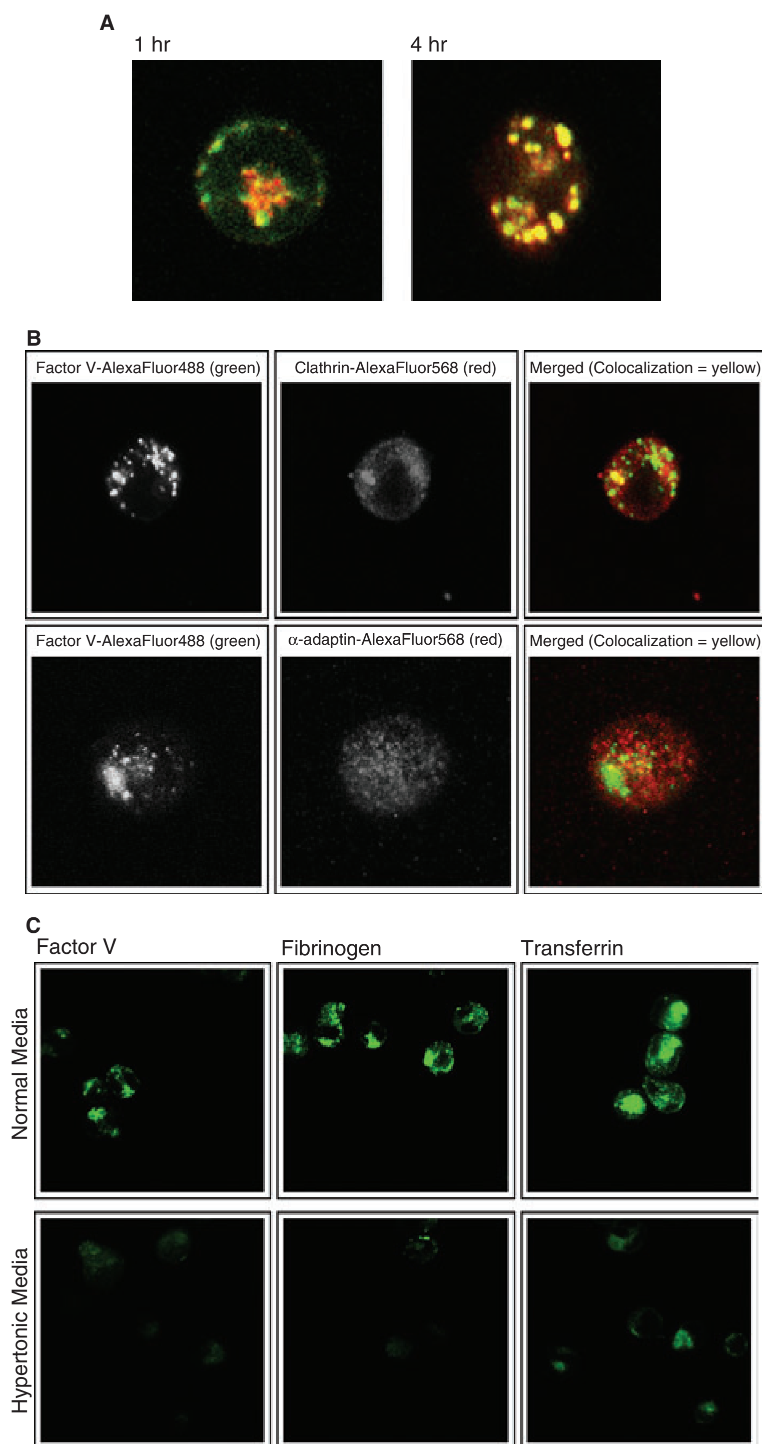

Fig. 6. Factor V endocytosis by megakaryocyte-like cells is a clathrin-dependent process. (A) PMA-stimulated CMK cells were cultured in the presence of AlexaFluor488-labeled factor V and AlexaFluor568-labeled transferrin for 1 or 4 h. The merged images are shown. (B) PMA-stimulated CMK cells were also cultured in the presence of AlexaFluor488-labeled factor V (green fluorescence) for 4 h, and subsequently immunostained for clathrin or α-adaptin, which was detected using a goat anti-mouse IgG-AlexaFluor568 (red fluorescence). The individual fluorescence images are shown in grey scale, and the merged images are shown in color. Similar results were obtained using MEG-01 cells. (C) PMA-stimulated CMK cells were cultured in normal tissue culture media (top) or hypertonic tissue culture media (bottom) and AlexaFluor488-labeled factor V, fibrinogen, or transferrin. The fluorescence micrographs shown above were visualized and imaged using the same microscope settings.

concept that factor V endocytosis by megakaryocytes appears to be regulated by a clathrin-dependent process rather than a pinocytotic mechanism.

Exposure of cells to hypertonic media for 15–30 min at 37 °C reduces membrane-attached clathrin lattices to non-functional flat remnants, incapable of supporting clathrin-dependent,

© 2005 International Society on Thrombosis and Haemostasis

receptor-mediated endocytosis [44]. Culture of PMA-stimulated CMK cells, in media made hypertonic by the addition of 0.45 mol L^{-1} sucrose, while not affecting cell viability drastically reduced their ability to endocytose factor V, transferrin and fibrinogen (also known to be endocytosed via a clathrin-dependent, receptor-mediated mechanism [45]) (Fig. 6C, bottom vs. top). Flow cytometric analyses of and calculation of fluorescence indices for these cell populations demonstrated that the amounts of factor V, fibrinogen and transferrin endocytosed by cells cultured in hypertonic media were decreased by 86, 94 and 70%, respectively, as compared to cells cultured in normal media. Taken together, these observations clearly demonstrate that factor V is endocytosed by megakaryocytes via a clathrin-dependent process.

Discussion

