Properties of Procoagulant Platelets

Defining and Characterizing the Subpopulation Binding a Functional Prothrombinase*

Ammon M. Fager, Jeremy P. Wood, Beth A. Bouchard, Ping Feng, Paula B. Tracy

Objective—The goal of this study was to define and characterize the subpopulation of platelets capable of regulating the functional interactions of factors Va (FVa) and Xa (FXa) on the thrombin-activated platelet surface.

Methods and Results—Flow cytometric analyses were used to define and characterize platelet subpopulations. At a concentration of thrombin known to elicit maximal platelet activation, platelet-derived FVa release, and prothrombinase assembly/function, only a subpopulation of platelets was positive for FVa and FXa binding. An additional subpopulation bound lower levels of FVa but little, if any, FXa. Fluorescence microscopy analyses confirmed these data. Phenotypically, platelets capable of binding FXa were more highly reticulated and demonstrated significantly increased expression of several key adhesion molecules, including P-selectin, glycoprotein Ibα, and integrins αIIb and β3. This platelet subpopulation was also defined by the expression of a nondissociable, membrane-bound pool of functional platelet-derived FVa, which made up ≈35% to 50% of the total membrane-bound cofactor.

Conclusion—The ability of activated platelets to support thrombin generation is defined by a subpopulation of platelets expressing a nondissociable pool of platelet-derived FVa and increased adhesive receptor density. This subpopulation is hypothesized to play a significant role in regulating both normal hemostasis and pathological thrombus formation because the adherent properties of platelets and their ability to mount and sustain a procoagulant response are crucial steps in both of these processes. (Arterioscler Thromb Vasc Biol. 2010;30:2400-2407.)

Key Words: adhesion molecules ■ coagulation ■ platelets ■ thrombin ■ factor V

Thrombin generation is pivotal to many physiological and pathophysiologic processes, including but not limited to hemostasis,1 thrombosis,2 wound healing,3 and atherosclerosis.4 Thrombin is formed via prothrombinase, a Ca2+-dependent, stoichiometric complex of the cofactor factor Va (FVa) and the serine protease factor Xa (FXa), assembled on an appropriate membrane surface.1 Even though FXa effects the cleavage of prothrombin to thrombin, its ability to do so in a physiologically relevant time frame is absolutely dependent on the presence of both FVa and the membrane surface. Activated platelets not only provide this membrane surface in vivo but also express and release FVa.4 The platelet-derived pool of FVa originates from megakaryocyte endocytosis of the plasma-derived procofactor.5-6 Subsequent to its endocytosis, FV is phenotypically modified7 to produce a cofactor with increased and sustained procoagulant activity, distinguishing it from its plasma-derived counterpart.8,9

The amount of thrombin produced via prothrombinase assembled on the surface of activated platelets is dependent on both the level of platelet activation,10 which is defined by the agonist used, and the quantity and quality of bound FVa. Although it has long been known that platelets are heterogeneous with respect to volume, density, lipid peroxidation, and metabolism,11,12 heterogeneity in the ability of activated platelets to bind coagulation factors has only recently been observed.13-16 Alberio et al were the first to demonstrate that only a fraction of platelets activated simultaneously with low concentrations of thrombin (<5 nmol/L) and convulxin, an agonist of the collagen receptor glycoprotein (GP) VI retain high levels of platelet-derived FV/FVa at their membrane surface16 in a transglutaminase-dependent manner.15 Studies done by Kempton et al16 demonstrate the formation of 2 subpopulations of activated platelets differentiated by their ability to bind the constituents of both intrinsic tenase and prothrombinase. Subsequent studies by Panteleev et al demonstrated that a unique platelet subpopulation, formed subsequent to thrombin-induced activation, binds high levels of FIXa, FVIIIa, and FX.17

Even though all of these studies have described heterogeneity in the ability of activated platelet subpopulations to bind coagulation factors, these subpopulations have not been characterized extensively regarding their ability to generate thrombin via assembly of a functional prothrombinase or their expression of other membrane properties important for
platelet function. In contrast to previous studies using low-dose thrombin and convulxin/collagen as platelet agonists, thrombin was used as the sole agonist in this study as it has been shown to effect maximal prothrombinase assembly and function at the activated platelet surface. Our data indicate that subsequent to thrombin-catalyzed activation, not all platelet-bound FVa molecules are capable of supporting FXa binding. Rather, the ability of activated platelets to generate thrombin via prothrombinase is defined by a subpopulation of platelets expressing both nondissociable and dissociable pools of platelet-derived FVa, each capable of binding FXa, and expressing an increased density of adhesive receptors at their activated membrane surface. Because the adherent properties of platelets and their ability to mount and sustain a procoagulant response are crucial steps in effecting normal hemostasis as well as pathological thrombus formation, the identified platelet subpopulation should be significant in regulating and contributing to these processes.

Methods
Materials purchased, antibody descriptions, preparation of coagulation proteins, and methods with which we have substantial experience are detailed in the supplemental materials (available online at http://atvb.ahajournals.org). Techniques and protocols new to our laboratory and essential to this work are detailed here.

