The Role of Rac1 in Platelet Signal Transduction and Function

BY

MICHAEL KEEGAN DELANEY
B.S. in Biology, DePaul University, 2006
B.S. in Environmental Science, DePaul University, 2006

THESIS

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Defense Committee:

Xiaoping Du, Chair and Advisor
Randal Skidgel
Jaehyung Cho
Dolly Mehta
Steven Olson, Periodontics
This thesis is dedicated to my wife, Valerie Rose Delaney, who provided an overwhelming amount of love, support, and encouragement to finish my graduate studies. I would also like to dedicate this thesis to my Father, Michael Edward Delaney, who provided me with the very unique and privileged opportunity to pursue any path I desired.
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19. Rac1–/– mouse platelets have attenuated platelet aggregation and secretion

20. Rac1 regulates shear-induced PS exposure and microvesiculation independent of its role in stimulating platelet secretion and aggregation

21. Rac1 is important for the exposure of PS during thrombus formation in vitro under shear

22. Platelet Rac1 is important in promoting fibrin generation in vitro and in vivo

23. The role of Rac1 in platelet signal transduction and function
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<td>$\Delta \psi_m$</td>
<td>loss in mitochondrial membrane potential</td>
</tr>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>Abi</td>
<td>Abl-interactor</td>
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<td>ABP</td>
<td>Actin-binding protein</td>
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<tr>
<td>AC</td>
<td>adenylyl cyclase</td>
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<tr>
<td>ACD</td>
<td>acid citrate dextrose</td>
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<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
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<td>Arp2/3</td>
<td>actin-related protein 2/3</td>
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<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>ATPase</td>
<td>adenosine triphosphatase</td>
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<tr>
<td>Btk</td>
<td>bruton tyrosine kinase</td>
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<tr>
<td>$Ca^{2+}$</td>
<td>calcium ion</td>
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<tr>
<td>$[Ca^{2+}]_i$</td>
<td>concentration of intracellular ionic calcium</td>
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<td>CalDAG-GEFI</td>
<td>calcium and diacylglycerol-regulated guanine nucleotide exchange factor I</td>
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<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CHO</td>
<td>chinese hamster ovary</td>
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<td>COX</td>
<td>cyclooxygenase</td>
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<td>C5b-9</td>
<td>compliment protein 5b and 9 complex</td>
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<td>DAG</td>
<td>diacylglycerol</td>
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<td>DAP</td>
<td>discrete adhesion points</td>
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<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>DTS</td>
<td>dense tubular system</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<td>ERK</td>
<td>Extracellular-signal-regulated kinase</td>
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<td>FV</td>
<td>factor V</td>
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<td>FVa</td>
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<td>FcR$\gamma$</td>
<td>Fc receptor gamma</td>
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<td>Fc gamma receptor IIA</td>
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<td>G-protein</td>
<td>Guanosine nucleotide-binding protein</td>
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<td>GAP</td>
<td>GTPase activating protein</td>
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<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GDI</td>
<td>Guanosine nucleotide dissociation inhibitor</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
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<td>GFOGER</td>
<td>glycine-phenylalanine-hydroxyproline-glycine-glutamic acid-arginine</td>
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<td>immunoglobulin</td>
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<td>IP₃</td>
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<td>ITAM</td>
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<td>knockout</td>
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<td>LAT</td>
<td>linker for activated T cells</td>
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<td>LIM kinase</td>
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<td>Leucine rich repeat</td>
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<td>polyacrylamide gel electrophoresis</td>
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<td>phosphate buffered saline</td>
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<td>phospholipid</td>
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<td>PPP</td>
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<td>phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger-1</td>
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<td>platelet rich plasma</td>
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<td>Rho-dependent kinase</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>sodium dodecyl sulfate</td>
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<td>sarco/endoplasmic reticulum calcium-ATPase</td>
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<td>STIM1</td>
<td>stromal interaction molecule 1</td>
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LIST OF ABBREVIATIONS (continued)

S6K1  70 kDa ribosome S6 protein kinase
Thr   threonine
Tiam1 T-cell lymphoma invasion and metastasis-inducing protein 1
TF    tissue factor
TMEM16F transmembrane protein with unknown function 16F
TP    thromboxane prostanoid
TXA2  thromboxane A2
TXB2  thromboxane B2
Tyr   tyrosine
VWF   von willebrand factor
WASP  Wiskott-Aldrich syndrome protein
WAVE  Wiskott-Aldrich syndrome protein-family verprolin homology protein
WT    wild-type
SUMMARY

Platelets are the key players in the hemostatic response to vascular injury because they rapidly adhere to the injured vessel wall, aggregate, and secrete granular molecules that recruit and activate additional platelets to form primary thrombi that prevent blood loss and maintain vascular integrity. Under the high shear-rate flow conditions present in arteries and capillaries, initial platelet adhesion is mediated by the interaction between subendothelial matrix-bound von Willebrand factor (VWF) and its platelet receptor, the glycoprotein Ib-IX (GPIb-IX) complex. The interaction between VWF and GPIb-IX not only mediates transient platelet adhesion but also initiates a signal transduction cascade culminating in platelet activation. GPIb-IX-mediated platelet activation signaling is known to involve several intracellular signaling molecules and pathways, including the Src family kinase (SFK) Lyn, the phosphoinositide 3-kinase (PI3K)/Akt pathway, and the cGMP and mitogen-activated protein kinase (MAPK) pathways. Thus far, the identified most proximal step to GPIb-IX that propagates platelet activation signals is the activation of Lyn and Lyn-dependent activation of the PI3K/Akt pathway. Interestingly, although the PI3K/Akt pathway is activated downstream of Lyn and is critical for promoting GPIb-IX-mediated platelet activation, the exact molecular mechanisms governing this process remains unclear.

We tested the hypothesis that Rac1, a member of the Rho family of small GTPases, is involved in the signaling mechanism mediating GPIb-IX-induced platelet activation. We discovered that Rac1 is required for GPIb-IX-induced platelet activation, as platelet deficiency in Rac1 abolished GPIb-IX-mediated activation of integrin αIIbβ3, stable platelet adhesion to VWF
under shear stress, TXA$_2$ production and platelet aggregation. Ligation of GPIb-IX with VWF stimulated the activation of Rac1, which required Lyn but was independent of PI3K. We also identified Vav, a RhoGEF, in mediating GPIb-IX-induced activation of Rac1, as VWF stimulated the activation of Vav independent of both Rac1 and PI3K whereas Lyn was required. Finally, Rac1 was also found to be required for GPIb-IX-induced activation of Akt and p38 MAPK. Thus, our study reveals an important new link in the GPIb-IX signaling pathway by identifying a novel mechanism of Rac1-dependent PI3K/Akt activation. We show that a Lyn-Vav-Rac1-PI3K-Akt pathway mediates VWF-induced activation of integrin αIIbβ3 to promote GPIb-IX-dependent platelet activation.

Platelets not only participate in the generation of a primary thrombus, but they also contribute to blood coagulation by facilitating thrombin generation. The ability of platelets to promote thrombin generation is referred to as platelet procoagulant activity (PPA), which is mediated by phosphatidylserine (PS) exposure and the release of procoagulant microvesicles (MVs). These two events are assumed to be Ca$^{2+}$-dependent because both are elicited by agents that increase intracellular Ca$^{2+}$ concentrations. However, physiological platelet agonists alone, such as thrombin or collagen, weakly induce PPA compared to Ca$^{2+}$-mobilizing agents, which begs the question: why are physiological agonists alone unable to induce significant PPA? To date, in the absence of Ca$^{2+}$-mobilizing agents, PS exposure and microvesiculation are only observed using high concentrations of thrombin in conjunction with snake venom or synthetic agonists to stimulate GPVI, or platelets must be adherent on immobilized adhesive proteins.
Summary (continued)

The conclusion from years of research was that multiple receptors must be simultaneously activated to induce PPA, which is termed as Collagen and Thrombin-stimulated (COAT) platelets. However, platelets circulate in the vasculature under shear. To this extent, most previous work has focused on studying agonist-induced PPA under static conditions. We tested the hypothesis that shear is required for significant PPA to be observed in platelets stimulated with physiological agonists that are independent of VWF/GPIb-IX.

We show that thrombin- or collagen-stimulated platelets only display significant PS exposure and microvesiculation when exposed to shear. As shear enhanced PS exposure and microvesiculation in response to a variety of agonists, such as thrombin, PAR4AP, collagen and ionophore, it is likely a common mechanism required for platelet PS exposure and MV release. Furthermore, whereas the effects in enhancing agonist-induced PS exposure and microvesiculation increase correspondingly with increasing levels of shear, even physiological levels of shear (as low as 250 s\(^{-1}\)) is sufficient to significantly promote PS exposure and MV release. Interestingly, the PS exposed on the surface of agonist-stimulated platelets under shear was immediately released as PS-exposed MVs and was minimally retained on platelet surfaces. Importantly, we show that Rac1 serves as an important signaling mechanism mediating shear-induced PPA independent of its known function in stimulating platelet secretion and aggregation. Finally, we discovered that Rac1-mediated shear-dependent PPA plays a critical role in promoting coagulation \textit{in vitro} and \textit{in vivo}. 
1. LITERATURE REVIEW

1.1 Platelets

1.1.1 Platelet Morphology and Structure

Platelets are small anucleate cellular fragments derived from hematopoietic stem cells known as megakaryocytes. They are the smallest type of circulating blood cell and have a characteristic discoid shape. Their distinctive appearance was originally described as “small plates,” hence the name platelets (Osler 1886). Due to their small size and shape, they are pushed in close proximity to the vessel edge in blood flow, placing them in prime position to detect and respond to vessel injury. Although there is substantial variability in the size of platelets per individual (Holme et al., 1988), they have a mean diameter of 2 to 5 μm, mean thickness of 0.5 μm, a mean cell volume of 6 to 10 fL, and they circulate in the blood at a concentration ranging from 1.5 to 4 x 10⁹/mL blood for 7-10 days (White 2007).

1.1.1.1 Platelet Surface

The platelet plasma membrane is a lipid bilayer. It is a 20 nm thick trilaminar structure that incorporates a glycocalix around its outer surface (White and Clawson 1980a). This glycocalix is made of membrane glycoproteins, glycolipids, mucopolysaccharides and adsorbed plasma proteins, and is observed as a fuzzy coat via electron microscopy (White 1993). These
components are critical for the platelet’s ability to facilitate adhesion, trigger activation, promote aggregation, and accelerate clot retraction (Clemetson 1985; Kunicki 1988; Phillips 1985). Resting platelets maintain an asymmetric phospholipid distribution within their lipid bilayer where negatively charged phospholipids, such as phosphatidylserine (PS), are exclusively maintained and sequestered within the inner leaflet and neutrally charged phospholipid species are more evenly distributed between the inner and outer leaflets (Bevers et al., 1983). Surface externalized PS promotes blood coagulation because it provides the membrane surface required for assembly of the prothrombinase complex; therefore, it is important for platelets to sequester the procoagulant lipid until required to accelerate blood coagulation (Bevers et al., 1982b; Rosing et al., 1985; Schick 1978). Finally, sodium and calcium adenosine triphosphatase (ATPase) pumps are also expressed within the lipid bilayer and function to maintain ionic homeostasis (Simons ER 1987).

### 1.1.1.2 Membrane Systems

Platelets contain two distinctive membrane systems not found in other blood cells. The first is the surface-connected open canalicular system (OCS). The OCS derives from the platelet plasma membrane and also the demarcation membrane system of the megakaryocyte (White 2007). The OCS is a complex network of fenestrations that are connected to the surface plasma membrane and tunnel through the cytoplasm towards the center of the platelet (Behnke 1967; Stenberg et al., 1984; White and Clawson 1980b). This allows various chemicals, molecules, and constituents in the blood to reach the inner areas of the platelet (White 1972). Thus, the OCS
facilitates not only the uptake and transfer of such products but also the discharge of products stored in secretory organelles (Escolar and White 1991; White 1974; White and Escolar 1991). The OCS also functions as a reservoir of plasma membrane, where the membrane may be accessed to greatly expanding the total surface area of the platelet as needed. For example, following adhesion platelets undergo a transition from a discoid to spread shape, a process shown to involve the OCS by expanding the exposed surface up to 420% (Escolar et al., 1989).

The second distinctive membrane system is the dense tubular system (DTS). The DTS is made of the smooth endoplasmic reticulum from megakaryocytes (Behnke 1969; Daimon and Gotoh 1982). Although it is similar to the OCS in that it is a membrane system, it differs from the OCS because it is not open and exposed to the blood, but rather a closed-channel network (Behnke 1970). This channel system is important for initiating and modulating platelet activation, mainly because it is involved in calcium homeostasis and prostaglandin synthesis (Gerrard et al., 1978). The DTS, in a manner similar to the sarcoplasmic reticulum of muscle, sequesters and stores ionized calcium (Ca$^{2+}$) and releases it during platelet activation. This facilitates platelets shape change, and the centralization and concomitant secretion of granules (Robblee et al., 1973; Menashi et al., 1982). The DTS also functions to compartmentalize the synthesis of prostaglandins, which regulate platelet activation (Gerrard et al., 1976).
1.1.1.3 Cytoskeleton

The cytoskeleton functions as a cellular scaffolding system. It has proteins that act like struts and girders that not only define the shape of platelets but also maintain its integrity. The highly specialized platelet cytoskeleton has three major components: a membrane skeleton, a microtubule coil, and an actin cytoskeleton located in the cytoplasm. In response to vessel injury, the platelet cytoskeleton is capable of rapidly reorganizing and interacting with various signaling molecules, which is critical for the integration and spatial organization of signal transduction during platelet activation (Fox 2001).

Overall, two components of the platelet cytoskeleton are derived from actin filaments: the membrane skeleton and the cytoplasmic skeleton (Fox 1993, 2001). The membrane skeleton is a crucial component of the cytoskeleton because it coats the inner, cytoplasmic face of the plasma membrane to form a two-dimensional network that mediates the connection of the entire cytoskeleton to the lipid bilayer. It also recruits signaling molecules during platelet activation. The proteins that comprise the membrane skeleton include spectrin, talin, moesin, and filamin and they function to cross-link actin filaments, bind signaling molecules, and interact with membrane proteins (Fox 2001). Spectrin is an integral part of the membrane skeleton. It lines the intracellular face of the plasma membrane and interconnects the ends of actin filaments originating in the cytoplasm (Hartwig et al., 1999). The cytoplasmic cytoskeleton is an interconnected network of actin filaments that fills the cytoplasm and mediates contractile events (Fox 1993). In the resting platelet, nearly 40% of actin is in microfilaments (Nachmias 1980; Nachmias VT 1988). Intermediate filaments, which have large amounts of
vimentin, are also present and believed to be more stable and tolerate tension within the cytoplasm (Muszbek et al., 1987). During platelet activation, platelets become rounded via shape-change, extend filopodia, and the amount of F-actin increases dramatically, nearly 70% (Carlsson et al., 1979). Actin monomers quickly become incorporated into polymerizing filaments, specifically at the platelet periphery. These function to modulate changes in the morphology of the platelet membrane (Jennings et al., 1981; Hartwig 1992). Finally, phosphorylation of myosin light chain (MLC) leads to the interaction of myosin with actin, thereby producing the tension required for granule secretion and other retractile processes (Fox and Phillips 1982; Hartwig 2007).

The third component of the platelet cytoskeleton is the microtubule coil, which is where the characteristic discoid shape of the resting platelets derives from. This coil is transferred from the megakaryocyte to the platelet as it is formed. It is one long microtubule approximately 100 μm in length that is wound 8 to 12 times into a circular coil, where it rests below the plasma membrane (Hartwig 2007). Platelet microtubules are long, hollow cylinders made of 13 protofilaments of αβ tubulin dimers, which are always in a dynamic equilibrium with assembled microtubules, where reversible cycles of assembly and disassembly occur. Approximately 60% of platelet microtubules are present as polymers. This is because the critical concentration for tubulin polymerization is 5 μM and the platelet tubulin concentration is 70 μM (Hartwig 2007; Kenney and Linck 1985). There are several isoforms of β tubulin, of which platelets express β1, β2, β4 and β5. However, it is believed that β1 is the dominant isoform of tubulin that is specific to megakaryocytes and platelets (Schwer et al., 2001). This was exemplified in gene knockout experiments, wherein β1-deficient mice presented thrombocytopenia and abnormal platelet
and microtubule morphology (Schwer et al., 2001; Italiano et al., 2003). Finally, microtubules also disassemble and reassemble to facilitate shape-change during platelet activation (Hartwig 2007).

**1.1.1.4 Granules**

Secretion of granule contents from activated platelets at sites of vascular injury is critical for hemostasis and thrombosis (Reed 2007). Platelets contain three types of granules, which are defined by their unique molecular contents: α-granules, dense granules, and lysosomes. Dense granules and α-granules are unique to megakaryocytes and platelets whereas lysosomes may be found in a variety of cell types and are thus considered ubiquitous. Interestingly, most of the components of each granule originate from megakaryocytes during different stages of their development (Renda and Brohard-Bohn 2001). Platelet α-granules are the most abundant granule type, with approximately 50 to 80 per platelet that are between 200 and 500 nm in diameter (Sixma et al., 1989; Harrison et al., 1990). They contain adhesive molecules, coagulation factors, fibrinolytic regulators, growth factors, chemokines, and immunologic modulators (Reed 2007). Examples of α-granule contents include platelet-specific proteins such as platelet factor 4 (PF4) and β-thromboglobulin, adhesive glycoproteins such as fibrinogen (Fg) and von Willebrand factor (VWF), coagulation factors such as FV, FVIII and FXI, mitogenic factors such as platelet-derived growth factor (PDGF) and insulin-like growth factor I, and granule membrane-specific proteins such as P-selectin (Harrison and Cramer 1993). The release of α-granules from platelets results in the translocation and surface expression of P-selectin on
the platelet plasma membrane, for this reason P-selectin is routinely used to detect α-granule release during platelet activation. A second type of granule is the platelet dense granule, which are nearly 10-fold less abundant than α-granules as there are usually 3 to 8 dense granules per platelet (White 1969). Dense granules store small molecules such as the adenine nucleotides, ATP and ADP, calcium, magnesium, pyrophosphate, and other platelet agonists such as histamine, serotonin, and epinephrine (Reed 2007). Overall, the regulated release of contents from α- and dense granules is critical for the recruitment of circulating platelets to the site of vascular injury and functions as an important autocrine and paracrine amplification system to promote primary hemostasis (Li et al., 2010a). Platelets can also release cargo from small, acidified vesicles called lysosomes. Lysosomes contain acid hydrolases typical of these organelles and lysosome-associated membrane proteins. Interestingly, larger concentrations of agonists are required to induce the release of lysosomes than the release of other granules (Holmsen et al., 1979; Holmsen et al., 1982).

1.1.2 Platelets in thrombosis and hemostasis

Blood platelets play an essential role in the biological response to vascular injury by forming hemostatic thrombi to prevent blood loss and maintain vascular integrity (Li et al., 2010a). Upon injury to blood vessels, platelets rapidly adhere, aggregate, and secrete compounds that recruit and activate additional platelets culminating in the formation of primary thrombi. Platelets not only participate in primary thrombus formation but also
contribute to blood coagulation by facilitating thrombin generation. Therefore, platelets have a prothrombotic and procoagulant function in response to vascular injury.

Primary hemostasis is immediately initiated following vessel wall injury, where damage to the endothelium leads to the exposure of adhesive proteins within the extracellular matrix that are not usually present in the blood, such as collagen and collagen-bound VWF. Platelets subsequently detect and adhere to exposed extracellular matrix proteins via their respective receptors leading to the initiation stage of thrombosis. The ability of platelets to physically adhere to the injured vessel wall and become activated locally is critical for thrombosis. This is mediated via the interaction between integrin \( \alpha_2\beta_1 \) and GPVI with collagen and GPIb-IX-V with matrix-bound VWF. Once captured, platelets become activated and recruit additional circulating platelets through the process of thromboxane A2 (TXA\(_2\)) synthesis and the secretion of granule contents, which releases adhesive proteins and soluble platelet agonists. Vessel injury also leads to the activation of thrombin from prothrombin. Signal transduction cascades initiated through both adhesion receptors and receptors for soluble platelet agonists culminate in the activation of the ligand-binding function of the adhesion receptor integrin \( \alpha_{\text{IIb}}\beta_3 \). The inside-out activation of \( \alpha_{\text{IIb}}\beta_3 \) increases its affinity for ligands, such as fibrinogen and VWF, which functions to significantly accelerate integrin-dependent platelet aggregation, stable adhesion, and the overall growth of the primary thrombus (Li et al., 2010a).

Platelets not only participate in the formation of a primary thrombus at the site of vessel injury, but also contribute to blood coagulation locally by facilitating thrombin generation and the deposition of insoluble fibrin. Following platelet activation, a collapse of membrane asymmetry occurs (membrane scrambling) leading to the externalization of PS not
only on the surface of activated platelets but also submicron-sized microvesicles (MVs), shed from the activated platelet membrane (Morel et al., 2008). PS exposure and the release of PS-exposed MVs are the key processes that mediate the procoagulant function of platelets (Owens and Mackman 2011). This is because exposed PS provides the catalytic membrane surface required for the Ca\(^{2+}\)-dependent assembly of blood clotting factors on the membrane surface into key enzymatic complexes that propagate the coagulation cascade: the tenase and prothrombinase complex (Lentz 2003). Ultimately, this greatly facilitates the process of thrombin generation, which is a key protease that regulates blood coagulation. Thrombin subsequently converts plasma fibrinogen into fibrin, which functions to stabilize the platelet plug.

### 1.2 Platelet adhesion receptors and signaling

Platelets function as sentinels in the vascular compartment. The hemodynamics of blood flow pushes them in close proximity to the vessel wall, placing them in prime position to sense and respond to injury. Critical to the platelets’ ability to sense damage in the vessel wall is the expression of various adhesion receptors on their surface. Adhesion receptors are able to detect the presence of adhesive proteins within the subendothelial matrix of the damaged vessel or on the surface of activated endothelium. This facilitates the capture, tethering, and adhesion of platelets to sites of injury. Most importantly, ligation of these receptors induces platelet activation signals critical to thrombosis and hemostasis. The major platelet adhesion
receptors include integrin $\alpha_{\text{IIb}}\beta_3$, the GPIb-IX-V receptor complex, and GPVI. Their respective signal transduction pathways are summarized in Figure 1.

1.2.1 Integrin $\alpha_{\text{IIb}}\beta_3$

Integrins are a family of transmembrane adhesion receptors that function to attach a cell to its surroundings. Integrin function is not only limited to cell adhesion, however. Integrins also mediate bi-directional signaling to regulate important cellular processes such as spreading, retraction, migration, and proliferation. They are obligate heterodimers with two distinct subunits: a $\alpha$ and $\beta$ subunit. Platelets express integrin heterodimers $\alpha_{\text{IIb}}\beta_3$ (fibrinogen receptor), $\alpha_\nu\beta_3$ (vitronectin receptor), $\alpha_2\beta_1$ (collagen receptor), $\alpha_5\beta_1$ (fibronectin receptor), and $\alpha_6\beta_1$ (laminin receptor). $\alpha_{\text{IIb}}\beta_3$ is the most abundantly expressed of these heterodimers, with an average of 80,000 copies per platelet (Wagner et al., 1996). It is also the most highly expressed receptor on the platelet membrane, making it the major platelet adhesion receptor. In general, these integrins share similar signal transduction mechanisms in that they undergo “inside-out” and “outside-in” signaling. In the resting platelet, $\alpha_{\text{IIb}}\beta_3$ is maintained in a low-affinity state. However, intracellular signals induce a conformational change in $\alpha_{\text{IIb}}\beta_3$ causing the receptor to enter a high affinity state. This process is referred to as “inside-out” signaling. When ligands subsequently bind to activated integrin, the receptor transmits signals from outside to inside the cell. This process is referred to as “outside-in” signaling, and it mediated platelet adhesion, aggregation and thrombus formation. This bidirectional signaling mechanism of $\alpha_{\text{IIb}}\beta_3$ is tightly regulated and critical for thrombosis and hemostasis.
Figure 1. Platelet adhesion receptors and signal transduction pathways. A schematic of the major platelet adhesion receptors and the signaling mechanism they utilize to promote platelet activation. This figure has been modified from its original form published in Arteriosclerosis, Thrombosis and Vascular Biology (Li et al., 2010a).
1.2.1.1 Inside-out signaling

“Inside-out” signaling describes the intracellular signaling mechanism that induces the transformation of the extracellular ligand-binding domain of $\alpha_{IIb}\beta_3$ from a low- to high-affinity state (Coller and Shattil 2008; Shattil et al., 2010). This transition activates the ligand-binding function of integrin. The association of talin and kindlin, cytosolic integrin binding proteins, with the cytoplasmic domain of $\beta_3$ is believed to be a critical step for $\alpha_{IIb}\beta_3$ to enter the high-affinity state thereby activating its ligand-binding function (Tadokoro et al., 2003; Moser et al., 2008; Ma et al., 2008; Calderwood et al., 1999; Patil et al., 1999; Wegener et al., 2007; Malinin et al., 2009). Talin and kindlin bind to distinct sequences within the cytoplasmic domain of $\beta_3$ but are believed to play a coordinated role in “inside-out” signaling. For example, kindlins bind to sequences around the C-terminal NXXY motif, whereas talin binds to the membrane proximal region and the NPLY motif. Also, kindlins regulate the interaction of talin with $\beta_3$ and cooperate with talin to induce the conformational changes in the extracellular ligand-binding domain of the receptor. Other signaling molecules that regulate inside-out signaling include $\text{Ca}^{2+}$ and diacylglycerol-regulated guanine nucleotide exchange factor1 (CalDAG-GEF1), Rap1 and Rap1-GTP-interacting adapter molecule (RIAM) (Crittenden et al., 2004; Chrzanowska-Wodnicka et al., 2005; Lafuente et al., 2004). CalDAG-GEF1 stimulates the activity of the Ras family small GTPase Rap1, specifically the isoform Rap1b, by converting it from the guanosine diphosphate (GDP)- to the guanosine triphosphate (GTP)-bound form. Once active, Rap1 bound to GTP interacts with the adaptor RIAM to promote the association of talin with $\beta_3$, facilitating the activation of integrin. However, although CalDEG-GEF1 and Rap1b are important for inside-out
signaling, neither is required, indicating that other pathways also mediate integrin inside-out signaling independent of the CalDAG-GEF1/Rap1/RIAM pathway.