These studies are the first to directly demonstrate that megakaryocytes endocytose factor V. Thus, the acquisition of plasma-derived factor V by patients subsequent to liver transplant was not a function of the antirejection medication the patients were receiving, engraftment of stem cells derived from the transplanted liver, or an inability of the patients' megakaryocytes to synthesize protein. This endocytic process is developmentally regulated during megakaryocyte differentiation, is specific, and regulated at the membrane surface by a clathrin-dependent, and most likely receptor-mediated event, such that endocytosed factor V is subsequently trafficked to secretory granule-like structures. In these studies, endocytosed factor V partially colocalized with components of clathrin-coated vesicles and endocytosis could be inhibited by cell culture in hypertonic media, observations consistent with several studies performed in different cell systems demonstrating the clathrin-dependence of other endocytosed proteins [46–50]. Furthermore, plasma concentrations of transferrin and fibrinogen, two proteins known to be endocytosed by megakaryocytes, had no effect on the endocytosis of factor V. These data also indicate that endogenous synthesis of factor V accounts for an insignificant fraction of the total megakaryocyte intracellular pool, which is consistent with other studies that demonstrate that factor V mRNA is undetectable in megakaryocytes derived from peripheral blood [51]. Similarly, factor V antigen was undetectable in megakaryocytes derived from bone marrow cultured in its absence, indicating that less than 0.05% of the total platelet-derived factor V was endogenously synthesized by these cells [52]. In contrast to these observations, Gewirtz and colleagues, using metabolic labeling studies, demonstrated that mature human megakaryocytes synthesize factor V [53], and they subsequently identified factor V mRNA in pure megakaryocyte populations [54]. Interestingly, in the latter study, the factor V steady-state mRNA levels did not change as a result of cell culture with phorbol diester in the presence of serum, whereas factor V antigen levels increased under these same conditions. This latter observation is consistent with the results of the present study that clearly demonstrates endocytosis of factor V by megakaryocytes following their differentiation by cytokines and phorbol diester. These combined observations are analogous to the results of several studies demonstrating that the platelet fibrinogen pool is derived exclusively from megakaryocyte or platelet endocytosis of fibrinogen from the plasma [6–8], despite the presence of mRNA in these cells [9].