Generation and Characterization of Procoagulant Platelet Subpopulations
Platelets were isolated from human venous blood collected from healthy, nonmedicated individuals as detailed previously.20 Washed platelets (1 × 10^9/mL) were activated with thrombin (50 nmol/L) in the presence of Arg-Gly-Asp-Ser (RGDS 1 mmol/L) to prevent platelet aggregation. Alternatively, platelets were activated with a combination of low-dose thrombin (5 nmol/L) and convulxin (0.5 μg/mL). In some experiments, platelets were activated in the presence of dansylcadaverine (<200 μmol/L). Following the addition of hirudin (75 nmol/L), activated platelets were incubated with FXa (5 nmol/L). In some experiments, plasma-derived FVa (5 nmol/L) was also added to ensure saturation of all available prothrombinase sites. Following fixation to cross-link platelet-bound FVa and FXa, platelets expressing FVa, FXa, P-selectin, integrin αIb, GPIIb, or integrin αIIb were visualized by flow cytometry using specific or control fluorophore-conjugated monoclonal antibodies. In some experiments, the platelets were incubated with Retic-COUNT thiazole orange (TO) reagent before flow cytometric analyses. Platelet-bound proteins were also visualized by fluorescence microscopy. Flow cytometry and fluorescence microscopy protocols are detailed in the supplemental materials.

Determination of a Functional, Nondissociable, Platelet-Derived FVa Pool
Platelet suspensions (1 × 10^9/mL) containing RGDS (5 mmol/L) and Gly-Pro-Arg-Pro (2 mmol/L) were activated with thrombin (50 nmol/L). CaCl_2 was omitted from the buffer to disrupt Ca^2+ dependent interactions between the FVa heavy and light chains. The platelets were subsequently washed by centrifugation 1 to 5 times in buffer containing RGDS (5 mmol/L) and lacking Ca^2+. At each wash, platelet-bound FVa activity was determined using a 1-stage FV clotting assay, and retention of the FVa light and heavy chains was assessed by Western blotting. In other experiments, retention of the FVa heavy chain was assessed by flow cytometry. Details are provided in the supplemental materials.

Statistical Analyses
For Tables 1 and 2, unpaired t tests were used to calculate 2-tail probability values. All calculations were performed using GraphPad Prism software. Data are expressed as mean ± SEM.

### Table 1. Procoagulant Platelets Express Increased Adhesive Receptor Density

<table>
<thead>
<tr>
<th>Receptor</th>
<th>CD No.</th>
<th>FXa-Negative</th>
<th>FXa-Positive</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Integrin β3</td>
<td>61</td>
<td>1.6 ± 1.9</td>
<td>9.0 ± 3.0*</td>
<td>5.8 ± 1.6</td>
</tr>
<tr>
<td>P-Selectin</td>
<td>62P</td>
<td>5.1 ± 2.9</td>
<td>22.7 ± 4.2†</td>
<td>4.5 ± 0.8</td>
</tr>
<tr>
<td>GPIIb</td>
<td>42b</td>
<td>14.3 ± 2.6</td>
<td>87.5 ± 18.9†</td>
<td>6.1 ± 2.4</td>
</tr>
<tr>
<td>Integrin αIIb</td>
<td>41</td>
<td>35.8 ± 1.4</td>
<td>150 ± 5.4ψ</td>
<td>4.2 ± 0.3</td>
</tr>
</tbody>
</table>

FXa binding and platelet adhesion receptor expression were detected using appropriate fluorophore-conjugated monoclonal antibodies as described in Methods and shown in Figure 2. Fold increases in receptor density were determined as the ratio of mean fluorescence intensity (MFI) in the FXa-positive platelets to MFI in the FXa-negative platelets. Values are expressed as mean ± SEM. n = 2 to 109.

*P < 0.01; †P < 0.001; ‡P < 0.02 compared with FXa-negative platelets.

### Table 2. Age-Related Expression of Adhesive Platelet Membrane Receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>CD No.</th>
<th>Mature (TO—)</th>
<th>Young (TO+)</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Integrin β3</td>
<td>61</td>
<td>2.5 ± 1.1</td>
<td>14.7 ± 1.9*</td>
<td>6.0 ± 1.0</td>
</tr>
<tr>
<td>P-Selectin</td>
<td>62P</td>
<td>4.2 ± 1.5</td>
<td>24.8 ± 2.1*</td>
<td>6.0 ± 0.7</td>
</tr>
<tr>
<td>GPIIb</td>
<td>42b</td>
<td>7.7 ± 3.3</td>
<td>52.7 ± 4.8*</td>
<td>6.8 ± 0.9</td>
</tr>
</tbody>
</table>

Platelet adhesion receptor density was determined as described. Platelet age was assessed in all samples by analysis of TO staining as detailed in Methods. Fold increases in receptor density were determined as the ratio of mean fluorescence intensity (MFI) in the young (TO-positive) platelets to MFI in the mature (TO-negative) platelets. Values are expressed as mean ± SEM. n = 26 to 52.