1.2.1.2 Outside-in signaling

The binding of ligands to integrin $\alpha_{IIb}\beta_3$ leads to platelet adhesion, aggregation, and consequent intracellular signal transduction cascades culminating in platelet spreading, secretion, stable adhesion, and retraction (Shattil and Newman 2004). This signaling process is referred to as “outside-in” signaling. The most proximal event following ligation of integrin is the binding of the G-protein subunit $G\alpha 13$ to the cytoplasmic domain of $\beta 3$ (Gong et al., 2010). It was recently shown that $G\alpha 13$ and talin bind to mutually exclusive but distinct sites within the cytoplasmic domain of integrin $\beta 3$. Furthermore, it was also shown that $G\alpha 13$ and talin bind to $\beta 3$ in opposing waves (Shen et al., 2013). Interestingly, talin initially binds to $\beta 3$ to mediate inside-out signaling; however, integrin-ligand binding causes talin to dissociate from $\beta 3$. This is critical, as it allows $G\alpha 13$ to bind to an ExE motif within the $\beta 3$ tail to selectively mediate integrin outside-in signaling. The mechanism whereby $G\alpha 13$-$\beta 3$ interaction induces outside-in signaling is through the phosphorylation and activation of the SFK c-Src, which is associated with $\beta 3$ (Arias-Salgado et al., 2003; Obergfell et al., 2002). SFKs are critical to outside-in signaling for several reasons. First, Src phosphorylates and activates p190RhoGAP, a signaling molecule that inactivates RhoA, thereby inhibiting the RhoA-dependent retractile signaling pathway (Arthur and Burridge 2001; Flevaris et al., 2007). Src-dependent inhibition of RhoA-mediated retraction leads to cell spreading. Second, Src not only phosphorylates p190RhoGAP
but also the cytoplasmic domain of β3. Src specifically phosphorylates 2 NXXY motifs in the cytoplasmic domain of β3 to regulate outside-in signaling (Law et al., 1999). For example, Src-dependent phosphorylation of Tyr$^{747}$ and Tyr$^{759}$ negatively regulates talin binding and protects β3 from cleavage by calpain, respectively (Anthis et al., 2009; Xi et al., 2006). Moreover, Src-dependent phosphorylation of β3 has also been shown to facilitate the interaction of myosin heavy chain and the adapter Shc to promote outside-in signaling (Jenkins et al., 1998). Finally, SFKs also promote the activation of Syk and its incorporation into the integrin signaling complex (Boylan et al., 2008). This is achieved via phosphorylation of Fc gamma receptor IIA (FcγRIIA), which promotes ITAM-dependent signal transduction. Syk facilitates the activation of PLCγ2 and platelet activation similar to the ITAM signaling pathway stimulated by GPVI (Woodside et al., 2001; Abtahian et al., 2006). Overall, these signal transduction mechanisms regulate the early outside-in signaling pathway leading to secretion, aggregation, stable adhesion, and spreading (Li et al., 2010a).

The late phase of integrin outside-in signaling is characterized by retractile signaling (Shen et al., 2012). Cell retraction requires a retractile force generated by actin-myosin interaction and is defined as the inward movement of cell membranes and the cytoskeleton associated with it. The function of integrin-mediated retractile signaling is to facilitate the process of clot retraction. Platelet retractile forces are mediated through RhoA- and Rac1-dependent signaling pathways (Flevaris et al., 2009; Flevaris et al., 2007). Early in the process of outside-in signaling, Gα13 binds to the cytoplasmic domain of β3, inducing the activation of β3-bound c-Src (Gong et al., 2010; Shen et al., 2013). Activated c-Src then promotes cell spreading by inhibiting the RhoA-dependent retractile pathway. However, later during integrin outside-in
signaling and platelet spreading, increased intracellular calcium levels activate the calcium-regulated cysteine protease calpain. Once activated, calpain cleaves the cytoplasmic domain of β3 integrin at Tyr^{759} after it is dephosphorylated. Calpain cleavage of β3 releases the inhibitory effect of the Gα13/Src pathway on RhoA and thereby promotes RhoA-dependent platelet retraction (Du et al., 1995; Xi et al., 2003; Xi et al., 2006). RhoA subsequently becomes activated, which in turn stimulates the activity of Rho dependent kinase (ROCK) to facilitate the phosphorylation and subsequent inactivation of myosin light chain (MLC) phosphatase, providing an increase in MLC phosphorylation and consequent actin-myosin mediated retraction (Kimura et al., 1996). Interestingly, it was suggested that an additional mechanism independent of RhoA mediates clot retraction, as inhibition of calpain cleavage partially, but not fully, rescued clot retraction (Flevaris et al., 2007). It was later discovered that this alternate pathway is mediated by Rac1 (Flevaris et al., 2009). Rac1 was shown to mediate integrin outside-in signaling via regulation of the MAPK signaling pathway, which also stimulated phosphorylation of MLC similar to the ROCK-mediated mechanism thereby providing another mechanism to promote actin-myosin dependent retraction.

1.2.2 The Glycoprotein Ib-IX-V Complex (GPIb-IX-V)

The platelet VWF receptor, the GPIb-IX-V complex, is critical for platelet adhesion under high shear rate flow conditions (Du 2007). Under the condition of shear, initial and transient adhesion, which is mediated via a “catch or flex bond,” requires the interaction between immobilized VWF and GPIb-IX-V (Yago et al., 2008; Kim et al., 2010). Importantly, GPIb-IX not
only mediates the physical adherence of platelets to the vessel wall under shear, but also stimulates a signal transduction cascade culminating in platelet activation and secretion leading to integrin activation and integrin-dependent stable platelet adhesion and aggregation. Accordingly, the GPIb-IX-V complex is pivotal in initiating and propagating thrombosis and hemostasis.

1.2.2.1 Structure and function

Approximately 25,000 copies of the platelet VWF receptor, the GPIb-IX-V receptor complex (GPIb-IX-V), are expressed on the platelet surface, making it the second most common receptor to be expressed in comparison to integrin αIIbβ3 (Modderman et al., 1992). GPIb-IX-V is composed of four transmembrane proteins: GPIbα, GPIbβ, GIV and GPV. They are expressed in the complex in a ratio of 2:2:2:1 (Ware 1998). GPIbα and GPIbβ are linked to each other via a disulfide bond to form GPIb. GPIX and GPV are noncovalently associated with the complex. GPIb-IX is presumed to be sufficient for mediating both the ligand-binding and signaling functions of the receptor (Du 2007). Each of the four subunits is a member of the leucine-rich repeat (LRR) superfamily, as they have LRRs that are approximately 24 amino acids in length flanked by conserved N- and C-terminal disulfide loop structures (Lopez 1994). Although GPIb-IX-V is well known for its role in mediating platelet adhesion, it also transduces signals leading to platelet activation. Furthermore, it is also known to be involved in the platelet response to low doses of thrombin.
GPIbα is the largest subunit of the complex and has various structural and functional domains. It is made of a total of 610 residues with a total molecular mass of 135 kDa (Canobbio et al., 2004). Residues 1-282 of the N-terminal domain is made of 8 LRRs and contains the binding sites for various ligands. These include VWF, Mac-1, P-selectin, thrombin, FXI, FXII, kininogen, and thrombospondin (Andrews R.K. et al., 2007). The LRRs 2 through 4 are important for the binding of VWF and to mediate platelet adhesion, and mutations in LRRs 2 and 5 through 7 lead to Bernard Soulier syndrome (Shen et al., 2002; Whisstock et al., 2002). The N-terminal domain is followed by a region with many negatively charged residues (residues 283-302), particularly 3 sulfated tyrosine residues important for the binding of both VWF and thrombin to the receptor (Dong et al., 1994; Marchese et al., 1995). The macroglycopeptide domain (residues 303-485), which is a long and highly glycosylated mucin-like region, connects the N-terminal domain and negatively charged region to a single transmembrane region (residues 486-515). The cytoplasmic tail of GPIbα is made of 96 residues (516-610) and has binding sites for intracellular signaling molecules including 14-3-3ζ and actin binding protein (ABP) or filamin (Du et al., 1996; Andrews and Fox 1992).

GPIbβ has a molecular mass of 25 kDa, is 181 residues, and has one LRR (Canobbio et al., 2004). As previously mentioned, it is also disulfide-linked to GPIbα, specifically at a cysteine residue close to the transmembrane region (Lopez et al., 1988). Importantly, a serine residue (Ser^{166}) within the 34 residue cytoplasmic tail of GPIbβ is also phosphorylated by cAMP-dependent protein kinase (PKA) and is known to interact with calmodulin and 14-3-3ζ (Wardell et al., 1989; Andrews et al., 2001; Andrews et al., 1998). Similar to GPIbβ, GPIX also has only one LRR. It also has a similar molecular weight (22 kDa) and number of residues (160) and has a
very short cytoplasmic tail (5 residues) (Canobbio et al., 2004). GPV is larger than GPIb\(\beta\) and GPIX, with a molecular mass of 82 kDa and 544 residues (Canobbio et al., 2004). It has 15 LRRs within its extracellular domain, a single transmembrane domain, and cytoplasmic tail of 16 amino acids (Hickey et al., 1993; Lanza et al., 1993). GPV flanks GPIb-IX complexes through an interaction with GPIb\(\alpha\) (Li et al., 1995). Similar to GPIb\(\beta\), the cytoplasmic domain of GPV binds to calmodulin and 14-3-3\(\zeta\) (Andrews et al., 2001; Andrews et al., 1998). The extracellular domain of GPV has been shown to bind the platelet agonist collagen and thrombin (Moog et al., 2001; Dong et al., 1997).

### 1.2.2.2 Regulation of the VWF binding function of GPIb-IX

Two mechanisms regulate the interaction between VWF and GPIb-IX (Du 2007). The first mechanism involves conformational changes within VWF, the ligand for GPIb-IX. Soluble VWF cannot bind GPIb\(\alpha\). This is because soluble VWF requires a conformational change within its A1 domain such that its affinity for GPIb-IX-V may increase enough to facilitate ligand-receptor interaction (Miyata et al., 1996). This aforementioned active conformation is only attained under physiological conditions when VWF is physically bound to matrix proteins, such as collagen, or under high shear (Siedlecki et al., 1996). The lack of affinity of soluble VWF for GPIb-IX-V may be overcome in the presence of the non-physiological modulators ristocetin or botrocetin (Scott et al., 1991; Read et al., 1989). Ristocetin, an antibiotic, and botrocetin, a snake venom protein, stimulate the binding of soluble VWF to GPIb-IX. Botrocetin and ristocetin are used to induce GPIb-mediated platelet agglutination and aggregation.
The other mechanism that regulates the interaction of VWF with GPIb-IX-V involves the intracellular regulation of the receptor (Du 2007). Indeed, the ligand-binding function of GPIb-IX has been shown to be regulated by intracellular signaling molecules. 14-3-3ζ is an intracellular adapter and signaling molecule expressed as a homodimer. Moreover, it has two ligand binding sites recognizing phosphorylated serine residues within GPIbα (Dai et al., 2005). Ser^{609} of GPIbα is constitutively phosphorylated and was shown to be required for the high affinity binding of 14-3-3ζ to GPIb-IX (Du et al., 1996; Bodnar et al., 1999). 14-3-3ζ also binds to GPIbα at other regions, specifically at the residues 557-575 and the residues 580-590 (Andrews et al., 1998; Mangin et al., 2004). As stated previously, 14-3-3ζ not only binds to GPIbα, but also to GPIbβ, specifically at Ser^{166}, which is phosphorylated by PKA (Andrews et al., 1998). Therefore, it is possible that a dimer of 14-3-3ζ may potentially interact with GPIbα alone or also with GPIbα in addition to GPIbβ simultaneously. These multiple interactions have lead to the development of a “toggle switch” model that describes the mechanism regulating the ligand-binding function of GPIb-IX-V (Du 2007). In this model, Ser^{166} on GPIbβ becomes phosphorylated by PKA, which in turn facilitates the interaction of 14-3-3ζ not only with GPIbα, but also GPIbβ. Simultaneous interaction of 14-3-3ζ with GPIbα and GPIbβ this turns the toggle switch off and locks GPIb-IX in the resting state, where it is unable to interact with VWF. However, a decrease in cAMP within the platelet cytosol leads to the dissociation of 14-3-3ζ from GPIbβ, although it still remains bound to GPIbα. This turns the toggle switch on, in turn activating the ligand-binding function of GPIb-IX and facilitating ligand-receptor interaction (Dai et al., 2005).
1.2.2.3 GPIb-IX-mediated platelet activation signaling

Signal transduction mediated through GPIb-IX is important for the inside-out activation of integrin $\alpha_{\text{IIb}}\beta_3$, facilitating integrin-dependent stable platelet adhesion and aggregation (Du 2007). Signaling events that mediate these functions include an increase in intracellular Ca$^{2+}$, nitric oxide (NO) and cyclic guanosine monophosphate (cGMP), protein phosphorylation, secretion of ADP, and synthesis of TXA$_2$.

The most proximal step to the GPIb-IX-V receptor complex that mediate signal transduction is the activation of the SFK Lyn, and Lyn-dependent activation of the PI3K/Akt signaling pathway. The interaction of Glb-IX with VWF stimulates the association of SFKs and PI3K with the receptor complex (Wu et al., 2001; Mu et al., 2010). Moreover, PI3K, through its p85 subunit, was shown to be required for the association of SFKs with GPIb-IX following stimulation with VWF. This suggests that a complex forms between SFKs and PI3K to mediate GPIb-induced platelet activation. Interestingly, the association between PI3K and GPIb-IX was also shown to be mediated through the signaling adapter 14-3-3ζ. Thus, it is possible that a signaling complex assembles during GPIb-IX-induced platelet activation where 14-3-3ζ, PI3K, and SFKs become recruited to the receptor complex (Munday et al., 2000). SFKs and PI3K are thought to transmit “early” activation signals from GPIb-IX, meaning they do not require signal amplification mechanisms from other receptors to induce platelet activation through GPIb-IX.

For example, SFKs and PI3K were shown to be critical for GPIb-IX-induced increase in intracellular Ca$^{2+}$ levels ([Ca$^{2+}$]$_i$) and the “inside-out” activation of integrin in a manner independent of other receptors (Gu et al., 1999; Li et al., 2001; Liu et al., 2005; Kasirer-Friede et
VWF-induced platelet activation was also shown to be abolished by inhibitors of SFK, knockdown of Lyn, and inhibitors of PI3K, verifying that SFKs and PI3K are critical for GPIb-IX-induced platelet activation (Liu et al., 2005; Yap et al., 2002; Yin et al., 2008a). SFKs, specifically Lyn, were also shown to be upstream of PI3K and PI3K-dependent activation of Akt in the GPIb signaling pathway, resulting in the activation of integrin α<sub>Ⅱb</sub>β<sub>3</sub> (Yin et al., 2008b). Ultimately, Lyn-dependent activation of the PI3K/Akt pathway was shown to mediate GPIb-IX-induced platelet activation by stimulating the activity of NO-synthase. This leads to an increase in NO, NO-dependent activation of soluble guanylyl cyclase and elevation of cGMP, the activation of cGMP-dependent protein kinase (PKG), and PKG-dependent activation of the MAPK signaling pathway (Li et al., 2001; Li et al., 2006; Yin et al., 2008a; Yin et al., 2008b; Li et al., 2003b). Together, these data demonstrate that a Lyn/PI3K/Akt/NO/cGMP/PKG/MAPK signal transduction pathway mediates GPIb-IX-induced platelet activation by stimulating the activation of integrin α<sub>Ⅱb</sub>β<sub>3</sub>.

Signal amplification mechanisms induced by the secondary mediators ADP and TXA<sub>2</sub> are required for “complete” or maximal platelet activation to occur during GPIb-IX-induced signal transduction (Du 2007). This is exemplified by the role of ADP and TXA<sub>2</sub> in the second wave of VWF-induced platelet aggregation. It is known that in vitro VWF-induced platelet aggregation is characterized by two distinct waves. The first wave is comprised mainly of platelet agglutination mediated by the cross-linking of platelets via the interaction between VWF and GPIb-IX; however, it also involves GPIb-induced, integrin-dependent platelet aggregation, but this is believed to play a minor role (Li et al., 2001; Li et al., 2006). The second wave of VWF-induced
platelet aggregation represents TXA₂-, secretion-, and integrin-dependent platelet aggregation (Du 2007). This is because the first wave leads to GPIb-IX-induced TXA₂ synthesis, leading to TXA₂-dependent secretion of ADP and concomitant activation of integrin α₃β₃, which mediates irreversible integrin-dependent aggregation. For example, inhibitors of TXA₂ synthesis, genetic deletion of TP, and antagonists of ADP receptors block the second wave of VWF-induced aggregation, indicating their respective signaling pathways are required (Garcia et al., 2005a; Liu et al., 2004). Likewise, integrin α₃β₃ is also required for the second wave, as demonstrated by β₃⁻/⁻ mouse platelets (Liu et al., 2004). A further role for integrin α₃β₃ was suggested by the fact that GPIb-IX-induced synthesis of TXA₂ is attenuated by integrin antagonists, indicating that a significant amount of GPIb-IX-induced TXA₂ synthesis actually requires signal transduction through integrin following the early phase of VWF-induced platelet aggregation (Liu et al., 2005). Moreover, although TXA₂ is known to be essential to VWF-induced aggregation, GPIb-IX-induced activation of integrin may occur independent of TXA₂ (Li et al., 2001). Thus, the signaling pathways stimulated by the secondary mediators ADP and TXA₂ are critical amplification mechanisms that enhance platelet activation mediated by GPIb-IX.

Mitogen-activated protein kinases (MAPKs) are also known to mediate GPIb-IX-induced signal transduction. Evidence of this derives from the discovery that extracellular-signal-regulated kinase (ERK), a MAPK, becomes activated following ligand occupancy of GPIb-IX with VWF, and that ERK is critical for GPIb-IX-mediated platelet activation in vitro and in vivo (Garcia et al., 2005a; Li et al., 2001; Mazharian et al., 2005; Li et al., 2006; Oury et al., 2006). For example, pharmacological blockade of the ERK signaling pathway inhibits GPIb-IX-induced TXA₂ synthesis and the second wave of VWF-induced platelet aggregation (Garcia et al., 2005a; Li et
Interestingly, inhibitors of ERK signaling also attenuate the GPIb-IX-induced, integrin-dependent first-wave of VWF-dependent platelet aggregation, even in the presence of COX inhibitors (Li et al., 2001). To this extent, loss-of-function mutants of upstream signaling molecules that activate ERK, such as c-Raf and MEK, inhibited the activation of $\alpha_{\text{IIb}}\beta_3$ in Chinese hamster ovary (CHO) cells following stimulation with VWF and ristocetin (Li et al., 2001). Therefore, ERK is not only critical for stimulating the TXA$_2$-dependent signaling pathway during GPIb-IX-induced platelet activation, but also for the signaling pathway mediating GPIb-IX-induced, TXA$_2$-independent activation of integrin (Du 2007). Similar to ERK, P38 is another MAPK that is required for GPIb-IX-induced platelet activation (Li et al., 2006). Most importantly, it was discovered that P38 is upstream of and required for the activation of ERK during VWF-induced platelet activation. It was also shown that GPIb-IX-induced activation of P38 and ERK requires SFK/PI3K/Akt/cGMP/PKG signaling pathway (Yin et al., 2008a; Li et al., 2006). Thus, P38 and ERK play an important role in mediating the early signal transduction pathway elicited through GPIb-IX downstream of SFKs and PKG to activate integrin $\alpha_{\text{IIb}}\beta_3$.

The immunoreceptor tyrosine-based activation motif (ITAM) receptors, Fc receptor $\gamma$ chain (FcR$\gamma$) and FC$\gamma$ receptor IIA (Fc$\gamma$RIIA), have also been shown to amplify signal transduction pathways originating from GPIb-IX. This is exemplified by the fact that Fc$\gamma$RIIA and FcR$\gamma$ have been found to associate with the GPIb-IX-V receptor complex. Furthermore, mice deficient in FcR$\gamma$ or ITAM regulated signaling molecules, such as Syk, linker for activated T cells (LAT), Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa (SLP-76) and Bruton tyrosine kinase (Btk), have a defect in the second wave of VWF-induced platelet aggregation (Sullam et al., 1998; Wu et al., 2001; Liu et al., 2005; Liu et al., 2006). Interestingly, GPIb-IX-
induced TXA₂ synthesis and inside-out activation of integrin α₁β₃ were normal in transgenic mice lacking expression of either FcRγ or LAT, suggesting they are not directly involved in GPIb-IX-mediated signal transduction but downstream of secondary amplification events induced by GPIb, such as TXA₂ synthesis and integrin signaling. Consistent with this observation, Syk was also not required for GPIb-IX-induced, integrin-dependent stable platelet adhesion, which is known to require GPIb-IX-mediated activation of integrin α₁β₃ (Liu et al., 2005; Kasirer-Friede et al., 2004; Yin et al., 2008b). Altogether, these findings suggest that the ITAM signaling pathway may not be required for GPIb-induced signal transduction, but rather plays a critical role in amplifying platelet activation signals elicited by GPIb.

1.2.3 Collagen receptors

Collagen is an important prothrombotic adhesive protein that becomes exposed to flowing blood following injury to the vessel wall. Platelets are able to detect collagen via the surface receptors α₂β₁ and GPVI, which facilitates platelet adhesion, activation and aggregation (Clemetson et al., 1999; Clemetson and Clemetson 2001; Nieswandt and Watson 2003). GPVI is believed to be the major platelet receptor for collagen and has also been shown to be required for collagen-induced platelet activation. GPVI is a member of the immunoglobulin (IgG) superfamily and has a short cytoplasmic tail made of 51 amino acids, a transmembrane region, a mucin-like stalk, and 2 IgG domains (Clemetson 2007). Interestingly, GPVI is noncovalently associated with a homodimer of another receptor, FcRγ. FcRγ is not only required for the expression of GPVI on the platelet surface but also for signal transduction mediated through
GPVI (Kato et al., 2003; Poole et al., 1997). The FcRγ chain signals through its conserved ITAM domain. Upon the interaction of GPVI with collagen, GPVI receptor clustering and cross-linking occurs, which induces Lyn- and Fyn-dependent phosphorylation of tyrosine residues within the ITAM domain of FcRγ (Gibbins et al., 1996; Ezumi et al., 1998; Quek et al., 2000). These SFKs are constitutively bound to the cytoplasmic domain of GPVI and their kinase activity plays a critical role in stimulating GPVI-induced platelet activation (Senis et al., 2009). The tyrosine kinase Syk is then recruited to ITAM via its phospho-tyrosine residues where it modulates GPVI-induced signal transduction (Yanaga et al., 1995). Syk initiates a downstream signaling cascade involving the recruitment of two cytosolic adapters, SLP76 and Gads, and a transmembrane adapter, LAT (Watson et al., 2001). This leads to the recruitment of Btk, PI3K and PLCγ2, and Vav1 and Vav3 to this newly assembled signaling complex (Quek et al., 1998; Pasquet et al., 1999; Pearce et al., 2004; Watson et al., 2005). Ultimately, this signaling complex propagates GPVI-induced platelet activation leading to calcium mobilization, activation of PKC, TXA2 synthesis, and granule secretion. Akt was also shown to be activated downstream of PI3K to mediate GPVI-induced platelet activation, specifically through the NO/cGMP/PKG/MAPK signaling pathway (Chen et al., 2004; Stojanovic et al., 2006). It should also be noted that ITAM signaling is negatively regulated by platelet endothelial cell adhesion molecule-1 (PECAM-1), specifically through the activation of phosphatases (Newman and Newman 2003).

Another platelet receptor for collagen is the integrin α2β1; however, its role in collagen-induced platelet activation remains controversial (Nieswandt and Watson 2003). For example, β1−/− mouse platelets have a relatively mild defect in collagen-induced aggregation relative to the defect observed in GPVI−/− mouse platelets (Nieswandt et al., 2001). Evidence does exist to
contest the role of $\alpha_2\beta_1$ in platelet signal transduction. An $\alpha_2\beta_1$-specific ligand, the GFOGER peptide, had no effect on platelet functional responses or tyrosine phosphorylation (Knight et al., 1999). Similarly, other reports have shown that $\alpha_2\beta_1$ is not required for collagen-induced tyrosine phosphorylation (Hers et al., 2000). However, investigators showed that platelets generate filopodia and lamellapodia when allowed to adhere on surfaces coated with GFOGER, even in the presence of inhibitors of secondary mediators (Inoue et al., 2003). Interestingly, these platelets did not respond to GFOGER in suspension. Further investigation revealed that $\alpha_2\beta_1$ does mediate platelet adhesion to and spreading on GFOGER via mechanisms similar to integrin $\alpha_{IIb}\beta_3$ and that $\alpha_2\beta_1^{-/-}$ platelets have a defect in adhesion to type-I collagen (Wonerow et al., 2002; Chen et al., 2002). Thus, although it appears that the role of $\alpha_2\beta_1$ in collagen-induced platelet activation signaling is controversial, especially in regards to the experimental system employed, $\alpha_2\beta_1$ does play some role. More recently, it was proposed that $\alpha_2\beta_1$ is critically involved in mediating signals that function to stabilize thrombi growing on collagen (Kuijpers et al., 2003; Nieswandt and Watson 2003).

