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Mechanisms Regulating Acquisition of Platelet-Derived Factor V/Va by Megakaryocytes

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ABSTRACT

Factor Va serves as the nonenzymatic protein cofactor for the prothrombinase complex, which converts prothrombin to thrombin in the events leading to formation of a hemostatic plug. Several observations support the concept that platelet-derived factor V/Va is physically and functionally distinct and plays a more important role in thrombin generation at sites of vascular injury as compared to its plasma counterpart. Platelet-derived factor V/Va is generated following endocytosis of the plasma-derived molecule by the platelet precursor cells, megakaryocytes, via a two receptor system consisting of low density lipoprotein (LDL) receptor-related protein-1 (LRP-1) and an unidentified specific "binding site". More recently, it was suggested that a cell surface-expressed β-galactoside binding protein, galectin-8, was involved in factor V endocytosis. Endocytosed factor V is trafficked through the cell and retailored prior to its storage in α-granules. Given the essential role of platelet-derived factor Va in clot formation, understanding the cellular and molecular mechanisms that regulate how platelets acquire this molecule will be important for the treatment of excessive bleeding or clotting. J. Cell. Biochem. 116: 2121–2126, 2015.

KEY WORDS: FACTOR V; PLATELETS; MEGAKARYOCYTES; LRP-1; THROMBIN

Factor V is an essential coagulation protein synthesized by the liver as a single chain 330 kD molecule. Upon thrombin cleavage, factor V is activated to factor Va, consisting of a heavy (94 kD) and light (74 kD) chain noncovalently associated through a calcium (Ca^{2+}) ion (Fig. 1). Factor Va functions as the critical, nonenzymatic cofactor of the coagulation enzyme complex prothrombinase, which activates the zymogen prothrombin to the active serine protease thrombin (Nesheim et al., 1979; Mann et al., 1988). The prothrombinase complex is composed of factor Va and the serine protease factor Xa assembled on an appropriate membrane surface, such as that provided by the activated platelet membrane, in the presence of Ca^{2+} (Mann et al., 1988, 1990) (Fig. 2). Thrombin, once formed, not only cleaves fibrinogen to form the insoluble fibrin clot, it also modulates its own formation through the activation of platelets and the coagulation proteins factors V, VIII and XI, and through the formation of the potent anticoagulant, activated protein C. Beyond its role in coagulation, thrombin also has chemotactic and mitogenic properties enabling it to participate in inflammatory processes, cellular growth, and brain development [Narayanan 1999] through its activation of cellular protease-activated receptors (PARs) [Coughlin 2000]. Generation of thrombin can be achieved by cleavage of prothrombin by factor Xa alone; however, the complete prothrombinase complex is 300,000 times more efficient at thrombin generation (Nesheim et al., 1979) highlighting the importance of factor Va. Indeed, clinical observations demonstrate that deficiencies of factor Va can result in a severe bleeding tendency in the affected individual [Camire 2011]. In stark contrast, inefficient inactivation of the cofactor by activated protein C, as is seen with factor Va^{Leiden}, can lead to thrombosis (Dahlback et al., 1996, Kujovich 2011).

PLATELET-DERIVED FACTOR V/VA FORMS THE MORE HEMOSTATICALLY RELEVANT COFACTOR MOLECULE

The procofactor, factor V, exists in two whole blood pools: 75-80% circulates in the plasma while the remaining 20-25% is stored in platelet α-granules from where it is released upon platelet activation at sites of vascular injury (Tracy and Eide, 1982). Several observations demonstrate that plasma- and platelet-derived factor V and Va are physically and functionally distinct; these differences impart a more procoagulant phenotype on the platelet cofactor

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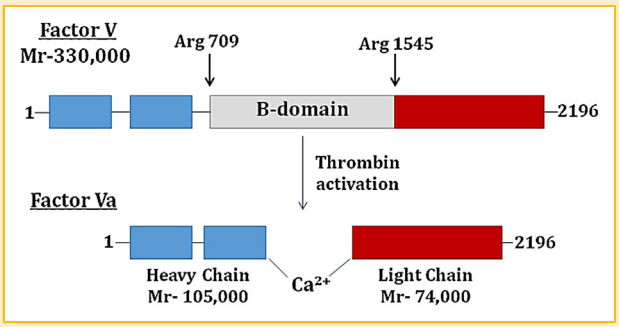