*P < 0.001 compared with mature platelets.
demonstrating no binding (I), an intermediate level of binding (II), or substantial (ie, high) FVa binding (III). The mean fluorescence intensity of the platelets binding “intermediate” levels of FVa was consistently 5% to 8% of that expressed by those platelets binding “high” levels of FVa (5.8 ± 0.5%, n = 28). In duplicate reaction mixtures, assessment of FXa binding characteristics (Figure 1A, lower panel) indicated that platelets segregate into only 2 populations: those demonstrating substantial FXa binding (III) and those demonstrating no binding whatsoever (I). Similar analyses, with 28 different platelet donors (aged 33 to 91 years old; 54% female), indicated that the percentage of platelets capable of binding high levels of FVa was 33.5% ± 13.6% for each individual. Neither donor gender nor donor age showed a correlation with the percentage of platelets comprising the procoagulant subpopulation. As the monoclonal antibodies demonstrating no binding (I), an intermediate level of binding (II), or substantial (ie, high) FVa binding (III). The mean fluorescence intensity of the platelets binding “intermediate” levels of FVa was consistently 5% to 8% of that expressed by those platelets binding “high” levels of FVa (5.8 ± 0.5%, n = 28). In duplicate reaction mixtures, assessment of FXa binding characteristics (Figure 1A, lower panel) indicated that platelets segregate into only 2 populations: those demonstrating substantial FXa binding (III) and those demonstrating no binding whatsoever (I). Similar analyses, with 28 different platelet donors (aged 33 to 91 years old; 54% female), indicated that the percentage of platelets capable of binding high levels of FVa was 33.5% ± 13.6% for each individual. Neither donor gender nor donor age showed a correlation with the percentage of platelets comprising the procoagulant subpopulation. As the monoclonal antibodies against both FVa and FXa had the same fluorescence-to-protein ratio and yielded similarly sized subpopulations, it appears that those platelets defined as high binders may bind FVa and FXa equivalently.

To determine whether the platelets capable of high FVa binding comprised the same platelet subpopulation as those capable of binding FXa, 2 approaches were taken. First, flow cytometric analyses of activated platelets, dual labeled to assess both FVa (anti-FVa–Alexa Fluor 647) and FXa (anti-FXa–Alexa Fluor 488) binding, yielded platelet subpopulations (Supplemental Figure II) similar to those shown in Figure 1A in that separate subpopulations bound intermediate and high levels of FVa, whereas a single subpopulation bound high levels of FXa. Additional quantification of FVa and FXa binding to the CD62P platelet population (Figure 1B) indicated that 88.0% ± 1.1% (n = 6) of platelets capable of binding FXa also showed high levels of FVa binding (Figure 1B, III) and represented the majority of the procoagulant platelet population.
population. Likewise, the majority of activated platelets binding high levels of FVa (93 ± 0.5%; n = 6) were defined by FXa binding. A small percentage of those platelets originally defined as binding intermediate levels of FVa were able to bind FXa (mean = 11.8 ± 1.1%; n = 6) (Figure 1B, II), but these platelets represent those binding the greatest amount of FVa in that population. Likewise, a small percentage of those platelets defined as high FVa binders did not appear to bind FXa (mean = 7.0 ± 0.5%; n = 6) (Figure 1B, VI). Similar data were obtained in 3 additional experiments, each using platelets from different donors (data not shown).

Confocal microscopic analyses of activated platelets immunostained with anti-FVα2-Cy5, anti-FXa–Alexa Fluor 488, and anti-CD62-phycoerythrin confirmed these data (Figure 1C). A cell-by-cell comparison of these 3 images indicated that although not all activated platelets bound FVa and FXa, significant levels of FVa and FXa binding colocalized to the same subset of activated platelets, in support of the concept that, with few exceptions (Figure 1C, arrow), the majority of platelets expressing high levels of FVa are those that bind FXa. These combined data indicate that the assembly of a functional prothrombinase is primarily defined by a discrete subpopulation of activated platelets and, equally important, that all platelet-bound FVa molecules are not equal in their ability to support FXa binding.

Additional experiments were done to determine whether this subset of procoagulant platelets mimicked that previously reported by Alberio et al. Indeed, a similar subset could be generated with the simultaneous addition of low-dose thrombin (5 nmol/L) and the GPVI agonist convulxin (data not shown); however, the addition of the transglutaminase inhibitor dapsylcaderine (≥200 μmol/L) was without effect on FVa binding to the activated platelet (Supplemental Figure III), suggesting a different mechanism for procoagulant subpopulation formation than that proposed by Dale et al.

Characterization of the Adhesive Properties of the Procoagulant Platelet Subpopulation

Experiments were done to characterize the adhesive properties of the procoagulant platelet subpopulation capable of binding a functional prothrombinase by quantifying the membrane densities of P-selectin (CD62P) and GPⅠbα (CD42b), as well as integrins αⅠb (CD41) and β3 (CD61), using specific fluorophore-conjugated monoclonal antibodies (Supplemental Figure IV). These experiments were performed in the presence of added plasma-derived FVa to define the entire population of platelets capable of binding FXa. In a representative experiment (Figure 2), procoagulant platelets demonstrated a 5.8 ± 1.6-fold (n = 93) higher level of integrin β3 expression compared with those platelets unable to bind FXa. Likewise, higher levels of expression were observed for P-selectin (4.5 ± 0.8-fold, n = 109), integrin αⅠb (4.2 ± 0.3-fold, n = 2), and GPⅠbα (6.1 ± 2.4-fold, n = 18) (Table 1). In additional flow cytometric analyses, which simultaneously compared both FVa and FXa binding characteristics, the increased expression of P-selectin previously seen in the FXa-positive platelets was also seen in those platelets capable of binding high levels of FVa (Supplemental Figure V), consistent with the existence of a single procoagulant platelet subpopulation expressing increased adhesive receptor density.