### 1.3 Platelet G-protein couples receptors and signaling

Many soluble platelet agonist signal through G-protein coupled receptors (GPCRs) to elicit platelet activation during thrombosis, including thrombin, ADP and TXA$_2$. GPCRs are a class of seven transmembrane receptors that transmit intracellular signals by coupling to heterotrimeric guanosine nucleotide-binding proteins (G-proteins) (Tuteja 2009). Heterotrimeric G-proteins include $\alpha$, $\beta$ and $\gamma$ subunits, and they are constitutively associated
with GPCRs. Ligand occupancy of GPCRs induces a conformational change that stimulates their inherent guanosine nucleotide exchange factor (GEF) activity. Conformation-induced increase in GEF activity catalyzes the α subunit to exchange GDP for GTP, which causes the α subunit to dissociate from the GPCR and the β/γ complex so that it may interact with downstream effectors. The β/γ complex can also dissociate from the activated GPCR to interact with downstream effectors following the aforementioned sequence of events. There are four major families of GPCRs: Gq/11, Gi/z, Gs, and G12/13 (Offermanns 2006). Platelets express Gq, G12/13, Gi/Gz, and Gs. Gs is known to play an inhibitory role in platelet activation by stimulating the activation of adenyl cyclase (AC), leading to an increase in cAMP and concomitant activation of PKA (Kobayashi et al., 2000; Gorman et al., 1977; Tateson et al., 1977). Gi is known to promote platelet activation by inhibiting AC, which thereby constrains the inhibitory effect of cAMP and PKA (Ohlmann et al., 1995; Ohlmann et al., 2000). Also, activation of Gi-coupled receptors induces Gβγ-dependent activation of PI3K, which thereby promotes secretion and the amplification of integrin activation (Li et al., 2003a). Gq positively regulates platelet activation by stimulating the activity of PLCβ leading to an increase in intracellular Ca^{2+}, shape change, granule secretion, activation of integrin, and aggregation (Offermanns et al., 1997). Gq also stimulates Ca^{2+}/calmodulin- and RhoA-dependent contractile signaling (Vogt et al., 2003). Although platelets express both Gα12 and Gα13, only Gα13 is believed to important for platelet function (Offermanns et al., 1994; Moers et al., 2003). G13 promotes platelet activation via the p115RhoGEF/RhoA/ROCK-dependent signaling pathway, which promotes platelet shape change and granule secretion (Suzuki et al., 2003; Klages et al., 1999). Gα13 also binds to the cytoplasmic domain of integrin β3 to upregulate integrin outside-in signaling (Gong
et al., 2010; Shen et al., 2012; Shen et al., 2013). Finally, it is recognized that in order to achieve full platelet activation, signal transduction must occur through both Gi- and either Gq- or G12/13-coupled receptors (Jin and Kunapuli 1998; Moers et al., 2003). The platelet GPCRs and their respective signal transduction mechanisms are summarized in Figure 2.
Figure 2. Platelet G-protein coupled receptors and signal transduction pathways. A schematic of the major platelet GPCRs and the signaling mechanism they utilize to promote platelet activation. This figure has been modified from its original form published in Arteriosclerosis, Thrombosis and Vascular Biology (Li et al., 2010a). Gi, Gq, and G13 signaling pathways are shown in red, blue and pink, respectively.
1.3.1 Thrombin receptors

Thrombin is a potent platelet agonist both in vivo and in vitro that is critical to thrombosis and hemostasis (Bahou 2007). It is a serine protease that becomes activated from the inactive zymogen prothrombin following initiation of the coagulation cascade locally at sites of vascular injury via the extrinsic or intrinsic prothrombinase complex (Coughlin 2005). Thrombin activates platelets via protease activated receptors (PARs), of which human platelets express PAR1 and PAR4 and mouse platelets express PAR3 and PAR4 (Kahn et al., 1999; Kahn et al., 1998). Thrombin activates PARs via proteolytic cleavage of the N-terminus of the receptor, creating a new N-terminus that functions as a “tethered ligand” that binds to another region within the GPCR to induce intracellular signaling (Vu et al., 1991). PAR1 is coupled to G12/13, Gq, and Gi/z families of heterotrimeric G proteins. PAR4 is coupled to Gq and G12/13. Although human platelets express both PAR1 and PAR4 and activation of either receptor induces platelet secretion and aggregation, PAR1 is believed to be more important for the physiological platelet response to thrombin. First, PAR4 is 1 to 2 logs less responsive to thrombin when compared to PAR1, such that PAR1 has higher affinity for thrombin binding (Xu et al., 1998). This has also been demonstrated functionally, where antibodies that block the interaction of thrombin with PAR1 and PAR1 antagonists were shown to inhibit low-dose thrombin-induced platelet activation; however, antibodies that blocked thrombin activity on PAR4 had no effect on low-dose thrombin-induced platelet activation. Interestingly, blockade of both PAR1 and PAR4 were able to inhibit high-dose thrombin-induced platelet activation (Brass et al., 1992; Bernatowicz et al., 1996; Kahn et al., 1999). This suggests that although PAR1 responds more readily to
lower doses of thrombin and therefore mediates a more substantial portion of thrombin signaling under those conditions, PAR4 contributes to platelet activation at high doses of thrombin. Although it appears that PAR4 may be redundant to PAR1 and have lower affinity for thrombin, it is possible that qualitative differences in their signal transduction mechanisms make important contributions to overall platelet activation. For example, there are differences in G-protein coupling between PAR1 and PAR4, the receptors have different kinetics at which their respective signal is inactivated (receptor desensitization), and the kinetics of thrombin-induced Ca\textsuperscript{2+}-mobilization is regulated by both PAR1 and PAR4 such that sustained cytosolic Ca\textsuperscript{2+} responses requires both receptors: PAR1 for the early response and PAR4 for a late sustained response (Covic et al., 2000; Shapiro et al., 2000). Thus, it appears that the signal transduction mechanisms of PAR1 and PAR4 are not redundant, and their differential affinity for thrombin and kinetics of inactivation in fact allow thrombin to induce signals with distinct temporal characteristics. There are also differences between the PAR receptors in mouse platelets. For example, although PAR3 and PAR4 appear to function as thrombin receptors in mouse platelets and both PAR3 and PAR4 deficient mice have a bleeding and thrombosis defect \textit{in vivo}, PAR4 was shown to be the primary receptor mediating signaling whereas PAR3 functions as a cofactor that facilitates the thrombin-dependent cleavage of PAR4 but does not signal independently of PAR4 (Kahn et al., 1998; Weiss et al., 2002; Nakanishi-Matsui et al., 2000; Sambrano et al., 2001). Thus, thrombin-induced activation of mouse platelets is dependent on PAR4. Activation of PAR1 and PAR4 leads to shape change, Ca\textsuperscript{2+}-mobilization, inhibition of cAMP formation, and the activation of several signaling molecules, such as PLC, PI3K, and the small GTPases Rho, Rac and Rap1 (Coughlin 2005). Interestingly, thrombin has
also been shown to bind to GPIbα; however, the physiological relevance of GPIbα-thrombin interaction remains debatable (Okumura et al., 1978; Ruggeri et al., 2010).

1.3.2 **Purinergic receptors**

Purine and pyrimidine nucleotides, such as ADP and ATP, are released from damaged cells and secreted from activated platelets following vascular injury. They play a critical role in stimulating platelet activation during thrombosis (Cattaneo 2007). The adenine-nucleotide receptors on platelets are called P2 receptors and they are divided into two families: GPCRs termed P2Y and ligand-gated ion channels termed P2X. Platelets express two P2Y receptors, P2Y₁ and P2Y₁₂, and one P2X receptor, P2X₁ (Wang et al., 2003). P2Y₁ couples to heterotrimeric G-proteins containing Gαₙ, and therefore induces platelet activation by stimulating the activity of PLC, and PLC-dependent release of DAG and IP₃ to induce PKC activation and Ca²⁺-mobilization, respectively (Fabre et al., 1999; Murugappa and Kunapuli 2006). P2Y₁⁻/⁻ mice are defective in ADP-induced platelet shape change and aggregation, and exhibit a thrombosis defect in vivo (Leon et al., 1999). P2Y₁₂ couples to heterotrimeric G-proteins containing Gi, and therefore contributes to platelet activation by inhibiting the activity of AC, thereby reducing the synthesis of cAMP and its negative effect on platelet activation (Ohlmann et al., 1995; Murugappa and Kunapuli 2006). Experiments with P2Y₁₂⁻/⁻ mice demonstrated a bleeding defect and reduced ADP-induced aggregation. However, ADP-induced shape change and Ca²⁺-mobilization remained unaffected (Foster et al., 2001). Moreover, P2Y₁₂ was also shown to participate in multiple steps during the process of thrombosis, including platelet adhesion,
activation, and the growth and stability of the thrombus (Remijn et al., 2002). Although P2Y_{12} is
demonstrated to be important for thrombosis, signaling through the Gi-coupled receptor alone
is insufficient to fully activate platelets. In this respect, P2Y_{1} is mainly believed to be important
for synergizing with other platelet activation signaling pathways to facilitate platelet activation
rather than induce platelet activation. The fact that P2Y receptors synergize with other receptor
signaling pathways to induce platelet activation is exemplified by the fact that ADP-induced
platelet activation requires synergistic effects between both the P2Y_{1} and P2Y_{12} signaling
pathways and that both P2Y_{1}^{-/-} and P2Y_{12}^{-/-} mice have attenuated responses to ADP-
independent agonists, such as thrombin and collagen (Dorsam et al., 2002; Nieswandt et al.,
2002; Foster et al., 2001; Leon et al., 1999). This demonstrates that P2Y receptor signaling is
critical for the secondary amplification of platelet activation signals that are required for the
stabilization of platelet aggregation and thrombus growth and for optimum platelet activation
to be attained.

P2X_{1} is the third purine nucleotide receptor on platelets and it is distinct from the P2Y
receptors in that it functions as a ligand-gated ion channel rather than a GPCR (Cattaneo 2007).
Upon interaction with its respective ligand, ATP, P2X_{1} mediates rapid changes in membrane
permeability to cations causing a rapid influx of Ca^{2+} (Mahaut-Smith et al., 2000; Rolf and
Mahaut-Smith 2002). Although P2X_{1} functions as an ATP-gated Ca^{2+} channel, it only transiently
elevates [Ca^{2+}]_{i} and the magnitude of the increase in [Ca^{2+}]_{i} is less than that induced by P2Y_{1}
(Vial et al., 2002). Because of this limitation, P2X_{1}-dependent Ca^{2+} influx alone is insufficient to
stimulate platelet aggregation and full platelet activation, and only induces platelet shape-
change (Takano et al., 1999). Rather, this receptor is believed to synergize with P2Y_{1} and P2Y_{12}
to increase [Ca\(^{2+}\)] and activate ERK (Oury et al., 2002; Vial et al., 2002). An important role for P2X\(_{1}\) in platelet function was recently verified using a genetic approach. Transgenic mice lacking expression of P2X\(_{1}\) were shown to have impaired thrombus formation \textit{in vivo}, and overexpression of P2X\(_{1}\) produced a prothrombotic phenotype (Hechler et al., 2003; Oury et al., 2003). Thus, although P2X\(_{1}\) itself is insufficient to induce platelet activation, it does play a role similar to the P2Y receptors in mediating signal amplification and synergizing with other platelet activation signaling pathways.

### 1.3.3 Thromboxane receptors

TXA\(_{2}\) is a short-lived lipid secondary mediator and agonist synthesized by activated platelets. Various platelet agonists stimulate the release of arachidonic acid (AA) from phospholipids in the plasma membrane, where it is subsequently metabolized by cyclooxygenases (COXs) and synthetases to generate prostaglandins (PG) and thromboxanes (TX) (Marcus 1987). COX1 metabolizes AA to PGG\(_{2}\), which is converted to PGH\(_{2}\) (Crofford 1997; Warner and Mitchell 2004). Thromboxane synthase then converts PGH\(_{2}\) to TXA\(_{2}\) (Dubois et al., 1998). Once synthesized, TXA\(_{2}\) may diffuse out of the platelet where it functions as an autocrine and paracrine secondary mediator that recruits additional platelets to the growing thrombus and enhances platelet activation and aggregation (Svensson et al., 1976). COX inhibitors, such as aspirin, inhibit platelet function by inhibiting the enzymatic activity of COX1, and thus block the synthesis of TXA\(_{2}\). TXA\(_{2}\) is a potent platelet agonist that binds to two receptors of the thromboxane prostanoid (TP) receptor family of GPCRs: TP\(\alpha\) and TP\(\beta\). Interestingly, mRNA
transcripts for TPα and TPβ have been detected in platelet lysates; however, the dominant isoform appears to be TPα rather than TPβ (Habib et al., 1999). TPα is coupled to heterotrimeric G-proteins containing G12/13 and Gq. Therefore, the TXA₂ receptor is capable of inducing platelet activation via both the canonical G12/13 and Gq signaling pathways in platelets (Djellas et al., 1999; Knezevic et al., 1993; Offermanns 2006). As such, ligation of TPα activates the Gq signaling pathway resulting in the activation of PLC, Ca²⁺ mobilization and activation of PKC, which stimulates platelet aggregation and secretion. Ligation of TPα also activates the G12/13 pathway, resulting in the activation of the RhoA pathway, which stimulates platelet shape-change and aggregation. Tp⁻/⁻ mice have defects in platelet aggregation to multiple agonists and a defect in hemostasis in vivo. Thus, TP-mediated signal transduction is critical to thrombosis and hemostasis (Thomas et al., 1998).

1.4 Platelet Procoagulant Activity

Platelet procoagulant activity (PPA) is defined by the ability of platelets to facilitate thrombin generation and the deposition of insoluble fibrin (Zwaal et al., 1992). The ability of platelets to facilitate thrombin generation derives from two highly related physiological processes: the exposure of PS and the release of MVs with surface-externalized PS. The availability of platelet-derived, surface-externalized PS is critical to the hemostatic response to vascular injury.
1.4.1 Mechanisms of PS exposure in platelets

1.4.1.1 Flippase, floppase, and scramblase

Resting platelets maintain an asymmetric phospholipid (PL) distribution, where the outer membrane surface is made almost exclusively of choline PLs that have little to no procoagulant potential, such as sphingomyelin (SPH) and phosphatidylcholine (PC). Conversely, the procoagulant lipid PS is exclusively sequestered within the inner membrane leaflet (Lhermusier et al., 2011). This asymmetry allows platelets to sequester the procoagulant lipid until its surface externalization is required to accelerate blood coagulation. It is established that the asymmetric distribution of PLs between the lipid bilayer is maintained via energy-dependent lipid transporters, such as flippase and floppase. These transporters utilize ATP hydrolysis to perform unidirectional transport of PLs against a concentration gradient. P4 adenosine triphosphatase (ATPase) functions as the flippase that transports PLs from the outer to inner membrane leaflet (Tang et al., 1996; Paulusma and Elferink 2010). Multi-drug resistant protein-1 (MRP1), a member of the ATP-binding cassette (ABC) transporter family, functions as the floppase that transports PLs from the inner to outer membrane leaflet (Kamp and Haest 1998; Dekkers et al., 1998). Most importantly, platelets utilize an energy-independent mechanism that requires high [Ca$^{2+}$]i to execute bidirectional movement of PLs, known as scramblase. When μM [Ca$^{2+}$], concentrations are achieved during platelet activation, calcium inhibits floppase while activating scramblase (Zwaal and Schroit 1997). Scramblase activity leads to the rapid collapse of the lipid asymmetry that is actively maintained in resting platelets,
and this process is required for the exposure of PS on the surface of platelets. Platelet scramblase activity requires the transmembrane protein with unknown function 16F (TMEM16F) (Yang et al., 2012).

### 1.4.1.2 Agonist-induced PS exposure

In general, elevations of $[\text{Ca}^{2+}]_i$ regulate nearly every functional response of platelets to prothrombotic stimuli. It is thus not surprising that calcium not only regulates the surface externalization of PS in platelets, but is also required for the process. In the presence of extracellular calcium, calcium ionophore (A23187), which artificially elevates $[\text{Ca}^{2+}]_i$, independent of receptor-mediated signal transduction mechanisms, is the most potent inducer of PPA, even more than physiological platelet agonists (Bevers et al., 1985; Dachary-Prigent et al., 1993). This led to the discovery that a sustained rise in $[\text{Ca}^{2+}]_i$, specifically within the μM range, is required for PS exposure in platelets (Heemskerk et al., 1997b; Pasquet et al., 1996). Interestingly, although various agonists may induce PS exposure, they do so with varying efficacy, which is suggested to be correlated to their respective ability to sufficiently elevate the level of $[\text{Ca}^{2+}]_i$ (Heemskerk et al., 2002). It has been postulated that different platelet functions are differentially regulated by $[\text{Ca}^{2+}]_i$, and that only when high enough levels of $[\text{Ca}^{2+}]_i$ are attained, platelets will exhibit a procoagulant response. For example, a high and sustained rise in $[\text{Ca}^{2+}]_i$ induces PPA; however, moderate, transient and spiking elevations in $[\text{Ca}^{2+}]_i$ mediate a different set of functional responses independent of PS exposure (Heemskerk et al., 2002; Heemskerk et al., 1997a). These include platelet shape change, integrin activation, granule
secretion, and TXA₂ synthesis. GPCR agonists, such as ADP, thrombin and TXA₂, were believed to weakly induce PS exposure (Leon et al., 2003; Heemskerk et al., 2005). This contrasts the greater procoagulant response observed by these investigators when using agonists that activate GPVI, a tyrosine kinase-linked receptor, either through stimulation with soluble agonists or adherence to immobilized ligand (Thiagarajan and Tait 1991; Siljander et al., 2001). Interestingly, thrombin was first identified as being unable to induce PPA, and platelets required co-stimulation with collagen in addition to thrombin to induce PPA (Bevers et al., 1983). In result, investigators typically used thrombin in combination with GPVI-specific agonists to study agonist-induced PPA, as this is the only way they could induce a sufficient response. However, it was more recently shown that activation of adhesion receptors is also a potent mechanism that amplifies the signals required for agonist-induced PPA (Heemskerk et al., 2005). This not only exemplifies the divergence of the platelet aggregation and procoagulant response, but also that different agonists are differentially able to induce PPA. However, it must be noted here that the aforementioned studies were performed in the absence of shear stress, an inherent physiological force present in flowing blood, but under static conditions. Thus, these conclusions are rather limited because the efficacy of platelet agonists to induce PPA was not evaluated under conditions where physiological levels of shear are present (see Chapter 5).

It was postulated that, under static conditions, the biochemical explanation for the differential efficacy of agonists to induce PPA is that thrombin, signaling through Gq-coupled receptors, transiently activates PLCβ leading to the production of inositol 1,4,5-trisphosphate (IP₃) and a short, “spiking” Ca²⁺ signal (Heemskerk et al., 1993; Heemskerk et al., 2001). It was
suggested that, similar to other GPCR agonists, this Ca$^{2+}$ response is not large enough to induce appreciable PS exposure and that thrombin only induces a sustained elevation of [Ca$^{2+}$]$_i$ to the level required to induce PS exposure at extremely high doses, of which are not commonly used to evaluate platelet function (Heemskerk et al., 1997a). The fact that low to moderate doses of thrombin do not elicit enough of a Ca$^{2+}$ response to induce PPA is suggested by the fact that thapsigargin, an inhibitor of the sarco/endoplasmic reticulum calcium-ATPase (SERCA), strongly potentiates and prolongs the Ca$^{2+}$ response to thrombin resulting in the reversal of its weak induction on PS exposure (Dachary-Prigent et al., 1995; Smeets et al., 1993). On the contrary, it was also proposed that GPVI-specific agonists or adhesion to GPVI-specific ligands more effectively elevates [Ca$^{2+}$]$_i$, resulting in a sustained and proportionately larger Ca$^{2+}$ response. This is presumed to result from greater activity of PLC, specifically PLC$\gamma$2, leading to IP$_3$-mediated Ca$^{2+}$-mobilization, and, most importantly, store-operated Ca$^{2+}$ entry (SOCE). The process of SOCE is thought to be required for agonist-induced PPA, because it is the mechanism responsible for sufficiently elevating [Ca$^{2+}$]$_i$ to the level required to induce scramblase activity (Thiagarajan and Tait 1991; Bevers et al., 1982b; Dachary-Prigent et al., 1995). Therefore, although most agonists alone poorly induce PPA under static conditions, it appeared that the optimal procoagulant response only occurs when multiple receptors, either signaling or adhesion receptors, are simultaneously occupied and signaling in the absence of shear (Heemskerk et al., 2000). Perhaps this functions to localize thrombin generation at specific areas in the vasculature where it is most necessary, such that soluble platelet agonists will not cause aberrant PPA but the presence of adhesive proteins and soluble agonists together at a localized area will induce the PPA required for physiologically relevant thrombin generation to
occur. However, it is strongly recommended that such experiments be repeated and their implications re-evaluated under more relevant conditions to that of flowing blood, such as in the presence of shear (see Chapter 5).

1.4.1.3 Agonist-induced versus apoptosis-induced PS exposure

Although platelet agonists induce PS exposure, it has also been demonstrated that platelets undergo apoptosis and that apoptotic platelets externalize PS and exhibit procoagulant activity (Schoenwaelder et al., 2009; White et al., 2012). Platelets contain many signaling molecules that regulate apoptosis. This includes the pro-apoptotic Bcl-2 family members Bid, Bax and Bak, the anti-apoptotic Bcl-2 family member Bcl-X\textsubscript{L}, and caspase-3 and -9. Platelet apoptosis is studied using ABT-737, a BH3 mimetic small molecule inhibitor of the anti-apoptotic survival BcL-X\textsubscript{L} that promotes Bad/Bax-driven mitochondrial damage, concomitant activation of caspases, and caspase-dependent PS exposure (Mason et al., 2007; Schoenwaelder et al., 2009; White et al., 2012; Zhang et al., 2007). Interestingly, although both agonist-induced signaling pathways and the apoptosis pathway lead to PS exposure and procoagulant activity in platelets, evidence exists demonstrating that both of these mechanisms are differentially regulated (Schoenwaelder et al., 2009; White et al., 2012). For example, agonist-induced PS exposure is unaffected by either genetic or pharmacological targeting of the apoptotic machinery. Bak\textsuperscript{-/-}, Bax\textsuperscript{-/-}, caspase-9\textsuperscript{-/-}, and caspase inhibitor-treated platelets are not defective in agonist-induced PS exposure but are defective in apoptosis-induced PS exposure. Furthermore, agents that elevate cAMP, which inhibits the signaling mechanisms that stimulate
platelet activation in a PKA-dependent manner, block agonist-induced but not apoptosis-induced PS exposure (Schoenwaelder et al., 2009). Another distinction is that proteolytic cleavage of cytoskeletal proteins, such as filamin and gelsolin, in apoptotic platelets occurs independent of \([\text{Ca}^{2+}]_i\), whereas agonist-induced cleavage of these cytoskeletal proteins is dependent upon \([\text{Ca}^{2+}]_i\) and the calcium-dependent protease calpain. This suggests that although both physiological agonists and apoptosis induce PS exposure in platelets, they do so with distinct molecular mechanisms. This is in agreement with data obtained from lymphocytes isolated from patients with Scott’s syndrome that lack the scramblase TMEM16F (Williamson et al., 2001). Apoptosis-induced PS exposure was intact but \(\text{Ca}^{2+}\)-induced PS exposure was abolished. This was later verified in TMEM16F\(^{-/-}\) platelets, where it was demonstrated that apoptosis-induced PS exposure is independent of TMEM16F but agonist-induced PS exposure is TMEM16F-dependent (van Kruchten et al., 2013). Together, it appears that multiple mechanisms exist in platelets to induce PS exposure, but during thrombosis, the primary mechanism is presumed to involve agonist-induced signaling pathways and activation of TMEM16F.

### 1.4.2 Function of PS in blood coagulation

The surface externalization of PS on platelets and platelet-derived MVs is critical for blood coagulation and provides a mechanism for thrombin generation to occur specifically at sites where platelets become activated during vascular injury (Bouchard et al., 2007). Under normal conditions, kinetic restraints limit the ability of key blood coagulation enzymes to
produce sufficient concentrations of thrombin to attain hemostasis. However, this is precisely why platelets are essential to coagulation: they provide the catalytic membrane surface required to surpass these constraints allowing for optimal thrombin generation to occur within a specific part of the vasculature. Following vascular injury, coagulation is initiated by the presence of tissue factor (TF) on the outer surface of cells and MVs. This results in the assembly of the extrinsic tenase complex, where circulating FVIIa colocalizes with TF on the PL surface (TF-FVIIa complex) (Furie and Furie 2008). The extrinsic tenase is mainly responsible for activating FX (FX to FXa) but it also activates FIX (FIX to FIXa) (Lawson and Mann 1991). The limited amount of FXa formed subsequently activates very small amounts of thrombin (FIIa) in the pM range of concentration, which functions to activate FV (FV to FVa), FVIII (FVIII to FVIIIa) and FXI (FXI to FXIa) (Hoffman and Monroe 2007). After this small amount of thrombin is generated, platelets begin to respond and amplify the coagulation cascade (Bouchard et al., 2007). Thrombin triggers the activation of platelets, leading to the regulated surface exposure of the procoagulant lipid PS. PS is negatively charged, and its translocation to the outer surface of platelets provides a negatively charged membrane surface. This facilitates the Ca\(^{2+}\)-dependent binding of coagulation factors to the lipid surface, specifically through a high affinity calcium binding domain, the \(\gamma\)-carboxyglutamate (Gla) domain (Huang et al., 2003). Surface exposed PS is required for the assembly of two key complexes on platelet-derived PL surfaces: the intrinsic tenase complex and the prothrombinase complex. FVIIIa and FIXa, which is activated by FXIa, colocalize on the negatively charged PL surface provided by PS in a Ca\(^{2+}\)-dependent manner to form the intrinsic tenase, which catalyzes the activation of FX (FX to FXa) (Mann et al., 2006). In fact, the intrinsic tenase activates FX 50 to 100 times faster than the
extrinsic tenase complex, and thus provides an important amplification mechanism to accelerate the formation of the prothrombinase complex (Bouchard et al., 2007). FXa then colocalizes with FVα on the negatively charged PL surface provided by PS in a $\text{Ca}^{2+}$-dependent manner to form prothrombinase, which activates thrombin from prothrombin (FII to FIIa). At this time, the enzymatic reactions of the coagulation cascade are primed and ready for propagation in order to produce physiological relevant amounts of thrombin. Most importantly, in order for this to occur, a negatively charged PL surface (PS) must be provided by activated platelets being recruited into the site of vascular injury. During this phase, the rate of thrombin generation is limited by the rate at which Xa is generated, and the assembly of the intrinsic tenase on platelet-derived, surface-externalized PS results in a 24 million-fold acceleration of the rate of Xa formation. Also, inhibiting the binding of coagulation factors to PS-exposed membranes during this phase decreases the catalytic efficiency of both the tenase and prothrombinase complexes by up to 1000-fold (Bouchard et al., 2007). Thus, the catalytic membrane surfaces provided by platelets in the form of surface-externalized PS functions as a mechanism for the localized activity of the intrinsic tenase and prothrombinase complex, which results in the optimization of thrombin generation. This culminates in a rapid burst in thrombin generation (Mann et al., 2006). Finally, thrombin cleaves fibrinogen to soluble fibrin monomers, which subsequently spontaneously polymerize to form soluble polymers of fibrin. These polymers are then cross-linked by factor XIII, which is activated also by thrombin (FXIII to FXIIIa) to form an insoluble fibrin meshwork that stabilizes the platelet plug (Mann et al., 2003).
1.4.3 Platelet microvesicles

The existence of platelet microvesicles (MVs) was first alluded to in the 1940s in studies that compared the clotting times of platelet-containing plasma, platelet-poor plasma (PPP), and the supernatant of PPP following high speed centrifugation. PPP had prolonged clotting times relative to normal plasma, indicating that platelets are important for blood coagulation. However, the clotting time of PPP subjected to high speed centrifugation was further prolonged relative to PPP. This indicated that the PPP contained some subcellular factor that promoted clotting (Chargaff and West 1946). In 1967, this material was thought to be membrane fragments originating from platelets, and was thus called “platelet dust” (Wolf 1967). It was eventually discovered that “platelet dust” is in fact platelet-derived MVs that possess high procoagulant activity, and these MVs explain the coagulant activity of PPP.

Platelet-derived MVs are a heterogeneous population of submicron, membrane vesicles ranging from 0.1 to 1 μm in size (Morel et al., 2008). They have a lipid bilayer with transmembrane proteins and receptors characteristic of the platelets they are derived from. For example, platelet MVs express GPIb-IX-V, αIIbβ3, P-selectin, and PECAM-1 in addition to other platelet-specific receptors and chemokine receptors (Garcia et al., 2005b). They also have PAF, β-amyloid precursor protein, active calpain, and AA (Nomura et al., 2008). Platelet MVs are shed from the platelet membrane following exposure to very high levels of shear, contact with surfaces of foreign bodies, complement-binding proteins or Ca²⁺ ionophore, low temperature, incubation with physiological platelet agonists, and even storage. Most importantly, the
common denominator between the platelet activation mechanisms that induce the generation of MVs is a dramatic increase in $[\text{Ca}^{2+}]_i$ (Horstman and Ahn 1999).