Fig. 1. Factor V activation by thrombin. Factor V is synthesized and circulates in plasma as a single chain, inactive procofactor molecule. Upon limited proteolysis by thrombin at Arg 709, 1018, and 1545, factor V is converted to its active form, factor Va, by removal of the B-domain. The active cofactor consists of a heavy chain and a light chain noncovalently associated through a Ca^{2+} ion. Mr, mass of factor V, the factor Va heavy chain, and the factor Va light chain in Da.

(Lee and Mann, 1989; Monkovic and Tracy, 1990; Kalafatis et al., 1994; Camire et al., 1995; Conlon et al., 1997; Camire et al., 1998; Gould et al., 2004; Gould et al., 2005; Wood et al., 2008; Tracy et al., 2013). For example, plasma-derived factor V circulates as a large, single chain molecule expressing no procogulant activity prior to its activation by thrombin (Mann et al., 1990). In contrast, the platelet-derived form is released in a partially proteolytically-activated state expressing substantial cofactor activity (denoted from here on in as factor V/Va) (Monkovic and Tracy, 1990; Gould et al., 2004). These observations are supported by clinical studies demonstrating that the platelet-derived factor V/Va molecule is the more hemostatically relevant cofactor in thrombin generation at sites of vascular injury.

An individual with an inhibitor to plasma-derived factor V, yielding less than 1% activity, but whose platelet-derived factor V/Va is unaffected experienced no adverse bleeding and maintained adequate hemostasis during surgery suggesting that that patient's platelet-derived factor V/Va pool, which was protected from the inhibitory antibody, was sufficient for normal hemostasis (Nesheim et al., 1986). An essential role for platelet factor V/Va is further highlighted by the severe bleeding problems associated with factor V New York, a disorder characterized by an intrinsic defect in platelet-derived factor V/Va and normal plasma-derived factor V levels (~79%) (Weiss et al., 2001). More recently, it was demonstrated that the platelet-derived factor V/Va acquired and retained following administration of fresh frozen plasma to an individual with a complete, congenital deficiency in both the plasma- and platelet-derived cofactor molecules confers hemostasis and prevents gastro-intestinal bleeding even following depletion of the plasma pool (Bouchard et al., 2009). Other reports describe the success of platelet transfusions in the cessation of severe bleeding resulting from factor V deficiency (Borchgrevink and Owren, 1961; Salooja et al., 2000) or factor V inhibitors (Chediak et al., 1980; Brandt et al., 1986; Raman et al., 1995).

FACTOR V ENDOCYTOSIS BY MEGAKARYOCYTES IS MEDIATED BY A TWO RECEPTOR SYSTEM

Platelet-derived factor V/Va is acquired via endocytosis of the plasma procofactor by megakaryocytes, the platelet precursor cells (Bouchard et al., 2005; Gould et al., 2005; Suehiro et al., 2005, Rowley et al., 2011). Bouchard et al. demonstrated for the first time that megakaryocytes express low density lipoprotein (LDL) receptor-related protein-1 (LRP-1), and that LRP-1 mediates factor V endocytosis (Bouchard et al., 2008). These observations were supported by Lambert et al., who also demonstrated that LRP-1

