SIGNALING MECHANISMS ASSOCIATED WITH THE PLATELET COLLAGEN RECEPTORS

By

Robin James Marjoram

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Approved:

Samuel A. Santoro, M.D., Ph.D.

Mary M. Zutter, M.D.

David B. Friedman, Ph.D.

Billy G. Hudson, Ph.D.

W. Gray Jerome, Ph.D.

Andries Zijlstra, Ph.D.

To my friends and family that have truly supported me during this journey.

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CHAPTER I

OVERVIEW

Cellular adhesions are important interactions formed between a cell and a neighboring cell or between a cell and the extracellular matrix (ECM). These cellular connections contribute to the form and function of cells in metazoans. In this dissertation, I use the interaction of platelets (a type of blood cell) with collagens (ECM proteins), an interaction vital in hemostasis, as a model of cellular adhesion. To analyze this interaction, I examined the roles of the two surface receptors for collagens on platelets: $\alpha_2\beta_1$ integrin ($\alpha_2\beta_1$) and glycoprotein VI (GPVI)/Fc receptor γ-chain (FcRγ) complex. The two central questions I address are: 1) What are the contributions of integrins on the cell-surface to cellular adhesion to the ECM? 2) Is cellular adhesion to the ECM through integrins influenced by the activation of other cell-surface receptors such as G protein-coupled receptors (GPCRs)? My studies on $\alpha_2\beta_1$, GPVI/FcRγ, and GPCRs address these questions and include novel observations on the interactions of platelets with collagens that could have broader implications on cell adhesion in general.

Through my studies, I identified a novel role of platelet surface receptors (GPCRs and GPVI/FcRγ) in increasing platelet adhesion to collagens. The increase in platelet adhesion occurs through a priming mechanism of $\alpha_2\beta_1$ that is stimulated by $G\alpha_q$-linked GPCRs or GPVI/FcRγ and increases the resting platelets' adhesiveness (avidity) towards collagens through phospholipase C (PLC) signaling pathways. Mechanistically, modulation of integrin avidity on the cell surface can happen in several ways: changing

the affinity of individual integrins for their ligand, increasing the surface expression of the integrin, or altering the number of integrins participating in the ligation without increasing the total surface population. The priming mechanism of $\alpha_2\beta_1$ stimulated by suboptimal activation of $G\alpha_q$-linked GPCRs seems to be an actin-guided redistribution of the integrin on the platelet surface before the integrin is converted into its high affinity conformer. This effect of $\alpha_2\beta_1$ on platelets could have relevance in other cell types for increasing the adhesiveness during formation of cell adhesions or in strengthening preexisting adhesions. The stimulated increase in platelet avidity for collagens occurs independently of integrin activation but does seem to be associated with a change in cell morphology of the platelet and a physical connection between $\alpha_2\beta_1$ and the actin cytoskeleton.

I postulate that the priming mechanism of $\alpha_2\beta_1$ on platelets modulates platelet avidity for collagens through a redistribution of $\alpha_2\beta_1$ on the cell surface that is directed by the actin cytoskeletal dynamics associated with reshaping of the cell. This priming mechanism of $\alpha_2\beta_1$ could apply to other types of integrins and have relevance in adhesions formed by migrating cells (e.g. leukocytes and metastatic cancer cells) as well as the dynamic maintenance of adhesions in non-migrating cells (e.g. muscle cells and endothelial cells). It is also intriguing to consider the application of this paradigm to adhesive interactions formed by cells within a developing embryo where cell-cell and cell-ECM interactions are present in a continuously changing environment.

My identification and analysis of this $\alpha_2\beta_1$ priming mechanism in platelet adhesion to collagens, as well as my studies on the role of GPVI/FcRγ in this interaction, are detailed in chapters II-VI. An introduction to hemostasis and platelet adhesion to the

ECM at vascular wound sites and the key surface receptors involved in this complex process are highlighted in chapter II. I show in chapter III that $\alpha_2\beta_1$-mediated platelet adhesion to collagens can be increased through suboptimal activation (levels where $\alpha_{IIb}\beta_3$ is not activated) of $G\alpha_q$-linked GPCRs (specifically protease-activated receptor 4; PAR4) utilizing a PLC-dependent signaling pathway, and the material in chapter III is adapted from my paper published in the *Journal of Biological Chemistry*. I further analyze this PAR4-induced $\alpha_2\beta_1$ priming mechanism of platelet adhesion to collagen in chapter IV and show that PAR4 induces a modulation of $\alpha_2\beta_1$ avidity that is not caused by a change in $\alpha_2\beta_1$ conformation or its affinity for collagen. Rather, this modulation seems to be linked with a possible change in $\alpha_2\beta_1$ valency through a transient association with the platelet actin cytoskeleton. In chapter V, I analyzed aspects of GPVI/FcRγ-mediated platelet processes involved in hemostasis and thrombosis and show the importance of GPVI/FcRγ as the primary signaling receptor for collagen and show that direct activation of GPVI/FcRγ by convulxin can cause enhanced $\alpha_2\beta_1$-mediated platelet adhesion. I conclude in chapter VI by discussing future research directions and proposing an amended mechanism for platelet adhesion to collagens. I believe this work will have broader implications in biology in general and in the design of better anti-platelet drugs.

CHAPTER II

INTRODUCTION

Blood is a vital tissue of vertebrates that exists as a colloidal fluid composed of cells (erythrocytes, leukocytes, and platelets) suspended in plasma, a complex aqueous solution of salts and organic molecules. The liquid state of blood and an intact vasculature through which it circulates are critical for normal functions and viability of an organism. Platelets are crucial elements for maintaining an intact circulatory system, but also have a significant role in thrombotic pathologies. This dissertation aims to determine the contributions that the two platelet surface receptors, $\alpha_2\beta_1$ integrin ($\alpha_2\beta_1$) and glycoprotein VI (GPVI)/Fc receptor γ-chain (FcRγ) complex, make toward the platelet's interaction with collagens, specifically collagen type I, at vascular wound sites. A discussion is included on platelet adhesion and signaling involved in hemostasis and thrombosis, substrate/integrin interactions, and anti-platelet therapies.

Hemostasis and Thrombosis

Maintenance of intact and unblocked blood vessels is vital to sustain life in humans and other vertebrates. The blood's fluidity enables the heart to pump and circulate it through the series of tubes made up of arteries, veins, and capillaries that form the vascular network. This network provides direct and indirect links to all the cells of the body, which is necessary for transport of nutrients, oxygen, and molecular signals to the cells and to remove cellular secretions like metabolic waste (carbon dioxide).

Perfusion of blood throughout the tissues of an organism is critical for cell survival and normal tissue function. When perfusion is stopped through arteries or veins, the organism must restore the ability of blood to permeate into the damaged tissue or pathologies can develop such as myocardial infarction, stroke, or venous thrombosis causing morbidity or mortality.

Hemostasis is the process that has evolved in vertebrates to stop hemorrhage due to vascular injury by producing a thrombus, or blood clot, at the wound site and involves an intricate balance between antithrombotic and prothrombotic cellular and molecular components (1,2). The cellular components in this complex process include blood cells, primarily thrombocytes (also called platelets), the endothelial cells (ECs) that form the luminal surface of the vascular wall, and the smooth muscle cells (SMCs) that form the muscular lining of blood vessels (Figure 2-1A). Under normal blood circulation conditions, ECs lining the blood vessels provide a smooth surface for the blood to flow over as well as produce antithrombotic agents, which create an inhibitory environmental barrier for thrombus formation. Some antithrombotic agents ECs produce are nitric oxide (NO), prostacyclin (PGI_2), and ecto-ADPases (CD39) to inhibit platelet activation as well as thrombomodulin (TM) and heparan sulfate to modulate the coagulase activity of thrombin (2,3). Upon vascular injury, endothelial cells are damaged causing a change in their cellular activities that promotes vasoconstriction by SMCs; platelet adhesion, activation, and aggregation; thrombin activation and fibrin formation and the underlying extracellular matrix (ECM) of the vessel wall is exposed, which all promote the production of a thrombus at the wound site (2,4).

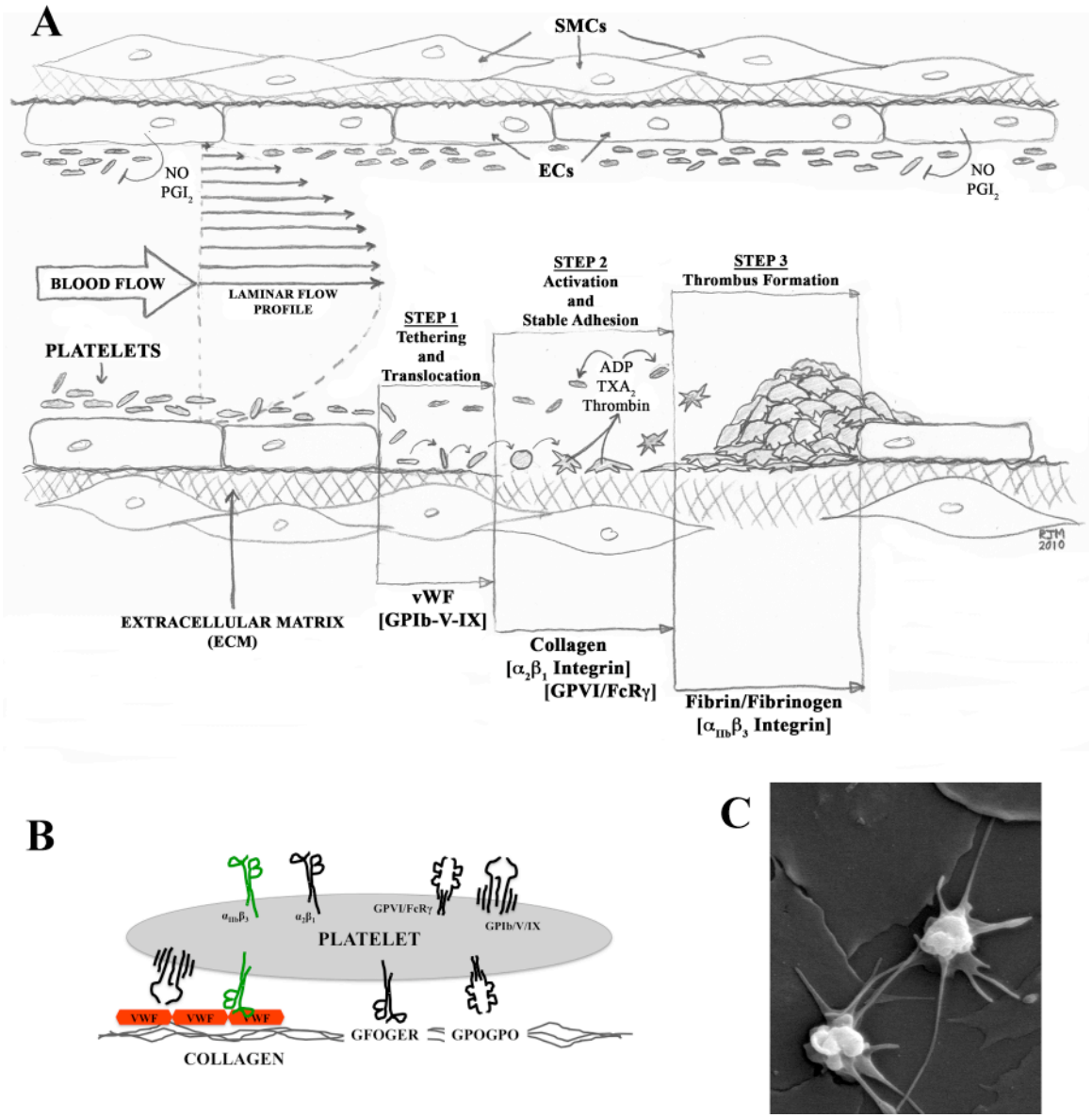

FIGURE 2-1: **Arterial hemostasis and platelet adhesion to collagen.** *A*, Arterial hemostasis is a complex process involving multiple steps in forming a stable thrombus at the site of the vascular injury under the shear stresses created by laminar flow of blood. Platelets are forced to the periphery of the vessel where they normally flow over the endothelial layer in inactive state supported by secretion of inhibitors (NO and PGI_2). At vascular injury sites, endothelial cells are damaged causing exposure of the subendothelial ECM, which platelets interact with in three steps that involve ligation of adhesion receptors on the platelet surface. These interactions as well as the actions of other platelet activators (ADP, TXA_2, and thrombin) form a stable platelet aggregate at the wound site. *B*, Platelet adhesion to collagen involves the direct interaction of $\alpha_2\beta_1$ and GPVI/FcRγ with specific collagen binding sites. Indirect platelet adhesion to collagen occurs through the association of VWF with collagen that allows GPIb/V/IX and $\alpha_{IIb}\beta_3$ to bind VWF. *C*, Scanning electron micrograph of human platelets adhering to collagen I and illustrating the dramatic morphological changes platelets undergo during the adhesive process.

Thrombus development intimately involves platelets and the blood coagulation system, however the role platelets serve is dependent on where the thrombus formation occurs in the vasculature. Platelets are more relevant for thrombus formation in arteries and capillaries where blood flow conditions produce higher shear stresses and are less relevant for venous thrombus formation, which seems to rely more on the coagulation pathway (1,5). When the prothrombotic activity outweighs the antithrombotic activity, hemostasis is induced at the site of the vessel injury and proceeds through three successive but integrated steps (Fig. 2-1A) (2,6-8).

The first step in hemostasis involves the translocation of platelets over the exposed ECM through the interaction of the platelet receptor GPIb/V/IX complex with von Willebrand factor (VWF). VWF is a large soluble serum protein that can form polymers and bind to collagen I contained in the ECM, which immobilizes VWF and, with shear stress alters its conformation to create a surface that platelets can loosely interact with (9). This labile interaction causes the platelets to roll across the vessel subendothelium and decelerate from the rate of the blood flow (6,10,11). The tethering of platelets promotes binding of their collagen receptors, $\alpha_2\beta_1$ and GPVI/FcRγ, to collagen I and leads to step 2: stable adhesion and activation of the platelets (6,7,12,13).

Step 2 involves platelet adhesion and activation at the vascular wound site and this part of hemostasis will be the focus of this dissertation. Stable adhesion of platelets to the ECM immobilizes them and promotes their activation through agonists in the microenvironment (collagen I, thrombin, ADP, and thromboxane A_2). Evidence suggests that the stable adhesion of platelets to subendothelial collagens especially under high shear stress conditions is mediated by $\alpha_2\beta_1$, whereas the activation of platelets is

transduced through GPVI/FcRγ signaling. However, the exclusivity of these roles and the sequence of the collagen interactions of $\alpha_2\beta_1$ and GPVI/FcRγ between themselves and with other surface receptors ($\alpha_{IIb}\beta_3$ and GPIb/V/IX) have cause for dispute in the field (Figure 2-1B). Platelet activation is characterized by a change in cell morphology, activated $\alpha_{IIb}\beta_3$, generation and release of thromboxane A_2 (TXA_2), secretion of dense and α-granules, and microvesicle formation that lead to step 3: thrombus formation (5,14,15).

