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The factor V light chain mediates the binding and endocytosis of plasma-derived factor V by megakaryocytes

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Plasma- and platelet-derived factor (F) Va are essential for thrombin generation catalyzed by Prothrombinase assembled on the activated platelet surface [1]. While both the plasma- and platelet-derived molecules can form a functional complex, several studies demonstrate that the two cofactor pools are physically and functionally distinct [2–7]. Despite these differences, the entire platelet-derived pool of the procofactor, FV, is endocytosed from the plasma by megakaryocytes [4,8] via a two-receptor system consisting of a specific, unknown FV receptor and low density lipoprotein (LDL) receptor-related protein-1 (LRP-1) [9], an endocytic receptor belonging to a super-family of proteins related to the LDL receptor [10]. Flow cytometric analyses and fluorescence microscopy indicate that all megakaryocytes express LRP-1 [9,11]. However, cells that endocytose FV must express both LRP-1 and an unidentified, specific FV receptor. FV binds to the specific FV receptor in a Ca^{2+} -independent manner [9]. This binding interaction facilitates binding of another FV molecule to LRP-1, which mediates endocytosis through Ca^{2+} - [9] and clathrin-dependent [8] mechanisms. Endocytosed FV is modified intracellularly to form the unique platelet-derived cofactor molecule [2–7] and trafficked to α -granules. The platelet-derived cofactor molecule plays the predominant hemostatic role at sites of vascular injury [12]; therefore, elucidation of the mechanisms regulating its endocytosis, modification and storage in α -granules will provide insight into the events leading to formation of a fibrin clot.

The goal of these studies was to identify the specific region(s) of FV involved in its interactions with the membrane receptors that mediate its endocytosis. Plasma FV is converted to FVa via cleavage by thrombin at Arg⁷⁰⁹, Arg¹⁰¹⁸ and Arg¹⁵⁴⁵, to release the large central B domain and form the active cofactor [1]. FVa consists of a heavy chain (residues 1–709) and light chain (residues 1546–2196) non-covalently linked with Ca^{2+} (Fig. 1A). Endocytosis assays were performed using iodine 125-labeled FV (¹²⁵I-FV), which was prepared and characterized as described previously [9]. Pre-incubation of megakaryocyte-like CMK cells with 50-fold molar excesses of unlabeled, human and bovine FV and FVa inhibited endocytosis of ¹²⁵I-FV nearly 100% (Fig. 1B). Similarly, a recombinant human FV single chain construct missing most of the B-domain (recombinant FV_{de1811-1491}; rFV-DT) [13] (see Fig. 1A) inhibited endocytosis by 86% ± 0%, suggesting that the B domain is not involved. Pre-incubation of cells with the isolated light chain [14]

inhibited endocytosis of ^{125}I -FV by $84\% \pm 2\%$. In contrast, the isolated heavy chain [14] had a small effect on endocytosis ($19\% \pm 15\%$ inhibition). Inhibition of ^{125}I -FV endocytosis by various anti-FV antibodies provided and characterized by Dr Kenneth Mann (University of Vermont, Burlington, VT, USA) was determined in parallel. Antibodies directed against the FV light chain (E9, #2 and #9) or FV heavy chain (#17) [15] were pre-incubated with ^{125}I -FV prior to the addition of CMK cells. Consistent with the results described above, anti-FV light chain antibodies, E9 and anti-FV#2 inhibited FV endocytosis by megakaryocytes by greater than 80% (Fig. 1C). Anti-FV#9 as well as anti-FV#17 had little or no effect on FV endocytosis ($< 20\%$ inhibition).

One of the anti-FV light chain antibodies, E9, inhibited ^{125}I -FV endocytosis to the same extent as unlabeled FV. As the two-receptor model of endocytosis dictates that FV binding to the specific FV receptor is necessary for and precedes FV binding to LRP-1 [9], additional experiments were performed in an attempt to define which of the receptor-FV interactions E9 inhibits. Previously, it was demonstrated that ^{125}I -FV binding was inhibited $\sim 50\%$ by receptor-associated protein, a known ligand of LRP-1 [9]. In contrast, unlabeled FV inhibited binding by $\sim 90\%$. These experiments were performed in the absence of Ca^{2+} , which is essential for FV endocytosis but not binding [9]. Similar experiments were performed using E9. Inhibition of FV binding by $\sim 50\%$ would suggest that E9, like RAP, is inhibiting FV binding to LRP-1 alone. One of two scenarios is possible if complete inhibition is observed: E9 is inhibiting binding to the specific FV receptor alone and, therefore, binding to LRP-1 cannot occur; or, similar to the observations made using FV [9], E9 is directly inhibiting binding of ^{125}I -FV to both receptors. As the E9 concentration increased, steady state binding of ^{125}I -FV decreased with inhibition, nearing 100% at a 3.3-fold molar excess of E9 (Fig. 1D). These overall data suggest that amino acid residues in the FV light chain mediate the binding to and endocytosis of FV by megakaryocytes and are likely to encompass all or part of the E9 epitope. Experiments are currently being performed to identify this epitope. As the FV heavy chain and B domain appear to have no role in the endocytic process, our combined data suggest that the FV light chain mediates FV's interactions with both receptors at the megakaryocyte cell surface.

Addendum

B.A. Bouchard conceived the research, performed experiments, analyzed data and prepared the manuscript. S. Abdalla performed experiments and analyzed data. P.B. Tracy conceived the research, analyzed data and reviewed the manuscript.

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Fig. 1.

The FV light chain mediates its binding to and endocytosis by megakaryocytes. (A) The domain structures of FV, rFV-DT and FVa. (B) CMK cells ($1 \times 10^6 \text{ mL}^{-1}$) were pre-incubated (1 h, 37°C) with unlabeled, human and bovine FV and FVa, isolated human FV light chain (LC) and heavy chain (HC), or FV-DT ($0.5 \mu \text{M}$ each), followed by the addition of ^{125}I -FV (10 nM , 1 h, 37°C). Excess and cell surface-bound ^{125}I -FV were removed by washing followed by centrifugation. Inhibition of ^{125}I -FV endocytosis was quantified by comparing the cpm associated with the dry cell pellets in the presence vs. absence of competitor. The data represent the mean $\pm$ SEM of three separate experiments in which triplicate determinations were performed. (C) ^{125}I -FV (5 nM) was incubated with anti-FV light chain (E9, #2 and #9) or heavy chain (#17) antibodies ($1 \mu \text{M}$, 1 h, 37°C) prior to the addition of CMK cells. Inhibition of ^{125}I -FV endocytosis was quantified as described above. Unlabeled, human FV (250 nM) served as the control. The data represent the mean $\pm$ SEM of two separate experiments in which triplicate determinations were performed. (D) ^{125}I -FV (30 nM) was incubated with E9 ($0-1 \mu \text{M}$) prior to the addition of CMK cells (30 min, 37°C) in the absence of Ca^{2+} to prevent endocytosis. Cell surface-bound protein was separated from unbound protein by centrifugation through oil, and the amount of radioactivity associated with the cell pellets determined. Non-specific binding, determined in the presence of unlabeled FV ($1.5 \mu \text{M}$), was subtracted from the total binding to estimate specific binding. The data represent the mean $\pm$ SD of the specific binding of a single experiment in which quadruplicate determinations were performed.