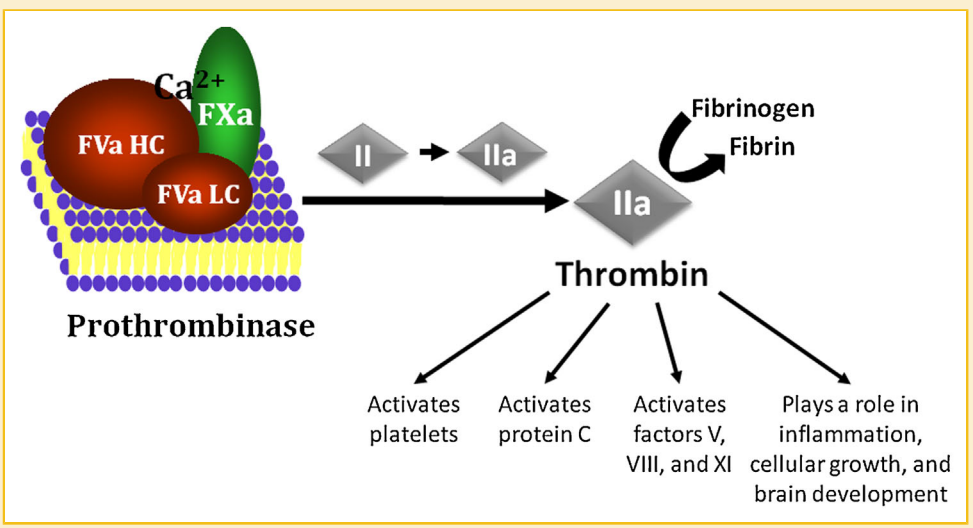

Fig. 2. The essential role of factor Va in thrombin generation. The prothrombinase complex consists of the nonenzymatic cofactor factor Va and the serine protease Xa, which, when bound to an appropriate cell membrane such as the activated platelet surface in the presence of Ca^{2+} ions, converts prothrombin to thrombin. As indicated, thrombin plays multiple roles in the hemostatic response through the activation of platelets and coagulation proteins and the down regulation of blood coagulation through the formation of the potent anticoagulant, activated protein C, and plays prominent roles in inflammation, cellular growth, and brain development.

was expressed differentially on developing megakaryocytes and mature platelets (Lambert et al., 2009). LRP-1 is a member of the LDL receptor family (Strickland et al., 2002). It is a ubiquitous receptor whose canonical role is to bind to and deliver ligands to lysosomes for degradation including lipoproteins and coagulation/fibrinolytic proteins, such as factor VIII (Saenko et al., 1999; Sarafanov et al., 2001), a coagulation protein that is highly homologous to factor V (Kane and Davie, 1986; Mann et al., 1988). LRP-1 is composed of an 85 kDa membrane spanning C-terminal region that is noncovalently associated with a 515 kDa N-terminal extracellular region (Herz et al., 1990). Ligands bind to LRP-1 via its extracellular domain, while the cytoplasmic domain binds to adaptor proteins that serve as a link between the receptor and other membrane proteins (Gotthardt et al., 2000). Within the extracellular domain are four ligand binding domains, clusters I, II, III and IV (Willnow et al., 1994), composed of arrays of cysteine-rich Ca^{2+}-binding regions known as complement-type repeats. Studies have also shown that the lysine side chains are important for ligand binding to LRP-1 (Lillis et al., 2008). More recently, residues Lys 2092 and Phe 2093 of the light chain of the coagulation protein factor VIII, which is ~40% homologous to factor V (Kane and Davie, 1986; Jenny et al., 1987), have been found to be important for endocytic uptake by LRP-1 (Saenko et al., 1999; Sarafanov et al., 2001). Much is known about the structure of LRP-1; however, the region where factor V binds is currently unknown.

While a role for LRP-1 in factor V endocytosis by megakaryocytes has been clearly established, LRP-1 expression alone is not sufficient for factor V uptake (Bouchard et al., 2008). Cells that endocytose factor V express both LRP-1 and an additional, as yet, uncharacterized specific factor V "binding site" on their cell surface (Fig. 3). Factor V binds to the specific factor V "binding site" in a Ca^{2+}-independent manner. This facilitates binding of another factor V molecule to LRP-1. In studies using various factor V molecules and monoclonal antibodies, it was demonstrated that factor V binding to and endocytosis by megakaryocytes are mediated by the factor V light chain (Bouchard et al., 2013) although the specific factor V regions and amino acid residues involved are unknown. Following its binding to LRP-1, factor V is endocytosed in Ca^{2+}- and clathrin-dependent manners. It is subsequently modified intracellularly to form the unique platelet-derived cofactor molecule prior to its trafficking to α-granules (Bouchard et al., 2006). This notion is supported by the observation that, subsequent to its endocytosis, factor V undergoes proteolysis by one or more specific megakaryocyte protease(s) to form the partially-activated platelet-derived pool (Osterud et al., 1977, Monkovic and Tracy, 1990; Gould et al., 2004; Ayombil et al., 2013).