Thrombus formation is the third step in hemostasis and involves platelet aggregation and fibrin production. Thrombin proteolytically cleaves serum fibrinogen into fibrin that polymerizes to form an insoluble network of fibers (16). The aggregation of platelets predominantly occurs through integrin ligation, primarily by activated $\alpha_{IIb}\beta_3$ binding to fibrinogen and fibrin, which crosslinks platelets to produce the blood clot (5,15). This is the current model of hemostasis but there are still significant gaps in knowledge of how the hemostatic process works. Under normal hemostatic conditions, all of the above processes of thrombus formation are spatially and temporally contained to the wound site, but when this does not occur, pathologies can develop (4).

Thrombosis is the pathological formation of a blood clot within the vascular system and is caused by a dysregulation in the hemostatic processes. Like hemostasis, thrombosis involves the same elements used in clot formation, but unlike hemostasis, the clot production exceeds normal limits and causes problems by obstructing or fully occluding an artery or vein. These aberrant clots can produce severe pathologies such as myocardial infarction (heart disease) or stroke, which are the first and third leading causes of death in the U.S., respectively (17). Both diseases account for a large

percentage of the national health expenditure and it is estimated that the direct and indirect costs for cardiovascular disease (CVD) and stroke in the United States in 2010 will total $503.2 billion (17,18). For these reasons, it is important to understand the mechanisms involved in hemostasis and thrombosis, so that better treatments and preventatives can be developed.

Blood Rheology

Blood rheology (i.e. the physics of blood flow) has a significant impact on cellular and molecular events during both normal and pathological thrombus development (19). The focus here will be on the characteristics of blood flow found in arterial hemostasis and thrombosis as this is where platelets have a crucial role because of the higher flow rates and shear stresses present in these vessels.

The fluid dynamics of liquid blood are defined as non-Newtonian, where the liquid viscosity is dependent on the shear rates between adjacent fluid layers in a laminar flow system due in part to the different cell types that create a complex viscosity profile (19). However, if analysis is focused at the vascular wall where platelets are selectively concentrated by the laminar flow, the dynamics can be described as a simple Newtonian fluid and independent of the liquid viscosity (19,20). Under these parameters, the laminar flow of blood through an artery produces a flow profile having the maximum flow velocity at the center of the vessel that decreases to the slowest velocity at the wall (Figure 2-1A). The difference in flow velocities between parallel fluid layers produces a shear stress (the force per unit area between adjacent fluid layers) and a shear rate (the relative change in velocity between adjacent fluid layers) both of which are highest at the

blood/artery wall interface (19). Shear rates at the arterial wall are in the range of 1,000-10,000 s^{-1} and can be significantly increased at sites of pathological stenoses caused by an occlusive thrombus or atherosclerotic plaque (shear rates > 10,000 s^{-1}) (21). Stenosis of an artery also produces turbulent flow that diverges from laminar flow producing zones of flow deceleration and acceleration, streamline separation, and flow vortices that impact thrombus development (19).

Blood rheology is a significant factor in regulating platelet adhesion, aggregation, and thrombus formation. A study using rabbit blood flowed over aortic subendothelium showed a direct dependence on shear rate and thrombus formation (22). This dependence on shear rate was also evident in a study on patients' blood who suffered from platelet aggregation diseases: Bernard-Soulier Syndrome (BSS) (loss of platelet glycoprotein (GP) Ib-V-IX complex), Glanzmann's thrombasthenia (GT) (loss of platelet $\alpha_{IIb}\beta_3$ integrin), type 3 von Willebrand disease (VWD) (loss of von Willebrand factor (VWF)), and congenital afibrogenemia (Af) (loss of fibrinogen) (23). This study showed that when blood from a normal donor was flowed over a subendothelial ECM protein (collagen) platelet adhesion and aggregation was enhanced with increasing shear rate. Using blood from BSS, GT, vWD, or Af patients, they further showed a role for the platelet receptor GPIb-V-IX interaction with VWF in initiating and promoting stable platelet adhesion to collagen as well as a stabilizing role for the interaction between platelet $\alpha_{IIb}\beta_3$ integrin and fibrinogen in platelet aggregation (23). Another study identified platelet aggregation to be shear gradient-dependent during *in vivo* thrombus development at wound sites (24). There is still a lot to be learned about what effects the

physical forces of blood flow have on thrombus development but it is clear that platelets play a vital role in the hemodynamic environment.

The Vascular Subendothelial Extracellular Matrix and Collagens

Upon vascular injury, the subendothelial ECM, which is normally masked by the ECs, is exposed to the blood. The rheologic properties of blood and the forces produced by its flow promote the interaction of the unmasked ECM with platelets. This interaction is crucial for the development of a platelet thrombus at an arterial wound site.

The subendothelial ECM of an artery is an organized and very insoluble structural support system composed of various proteins that the cells interact with through surface receptors. The wall contains two types of ECM: the basement membrane and the interstitial ECM. The basement membrane (BM) directly underlies the ECs lining the luminal surface of the blood vessel and is comprised mainly of collagens (type IV, XV, and XVIII), laminin, nidogen, and perlecan (25,26). Below the BM, the interstitial ECM envelops the SMCs and is made up of fibrillar collagens (type I and III), fibronectins, tenascins, vitronectin, proteoglycans, and elastin (25). Fibrillar collagen type I is the most abundant protein in humans and is prevalent in the ECM of the arterial wall where it is known to be a potent activator of platelets (27,28). When ECs are damaged, these subendothelial ECM proteins including collagen I are exposed to the blood and help stimulate thrombus formation.

Collagens are a family of 28 extracellular matrix proteins that exist as trimers of α-chains that contain triple helical domains. The collagen α-chains have amino acid sequences consisting of regions of a repeating glycine-X-Y motif (-GXY-; X and Y being

any amino acid) where oftentimes proline (P) is at the X position and 4-hydroxyproline (O) is at the Y position (29). The repetitive -GXY- motif as well as the association of the noncollagenous domains promotes the formation of the triple helical protomer that can further oligomerize to form suprastructures such as fibrils, networks, and microfibrils (30). Collagen I and III are fibril-forming collagens where as collagen IV of the BM forms a two-dimensional network, and all serve to help construct particular microenvironments for cellular functions (29,30). When these ideally positioned collagens (I, III, and IV) in the vessel wall are exposed to blood at vascular injury sites, platelets and serum proteins are able to interact with them and help activate hemostasis (Figure 2-1A and B).

The Platelet

The first accurate description of platelets from human blood was by Max Schultze in 1865; he described platelets as normal constituents of blood that are colorless little spherules with the potential to clump and extend protrusions in response to the coagulation of fibrous material (31). Schultze realized the importance of these spherules and recommended further study of this blood component. In an 1882 paper, Giulio Bizzozero further supported the idea that platelets were a normal cellular component of circulating blood, but most importantly through *in vivo* and *in vitro* microscopic analyses, he determined that platelet adhesion and aggregation at vascular wound sites was involved in thrombus formation (31). These groundbreaking studies elucidated the platelet's function, and since then the platelet has been a major focus of studies on hemostasis and thrombosis.

Under normal conditions, platelets circulate in blood as small discoid, anuclear cytoplasts (average d × h of 3.0 × 0.5μm) at a concentration range of 150-350 × 10^9 platelets/L for about 10 days before being removed by the spleen or liver (14,32). Platelets are produced by megakaryocytes in the bone marrow through a unique terminal differentiation event where the megakaryocyte transforms into a very large cell with a polyploid nucleus and highly active gene transcription and translation that sheds many cytoplasts into the blood stream (33). The amount of platelets in circulation is critical for its function in hemostasis and is precisely controlled through the hormone, thrombopoietin (TPO), by regulating the proliferation and maturation of megakaryocytes (32,33). During platelet formation, the megakaryocyte packages about 10^3 platelets with all the organelles, granules, and molecular components necessary to perform their cellular functions (34).

Inactive discoid platelets circulate through about 60,000 miles of blood vessels in the human body until they come into contact with a vascular injury site. At arterial injury sites, platelets are forced by the characteristics of blood flow to interact with the damaged region (Figure 2-1A). This causes platelets to weakly adhere and translocate over the injury site leading to deceleration and formation of stable adhesions that promote platelet activation and secretion followed by platelet aggregation and thrombus formation (6,15). Within the region of injury, platelets are exposed to multiple cell activators, which are secreted by endothelial cells and activated platelets or deposited from the serum. However, the serum coagulase, thrombin, and fibrillar collagens of the subendothelial ECM are arguably the most potent activators of platelets. Upon platelet activation, the cell morphology rapidly and dramatically changes from the inactive discoid to a sphere

that extends filopodia which the platelet mass spreads out over through dynamic rearrangement of the actin cytoskeleton (Figure 2-1C). These morphological changes of the platelet result in coverage of the wound site by the platelet plug to prevent any further blood loss from the vasculature.

Along with cell morphology changes, activated platelets secrete molecules that serve to promote and amplify hemostasis by recruiting more platelets to the wound site (Figure 2-1A). Activated platelets generate thromboxane A_2 (TXA_2) in their cytoplasm by a cyclooxygenase-1 (COX-1)-dependent mechanism and release TXA_2 by passive diffusion across the plasma membrane to the extracellular space where it activates platelets further (1). Other molecules are released by platelets through three types of secretory vesicles: α-granules, dense granules, and lysosomes (35). The α-granules contain a variety of soluble and membrane-associated proteins such as adhesion molecules (P-selectin, vWF, $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ integrins, and fibrinogen), chemokines (CXCL4, CCL5, and CXCL8), coagulation factors (Prothrombin, factor V and VIII), fibrinolytic factors (antithrombin III, plasminogen, and PAI-1) as well as many other proteins (35,36). The dense granules hold small molecules like ions (Ca^{2+} and Mg^{2+}), nucleotides (ADP and ATP), and serotonin and have a lot fewer proteins associated with them (35). The lysosomes in platelets are homologous to lysosomes of other cell types and contain acid hydrolases and cathepsins (35). The dense and α-granules are found only in megakaryocytes and platelets, and the secretion of these granules by platelets is important in promoting their hemostatic functions.

Platelet Surface Receptors

For platelets to interact with agonists and substrates within the wound environment, a variety of cell surface receptors are expressed on platelets. These receptors consist of integrins, immune-like receptors, and G protein-coupled receptors (GPCRs). There are five integrins ($\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$) present on the surface of platelets, which have varying substrates (Figure 2-2). The two most studied immune-like receptors on the platelet surface are GPVI/FcRγ and GPIb/V/IX complexes. GPCRs on the platelet surface act as receptors for various agonists such as thrombin, ADP, TXA_2, epinephrine, and PGI_2. All these receptors serve important functions in heomstasis and are potential drug targets for thrombotic diseases.

Platelet Collagen Receptors

Platelets have two known membrane receptors that bind directly to collagen: $\alpha_2\beta_1$ and GPVI/FcRγ complex. Both are platelet surface glycoproteins, however, $\alpha_2\beta_1$ is a member of the integrin family and the GPVI/FcRγ complex is associated with the immunoglobulin superfamily of proteins. From the amassed data on these two collagen receptors, it appears that for the platelet/collagen interaction, $\alpha_2\beta_1$ serves as the primary adhesive receptor and GPVI/FcRγ transduces the activation signal.

$\alpha_2\beta_1$ Integrin

$\alpha_2\beta_1$ (also known as GPIa/IIa, VLA-2, or CD49b/CD29) is an integrin that functions as an ECM receptor for collagens and laminins and is expressed in many different cell types as a noncovalently-linked heterodimer composed of α and β subunits

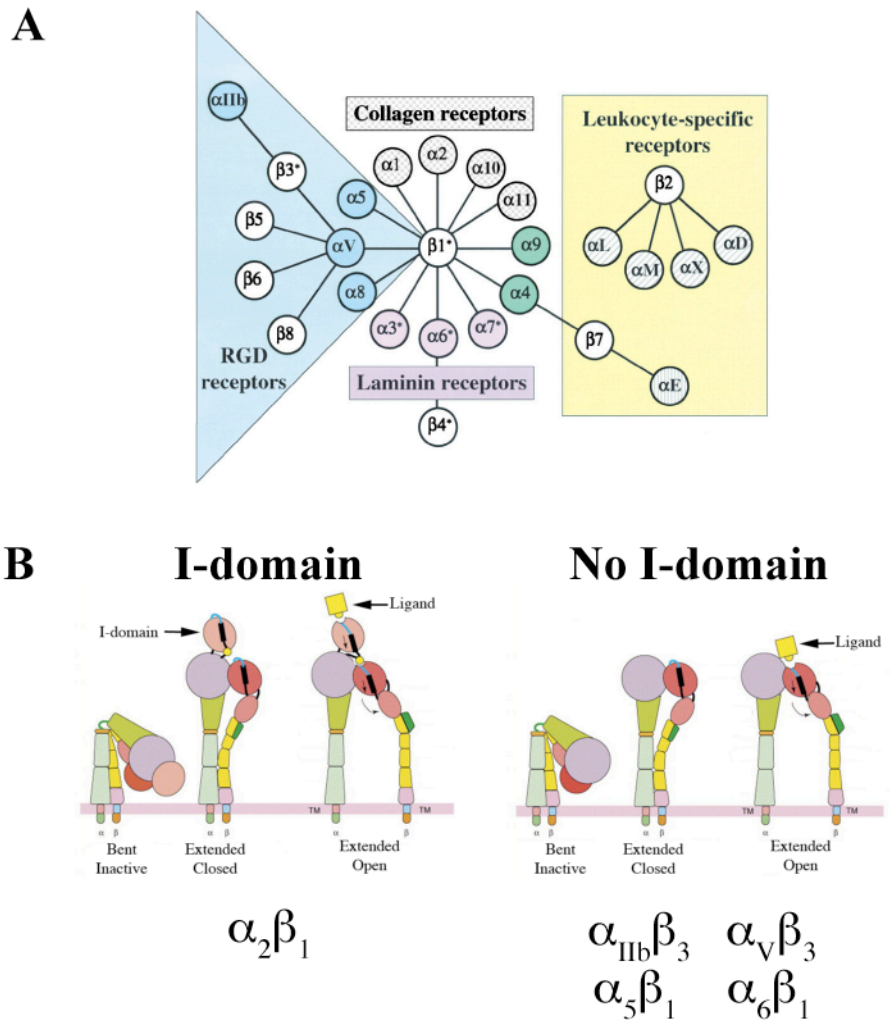

FIGURE 2-2: **The Integrin Family and Platelet Integrins.** *A*, The 24 disitinct integrins function as cell adhesion receptors and are composed of heterodimers of α and β subunits. The integrins can be further subgrouped into RGD-binding, collagen-binding, laminin-binding, or leukocyte-specific receptors. The collagen-binding and leukocyte-specific integrins contain an extra inserted-domain (I-domain) in their α subunits, which mediates their ligand binding. *B*, Platelets express an I-domain containing integrin ($\alpha_2\beta_1$) and several integrins that lack an I-domain ($\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$). Integrin "activation" and transition to a receptor conformation with high affinity for the ligand occurs by extension from the bent conformation and opening of the headpiece binding domain through the separation of the transmembrane (TM) regions of the α and β subunits. Adapted from Hynes, RO, *Cell*, 2002 and Luo, BH and Springer, TA, *Immunol Rev*, 2007.