1.4.3.1 Formation of platelet microvesicles

Platelet MVs are believed to form following an interesting sequence of activation-induced morphological changes. During platelet activation, platelets undergo “shape-change” wherein their morphology turns from the typical discoid shape to a spherical, “balloon-like” shape (Heemskerk et al., 2000). Activated platelets then begin to form pseudopodia, which are membrane protrusions extending outward and away from the center of the rounded platelet (Hartwig 2006). These pseudopodia undergo further morphological changes by generating membrane blebs, which may be observed as spherical buds of lipid beginning to emerge and bulge out of the platelet membrane (Polasek 1982; Fackler and Grosse 2008). These blebs have been shown to have high levels of surface-externalized PS and their morphology closely resembles that of platelet-derived MVs (Heemskerk et al., 2005; Siljander et al., 2001).

Interestingly, these PS-exposed blebs are thought to be the precursor to platelet-derived MVs (Horstman and Ahn 1999). This is not only suggested by the fact that platelet membrane blebs are morphologically very similar to platelet-derived MVs, but also because pseudopodia with multiple blebs have been observed fragmenting and vesiculating into platelet-derived MVs (Basse et al., 1994; Ariyoshi and Salzman 1996; Yano et al., 1994). Although the morphological changes that lead to platelet microvesication have been visualized, the exact molecular mechanisms governing these changes are not fully understood.
It is well accepted that the molecular mechanisms that regulate the release of procoagulant MVs involve a dramatic and sustained increase in $[\text{Ca}^{2+}]$, and the proteolytic activity of calpain. In order for membrane blebbing to occur, the platelet membrane must detach from the membrane cytoskeleton (Flaumenhaft 2006; Fackler and Grosse 2008). In fact, it is proposed that the resting membrane skeleton of platelets prevents or stabilizes against microvesiculation, and that during platelet activation distinct changes occur in the platelet cytoskeleton to facilitate MV release (Fox et al., 1990). The most critical event to occur is a dramatic increase in $[\text{Ca}^{2+}]$ (Lhermusier et al., 2011). This is critical for 2 reasons: it leads to the activation of TMEM16F (scramblase activity) and also activation of the $\text{Ca}^{2+}$-dependent protease calpain. Calpain is a key regulator of activation-induced microvesiculation, and has been shown to be required for the process (Yano et al., 1993; Basse et al., 1994; Pasquet et al., 1996). The primary mechanism for its involvement is its ability to cleave a multitude of proteins that link the cytoskeleton to the membrane when $[\text{Ca}^{2+}]$ attains a critical concentration during platelet activation. This in effect destroys the anchoring and linking function of the membrane cytoskeleton allowing the plasma membrane to physically dissociate from the cytoskeleton. This relieves the tension that restricts microvesiculation, in turn facilitating the outward movement of cytosol into membrane blebs and their consequent shedding. Together, there is a large body of evidence that indicates the level of microvesiculation that occurs in activated platelet is correlated to the degree of proteolytic cleavage of cytoskeletal proteins (Yano et al., 1993; Pasquet et al., 1996; Fox 2001; Fox et al., 1990; Fox et al., 1991). As stated previously, electron micrographs show that membrane extensions indeed fragment into platelet MVs, and that the formation of the membrane extensions requires calpain, as they are inhibited by small
molecule inhibitors of calpain. An increase in $[Ca^{2+}]_i$ was also detected within these membrane protrusions and a sustained increase in $[Ca^{2+}]_i$ is required. It should be noted; however, that the process of microvesiculation and PS exposure are distinct but are very much related. For example, inhibition of calpain specifically blocks bleb formation and MV release but not PS exposure following stimulation with A23187 (Dachary-Prigent et al., 1995). Moreover, platelet PS exposure can be induced in the absence of MV release, but MV release has not been demonstrated in the absence of PS exposure. Importantly, although it is known that PS exposure itself is insufficient to induce microvesiculation, the processes are highly related as evidenced by platelets from Scott’s syndrome patients, which have defects in both PS exposure and MV release (Lhermusier et al., 2011). However, Scott’s syndrome platelets have normal calpain activity and $[Ca^{2+}]_i$ elevations. This demonstrates that $Ca^{2+}$ and calpain are not the most proximal step to MV release and that $Ca^{2+}$ must act on a downstream target to induce procoagulant activity. This downstream target was recently discovered to be TMEM16F, a protein critical for $Ca^{2+}$-dependent lipid scrambling (Kunzelmann et al., 2013). TMEM16F was shown to be required for agonist-induced PS exposure, but its role in platelet-derived MV formation has not been investigated thoroughly. It is unknown whether loss of TMEM16F leads to a loss of function in MV release directly or indirectly because PS exposure is abolished in TMEM16F null cells but also required for the process of microvesiculation.

Other molecular mechanisms that regulate the release of procoagulant MVs involve integrin $\alpha_{IIb}\beta_3$ and an increase in mitochondrial permeability. Integrin $\alpha_{IIb}\beta_3$ plays an essential and general role in stimulating agonist-induced MV release (Gemmell et al., 1993). This is evidenced by the fact that agonist-induced MV release is blocked by integrin antagonists and in
Glanzmann’s thrombasthenia platelets. It should be noted; however, that integrin αIIbβ3 is not required when platelets are stimulated with ionophore or complement proteins (Holme et al., 1995). This demonstrates that integrin signaling plays an essential role, but that its function may be bypassed by artificially elevating [Ca\(^{2+}\)]. This also suggests that the mechanism whereby integrin induces MV release is via elevation of [Ca\(^{2+}\)]. Yet another mechanism that helps facilitate MV shedding is depolarization of the mitochondrial membrane. The mitochondrial permeability transition pore (MPTP) is a nonselective pore made of several proteins that forms in the mitochondrial membrane during the cellular response to certain stimuli (Halestrap 2009). Its main function is to stimulate a rapid loss in mitochondrial membrane potential (Δψ\(_m\)). Interestingly, it was shown that only strong stimuli, such as high concentrations of thrombin with convulxin or collagen, leads to mitochondrial-dependent MV release (Jobe et al., 2008). This may help explain previous work, where only simultaneous activation with multiple platelet agonists leads to the development of PPA (Heemskerk et al., 2000), but it ignores other important factors such as the shear forces present in blood flow. For example, it was shown that low doses of agonists may activate platelets leading to shape-change, release of granule contents, platelet spreading and activation of integrin αIIbβ3, but do not lead to formation of the MPTP, Δψ\(_m\), PS externalization and MV release. It was thought that platelets become procoagulant only when stimulated with enough agonist concentrations to form the MPTP and Δψ\(_m\), which was demonstrated to require multiple platelet agonists rather than just one agonist alone (Jobe et al., 2008). The MPTP and Δψ\(_m\) were shown to bring platelets from just the activated state, to a “highly-activated state” where they externalize PS and shed MVs. Similar to integrin αIIbβ3, the mitochondria may function to induce MV release through an increase in
\([\text{Ca}^{2+}]_i\), as addition of ionophore to cyclophilin D (CypD) knockout platelets, which cannot form the MPTP, rescues the defect in PS and MV release, a result similar to that observed in Glanzmann's thrombasthenia platelets (Gemmell et al., 1993). It was later verified that when platelets are activated with strong enough agonist concentrations to induce \(\Delta \psi_m\), \text{Ca}^{2+} \text{ released from the activated mitochondria was indeed critical for bringing the } [\text{Ca}^{2+}]_i \text{ to the level required to stimulate agonist-induced PPA (Choo et al., 2012). However, it still remains unclear why very large concentrations of agonists, or multiple agonists together, are required to induce mitochondrial-dependent PS exposure under static conditions. Also, whether the mitochondrion is actually involved in agonist-induced microvesiculation, a consequence of PS exposure, remains undetermined. Likewise, whether the mitochondrion regulates PPA under more relevant conditions, such as that under the shear forces of blood flow, is unknown.

### 1.4.3.2 Shear and platelet microvesicles

The hemodynamics of blood flow, particularly shear, plays an important role in modulating platelet activation leading to the release of procoagulant MVs. Circulating platelets are able to sense levels of shear close to the vessel wall and respond accordingly by promoting hemostasis. Critical to this function is the ability of platelets to not become activated in response to the shear stress found in normal blood flow, but respond to the extremely high levels of shear stress generated in stenotic lesions, atherosclerotic small arteries or arterioles suffering from vasospasm. The pathological constriction of blood vessels significantly alters blood flow, which is associated with an increase in shear rates from the typical range of 20 to
2000 s$^{-1}$ to rates that are 100-fold higher than in the absence of obstruction and 10-fold higher than in microarterioles (Goldsmith and Turitto 1986; Strony et al., 1993; Tangelder et al., 1988). Such high shear conditions lead to spontaneous platelet activation and aggregation, and also stimulates the release of procoagulant MVs (Miyazaki et al., 1996). Shear-induced platelet aggregation (SIPA) in PRP or whole blood is thought to involve the interaction of VWF with GPIb-IX-V, leading to Ca$^{2+}$ mobilization, integrin activation, and VWF/$\alpha_{IIb}\beta_3$-dependent aggregation (Ikeda et al., 1991; Ikeda et al., 1993; Ruggeri 1993; Moake et al., 1986; Moake et al., 1988). The mechanism of high shear-induced MV release was shown to be consequential to SIPA and to require an increase in [Ca$^{2+}$], and concomitant activation of calpain (Miyazaki et al., 1996). GPIb-IX-induced activation of $\alpha_{IIb}\beta_3$ is also reported to play a role, as integrin antagonists abolish shear-induced MV release (Goto et al., 2003). Investigators have shown that the minimum shear rate required for agonist-independent MV release is 10,500 s$^{-1}$ and that optimal shear-induced MV release occurs at shear rates greater than 40,000 s$^{-1}$ (Holme et al., 1997; Reininger et al., 2006). This further demonstrates that PPA may not only be regulated by high concentrations of agonists localized at the site of vascular injury, but also the presence of pathological shear at these sites. Under such high-shear conditions, the release of procoagulant MVs was shown to involve a membrane tethering mechanism formed via a bond between immobilized VWF and GPIb-IX-V (Reininger et al., 2006). Under very high shear, the platelet adheres at discrete adhesion points (DAP) via a bond formed between immobilized VWF and GPIb-IX-V. Because of the hydrodynamic drag caused by excessive shear, the tether was shown to elongate and eventually fragment into platelet-derived MVs with exposed PS. The process is believed to require VWF-GIb-IX interaction, because pharmacological blockade of this
interaction blocked shear-induced MV release. This tethering-induced process of microvesiculation is morphologically very similar to the previously described process, where PS-exposed membrane blebs are shed from pseudopodia. It should be noted; however, that under the physiological conditions of normal blood flow, such as shear rates below 2000 s\(^{-1}\), platelets do not become procoagulant (Holme et al., 1997; Reininger et al., 2006). Thus, studies have only limited their evaluation of pathological levels of shear stress, but not physiological levels of stress (see Chapter 5), on agonist-induced MV release. Taken together, an essential component to platelet function is to respond to localized areas where high shear exists, such as atherosclerotic arteries, and release procoagulant MVs to facilitate arterial thrombosis and blood coagulation.

1.4.3.3 Function of platelet microvesicles

MVs are present in the blood of normal individuals, but MVs derived from platelets are believed to be the main source of procoagulant lipid material in the circulation, especially because 80-90% of the detected MVs in blood are platelet-derived (Berckmans et al., 2001). Evidence that platelet MVs are physiologically relevant to thrombosis and hemostasis \textit{in vivo} derives from the fact that they are associated with a thrombotic tendency. For example, levels of circulating platelet-derived MVs are significantly elevated in various diseases that are associated with hypercoagulation and an elevated risk of thrombosis. Elevated circulating platelet MVs are associated with rheumatoid arthritis, uremia, malignancy, sickle cell disease, heparin-induced thrombocytopenia, arterial thrombosis, idiopathic thrombocytopenic purpura,
and thrombotic thrombocytopenia (Boilard et al., 2010; Ando et al., 2002; Varon and Shai 2009; Tomer et al., 2001; Hughes et al., 2000; Lee et al., 1993; Galli et al., 1996). Platelet MVs have also been implicated in the development of atherosclerosis in addition to the regulation of angiogenesis (Tan and Lip 2005; Kim et al., 2004). Most importantly, the involvement of platelet MVs in thrombosis and hemostasis is undisputed given the discovery of rare hereditary bleeding disorders that are caused by a defect in platelet microvesiculation. These diseases include Scott’s Syndrome and Castamans defect (Satta et al., 1997; Yang et al., 2012; Castaman et al., 1996; Castaman et al., 1997). Platelets from Scott syndrome patients, a disease caused by a loss-of-function mutation in TMEM16F, are defective in agonist-induced PS exposure, MV release, and the ability to support the formation of the tenase and prothrombinase complex. Another bleeding disorder, Castaman’s defect, is characterized by a loss-of-function specifically in the generation of platelet-derived MVs. Thus, both of these disorders are caused by an inability to produce platelet-derived MVs, which causes bleeding diathesis.

Platelet MVs share similar functions to platelets, but their primary function is to promote coagulation locally at sites of vascular injury. Platelet-derived MVs are highly procoagulant, and have actually been shown to have 50- to 100-fold higher procoagulant activity relative to platelets (Sinauridze et al., 2007). The remarkable procoagulant nature of platelet-derived MVs derives from one defining characteristic: the presence of large amounts of PS within their outer membrane leaflet. In fact, platelet MVs have up to a 10-fold greater density of PS on their outer surface relative to platelets (Dachary-Prigent et al., 1993). PS is a negatively charged lipid, and its presence on the outer surface of cells and MVs is absolutely essential to promoting coagulation. This is because the coagulant response is directly regulated
by the formation of enzymatic complexes that initiate, amplify, and propagate the coagulation cascade. These enzymatic complexes only assemble in the presence of a negatively charged membrane surface. Because platelet-derived MVs have very high levels of surface-exposed PS, which is negatively charged, they significantly accelerate the Ca$^{2+}$-dependent assembly of vitamin K-dependent proteases and cofactors into these enzymatic complexes. The two enzyme complexes that are essential for platelet MVs to contribute to blood coagulation include the tenase and prothrombinase complex (Bouchard et al., 2007). Platelet MVs form the tenase complex following the colocalization of FVIIIa and FIXa on their PS-exposed surface, which catalyzes the formation of FXa. This facilitates the formation of the prothrombinase complex, which consists of FXa and FVa and stimulates the activation of thrombin from prothrombin. The presence of PS is critical for accelerating the enzyme kinetics required for physiologically relevant thrombin generation, and has been shown to accelerate prothrombinase activity more than 1000-fold (Rosing et al., 1980). In addition, the rate at which FXa and thrombin are generated following vascular injury controls the overall kinetic rate of the coagulation process (Bouchard et al., 2007). Because of this, platelet-derived MVs clearly play an essential role in supporting and maintaining coagulation.

Another important function of platelet-derived MVs is to localize coagulation at the site of injury through their adhesive properties. Platelet MVs are able to adhere to immobilized fibrinogen, bind to soluble fibrinogen, and adhere to the subendothelial matrix in an integrin $\alpha_{IIb}\beta_3$-dependent manner (Holme et al., 1998). Thus, platelet MVs are able to co-aggregate with aggregating platelets that are accumulating in growing thrombi, adhere to the damaged vessel wall, and bind to accumulating fibrin that actively becomes incorporated into the growing
thrombus. This facilitates the localized deposition of PS at sites of injury where platelets become activated. Furthermore, platelet-derived MVs also function to help promote the deposition of TF at the site of injury. TF is a cofactor that is important for propagating the coagulation cascade (Mann et al., 2006). It is a surface receptor for FVIIa, and the presence of TF at the site of injury greatly accelerates thrombin generation. This is because TF and FVIIa colocalize on PL surfaces to form the extrinsic tenase, which catalyzes the formation of FX to FXa thereby increasing the rate of assembly of the prothrominase complex. Circulating monocytes and leukocytes in addition to MVs derived from monocytes, leukocytes and endothelial cells function as a circulating storage pool of TF (Reid and Webster 2012). Furthermore, each of these cellular-derived entities may become incorporated into a rapidly growing thrombus following injury to the vessel wall (Nomura et al., 2008). This is because P-selectin glycoprotein-1 (PSGL-1) is expressed on these cells and MVs, and once platelet-derived MVs expressing P-selectin, an adhesion receptor for PSGL-1, become incorporated into growing thrombi they recruit PSGL-1 expressing leukocytes, monocytes and their associated MVs that have TF (Forlow et al., 2000; Falati et al., 2003). P-selectin dependent recruitment of these PSGL-1 expressing cells and MVs results in the incorporation of TF into the growing thrombus, which has been shown to be critical for facilitating platelet aggregation and the formation of a fibrin plug (Chou et al., 2004). This promotes the accumulation of TF at the site of injury and further helps to localize thrombin generation. Therefore, the adhesive properties of platelets mediated by integrin $\alpha_{IIb}\beta_3$ and P-selectin play a critical role in the ability of platelet-derived MVs to localize thrombin generation and fibrin deposition at sites of vessel wall injury.
1.5 Rac1 GTPase

Rac1 is a member of the Rac-related subfamily of the Rho-family small GTPases (Wennerberg and Der 2004). There are 3 isoforms of Rac: Rac1, Rac2 and Rac3. They have similar structural characteristics and share significant sequence identity (~88%). Rac1 is abundantly expressed in platelets and believed to be the predominant isoform, mainly because it is the only isoform that has been detected and Rac2−/− platelets have no detectable defects in platelet function (Polakis et al., 1989; McCarty et al., 2005). Rac1 is regulated in a dynamic and complex manner. Furthermore, the mechanisms regulating the activity of Rac1 have proven to be critical for platelet function, thrombosis, and hemostasis. Figure 3 summarizes the mechanisms that regulate the activity of Rac1. Figure 4 summarizes the general functions and signal transductions pathways mediated by Rac1.
Figure 3. Regulation of Rac1. A schematic of the mechanisms regulating the activity of Rac1.
1.5.1 Structure and function

Rac1 was originally identified as Ras-related botulinum toxin substrate 1 with an approximate molecular mass of ~21 kDa (Didsbury et al., 1989). Being that Rac1 is a small GTPase, it functions as a molecular switch cycling between two conformational states. These states are regulated by the binding of the guanine nucleotides GTP or GDP to Rac1 (Etienne-Manneville and Hall 2002). In general, GDP-bound Rac1 is inactive or turned “OFF” whereas GTP-bound Rac1 is active and turned “ON.” In order for Rac1 to become activated, a nucleotide exchange must occur where the bound GDP is dissociated and exchanged for GTP. This produces the GTP-bound form of Rac1, which is catalyzed by guanine nucleotide exchange factors (GEFs). In order for Rac1 to be turned “OFF” or inactivated, the bound GTP must be hydrolyzed to GDP. This is an irreversible process that is referred to as the guanosine triphosphatase (GTPase) reaction. Rac1 has intrinsic GTPase activity, but it is very low; therefore, GTPase-activating proteins (GAPs) are required to accelerate the reaction and turn Rac1 “OFF.” Interestingly, guanine dissociation inhibitors (GDIs) bind to Rac1 when it is bound to GDP. This inhibits the nucleotide exchange of GDP for GTP, which maintains Rac1 in the “OFF” state thereby preventing it from localizing to the cytoplasmic side of the membrane. Therefore, the activity of Rac1 is tightly regulated through the interaction of several intracellular signaling molecules that associate with it depending on whether it is bound to GDP or GTP.

Rac1 is not only regulated by the binding of guanine nucleotides, but also its subcellular localization. In general, Rac1 is presumed to be primarily inactive when localized in the cytosol
but active when it becomes recruited to the plasma membrane. An important amino acid sequence that confers the ability of Rac1 to translocate to the plasma membrane is a conserved CAAX box located at the C-terminus of Rac1, which is post-translationally modified via the attachment of a geranyl-geranyl (GG) moiety (Seabra 1998). During the process whereby Rac1 becomes activated, it is translocated to the plasma membrane where the isoprenoid moiety (GG) becomes inserted into the lipid bilayer. This functions to anchor Rac1 to the membrane, which maintains its membrane proximal localization. Interestingly, GDIs not only regulate Rac1 by preventing nucleotide exchange of GDP for GTP, but also prevent the recruitment of Rac1 to the plasma membrane. Thus, GDIs negatively regulate Rac1 by preventing GTP-loading and maintaining its localization to the cytosol (Olofsson 1999; Keep et al., 1997).

Rac1 has two sites that are important for interaction with GDIs: the GG moiety and the switch regions (Scheffzek et al., 2000; Hoffman et al., 2000; Lian et al., 2000; Longenecker et al., 1999). GDIs actually bind to the GG moiety located in the C-terminus of Rac1 and insert it into a hydrophobic pocket located within the GDI. This functions to sequester the lipid moiety and prevent it from interacting with the membrane. Furthermore, GDIs may also remove Rac1 from the membrane to terminate Rac1-dependent signal transduction. Switch I (residues 26-45) and Switch II (residues 59-74) are important for association with GDIs, which are also binding sites for guanine nucleotides, effectors (PAK and NADPH oxidase), GAPs, and GEFs. Therefore, GDIs prevent nucleotide exchange, the hydrolysis of GTP to GDP, and membrane localization (Del Pozo et al., 2002). Interestingly, an additional sequence in Rac1 that is important for targeting it to the membrane is a polybasic region (KKRKRK), which is proximal to the C-terminus of Rac1. This polybasic region and the GG moiety are required for membrane translocation of Rac1,
which may also occur independent of the binding of effectors (Riento et al., 2005). Upon translocation of Rac1 to the membrane, Rac1 binds GTP and enters the active state. This is because the physical association between Rac1 and the membrane releases the C-terminal GG moiety from the hydrophobic pocket of the bound GDI, therefore enabling it to become inserted into the plasma membrane. Following insertion of the GG moiety, the GDI becomes displaced from Rac1, which in turn facilitates Rac1-GEF interaction, the binding of GTP, and the interaction of GTP-loaded Rac1 with downstream effectors. The subcellular localization of Rac1 to the plasma membrane is known to be critical for signal transduction. This is because it has been shown to be required for Rac1 to interact with and activate downstream effectors, specifically p21-activated kinase (PAK) (del Pozo et al., 2000; Symons 2000; del Pozo et al., 2004; Guan 2004).

The most well characterized function of Rac1 is its role in stimulating actin polymerization, specifically at the cell membrane to produce sheet-like protrusions of membrane, known as lamellipodia, that are thin and extensive and are made of a network of branching actin filaments (Ridley et al., 1992). Rac1 stimulates actin polymerization via phosphatidylinositol-4-phosphate 5-kinase (PIP5K), PAK and suppressor of cAMP receptor (SCAR)/Wiskott-Aldrich syndrome protein-family verprolin homology protein (WAVE) (Tolias et al., 1995). Active GTP-bound Rac1 binds to and activates PIP5K, which subsequently catalyzes the formation of phosphatidylinositol-4,5-bisphosphate (PIP$_2$). PIP$_2$ regulates actin-binding proteins, such as coflin and gelsolin, to facilitate the uncapping of actin filament barbed ends (Oude Weernink et al., 2004). SCAR/WAVE is a member of the conserved Wiskott-Aldrich syndrome protein (WASP) family of cytoskeletal regulators. The primary function of WASPs is to
stimulate actin-related protein 2/3 (Arp2/3)-dependent actin nucleation and the formation of a dendritic, branched actin filament network (Burridge and Wennerberg 2004). In the resting cell, Arp2/3 is kept in an inactive state via its subcellular localization or because its activator, SCAR/WAVE, is associated with an inhibitory protein complex. The aforementioned inhibitory complex consists of SCAR/WAVE, p53-inducible mRNA 121 (PIR121), Nck-associated protein (Nap), Abl-interactor (Abi) and haematopoietic stem progenitor cell 300 (HSPC300) (Eden et al., 2002). Following stimulation, Rac1 binds GTP, becomes active, and stimulates SCAR/WAVE/Arp-dependent actin polymerization via three potential mechanisms (Ibarra et al., 2005). One proposed mechanism is that the binding of active GTP-bound Rac1 to the inhibitory complex physically displaces SCAR/WAVE. This facilitates the subsequent interaction with and activation of Arp2/3. The other proposed mechanism is that Rac1-GTP becomes incorporated into the complex, resulting in the formation of a pentameric signaling unit. This signaling unit is proposed to subsequently activate Arp2/3. The third mechanism involves the direct interaction between GTP-loaded Rac1 and a signaling adapter, insulin receptor phosphotyrosine 53 kDa substrate (IRSp53). GTP-loaded Rac1 was found to bind to IRSp53, which functions as an adapter between Rac1 and SCAR/WAVE. Thus, GTP-loaded Rac1 binds to IRSp53, which is associated with SCAR/WAVE, and subsequently induces Arp2/3-dependent actin polymerization (Miki et al., 1998).
Figure 4. The function of Rac1 and its signaling mechanisms. A schematic summarizing the various functions of Rac1 and its respective signaling pathways. This figure is adapted from its original version in Cell (Burridge and Wennerberg 2004).
PAK modulates the cytoskeleton through its main effector LIM kinase (LIMK), and also filamin. The primary mechanism whereby PAK facilitates actin polymerization is through the phosphorylation and subsequent activation of LIMK (Edwards et al., 1999). Once active, LIMK phosphorylates and inactivates cofilin. Cofilin promotes the depolymerization of F-actin; therefore, PAK-dependent activation of LIMK functions to stabilize actin filaments. PAK also binds to and phosphorylates filamin (Vadlamudi et al., 2002). Filamin has been shown to not only support membrane protrusions through the process of crosslinking F-actin and stabilizing networks of actin, it also has a scaffold function where it recruits PAK to the membrane (Stossel et al., 2001).

Another well characterized role for Rac1 is its ability to stimulate the generation of reactive oxygen species (ROS), specifically by activating the membrane bound enzyme complex NADPH oxidase (NOX) (Brown and Griendling 2009). NOXs are transmembrane proteins that function to generate the superoxide anion radical (O$_2^•$), which is critical for the generation of various ROS. Rac1-mediated activation of NOX plays essential roles in regulating a variety of processes in cell biology, such as cytoskeletal reorganization, gene expression, proliferation, differentiation, migration and apoptosis (Bedard and Krause 2007). NOXs are multimeric enzymes. They are formed from a complex consisting of two transmembrane proteins, including p22$^{\text{phox}}$ and gp91$^{\text{phox}}$, and several cytosolic components, including p67$^{\text{phox}}$, p47$^{\text{phox}}$ and p40$^{\text{phox}}$. With the appropriate extracellular stimuli, Rac1 becomes activated and localizes with the aforementioned cytosolic components to the membrane. It then assembles an enzymatic complex with the transmembrane proteins p22$^{\text{phox}}$ and gp91$^{\text{phox}}$, which subsequently
stimulates the enzymatic activity of the complex. GTP-loaded Rac1 has been shown to be required for the activation of NOX enzymatic activity, specifically the isoforms NOX1, NOX2 and NOX3. Interestingly activation of the isoforms NOX4, NOX5 and Duox are believed to occur independent of Rac1 (Bedard and Krause 2007). Once activated, Rac1 also plays a critical role in both the activation and recruitment of cytosolic components to the membrane (Sarfstein et al., 2004; Diekmann et al., 1994). The effector region, residues 26-45, and the insert region, residues 124-135, of Rac1 are required for it to induce NOX-dependent superoxide generation (Nisimoto et al., 1997).