It was recently reported by Zappelli et al. that galectin-8, a protein that binds glycans containing β-galactosides such as lactose, was involved in factor V endocytosis (Zappelli et al., 2012). Traditionally cytosolic proteins, galectins were shown to be secreted from several cell types including CHO, BHK, endothelial, Th1, and macrophages via transporters, vesicles, or exocytosis [Hughes, 1999]. Once secreted, galectins promote cell-matrix or cell-cell interactions by bridging cell membrane receptors with extracellular matrix proteins or receptors on nearby cells (Elola et al., 2007). It is via this mechanism that galectin-8 and galectin-1 are capable of activating platelets (Pacienza et al., 2008; Romaniuk et al., 2010). Peptide mass fingerprinting of platelet lysates identified several putative binding partners of galectin-8 including factor V (Romaniuk et al., 2010). This interaction is presumably mediated by the abundant N-linked glycans present on factor V (Kim et al., 1999; Nicolaes et al., 1999). Zappelli et al. confirmed a direct interaction between galectin-8 and factor V using surface plasmon resonance analysis and solid phase binding assays (Zappelli et al., 2012). Galectin-8 binding to immobilized factor V is reversible and dose-dependent, and can be inhibited by β-galactoside-containing sugars. The K_d for this interaction is ~30 nM, which is identical to the plasma concentration of factor V. Furthermore, when a megakaryocyte-like cell line, DAMI, was preincubated with known galectin-8 ligands or an anti-galectin-8 antibody that blocks galectin-8 interactions with its ligands, factor V endocytosis was inhibited by more than two-fold. A similar observation was made following knockdown of galectin-8 with RNAi. Based on these combined results the authors concluded that galectin-8 is involved factor V endocytosis and may serve as the unidentified specific factor V "binding site".

The fate of LRP-1 following factor V endocytosis is unknown. No comprehensive study to define LRP-1 trafficking has been performed to date. However, much can be inferred from what is known about the intracellular trafficking of other similar receptors, including the LDL receptor that has been extensively studied and is used as a model of the endocytic pathway (Goldstein et al., 1985; Mellman 1996).

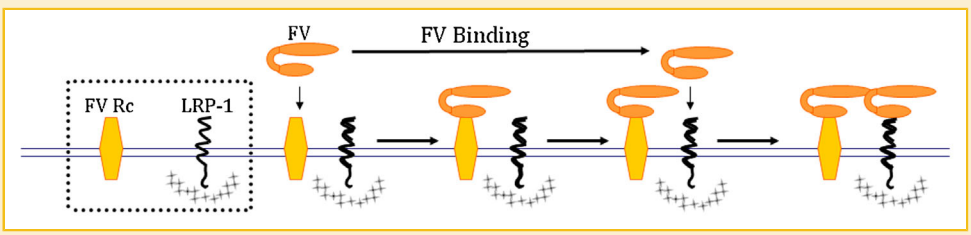

Fig. 3. A model describing the megakaryocyte cell surface events mediating factor V endocytosis. Two receptors, including low density lipoprotein (LDL) receptor-related protein-1 (LRP-1), a well- characterized receptor, and an as of yet unidentified factor V "binding site" (FV Rc), mediate factor V (FV) endocytosis by megakaryocytes. The initial binding of factor V to the FV Rc facilitates binding of a second molecule of factor V to LRP-1. Factor V is subsequently endocytosed in Ca^{2+}- and clathrin-dependent manners, physically and functionally modified, and trafficked to α-granules. Figure 3 was modified from The Journal of Thrombosis and Haemostasis, Vol. 6, Beth A. Bouchard, Natalie T. Meisler, Michael E. Nesheim, Chun-Xiang Liu, Dudley K. Strickland, and Paula B. Tracy, A unique function for LRP-1: A component of a two receptor system mediating specific endocytosis of plasma-derived factor V by megakaryocytes, pp. 638-644, 2008, with permission from John Wiley & Sons Ltd.