(Figure 2-2)(37,38). $\alpha_2\beta_1$ is part of the subgroup of integrins that bind to ligands through an inserted-domain (I-domain) within the α subunit. The $\alpha_2\beta_1$ I-domain contains a metal ion dependent adhesion site (MIDAS) motif that requires the coordination of a metal ion (Mg^{2+} or Mn^{2+}) to bind collagen (39-41). On human platelets, the average receptor density of $\alpha_2\beta_1$ is about 1731 ± 432 sites per platelet (42). Platelets adhere to collagens I-VIII through $\alpha_2\beta_1$ (43). Integrins are thought to be bidirectional signaling receptors (inside-out and outside-in signaling mechanisms) that connect the ECM to a cell's actin cytoskeleton and are present in inactive conformations at the surface of cells but convert to low or high affinity conformational states upon ligation or through intracellular signals (44,45). $\alpha_2\beta_1$ has been shown to have two binding affinities (high and low) for collagen ($K_{D(high)}$= 9.9 nM; $K_{D(low)}$= 58 nM) and has the highest affinity for collagen of the two platelet collagen receptors (46,47). The amino acid sequence -GER- in collagen is essential for $\alpha_2\beta_1$ binding, and the sequence -GFOGER- (O = hydroxyproline) represents the high affinity $\alpha_2\beta_1$ binding site in collagens (48,49).

$\alpha_2\beta_1$ was initially suggested to be a platelet collagen receptor when a patient with a bleeding disorder possessed platelets that did not respond to collagen and were shown to lack $\alpha_2\beta_1$ (50). It has also been shown that the heterogeneity of $\alpha_2\beta_1$ expression can be connected to two linked polymorphisms within the gene at nucleotides 807 (T or C) and 873 (A or G), and these may contribute to clinical phenotypes. The 807C/873G pair is associated with low $\alpha_2\beta_1$ expression levels, whereas the 807T/873A pair is associated with high expression levels (51,52). This heterogeneity of $\alpha_2\beta_1$ expression due to genetic variation is associated with altered platelet responses to collagen as seen between different mouse strains (53).

GPVI/FcRγ Complex

GPVI is an immunoglobulin (Ig) plasma membrane protein that contains two Ig-like domains and a mucin-like Ser/Thr-rich region in its extracellular region and, in contrast to $\alpha_2\beta_1$, is only expressed in platelets and megakaryocytes (Figure 2-3) (47,54,55). In platelets, through a salt bridge in the transmembrane domain, GPVI constitutively associates with another membrane protein, Fc Receptor γ-chain (FcRγ), which contains an immunoreceptor tyrosine-based activation motif (ITAM) (56,57). The average receptor density of GPVI/FcRγ on platelets is 3730 ± 453 sites per platelet (42). GPVI/FcRγ is the lower affinity collagen receptor on platelets and requires receptor dimerization to measure a binding affinity ($K_{D(dimer)}$= 578 nM) (47,58,59). GPVI/FcRγ binds to collagen at -$(GPO)_n$- repeats and requires at least two in a series (60,61). Collagen I and III contain repeats of -$(GPO)_n$- making up ~10% of the total amino acid sequence and containing series of n = 5 and 3, respectively (27,62). Similarly to $\alpha_2\beta_1$, GPVI/FcRγ was suggested to be a platelet collagen receptor through analysis of a patient with a bleeding disorder whose platelets lacked a 62 kDa protein (63). The association of GPVI and FcRγ is necessary to form the receptor on platelets and binding of collagen leads to the phosphorylation and activation of the ITAM domain of FcRγ, which produces the majority of the collagen signal that activates platelets (56,64,65). Besides GPVI/FcRγ, other receptors on the surface of platelets are able to activate platelets.

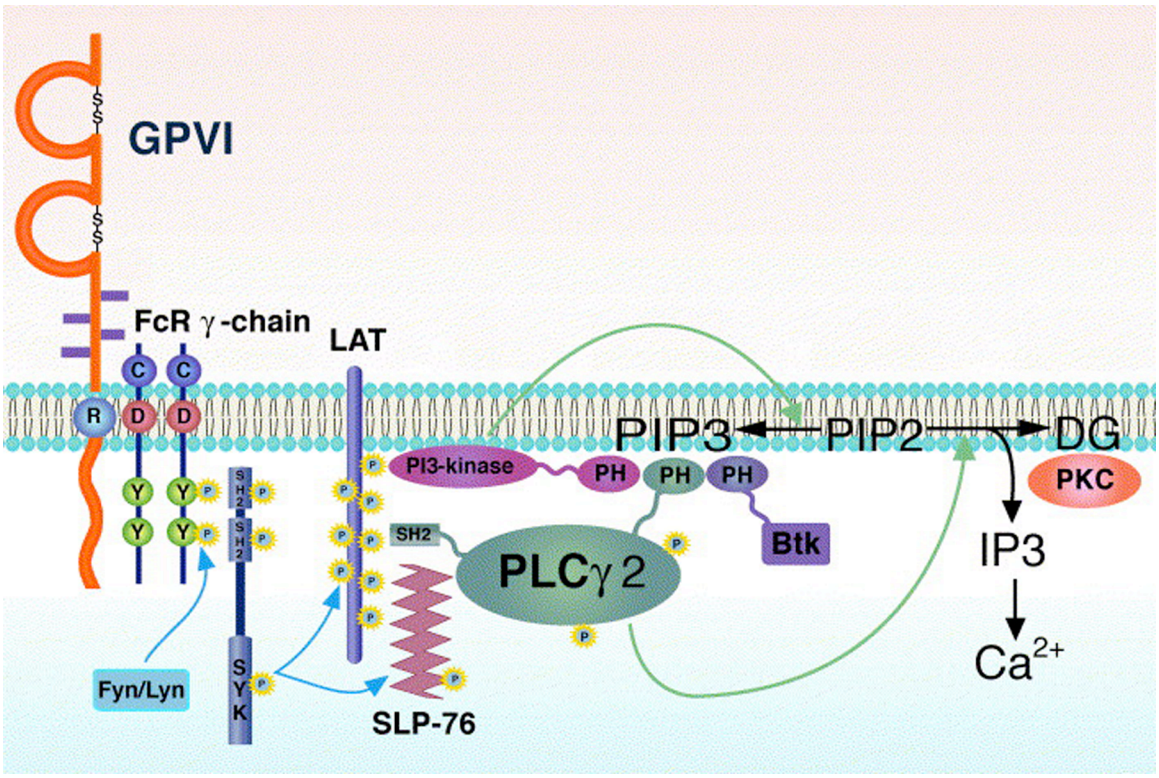

FIGURE 2-3: **GPVI/FcRγ Complex and Associated Signaling Pathways.** GPVI/FcRγ is thought to exist as a complex of two GPVI molecules associated through salt bridges with two covalently linked FcRγ molecules. Clustering of the GPVI/FcRγ complexes by multivalent ligands induces receptor activation and signaling through tyrosine phosphorylation of the FcRγ ITAM domain by Src kinases (Fyn/Lyn). This leads to recruitment and activation of Syk, which further propagates the downstream signal leading to activation of PLCγ2 and PI3K. Adapted from Moroi, M and Jung, SM, *Thromb Res*, 2004.

Platelet G Protein-Coupled Receptors

Platelets express several GPCRs on their surfaces that serve as receptors for platelet activators (thrombin, ADP, and TXA_2) as well as for the inhibitor, PGI_2 (Figure 2-4). GPCRs form a large family (~1000 human genes) of seven-transmembrane receptors that are involved in all physiological processes and couple to guanine-nucleotide regulatory heterotrimeric protein complexes (G-proteins) composed of α and $\beta\gamma$ subunits that transduce their signals (66). GPCRs are major targets for the development of drugs by pharmaceutical companies especially in therapeutics for thrombotic diseases. Human platelets have two thrombin GPCRs on their surface called protease-activated receptors (PARs).

Protease-Activated Receptors

The serine protease, thrombin, is known for its role in the coagulation cascade for cleaving fibrinogen to form insoluble fibrin, but this coagulase is also a potent activator of platelets linking coagulation and hemostasis. Thrombin stimulates the platelet response through two surface receptors, PAR1 and PAR4, by proteolysis of the extracellular N-termini of the PARs to form tethered ligands that bind and activate the receptors (67). There are four known PARs (PAR1-4) in humans that are expressed in many cell types including platelets, ECs, and SMCs and respond to multiple extracellular proteases. PAR1 and PAR4 are primarily activated by thrombin, and both associate with the G-proteins, $G\alpha_q$ and $G\alpha_{12/13}$, where as only PAR1 associates with $G\alpha_i$ (68). The generation of active thrombin at vascular wound sites combined with the permanent

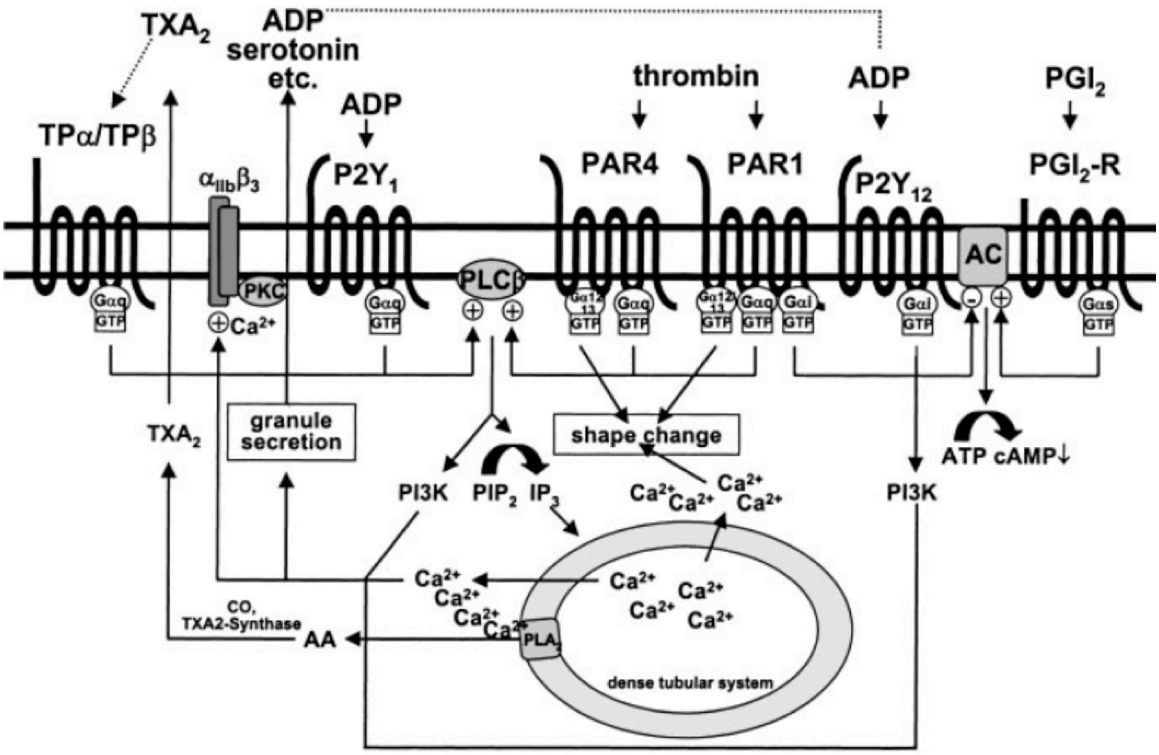

FIGURE 2-4: **Platelet GPCRs and Associated Signaling Pathways.** Platelets express several GPCRs on their surface important in hemostatic functions. Thrombin activates PAR1 and PAR4 through proteolytic cleavage of the extracellular N-terminal tail of the PAR receptor to form a tethered ligand that activates the PAR receptor. PAR1 couples to the G proteins $G\alpha_q$ (activates PLCβ), $G\alpha_i$ (inhibits adenylyl cyclase (AC)), and $G\alpha_{12/13}$ (activates small GTPases) whereas PAR4 couples to $G\alpha_q$ and $G\alpha_{12/13}$. Activated platelets secrete ADP and TXA_2, and these agonists can activate platelets through GPCRs. ADP is an agonist for $P2Y_1$ that couples to $G\alpha_q$ and $P2Y_{12}$ that couples to $G\alpha_i$. TXA_2 activates two receptors TPα and TPβ that both couple to $G\alpha_q$. These GPCR signaling pathways can stimulate processes like platelet shape change, granule secretion, and $\alpha_{IIb}\beta_3$ activation that all have a part in platelet activation. PGI_2 (an inhibitor of platelet activation) has its affect through PGI_2-R that couples to $G\alpha_s$ (activates adenylyl cyclase). Adapted from Jurk, K., and Kehrel, B. E. *Semin Thromb Hemost*, 2005.

nature of the proteolytic activation of PARs and the production of a ligand connected to its receptor makes thrombin a potent activator of platelets.

Platelet Receptor Knockout Mice

Knockout (-/-) mice have been very useful models in elucidating the molecular mechanisms involved in hemostasis and thrombosis through *in vivo* and *in vitro* anlyses (8). Mice deficient in either $\alpha_2\beta_1$ or GPVI/FcRγ have loss of functions to collagen *in vitro*, however the data from *in vivo* analyses are conflicting. $\alpha2^{-/-}$ mice have lost $\alpha_2\beta_1$ expression and show attenuated platelet aggregation to soluble collagen and loss of binding to collagen under flow and static conditions *in vitro* (69,70). Upon *in vivo* photochemical injury of the carotid artery, the $\alpha2^{-/-}$ mice show attenuated thrombus formation (71). However, another report looking at *in vivo* thrombus formation induced by ligation of the carotid artery showed $\beta1^{-/-}$ and $\alpha2^{-/-}$ mice were similar to wild-tpye (WT) and suggested other platelet receptors could compensate for loss of $\alpha_2\beta_1$ (72).