Several studies from our laboratory indicate that platelet-derived factor V is both physically and functionally distinct from its plasma counterpart [13,16,17]. Platelet-derived factor V is resistant to phosphorylation catalyzed by a platelet-associated casein kinase II-like enzyme. In contrast to plasma-derived factor V, which circulates as a single-chain molecule, platelet-derived factor V is stored in α-granules in a partially proteolytically activated state. Characterization of purified platelet-derived factor V indicated that while the heavy chain is intact, the platelet-derived factor V light chain has a unique cleavage at Tyr1543, which leads to functional cofactor activity. Furthermore, the platelet-derived factor V heavy chain possesses a unique *O*-linked glycoform on Thr402. Based on these and other observations, we hypothesize that prior to its targeting to α-granules, endocytosed plasma factor V is 'retailored' post-translationally to yield the functional cofactor molecule.

Our laboratory's observations are the first to demonstrate modifications of a protein subsequent to its endocytosis that alter its function. Thus, the intracellular trafficking pathways that direct endocytosed factor V to the α-granule are of interest. In cultured human megakaryocytes and the megakaryocyte-like cell line, CHRF-288, Sixma and colleagues distinguished three types of granules by electron microscopy: multivesicular bodies type I (MVB I), MVB II, and α-granules [55]. Using gold-labeled albumin and fibrinogen, they demonstrated that MVBs appear to be endocytic compartments, which may function as precursors to α-granules. Furthermore, because endogenously synthesized megakaryocyte proteins such as VWF were also found in MVBs, proteins may be trafficked directly from the Golgi to MVBs [55]. An alternative hypothesis based on the collective data is that subsequent to their endocytosis by megakaryocytes, proteins are trafficked from the endosome to the Golgi to MVBs, which would allow for modification of the endocytosed protein. Based on the data obtained to date, we believe that $CD34^+$ cell-derived megakaryocytes, as well as MEG-01 and CMK cells, will be useful as model systems to begin to test these hypotheses. Since the concentration of platelet-derived factor V can be < 100 times that of its plasma counterpart at a site of injury [24], its unique functional properties will have a distinct advantage over plasma-derived factor V. Thus, studies of the origin and unique characteristics of platelet-derived factor V are essential for a relevant understanding of procoagulant events. Furthermore, identification of the megakaryocyte membrane receptor that mediates factor V binding and its subsequent endocytosis is also of great interest as endocytosis of factor V by megakaryocytes, but not platelets, represents a novel mechanism whereby platelets acquire an essential hemostatic protein.

© 2005 International Society on Thrombosis and Haemostasis

Acknowledgements

These studies would not have been possible without the generous donations of CMK and CHRF-288 cells by Drs Hava Avraham (Harvard Medical School) and Michael Lieberman (University of Cincinnati College of Medicine), respectively. The authors would also like to thank Lorna Seifert and Jennifer Bombard for their technical assistance. This work was supported by HL70826 (to PBT), HL46703 (Project 4) (to PBT) and the Department of Biochemistry, University of Vermont College of Medicine.