TO, a dye frequently used to stain nucleic acid and previously shown to identify the youngest platelets in circulation, was used to evaluate the effect of platelet age on FVa and FXa binding to activated platelets, as well as adhesive receptor density expressed by activated platelets. Dual-labeling studies demonstrated that 56.3 ± 5.4% (n = 16) of TO-positive platelets were able to bind high levels of FVa, whereas 63.2 ± 2.8% (n = 20) were able to bind FXa. Conversely, 75.1 ± 5.4% (n = 16) of platelets able to bind high levels of FVa and 68.7 ± 3.8% (n = 20) of platelets able to bind FXa were TO-positive. Evaluation of adhesive receptor expression as a function of platelet age demonstrated a 5.9 ± 0.7-fold (n = 52) higher level of P-selectin expression in the youngest (TO-positive) platelets compared with their more mature (TO-negative) counterparts (Table 2). In addition, expression of integrin β3 and GPⅠbα were increased 6.0 ± 1.0-fold (n = 26) and 6.8 ± 0.9-fold (n = 26) in TO-positive platelets, respectively (Table 2).

The Procoagulant Platelet Subpopulation Is Characterized by a Nondissociable Pool of Platelet-Derived FVa

During the course of these studies, observations were made indicating that a significant fraction of the membrane-bound,
platelet-derived FVa pool expressed subsequent to platelet activation could not be removed from the platelet membrane despite repeated washing. Thrombin-catalyzed activation of washed human platelets in the presence of RGDS and Gly-Pro-Arg-Pro, to prevent platelet aggregation and polymerization of platelet-derived fibrin, allowed repeated washing of the activated platelets, providing that RGDS was included in the wash buffers. When washing was done in the presence of EDTA to disrupt the Ca\(^{2+}\)-dependent interaction between the FVa heavy and light chains, flow cytometric analyses using a monoclonal antibody specific for the FVa heavy chain indicated that approximately 35% of the heavy chain remained bound to the activated platelet membrane even though its interaction with the light chain had been abolished (Supplemental Figure VI). The amount of bound light chain remained unchanged, consistent with its Ca\(^{2+}\)-independent membrane binding characteristics (data not shown). Near-identical results were obtained when retention of the platelet-derived FVa heavy and light chains was assessed by Western blotting (Figure 3A and 3B). Densitometric analyses indicated that the majority of the light chain remained associated with the platelet surface, as did \(~30\%\) of the heavy chain. Thus, platelet activation resulted in the formation of a substantial, nondissociably bound pool of FVa. This nondissociably bound cofactor pool was capable of binding FXa because clotting assays indicated that \(37.6\pm11.2\%\) (range \(14.6\) to \(62.0\%), \(n=7\)) of the FVa cofactor activity remained associated with the activated platelet membrane following 5 washes (Figure 3C) in either the presence or the absence of Ca\(^{2+}\) (Figure 3C, inset).
Consequently, experiments were done to determine whether and how this nondissociable pool of platelet-derived FVa contributed to the formation of the procoagulant platelet subpopulation. Flow cytometric analyses were used to quantify FVa and FXa binding to thrombin-activated platelets in the presence and absence of plasma-derived FVa. In the absence of added FVa, only the platelet-derived cofactor is expressed/bound to the activated platelet membrane, and it is defined by both nondissociable and dissociable cofactor pools, each of which is capable of binding FXa as determined by functional assays. A representative experiment (n = 6) is shown in Figure 4, where FVa binding is depicted in the upper histograms and FXa binding in the lower histograms. In the absence of added FVa, the percentage of platelets intensively positive for FVa represented approximately 16% of the entire activated platelet population. FXa binding under identical conditions likewise represented 16% of the activated platelet population. When FVa was added to ensure saturation of all available binding sites, the percentage of platelets intensively positive for both FVa and FXa remained virtually the same (18% versus 17%), whereas the amount of bound proteins increased as indicated by the increased fluorescence associated with this population of activated platelets. These data are consistent with the formation of a single platelet subpopulation responsible for regulating functional prothrombinase assembly.

In all 6 experiments, the percentage of activated platelets binding both FVa and FXa was not significantly affected by the presence or absence of additional plasma-derived FVa, though the amount of FXa bound increased by 15% to 59% in the presence of saturating plasma-derived FVa. Differences were also observed between experiments, as the percentage of platelets representing the procoagulant subpopulation varied from as little as 8% to as much as 54% depending on the platelet donor.