$GPVI^{-/-}$ and $FcR\gamma^{-/-}$ mice have a loss of surface GPVI, and platelets from both show an impaired aggregation to collagen *in vitro* under static and flow conditions but maintain platelet adhesion as a monolayer (73,74). $FcR\gamma^{-/-}$ mice did have an attenuated thrombus formation upon severe (10%) or moderate (8%) $FeCl_3$ injury to mesenteric arterioles but did not to laser injury of the arterioles, and this difference was attributed to the exposure of collagen at the wound site caused by $FeCl_3$ but not by the laser (74). Another study compared the loss of GPVI combined with a loss of $G\alpha_q$ or $G\alpha_{13}$ in the ability of mouse platelets to adhere to collagen I under flow conditions and found that the signaling stimulated by TXA_2 through its GPCRs was essential for platelet adhesion to

collagen in the absence of GPVI/FcRγ (75). These data suggest that $\alpha_2\beta_1$ and GPVI/FcRγ are important in platelet functions but not essential, and that there is cooperation between the collagen receptors themselves as well as other surface receptors such as GPCRs in the platelet/collagen interaction.

Mice deficient in several platelet GPCRs (PAR3, PAR4, P2Y1, or TPα/β) have been generated and tested for hemostatic defects. The thrombin receptors PAR3 and PAR4 are expressed on the surface of mouse platelets, which differs from human platelets. The $PAR4^{-/-}$ mice showed attenuated platelet responses to thrombin *in vitro* and increased bleeding times and protection from arteriolar thrombosis *in vivo* (76). Mouse PAR3 is a receptor that does not transduce a signal when engaged by thrombin but acts as a cofactor for PAR4 activation (77). Interestingly, the $PAR3^{-/-}$ mice showed attenuated platelet responses *in vitro* and *in vivo* supporting a role in hemostasis for cofactors in thrombin receptor activation (78,79). Other GPCRs that activate platelets, P2Y1 a receptor for ADP and TPα/β receptors for TXA_2, have been knocked out in mice. Both the $P2Y1^{-/-}$ and the $TP^{-/-}$ mice had attenuated platelet responses to their respective agonists *in vitro* and had protection from *in vivo* thrombosis assays. These results taken together suggest that platelet surface receptors have overlapping influences on platelet activities involved in the complex processes of hemostasis and thrombosis.

The Two-Step, Two-Site Model of Platelet Adhesion to Collagen

Blood clot formation is the product of a complex cascade of cellular and molecular events, and stable platelet adhesion and activation at the site of the vascular injury is a crucial step. A blood vessel injury that damages the ECs and exposes the

vessel wall subendothelial ECM to the blood enables the platelet to interact with prothrombotic cellular and molecular factors that initially outweigh the antithrombotic factors. The collagens of the subendothelial ECM are potent thrombogenic molecular components of the vessel wall, and fibrillar collagen I is the most abundant.

The order in which $\alpha_2\beta_1$ and GPVI/FcRγ bind to collagens and the signaling contributions they make towards platelet activation are intensely debated. The initial model for platelet collagen activation, proposed by our laboratory, involved a two-step, two-site mechanism that was shown to involve $\alpha_2\beta_1$ and, later, GPVI/FcRγ (40,41,56,58,80). The existing knowledge at present supports two variations that fit the two-site, two-step model (Figure 2-1B). One variation of the model proceeds with $\alpha_2\beta_1$ binding to collagen I first producing stable adhesion of the platelet leading to $\alpha_2\beta_1$-specific signals. This is followed by GPVI/FcRγ binding to collagen I and producing GPVI-specific signals that are required to stimulate platelet activation. The other variation has GPVI/FcRγ binding to collagen I first and producing GPVI-specific signals that activate the platelet and the integrins through inside-out signaling. This is followed by $\alpha_2\beta_1$ binding to collagen I to form a stable adhesion between the platelet and substrate. There is evidence for each scheme to fit the two-site, two-step model (42,81-84), and one study proposed that both schemes can occur depending on environmental conditions and may also reflect platelet receptor heterogeneity (7). Other evidence suggests that the direct interaction of $\alpha_2\beta_1$ and GPVI/FcRγ with collagen I plays a supportive role to the indirect interactions of GPIb/V/IX and $\alpha_{IIb}\beta_3$ binding to VWF-associated collagen (Figure 2-1B)(4). This contradictory data shows that there is still a lot to be learned about platelet adhesion to the ECM at a vascular wound site.

Platelet Surface Receptor Signaling

GPVI/FcRγ has been shown to be the primary signaling receptor for collagens on platelets, and its signal transduction pathways have been studied extensively (Figure 2-3). There is a significant gap in the knowledge of what signals $\alpha_2\beta_1$ contributes to platelet activation by collagen (outside-in signaling), as well as how signals from other surface receptors (GPCRs and GPVI/FcRγ) affect $\alpha_2\beta_1$ and its interaction with collagen. There is evidence that $\alpha_2\beta_1$ could contribute separate, additive, and synergistic collagen signals in platelets (7,83-88). Collagen signaling in platelets is a very complex process since platelet-activating receptors can bind to collagen directly ($\alpha_2\beta_1$ and GPVI/FcRγ) or indirectly (GPIb/IX/V and $\alpha_{IIb}\beta_3$), and secondary activation can occur through platelet granule secretion (ADP and TXA_2) and thrombin generation. However, through the use of receptor-specific collagen-related peptides (CRPs), specific molecular inhibitors, and knockout mice, a better understanding of platelet collagen signaling has been gained.

The majority of signals produced through collagen ligation of GPVI/FcRγ emanate from the clustering of these receptors and the tyrosine phosphorylation of the ITAM domain of FcRγ subunits by the Src kinases, Lyn and Fyn (Figure 2-3)(57,64,89). Phosphorylation of ITAM promotes association with Syk ($p72^{syk}$; a cytoplasmic tyrosine kinase) and activation by Fyn/Lyn (57,90). Activated Syk can then phosphorylate LAT which forms a membrane-tethered scaffolding complex with Gads and SLP-76 which recruits and promotes activation of phosphoinositide-3-kinase (PI3K) and phospholipase C-γ-2 (PLCγ2) (90-92). PLCγ2 cleaves the membrane phospholipid, PIP_2, into soluble IP_3 and membrane-linked DAG, which activates other downstream effectors and

stimulates Ca^{2+} release into the cytosol that amplifies the signal and leads to platelet shape change, granule secretion, and aggregation (15,57). There is evidence that GPVI produces FcRγ-independent signals through Calmodulin and this pathway is necessary for normal GPVI-collagen signaling (93). Collagen activation of GPVI/FcRγ has been shown to signal through Protein Kinase B (PKB), c-Src, Focal Adhesion Kinase (FAK), and p38 mitogen-activated protein kinase (p38 MAPK), which are important for normal platelet responses (94-97). Most of these findings have utilized a GPVI/FcRγ-specific, triple helical, cross-linked CRP (CRP-XL) to confirm GPVI signaling. A caveat with CRP-XL is it may not be biologically relevant since it contains the amino acid sequence -$(GPO)_{10}$- that is not present in collagens, thus CRP-XL may act as a super-activator of platelets and produce irrelevant signals (98). Our laboratory has redesigned the CRP for GPVI (GPVI-CRP) as well as designed an $\alpha_2\beta_1$-specific CRP (α2-CRP) to make them more biologically relevant.

The collagen signals $\alpha_2\beta_1$ generates in platelets through outside-in signaling do not induce platelet granule secretion or aggregation on their own. Syk and PLCγ2 activity in collagen treated platelets is inhibited by jararhagin (a snake venom that specifically cleaves the β_1 subunit) and by antibodies that block $\alpha_2\beta_1$ adhesion (99,100). Src, Syk, and PLCγ2 are activated in platelets adhered to a GFOGER containing CRP that specifically binds $\alpha_2\beta_1$ (85). Also, $\alpha_2\beta_1$ activates c-Src and Lyn in platelets stimulated by rhodocytin (a snake venom that specifically binds $\alpha_2\beta_1$ and causes platelet aggregation) or in human platelets lacking GPVI/FcRγ that were stimulated with collagen (101,102). FAK is activated by collagen in human platelets under arterial flow conditions and has a dependence on $\alpha_2\beta_1$, and FAK activation is seen in platelets

adherent to a GFOGER containing CRP (85,103,104). Signaling by $\alpha_2\beta_1$ in platelets adhering to a low collagen density under flow conditions stimulates p38 MAPK activity (105). These data suggest that $\alpha_2\beta_1$ and GPVI/FcRγ have the potential to cooperate in platelet collagen adhesion and activation through additive or synergistic signaling.

A general characteristic of integrins is their ability to transmit signals bidirectionally across the plasma membrane of cells to perform their function as dynamic links between the cell body and the extracellular environment. Inside-out signaling initiated by other surface receptors (e.g. GPCRs) and affecting integrin activities (ligand affinity and integrin clustering) has been a major research focus, especially for $\alpha_{IIb}\beta_3$ and the β_2 subgroup of leukocyte-specific integrins (44,106). Signaling from GPVI/FcRγ, GPIb/V/IX, and GPCRs have been shown to cause "activation" of $\alpha_{IIb}\beta_3$ (defined as a change in $\alpha_{IIb}\beta_3$ conformation leading to an increased affinity for its ligand) on platelets (Figure 2-2B)(107). The "activation" of $\alpha_{IIb}\beta_3$ occurs through stimulation of PLC and Rap1 that cause Talin to bind to the c-tail of the β_3 subunit and inducing the integrin α/β subunits to separate and convert the integrin from a bent to an extended conformation with an increased affinity for fibrinogen/fibrin (108). Clustering of $\alpha_{IIb}\beta_3$ (modulation of integrin avidity) has been shown to occur along with the affinity change in integrin "activation" but remains very controversial (109). These aspects of integrin biology have been explored for $\alpha_2\beta_1$ but still remain unclear, especially in relation to platelet functions.

Summary

The interactions of platelets with subendothelial collagens exposed at vascular wound sites is an important step in the complex process of hemostasis occurring under

the high forces of arterial blood flow. $\alpha_2\beta_1$ and GPVI/FcRγ are the receptors on platelets that bind to these exposed collagens and transmit its stimulatory signal. This dissertation will address the functions of $\alpha_2\beta_1$ and GPVI/FcRγ in the platelet-collagen interaction that are involved in hemostasis and thrombosis. The goal of this research was to elucidate and define the molecular mechanism(s) of $\alpha_2\beta_1$ and GPVI/FcRγ during the platelet's interaction with collagen I. Our hypothesis was that $\alpha_2\beta_1$ and GPVI/FcRγ have cooperative signaling mechanisms that are important for platelet adhesion to collagen I, but that $\alpha_2\beta_1$ is the primary receptor required for firm adhesion of the platelets to collagen whereas GPVI/FcRγ has a supporting role. This hypothesis was evaluated by utilizing receptor-specific CRPs designed to mimic $\alpha_2\beta_1$ or GPVI/FcRγ binding sites in collagen I in cellular, molecular, and biochemical assays.

The research discussed in this dissertation identifies a priming mechanism for $\alpha_2\beta_1$ binding to collagen I that increases platelet adhesion to the substrate. I show that this priming mechanism is stimulated by $G\alpha_q$-linked GPCRs, specifically PAR4, and can occur at suboptimal levels of GPCR activation (levels that do not "activate" $\alpha_{IIb}\beta_3$ causing platelet aggregation). Inhibition of GPCR-induced PLC signaling attenuates the priming of $\alpha_2\beta_1$ and reduces the platelet-collagen adhesion to basal levels. Suboptimal activation of PAR4 on mouse platelets corroborates the priming mechanism of $\alpha_2\beta_1$ observed in human platelets. I then focus on how suboptimal PAR4 activation causes increased platelet adhesion to collagen through $\alpha_2\beta_1$ and demonstrte that it is not caused by an affinity change for collagen or an increase in $\alpha_2\beta_1$ at the surface of platelets but seems to be caused by a change in $\alpha_2\beta_1$ avidity (integrin clustering). I then analyze the role of GPVI/FcRγ in platelet adhesion to collagen and show support for the concept that

GPVI/FcRγ serves primarily as a signaling receptor and a minor role as an adhesive receptor. My data also shows that GPVI/FcRγ activation through convulxin (Cvx; a snale venom that activates GPVI/FcRγ) can also increase platelet adhesion through $\alpha_2\beta_1$, similar to GPCRs. I end this dissertation with a discussion of this research, how it fits into what is known about the roles of the platelet collagen receptors, and future directions for research on $\alpha_2\beta_1$ and GPVI/FcRγ on platelets.

CHAPTER III

SUBOPTIMAL ACTIVATION OF PROTEASE-ACTIVATED RECEPTORS ENHANCES $\alpha_2\beta_1$ INTEGRIN-MEDIATED PLATELET ADHESION TO COLLAGEN[1]

Thrombin and fibrillar collagen are potent activators of platelets at sites of vascular injury. Both agonists cause platelet shape change, granule secretion, and aggregation to form the primary hemostatic plug. Human platelets express two thrombin receptors, protease-activated receptors 1 and 4 (PAR1 and PAR4) and two collagen receptors, the $\alpha_2\beta_1$ integrin ($\alpha_2\beta_1$) and the glycoprotein VI (GPVI)/FcRγ chain complex. Although these receptors and their signaling mechanisms have been intensely studied, it is not known if and how these receptors cooperate in the hemostatic function of platelets. This study examined cooperation between the thrombin and collagen receptors in platelet adhesion by utilizing a collagen-related peptide (α2-CRP) containing the $\alpha_2\beta_1$-specific binding motif, GFOGER, in conjunction with PAR activating peptides. We demonstrate that platelet adhesion to α2-CRP is substantially enhanced by suboptimal PAR activation (agonist concentrations that do not stimulate platelet aggregation) using the PAR4 agonist peptide and thrombin. The enhanced adhesion induced by suboptimal PAR4 activation was $\alpha_2\beta_1$-dependent and GPVI/FcRγ-independent as revealed in experiments with $\alpha_2\beta_1$- or FcRγ-deficient mouse platelets. We further show that suboptimal activation of other platelet G_q-linked G protein-coupled receptors (GPCRs) produces enhanced platelet

[1] This chapter has been adapted from the paper: Marjoram RJ, Voss B, Pan Y, Dickeson SK, Zutter MM, Hamm HE, Santoro SA. Suboptimal activation of protease-activated receptors enhances alpha2beta1 integrin-mediated platelet adhesion to collagen. *J Biol Chem*. 2009 Dec 11;284(50):34640-7.

adhesion to α2-CRP. The enhanced $\alpha_2\beta_1$-mediated platelet adhesion is controlled by phospholipase C (PLC), but is not dependent on granule secretion, activation of $\alpha_{IIb}\beta_3$ integrin, or on phosphoinositol-3 kinase (PI3K) activity. In conclusion, we demonstrate a platelet priming mechanism initiated by suboptimal activation of PAR4 or other platelet G_q-linked GPCRs through a PLC-dependent signaling cascade that promotes enhanced $\alpha_2\beta_1$ binding to collagens containing GFOGER sites.