References

1 Sporn LA, Chavin SI, Marder VJ, Wagner DD. Biosynthesis of von Willebrand protein by human megakaryocytes. *J Clin Invest* 1985; **76**: 1102–6.
2 Sitar G, Borroni R, Pesce G. Synthesis of thrombospondin and glycoprotein IIb-IIIa by a purified megakaryocyte human bone marrow culture. *Haematologica* 1986; **71**: 389–93.
3 Konkle BA, Schick PK, He X, Liu RJ, Mazur EM. Plasminogen activator inhibitor-1 mRNA is expressed in platelets and megakaryocytes and the megakaryoblastic cell line CHRF-288. *Arterioscler Thromb* 1993; **13**: 669–74.
4 Hill SSSG, Joshua P, Ribau J, Austin RC, Podor TJ. Differential mechanisms targeting type I plasminogen activator inhibitor and vitronectin into the storage granules of a human megakaryocytic cell line. *Blood* 1996; **87**: 5061–73.
5 Roussi JDL, Sigman J, Vaiman M, Pignaud G, Bonneau M, Martin Cramer E. Absence of incorporation of plasma von Willebrand factor into porcine platelet α-granules. *Br J Haematol* 1995; **90**: 661–8.
6 Cramer EM, Debili N, Martin JF, Gladwin AM, Breton-Gorius J, Harrison P, Savidge GF, Vainchenker W. Uncoordinated expression of fibrinogen compared with thrombospondin and von Willebrand factor in maturing human megakaryocytes. *Blood* 1989; **73**: 1123–9.
7 Handagama P, Scarborough RM, Shuman MA, Bainton DF. Endocytosis of fibrinogen into megakaryocyte and platelet alpha-granules is mediated by alpha IIb beta 3 (glycoprotein IIb-IIIa) [published erratum appears in *Blood* 1993; 82 (9): 2936]. *Blood* 1993; **82**: 135–8.
8 Louache F, Debili N, Cramer E, Breton-Gorius J, Vainchenker W. Fibrinogen is not synthesized by human megakaryocytes. *Blood* 1991; **77**: 311–16.
9 Podolak-Dawidziak M, Hancock V, Lelchuk R, Kotlarek-Haus S, Martin JF. The expression of mRNA for fibrinogen in megakaryocytes isolated from patients with T-cell lymphoma. *Br J Haematol* 1995; **91**: 362–6.
10 Poujol C, Nurden AT, Nurden P. Ultrastructural analysis of the distribution of the vitronectin receptor (alpha v beta 3) in human platelets and megakaryocytes reveals an intracellular pool and labelling of the alpha-granule membrane. *Br J Haematol* 1997; **96**: 823–35.
11 Camire RM, Pollak ES, Kaushansky K, Tracy PB. Secretable human platelet-derived factor V originates from the plasma pool. *Blood* 1998; **92**: 3035–41.
12 Christella M, Thomassen LG, Castoldi E, Tans G, Magdeleyns EJ, Delaunoit C, Debusscher L, Van Assche KJ, Rosing J. Endogenous factor V synthesis in megakaryocytes contributes negligibly to the platelet factor V pool. *Haematologica* 2003; **88**: 1150–6.
13 Gould WR, Simioni P, Silveira JR, Tormene D, Kalafatis M, Tracy PB. Megakaryocytes endocytose and subsequently modify human factor V *in vivo* to form the entire pool of a unique platelet-derived cofactor. *J Thromb Haemost* 2005; **3**: 450–6.
14 Tracy PB, Eide LL, Mann KG. Human prothrombinase complex assembly and function on isolated peripheral blood cell populations. *J Biol Chem* 1985; **260**: 2119–24.
15 Mazzorana M, Baffet G, Kneip B, Launois B, Guguen-Guillouzo C. Expression of coagulation factor V gene by normal adult human hepatocytes in primary culture. *Br J Haematol* 1991; **78**: 229–35.
16 Gould WR, Silveira JR, Tracy PB. Unique *in vivo* modifications of coagulation factor V produce a physically and functionally distinct platelet-derived cofactor: characterization of purified platelet-derived factor V/Va. *J Biol Chem* 2004; **279**: 2383–93.
