SUPPLEMENTAL MATERIALS & METHODS

Assessment of platelet endocytosis: Platelet-rich plasma (2x10^8 platelets/mL) was incubated with plasma-derived factor V (FV) (2 nM or 20 nM), AlexaFluor647-conjugated fibrinogen, or AlexaFluor647-conjugated factor IX (FIX) (negative control) (90 nM)\(^1\) (37°C). At each time point (0, 2, 4, and 6 hrs), aliquots of each reaction mixture were removed. For the samples incubated with FV, prostaglandin E\(_1\) (5 μM) was added and the platelets pelleted by centrifugation (660 x g, 3 min). Platelet pellets were resuspended in 20 mM Hepes, 0.15 M NaCl, pH 7.4, containing 0.1% polyethylene glycol (HBS/Ca\(^{2+}/\)PEG) containing prostaglandin E\(_1\), and adjusted to 1x10^9/mL. Platelets lysates were prepared as described above for quantitative western blotting analyses.

Aliquots of the platelets incubated with fluorescently-labeled fibrinogen or FIX were incubated with an equal volume of Optilyse C to lyse any residual red blood cells and fix the platelets (10 min, ambient temperature). HBS/Ca\(^{2+}/\)PEG was added and the samples stored at 4°C, protected from light. Binding and endocytosis of fluorescently-labeled fibrinogen by platelets was analyzed by flow cytometry using a BDLSRII Flow Cytometer. Platelets were identified by their forward and side scatter characteristics. The positive analyses regions were defined such that ≥98% of the platelets incubated with FIX-AlexaFluor647 were negative.

Agonist-induced platelet prothrombinase complex assembly and function: To assess the ability of protease-activated receptor (PAR) 1 (SFLLRN-NH\(_2\)) and PAR4 (AYPGKF-NH\(_2\)) agonist peptides to elicit thrombin generation, washed platelets (2 x 10^8/mL) were activated with varying concentrations of thrombin (0-100 nM), PAR1 (0–1 mM), and/or PAR4 (0-1 mM) for 2 min at ambient temperature. A 1.5 fold molar excess of hirudin was added to terminate the thrombin activation reactions. FVa and factor Xa (FXa) (5 nM each) were added and incubated with the
platelets for 2 min at ambient temperature. The reactions were initiated by the addition of an equal volume of a prothrombin and dansylarginine N-(3-3thyl-1,5-pentanediyl)amide (DAPA) mixture (1.4 µM and 3 µM final concentrations, respectively). Thrombin formation was assessed using a discontinuous assay as described previously.²
SUPPLEMENTAL LITERATURE CITED


SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Platelets isolated from a FV deficient patient bind to and endocytose fluorescently-labeled fibrinogen. Platelet-rich plasma (2x10^8 platelets/mL) was incubated (2 hr, 37°C) with (A) FIX conjugated to AlexaFluor647 or (B) fibrinogen conjugated to AlexaFluor647. Following fixation of the cells at 2, 4, and 6 hr, fluorescence from 10,000 platelets was analyzed by flow cytometry as described in the Supplemental Materials & Methods. The analysis region, P2, was defined using the platelets incubated with labeled FIX such that ≤2% of the platelets were positive. The data depict the results obtained after a 2 hr incubation. Result: Consistent with previous observations made in unaffected individuals, the patient’s platelets endocytosed fibrinogen,3,4 but not FV5 (data not shown). This is also consistent with the inability of platelets to bind FV.6 These combined observations suggest that the patient’s platelet-derived FV/Va originated following endocytosis from plasma.

Supplemental Figure 2. Subsequent to plasma transfusion, acquired platelet-derived FV is stored as a partially proteolytically-activated cofactor molecule. Washed platelets from the patient and an unaffected individual (Control) were lysed with triton X-100 in the presence of leupeptin. Western blotting analysis was performed as described in Materials & Methods. The positions of the molecular weight markers are indicated on the left of the figure. The position of single chain FV is noted (SC). Result: Similar to previous observations made in unaffected individuals,7-10 the patient’s platelet-derived FV/Va is stored in α-granules in a partially-activated state following endocytosis.
**Supplemental Figure 3. Comparison of thrombin-, PAR1 peptide-, and PAR4 peptide-induced platelet prothrombinase complex assembly and function.** A. Washed platelets (2 x 10^8/mL) were activated with thrombin (0 – 100 nM, circles), PAR1 peptide (0 – 1 mM, squares), or PAR4 peptide (0 – 1 mM, triangles) for 2 min at ambient temperature without stirring. Prothrombin activation mixtures contained activated platelets (1 x 10^8/mL), 5 nM FVa, 5 nM FXa, 1.39 µM prothrombin and 3 µM DAPA. Thrombin generation was measured as described in Supplemental Materials & Methods. The data represent the mean +/- SD. B. Washed platelets were activated with thrombin (0.1 µM), PAR1 peptide (100 µM), and/or PAR4 peptide (500 µM). Thrombin generation via prothrombinase was measured as described. The data represent the mean +/- SD and are shown as a percentage of the activity achieved with 0.1 µM thrombin. Result: Activation of platelets with PAR agonist peptides results in a prothrombinase activity equal to that generated following maximal stimulation by thrombin.

**Supplemental Figure 4. Intentional platelet activation with PAR peptides has no effect on thrombin generation in a tissue factor-initiated whole blood clotting model in an unaffected individual.** (A) Whole blood (1mL) from an unaffected individual was incubated (37°C with rocking) with TF (5 pM) alone (open squares) or TF (5 pM) plus PARS 1 (100 µM) and 4 (500 µM) agonist peptides (closed squares). TAT formation was measured by ELISA as described in Materials & Methods. Result: Platelet FV/Va release following platelet activation has no effect on thrombin generation in a tissue factor-initiated whole blood clotting model in an individual with normal concentrations of plasma- and platelet-derived FV.
Supplemental Figure 1A
Supplemental Figure 1B
Supplemental Figure 3B