Discussion

In this study, the subpopulation of activated platelets capable of regulating the functional interactions of FVa and FXa on their membrane surface was defined and characterized, under conditions ensuring maximal prothrombinase binding. Thus, the binding characteristics of FVa and FXa to thrombin-activated platelets could be unequivocally characterized, thereby extending previous studies in which it was assumed that activated platelets are homogeneous regarding their procoagulant potential. The ability of thrombin-activated platelets to subsequently generate additional thrombin via a functional prothrombinase was defined by a subpopulation of predominantly “young” platelets expressing significantly increased adhesive receptor density, as well as a nondissociable pool of platelet-derived FVa in which the heavy chain of the functional cofactor is “tethered” to the platelet membrane, most likely through a GPI anchor. This procoagulant platelet subpopulation was also capable of binding additional platelet- and plasma-derived FVa in a freely dissociable manner to facilitate the saturation of all available prothrombinase binding sites on the activated platelet membrane. This observation is consistent with earlier studies defining prothrombinase assembly and function in the presence and absence of added plasma-derived FVa, which indicated that the amount of platelet-derived cofactor expressed or released...
by the activated platelet membrane is not sufficient to saturate all available prothrombinase sites. In those studies, addition of plasma-derived FVa consistently increased rates of thrombin generation by 1.2- to 3-fold, consistent with an increase in FXa binding.24

In the same studies, saturation of the activated platelet membrane with FVa and FXa resulted in the binding of approximately 6000 molecules of FVa/platelet, whereas only 3000 molecules of bound FXa were observed,24 suggesting that only approximately half of the platelet-bound FXa would support a FXa binding interaction. Those data are now confirmed by the identification of a subpopulation of platelets that binds FVa but does not bind FXa, and they allow us to conclude that FVa and FXa form a 1:1 complex on the procoagulant platelet membrane capable of assembling prothrombinase. Taken together, these data also confirm that the binding of FVa and FXa to the activated platelet surface is independently regulated and add to a growing body of literature that argues for the existence of activation-dependent “receptors” for FVa or FXa at the human platelet surface.10,25

This procoagulant platelet subpopulation demonstrated a 4- to 6-fold increase in P-selectin, integrin β3, integrin αIIb, and GPⅠbα expression, thereby placing these platelets in a unique position to contribute to both hemostatic and thrombotic events, with increased adhesive receptor density guaranteeing the adhesion of these procoagulant platelets to subendothelial von Willebrand factor or von Willebrand factor released by activated endothelial cells in a number of pathological processes. Following shear-induced platelet activation, platelet-derived FVa is released and expressed on the activated membrane surface. FXa, formed via the tissue factor/FVIIa complex, assembles with platelet-bound FVa to form prothrombinase and generate small amounts of thrombin, which subsequently activate more platelets. Recruitment and incorporation of procoagulant platelet subpopulations into the growing thrombus via their increased expression of αⅡbβ3 will follow. These activated platelets also express a non-disociable, functional pool of platelet-derived FVa comprising approximately 35% to 50% of the total membrane-bound cofactor contributed by the activated platelet. Thus, this series of events will be as likely to promote thrombosis as it is to prevent hemorrhage. Likewise, the role of these platelets in the processes of wound healing, atherosclerosis, and inflammation will be favored by their increased expression of P-selectin, which will facilitate not only platelet binding to the activated endothelium but also the recruitment of monocytes/macrophages and neutrophils via their expression of P-selectin glycoprotein ligand-1.26

Our data support the concept that not all platelets express the same procoagulant potential, a notion that is supported by several studies.13-16 Yet studies regarding prothrombinase are limited to 2 laboratories.13,16 Among these, the work of Kempton et al16 indicates that platelet activation with low concentrations of thrombin and convulxin/collagen, termed COAT platelets, express high levels of platelet-derived FV/FVa at their membrane surface.13 The formation of these COAT platelets is hypothesized to occur when serotonin, released by dense granules, cross-links platelet-derived coagulation proteins, such as FV, to fibrinogen or thrombospondin bound to their receptors (αⅡbβ3 or CD36, respectively). Based on the ability of dansylcadaverine to inhibit COAT platelet formation, this cross-linking appeared to be mediated by platelet-derived FXIIIa.15,27 In contrast to COAT platelets, the presence of dansylcadaverine had no effect on the formation of the procoagulant platelet subpopulation described here. This is not surprising for the following reason: even though FV is a substrate for FXIIIa,28 the glutamine residues required for the transamidation reaction are all located within the activation peptide that is released when FV is activated to FVa.

The idea that platelets are not all created equal is not restricted to the human system.22,29,30 For example, Thattaliyath et al identified 2 populations of zebrafish thrombocytes distinguishable by age and lipophilicity.25 Consistent with our data, the younger, more lipophilic thrombocytes, which appear to be responsible for the initiation of arterial thrombus formation, constitutively express a higher density of adhesive receptors and, following activation, express more P-selectin and anionic phospholipid than their more mature counterparts.22

Considering their complexity, perhaps the heterogeneous nature of activated platelets should be expected. Platelets participate in a wide variety of physiological and pathophysiological processes. In some, thrombin generation may not be as important as the release of granular contents, which appear to be heterogeneous as well.31,32 Differential packaging of the α-granule proteins von Willebrand factor and fibrinogen allows their differential release.32 In addition, pro- and antiaangiogenic proteins appear to be organized into separate platelet granules, which are also differentially released.31 Thus, platelets appear to express very different phenotypes and therefore are most likely under distinct hematopoietic regulation via megakaryocyte differentiation.

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The authors acknowledge Nicole Maille and John Malcolm for technical assistance and Jay R. Silveira, PhD, for critical reading of the manuscript.