Introduction

Platelets are small, anuclear cellular elements that play a central role in hemostasis and contribute to vascular pathology. At sites of intravascular injury, platelets are exposed to multiple pro-thrombotic factors that promote thrombus formation and trigger firm adhesion of platelets to the subendothelial extracellular matrix. Thrombin and fibrillar collagen are two of the more potent stimuli (110,111).

Thrombin is an essential serine protease in the coagulation cascade that ultimately converts fibrinogen to fibrin. Thrombin also has a direct signaling effect on cells through protease-activated receptors (PARs) which are G protein-coupled receptors (GPCRs) that are activated by enzymatic cleavage of the amino-terminus of the receptor to produce a tethered ligand (67). Of the four known PAR isoforms, human platelets express PAR1 and PAR4. In platelets, these receptors show different signaling kinetics; PAR1 is activated at low thrombin concentrations with a quick signal shut off, whereas, PAR4 is activated at higher thrombin concentrations with a slow signal shut off (112-114). Thrombin-induced signaling has been shown to modulate ligand affinity of the platelet membrane $\alpha_{IIb}\beta_3$ integrin ($\alpha_{IIb}\beta_3$) to promote $\alpha_{IIb}\beta_3$ activation and induce platelet

aggregation (115-117). It is still unclear how thrombin activation of PAR1 and/or PAR4 might cooperate with the platelet collagen receptors to influence platelet interactions with collagens.

Platelets are exposed to fibrillar collagens following damage to the vessel wall endothelial cells. Platelets express two receptors for collagens: the $\alpha_2\beta_1$ integrin ($\alpha_2\beta_1$) and glycoprotein VI (GPVI)/FcRγ chain complex (41,63). Both have important roles in the platelet-collagen interaction. $\alpha_2\beta_1$ is important for stable platelet adhesion under shear stress at the injury site, and GPVI/FcRγ complex is required for platelet activation (65,71,73,118). However, there is still considerable debate regarding the precise roles and individual contributions of the two receptors.

The use of collagen-related peptides (CRPs), short peptides that contain repetitive collagen-like sequences (GXX') capable of forming triple helices similar to native collagens, represents a major technical advance in the study of the platelet-collagen interaction. $\alpha_2\beta_1$ has been shown to bind to the consensus sequence GXX'GER, whereas, GPVI binds to the collagen sequence $(GPO)_n$, where $n \geq 2$ and O = 4-hydroxyproline but not to $(GPP)_n$ sequences (119-121). For this study, we designed a 42 amino acid CRP (α2-CRP) that contains the high affinity binding motif for $\alpha_2\beta_1$, GFOGER, to analyze specifically the platelet $\alpha_2\beta_1$ integrin-collagen interaction.

We show that $\alpha_2\beta_1$ integrin-mediated platelet adhesion is enhanced by suboptimal stimulation (agonist concentrations that do not initiate platelet aggregation) of PAR4, and to a lesser extent PAR1, by PAR activating-peptides or thrombin. We also show that suboptimal activation of other G_q-linked GPCRs on platelets ($P2Y_1$, TPα, and TPβ) can stimulate enhanced $\alpha_2\beta_1$ integrin-mediated platelet adhesion. The enhanced platelet

adhesion to α2-CRP substrate is mediated through a phospholipase C (PLC)-dependent pathway, but not through a PI3K-dependent pathway. Neither activation of $\alpha_{IIb}\beta_3$ nor granule secretion is required. We confirm the $\alpha_2\beta_1$ dependence of the process by showing that $\alpha_2\beta_1$-deficient mouse platelets lose the ability to adhere to α2-CRP, whereas both wild-type and FcRγ-deficient mouse platelets exhibit both basal and PAR4 enhanced adhesion.

Experimental Procedures

Materials and Animals

Collagen I from rat-tail tendon was purchased from Upstate Cell Signaling Solutions. α2-CRP was synthesized by Celtek Peptides. U73122, U73343, and wortmannin were purchased from Calbiochem. Bovine serum albumin (BSA), $MgCl_2$, EDTA, DMSO, PGE_1, ADP, *p*-nitrophenol-*N*-acetyl-*β*-D-glucosaminide, RGDS peptide, Apyrase, 3,3',5,5'-tetramethylbenzidine dihydrochloride, and other chemicals were purchased from Sigma Aldrich. Activating peptides for PAR1 (PAR1-AP; SFLLRN) and PAR4 (PAR4-AP; AYPGKF) were purchased from GL Biochem. Human α-thrombin was purchased from Enzyme Research Labs. U46619 and SQ29,584 were purchased from Cayman Chemical. Anti-human α2 integrin monoclonal antibody (6F1) was a generous gift from Dr. Barry S. Coller (The Rockefeller University). Anti-human α2 integrin I-domain monoclonal antibody (12F1) was purchased from BD Pharmingen. Goat anti-mouse secondary antibody conjugated to horseradish peroxidase was purchased from Pierce. Fura2-AM was purchased from Molecular Probes. Hank's Balanced Salt Solution lacking divalent cations (HBSS-) was purchased from Invitrogen. The α2

integrin subunit-deficient mice, originally generated on a C57Bl/6 X 129/SvJ background were backcrossed 8 times to the C57Bl/6 background using a microsatellite marker-assisted selection ("speed congenics"), as previously described (69). Knock-out mice for FcRγ on the C57Bl/6 background were purchased from Jackson Labs. Animals were housed in pathogen-free conditions at Vanderbilt University Medical Center in compliance with IACUC regulations. All animals were appropriately age and sex matched.

Methods

Platelet Isolation– Platelet-rich plasma (PRP) and washed platelets from human blood were prepared from blood obtained from healthy volunteers on the day of the experiment according to a protocol approved by the Institutional Review Board as described previously (122). Murine blood was drawn by cardiac puncture of the left ventricle after carbon dioxide asphyxiation and mixed 10:1 with acid citrate dextrose (ACD; 39 mM citric acid, 75 mM trisodium citrate, 135 mM dextrose) anticoagulant. Mouse platelets were isolated similarly as previously described (69). Human and mouse platelets were adjusted to 1-4×10^8 platelets/mL in adhesion buffer (0.5 % BSA in HBSS-).

Platelet Aggregation– Aggregation assays using PRP were performed on a BIO/DATA Corporation PAP-4 aggregometer at 37°C with stirring (1200 rpm) as described (69). Agonists were added at designated final concentrations.

Platelet Adhesion– Adhesion assays were carried out using washed, isolated human or mouse platelets (1×10^8 platelets/mL) in the presence of 2 mM $MgCl_2$ or 5 mM EDTA in adhesion buffer (0.5 % BSA in HBSS-). Substrates were coated to wells of 96-well

Immulon 2HB microtiter plates at concentration of 30 μg/mL unless otherwise denoted and blocked with adhesion buffer. When extracellular inhibitors (Apyrase, SQ29,548, RGDS, or α2-inhibitory antibody) or intracellular inhibitors (U73122, U73343, or wortmannin) were used, platelets were treated for 10 minutes at 21°C before agonist treatment. When agonists (PAR1-AP, PAR4-AP, ADP, U46619, or α-thrombin) were used, platelets were treated for 3 minutes at 21°C. After treatments, platelets were allowed to adhere to the substrates for 60 minutes unless otherwise denoted at 37°C. Wells were vigorously washed with adhesion buffer plus 2 mM $MgCl_2$ or 5 mM EDTA, and images of adherent platelets were taken using a Nikon Eclipse TS100 microscope with a Nikon Coolpix P5000 digital camera. Adherent platelets were quantified by a hexosaminidase colorimetric assay using 3.75 mM *p*-nitrophenol-*N*-acetyl-*β*-D-glucosaminide as the substrate as previously described (122). The chromogenic reactions were stopped by adding 1.5 volumes of 50 mM glycine, 5 mM EDTA (pH 10.4) buffer, and the well absorbances were read at 405 nm using a Molecular Devices Emax microplate reader. Each data point was performed in triplicate measurements.

Calcium Mobilization– Changes in intracellular calcium concentrations were measured as described previously (123). Briefly, washed isolated human platelets were incubated with 2.5 μM Fura2-AM at 37°C for 30 minutes and washed. Platelets were resuspended in Tyrodes buffer, and agonist-stimulated calcium mobilization was assayed at 37°C with stirring using a Varian Eclipse fluorometer to measure fluorescence intensity changes at excitation wavelengths of 340 and 380 nm.

Cloning, expression and purification of α2 Integrin I Domains– The cloning and expression of the α2 integrin I domain was similar to our previously described methods

(124). A cDNA encoding the α2 integrin I domain (P141-G337) was amplified by PCR, and cloned into pGEX-5X-1 (GE Healthcare) between BamHI and XhoI sites. QuikChange mutagenesis (Stratagene) was used to replace the factor Xa cleavage site with the human rhinovirus 14 3C protease cleavage site (125). The GST fusion protein was purified on Glutathione Sepharose FF following the manufacturers instructions (GE Healthcare). The purified fusion protein was cleaved overnight at 4˚C in Cleavage Buffer (50 mM Tris-HCl pH 7.0, 0.15 M NaCl, 1 mM EDTA, 1 mM DTT) containing rhinovirus 14 3C protease (1/50, w/w). The mixture was passed through a second Glutathione Sepharose FF column to remove cleaved GST, any remaining uncleaved fusion protein, and 3C protease (also a GST fusion protein). Flow through fractions containing I domain were pooled and dialyzed against 50 mM sodium acetate pH 4.5, 1 mM DTT. The dialyzed material was applied to an SP Sepharose HP column, washed extensively with 50 mM sodium acetate pH 4.5, 1 mM DTT, and eluted in a 0-1 M linear NaCl gradient. The I domain eluted at approximately 300 mM NaCl. Pooled fractions were dialyzed at 4˚C against TBS (50 mM Tris-HCl, pH 7.4, 150 mM NaCl).

α2 I-domain Binding– Recombinant α2 I-domain binding was determined by a solid-phase binding assay as previously described (126). Briefly, substrate coated 96-well plates were prepared as described above, and wells were blocked with Tris-buffered saline (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) with 300 μg/ml BSA. Purified recombinant α2 I-domain was diluted to 100 nM in wash buffer (Tris-buffered saline containing 0.05% Tween 20, 30 μg/ml BSA, and 2 mM $MgCl_2$ or 5 mM EDTA) and allowed to bind to the substrates at 21°C. The wells were washed with the appropriate wash buffer, and a 1:500 dilution of anti-α2 I-domain antibody (12F1) in the appropriate

wash buffer was added and incubated at 21°C. The wells were washed three times, and a 1:750 dilution of goat anti-mouse IgG secondary antibody conjugated to horseradish peroxidase in the appropriate wash buffer was added and incubated at 21°C. The wells were washed three times, and a solution of tetramethylbenzidine dihydrochloride, prepared according to the manufacturer's instructions, was added to the wells. The chromogenic reactions were stopped with addition of 4 N H_2SO_4, and the well absorbances were read at 450 nm using a Molecular Devices Emax microplate reader.

Statistical Analyses– Means, standard deviations (SD), standard error of the means (SEM), two-way ANOVA for column statistics, and nonlinear curve fits were calculated using GraphPad Prism 4 software.

Results

Design and Characterization of the $\alpha_2\beta_1$-Specific Collagen-Related Peptide (α2-CRP).

To examine the cooperation between thrombin receptors and the collagen receptor, $\alpha_2\beta_1$ integrin, on platelets, we designed a CRP to which $\alpha_2\beta_1$ specifically binds (α2-CRP) in order to isolate the integrin-collagen interaction (Figure 3-1A). CRPs have been extensively characterized and are commonly used to analyze the platelet's interaction with collagen (120). Based on previous studies, we designed α2-CRP to contain a binding sequence for $\alpha_2\beta_1$ (49,119). α2-CRP was designed as a 42 amino acid peptide containing GPP repeats necessary for triple helix formation and the high affinity $\alpha_2\beta_1$-specific binding sequence, GFOGER (O = 4-hydroxyproline). GPVI binds specifically to the sequence $-(GPO)_n-$, where $n \geq 2$, and GPVI loses the ability to bind

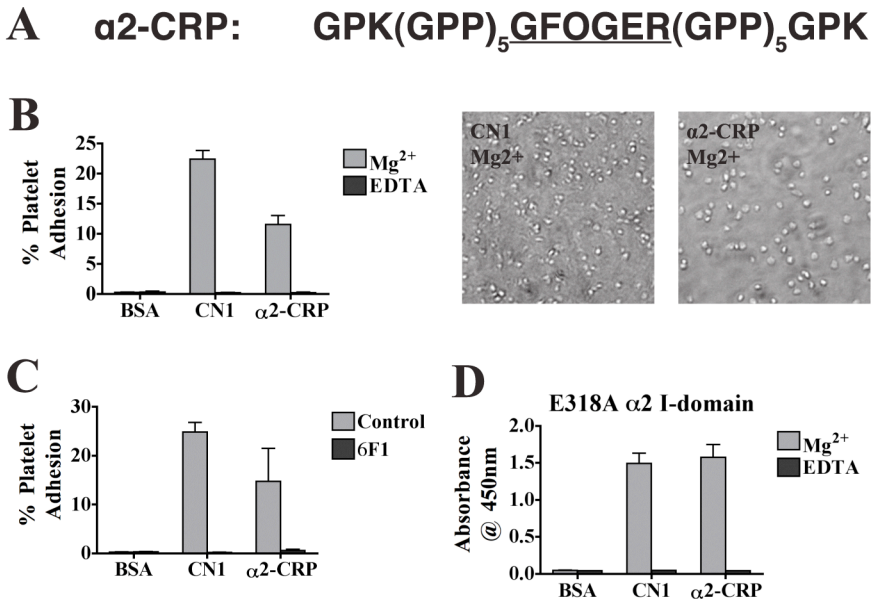

FIGURE 3-1: **Characterization of collagen receptor-specific CRPs.** *A*, the amino acid sequence of the 42 mer CRP designed to bind specifically to $\alpha_2\beta_1$ integrin ($\alpha_2\beta_1$ binding motif is underlined). *B*, platelet adhesion assay to BSA, CNI, or α2-CRP using isolated human platelets in the presence of 2 mM $MgCl_2$ or 5 mM EDTA; on the right, representative microscopic images (40X) of adherent platelets from adhesion assay. *C*, platelet adhesion assay to BSA, CNI, or α2-CRP using isolated human platelets with or without 10μg/mL inhibitory α2 integrin antibody (6F1). *D*, α2 integrin I-domain binding assay using BSA, CNI, or α2-CRP as substrates for the recombinant E318A α2 I-domain. *CNI*, collagen I; *BSA*, bovine serum albumin; Mg^{2+}, $MgCl_2$. Results are percentages of adherent platelets or absorbances at 450 nm (mean ± SEM of 3 independent experiments performed in triplicate).

when the hydroxyproline residues are changed to prolines (119). We tested the ability of isolated human platelets to adhere in a Mg^{2+}-dependent fashion to α2-CRP, collagen I, and BSA substrates (Figure 3-1B). BSA was used as a negative control and showed no platelet adhesion. In the presence of Mg^{2+}, about 22% of the platelets adhered to collagen I, compared to 11% to α2-CRP. In the presence of EDTA, platelets did not adhere to collagen I or α2-CRP. Light microscopic analysis of platelets adherent to α2-CRP and collagen I substrates showed platelets adhered evenly as a monolayer in the presence of Mg^{2+} (Figure 3-1B). Platelet aggregates were not present.