1.5.2 Rac1 in platelet activation

Unlike other cells of hematopoietic origin, where Rac2 is the major isoform, Rac1 is believed to be the predominant isoform in platelets. This is because it is the only isoform of Rac GTPase that has been detected in platelets (McCarty et al., 2005). The GTP-loading of Rac1 is induced by a number of platelet agonists that stimulate different classes of receptors on the platelet surface. For example, the tyrosine-kinase linked receptor GPVI and GPCRs, such as those activated by thrombin (PARs) and TXA2 (TP receptor), induce the activation of Rac1 and its translocation to the plasma membrane and where it interacts with the cytoskeleton (Soulet et al., 2001; Dash et al., 1995; Azim et al., 2000; Qian et al., 2012). Recent advancements have shed light on the mechanisms that regulate the activity of Rac1 in platelets following stimulation with agonist. Although there are over 60 known GEFs for Rho GTPases, studies of their expression and function in platelets are limited. To date, platelets have been shown to
express the following Rac-GEFs: T-cell lymphoma invasion and metastasis-inducing protein 1 (Tiam1), Vav, and phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger-1 (P-Rex1) (Aslan et al., 2011; Pearce et al., 2007; Qian et al., 2012). A role for P-Rex1 in the regulation of platelet Rac1 activity was identified using platelets from P-Rex1−/− mice, which were defective in GPCR-mediated activation of Rac1, specifically through Gq- and G12/13-coupled receptors (Qian et al., 2012). Likewise, Vav1 was also shown to regulate GPVI-induced, but not GPCR-induced, activation of Rac1 in platelets, although Vav3 may also be involved (Pearce et al., 2007; Pearce et al., 2002). Finally, Tiam1 was also shown to stimulate the GTP-loading and activation of Rac1 via a pathway involving mammalian target of rapamycin (mTOR) and 70 kDa ribosome S6 protein kinase (S6K1) (Aslan et al., 2011). Unlike RhoA, which is activated by Gα13, Rac1 is predominantly activated downstream of the Gαq signaling pathway and was shown to not be involved in platelet shape change (Gratacap et al., 2001). Likewise, agonist-induced activation of Rac1 appears to require intracellular Ca2+ signaling (Soulet et al., 2001; Gratacap et al., 2001). Also, because thrombin, ADP or the combination of ADP and U46619, a TXA2 analog that activates the TP receptor, failed to activate Rac1 in Gαq−/− mouse platelets, it appears that signaling through Gi-coupled receptors alone is insufficient to stimulate the activation of Rac1. Rather, full activation of Rac1 was shown to require Gαq-dependent signaling in addition to the release of Gi-coupled receptor agonists (granule secretion) induced by Gαq-coupled pathways (Gratacap et al., 2001; Soulet et al., 2005).

Initial studies in fibroblasts demonstrated that Rac promotes actin polymerization at the cell membrane to specifically induce lamellipodia formation and membrane ruffles (Ridley et al., 1992). Therefore, early investigations sought to verify this function in platelets. Indeed, it
was shown that Rac1, and its well known effector PAK, become activated when platelets are allowed to spread on collagen coated surfaces. Also, inhibition of either Rac1 or PAK attenuated platelet lamellae formation on collagen whereas inhibition of ROCK, a known effector of RhoA, only attenuated platelet stress fiber formation but not lamellae formation (Suzuki-Inoue et al., 2001; Vidal et al., 2002). These results implicated Rac1 in the regulation of platelet lamellipodia formation. It was subsequently shown that Rac1 is indeed involved in regulating platelet spreading and the formation of lamellipodia, specifically on VWF and fibrillar collagen but not the integrin ligands laminin and fibrinogen (McCarty et al., 2006; McCarty et al., 2005).

Exogenous GPCR agonists, such as thrombin or ADP, were required for integrin-mediated cytoskeletal rearrangement to occur when platelets were adherent to integrin ligands (McCarty et al., 2005). However, a limitation with these studies derives from the fact that the platelets themselves were unable to spread well on fibrinogen in the absence of exogenous platelet agonists. However, it was later shown that platelet spreading on fibrinogen requires Rac1, and that ligand occupancy of $\alpha_{\text{IIb}}\beta_3$ leads to the activation of Rac1 in a SFK-dependent manner (Aslan et al., 2011; Jirouskova et al., 2007). This is harmonious with a study that demonstrated that the adhesion of $\alpha_{\text{IIb}}\beta_3$-transfected CHO cells to fibrinogen lead to a 10-fold increase in Rac activation leading to lamellipodia formation (Obergfell et al., 2001). The involvement of Rac1 in integrin outside-in signaling is not limited to the early pathway mediating spreading. Rac1 also regulates the late integrin outside-in signaling pathway leading to clot retraction (Flevaris et al., 2009). Rac1 was shown to regulate integrin-mediated p38 and ERK phosphorylation, which stimulates an increase in MLC phosphorylation independent of the RhoA and ROCK pathway to promote platelet retractile function and clot retraction.
In addition to regulating the process of cytoskeletal rearrangement during platelet spreading, Rac1 is also involved in the process of granule secretion, which is critical for the secondary signal amplification of platelet activation. This is evidenced by the fact that Rac1−/− mouse platelets have a general defect in agonist-induced granule secretion, causing unstable secondary platelet aggregation (Akbar et al., 2007). Rac1 was shown to be required for granule secretion induced by all platelet agonists tested, such as ADP, thrombin and U46619. Furthermore, defects in thrombus formation in Rac1−/− mice were rescued via supplementation of exogenous granule content ADP, verifying that one of mechanisms whereby Rac1 stimulates platelet activation is via granule secretion (Pleines et al., 2009). The general role of Rac1 in stimulating platelet granule secretion may explain why platelets deficient in Rac1 have a bleeding defect, exhibit instability in aggregate formation under conditions of shear, and a severe defect in thrombus formation in vivo (Akbar et al., 2007; McCarty et al., 2005; Pleines et al., 2009).

I acknowledge that parts of this literature review (Figures 1 and 2) were adapted from its original publication in Arteriosclerosis, Thrombosis, and Vascular Biology: December 2010 - Volume 30 - Issue 12 - p 2341-2349. Wolters Kluwer Health Lippincott Williams & Wilkins©. No permission letter is needed for reuse (see APPENDIX).
2. Purpose of Study

Under the high shear-rate flow conditions present in arteries and capillaries, platelet adhesion to the injured vessel wall is mediated by the interaction between subendothelial-bound VWF and the GPIb-IX-V receptor complex (Du 2007; Lopez 1994; Ruggeri and Mendolicchio 2007). Upon adhesion, GPIb-IX initiates a signal transduction cascade culminating in the activation of integrin αIIbβ3, leading to stable platelet adhesion, spreading, and aggregation (Du 2007; De Marco et al., 1985; Grainick et al., 1985; Savage et al., 1998). This signaling mechanism is essential for thrombosis under high shear conditions and is known to involve several intracellular signaling molecules and pathways. These include the SFK Lyn, the PI3K/Akt pathway, and the cGMP and MAPK pathways (Du 2007; Garcia et al., 2005a; Li et al., 2010a; Li et al., 2001; Li et al., 2003b; Li et al., 2010b; Yap et al., 2002; Yin et al., 2008a; Yin et al., 2008b). To date, it is understood that ligand occupancy of GPIb-IX stimulates the sequential activation of Lyn, PI3K and Akt leading to cGMP-dependent activation of the MAPK pathway. The identified most proximal step to GPIb-IX that propagates this signaling cascade is the activation of Lyn and Lyn-dependent activation of the PI3K/Akt pathway; however, the exact molecular mechanisms governing this process remained unclear. The purpose of this study was to elucidate how Lyn activates the PI3K/Akt pathway following ligand occupancy of GPIb-IX. Most importantly, we hypothesized that Rac1 was involved in GPIb-induced platelet activation, mainly because it was previously demonstrated to be important for platelet spreading on VWF (McCarty et al. 2006) and arterial thrombosis in vivo (McCarty et al. 2005). We also theorized that Rac1 may be involved in the molecular mechanism linking Lyn to PI3K. In order to study the
role of Rac1 in GPIb-induced platelet activation, we generated megakaryocyte- and platelet-specific Rac1−/− mice and utilized a small molecule inhibitor of Rac1 (NSC23766) to block its activity in platelets. This allowed us to use both a genetic and pharmacological approach to study whether Rac1 is involved in GPIb-induced platelet activation and how it regulates the GPIb signaling pathway.

Another objective of our study was to elucidate the mechanisms that regulate the procoagulant activity of platelets stimulated by physiological agonists. The ability of platelets to promote thrombin generation and the deposition of insoluble fibrin at sites of vascular injury is referred to as PPA (Bevers et al., 1991). Platelets have two fundamental mechanisms that allow them to become procoagulant: PS exposure and the release of MVs (microvesiculation). The process of PS exposure and microvesiculation is believed to be dependent on Ca2+ (Bevers et al., 1982b; Thiagarajan and Tait 1991; Dachary-Prigent et al., 1995; Smeets et al., 1993; Sims et al., 1989; Wiedmer et al., 1990), the Ca2+-dependent protease calpain (Verhallen et al., 1987), TMEM16F (Yang et al., 2012), and other signaling molecules such as integrin αIIbβ3, GPVI, MAPKs, PIP2, Orai1 and STIM1 (Gemmell et al., 1993; Heemskerk et al., 2005; Flaumenhaft 2006; Gilio et al., 2010). The mitochondria are also thought to play a role (Choo et al., 2012; Jobe et al., 2008). More controversially, the apoptotic machinery has also been implicated in agonist-induced PS exposure, specifically caspases (Boing et al., 2008; Shcherbina and Remold-O'Donnell 1999). However, although the aforementioned signaling molecules are known to play a role in agonist-induced PPA, it still remains to be determined why physiological agonists alone, especially thrombin or collagen, minimally induce PS exposure and microvesiculation (Bevers et al., 1982b), even though they effectively induce platelet activation. We suggest that
a physiological parameter has been overlooked in regulating PPA, especially due to the established role of agonist-stimulated platelets and platelet-derived MVs in promoting and amplifying coagulation.

Nearly all of the studies evaluating PPA lack one essential physiological component in their experimental conditions that is present in the circulation: shear. Therefore, the purpose of our study was to determine whether shear was involved in the procoagulant response of platelets to physiological agonists. We hypothesized that shear was involved in PPA because platelets circulate in the vasculature under shear stress close to the vessel wall, and hemodynamics modulates platelet adhesion and activation. Furthermore, other studies had demonstrated that pathological shear with rates exceeding 40,000 s\(^{-1}\) induce platelet microvesiculation (Holme et al., 1997; Goto et al., 2003). GPIb-IX-dependent transient platelet adhesion under pathologically extremely high shear stress has also been shown to induce microvesiculation (Reininger et al., 2006). However, we wanted to evaluate agonist-induced PPA under the shear stress of normal blood flow independent of VWF-GPIb interaction. To do this we utilized a cone-plate rheometer to evaluate whether defined levels of shear (250 to 6000 s\(^{-1}\)) had an effect on agonist-induced PS exposure and MV release in platelet suspensions using flow cytometry. Furthermore, we evaluated whether Rac1 was involved in the procoagulant response to shear using platelet-specific Rac1\(^{-/-}\) mice and the Rac1 inhibitor, NSC23766. This allowed to evaluate whether shear was involved in the platelet procoagulant response and whether Rac1 was the signaling molecule mediating this response.
3. Methods

3.1 Generation of mice with Rac1\(^{-/-}\) platelets

Mice containing the Rac1 conditional allele (Rac1\(^{loxP/loxP}\)) were crossed with mice carrying the PF4-Cre transgene (PF4-Cre\(^{+}\))(Akbar et al., 2007; Tiedt et al., 2007). PF4-Cre\(^{+}\)/Rac1\(^{loxP/loxP}\) mice are notated as Rac1\(^{-/-}\) and PF4-Cre\(^{-}\)/Rac1\(^{loxP/loxP}\) mice as WT. Mice were maintained on a mixed SV/129/C57/Bl-6 background, and littermates were used as control. Animal usage and protocol were approved by the institutional animal care committee of the University of Illinois at Chicago.

3.2 Preparation of washed platelets

Human platelets were isolated from healthy donors as previously described with informed consent obtained in accordance with the Declaration of Helsinki (Li et al., 2003c). Institutional Review Board approval was obtained from the University of Illinois at Chicago. Fresh blood was drawn via venipuncture and anticoagulated in human acide citrate dextrose (ACD: 2.5% trisodium citrate, 2% dextrose and 1.5% citric acid) at a ratio of 1.4 mL ACD per 10 mL blood. The whole blood, containing 0.1 \(\mu\)g/mL PGE\(_1\), was centrifuged at 300g for 20 minutes at room temperature to obtain platelet-rich plasma (PRP). Platelets were then obtained via centrifugation at 1000g for 15 minutes at room temperature. Murine platelets were isolated as described previously with some modifications (Marjanovic et al., 2005). Blood was collected
from the abdominal vena cava of isoflurane-anesthetized mice into syringes containing 100 µL mouse ACD (2.5% trisodium citrate, 1.5% D-glucose, 0.4% citric acid). The whole blood was combined from multiple mice, from the same genotype if necessary, and 0.9% NaCl was added in order to achieve sufficient total volume (≥ 5 mL) prior to centrifugation. 1 U/mL apyrase and 0.1 µg/mL PGE1 was added and the blood centrifuged at 200g for 10 minutes at room temperature. The PRP was decanted, and 0.9% NaCl added to the whole blood again, which was centrifuged at 200g. The PRP from both steps was combined, 5 mM EDTA and 1 U/mL apyrase was added, and the platelets obtained via centrifugation at 700g for 8 minutes. Human and murine platelets were washed twice in CGS buffer (120 mM NaCl, 12.9 mM trisodium citrate, 30 mM D-glucose, pH 6.5) with the above described buffers at 700g for 10 minutes at ambient temperature. Washed platelets were resuspended in modified Tyrode’s buffer (12 mM NaHCO3, 138 mM NaCl, 5.5 mM D-glucose, 2.9 mM KCl, 0.42 mM NaH2PO4, 10 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) pH 7.4) to a concentration of 3x10^8/mL (unless otherwise noted), 1 mM MgCl2 and 1 mM CaCl2 was added, and the platelet suspension allowed to recover to resting state at room temperature for 1 hour. CaCl2 was not added to platelet suspensions when performing VWF-induced agglutination and aggregation in the presence of botrocetin or ristocetin. In some experiments, platelets were prepared as described previously (Lie et al., 2005), wherein platelet washing was performed using modified Tyrode’s buffer containing 5 mM EDTA (ethylenediaminetetraacetic acid) and 1 U/mL apyrase.
3.3 Platelet spreading on immobilized VWF

Spreading assays were performed as described previously (Yin et al., 2008a; Yin et al., 2008b). Briefly, microscope cover glass (Fisher Scientific) was placed in 24 well MULTIWELL plates (Becton Dickinson Falcon) and coated with 30 µg/mL purified VWF from human plasma in 0.1 M NaHCO₃ (pH 8.3) at 4°C overnight. Washed human (1x10⁷ platelets/mL) or mouse platelets (1.5x10⁷ platelets/mL) in modified Tyrode’s buffer containing 1 mM CaCl₂ and 1 mM MgCl₂ were allowed to adhere and spread on VWF-coated wells at 37°C and botrocetin or ristocetin was added. The Tyrode’s buffer was aspirated and the platelets fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS: 0.01 M NaH₂PO₄, 0.15 M NaCl, pH 7.4) for 10 minutes. Following fixation, the wells were washed twice in PBS and permeabilized in PBS containing 0.1% Triton X-100 for 5 minutes. The wells were again washed twice with PBS and the platelets stained with 1 U/mL Alexa fluor 546 labeled phalloidin (Invitrogen). Adherent platelets were viewed with a Leica DMI RB fluorescence microscope (Leica) using a HCX PLFLUOTAR 100 X 1.30 NA PH3 oil objective lens with 1.5X magnification. Images were acquired using a CoolSNAP HQ CCD camera (Photometrics) and processed with RS Image version 1.4 software (Photometrics). The spreading area of single platelets was measured using ImageJ software (National Institutes of Health) with pixel numbers as unit of size.
3.4 Fibrinogen binding assay

Buffers contained 1% bovine serum albumen (BSA) and 2 mM MgCl$_2$. Washed human (1×10$^8$/mL) and mouse (2×10$^8$/mL) platelets in modified Tyrode’s buffer were stimulated with ristocetin/botrocetin ± VWF in the presence of 5 or 2 μg/mL Oregon Green-labeled fibrinogen (Molecular Probes) for 20 or 10 minutes, respectively. Integrilin (MERCK) and RGDS peptide were used to determine the level of nonspecific binding of Oregon Green-labeled fibrinogen to the platelet surface. Platelets were diluted 1:20 in modified Tyrode’s buffer and analyzed with a BD Accuri C6 flow cytometer. Specific fibrinogen binding was determined by subtracting the geometric means of fluorescence intensity of the nonspecific binding (integrin antagonist present) from the total binding (integrin antagonist absent).

3.5 VWF binding assay

2 mM MgCl$_2$, 1% BSA, and 10 mM EDTA was added to all buffers. Washed human and mouse platelets (1×10$^8$/mL) in modified Tyrode’s buffer were incubated 5 or 10 minutes with VWF ± ristocetin/botrocetin, respectively. Platelets were fixed with paraformaldehyde (PFA) at a final concentration of 1%, prepared in HEPES-saline (2% PFA, 150 mM NaCl, 10 mM HEPES, pH 7.4) buffer for 20 minutes, centrifuged at 100g for 1 minute, and the pellet stained for 30 minutes in 0.1 mL modified Tyrode’s buffer containing 4 μg/mL SZ-29-FITC (anti-VWF antibody). Samples were diluted 1:20 with modified Tyrode buffer and analyzed using a BD Accuri C6 flow cytometer. Specific VWF binding was determined by subtracting the geometric means of
fluorescence intensity of VWF binding in the presence of botrocetin/ristocetin alone from the VWF binding in the absence of botrocetin/ristocetin.

3.6 Platelet aggregation and secretion

VWF/GPIb-IX-dependent platelet aggregation was monitored in a Chrono-log luminaggregometer at 37°C with stirring (1000 rpm) using washed human or mouse platelets (3x10^8/mL) containing 1 mM MgCl_2 (Liu et al., 2004). Ristocetin or purified botrocetin was added, following the addition of purified human VWF. Aggregation and secretion of granule ATP secretion using VWF-independent platelet agonists was studied as previously described (Stojanovic et al., 2006). Luciferin-luciferase/CHRONO-LUME reagent was used to monitor platelet secretion during aggregation. 50 and 100 μM NSC23766 was used in experiments with PAR4 peptide AYPGKF and PAR1 peptide SFLLRN, respectively.

3.7 Thromboxane generation assay

VWF-induced agglutination and aggregation of washed human and murine platelets (3x10^8 platelets/mL) was initiated) after addition of appropriate agonist (VWF in presence of botrocetin/ristocetin ± VWF). The reaction was stopped via addition of 3 mM aspirin and 10 mM EDTA at the time of full platelet aggregation (8 minutes). Samples were stored on ice, centrifuged at 6000 g for 1 minute in a microfuge, and the supernatant isolated and stored at -70°C until analysis. The TXB_2 EIA Kit (Assay Designs) was used to determine the level of TXB_2 in
each sample (Liu et al., 2005; Liu et al., 2004). TXB$_2$ is used to indicate levels of TXA2. All reagents were prepared as described in the package insert. Human platelet samples were diluted 1:50 and mouse platelet samples 1:10 or 1:20 in assay buffer. Data are from ≥3 experiments and expressed as mean ± SEM. Statistical significance was determined using the Student t test.

### 3.8 Platelet adhesion under shear stress

Platelet adhesion assays were performed as described previously (Yin et al., 2008a; Yin et al., 2008b). Briefly, glass slides were coated with VWF (30 µg/mL) in 0.1 M NaHCO$_3$ (pH 8.3) at 4°C overnight. Washed human or mouse platelets (3 x 10$^8$ platelets/mL), suspended in Modified Tyrode’s buffer containing 1 mM CaCl$_2$ and 1 mM MgCl$_2$, were treated with vehicle or inhibitors. These included dimethyl sulfoxide (DMSO) vehicle (Sigma-Aldrich), Arg-Gly-Asp-Ser (RGDS) peptide (Bachem), NSC23766 (Calbiochem) or aspirin (Sigma-Aldrich) for 5 minutes, labeled with 10 µM of the fluorescence dye mepacrine (Sigma-Aldrich), and loaded onto the slides. A cone-plate rheometer (Thermo Scientific Haake) was used to apply a constant shear rate (human: 400 s$^{-1}$; mouse 800 s$^{-1}$) to the platelets for 5 minutes. Slides were washed with phosphate-buffered saline (PBS) and viewed with a Leica DMI RB inverted fluorescence microscope (Leica Microsystems) using an N PLAN L 40X/0.55 NA [infinity]/0-2/C objective lens. Images were acquired using a Cool SNAP HQ CCD Camera (Photometrics) and processed with RS Image software, version 1.4 (Photometrics). Stable adherent platelets were counted in 10
randomly selected microscope fields. Data are representative of ≥3 experiments, and statistical significance was determined via ANOVA and posttest.

3.9 Immunoblot detection of SFKs, Vav, Akt, and p38 MAPK

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of phosphorylated signaling proteins was performed as described previously (Yin et al., 2008b). Anti-pan phospho SFK (No. 2101, Tyr416), which reacts with phosphorylated Lyn, anti-phospho P38 (No. 9211, Thr180/Tyr182), anti-phospho Akt (No. 4058, Ser473), and anti-pan Akt (No. 2920) antibodies are from Cell Signaling Technology. Anti-pan phospho Vav (sc-16408-R), anti-pan Vav (sc-55482), anti-Lyn (sc-7274), and anti-P38 (sc-535) antibodies are from Santa Cruz Biotechnology.

3.10 Immunoblot detection of activated Rac1 GTPase

To detect levels of GTP-bound Rac1, pull-down assays were performed as described previously (Akbar et al., 2007; Gong et al., 2010). Briefly, washed platelet suspensions were lysed 1:1 with ice-cold 2X lysis buffer (100 mM Tris, pH 7.2, 20 mM MgCl2, 1 M NaCl, 2% Triton X-100, 0.2% SDS, 10 μg/mL each of aprotinin and leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF)) and the lysates cleared at 22,000g for 5 min at 4°C. The supernatants were incubated with glutathione sepharose bead-conjugated GST-Rac1 binding domain fusion protein for 1 hour at 4°C with gentle rocking. The beads were washed four times with ice-cold
wash buffer (50 mM Tris, pH 7.2, 10 mM MgCl$_2$, 150 mM NaCl, 0.1% Triton X-100 with 10 μg/mL each of aprotinin and leupeptin, 1 mM PMSF), and analyzed via SDS-PAGE and Western Blot using anti-Rac1 antibody (No. 61051; BD Biosciences).

3.11 Flow cytometric analysis of PS exposure and MV release

Flow cytometry data was acquired with a FACSCalibur or Accuri C6 flow cytometer (BD Biosciences). Analysis was performed with CFlow® Plus (BD Biosciences) and FlowJo (Tree Star Inc.) software. Graphs for figures were made using FCS Express 4 (De Novo™). In all experiments, washed platelets (1 X 10$^8$/mL) were suspended in modified Tyrode’s containing 1 mM CaCl$_2$ and 1 mM MgCl$_2$. Washed platelets were stimulated with thrombin (Enzyme Research Laboratories), type-I fibrillar collagen prepared according to the manufacturer’s recommendations (Chrono-Log), or A23187 (Sigma-Aldrich) for 8 minutes. ABT-737 (Calbiochem) induced annexin V binding was performed as described previously (Schoenwaelder et al., 2009). In some experiments, platelets were stimulated with ABT-737 for various lengths of time at 37°C. A clone-plate rheometer (Thermo Scientific Haake) was used to apply shear ranging from 250 to 6000 s$^{-1}$ for 8 minutes to platelet suspensions in a 60 x 15mm polystyrene dish. Unless otherwise stated, human platelets were subjected to 1000 s$^{-1}$ and mouse platelets 3000 s$^{-1}$. Exposed PS was detected using Alexa Fluor®-488 conjugated annexin V (Invitrogen) according to the manufacturer’s specifications. Samples were diluted 1:5 to stain with annexin V conjugate for 15 minutes in the dark. Samples were immediately analyzed using flow cytometry. MVs and platelets were distinguished according to their light scattering pattern.
(Boilard et al., 2010). Size standard beads were supplied by BIOCYTEX (Megamix) to verify the size of MVs according to ISTH standards (Lacroix et al., 2010). PS-positive events were quantified by gating the fluorescent annexin V positive events in fluorescence histograms. C3-toxin (Cytoskeleton Inc.) was reconstituted in H$_2$O and incubated with platelets for 45 minutes or appropriate vehicle. Platelets were incubated with Q-VD-OPh, Z-DEVD-FMK, NSC23766 (all from Calbiochem) or DMSO control for 5 minutes.

3.12 Clotting (recalcification time)

Recalcification of citrated PRP was monitored in a cone-plate rheometer (Thermo Scientific Haake) or a whole-blood turbidimetric lumi-aggregometer (Chrono-Log). To obtain human platelet-rich plasma (PRP) and platelet-poor plasma (PPP), whole blood was anticoagulated with 3.8% citrate at 1:10 volume, and centrifuged at 400g for 30 minutes at ambient temperature to obtain PRP. PRP was centrifuged at 1200g for 20 minutes, and the supernatant further centrifuged at 2000g for 20 minutes to obtain PPP. Using a cone-plate rheometer, 250 µL of platelet suspension was pipetted onto a 60 x 15mm polystyrene dish and 6000 s$^{-1}$ shear was applied at room temperature (23°C). The time from addition of 10 mM CaCl$_2$ to the formation of an insoluble fibrin clot was indicated by a sharp increase in viscosity and defined as recalcification time. Time was stopped for samples that failed to form a clot after 60 minutes. Using the turbidimetric method, the recalcification time of PRP was determined by monitoring turbidity after addition of CaCl$_2$ at 37°C with stirring (100 rpm) using a turbidimetric lumi-aggregometer. PRP was used as the baseline reference (100% light transmittance). The
time from addition of CaCl$_2$ to the formation of an insoluble fibrin clot was indicated by a sharp increase in turbidity and defined recalcification time. 8 and 5 mM CaCl$_2$ was used to recalcify human PRP and mouse platelet supplemented human PPP, respectively.