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Disclosures
None.
References


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SUPPLEMENTAL MATERIAL:

METHODS

Materials. Recrystallized bovine serum albumin (BSA) was purchased from MP Biomedical (Solon, OH). Hirudin (recombinant) was obtained from Calbiochem (La Jolla, CA). The fluorescent thrombin inhibitor dansylarginine N-(3-ethyl-1,5-pentanediyl)amide (DAPA)\(^1\) was obtained from Haematologic Technologies (Essex Junction, VT). AlexaFluor488 and AlexaFluor647 Protein Labeling Kits were purchased from Invitrogen (Carlsbad, CA). Cy5 Protein Labeling Kit was purchased from Amersham Biosciences (Piscataway, New Jersey). Dansylcadaverine and Triton X-100 were obtained from Sigma (St. Louis, MO). Convulxin was purchased from Centerchem, Inc. (Norwalk, CT). Thiazole orange was purchased as Retic-COUNT\(^\text{TM}\) Reagent from Becton Dickinson Biosciences (San Jose, CA). Aqua Poly/Mount anti-fade reagent was purchased from Polysciences, Inc. (Warrington PA). Spectrozyme TH was obtained from American Diagnostica (Stamford, CT). Western Lightning chemiluminescence reagent was obtained from PerkinElmer (Boston, MA). Arg-Gly-Asp-Ser (RGDS), Gly-Pro-Arg-Pro (GPRP), the PAR-1 agonist peptide (SFLLRN-NH\(_2\)), and the PAR-4 agonist peptide (AYPGKF-NH\(_2\)) were provided by the Protein Core Facility, Department of Biochemistry, University of Vermont (Burlington, VT).

Preparation of coagulation proteins. Human factor Xa, prothrombin, and thrombin were purchased from Haematologic Technologies (Essex Junction, VT). Human, plasma-derived factor V was purified by affinity chromatography from fresh frozen plasma as described,\(^2\) and judged to be >95% pure by SDS-PAGE before and after disulfide bond reduction, according to the method of Laemmli.\(^3\) FVa was prepared by activation of the plasma-derived FV (1µM), in 20mM HEPES, 150mM NaCl, 5mM CaCl\(_2\), pH 7.4 (HBS/Ca\(^{2+}\)) containing 0.1% PEG-8000, with 20mM thrombin at 37°C for 10 min to ensure complete activation, then quenched with hirudin (30nM). Protein concentrations were determined by absorbance at 280nm using the following molecular weights and extinction coefficients (E\(^1\%\)\( \text{cm}^{-1}\)): prothrombin, 72,000, 13.8;\(^4\) thrombin, 38,500, 18.3;\(^5\) FVa, 330,000, 9.6;\(^6\) and FXa, 46,000, 11.6.\(^7\)

Antibodies. Anti-CD61 (integrin β3) conjugated to peridinin chlorophyll protein (PerCP), anti-CD42b (glycoprotein Ibα) conjugated to allophycocyanin (APC), and anti-CD62P (P-selectin) conjugated to phycoerythrin (PE) were obtained from Becton Dickinson Biosciences (San Jose, CA). Anti-CD41 (integrin αIIb) conjugated to PE was purchased from Dako (Carpinteria, CA). Anti-human factor V\#2 (anti-FV\#2) against the FVa light chain, anti-human factor V\#17 (anti-FV\#17) against the FVa heavy chain, anti-human factor V\#9 (anti-FV\#9) against the light chain, and anti-human factor X\#27-5 (anti-FXa) monoclonal antibodies, which do not interfere with platelet prothrombinase complex assembly and function, were obtained from the Antibody Core Facility, Department of Biochemistry, University of Vermont (Burlington, VT). Anti-FV\#2, and anti-FXa were labeled with AlexaFluor488 or Cy5 per the manufacturer’s directions. Horse anti-mouse IgG coupled to horseradish peroxidase (HRP) was purchased from Vector Laboratories ( Burlingame, CA). Goat anti-mouse IgG coupled to AlexaFluor488 was obtained from Invitrogen (Carlsbad, CA).

Preparation of washed platelets. Platelets were isolated from human venous blood collected from healthy non-medicated individuals using a modification of the method of Mustard et al.\(^8\) All subjects were
recruited through open advertisement and provided informed written consent prior to participation in this study, which was approved by the University of Vermont/Fletcher Allen Health Care Committee on Human Research. Apyrase was omitted from all washing steps and 5mM HEPES-buffered Tyrode’s albumin, pH 7.4 (0.14M NaCl, 2.7mM KCl, 12mM NaHCO3, 0.42mM NaH2PO4-H2O, 1mM MgCl2, 2mM CaCl2, 5mM dextrose, 0.35% BSA, 5mM Hepes) (HTA) was used as the final platelet suspension buffer. Platelet concentrations were determined using a Coulter Z1 particle counter (Beckman Coulter, Fullerton, CA).