To further support the receptor specificity of the α2-CRP, we analyzed the effect of an inhibitory monoclonal antibody (6F1) directed against the $\alpha_2\beta_1$ integrin (Figure 3-1C). In the presence of the inhibitory antibody, adhesion to collagen I and α2-CRP was largely attenuated. We also determined $\alpha_2\beta_1$ integrin binding specificity by using a purified activated-mutant α2 integrin inserted-domain (E318A α2 I-domain) (127). When Mg^{2+} was present, the E318A α2 I-domain bound to both collagen I and α2-CRP but not to BSA (Figure 3-1D). No I-domain binding was detected when EDTA was present.

Suboptimal PAR Stimulation.

PAR1 and PAR4 activating peptides (PAR1-AP and PAR4-AP, respectively) have been well characterized and are important tools used to study the contributions of the two thrombin receptors (117,128). The amino acid sequence for PAR1-AP is SFLLRN and for PAR4-AP is AYPGKF. We utilized these specific PAR agonists in preliminary experiments to identify concentrations below, which they did not stimulate

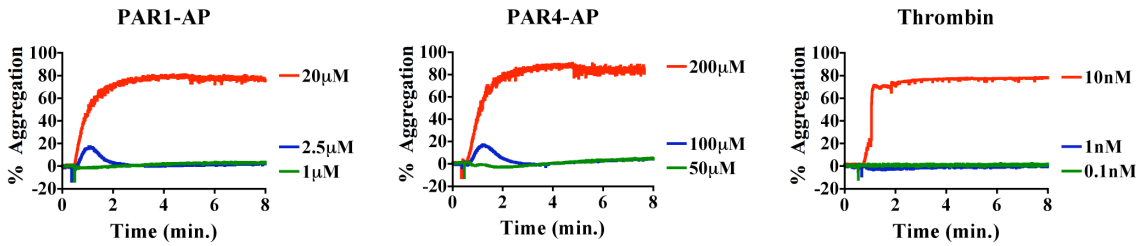

FIGURE 3-2: **Analysis of suboptimal PAR activation.** Platelet aggregation assays were done using human PRP to determine suboptimal concentrations of PAR1-AP, PAR4-AP, and α-thrombin. Tested concentrations were: 20, 2.5, and 1 μM for PAR1-AP; 200, 100, and 50 μM for PAR4-AP; 10, 1, 0.1 nM α-thrombin. Shown are representative data of three independent experiments.

platelet aggregation in PRP (Figure 3-2). For PAR1-AP, 2.5μM initiated aggregation of about 15% but by 3 minutes the platelets had disaggregated and returned to baseline; 1μM did not initiate aggregation but could stimulate platelet shape change. For PAR4-AP, 100 μM initiated aggregation of about 15% but by 3 minutes the platelets had disaggregated and returned to baseline; 50 μM did not initiate aggregation but could stimulate platelet shape change. We therefore, considered concentrations ≤ 2.5 μM PAR1-AP and ≤ 100 μM PAR4-AP to be suboptimal. In a similar manner, we determined suboptimal concentrations of α-thrombin to be ≤ 1nM. We also determined suboptimal concentrations for ADP (≤ 500 nM) and a TXA_2 analog, U46619 (≤ 250 nM) (data not shown). To address variability between individual blood donors, we compared PRP from six healthy individuals for their ability to aggregate to 1 μM or 20 μM PAR1-AP and to 50 μM or 200 μM PAR4-AP (Figure 3-3A). For PAR1-AP, the suboptimal concentration (1 μM) induced a final mean platelet aggregation of 3.0% ± 3.7, and 20 μM produced a final mean aggregation of 81.5% ± 13.1. For PAR4-AP, the suboptimal concentration (50 μM) had a final mean platelet aggregation of 1.2% ± 3.0, and 200 μM produced a final mean aggregation of 65.5% ± 33.2.

Suboptimal PAR Activation Enhances Platelet Adhesion to α2-CRP.

Knowing that the suboptimal PAR activation was producing platelet shape change but not aggregation, we analyzed whether this suboptimal stimulation could affect platelet adhesion through $\alpha_2\beta_1$. To address this, we pretreated isolated human platelets with 1 μM PAR1-AP or 50 μM PAR4-AP and allowed the platelets to adhere to α2-CRP

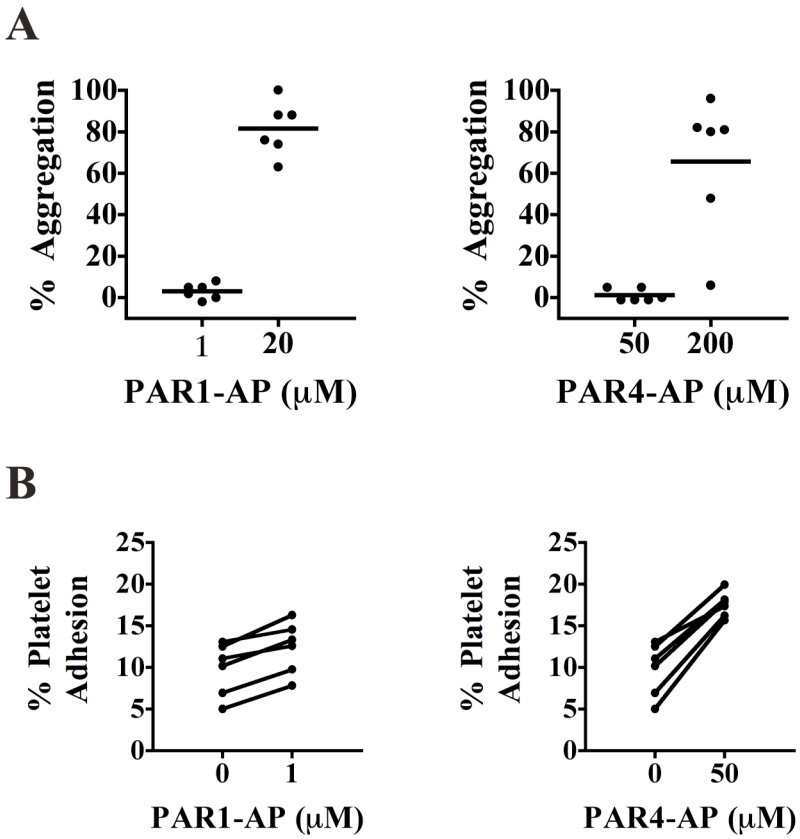

Figure 3-3: **Analysis of variability between blood donors.** *A*, platelet aggregation assays were done using human PRP from six healthy donors to determine the variance between the blood donors' platelets by analyzing their ability to aggregate to suboptimal concentrations of PAR1-AP (1 μM) or PAR4-AP (50 μM) in comparison to optimal concentrations of PAR1-AP (20 μM) or PAR4-AP (200 μM). Results are presented as the maximum final % aggregation after 8 minutes for each individual (n=6) with the bar representing the mean. *B*, human platelet adhesion assays were done using platelets isolated from the blood of the same six donors as above to determine the variability of enhanced platelet adhesion to α2-CRP induced by suboptimal activation of PAR1 or PAR4. This allowed us to correlate the enhancement of platelet adhesion with the absence of platelet aggregation during suboptimal activation of platelet PARs. Platelets were treated with 1 μM PAR1-AP or 50 μM PAR4-AP in the presence of apyrase (1 U) + SQ29,548 (1 μM) and allowed to adhere to α2-CRP. Results are percentages of adherent platelets for each individual (n=6) with a connecting line between the two different treatments to illustrate the enhancement of platelet adhesion.

with or without apyrase and SQ29,548 to prevent platelet activation by secreted ADP and TXA_2, respectively (Figure 3-4A). In the presence and absence of the inhibitors, both 1 μM PAR1-AP and 50 μM PAR4-AP increased platelet adhesion to α2-CRP above basal levels, though the presence of apyrase and SQ29,548 slightly decreased adhesion levels suggesting that some secondary platelet stimulation occurred. We therefore, used apyrase and SQ29,548 treatment in all the following assays unless otherwise specified. The increases in platelet adhesion stimulated by PAR4-AP compared to basal levels were statistically significant ($p < 0.001$). To further address variability between individual blood donors, we analyzed the platelets' ability to adhere to the α2-CRP in the presence or absence of suboptimal concentrations of the PAR-APs from the same six individuals we analyzed for variance in platelet aggregation (Figure 3-3B). All of the individuals' platelets showed an increase in adhesion to α2-CRP over basal levels when treated with 1 μM PAR1-AP or 50 μM PAR4-AP, and PAR4 activity produced the greatest increase in adhesion in all donors.

To show that the increase in platelet adhesion to the CRP substrate was not due to activation of $\alpha_{IIb}\beta_3$ integrin ($\alpha_{IIb}\beta_3$) and adhesion of platelet aggregates, we determined whether inhibiting $\alpha_{IIb}\beta_3$ by using a small peptide, RGDS, affected the enhanced platelet adhesion to α2-CRP stimulated by 1 μM PAR1-AP or 50 μM PAR4-AP (Figure 3-4B). The presence of RGDS did not affect the enhancement of platelet adhesion to α2-CRP stimulated by 1 μM PAR1-AP or 50 μM PAR4-AP. The increases in platelet adhesion stimulated by PAR4-AP compared to basal levels were statistically significant ($p < 0.05$). This finding was consistent with our microscopic examination showing the absence of platelet aggregates (data not shown).

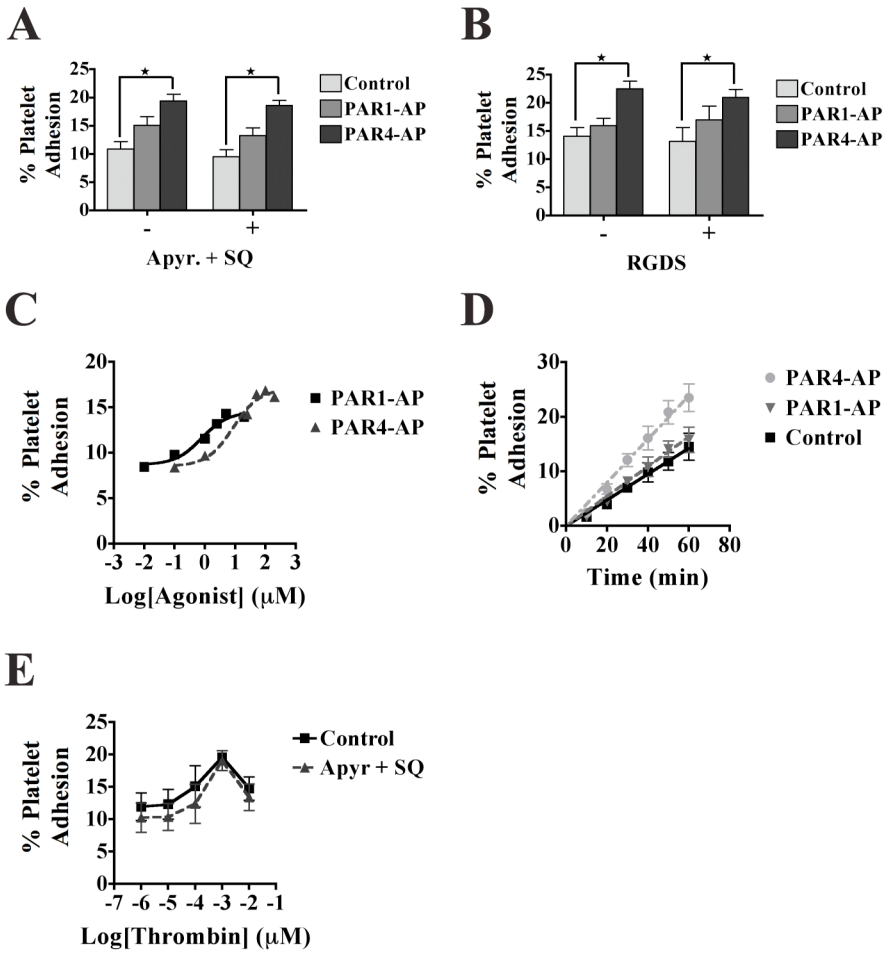

FIGURE 3-4: **Suboptimal PAR activation enhances platelet adhesion to α2-CRP.** *A*, human platelet adhesion assays were done to analyze human platelet adhesion to BSA, CNI, or α2-CRP when platelets were treated with 1 μM PAR1-AP or 50 μM PAR4-AP with or without apyrase (1 U) + SQ29,548 (1 μM). Results are percentages of adherent platelets (mean ± SEM of 4 independent experiments performed in triplicate, $^{*}P < 0.001$). *B*, human platelet adhesion assays were done to analyze human platelet adhesion to α2-CRP when platelets in the presence of apyrase (1 U) + SQ29,548 (1 μM) were treated with 1 μM PAR1-AP or 50 μM PAR4-AP with or without 2 mM RGDS peptide. Results are percentages of adherent platelets (mean ± SEM of 2 independent experiments done in triplicate, $^{*}P < 0.05$). *C*, dose-response curves for a range of PAR1-AP or PAR4-AP concentrations in relation to human platelet adhesion to α2-CRP in the presence of apyrase (1 U) + SQ29,548 (1 μM). Results are percentages of adherent platelets (mean of 4 independent experiments performed in triplicate). *D*, time-courses for human platelet adhesion were done to analyze changes in human platelet adhesion to α2-CRP over time when platelets in the presence of apyrase (1 U) + SQ29,548 (1 μM) were treated with 1 μM PAR1-AP or 50 μM PAR4-AP. Results are percentages of adherent platelets (mean of 5 independent experiments done in triplicate). *E*, dose-response curve for a range of α-thrombin concentrations in relation to human platelet adhesion to α2-CRP in the presence of apyrase (1 U) + SQ29,548 (1 μM). Results are percentages of adherent platelets (mean ± SEM of 3 independent experiments each done in triplicate). *CNI*, collagen I; *BSA*, bovine serum albumin; *Apyr*, Apyrase; *SQ*, SQ29,548.