Following endocytosis of the LDL–LDL receptor complex in a clathrin-dependent manner, the endocytic vesicle fuses with the early or sorting endosome and LDL dissociates in the acidic environment of the early endosome. The membrane-associated LDL receptors are sorted into recycling endosomes and returned to the cell surface while LDL proceeds to the lysosome (Goldstein et al., 1985; Mellman 1996). Studies by Stockinger and colleagues demonstrated that sorting nexin 17 (Snx17), a protein important for receptor membrane recycling and sorting, binds to the intracellular domains of several members of the LDL receptor family, including LRP-1, and regulates endocytosis of the LDL receptor (Stockinger et al., 2002). Interestingly, Snx17 was originally identified as an intracellular binding protein for the platelet/megakaryocyte-specific protein P-selectin (Florian et al., 2001), which accelerates P-selectin internalization and inhibits its lysosomal degradation (Williams et al., 2004). Snx17 resides on distinct vesicular structures partially overlapping with endosomal compartments characterized by the presence of early endosomal antigen 1 and Rab4 (Stockinger et al., 2002). Following endocytosis, LDL passes through Snx17-positive compartments, and Snx17 enhances the endocytosis rate of this receptor (Stockinger et al., 2002). More recently, Bu and colleagues demonstrated that Snx17 is important for LRP-1 recycling following endocytosis and interacts with the NPxY motif in the cytoplasmic tail of LRP-1 proximal to the cell membrane (van Kerkhof et al., 2005). Disruption of the interaction between LRP-1 and Snx17 had no effect on endocytosis but decreased LRP-1 recycling from endosomes and increased its lysosomal degradation suggesting that Snx17 mediates LRP-1 sorting to the recycling pathway in early endosomes. However, as endocytosis of a protein not destined for lysosomal degradation ascribes a novel function to LRP-1 (Strickland et al., 2002), it can be hypothesized that following endocytosis LRP-1 escorts factor V through the megakaryocyte for modification and storage in α-granules. A goal of future studies should be to test this hypothesis.

PERSPECTIVES AND FUTURE DIRECTIONS

It is now widely accepted that the platelet-derived factor V/Va pool in humans originates through endocytosis of the plasma molecule by megakaryocytes. Subsequent to endocytosis it is physically and functionally modified to yield a unique platelet-derived pool. It is this platelet-derived pool that predominates at sites of vascular injury following its release from activated platelets. While much is known about factors V and Va structure and function, research on the mechanism by which factor V is endocytosed by the megakaryocyte and trafficked to the platelet has only just begun. Knowledge about the specific residues or regions of factor V and LRP-1 that mediate their interaction and the role of factor V glycans in endocytosis would help us better understand the endocytic mechanism. In addition, studies assessing the stage of megakaryocyte differentiation when factor V endocytosis begins or ends and the cellular and molecular signals that guide factor V through the cell would provide insight into α-granule development and platelet production. Advancements in our understanding of how megakaryocytes regulate acquisition and formation of the platelet-derived factor V/Va pool may ultimately lead to development of novel therapies to treat bleeding or clotting by modulating platelet-derived factor V/Va levels. Even after decades of research, bleeding and clotting are still significant causes of morbidity and mortality. Thus, there is a critical need for the development of new options for the treatment of hemostatic disorders that are safe and effective. As the local concentration of platelet-derived factor V/Va at sites of vascular injury and platelet release is ~100-fold higher than its plasma counterpart (Nesheim et al., 1986), even small changes in platelet-derived factor V/Va concentration or function will profoundly influence thrombin generation and fibrin clot formation. A reduction in the levels of platelet-derived factor V/Va at sites of vascular injury in individuals with cardiovascular disease or prone to thrombosis may dampen thrombin generation and subsequent clot formation. Likewise, increased formation and concentrations of the platelet-derived cofactor may confer hemostatic competence in individuals with bleeding disorders. Modulation of platelet-derived factor V/Va levels at sites of vascular injury by inhibiting or increasing megakaryocyte formation of this cofactor pool represents a novel and targeted approach to treatment of bleeding or thrombosis.

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