is one of the most abundant F-BAR proteins in platelets and propose that it contributes to the formation of the DMS in megakaryocytes. In platelets, PACSIN2 associates with filamin A (Flna), a cytoskeletal protein that stabilizes platelet membranes subjected to shear stress and promotes platelet adhesion to the von Willebrand factor by bridging the adhesion glycoprotein GPIbα to the actin cytoskeleton.\(^5\) Interestingly, although ~50% of Flna is associated with the cytoskeleton, PACSIN2 is selectively associated with soluble Flna. This interaction involves a putative Flna binding motif located between PACSIN2 amino acids 174 and 182, near a loop at the tip of the F-BAR domain necessary for membrane tubulation and Flna repeat 20. Although the localization of the Flna binding motif suggests that the interaction between PACSIN2 and Flna might disrupt PACSIN2 membrane-binding and tubulating activities, Flna binding enhances the membrane-scultping activity of PACSIN2 in vitro and dissociates from membrane-bound PACSIN2 during tubulation. These data uncover a novel mechanism for activation of PACSIN2.

To explore the biological relevance of this finding, the authors used super-resolution structured illumination microscopy to examine the localization of PACSIN2, Flna, and GPIbα in mouse and human platelets and megakaryocytes. PACSIN2 and GPIbα colocalize in clusters resembling the focal membrane invaginations associated with the open canalicular system of platelets or the DMS of megakaryocytes,\(^5\) suggesting that PACSIN2 is involved in the initiation of membrane invagination during the formation of these structures. Although in most wild-type platelets PACSIN2 was concentrated in a single focus per cell, in Flna-null platelets, PACSIN2 formed multiple foci. The cause of the formation of multiple foci is unclear. Because the amount of PACSIN2 was increased in Flna-null platelets, this may reflect a propensity of PACSIN2 to aggregate at sites of high concentration (eg, sites of membrane tubulation). In contrast, in Flna-null megakaryocytes, PACSIN2 levels were normal, PACSIN2 was dispersed throughout the cell, and PACSIN2-coated membrane tubules were substantially decreased. Similar results were obtained by expressing a PACSIN2 mutant unable to interact with Flna, indicating that Flna binding promotes PACSIN2-mediated membrane tubulation in megakaryocytes. Based on these data and on the fact that Flna-null megakaryocytes exhibit defects in DMS formation, the authors suggest that PACSIN2 contributes to the formation of the DMS.

The findings of Begonja et al raise important new questions concerning the role of PACSIN2 in platelets that can only be answered by examining platelet and megakaryocyte membrane organization and mechanical strength following targeted inactivation of Pcasin2 or following short hairpin RNA-mediated depletion in megakaryocytic cell lines.\(^6\) Indeed, through its roles in membrane tubulation and stimulation of actin polymerization, PACSIN2 may contribute to the mechanical stability of platelets and to the formation of the DMS (see figure).

Eckly et al\(^5\) suggested that formation of the DMS is coupled to the process of abortive cytokinesis and that the tubular membrane invaginations of the DMS represent a megakaryocyte-specific pseudo-cleavage furrow. As Drosophila PACSIN regulates cleavage furrow assembly\(^9\) and Flna inhibits actomyosin activity in platelets,\(^10\) determining how the interaction of PACSIN2 with Flna regulates megakaryocyte ploidy is of particular interest.

Conflict-of-interest disclosure: The authors declare no competing financial interests.\(^3\)

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Comment on Ivanciu and Camire, page 94

**FXa variants advance toward a therapy for bleeding**

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In this issue of Blood, Ivanciu and Camire describe engineered factor (F)Xa variants with a spectrum of properties that broaden the utility of FXa as a treatment for bleeding.\(^1\)