We further analyzed the PAR-stimulated enhancement of platelet adhesion to α2-CRP by dose-response and time-course analyses. The dose-response curves for PAR1-AP and PAR4-AP enhancement (Figure 3-4C) show that a range of suboptimal concentrations for both PAR1-AP and PAR4-AP can stimulate greater adhesion of platelets to α2-CRP. Suboptimal stimulation of PAR4 produces a greater maximal enhancement than does PAR1. PAR4-AP initiated enhancement of platelet adhesion at 1 μM and had a maximum enhancement of 2.1 fold at 100 μM. PAR1-AP started to show enhancement of platelet adhesion at 0.1 μM and had a maximum enhancement of 1.7 fold at 5 μM. This difference between PAR1 and PAR4 is also evident in the time-course analysis (Figure 3-4D). We examined adhesion of PAR1-AP (1 μM) or PAR4-AP (50 μM) treated platelets to α2-CRP over 60 minutes, and we observed that PAR4-AP treated platelets exhibited an increased rate of adhesion to α2-CRP compared to PAR1-AP treated and untreated platelets. PAR1-AP treatment had a minimal increase in rate of platelet adherence compared to the basal rate. To link this PAR activity to their physiological activator, thrombin, we determined the dose-response curve for α-thrombin in relation to platelet adhesion to α2-CRP (Figure 3-4E). Previously, we determined suboptimal concentrations of α-thrombin to be ≤ 1 nM (Figure 3-2). We then used a range of suboptimal concentrations of α-thrombin to treat platelets and analyze their adhesion to α2-CRP, and we show 0.1 and 1 nM α-thrombin can also stimulate enhancement of platelet adhesion to the CRP. Interestingly, the enhancement decreased at the highest concentration (10 nM) of thrombin tested.

Enhanced Platelet Adhesion to α2-CRP is PLC-Dependent and also Induced by Other Platelet G_q-Linked GPCRs.

Since both PAR1 and PAR4 can link and signal through the G-protein G_q, we analyzed suboptimal PAR activation by determining if 1 μM PAR1-AP or 50 μM PAR4-AP could stimulate intracellular Ca^{2+} mobilization (Figure 3-5A). Both 1 μM PAR1-AP and 50 μM PAR4-AP stimulated intracellular Ca^{2+} mobilization with the PAR4 response being greater and longer in duration than PAR1. 1 μM PAR1-AP resulted in a limited extent of calcium mobilization compared to that produced by 20 μM PAR1-AP, whereas, 50 μM PAR4-AP stimulated a calcium mobilization similar to that produced by 200 μM PAR4-AP in intensity but which was not sustained over time. 10 nM thrombin was used as a positive control.

Since suboptimal activation of both PAR1 and PAR4 stimulated intracellular Ca^{2+} mobilization, we determined whether these GPCRs were producing the enhanced platelet adhesion via G_q and PLCβ2/β3 signaling pathway (Figure 3-5B). Platelets treated with the solvent (DMSO) maintained enhanced adhesion when stimulated by 1 μM PAR1-AP or 50 μM PAR4-AP. When platelets were treated with 10 μM U73122, a PLC inhibitor, the enhanced platelet adhesion stimulated by 1 μM PAR1-AP or 50 μM PAR4-AP was attenuated and decreased to basal levels. When platelets were treated with 10 μM U73343, a U73122 negative control molecule (differs from U73122 by an absence of a double bond), the enhanced platelet adhesion stimulated by 1 μM PAR1-AP or 50 μM PAR4-AP remained intact. We also determined if PI3K played a role in the enhanced platelet adhesion using the PI3K inhibitor, wortmannin. When platelets were treated with

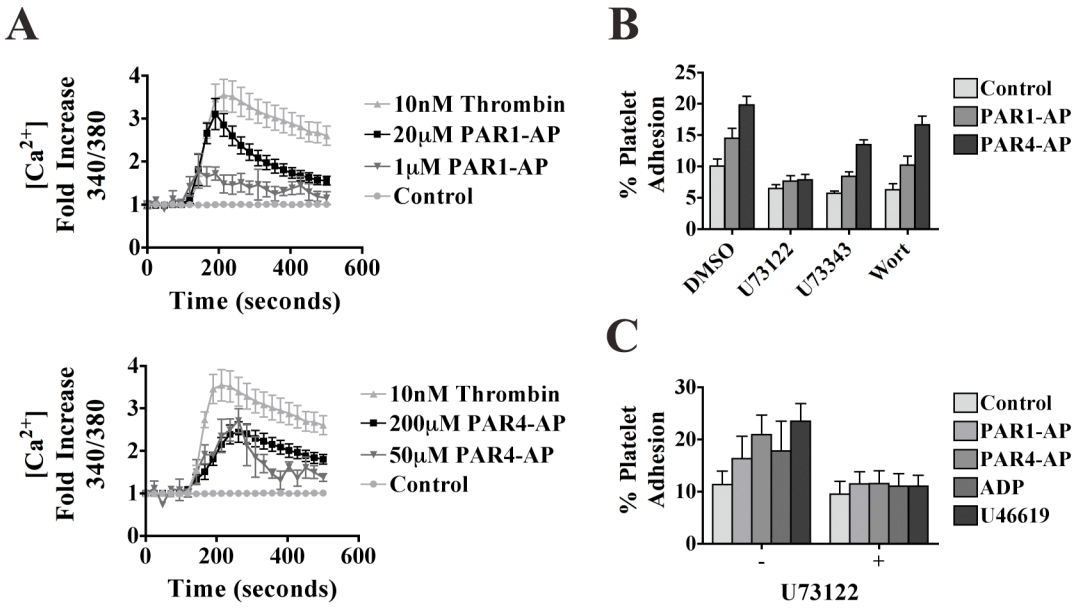

FIGURE 3-5: **Enhanced platelet adhesion to α2-CRP is PLC-dependent and also enhanced by other platelet G_q-linked GPCRs.** *A*, human platelet intracellular Ca^{2+} mobilization assays were done to analyze changes in intracellular [Ca^{2+}] of platelets loaded with FURA-2-AM and stimulated by 1 μM PAR1-AP or 50 μM PAR4-AP in comparison to optimal concentrations (20 μM PAR1-AP or 200 μM PAR4-AP), 10 nM thrombin, and no treatment (control). Results are the ratio of measured absorbances 340 nm/380 nm (mean ± SEM of 3 independent experiments done in triplicate). *B*, human platelet adhesion assays were done to analyze adhesion to α2-CRP when platelets in the presence of apyrase (1 U) + SQ29,548 (1 μM) with or without 10 μM U73122, 10 μM U73343, or 1 μM wortmannin were treated with 1 μM PAR1-AP or 50 μM PAR4-AP. Results are percentages of adherent platelets (mean ± SEM of 5 independent experiments carried out in triplicate). *C*, human platelet adhesion assays were done to analyze adhesion to α2-CRP when platelets in the absence of apyrase + SQ29548 and with or without 10 μM U73122 were treated with 1 μM PAR1-AP, 50 μM PAR4-AP, 0.5 μM ADP, or 0.25 μM U46619. Results are the mean percentages of adherent platelets (mean ± SEM of 4 independent experiments done in triplicate). *DMSO*, dimethyl sulfoxide; *Wort*, wortmannin.

100 nM wortmannin the enhanced platelet adhesion stimulated by 1 μM PAR1-AP or 50 μM PAR4-AP was not affected.

The ADP receptor, P2Y1, and the TXA_2 receptors, TPα and TPβ, are also platelet GPCRs that signal through G_q proteins. To investigate their effects on platelet adhesion to α2-CRP, we employed an approach similar to that which we used to study the PAR receptors except apyrase and SQ29,548 were excluded from the adhesion assay. We determined suboptimal concentrations of ADP and U46619 (a TXA_2 analog) to be 500 nM and 250 nM, respectively (data not shown). Platelets in the absence of apyrase and SQ29,548 showed enhanced adhesion compared to basal when stimulated by 1 μM PAR1-AP, 50 μM PAR4-AP, 500 nM ADP, or 250 nM U46619 (Figure 3-5C). When platelets were treated with 10 μM U73122 the enhanced platelet adhesion stimulated by 1 μM PAR1-AP, 50 μM PAR4-AP, 500 nM ADP, or 250 nM U46619 was attenuated, but platelets still bound at basal levels.

PAR4 Stimulated Enhanced Adhesion of Mouse Platelets.

To further support a PAR-driven priming mechanism for enhanced $\alpha_2\beta_1$ integrin-mediated adhesion, we analyzed the adhesion of platelets from wild-type ($\alpha2^{+/+}$), $\alpha_2\beta_1$-deficient ($\alpha2^{-/-}$) and FcRγ-deficient ($\alpha2^{+/+}$ $FcR\gamma^{-/-}$) mice on the pure C57Bl/6 background to collagen I and α2-CRP (Figure 3-6). First, we determined adhesion levels to BSA, collagen I, or α2-CRP by wild-type, α2-deficient, or FcRγ-deficient mouse platelets (Figure 3-6A). Both wild-type platelets and FcRγ-deficient platelets adhered to collagen I and α2-CRP, but platelet adhesion to the substrates was completely lost in α2-deficient platelets. No platelet adhesion was seen on BSA substrates. Next, we analyzed the effect

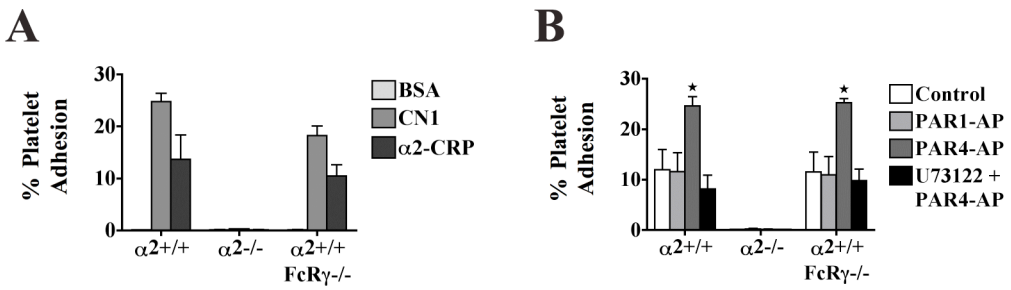

FIGURE 3-6: **PAR4 stimulated enhanced adhesion of mouse platelets.** *A*, wild-type (α2+/+), α2-deficient (α2-/-), and FcRγ-deficient (α2+/+ FcRγ-/-) mouse platelets on a pure C57/BL6 background were used in adhesion assays to analyze cell adhesion to BSA, CNI, or α2-CRP in the presence of apyrase (1 U) + SQ29,548 (1 μM). *B*, wild-type (α2+/+), α2-deficient (α2-/-), and FcRγ-deficient (α2+/+ FcRγ-/-) mouse platelets were used in adhesion assays to analyze adhesion to α2-CRP when platelets in the presence of apyrase (1 U) + SQ29,548 (1 μM) were treated with 1 μM PAR1-AP or 50 μM PAR4-AP. 10 μM U73122 was used to inhibit PLC activity. Results are the mean percentages of adherent platelets (mean ± SEM of 4 independent experiments done with duplicate measurements, $^{*}P < 0.01$).

of suboptimal activation of PAR4 (mice do not express PAR1 on platelets) on wild-type, α2-deficient, or FcRγ-deficient mouse platelets on adhesion to α2-CRP (Figure 3-6B). Similar to human platelets, treatment of wild-type mouse platelets with 50μM PAR4-AP did not produce platelet aggregation and was determined to be suboptimal (data not shown). Wild-type, α2-deficient, or FcRγ-deficient mouse platelets were treated with 50 μM PAR4-AP, 1 μM PAR1-AP (negative control) or vehicle and adhesion to α2-CRP was measured. Wild-type platelets showed a basal level of adhesion when treated with vehicle or 1 μM PAR1-AP, but when treated with 50 μM PAR4-AP, platelet adhesion substantially increased. α2-deficient platelets exhibited no adhesion even when treated with 50 μM PAR4-AP. FcRγ-deficient platelets showed adhesion similar to that of wild-type platelets. They adhered at a basal level when treated with vehicle, but with 50 μM PAR4-AP treatment, platelet adhesion increased markedly. The PAR4-AP stimulated enhancement of wild-type and FcRγ-deficient platelets to α2-CRP was abolished by the PLC inhibitor, U73122 (Figure 3-6B). These experiments were also carried out with wild-type ($\alpha2^{+/+}$), $\alpha_2\beta_1$-deficient ($\alpha2^{-/-}$) and GPVI-deficient ($\alpha2^{+/+}$ $GPVI^{-/-}$) mice on a mixed background (C57Bl/6 X 129/SvJ). The results of these studies were entirely concordant with those presented above for mice on the pure C57Bl/6 background (data not shown).

Discussion

This study provides further insight into the mechanisms giving rise to stable adhesion of platelets to collagen at sites of vascular injury. The data reveal cooperation between the platelet receptors for thrombin, ADP, and TXA_2 with the collagen receptor,

$\alpha_2\beta_1$ integrin. Specifically, the results show that suboptimal stimulation of PAR4, and other platelet G_q-linked GPCRs ($P2Y_1$, TPα/β, and to a lesser degree PAR1) can trigger a PLC-dependent priming mechanism of $\alpha_2\beta_1$ integrin that enhances platelet adhesion to collagen. This GPCR-mediated enhancement of platelet adhesion through the $\alpha_2\beta_1$ integrin suggests the presence of an additional, novel GPVI-independent mechanism to the multi-stage model of platelet adhesion to collagen at vascular injury sites.

For this study, we utilized the α2-CRP as a platelet adhesion substrate that exclusively utilizes the $\alpha_2\beta_1$ integrin-specific interaction (GFOGER) and mimics the triple-helical structure of collagens while eliminating the GPVI/FcRγ complex interaction and other indirect platelet-collagen interactions. This separation of interactions was necessary because the individual roles of $\alpha_2\beta_1$ integrin and GPVI/FcRγ complex during platelet collagen adhesion and their relevance in hemostasis have been debated intensely (83,121,129,130). The GFOGER sequence is primarily found in collagens I, II, and IV, which are constituents of the vascular subendothelial extracellular matrix (120). α2-CRP supported $\alpha_2\beta_1$ integrin-specific binding with a metal ion-dependence consistent with previous studies utilizing similar CRPs (85,120,131). Using the α2-CRP substrate, we demonstrated that suboptimal concentrations of PAR1-AP, PAR-4AP, α-thrombin, ADP, and U46619 enhanced platelet adhesion mediated by the $\alpha_2\beta_1$ integrin. Suboptimal PAR activation enhanced platelet adhesion independently of platelet secretion or $\alpha_{IIb}\beta_3$ integrin activation. The apparent activation of $\alpha_2\beta_1$ integrin and not $\alpha_{IIb}\beta_3$ integrin was surprising and suggests there are different activation mechanisms or differences in the threshold for activation between these two platelet integrins.