**Generation and analyses of procoagulant platelet subpopulations.** Washed platelets (1x10⁹/mL) were activated with thrombin (50nM) (5 min, ambient temperature) in the presence of RGDS (1mM) to prevent platelet aggregation. In some experiments, platelets were activated in the presence of dansylcadaverine (0 – 200µM), or in the absence of RGDS. In other experiments, platelets were activated by a combination of low dose thrombin (5nM) and convulxin (0.5 µg/mL). Following the addition of hirudin (75nM), activated platelets were incubated with plasma-derived FVa and/or FXa (5nM each, 10 min, ambient temperature). Aliquots of the reaction mixtures (2.5x10⁷ platelets) were diluted 1:1 with 4% paraformaldehyde and incubated (30 min, ambient temperature) to cross-link the platelet-bound factors Va and Xa. Following centrifugation (660xg, 3 min) to sediment the platelets, the platelets were washed two times by resuspension with 1 mL HTA followed by centrifugation. Non-specific binding sites were blocked with 8% bovine serum albumin (30 min, ambient temperature). Following centrifugation, platelets were incubated with a variety of fluorophore conjugated monoclonal antibodies including: anti-CD61-PerCP (5nM), anti-CD62-PE (0.4nM), anti-CD42b-APC (15 µL), anti-CD41-PE (100nM), anti-FV#2 conjugated to AlexaFluor488, Cy5, or AlexaFluor647, and/or anti-FXa conjugated to AlexaFluor488 (0.1µM each, 45 min, ambient temperature). In some experiments, platelets were incubated with anti-FV#17, detected with goat anti-mouse IgG conjugated to AlexaFluor488. Non-specific antibody interactions were assessed by incubation with similarly labeled equimolar concentrations of isotype-matched, non-immune antibodies, or with anti-FXa-AlexaFluor488 in the absence of added FXa. Excess antibody was removed by the addition of 0.5 mL of HTA followed by centrifugation. The platelets were resuspended with 2% paraformaldehyde (0.5 mL), and stored at 4°C until flow cytometric analyses. Platelet-bound proteins were also visualized by fluorescence microscopy. In some experiments, the platelets were incubated with 1 mL Retic-COUNT™ thiazole orange (TO) reagent prior to flow cytometric analyses (60 min, ambient temperature).

**Flow Cytometry.** For flow cytometric analyses of platelet-bound proteins, the fluorescence from 10,000 platelets was analyzed on a Coulter EPICS Elite Flow Cytometer (Beckman Coulter Inc., Fullerton, CA) or a BD LSRII flow cytometer (Becton Dickinson, San Jose CA). Two-color and three-color analyses were accompanied by single-antibody staining to allow adequate compensation of each fluorescence detector. The positive gate was set such that ≥98% of the cells stained with appropriate control antibodies were negative. For flow cytometric analyses of thiazole orange staining, positive gates were set such that ≥98% of unstained platelets from the same donor were negative.

**Fluorescence Microscopy.** For fluorescence microscopy analyses, 10,000 platelets were cytocentrifuged onto poly-L-lysine (2
mg/mL) coated slides, mounted using Aqua Poly/Mount anti-fade reagent, and visualized using a BioRad MRC 1024ES Confocal Microscope. The fluorophores were excited at 488, 568, or 647nM with a krypton/argon laser.

**Kinetic analyses of platelet prothrombinase complex assembly and function.** Subsequent to platelet activation, FVa and FXa (5nM 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/DAPA mixture (1.4µM and 3µM final concentrations, respectively). Fifty µL aliquots of the reaction mixtures were removed at 10, 20, 30, 40, 50, and 60 sec and added to 100µL of 20mM Heps, 0.15M NaCl, pH 7.4, containing 50mM EDTA and 0.1% polyethylene glycol. The samples were stored at −20°C until thrombin assays were performed.

**Thrombin assays.** Thrombin assays were performed in duplicate to determine the amount of thrombin generated over the time course of the reaction. The thrombin concentrations in the unknown samples were compared to a standard curve prepared daily using known amounts of thrombin (200 to 0.78nM). Eighty µL of Spectrozyme TH (0.4mM) was added to 20 µL of standard or unknown and the optical density at 405nM was monitored over 10 min using a ThermoMax kinetic plate reader (Molecular Devices Corporation, Sunnyvale CA). Under these conditions, the initial rate of thrombin generation is linear and no more than 10% of the substrate prothrombin was consumed during the course of the assay.