3.13 Fluorescence intravital microscopy

Laser-induced injury of mouse cremaster arterioles was visualized microscopically in littermate WT control or platelet-specific Rac1$^{-/-}$ mice (6-8 weeks old). Mice were anesthetized via intraperitoneal injection of ketamine and xylazine and placed on a thermo-controlled blanket (37°C). The cremaster muscle was exteriorized and superfused with thermo-controlled (37°C) bicarbonate-buffered saline throughout the experiment. Fluorescence and brightfield images were recorded using an Olympus BX61W microscope with a 60 x/1.0 NA water immersion objective and a high speed camera (Hamamatsu C9300) through an intensifier (Video Scope International). Fluorescence images were captured at 20 frames per second, and data were analyzed using Slidebook v5.5 (Intelligent Imaging Innovations). Arteriolar wall injury was induced with a micropoint laser ablation system (Photonics Instruments). Platelet accumulation was visualized by infusion of Dylight 649-labeled anti-mouse CD42c (emfret analytics) at 0.05 µg/g body weight. Fibrin accumulation was visualized using Alexa Fluor® 488-conjugated anti-fibrin antibody 59D8 at 0.3 µg/g body weight, kindly provided by Dr. Hartmut Weiler, the Blood Center of Wisconsin, as described previously (Atkinson et al., 2010). Five to seven thrombi were generated in 2-4 different arterioles with a diameter of 30-45 µm in one mouse, with new thrombi formed upstream of earlier thrombi. Data were collected for 4
minutes following laser injury. ImageJ (National Institutes of Health (NIH)) was used to calculate the surface area of platelets and fibrin in the thrombus following laser-induced injury at 60, 120 and 240 seconds in approximately 30 thrombi in 3-6 mice per group.

3.14 Fluorescence microscopy following application of shear

1 mL of washed human or mouse platelets (1 X 10^8/mL) suspended in modified Tyrode’s buffer containing 1 mM CaCl₂ and 1 mM MgCl₂ were stimulated with thrombin for 8 minutes and pipetted onto 50 mm glass bottom dishes (MaTek Corporation) coated with 30 µg/mL fibrinogen. The platelet suspensions were either left static, or subjected to shear (6000 s⁻¹) using a cone-plate rheometer for 8 minutes. After the application of shear, or no shear, the platelet suspension was aspirated and the surface washed twice with annexin-binding buffer. 1 mL of annexin-binding buffer was added containing either 5 µg/mL anti-GPIb-IX antibody (SZ1) (Du et al., 1987) to label human platelets or 5 µg/mL anti-mouse αIIb (CD41) antibody (eBioscience) to label mouse platelets, and 50 µL Alexa Fluor®-488 conjugated annexin-V (Invitrogen) to label exposed PS. Staining proceeded for 20 minutes, the surface washed twice with annexin-binding buffer, and 1 mL annexin-binding buffer containing 2.5 µL of Alexa Fluor®-546 conjugated goat anti-mouse or goat anti-rat antibody (Invitrogen) was incubated for 20 minutes. The sample was washed twice with annexin-binding buffer, and 1 mL annexin-binding buffer was added for visualization using fluorescence microscopy using a Leica DMI RB fluorescence microscope (Leica) and a HCX PL FLUOTAR 100 X 1.30 NA PH3 oil objective lens. Images were acquired using a CoolSNAP HQ CCD camera (Photometrics) and processed with
Micro-Manager software (NIH). Thrombus surface area per field, and the surface area of both the red channel (anti-platelet stain) and green channel (exposed PS stain) were measured using ImageJ software (NIH). For 3D imaging of the thrombi, a Zeiss LSM510 META confocal microscope was used to obtain Z-stack images every 1.21 \( \mu \text{m} \) along the optical \( y \)-axis, with the contact of thrombus and the fibrinogen-coated slide as \( y=0 \). The Z-stack data set was analyzed and plotted using the bioView3D software (the Center for Bio-Image Informatics, UCSB).

### 3.15 Shear-induced secretion

Washed mouse platelets (1 \( \times 10^8 \)/mL) suspended in modified Tyrode’s buffer containing 1 mM CaCl\(_2\) and 1 mM MgCl\(_2\) were either left static or a clone-plate rheometer was used to apply shear (6000 s\(^{-1}\)) for 8 minutes to 250 \( \mu \)L of platelet suspension in a 60 x 15mm polystyrene dish. Samples were then transferred to a glass cuvette to measure secreted ATP using a whole-blood turbidimetric lumi-aggregometer (Chrono-Log) and the luminescent chrono-lume\(^*\) reagent (Chrono-Log).

### 3.16 Statistical analysis

Statistical analysis was performed in GraphPad Prism 5. All quantitative data are expressed as mean plus standard error of the mean (SEM), or median plus interquartile range (IQR) when the populations were not normally distributed, unless otherwise noted in the figure legend. Comparison between two groups was performed using appropriate Students t-test
based on equal variances. One-way ANOVA and Bonferroni post-test were used to compare more than two groups. The Kruskal-Wallis test and Dunn’s Multiple Comparison Test was used to compare intravital microscopy data. For all statistical comparisons: *, **, and *** represents statistical significance of $P < 0.05$, 0.01, and 0.001, respectively.
4. THE ROLE OF RAC1 IN GLYCOPROTEIN IB-IX-MEDIATED SIGNALTRANSDUCTION AND INTEGRIN ACTIVATION

4.1 Generation of megakaryocyte/platelet-specific Rac1<sup>−/−</sup> mice.

To study the role of Rac1 in GPIb-IX–induced platelet activation, we generated mice that lack expression of Rac1 exclusively in megakaryocytes and platelets and used a small-molecule inhibitor of Rac1, NSC23766. To obtain megakaryocyte- and platelet-specific conditional Rac1 knockout mice, Pf4-Cre transgenic mice were crossbred with mice containing the Rac1<sub>loxP/loxP</sub> allele (Akbar et al., 2007; Tiedt et al., 2007). Deletion of Rac1 in Rac1<sup>−/−</sup> mouse platelets was verified via Western Blot analysis of platelet lysates (Figure 5). Rac1<sup>−/−</sup> mouse platelets did not show any noticeable difference in routine hematologic parameters, including morphology and counts, compared with WT platelets. This is similar to previous reports, where Rac1<sup>−/−</sup> mouse platelets were generated using the Mx1-Cre transgene (McCarty et al., 2005; Pleines et al., 2009).

4.2 The role of Rac1 in stable platelet adhesion to VWF under shear

We initially evaluated the effect of Rac1 deficiency on GPIb-IX–induced, integrin-dependent stable platelet adhesion to VWF under shear stress, which was created using a cone-plate rheometer. As shown in Figure 5, stable adhesion of human and mouse platelets to VWF was completely inhibited by the integrin antagonist Arg-Gly-Asp-Ser (RGDS) peptide (Figure 5B-
D). This is consistent with our previous results that stable platelet adhesion to VWF under shear flow conditions requires not only GPIb-IX but also GPIb-IX-dependent activation of integrin α<sub>IIb</sub>β<sub>3</sub> (Yin et al., 2008a; Yin et al., 2008b). Stable platelet adhesion to VWF under flow is partially inhibited by the COX inhibitor aspirin, which is also consistent with our previous data that the COX pathway plays an amplifying role in promoting platelet adhesion under shear stress (Yin et al., 2008a; Yin et al., 2008b). Interestingly, stable platelet adhesion to VWF was dramatically impaired in Rac<sup>1−/−</sup> mouse platelets or NSC23766-treated human platelets (Figure 5B-D). These data indicate that Rac1 plays a critical role in GPIb-IX–mediated, integrin-dependent platelet adhesion to VWF under shear stress. Importantly, deficiency of Rac1 led to significantly greater inhibition of stable platelet adhesion than aspirin (Figure 5B–1D), indicating that the role of Rac1 in GPIb-IX–mediated platelet activation goes beyond the cyclooxygenase-dependent amplification signaling pathway.
Figure 5. The role of Rac1 in integrin-dependent stable platelet adhesion to VWF under shear. (A) Washed WT and Rac1$^{-/-}$ mouse platelets were solubilized in SDS buffer, and protein expression of Rac1 and β3 was analyzed via SDS-PAGE and Western blot. (B) Washed WT and Rac1$^{-/-}$ mouse platelets, pretreated with 2 mM RGDS or 1 mM aspirin for 5 minutes, or washed human platelets, pretreated with 0.1% DMSO, 100 μM NSC23766, 2 mM RGDS, or 1 mM aspirin for 5 minutes, were loaded onto VWF-coated glass slides in the presence of 10 μM mepacrine. Platelets were subjected to a constant shear rate for 5 minutes. After washing, adherent platelets were viewed and photographed. The number of adherent platelets per field was quantified and shown as mean ± SEM. (C) Representative images of adherent WT and Rac1$^{-/-}$ mouse platelets from (B). (D) Representative images of adherent human platelets from (B).
4.3 The role of Rac1 in platelet spreading on immobilized VWF

We next wanted to determine whether Rac1 is important for platelet spreading on immobilized VWF (Figure 6A,B). WT mouse platelets spread well on VWF, forming substantial lamellipodial structures. Although Rac1−/− mouse platelets did undergo limited GPIb-IX-induced cytoskeletal rearrangement, as evidenced by their ability to undergo filopodia extension, Rac1−/− platelets were defective in spreading on VWF and failed to form lamellipodia. Rac1 is thus critical for GPIb-IX-induced platelet spreading on VWF in mouse platelets. To determine whether Rac1 is important for the spreading of human platelets on VWF, washed human platelets were treated with either DMSO control or increasing doses of the Rac1 inhibitor NSC23766, ranging from 20 µM to 200 µM. NSC23766 dose-dependently inhibited human platelet spreading on VWF (Figure 6B-D). These data demonstrate that Rac1 is important for GPIb-IX-induced platelet spreading on VWF, and suggests that Rac1 plays an important stimulatory role in GPIb-IX induced integrin inside-out signaling.
Figure 6. The role of Rac1 in platelet spreading on immobilized VWF. (A) Washed WT and Rac1⁻/⁻ mouse platelets were allowed to adhere and spread on VWF for 120 minutes in the presence of 1 μg/mL botrocetin. (B) Washed human platelets were treated with 0.1% DMSO or 20 μM, 80 μM, and 200 μM NSC23766 for 5 minutes and allowed to adhere and spread on VWF for 60 minutes in the presence of 1 mg/mL ristocetin. Shown are representative pictures from one of 3 experiments with similar results. (C,D) Data in bar graphs represent the mean surface area (+ SEM) of individual platelets per field. A minimum of 150 platelets were sampled per treatment. Statistical significance was determined by Student’s t-test or One-way ANOVA and post-test.
4.4 **Rac1 is important for GPIb-IX-induced integrin activation**

Shear-induced platelet adhesion and spreading on immobilized VWF requires GPIb-IX–induced activation of integrin α\textsubscript{Ib}β\textsubscript{3}, which is defective in platelets deficient in Rac1. Thus, we wanted to verify that Rac1 plays a role in GPIb-IX–induced activation of integrin α\textsubscript{Ib}β\textsubscript{3}. WT and Rac1\textsuperscript{−/−} mouse platelets were stimulated with VWF in the presence of botrocetin and analyzed for the binding of Oregon Green-labeled fibrinogen using flow cytometry. VWF/botrocetin-induced fibrinogen binding was diminished in Rac1\textsuperscript{−/−} mouse platelets compared with WT platelets, indicating that Rac1\textsuperscript{−/−} mouse platelets were defective in GPIb-IX–induced activation of α\textsubscript{Ib}β\textsubscript{3} (Figure 7A and 7B). These results were recapitulated in human platelets, where VWF/ristocetin-induced fibrinogen binding was abolished by Rac1 inhibitor treatment compared with the control solvent DMSO (Figure 7C and 7D). These results demonstrate that Rac1 plays an important role in the signaling pathway of GPIb-IX–dependent integrin activation.
Figure 7. Rac1 is important for glycoprotein Ib-IX-induced integrin activation. (A) Flow cytometric analysis of the binding of Oregon Green-conjugated fibrinogen (Oregon Green-Fg) to WT and Rac1−/− mouse platelets stimulated with 2 μg/mL botrocetin (Bot.) ± 10 μg/mL VWF in the presence (gray filled) or absence (black line) of 2 mM of the integrin receptor antagonist RGDS. (B) The quantitative results from 3 independent experiments as described in (A). (C) Flow cytometric analysis of the binding of Oregon Green-Fg to washed human platelets treated with 0.1% DMSO or 100 μM NSC23766 for 5 minutes and subsequently stimulated with 0.6 mg/mL ristocetin (Rist.) and 10 μg/mL VWF in the presence (gray filled) or absence (black line) of 40 μg/mL of the integrin receptor antagonist integrilin. D, The quantitative results from 3 independent experiments as described in C. ***, **, and * represent statistical significance (P<0.0001, P<0.001, and P<0.05, respectively) as determined by Student t test. Data are shown as mean ± SEM.
4.5 **Deficiency in Rac1 does not affect the VWF-binding function of GPIb-IX**

To exclude the possibility that the functional defects observed in Rac1-deficient platelets derive from a defect in the VWF-binding function of GPIb-IX, washed WT and Rac1\(^{-/-}\) mouse platelets or washed human platelets treated with DMSO or NSC23766 were incubated with human VWF in the presence of botrocetin or ristocetin, respectively, to induce the binding of VWF to GPIb-IX. The platelets were then fixed, stained with a FITC-labeled anti-VWF antibody, and analyzed via flow cytometry. As expected, botrocetin or ristocetin induced the binding of soluble VWF to platelets. There was no difference in botrocetin-induced binding of VWF to WT and Rac1\(^{-/-}\) mouse platelets (Figure 8A and 8B) or ristocetin-induced binding of VWF to DMSO- and NSC23766-treated human platelets (Figure 8C and 8D). Therefore, Rac1 is not involved in regulating the VWF-binding function of GPIb-IX but rather is important in GPIb-IX–dependent signal transduction.
Figure 8. Platelet deficiency in Rac1 does not affect the VWF-binding function of GPIb-IX. (A) Flow cytometric analysis of VWF binding, as determined by the binding of FITC-labeled anti-VWF antibody, to washed WT and Rac1<sup>−/−</sup> mouse platelets after addition of 20 μg/mL VWF in the presence (black line) or absence (gray filled) of 1.25 μg/mL botrocetin for 10 minutes. (B) Quantification of specific VWF binding from (A). (C) Flow cytometric analysis of VWF binding to washed human platelets that were pretreated with either 0.1% DMSO or 50 μM or 200 μM NSC23766 and stimulated with 20 μg/mL VWF in the presence (black line) or absence (gray filled) of 0.5 mg/mL ristocetin for 5 minutes. (D) Quantification of specific VWF binding from (C). Data are shown as mean ± SEM and are from 3 experiments.
4.6 Rac1 is required for GPIb/VWF-mediated platelet aggregation and TXA2 production

To further confirm the role of Rac1 in GPIb-IX–mediated platelet activation, we also evaluated the effect of Rac1 deficiency on VWF-induced platelet aggregation. VWF-induced platelet aggregation is characterized by 2 distinct waves (Du 2007; Li et al., 2010a). The first wave mainly comprises VWF/GPIb-IX–dependent platelet agglutination and an often minor component of GPIb-IX–induced integrin-dependent platelet aggregation (Li et al., 2001; Li et al., 2006). The second wave represents TXA2-, secretion-, and integrin-dependent platelet aggregation. It requires GPIb-IX–induced activation of integrin αIIbβ3 and subsequent TXA2 synthesis, which initiates integrin- and TXA2-dependent granule secretion and secretion-dependent amplification of platelet activation. As expected, stimulation of WT mouse platelets with VWF/botrocetin or stimulation of human platelets with VWF /ristocetin lead to 2 waves of platelet aggregation (Figure 9A and 9B). However, Rac1−/− platelets and WT mouse platelets treated with NSC23766 had a defect in the second wave of VWF-induced aggregation (Figure 9A). Similarly, NSC23766-treated human platelets were also defective in the second wave of VWF-induced platelet aggregation (Figure 9B). Thus, these results further verify that Rac1 is required for VWF/GPIb-IX–mediated platelet activation in both human and mouse platelets. In addition, Rac1−/− mouse platelets (Figure 9C) or NSC23766-treated human platelets (Figure 9D) were defective in GPIb-IX–mediated TXA2 synthesis, indicating that Rac1 is important in the early GPIb-IX–mediated signaling pathway that is upstream of TXA2 synthesis.
Figure 9. Rac1 is required for VWF-induced platelet aggregation and TXA₂ synthesis. (A) Washed WT platelets, treated with 100 μM NSC23766 or Rac1⁻/⁻ platelets, were stimulated with 5 μg/mL and 1 μg/mL botrocetin to induce aggregation in a lumi-aggregometer. (B) Washed human platelets, preincubated with 0.1% DMSO, 100 μM NSC23766, 10 μM, or 20 μM LY294002, were stimulated with 5 μg/mL VWF and 0.3 mg/mL ristocetin to induce aggregation in a lumi-aggregometer. (C) and (D) The amount of TXB₂ was determined in WT and Rac1⁻/⁻ mouse platelets or human platelets treated with 0.1% DMSO or 100 μM NSC23766 after platelet aggregation stimulated by 1 μg/mL botrocetin or 0.3 mg/mL ristocetin, respectively, ±5 μg/mL VWF.
4.7 Rac1 is required for GPIb-IX-mediated activation of the PI3K/Akt pathway

Previous studies from our laboratory and others have shown that GPIb-IX–mediated early signaling leading to integrin activation involves GPIb-IX–dependent activation of the SFK Lyn and Lyn-dependent activation of the PI3K/Akt pathway, which subsequently stimulates the cGMP and MAPK pathways (Li et al., 2001; Li et al., 2003b; Yin et al., 2008a; Yin et al., 2008b). To determine how Rac1 is involved in GPIb-IX–mediated platelet activation, we determined whether and how loss of function of Rac1 affected this signaling pathway by evaluating VWF/GPIb-IX–mediated phosphorylation of SFKs, Akt, and p38 MAPK during the early agglutination phase of VWF-induced platelet aggregation (2-minute time point) before the second wave of platelet aggregation (Du 2007; Li et al., 2010a). VWF/GPIb-IX–induced phosphorylation of Akt was abolished in Rac1−/− mouse platelets (Figure 10A) or NSC23766-treated human platelets (Figure 10B), indicating that Rac1 is upstream of Akt in the GPIb-IX signaling pathway. Furthermore, platelet deficiency in Rac1 also abolished GPIb-IX–mediated phosphorylation of p38 MAPK (Figure 10A and 10B). These data suggest that Rac1 is required for activation of the PI3K/Akt pathway as well as the downstream p38 MAPK pathway. Consistent with the observation that Rac1 functions upstream of the TXA2 synthesis pathway (Figure 9), aspirin had no effect on GPIb-IX–mediated phosphorylation of Akt and P38, which was fully inhibited by NSC23766 in the presence of aspirin. In contrast, VWF/GPIb-IX–induced phosphorylation of SFKs was not negatively affected by deficiency of Rac1 (Figure 10A and 10B). Thus, Rac1 is not required for GPIb-IX–dependent activation of SFKs.
Figure 10. Rac1 is required for GPIb-IX-mediated activation of the PI3K/Akt pathway. (A) Washed WT and Rac1−/− mouse platelets or (B) washed human platelets treated with 0.1% DMSO, 10 μM PP2, 20 μM LY294002, 100 μM NSC23766, 1 mM aspirin (ASA), or 100 μM NSC23766+1 mM ASA were stimulated with 1 μg/mL botrocetin (Bot) or 0.3 mg/mL ristocetin (Rist), respectively, with or without 5 μg/mL VWF in a lumi-aggregometer. (A-B) The amount of phosphorylated SFK (Tyr416), Akt (Ser473), and P38 MAPK (Thr180/Tyr182) was determined using SDS-PAGE and Western blot with appropriate antibodies. Total Lyn, Akt, and P38 are shown as loading controls.
4.8 Activation of Vav and Rac1 during GPIb-IX signaling requires the SFK Lyn

To assess whether Lyn and PI3K were involved in GPIb-IX–mediated activation of Rac1, the levels of GTP-bound Rac1 in human platelets, treated with the SFK inhibitor PP2 or PI3K inhibitor LY294002, or in Lyn−/− mouse platelets were assayed after ligation of GPIb-IX. Rac1 was activated within 1 minute and remained active throughout VWF-induced platelet aggregation in WT platelets (Figure 11A). However, VWF-induced GTP loading of Rac1 was abolished in Lyn−/− (Figure 11A) and PP2-treated platelets (Figure 11B), indicating that Rac1 is activated downstream of Lyn in the GPIb-IX signaling pathway. Interestingly, VWF-induced GTP loading of Rac1 remained unaffected by LY294002 (Figure 11B and 11C), indicating that Rac1 activation occurs independent of PI3K. In fact, under the same conditions, LY294002- and NSC23766-treated platelets were both defective in GPIb-IX–mediated activation of Akt and p38 MAPK but not SFKs (Figure 10B). Taken together, these results indicate not only that Rac1 becomes activated during GPIb-IX–dependent platelet activation but also that Rac1 is activated downstream of Lyn and functions upstream of the PI3K/Akt/MAPK pathway.

Rac1 can be activated by multiple guanine nucleotide exchange factors, including Vav. Recent studies suggest that Vav can be activated by Lyn-dependent phosphorylation at Tyr174 (Pearce et al., 2004). To evaluate whether Lyn may mediate GPIb-IX–dependent activation of Rac1 via phosphorylation of Vav, we determined whether ligation of GPIb-IX induces Vav phosphorylation and whether GPIb-IX–dependent Vav phosphorylation is affected in Lyn−/− mouse platelets or PP2-treated platelets during the early phase of VWF-induced platelet agglutination/aggregation before the second wave of platelet aggregation. Indeed, ligation of
GPIb-IX induced Vav activation, which was abolished in Lyn−/− mouse platelets (Figure 11D) or PP2-treated human platelets (Figure 11E). In contrast, activation of Vav was unaffected by either LY294002 or NSC23766 (Figure 11E). Thus, Lyn mediates GPIb-IX–induced activation of Vav and Rac1, independent of the PI3K pathway. Collectively, we have demonstrated that a novel Lyn-Vav-Rac1-PI3K-Akt signaling pathway is important in the early phase of GPIb-IX–mediated signal transduction leading to platelet activation (Figure 12).
Figure 11. Activation of Vav and Rac1 during GPIb-IX signaling requires the SFK Lyn. (A) Washed WT and Lyn−/− mouse platelets, (B) washed human platelets treated with 0.1% DMSO, 10 μM PP2, or 20 μM LY294002, and (C) washed WT mouse platelets treated with 0.1% DMSO or 20 μM LY294002 were stimulated with 5 μg/mL VWF together with 1 μg/mL botrocetin (Bot) or 0.3 mg/mL ristocetin (Rist) in a lumi-aggregometer. The amount of GTP-bound Rac1 was determined using the Rac1 activation assay, SDS-PAGE, and Western blot. Total Rac1 is shown as loading control. (D) Washed WT and Lyn−/− mouse platelets or (E) human platelets treated with 0.1% DMSO, 10 μM PP2, 20 μM LY294002, or 100 μM NSC23766 were stimulated with 1 μg/mL botrocetin (Bot) or 0.3 mg/mL ristocetin (Rist), respectively, with or without 5 μg/mL VWF. The level of phosphorylated Vav (Tyr174) was determined using SDS-PAGE and Western blot. Total Vav is shown as loading control.
Figure 12. A Lyn-Vav-Rac1-PI3K-Akt pathway mediates GPIb-IX-induced platelet activation. A schematic of the GPIb-IX signaling pathway is shown. VWF binding to GPIb-IX sequentially activates Lyn, Vav, Rac1, PI3K, and Akt. Akt stimulates cGMP-dependent activation of MAPKs, leading to integrin activation and stable platelet adhesion. This further leads to activation of signal amplification pathways, in which Rac1 may also play a role. Encircled signaling molecules were investigated in this study.
4.9 Discussion

In this study, we demonstrate that Rac1 plays a critical role in stimulating GPIb-IX–dependent platelet activation. We further show that Rac1 is important in the early GPIb-IX signaling pathway, leading to activation of integrin α_{IIb}β_{3} and stable platelet adhesion under flow. Importantly, we have discovered that Rac1 and its GEF, Vav, are activated downstream from Lyn and that Rac1 is required for activating the PI3K/Akt pathway. Thus, our study not only reveals a novel role for Rac1 in platelet activation but also as an important mediator of GPIb-IX–induced, Lyn-dependent activation of the PI3K/Akt signaling pathway, leading to integrin activation.

We conclude that Rac1 is required for GPIb-IX–mediated platelet activation. This conclusion is supported by the data that Rac1^{−/−} mouse platelets and NSC23766-treated human platelets were defective in GPIb-IX–dependent integrin activation and stable platelet adhesion to VWF under flow. Furthermore, the TXA_{2}, secretion- and integrin-dependent second wave of platelet aggregation was abolished in Rac1-deficient platelets without affecting VWF binding, further supporting an important role for Rac1 in GPIb-IX–mediated platelet activation signaling. The platelet activation process induced by GPIb-IX can be divided into early-phase GPIb-IX–specific signaling events and late-phase amplification signaling pathways shared by all other platelet agonists (Du 2007; Li et al., 2010a). Several signaling molecules and events have been shown to be important in the early GPIb-IX signaling pathway leading to integrin activation and stable platelet adhesion, including the SFKs c-Src and Lyn, the PI3K/Akt pathway, [Ca^{2+}]_{i} oscillation, cGMP-dependent protein kinase, and p38/ERK MAPKs (Du 2007; Li et al., 2010a; Li
et al., 2003b; Yap et al., 2002; Garcia et al., 2005a; Wu et al., 2001; Kasirer-Friede et al., 2004; Kroll et al., 1991; Ozaki et al., 1995). Some other signaling molecules, such as components of the ITAM signaling pathway, have been shown to be important in the late signal amplification phase of GPIb-IX–induced platelet activation, which facilitates aggregation and recruitment of additional platelets to the growing thrombus (Du 2007). However, the distinct role of many other signaling molecules in either the early or late phase of GPIb-IX–induced platelet activation remains poorly characterized. This is because the aggregation response of platelets to GPIb-IX–specific early phase receptor signaling is often masked by VWF-mediated platelet agglutination, and the second phase of VWF-induced platelet aggregation is complicated by the platelet response to amplification signals induced via the integrin, ITAM, TXA2, and ADP signaling pathways. It is thus difficult to use the routine platelet aggregation assay to effectively specify the role of a particular signaling molecule in GPIb-IX–specific platelet responses. We thus determined the specific role of Rac1 in early GPIb-IX signaling by analyzing the effect of Rac1 deficiency in platelet adhesion to VWF under flow conditions. Under shear stress, stable platelet adhesion to immobilized VWF requires the early GPIb-IX–mediated signaling pathway that stimulates the inside-out activation of integrin, leading to integrin-dependent stable platelet adhesion. Stable platelet adhesion under shear stress does not require molecules important in the secondary signaling pathways, such as Syk and immunoreceptor tyrosine-based activation motif, although it is amplified by TXA2 (Yin et al., 2008a; Yin et al., 2008b). Our data demonstrate that stable platelet adhesion to VWF under shear stress was dramatically impaired in Rac1−/− mouse platelets and NSC23766-treated human platelets. Furthermore, the impairment in Rac1−/− mouse platelets and NSC23766-treated platelets is significantly greater
than the inhibitory effects of saturable concentrations of aspirin. These data indicate that the role of Rac1 in stable platelet adhesion is not limited to its role in TXA₂ synthesis and signal amplification but rather that Rac1 plays an important role in the early, TXA₂-independent GPIb-IX–dependent signaling pathway that activates α₁Iβ₃. This conclusion is further supported by the data showing that Rac1 stimulates activation of Akt and p38 MAPK independent of the TXA₂ pathway (Figure 10B) and that Rac1 is also upstream of the TXA₂ synthesis pathway (Figure 9C and 9D). Importantly, we have provided direct evidence that Rac1 is critical for GPIb-IX–dependent integrin activation (Figure 7). These results, for the first time, indicate a new small GTPase-dependent signaling mechanism in the early GPIb-IX signaling pathway, leading to integrin activation and stable platelet adhesion under shear stress. These results, however, do not exclude an important role for Rac1 also in the secondary platelet amplification pathways, such as granule secretion and integrin outside-in signaling, which have been demonstrated previously (Akbar et al., 2007; Aslan et al., 2011).