17 Monkovic DD, Tracy PB. Functional characterization of human platelet-released factor V and its activation by factor Xa and thrombin. *J Biol Chem* 1990; **265**: 17132–40.
18 Monkovic DD, Tracy PB. Activation of human factor V by factor Xa and thrombin. *Biochemistry* 1990; **29**: 1118–28.
19 Kalafatis M, Rand MD, Mann KG. The mechanism of inactivation of human factor V and human factor Va by activated protein C. *J Biol Chem* 1994; **269**: 31869–80.
20 Camire RM, Kalafatis M, Cushman M, Tracy RP, Mann KG, Tracy PB. The mechanism of inactivation of human platelet factor Va from normal and activated protein C-resistant individuals. *J Biol Chem* 1995; **270**: 20794–800.
21 Conlon SJ, Camire RM, Kalafatis M, Tracy PB. Cleavage of platelet-derived factor Va by plasmin results in increased and sustained co-factor activity on the thrombin-activated platelet surface. *Thromb Haemost* 1997; **77**: 2507a.
22 Camire RM, Kalafatis M, Simioni P, Girolami A, Tracy PB. Platelet-derived factor Va/Va Leiden cofactor activities are sustained on the surface of activated platelets despite the presence of activated protein C. *Blood* 1998; **91**: 2818–29.
23 Tracy PB, Mann KG. Abnormal formation of the prothrombinase complex: factor V deficiency and related disorders. *Hum Pathol* 1987; **18**: 162–9.
24 Nesheim ME, Nichols WL, Cole TL, Houston JG, Schenk RB, Mann KG, Bowie EJ. Isolation and study of an acquired inhibitor of human coagulation factor V. *J Clin Invest* 1986; **77**: 405–15.
25 Yang TL, Pipe SW, Yang A, Ginsburg D. Biosynthetic origin and functional significance of murine platelet factor V. *Blood* 2003; **102**: 2851–5.
26 Sun H, Yang TL, Yang A, Wang X, Ginsburg D. The murine platelet and plasma factor V pools are biosynthetically distinct and sufficient for minimal hemostasis. *Blood* 2003; **102**: 2856–61.
27 Chiu HC, Schick PK, Colman RW. Biosynthesis of factor V in isolated guinea pig megakaryocytes. *J Clin Invest* 1985; **75**: 339–46.
28 Tracy PB, Peterson JM, Nesheim ME, McDuffie FC, Mann KG. Interaction of coagulation factor V and factor Va with platelets. *J Biol Chem* 1979; **254**: 10354–61.
29 Cerveny TJ, Fass DN, Mann KG. Synthesis of coagulation factor V by cultured aortic endothelium. *Blood* 1984; **63**: 1467–74.
30 Jenny RJ, Pittman DD, Toole JJ, Kriz RW, Aldape RA, Hewick RM, Kaufman RJ, Mann KG. Complete cDNA and derived amino acid sequence of human factor V. *Proc Natl Acad Sci USA* 1987; **84**: 4846–50.
31 Ishihara H, Connolly AJ, Zeng D, Kahn ML, Zheng YW, Timmons C, Tram T, Coughlin SR. Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature* 1997; **386**: 502–6.
32 Katzmann JA, Nesheim ME, Hibbard LS, Mann KG. Isolation of functional human coagulation factor V by using a hybridoma antibody. *Proc Natl Acad Sci USA* 1981; **78**: 162–6.
33 Straughn W 3rd, Wagner RH. A simple method for preparing fibrinogen. *Thromb Diath Haemorrh* 1966; **16**: 198–206.
34 Williams JL, Pipia GG, Datta NS, Long MW. Thrombopoietin requires additional megakaryocyte-active cytokines for optimal *ex vivo* expansion of megakaryocyte precursor cells. *Blood* 1998; **91**: 4118–26.
35 Taniguchi Y, London R, Schinkmann K, Jiang S, Avraham H. The receptor protein tyrosine phosphatase, PTP-RO, is upregulated