Treatment of hemophilia A and B requires replacement of the deficient coagulation factor (FVIII or FIX, respectively) to restore thrombin generation at the site of tissue injury. This is successfully used for treatment of hemophilia but has the common problem of developing an inhibitory antibody against the infused protein. As an alternative strategy, FXa has been investigated as a means of bypassing the intrinsic pathway because FXa bound to damaged or activated endothelium in the presence of the cofactor FXa converts prothrombin to thrombin.\(^2\) One problem with this approach is that administering wild-type
FXa (wt-FXa) is ineffective because it has a very short half-life in plasma due to the high concentration of circulating inhibitors (particularly antithrombin III) that prevent passage to the activated surface. Strategies to improve these pharmacokinetic properties with protein engineering could involve mutation of surface residues or providing additional N-linked glycan residues. Ivanciu and colleagues have developed an innovative approach to tackle this problem by engineering alternative amino acids at the N-terminal tip of the highly conserved FXa activation loop.¹ The figure shows that two key amino acids (Val17 and Ile16 shown in purple) are buried in the hydrophobic core of the FXa enzyme. Ile16 does not contact the substrate prothrombin but is indirectly critical for formation of the oxyanion hole and FXa selectivity pocket by interacting with Asp194. This interaction alone is not sufficient to produce a mature FXa active site competent to cleave prothrombin, which requires the additional presence of the cofactor FVa.

Biochemical studies mutating FXa Val17 and Ile16 revealed that substitutions here are less efficient at forming the active site resulting in an enzyme that is zymogen-like.² How do the mutations at the tip of the activation loop work to improve FXa utility in the treatment of bleeding? Subsequent studies revealed that zymogen-like FXa tip variants do not bind circulating inhibitors, which effectively broadens the functional half-life and provides safe passage to the site of injury.³ This observation together with the ability of FVa to combine with the FXa variants to produce an active prothrombinase complex was the recipe for effectively bypassing the intrinsic pathway. These studies were then extended into a mouse model of hemophilia to demonstrate that the FXa Ile16Leu variant was more effective than FVIIa as a replacement therapy.⁴

Ivanciu and Camire¹ present data that further advance these studies to characterize a total of 11 variants mutating FXa residues Ile16 and Val17. Variants Val17Met, Ile16Leu, Ile16Met, Val17Thr, Val17Ser, and Ile16Thr are characterized in detail in terms of enzyme activity, plasma half-life, clotting times, and thrombin generation in hemophilic plasma. The results reveal that these variants were effective in mouse injury models and achieved a broadening of the spectrum of FXa zymogen-like activities. At one end of the spectrum, variants Val17Met and Val17Leu (termed group 1)⁴ are characterized as having high activity. This type of variant can be achieved only by modifying Val17, which is more remote to the oxyanion hole than Ile16 and only partially buried in the active FXa structure (see figure). In terms of circulating plasma half-life, the group 1 variants were characterized as having a 10-fold advantage compared with the wt-FXa. In the middle of the spectrum, variants Ile16Leu and Ile16Met (group 2) have an intermediate zymogenicity with a plasma half-life up to 100-fold greater than wt-FXa. Finally, at the other end of the spectrum, the Ile16Thr and Val17Ser variants (group 3) are the most zymogen-like and have a plasma half-life up to 350-fold greater than wt-FXa (6-7 hours). It is notable that this latter type of variant is achieved only by removing the hydrophobic nature of the amino acid whereby introduction of the small polar-uncharged amino acid (Thr or Ser) into the activation loop tip achieves the correct balance of inducing FXa zymogenicity without compromising the ability of FVa to rescue activity in the prothrombinase complex.

The time of administration was also investigated, and postinjury administration of high-activity FXa variants such as Val17Met were shown to be the most efficacious in the mouse model. Interestingly, variants involving the polar-uncharged amino acids (Ile16Thr, Val17Ser) with long functional half-lives are effective when administered before injury and may be suitable for prophylactic treatment. This provides a toolkit of FXa variants which can be tailor-made for different clinical contexts involving bleeding.⁵

Conflict-of-interest disclosure: The author declares no competing financial interests.

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FXa variants advance toward a therapy for bleeding

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