It is established that the SFK Lyn and the PI3K/Akt pathway are important in early GPIb-IX signaling (Yap et al., 2002; Yin et al., 2008a; Yin et al., 2008b; Gu et al., 1999; Liu et al., 2005). It remains unclear, however, as to how GPIb-IX activates these molecules and how these molecules are linked into a signaling pathway or network that mediates GPIb-IX signaling leading to integrin activation. Previous studies have shown the coimmunoprecipitation of GPIb-IX with PI3K and Lyn, and it was postulated that the GPIb-associated p85 subunit of PI3K functions as a scaffold that recruits SFKs to GPIb, thereby facilitating the activation of SFKs (Mu et al., 2010; Wu et al., 2003). Here, we show that GPIb-IX–mediated activation of the PI3K/Akt signaling pathway requires Rac1. We also show that GPIb-IX–mediated activation of Rac1
requires Lyn and likely involves Lyn-dependent phosphorylation of Vav. Furthermore, the catalytic function of PI3K is not required for GPIb-IX–mediated activation of Rac1, because LY294002 had no effect on GPIb-IX–dependent Rac1 activation. Thus, although we do not exclude the role of PI3K as a scaffold, our study reveals a novel signaling mechanism in which ligand binding to GPIb-IX induces the sequential activation of the SFK Lyn, Vav, and Rac1. Rac1 then mediates activation of the PI3K/Akt signaling pathway. Thus, this study represents a significant advance by identifying an important molecule in the early GPIb-IX signaling pathway leading to integrin activation.

The identification of Rac1 as a downstream effector of Lyn that stimulates the PI3K/Akt pathway is a novel finding not only to the GPIb-IX signaling pathway in platelets but also may have general implications to the mechanisms regulating PI3K activation. The SFKs Lyn/Fyn and Rac1 have both been implicated in stimulating the activity of PI3K by binding to the p85 subunit of PI3K (Pleiman et al., 1994; Bokoch et al., 1996; Tolias et al., 1995). Our data, however, indicate that Lyn activates the Rac1 GEF Vav and thus Rac1, and requires Rac1 to mediate activation of PI3K. It will be interesting to further investigate whether this new model of Lyn- and Rac1-dependent PI3K activation is a common mechanism in other cell types.

I acknowledge that this research (Figures 5, and 7 through; text verbatim from pages 83 to 103) was originally published in Arteriosclerosis, Thrombosis, and Vascular Biology: November 2012 - Volume 32 - Issue 11- p 2761-2768. Wolters Kluwer Health Lippincott Williams & Wilkins©. No permission letter is needed for reuse (see APPENDIX).
5. AGONIST-INDUCED PLATELET PROCOAGULANT ACTIVITY REQUIRES SHEAR AND A RAC1-DEPENDENT SIGNALING MECHANISM

5.1 Shear significantly enhances physiological agonist-induced platelet PS exposure and microvesiculation

Previous reports have shown that physiological agonists, such as thrombin and collagen, are weak inducers of PPA under static conditions (Alberio et al., 2000; Bevers et al., 1982a; Bevers et al., 1982b; Dachary-Prigent et al., 1993; Heemskerk et al., 2000). As platelets normally flow in blood vessels under shear stress, we studied platelet PS exposure induced by these agonists under defined flow shear rates in comparison with that under static conditions. We detected a very small number of PS-exposed events when platelets were activated by thrombin or collagen under static conditions (Figure 13A,B). Low numbers of PS-exposed events were observed even with the combination of thrombin and collagen, although more than platelets stimulated with a single agonist (Figure 13A,B). In contrast, we observed a dramatic increase in PS exposure in response to physiological agonists when platelets were subjected to shear (6000 s\(^{-1}\)) using a cone-plate rheometer. In fact, the PS-positive events reached more than 90% when platelets stimulated with thrombin + collagen were subjected to shear. Moreover, shear alone did not induce PS exposure under these conditions in the absence of agonists. Our results thus indicate that shear significantly enhances physiological agonist-induced PS exposure.

To determine whether agonist-stimulated platelets also release MVs (< 1 µm in diameter as defined by the ISTH standard (Lacroix et al., 2010)) under shear stress, we
evaluated the size of MVs in platelet suspension relative to 0.5 and 0.9 μm-diameter fluorescent beads (Figure 13C,D). Shear stress (even at 6000 s⁻¹) alone did not induce platelet microvesiculation. Thrombin stimulation alone induced the release of small amounts of MVs. When platelets were stimulated with thrombin and subjected to shear; however, a dramatic increase in microvesiculation was observed, which were associated with high levels of exposed PS (Figure 13C,D).

We further characterized the relationship between shear and the induction of PS exposure and microvesiculation in agonist-stimulated platelets. Washed mouse or human platelets were subjected to no shear or increasing levels of shear in the presence or absence of thrombin. Less than 5% of platelets showed PS exposure and microvesiculation when stimulated with thrombin in the absence of shear or when subjected to shear without thrombin (Figure 13E). In contrast, as little as 250 s⁻¹ shear led to a 3- and 5-fold increase in PS exposure and microvesiculation, respectively, in thrombin-stimulated human platelets. In mouse platelets, a 9-fold increase in PS exposure and 11-fold increase in MV release were induced by thrombin at 1000 s⁻¹ shear. Further increases in the levels of shear led to progressively increased PS exposure and microvesiculation in thrombin-stimulated platelets (Figure 13E). Thus, although thrombin induces Ca²⁺ mobilization and platelet activation under static conditions, it induces minimal PPA without shear (Sambrano et al., 2001). These results indicate that shear is a requirement for PS exposure and microvesiculation to occur in platelets stimulated by physiological agonists, and that agonist-induced PPA is shear level-dependently increased.
Shear-dependent PS exposure and microvesiculation is a common response to a variety of platelet activation pathways, as the shear-dependence is similar in platelets responding to thrombin as well as collagen or protease-activated receptor 4 agonist peptide (PAR4AP) (Figure 14). Shear also similarly enhanced PS exposure in platelets adherent to physiological integrin ligand fibrinogen, in the presence of a soluble agonist (Figure 15). Furthermore, although A23187 can induce microvesiculation and PS exposure in the absence of shear, shear even significantly amplified A23187-induced microvesiculation and PS exposure (Figure 13F, G). Together, these data indicate that shear is a critical common mechanism that promotes PS exposure and microvesiculation in activated platelets.
Figure 13. The role of shear in agonist-induced PS exposure and microvesiculation in platelets. (A) Typical flow cytometry histograms of washed mouse and human platelets stimulated with thrombin (mouse 0.05 U/mL, human 1 U/mL), collagen (mouse 2.5 μg/mL, human 6 μg/mL) or thrombin plus collagen, subjected to shear (6000 s⁻¹) or not, and stained with fluorescent annexin V. (B) Quantification of (A) (mean±SEM, n=3 for mouse; n=6 for human). (C-D) Flow cytometry analyses of platelets treated with or without thrombin (mouse 0.2 U/mL, human 10 U/mL), subjected to shear and labeled with fluorescent annexin V. 0.5 μm (black arrow) and 0.9 μm (grey arrow) standard beads mark particle sizes, and blue lines passing through the size standards define the region between 0.5 μm and 0.9 μm. (C) SSC vs FSC. (D) FSC vs annexin V fluorescence. MVs are defined as having sizes below 1 μm in diameter by ISTH consensus. (E) Washed mouse (n=3) and human (n=4) platelets were stimulated with 0.1 and 1 U/mL thrombin, respectively, or unstimulated, and subjected to increasing levels of shear. Annexin V binding and MVs were analyzed using flow cytometry (mean±SEM). (F-G) Resting or A23187 (100 nM)-stimulated mouse platelets were subjected to shear or not, and analyzed for annexin V binding and MV release. (F) Typical SSC vs FSC density plots and histogram of annexin V binding. (G) Quantification of annexin V binding and microvesiculation (mean±SEM, n=3).
Figure 14. Shear-dependent PS exposure and microvesiculation is a common response to a variety of platelet activation pathways. Washed WT mouse platelets were treated as follows: unstimulated (resting), stimulated with 50 μM PAR4 (AYPGKF), or stimulated with 2 μg/mL collagen. Platelets were subsequently left static or subjected to shear. Samples were then analyzed via flow cytometry to detect the binding of annexin V and microvesiculation (MV release). (A) Representative fluorescence histograms of annexin V binding. (B) Quantification of annexin V-binding (C) and MV release (mean±SEM, n=4).
Figure 15. Shear-dependent PS exposure during platelet adhesion in the presence of soluble platelet agonist. Washed human platelets were stimulated with 1 U/mL thrombin and allowed to adhere to immobilized fibrinogen in the absence or presence of shear, which was applied using a cone-plate rheometer. Fluorescence microscopy was used to visualize platelets and exposed PS. Platelets were detected using anti-GPIb-IX antibody (SZ1) and subsequent staining with Alexa Fluor®-546 conjugated anti-mouse antibody. Surface externalized PS was detected using Alexa Fluor®-488 conjugated annexin-V. (A) Representative images at 100X magnification with green and red fluorescence merged. Scale bar represents 10μm. (B) Quantification of the surface area of green (annexin-V) and red (GPIb-IX) fluorescence in the absence or presence of shear. (mean±SEM, n=7). One-way ANOVA was used to determine statistical significance, where *** represents P<0.0001.
5.2 Shear-induced PS exposure stimulated by platelet agonists is coupled with the release of PS-exposed MVs

To determine the relationship between PS exposure and microvesiculation under shear, we analyzed the particle size of fluorescent annexin V-binding events in platelets stimulated with thrombin and shear (Figure 16A). Events with high, low, or no annexin V-binding were gated in G1, G2 and G3, respectively, and then plotted in SSC versus FSC density plots to evaluate their respective size and density profile (Figure 16B, C). This enabled us to evaluate the contribution of platelets and MVs to the total pool of exposed PS detected under shear. Interestingly, the vast majority of the highly fluorescent, PS-positive events (Gate: G1) was associated with MVs, which was dramatically increased following thrombin/shear stimulation (Figure 16B-D). In contrast, the majority of the events that had little to no exposed PS (Gate: G3) (Figure 16B-D) were platelets. Events with intermediate fluorescence (Gate G2) (Figure 16B-D) were a mixture of platelets and aggregates. Therefore, MVs generated under shear have higher levels of exposed PS than activated platelets. These results suggest that once PS is exposed on the surface of agonist-stimulated platelets under shear, it is released as PS-exposed MVs.
Figure 16. **Shear-dependent PS exposure and microvesiculation are tightly coupled.** Thrombin-stimulated mouse (0.2 U/mL) and human (10 U/mL) platelets were subjected to shear or thrombin alone or thrombin + shear and analyzed for PS exposure (annexin V binding) and MV release using flow cytometry. (A) Histograms showing annexin V binding and gating of events with high (G1), intermediate (G2), and baseline (G3) fluorescence intensity. (B, C) SSC vs FSC density plots showing the light scatter characteristics of the ungated and gated events from either mouse (B) or human (C) platelets. (D) Quantification of platelets and MVs that were within G1, G2 and G3 gates (mean±SEM, n=3 for mouse, n=5 for human).
5.3 Shear-induced PS exposure and microvesiculation are mediated via Rac1 in activated platelets.

During our investigation into the molecular mechanisms mediating shear-dependent PS exposure and microvesiculation in platelets, we discovered that NSC23766, a small molecule inhibitor of Rac1, inhibits shear-dependent microvesiculation and PS exposure induced by thrombin and collagen (Figure 17A-C). To specifically study the role of platelet Rac1 in shear-induced PPA, we generated mice that lack expression of Rac1 only in megakaryocytes and platelets using the PF4-Cre transgene and the floxed Rac1 allele (Delaney et al., 2012). Indeed, shear-induced PS exposure and microvesiculation was nearly abolished in Rac1−/− platelets stimulated with low-dose thrombin or collagen, as compared to wild-type (Figure 17D-F). These data demonstrate that Rac1 is critical for shear-induced PS exposure and microvesiculation in platelets stimulated with physiological agonists. Interestingly, this defect was not limited to physiological agonists, but also observed with A23187 (Figure 17G-I). This is consistent with the above-demonstrated general role of shear in enhancing PS exposure and microvesiculation, and suggests that Rac1 mediates a shear-dependent common platelet microvesiculation and PS exposure pathway. High concentrations of thrombin, however, rescued the defects of Rac1−/− platelets in microvesiculation and PS exposure, indicating the presence of Rac1-independent signaling pathways under these conditions (Figure 17D-F).
Figure 17. Flow cytometry analysis of the importance of Rac1 in shear-induced PS exposure and microvesiculation in activated platelets. (A-C) Washed human platelets (n=3) preincubated with 0.1% DMSO or 100 μM NSC23766 were treated as follows: ± 0.1 U/mL thrombin, ± 5 μg/mL collagen, and ± shear. The samples were analyzed for annexin V binding and MVs using flow cytometry. Histograms (A) and quantification (B) of annexin V binding as well as quantification of MV release (C) are shown (mean±SEM, n=3). (D-F) Washed WT and Rac1−/− platelets were treated as follows: ± 0.03 or 0.1 U/mL thrombin and ± shear. Samples were analyzed using flow cytometry. (D) Histograms of annexin V binding. (E) Quantification of PS exposure. (F) Quantification of MV release. (E and F, mean±SEM, n=4). (G-I) Washed WT and Rac1−/− platelets were treated as follows: ± 2 μg/mL collagen, ± 50 nM A23187, and ± shear (n=8 and 4, respectively). Samples were analyzed using flow cytometry to detect PS exposure and MV release. (G) Histograms of annexin V binding. (H) Quantification of annexin V binding. (I) Quantification of MV release (H and I, mean±SEM).
5.4 Rac1 regulates shear-induced PS exposure independent of the apoptosis pathway

To determine whether Rac1-dependent PS exposure involves the apoptosis signaling pathway, wild-type and Rac1−/− mouse platelets were stimulated with ABT-737, a Bcl-xt inhibitor that promotes Bad/Bax-driven mitochondrial damage, activation of caspases and caspase-dependent PS exposure, (Mason et al., 2007; Schoenwaelder et al., 2009; White et al., 2012; Zhang et al., 2007) and analyzed via flow cytometry. ABT-737 dose-dependently induced PS exposure in both wild-type and Rac1−/− platelets to the same level (Figure 18A,B), which was abolished by the pan-caspase inhibitor Q-VD-OPh (Figure 18A,B), indicating that Rac1 is not required for the PS exposure induced by caspase-dependent apoptosis. Importantly, high levels of PS is predominantly exposed on platelet surface following ABT-737 treatment (Figure 18C), which is in contrast to the predominant association of externalized PS with MVs when platelets were stimulated with physiological agonists under shear (Figure 16). To further verify that Rac1 is not involved in PS exposure during apoptosis in general, Jurkat cells were treated with vehicle control, NSC23766, or Q-VD-OPh and incubated with anti-FAS to induce extrinsic apoptosis. As expected, anti-FAS treated Jurkat cells exposed PS in a dose-dependent manner, which was abolished by Q-VD-OPh (Figure 18D,E). Inhibition of Rac1 did not attenuate but enhanced FAS-induced PS exposure (Figure 18D,E). Thus, Rac1 is dispensable for apoptosis-induced PS exposure and plays a distinct role in agonist-induced PS exposure under shear.
5.5 The distinct and additive roles of Rac1 and apoptosis signaling in mediating PPA under shear

To further determine whether Rac1 and caspases represent distinct signaling pathways in shear-induced PPA, we directly compared the effects of Rac1 knockout to caspase inhibition. Thus, wild-type and Rac1\(^{-/-}\) platelets were treated with Q-VD-Oph, the caspase-3 inhibitor Z-DEVD-FMK or DMSO control, activated with low to high doses of thrombin (0.02 to 0.2 U/mL) and subjected to shear (Figure 18F,G). As described above, low dose thrombin-induced, shear-dependent PS exposure and microvesiculation was predominantly Rac1-dependent, and the role of caspases was marginal (Figure 18F,G). Interestingly, the marginal inhibitory effect of the caspase inhibitors was retained with increasing thrombin concentrations, whereas the effect of Rac1 knockout was diminished at high thrombin concentrations (Figure 18F,G). At an intermediate concentration of thrombin (0.05 U/mL), PS exposure and MV release in Rac1\(^{-/-}\) platelets were about 20% and 15% of control wild-type platelets, which was further inhibited by caspase inhibitors to near zero, suggesting that the Rac1 pathway is distinct from the caspase-dependent apoptosis pathway and that these two pathways play additive roles in the process. This was further demonstrated at very high concentrations of thrombin, where microvesiculation and PS exposure were no longer Rac1-dependent but the partial inhibitory effects of caspase inhibitors remained similar. Together, our data suggest that the Rac1-dependent pathway of microvesiculation and PS exposure is distinct from the apoptosis pathway and specifically responsible for the agonist-stimulated shear-dependent platelet response, whereas the caspase pathway plays a minor role in this response.
Figure 18. Rac1 regulates shear-induced PS exposure independent of caspase-induced PS exposure. (A-C) WT and Rac1−/− platelets were pretreated with 0.1% DMSO or 20 μM Q-VD-OPh, stimulated with 0.1 or 1 μM ABT-737, and analyzed using flow cytometry to detect PS exposure. (A) Representative histograms of annexin V binding. (B) Quantification of total PS positive events (mean±SEM, n=3). (C) Percentage of platelets that bound annexin V (annexin V+) (mean±SEM, n=3). (D, E) Jurkat cells were treated with 0.1% DMSO, 20 μM Q-VD-OPh or 100 μM NSC23766, stimulated with 0.1 or 1 μg/mL Anti-FAS, and analyzed via flow cytometry for PS exposure. (D) Representative histograms of annexin V binding. (E) Quantification of annexin V-positive events (fold increase relative to the baseline (mean±SEM, n=7). (F, G) Washed WT and Rac1−/− mouse platelets treated with 0.1% DMSO, 20 μM Q-VD-O-ph or 40 μM Z-DEVD-FMK (in DMSO) were stimulated with increasing doses of thrombin, subjected to shear, and analyzed for (F) PS exposure and (G) MV release (mean±SEM, n=4). The response of thrombin-stimulated WT platelets treated with DMSO was defined as 100% for each thrombin dose.
5.6 **Rac1 regulates shear-induced PPA independent of its role in stimulating platelet secretion and aggregation**

Rac1 promotes platelet secretion and aggregation (Figure 19), as published previously (Akbar et al., 2007). Thus, it is possible that the stimulatory effect of Rac1 in the process of shear-dependent PPA is consequential to increases in platelet secretion and aggregation. If this were true, molecules that similarly promote thrombin-induced platelet aggregation should also promote thrombin-induced PPA under shear. Figure 20A shows that C3-toxin, an inhibitor of another Rho GTPase, RhoA, attenuates low dose thrombin-induced platelet aggregation similar to the Rac1 inhibitor or Rac1−/− platelets (Akbar et al., 2007). This result recapitulates the dose-dependent defect of RhoA−/− platelets in thrombin- and PAR4-induced platelet aggregation (Pleines et al., 2012). In contrast, while inhibition of Rac1 blocked shear-induced PS exposure and microvesiculation in thrombin-stimulated platelets (Figure 20B), inhibition of RhoA had no inhibitory effect (Figure 20B). Rather, C3-toxin significantly enhanced shear-induced PS exposure and microvesication (Figure 20B). Thus, Rac1 and RhoA similarly upregulate thrombin-induced platelet secretion and aggregation but play opposing roles in shear-induced PPA, suggesting that platelet aggregation per se is unlikely to be the reason for the role of Rac1. To exclude the possibility that the shear- (and thus Rac1-) dependent PPA is consequential to increased platelet granule secretion, we examined the relationship of shear with granule secretion and PS exposure/microvesiculation. Without platelet agonists, shear alone (up to 6000 s−1) neither induces significant granule secretion nor PS exposure/microvesiculation (Figure 20C). Under static conditions, thrombin induced significant granule release, but
minimally induced PS exposure. In the presence of shear, however, thrombin induced a
dramatic increase in PS exposure although levels of granule secretion were similar to that in the
absence of shear. Thus, shear (and thus Rac1-) dependent platelet PS exposure and
microvesiculation is unlikely to be caused by increased granule secretion. Indeed, the inhibitory
effect of the Rac1 inhibitor NSC23766 on platelet aggregation was corrected by supplementing
the granule content ADP (1 μM) (Figure 20D), indicating that Rac1-dependent ADP secretion is
responsible for enhanced platelet aggregation (Akbar et al., 2007). In contrast, supplementation
of 1 μM ADP failed to correct the inhibitory effect of NSC23766 on PAR1AP- and PAR4AP-
induced microvesiculation under shear (Figure 20E). Thus, the role of Rac1 in promoting shear-
induced PPA appears to be independent of its role in promoting platelet secretion and
aggregation.
Figure 19. Rac1−/− mouse platelets have attenuated platelet aggregation and secretion. Washed WT and Rac1−/− mouse platelets were stimulated with 0.02 U/mL thrombin, 50 μM PAR4 activating peptide (AYPGKF) or 1 μg/mL collagen and platelet aggregation and ATP secretion was monitored in a turbidimetric lumi-aggregometer over time.
Figure 20: Rac1 regulates shear-induced PS exposure and microvesiculation independent of its role in stimulating platelet secretion and aggregation. (A) Representative aggregation and ATP secretion traces of washed human platelets activated with 0.035 U/mL thrombin following treatment with 2 μg/mL C3-toxin, 100 μM NSC23766 or vehicle control. (B) Washed human platelets were treated with vehicle, 2 μg/mL C3-toxin (C3) or 100 μM NSC23766 (NSC), stimulated with 0.025 U/mL thrombin, subjected to shear, and analyzed for PS exposure and MV release. Data are expressed as mean±SEM, n=3. (C) Quantification of ATP secretion (left Y-axis) and PS exposure (right Y-axis) in washed mouse platelets, either left static or subjected to shear, in the presence or absence of thrombin (0.03 U/mL) (mean±SEM, n=3). (D) Aggregation of NSC23766- or DMSO-treated human platelets following stimulation with 60 μM AYPGKF (PAR4) or 2 μM SFLLRN (PAR1) in the presence or absence of 1 μM ADP. 1 μM ADP was added alone as a control. (E) Platelets treated under the same conditions as (C) were analyzed for the fold increase in shear-induced MV release from the resting baseline (mean±SEM, n=8).
5.7 The role of Rac1 in coagulation in vitro and in vivo.

To determine whether Rac1-dependent PS exposure occurs during thrombus formation under shear, wild-type and Rac1−/− platelets were stimulated with thrombin and subjected to shear over a fibrinogen-coated surface (Figure 21). Indeed, wild-type platelets formed large thrombi with associated exposed PS, as indicated by annexin V binding. 3D confocal imaging of thrombi suggests that the strongest PS labeling was mainly located in the lower flanks of a thrombus near the bottom. Notably, Rac1−/− platelet thrombi showed reduced size and diminished PS exposure (Figure 21A-D). These data indicate that shear-dependent PS exposure occurs in this in vitro thrombus formation model, and Rac1 is important in the process.

To determine whether the role for Rac1 in stimulating platelet PS exposure and microvesiculation is important to coagulation, we tested the effect of the Rac1 inhibitor (NSC23766) on the clotting time of citrated human PRP induced by adding back CaCl₂ (recalcification time) under stirring conditions (Figure 22A), which generate shear, or under defined shear using a cone-plate rheometer (Figure 22B). The Rac1 inhibitor significantly delayed clotting upon recalcification (Figure 22A, B). Similarly, platelet-depleted human plasma supplemented with Rac1−/− platelets showed a significant delay in clotting compared to platelet-depleted human plasma supplemented with wild-type platelets (Figure 22A). These results suggest that Rac1, by stimulating shear-dependent PS exposure and microvesiculation, plays an important role in facilitating coagulation under shear.