© 2005 International Society on Thrombosis and Haemostasis

during megakaryocyte differentiation and is associated with the c-Kit receptor. *Blood* 1999; **94**: 539–49.

36 Fugman DA, Witte DP, Jones CL, Aronow BJ, Lieberman MA. *In vitro* establishment and characterization of a human megakaryoblastic cell line. *Blood* 1990; **75**: 1252.

37 Chuang JL, Schleef RR. Adenovirus-mediated expression and packaging of tissue-type plasminogen activator in megakaryocytic cells. *Thromb Haemost* 2001; **85**: 1079–85.

38 Nagano T, Kishimoto Y, Kimura T, Yasunaga K, Adachi M, Ryo R, Sato T*et al*. Ultrastructural analysis of a human megakaryocytic leukemia cell line (CMK11-5) in response to platelet agonists. *Int J Hematol* 1993; **57**: 73–80.

39 Bouchard BA, Thanassi NM, Tracy PB. Factor V endocytosed and retained by megakaryocytes is proteolytically processed by an intracellular protease to form the platelet-derived factor V pool. *J Thrombosis Hemostasis* 2003; **1**: P1335.

40 Nagano T, Taniguchi H, Hamamoto K. Ultrastructural analysis of incorporation into organelles of CMK cells. *J Clin Electron Microscopy* 1989; **22**: 5–6.

41 Ogura M, Morishima Y, Okumura M, Hotta T, Takamoto S, Ohno R, Hirabayashi N, Nagura H, Saito N. Functional and morphological differentiation induction of a human megakaryoblastic leukemia cell line (MEG-01s) by phorbol diesters. *Blood* 1988; **72**: 49–60.

42 Harrison P, Cramer EM. Platelet α-granules. *Blood Rev*, 1993: 52–62.

43 Bayer N, Schober D, Huttinger M, Blaas D, Fuchs R. Inhibition of clathrin-dependent endocytosis has multiple effects on HRV2 cell entry. *J Biol Chem* 2000; **276**: 3952–62.

44 Heuser JE, Anderson RG. Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation. *J Cell Biol* 1989; **108**: 389–400.

45 Klinger MH, Kluter H. Immunocytochemical colocalization of adhesive proteins with clathrin in human blood platelets. Further evidence for coated vesicle-mediated transport of von Willebrand factor, fibrinogen and fibronectin. *Cell Tissue Res* 1995; **279**: 453–7.

46 Barlic J, Khandaker MH, Mahon E, Andrews J, DeVries ME, Mitchell GB, Rahimpour R, Tan CM, Ferguson SS, Kelvin DJ. β-arrestins regulate interleukin-8-induced CXCR1 internalization. *J Biol Chem* 1999; **274**: 16287–94.

47 Cao H, Orth JD, Chen J, Weller SG, Heuser JE, McNiven MA. Cortactin is a component of clathrin-coated pits and participates in receptor-mediated endocytosis. *Mol Cell Biol* 2003; **23**: 2162–70.

48 Teo M, Tan L, Lim L, Manser E. The tyrosine kinase ACK1 associates with clathrin-coated vesicles through a binding motif shared by arrestin and other adaptors. *J Biol Chem* 2001; **276**: 18392–8.

49 Wei ML, Bonzelius F, Scully RM, Kelly RB, Herman GA. GLUT4 and transferrin receptor are differentially sorted along the endocytic pathway in CHO cells. *J Cell Biol* 1998; **140**: 565–75.

50 Memmo LM, McKeown-Longo P. The alphavbeta5 integrin functions as an endocytic receptor for vitronectin. *J Cell Sci* 1998; **111**: 425–33.

51 Giampaolo A, Vulcano F, Macioce G, Mattia G, Barca A, Milazzo L, Orlando M, Hassan HJ. Factor V expression in human *in vitro* developed megakaryocytes. *Thromb Haemost* 2001; **86**: 542a.

52 Veljkovic D, Cramer EM, Alimardani G, Fichelson S, Masse JM, Hayward CPM. Studies of alpha-granule proteins in cultured human megakaryocytes. *Thromb Haemost* 2003; **90**: 844–52.

53 Gewirtz AM, Keefer M, Doshi K, Annamalai AE, Chiu HC, Colman RW. Biology of human megakaryocyte factor V. *Blood* 1986; **67**: 1639–48.

54 Gewirtz AM, Shapiro C, Shen YM, Boyd R, Colman RW. Cellular and molecular regulation of factor V expression in human megakaryocytes. *J Cell Phys* 1992; **153**: 277–87.

55 Heijnen HFG, Debili N, Vainchencker W, Breton-Gorius J, Geuze HJ, Sixma JJ. Multivesicular bodies are an intermediate stage in the formation of platelet α-granules. *Blood* 1998; **91**: 2313–25.

© 2005 International Society on Thrombosis and Haemostasis