**Determination of a functional, non-dissociable, platelet-derived factor Va pool.** Platelets (1x10⁹/mL) resuspended in HTA containing 5mM RGDS and 2mM GPRP were activated for 2 min with 50nM thrombin. CaCl₂ was omitted from the HTA in order to disrupt Ca²⁺-dependent interactions between the FVa heavy and light chains. Following the addition of hirudin (75nM), the platelets were washed by centrifugation (660xg, 3min) followed by gentle resuspension in 1/3 of the starting volume of HTA minus CaCl₂/5mM RGDS (Wash “0”). The platelet concentration was adjusted to 2x10⁹ platelets/mL and platelet-bound FVa activity determined by a one-stage factor V clotting assay. The remaining platelets were diluted to 1x10⁹/mL with HTA minus CaCl₂/5mM RGDS, and washing was repeated 1-5 times. All clotting times were compared to a standard curve generated using a normal plasma pool as the factor V source. Retention of FVa light chain and heavy chain was assessed by western blotting analyses. At each wash, platelets were diluted to 1x10⁹/mL and lysed by addition of 1% Triton X-100. Protein samples were diluted with 5X SDS-sample preparation buffer (SPB) (312.5mM Tris-HCl, 10% SDS, 50% glycerol, 0.005% bromphenol blue, pH 6.8), in the presence or absence of 2% (v/v) BME, to a final concentration of 1X SPB. The samples were then heated at 95°C for 2 min and subjected to SDS-PAGE as described by Laemmli.³ Protein was transferred to nitrocellulose as described by Towbin et al.⁹ FVa light chain and heavy chain were probed using a mixture of anti-FV#9 and anti-FV#17. Anti-factor V antibody reactivity was detected using an HRP-conjugated horse anti-mouse IgG followed by chemiluminescence using Western Lightning chemiluminescence reagent. Densitometric analysis was performed using ImageJ software (NIH, Bethesda, MD). In other experiments, retention of the FVa heavy chain on the platelet membrane was assessed by flow cytometry using anti-FV#17 and goat anti-mouse IgG conjugated to AlexaFluor488 as described above.
Supplemental Figure I. Distribution of the procoagulant platelet subpopulation among normal controls. The percent of platelets able to bind “high” levels of factor Xa, determined as described in Figure 1, is shown for a group of normal control individuals (n=28). The horizontal line represents the mean (31.1% FXa-positive platelets), with the median=29.0%.
Supplemental Figure II. Simultaneous evaluation of FVa and FXa binding to activated platelets. Washed platelets were activated with thrombin (50 nM) and subsequently incubated with saturating concentrations of plasma-derived FVa and FXa (5 nM) as detailed in Figure 1. Following fixation, platelets were simultaneously stained with PE-conjugated anti-CD62 to identify activated platelets (y-axes), AlexaFluor647-conjugated antibodies to detect FVa (A) and AlexaFluor488-conjugated antibodies to detect FXa (B) binding (x-axes). The subpopulation of activated platelets that I) do not bind FVa or FXa, II) bind intermediate levels of FVa, or III) bind substantial amounts of FVa and FXa are indicated. Subsequent assessments of FVa binding in platelets positive for FXa (Figure 1B) as well as FXa binding (Figure 1C) in platelets capable of binding substantial amounts of FVa were also performed as reported in the text.
Supplemental Figure III. **FVa expression on the activated platelet surface is not dependent on transglutaminase activity.** Flow cytometric analyses of FVa binding to thrombin-activated (50nM) human platelets in the presence of increasing amounts (≤ 200µM) of the transglutaminase inhibitor dansylcadaverine (Dc) were performed as outlined in Figure 1a and Methods. Following fixation and blocking of non-specific binding sites, platelets were stained with anti-FVα-AlexaFluor488 to detect FVa binding. The data are expressed as the percentage of platelets capable of binding FVa (FVa+) in the presence of the indicated concentration of dansylcadaverine. Experiments were performed in duplicate, and the error bars represent the data range.
Supplemental Figure IV. Adhesive receptor expression is increased on the surface of procoagulant platelets. Procoagulant platelets were initially identified by size and ability to bind FXa as described in Figure 1. The density of adhesive receptors in the total platelet population was subsequently compared to that seen in both FXa positive and FXa negative platelets as described in Methods. (A) Total expression (red histograms) of the indicated adhesive receptor in the entire platelet population. (B) Receptor expression in those platelets that are unable to bind FXa (blue histograms). (C) The portion of total receptor expression seen in the subpopulation of platelets capable of binding FXa (pink or green histograms). (D) The bottom panel is a merged image of A and C with the dashed line indicating the level of receptor expression contributed by the procoagulant subpopulation. These results are summarized in Table 1.
Supplemental Figure V. P-selectin expression is similarly increased on the surface of platelets binding FXa and on those binding “high” levels of FVa. P-selectin expression levels, as well as FVa and FXa binding, were defined as described as described in Supplemental Figure II. The density of CD62P (P-selectin) expressed on the surface of those platelets capable of binding “high” levels of FVa was subsequently compared to that seen in platelets capable of binding FXa. (A) Total expression (blue histogram) of CD62P in platelets capable of binding “high” levels of FVa. (B) Total P-selectin expression in platelets which are able to bind FXa (green histogram).
Supplemental Figure VI. Identification of a non-dissociable, platelet-derived FVa pool tethered to the platelet membrane via the heavy chain (HC). Washed platelets were resuspended in HTA minus CaCl\(_2\) containing 5mM RGDS and 2mM GPRP, activated with 50nM thrombin, and washed repeatedly as in Figure 3 and in Methods. CaCl\(_2\) was excluded from the buffer to disrupt the calcium-dependent interaction between the FVa heavy and light chains. The remaining amounts of FVa HC before (A), and after (B) repeated washing, were determined using flow cytometric analyses as in Figure 1a and Methods. Following fixation and blocking of non-specific binding sites, platelets were stained with the anti-FV\#17 monoclonal antibody, followed by detection with goat anti-mouse IgG-AlexaFluor488 to detect FVa HC. Panel (C) depicts a merged image of A & B with the transparent histogram indicating the amount of FVa HC bound to the platelet prior to repeated washing in the absence of CaCl\(_2\).

SUPPLEMENTAL REFERENCES