To investigate whether the role of Rac1 in stimulating shear-induced PPA is important for fibrin formation in vivo, platelet-specific Rac1−/− mice were compared to wild-type controls in
a model of laser-induced cremaster arteriolar thrombosis. Wild-type mice rapidly formed platelet-rich thrombi (Figure 22C, D) at the site of vascular injury. Initially (60-120 seconds), fibrin was only associated with a small region of the developing platelet thrombus. Later (240 seconds), the platelet thrombi lacking associated fibrin embolized amid the growing fibrin clot resulting in the overlap and surrounding of platelet thrombi by fibrin (Figure 22C, D). Platelet-specific Rac1−/− mice were defective in fibrin accumulation (Figure 22C-E). Although platelet thrombus formation was also reduced in platelet-specific Rac1−/− mice (Figure 22C-E), (McCarty et al., 2005) the fibrin clot was much smaller than the platelet thrombus at the early stages of clot formation (60 seconds), indicating that the initial defect in fibrin formation is not the result of a lack of platelet thrombi (Figure 22C,D: 60 seconds). Later, the platelet thrombi in Rac1−/− mice shrank and were overlapped with and surrounded by the fibrin clot in a manner similar to wild-type mice (Figure 22C,D: 240 seconds) but at a much reduced size. Together, these results suggest that platelets promote coagulation in vivo via a Rac1-dependent mechanism and that Rac1-dependent fibrin clot formation is important in stabilizing platelet thrombi and thus determining the ultimate size of stabilized platelet thrombi in vivo.
Figure 21. Rac1 is important for the exposure of PS during thrombus formation *in vitro* under shear. Washed WT and Rac1−/− platelets were stimulated with 0.03 U/mL thrombin and subjected to shear (6000 s⁻¹) over a fibrinogen-coated surface. Platelets (red) were stained using anti-mouse CD41 and Alexa Fluor®-546 conjugated anti-rat antibodies. Surface exposed PS (green) was stained using Alexa Fluor®-488 conjugated annexin V. (A) Representative fluorescence microscopy images showing wild-type and Rac1−/− platelet thrombi (red) and exposed PS associated with thrombi (green). Scale bar represents 10 µm. (B) Quantification of surface area of thrombi per field (mean±SEM, n=7). (C) Quantification of the annexin-V surface area per field (mean±SEM, n=7). (D) Representative 3D reconstruction of confocal Z-stack images of WT and Rac1−/− platelet thrombi and exposed PS. Scale bar represents 10 µm.
Figure 22. Platelet Rac1 is important in promoting fibrin generation *in vitro* and *in vivo*. (A) The mean recalcification time $\pm$ SEM of human citrated PRP treated with 100 μM NSC23766 (n=20) or vehicle control (n=10), and of washed WT and Rac1$^{-/-}$ mouse platelets (n=5) resuspended in citrated human PPP after addition of CaCl$_2$ under stirring conditions. (B) The recalcification time of human citrated PRP treated with 100 μM NSC23766 or DMSO was monitored using a cone-plate rheometer under defined shear (6000 s$^{-1}$) after addition of CaCl$_2$ (mean±SEM, n=4). (C-D) Intravital microscopy was used to monitor fibrin generation (green) and platelet thrombi (red) *in vivo* following laser-induced cremaster arteriole wall injury in WT and platelet-specific Rac1$^{-/-}$ mice. (C) The sizes of the platelet thrombus and fibrin clot were compared by calculating their respective median surface area at 60 and 240 seconds (median+IQR). (D) Representative images of fibrin generation (green) and platelet thrombi formation (red) and merged images. Arrows indicate direction of blood flow. (E) The median of the total fluorescence detected over time for fibrin or platelets (PLTs) is shown, calculated by integrating the area under the curve for 30 thrombi in WT and platelet-specific Rac1$^{-/-}$ mice (median+IQR). The median integrated fluorescence signal of fibrin (F) and platelets (G) from 30 thrombi in WT and platelet-specific Rac1$^{-/-}$ mice is shown as a function of time.
5.8 Discussion

In this study, we identified a mechanism that explains a longstanding enigma that, despite the known importance of activated platelets in facilitating coagulation, physiological platelet agonists (thrombin or collagen) poorly induce a procoagulant response in platelet suspensions (Heemskerk et al., 2002; Heemskerk et al., 2005; Heemskerk et al., 2000; Lhermusier et al., 2011). To date, platelets had to be stimulated simultaneously with extremely high doses of multiple platelet agonists to induce marginal levels of PS exposure and microvesiculation. Here we show that platelets become potently procoagulant by releasing MVs with high levels of exposed PS when stimulated with low doses of physiological agonists, but only in the presence of physiological levels of shear found in flowing blood. Although it was previously known that extremely high shear causes platelet microvesiculation and PS exposure during VWF/GPIb-IX-dependent adhesion and aggregation (Miyazaki et al., 1996; Reininger et al., 2006), physiological levels of shear in normal blood flow clearly do not induce PPA. However, we show that when platelets are activated by common physiological stimuli, this normally innocuous level of shear becomes a major factor that promotes PS exposure and the tightly coupled release of PS-exposed MVs independent of VWF-GPIb-IX interaction. Shear significantly enhances PPA even at very low concentrations of agonists, and increases PS exposure and microvesiculation by an order of magnitude even when platelets are stimulated by the combination of the most potent physiological platelet agonists, thrombin and collagen (Dachary-Prigent et al., 1993). Moreover, shear even enhances A23187-induced PS-exposure
and microvesiculation. Thus, we introduce a previously unidentified common requirement for shear in platelet PS exposure and microvesiculation.

Another important discovery of this work is that agonist-induced, shear-dependent PPA requires platelet Rac1, as platelet-specific knockout of Rac1 impairs agonist-induced, shear-dependent platelet PS exposure and microvesiculation leading to a defect in coagulation \textit{in vitro} and \textit{in vivo}. Furthermore, the effect of platelet-specific Rac1 knockout excludes the otherwise likelihood that NSC23766 nonspecifically attenuates agonist-induced PPA under shear. Rac1-dependent PS exposure is distinct from the known pathway of apoptosis-induced PS exposure. This conclusion is supported by the following evidence. First, shear-dependent PS exposure and microvesiculation is only slightly inhibited by caspase inhibitors, indicating that the capase-dependent apoptotic pathway is only a minor component of shear-dependent PS exposure and microvesiculation induced by platelet agonists, even though these agonists are known to activate the caspase-dependent apoptotic pathway (Leytin et al., 2006). Secondly, the minor effects of caspase inhibitors and potent effect of Rac1 deficiency are additive. Thirdly, Rac1 deficiency had no effect on apoptosis-mediated PS exposure, suggesting that the apoptotic pathway does not involve Rac1. Importantly, a notable difference between shear/Rac1-dependent and apoptosis-induced PS exposure is that shear/Rac1-dependent externalized PS is predominantly associated with MVs (>95%), indicating a close link between PS exposure and microvesiculation. In contrast, a significant portion of the apoptosis-related PS is associated with platelets or cells (Figure 4). This difference makes sense physiologically, as apoptotic cells expose PS to initiate their own phagocytosis, whereas platelets release procoagulant MVs to maximize the efficiency of coagulation not only on platelet surfaces but also in liquid phase at
the site of vascular injury. This major feature of shear-dependent PS exposure is consistent with previous knowledge that platelet-derived MVs have high procoagulant activity (Dachary-Prigent et al., 1993), and explains previous reports suggesting that fibrin formation is not necessarily associated with the site of platelet thrombus formation in vivo (Jasuja et al., 2010; Vandendries et al., 2007).

Several factors that are identified to be associated with platelet PS externalization and MVs so far have also been known to stimulate platelet granule secretion and aggregation. These include caspases (Cohen et al., 2004), intracellular calcium (Dachary-Prigent et al., 1995), calcium channels (Harper et al., 2013), and Rac1, as described here. Thus, it is necessary to determine whether their role in PS exposure and microvesiculation can be secondary to their role in platelet activation (Akbar et al., 2007). Our data indicate that shear/Rac1-dependent platelet PS exposure and MV release is not associated with platelet granule secretion and aggregation. Importantly, supplementation of the granule content ADP to Rac1-inhibited platelets rescued platelet aggregation but failed to rescue shear-induced PPA. Furthermore, shear did not affect thrombin-induced granule secretion but dramatically enhanced PPA. These data suggest that the role of Rac1 in mediating shear-dependent platelet PS exposure and microvesiculation is likely to be independent of its role in promoting platelet secretion and aggregation (Akbar et al., 2007). Importantly, consistent with these in vitro observations, we have further shown that the in vivo fibrin clot size in arterial flow is independent of the size of platelet thrombi. In contrast, no matter how large the platelet thrombus size, platelet thrombi are not stable without fibrin deposition. At the later stage in thrombosis, platelet thrombi not associated with the fibrin clot eventually embolize. Thus, the smaller fibrin clot in platelet-
specific Rac1−/− mice arterioles results in smaller ultimate platelet thrombi compared to wild-type. Hence, our data suggests that platelet-dependent Rac1 function facilitates coagulation in vivo and that platelet-associated fibrin helps to stabilize platelet thrombi.

Taken together, we have made a significant new finding that shear stress represents a previously unidentified critical factor that is required for PS exposure and microvesiculation stimulated by physiological platelet agonists independent of VWF-GPIb-IX interaction. Furthermore, we have discovered an important role for Rac1 as a novel mediator of shear-dependent PS externalization and the shedding of PS-exposed MVs, and in promoting coagulation in vitro and in vivo. These advancements have important general implications to the cardiovascular field, cell biology, and the development of new treatments for hemostatic, thrombotic and inflammatory disorders.

I acknowledge that this research (Figures 13 through 22; text verbatim from pages 104 to 133) was originally published in Blood Online: Agonist-induced platelet procoagulant activity requires shear and a Rac1-dependent signaling mechanism. Delaney MK, Liu J, Kim K, Shen B, Stojanovic-Terpo A, Zheng Y, Cho J and Du X. 2014 July 25 pii: blood-2014-03-560821. No permission letter is needed for reuse (see APPENDIX).
6. Conclusions

The results of our work provide a significant advancement to the field of thrombosis and hemostasis, with particular interest in the mechanisms regulating GPIb-IX-induced signal transduction in addition to agonist-induced platelet procoagulant activity. The objective of the first study (Chapter 4) was to elucidate how ligand occupancy of GPIb-IX leads to Lyn-dependent activation of the PI3K/Akt pathway. Furthermore, we investigated whether Rac1 was involved in GPIb-induced platelet activation and whether it plays a role in this signaling mechanism. Using platelet-specific Rac1$^{−/−}$ mice and the Rac1 inhibitor, NSC23766, we learned that Rac1 is required for GPIb-induced platelet activation. Loss-of-function of Rac1 in platelets abolished stable platelet adhesion to VWF under shear, spreading on VWF, GPIb-induced activation of integrin, and VWF-induced aggregation and TXA$_2$ synthesis independent of the ligand-binding function of GPIb-IX. We also characterized the molecular mechanism that explains these defects. We show that ligand occupancy of GPIb-IX leads to Lyn-dependent activation of Vav and Rac1, and that Rac1 activates the PI3K/Akt pathway to promote GPIb-induced platelet activation (Figure 23 Part I). Thus, we have discovered a novel role for Rac1 in platelet activation and further clarified the molecular mechanism of how Lyn mediates GPIb-IX-induced platelet activation.

Lyn, Fyn and Rac1 are known to stimulate the kinase activity of PI3K by binding to its p85 subunit (Pleiman et al., 1994; Bokoch et al., 1996; Tolias et al., 1995). However, our data indicate that GPIb-induced activation of PI3K occurs indirectly through Lyn, as we show that Lyn requires Rac1 to mediate activation of PI3K. This suggests that Rac1 binds to PI3K to stimulate
its kinase activity following ligand occupancy of GPIb-IX. This should be further investigated, which may be done via Western Blot to evaluate whether PI3K and Rac1 may be co-immunoprecipitated following VWF-GPIb interaction. We also show that the Rac1 GEF, Vav, is activated downstream of Lyn independent of PI3K and Rac1. This suggests that Vav activates Rac1 in this signaling pathway, but this is not entirely clear. Further experiments should be performed to evaluate the role of Vav in GPIb-induced platelet activation, and to verify that Vav is upstream of the newly established Rac1/PI3K/Akt/MAPK pathway of GPIb-induced platelet activation. Particular interest should be focused on the role of Vav1 and Vav3, of which knockout mice are available, as they were previously shown to play critical (and redundant) roles in platelet activation (Pearce et al., 2004). Furthermore, Vav not only acts as a GEF for Rac1, but also RhoA. Therefore, it is also possible that RhoA plays a role in GPIb-induced platelet activation. To this extent, platelet-specific RhoA−/− mice have also been recently generated and it was shown that RhoA is important for platelet activation (Pleines et al., 2012). Taken together, these future studies will build upon the discoveries made here and certainly shed further light on the mechanisms regulating GPIb-induced platelet activation.

We have also made an important contribution to the understanding of the processes regulating platelet hemostatic function, otherwise referred to as PPA. PPA is mediated by PS exposure and the release of platelet MVs. A longstanding dogma in the field is that physiological agonists are very weak inducers of PPA, and that multiple agonists must be used simultaneously at very large doses to induce PPA. The objective of our second study (Chapter 5) was to challenge this dogma and to determine why in over 30 years of research no one has answered the question as to why platelet agonists are ineffective inducers of PPA, especially
when they are able to effectively induce platelet activation. We discovered that platelet agonists are indeed potent inducers of platelet PS exposure and MV release, but only in the presence of shear. Furthermore, we show that agonist-stimulated platelets dose-dependently respond to shear to expose PS and shed MVs. Importantly, the levels of shear evaluated are physiological and do not induce PPA in the absence of agonists. We also discovered that when exposed to shear, agonist-stimulated platelets expose PS that is immediately released on the surface of platelet MVs and minimally retained on the platelet surface. We also discovered that Rac1 plays a general role in regulating the procoagulant response to shear independent of its known role in stimulating platelet secretion and aggregation and the apoptosis pathway. Finally, we demonstrate that platelets promote coagulation in vivo via a Rac1-dependent mechanism and that Rac1-dependent fibrin clot formation is important in stabilizing platelet thrombi and thus determining the ultimate size of stabilized platelet thrombi in vivo.

These findings represent a significant advance to our field. They explain why investigators for years have been unable to induce PS exposure and MV release in agonist-stimulated platelets. However, it still remains unclear as to how Rac1 mediates the procoagulant response to shear. There are several likely downstream candidates. Rac1 is known to activate PAK and the NOX isoforms NOX1, 2 and 3. Therefore, it is suggested that the role of PAK and NOXs in the platelet procoagulant response to shear is further evaluated. Perhaps the most likely candidate is TMEM16F. TMEM16F was recently shown to be required for agonist-induced platelet PS exposure independent of the apoptosis pathway and to promote coagulation (Yang et al., 2012; van Kruchten et al., 2013), which is similar to the role we established for Rac1 in this study. Furthermore, Rac1 is recruited to the platelet membrane
upon activation, indicating that it may come in close proximity of TMEM16F. Interestingly, no one has evaluated whether TMEM16F promotes the release of platelet MVs during platelet activation. Its role in platelet PS exposure has only been studied. Given that platelet PS exposure and MV release are highly related processes, it would be interesting to further evaluate the role of TMEM16F in agonist-induced MV release in addition to PS exposure. Most importantly, whether TMEM16F is involved in the platelet procoagulant response to shear is certainly warranted, given that we have established that this process is important for coagulation in vivo.

Our results demonstrate that Rac1 has many functions in platelets, which suggests that it may be a useful drug target to prevent thrombosis. However, the problem with most antithrombotics is that they lead to bleeding diathesis. This is because antithrombotics often inhibit not only thrombus formation but also blood coagulation (Shen et al., 2013). To this extent, we have shown that Rac1 is important not only for thrombosis but also hemostasis; thus, pharmacological blockade of Rac1 may be associated with adverse bleeding. However, an interesting observation is that Rac1 promotes platelet secretion and aggregation independent of its regulation of shear-induced PPA. Thus, it is attractive to speculate that you could pharmacologically target the pathway regulating Rac1-dependent thrombus formation yet still allow for coagulation to occur but at a reduced rate. This may produce a result similar as what we observed in vivo using platelet-specific Rac1−/− mice. These mice were able to form small thrombi following laser-induced injury to cremaster arterioles, suggesting that a small molecule inhibitor of Rac1 would not prevent adhesion and allow thrombi to form but at a significantly reduced rate and final size. Most importantly, platelet-dependent Rac1 function was shown to
reduce fibrin formation at the site of injury, but partially, not completely. Thus, it is theoretically possible that pharmacological blockade of Rac1 would prevent pathological thrombus formation but still allow platelets to adhere to and seal the wound, and coagulation would still occur following vessel injury but at a significantly reduced rate. In theory, this would produce a similar effect as a newly reported integrin antagonist, which inhibited arterial thrombosis without the adverse effect of bleeding (Shen et al., 2013).
Figure 23. The role of Rac1 in platelet signal transduction and function. A summary of the novel roles of Rac1 in GPIb-induced platelet activation and agonist-induced platelet procoagulant activity established in this study. (Part I) Rac1 is required for GPIb-induced platelet activation. Rac1 is activated downstream of Lyn and Vav, and stimulates the PI3K/Akt pathway. (Part II) Platelet agonists potently induce procoagulant activity, but only in the presence of shear. Rac1 mediates the procoagulant response to shear in platelets, independent of the apoptosis signaling pathway.
7. CITED LITERATURE


Moake, J. L., N. A. Turner, N. A. Stathopoulos, L. Nolasco, and J. D. Hellums. 1988. Shear-induced platelet aggregation can be mediated by vWF released from platelets, as well as by exogenous large or unusually large vWF multimers, requires adenosine diphosphate, and is resistant to aspirin. *Blood* 71 (5):1366-74.


Shattil, S. J., C. Kim, and M. H. Ginsberg. 2010. The final steps of integrin activation: the end game. Nat Rev Mol Cell Biol 11 (4):288-300.


Approval Notice
Continuing Review (Response To Modifications)

November 27, 2013

Xiaoping Du, MD, PhD
Pharmacology
835 S. Wolcott Avenue
403-E M.S.B., M/C 868
Chicago, IL 60612
Phone: (312) 355-0250 / Fax: (312) 996-1225

RE: Protocol # 1999-0610
“Mechanisms of Platelet Activation”

Dear Dr. Du:

Your Continuing Review (Response To Modifications) was reviewed and approved by the Expedited review process on November 22, 2013. You may now continue your research.

Please note the following information about your approved research protocol:

**Protocol Approval Period:** December 4, 2013 - December 4, 2014
**Approved Subject Enrollment #:** 1500
**Additional Determinations for Research Involving Minors:** These determinations have not been made for this study since it has not been approved for enrollment of minors.
**Performance Sites:** UIC
**Sponsor:** NIH, NHLBI/NIH, NIH-National Institutes of Health
**PAF#:** 2011-02188,2010-00091,2009-05545
**Grant/Contract No:** HL080264,R01 HL062350, 3R01HL068819-08S1
**Grant/Contract Title:** Outside-in signaling mechanisms of platelet integrin alpha-llb-beta3, Signaling mechanism of platelet glycoprotein 1b-IX, The cGMP-dependent protein kinase pathway in platelets

**Research Protocol:**
- a) Mechanisms of Platelet Activation, Version 4, 11/05/2012

**Recruitment Materials:**
- a) Flyer, "Volunteers Needed...," Version 2, 03/12/2002
- b) Protocol #1999-0610 - Advertisement for mass mail by Internet Version 2 05/06/2004
- c) Advertisement for mass mail by Internet for sickle cell research group, Version 1, 01/06/2011
- d) Flyer "Sickle cell patients needed to volunteer for research study", Version 1, 01/06/2011
Informed Consents:

a) "Mechanisms of Platelet Activation," Version 9, 11/16/2010
b) Consent/authorization for sickle cell patients, Version 1.0, 01/07/2011

HIPAA Authorization: Continue to use the following document as it has not expired.


Your research meets the criteria for expedited review as defined in 45 CFR 46.110(b)(1) under the following specific category:

(2) Collection of blood samples by finger stick, heel stick, ear stick, or venipuncture as follows:
(a) from healthy, nonpregnant adults who weigh at least 110 pounds. For these subjects, the amounts drawn may not exceed 550 ml in an 8 week period and collection may not occur more frequently than 2 times per week; or
(b) from other adults and children, considering the age, weight, and health of the subjects, the collection procedure, the amount of blood to be collected, and the frequency with which it will be collected. For these subjects, the amount drawn may not exceed the lesser of 50 ml or 3 ml per kg in an 8 week period and collection may not occur more frequently than 2 times per week.

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Please remember to:

⇒ Use your research protocol number (1999-0610) on any documents or correspondence with the IRB concerning your research protocol.
⇒ Review and comply with all requirements on the enclosure, "UIC Investigator Responsibilities, Protection of Human Research Subjects" (http://tigger.uic.edu/depts/ovcr/research/protocolreview/irb/policies/0924.pdf)

Please note that the UIC IRB has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

Please be aware that if the scope of work in the grant/project changes, the protocol must be amended and approved by the UIC IRB before the initiation of the change.
We wish you the best as you conduct your research. If you have any questions or need further help, please contact OPRS at (312) 996-1711 or me at (312) 355-1609. Please send any correspondence about this protocol to OPRS at 203 AOB, M/C 672.

Sincerely,

Rahab Mwangi, MPH
IRB Coordinator, IRB # 3
Office for the Protection of Research Subjects

Enclosures:

1. **Informed Consent Documents:**
   a) "Mechanisms of Platelet Activation," Version 9, 11/16/2010
   b) Consent/authorization for sickle cell patients, Version 1.0, 01/07/2011

2. **Recruiting Materials:**
   a) Flyer, "Volunteers Needed....," Version 2, 03/12/2002
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      05/06/2004
   c) Advertisement for mass mail by Internet for sickle cell research group,
      Version 1, 01/06/2011
   d) Flyer "Sickle cell patients needed to volunteer for research study", Version 1,
      01/06/2011

cc: Asrar Malik, Pharmacology, M/C 868
    OVCR Administration, M/C 672
May 12, 2014

Xiaoping Du
Pharmacology
M/C 868

Dear Dr. Du:

The protocol indicated below has been reviewed in accordance with the Institutional Biosafety Committee Policies of the University of Illinois at Chicago on 3/13/2014. The protocol was not initiated until final clarifications were reviewed and approved on 5/7/2014. Protocol expires 3 years from the date of review (3/13/2017). This protocol replaces protocol 11-014 which has been terminated.

Title of Application: Signaling Mechanisms of Platelet Activation

IBC Number: 14-008

Highest Biosafety Level: 2

Condition of Approval: The enclosed report indicates the training status for bloodborne pathogen (BBP) training. Only those personnel who have been trained and whose training has not expired are approved for work that may involve exposure to bloodborne pathogens. Please note that federal regulations require yearly training for BBP.

You may forward this letter of acceptable IBC verification of your research protocol to the funding agency considering this proposal. Please be advised that investigators must report significant changes in their research protocol to the IBC office via a letter addressed to the IBC chair prior to initiation of the change. If a protocol changes in such a manner as to require IBC approval, the change may not be initiated without IBC approval being granted.

Thank you for complying with the UIC’s Policies and Procedures.

Sincerely,

Randal C. Jaffe, Ph.D.
Chair, Institutional Biosafety Committee

RCI/mbb

Enclosures

Cc: IBC file, Aleksandra Stojanovic-Terpo
March 28, 2014

Xiaoping Du
Pharmacology
M/C 868

Dear Dr. Du:

The protocol indicated below was reviewed at a convened ACC meeting in accordance with the Animal Care Policies of the University of Illinois at Chicago on 2/18/2014. The protocol was not initiated until final clarifications were reviewed and approved on 3/28/2014. The protocol is approved for a period of 3 years with annual continuation.

**Title of Application:** Signaling Mechanisms of Platelet Activation

**ACC Number:** 14-017

**Initial Approval Period:** 3/28/2014 to 2/18/2015

**Current Funding:** Portions of this protocol are supported by the funding sources indicated in the table below.

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This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare (OLAW), NIH. This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the funding proposal are matched to this ACC protocol.

In addition, all investigators are responsible for ensuring compliance with all federal and institutional policies and regulations related to use of animals under this protocol and the funding sources listed on this protocol. Please use OLAW’s “What Investigators Need to Know about the Use of Animals” (http://grants.nih.gov/grants/olaw/InvestigatorsNeed2Know.pdf) as a reference guide. Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

[Signature]

Bradley Merrill, PhD
Chair, Animal Care Committee

BM/mbb

cc: BRL, ACC File, Aleksandra Stojanovic, PAF 201000091, 20110218, 201300887
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NAME: Michael Keegan Delaney

EDUCATION: Ph.D. in Pharmacology, University of Illinois at Chicago, Illinois. 2014

B.S. in Biology, DePaul University in Chicago, Illinois. 2006

B.S. in Environmental Science, DePaul University in Chicago, Illinois. 2006

TEACHING EXPERIENCE: Teaching Assistant: Department of Environmental Science, DePaul University in Chicago, Illinois. Laboratory for Undergraduate Education in Environmental Science. 2005

RESEARCH EXPERIENCE: Graduate Research Assistant: Department of Pharmacology, University of Illinois at Chicago, Illinois. 2007-2014 Laboratory of Dr. Du


Research Assistant: DePaul University, Chicago, Illinois. Department of Biological Sciences. 2005 Laboratory of Dr. Sparkes

Research Assistant: DePaul University, Chicago, Illinois. Department of Environmental Science. 2003-2004 Laboratory of Dr. Montgomery and Dr. Eames

GRANTS: American Heart Association Predoctoral Fellowship 2011-2013 National Institutes of Health Predoctoral Fellowship 2008-2010

HONORS: Travel Award for Young Investigators 2013. Arteriosclerosis, Thrombosis, and Vascular Biology

Achievement Award from Dr. Albert and Doris Woeltjen 2012. University of Illinois at Chicago.

Travel Award for Young Investigators 2012. Arteriosclerosis, Thrombosis, and Vascular Biology.

Abstract Achievement Award 2011. American Society of Hematology
Achievement Award from Dr. Albert and Doris Woeltjen 2011. University of Illinois at Chicago

Dean’s Award for Scholastic Excellence 2005. DePaul University

Teacher Assistant Recognition Award in Environmental Science 2005. DePaul University

Research Award in Environmental Science 2005. DePaul University

Participation Award for DePaul University Science, Math, and Technology Showcase 2005. DePaul University

Outstanding Junior Award in Environmental Science 2004. DePaul University

**ORAL PRESENTATIONS:**


**POSTER PRESENTATIONS:**

“Shear-Dependent Membrane Scrambling and Microvesiculation and an Important Role for Rac1.” 55th Annual Meeting of the American Society of Hematology in Dallas, TX. 2013. Dec. 8-11.

“A Role for Rac1 in Glycoprotein Ib-IX-Mediated Signal Transduction and Integrin Activation.” 14th Biennial Midwest Platelet Conference in Cleveland, Ohio. University Hospitals Case Medical Center and Case Western Reserve University School of Medicine. 2012. Oct 18-19th
“An Important Role for Rac1 in Glycoprotein Ib-IX Induced Platelet Activation.” Annual retreat: UIC Department of Pharmacology 2012. Lake Lawn Lodge, Delavan, WI.


“An Important Role for Rac1 in Glycoprotein Ib-IX Induced Platelet Activation.” Annual retreat: UIC Department of Pharmacology 2011. Lake Lawn Lodge, Delavan, WI.


“The small GTPases Rac1 and RhoA regulate platelet microparticle formation, phosphatidylserine exposure, and procoagulant activity.” Annual retreat: UIC Department of Pharmacology 2010. Lake Lawn Lodge, Delavan, WI.


“The role of Rac1 and RhoA in platelet-derived microparticle formation and phosphatidylserine exposure.” Annual retreat: UIC Department of Pharmacology 2009. Lake Lawn Lodge, Delavan, WI.

CONFERENCES ATTENDED:

Biennial Midwest Platelet Conference in Cleveland, Ohio. University Hospitals Case Medical Center and Case Western Reserve University School of Medicine. Oct 18-19th 2012.

Arteriosclerosis, Thrombosis, and Vascular Biology 2012 Scientific Sessions in Chicago, IL, April 17-20.

52nd Annual Meeting of the American Society of Hematology in Orlando, FL, Dec. 4-7, 2010.

American Heart Association Scientific Sessions in Chicago, IL. Nov. 13–17, 2010


American Society for Pharmacology and Experimental Therapeutics, Annual Great Lakes Chapter Meeting. Chicago Cultural Center, Chicago, June 13th, 2008.

2nd Annual Chicago Area Undergraduate Research Symposium, Cultural Center, Chicago, April 1st, 2006.


26th Annual Society of Wetlands Scientists International Conference, Charleston, South Carolina, June 6th, 2005.

**PUBLISHED ABSTRACTS:**


Delaney MK and Du X. “An Important Role for Rac1 in Glycoprotein Ib-IX Induced Platelet Activation.” *Blood.* 2011; 118: 3250.


**PUBLISHED PAPERS:**