Our observations and conclusions are entirely consistent with evidence presented by others. Jung *et al.* showed enhanced binding of soluble collagen III by platelets stimulated with optimal concentrations of thrombin, ADP, and U46619 (46,132). Siljander *et al.* (131) showed enhanced platelet adhesion to CRPs containing various lower affinity GXXGER motifs upon stimulation with ADP. Furthermore, Inoue *et al.* (85) showed ADP enhanced adhesion and spreading of FcRγ-deficient mouse platelets to a GFOGER containing CRP. Our data indicate that this enhancing or priming effect on platelet adhesion to collagen may be mediated by several different platelet GPCRs and occurs without full activation of the GPCR. The combined use of the CRP specific for the $\alpha_2\beta_1$ integrin and of platelets deficient in GPVI/FcRγ expression has allowed us to demonstrate that the enhanced adhesion to collagen results from an effect on the $\alpha_2\beta_1$ integrin and that GPVI/FcRγ is not required, either directly or indirectly, for the adhesion enhancing effect.

To understand how suboptimally activated PAR1 and PAR4 transmit signals to enhance $\alpha_2\beta_1$ integrin-mediated platelet adhesion, we analyzed whether the signal transductions were through the G_q pathway of PLC activation shared by these GPCRs. Analysis of platelets treated with optimal PAR1-AP, PAR4-AP, and thrombin verified intracellular Ca^{2+} mobilization and showed the expected differences between the kinetics of PAR1 and PAR4, in agreement with previous studies (112,114). A dramatic difference in intracellular Ca^{2+} mobilization was observed between suboptimal PAR1-AP and PAR4-AP; whereas 1 μM PAR1-AP produced weak calcium mobilization, 50 μM PAR4-AP resulted in a level of calcium mobilization similar to that produced by 200 μM of the activating peptide except the mobilization was not sustained. The calcium

mobilization correlated with the levels of enhanced adhesion produced by each PAR and suggested that PLC was being activated to induce the enhancement. Inhibition of PLC with U73122 completely eliminated the PAR-enhanced adhesion, but left the basal level of adhesion unaltered. In contrast, inhibition of PI3K had no effect on the enhancement of platelet adhesion. This latter observation is in contrast with the report of Jung *et al*. who observed in their analysis of activated platelet binding of soluble collagen III a dependence upon PI3K (46). This discrepancy may be due to fundamental differences between platelet adhesion to solid-phase substrates and the binding of soluble ligands as observed by our group and others (85). These data support a GPCR-mediated $\alpha_2\beta_1$ integrin priming mechanism for platelet adhesion to collagen through PLC. Other molecules involved in the priming mechanism remain to be determined. Future studies might provide insight into whether the enhanced adhesion is due to a change in affinity or avidity of $\alpha_2\beta_1$ integrin for collagen.

Both PAR1 and PAR4 are able to stimulate enhanced platelet adhesion to α2-CRP, but PAR4 is more effective. This could be attributed to the different signaling kinetics of PAR1 and PAR4 as evident in the intracellular Ca^{2+} mobilization data and supported by previous studies (113,114). Suboptimal concentrations of α-thrombin, the physiologic agonist of PAR1 and PAR4, also stimulated enhanced platelet adhesion to α2-CRP. The level of enhanced adhesion observed with suboptimal thrombin stimulation resembles that observed with PAR4, suggesting further cooperation between PAR1 and PAR4 on human platelets. Similar to PAR3 on mouse platelets, PAR1 may serve as a cofactor for PAR4 activation by thrombin as shown previously due to their ability to heterodimerize (77,78,133). Interestingly, the thrombin dose response curves

show less enhancement of adhesion at the highest concentration tested (10 nM). This could be due to PAR desensitization and internalization. Alternatively or additionally, inhibition of thrombin by platelet secreted factors (antithrombin) may contribute (134).

These data suggest that suboptimal activation of platelets by thrombin through PAR1 and PAR4 at vascular injury sites can prime platelets to adhere more avidly to collagens that contain the GFOGER binding sequence and in theory to other GXXGER sequences in collagens (121). This mechanism is further supported by the recent findings regarding the role of thrombin in platelet adhesion to collagen under conditions of flow. Van der Meijden *et al.* (135) described separate but complimentary roles of thrombin produced subsequent to the contact activation of factor XII by collagen, and GPVI/FcRγ signaling on human platelet adhesion to collagen. Furthermore, inhibition of thrombin resulted in diminished platelet adhesion to collagen. Mangin *et al.* (136) reported that thrombin was able to compensate for the effect of GPVI/FcRγ deficiency in mouse platelets in *in vivo* models of thrombosis. The mechanism we describe, selective enhancement of the adhesive activity of the $\alpha_2\beta_1$ integrin via PAR4 activation, is the likely explanation for the observations of van der Meijden *et al.* (135) and Mangin *et al.* (136).

Our group originally proposed a two-step, two-site model of platelet adhesion and activation to collagen in which the higher affinity collagen receptor, $\alpha_2\beta_1$ integrin, binds first followed by the subsequent engagement of a lower affinity, signal-transducing co-receptor (80) which was shown to be the GPVI/FcRγ complex (56,137). There has been much debate regarding the roles of $\alpha_2\beta_1$ integrin and GPVI/FcRγ complex in the two-step, two-site model (7,82). It has been suggested that engagement of GPVI may be

required for activation of the $\alpha_2\beta_1$ integrin (7,82,129). We have previously shown that platelets from $\alpha_2\beta_1$-deficient mice have delayed thrombus formation *in vivo* within the carotid artery (71), and that under flow conditions *in vitro,* GPVI/FcRγ complex and $\alpha_2\beta_1$ integrin both play independent and vital roles in platelet adhesion and aggregation to collagen (138). Data from the present study suggests that platelets exposed to low levels of thrombin, ADP, or TXA_2 prior to contact with collagen at sites of vascular injury would have an increased affinity or avidity towards exposed $\alpha_2\beta_1$ integrin binding sites on collagens as a result of inside-out activation of $\alpha_2\beta_1$ integrin induced by GPCR signals through PLC to promote more stable adhesion. This supports an alternate, GPVI/FcRγ-independent pathway towards stable adhesion of platelets to collagen at sites of vascular injury. Future studies under *in vitro* or *in vivo* flow conditions will be necessary to test this GPCR priming mechanism of $\alpha_2\beta_1$ integrin. A recent report (139) supports the concept. In Summary, we have shown that suboptimal activation of the thrombin receptors and other G_q-linked GPCRs can enhance adhesion through $\alpha_2\beta_1$ integrin to GFOGER sites of collagens via a PLC-dependent priming mechanism. The data shed new light on the contributions that GPCRs and PLC signaling make toward platelet integrin-extracellular matrix interactions and could have implications in the design and application of novel therapeutic agents that target platelet GPCRs.

CHAPTER IV

SUBOPTIMAL ACTIVATION OF PROTEASE-ACTIVATED RECEPTOR 4 ENHANCES $\alpha_2\beta_1$ INTEGRIN-MEDIATED PLATELET ADHESION TO COLLAGEN BY MODULATING INTEGRIN AVIDITY

Adhesion of platelets to exposed collagens of the subendothelial matrix at sites of vascular injury is a vital step in hemostasis. Under the shear conditions of arterial blood flow, platelets bind to fibrillar collagens through $\alpha_2\beta_1$ integrin ($\alpha_2\beta_1$) to induce stable adhesion. Thrombin is activated at injury sites and can stimulate enhanced platelet adhesion to collagen through protease-activated receptor 1 and 4 (PAR1 and PAR4). In this study, we analyzed how suboptimal activation of PAR4 (activity levels that do not cause platelet aggregation) produces enhanced platelet adhesion to collagen by binding through $\alpha_2\beta_1$. We utilized a specific PAR4 activating peptide (PAR4-AP; AYPGKF) and a platelet substrate, $\alpha_2\beta_1$-specific collagen-related peptide (α2-CRP), that contains the high affinity $\alpha_2\beta_1$ binding sequence, GFOGER. We analyzed the enhanced platelet adhesion stimulated by suboptimal activation of PAR4 by PAR4-AP in the presence of Mn^{2+} (an inducer of the high affinity conformation of integrins) and observed an increase in platelet adhesion over the basal level. The PAR4-induced enhanced adhesion is not caused by an increased sedimentation rate or interaction area of the platelet because no increase was seen on poly-lysine. An increase in the number of $\alpha_2\beta_1$ at the platelet surface was not observed with suboptimal PAR4 activation, however, an increase was seen with optimal activation of PARs. We determined through competitive and

noncompetitive inhibition of $\alpha_2\beta_1$ that the PAR4 enhanced adhesion is not due to an increase in affinity of $\alpha_2\beta_1$ for α2-CRP. PAR4-AP treated platelets bound to the same effective concentration of α2-CRP substrate. Suboptimal PAR4-AP did not activate $\alpha_2\beta_1$ on human platelets but did cause $\alpha_2\beta_1$ to transiently increase its association with the insoluble actin cytoskeleton. These data suggest that suboptimal PAR4 activation produces an increase in platelet adhesion to collagen through actin cytoskeleton dynamics that modulate $\alpha_2\beta_1$ avidity.

Introduction

Platelets are small, anuclear cytoplasts that play a central role in arterial hemostasis. Exposure of platelets to multiple pro-thrombotic factors occurs at vascular injury sites and triggers stable adhesion of platelets to the subendothelial extracellular matrix. Thrombin and fibrillar collagens are potent stimuli of platelets at sites of blood vessel injury (110,111).

Thrombin is a protease in the coagulation cascade that can also stimulate cellular activities through protease-activated receptors (PARs). The four PAR isoforms are G protein-coupled receptors (GPCRs) that are activated by enzymatic cleavage of the receptor amino-terminus to produce a tethered ligand (67). Human platelets express PAR1 and PAR4, and both of these receptors link to and signal through $G\alpha_q$ to stimulate PLC activation (67). In platelets, thrombin-induced signaling through PARs has been shown to activate and increase the affinity of $\alpha_{IIb}\beta_3$ integrin ($\alpha_{IIb}\beta_3$) for RGD-containing ligands that causes platelet aggregation (115,116). This type of integrin activation by

thrombin has also been demostrated to occur for $\alpha_2\beta_1$ and increase its affinity for collagen (140).

Platelets express two receptors for collagens: the $\alpha_2\beta_1$ integrin ($\alpha_2\beta_1$) and glycoprotein VI (GPVI)/FcRγ chain complex (41,63). $\alpha_2\beta_1$ is important for stable platelet adhesion to collagen under shear stress at the injury site, and GPVI/FcRγ complex is required for platelet activation by collagen (65,71,73,118). A useful tool in studying receptor-specific collagen interactions are collagen-related peptides (CRPs), short peptides that contain repetitive collagen-like sequences (GXX') capable of forming triple helices similar to native collagens. $\alpha_2\beta_1$ has been shown to bind to the consensus sequence GXX'GER, and the sequence GFOGER is a high affinity binding site (120). We designed a 42 amino acid CRP (α2-CRP) that contains this high affinity binding motif for $\alpha_2\beta_1$ within GPP repeats to specifically analyze the platelet $\alpha_2\beta_1$-collagen interaction.

Integrins are heterodimeric (composed of α and β subunits) adhesion receptors on the surface of cells that can be subgrouped by ligand binding specificity, leukocyte expression, or the presence of an inserted-domain (I-domain) on some of the α subunits that serves as the ligand-binding site. Platelets express five integrins that bind to various ligands: an I-domain containing integrin ($\alpha_2\beta_1$) and four integrins that lack I-domains ($\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$) (107). The activation of integrins through ligand binding or inside-out signaling changes their conformation from bent/inactive to extended/high ligand affinity states with intermediate affinity states detectable for some integrins (106,141-143). There is also data supporting cells regulating integrin valency for their ligand through redistribution of integrins (clustering) on the cell surface (109,144-146).

For $\alpha_2\beta_1$, the activation of the integrin by inside-out or outside-in signaling mechanisms has been shown to induce an intermediate and high affinity conformations that promote collagen binding (132,147), but there is also evidence for the redistribution of $\alpha_2\beta_1$ on the plasma membrane in promoting collagen binding (148,149). The level of involvement between modulation of integrin affinity and valency in cell adhesion still remains unclear, specifically in platelet adhesion to collagen.

Previously, we showed that suboptimal levels of thrombin (levels where $\alpha_{IIb}\beta_3$ integrin is not activated) triggered PAR signaling that increased platelet adhesion to collagen through an $\alpha_2\beta_1$-dependent priming mechanism (150). In this study we wanted to characterize the integrin activation that suboptimal PAR4 stimulation caused to enhance $\alpha_2\beta_1$-mediated platelet adhesion to collagen. We hypothesized that the sub-threshold PAR4 activity triggered a conformational change in $\alpha_2\beta_1$, which increased the affinity for the collagen substrate. However, we observed enhanced platelet adhesion to α2-CRP with suboptimal PAR4 activation when $\alpha_2\beta_1$ was activated by Mn^{2+}. We determined that the affinity of $\alpha_2\beta_1$ for α2-CRP was similar for platelets treated with or without 50 μM PAR4-AP. The activated conformation of $\alpha_2\beta_1$ was not stimulated by suboptimal activation of PAR4 as detected by HUTS-4 immunoprecipitation. We also ruled out an increased level of $\alpha_2\beta_1$ surface expression on platelets treated with 50 μM PAR4-AP. We analyzed this PAR4-priming mechanism on other substrates (collagen I, poly-lysine, or laminin) and showed the specificity of the mechanism for $\alpha_2\beta_1$ with a collagen substrate and revealed a role for GPVI/FcRγ in priming $\alpha_2\beta_1$ and signaling crosstalk with PAR4. Finally, we showed that suboptimal activation of PAR4 causes a temporary increase of $\alpha_2\beta_1$ that is associated with the actin cytoskeleton of the platelet.

Taken together, the data supports an $\alpha_2\beta_1$-specific priming mechanism triggered by suboptimal activation of PAR4 that modulates integrin avidity through interactions with the actin cytoskeleton that do not cause an increase in the affinity of $\alpha_2\beta_1$ for collagen.

Experimental Procedures

Materials and Animals

Collagen I from rat-tail tendon was purchased from Upstate Cell Signaling Solutions. The α2-CRP, GPK(GPP)$_5$GFOGER(GPP)$_5$GPK, was synthesized by Celtek Peptides. Bovine serum albumin (BSA), Laminin, poly-lysine, $MgCl_2$, $MnCl_2$, PGE_1, p-nitrophenol-N-acetyl-β-D-glucosaminide, Apyrase, and other chemicals were purchased from Sigma Aldrich. Activating peptides for PAR1 (PAR1-AP; SFLLRN) and PAR4 (PAR4-AP; AYPGKF) were purchased from GL Biochem. Human α-thrombin was purchased from Enzyme Research Labs. SQ29,584 were purchased from Cayman Chemical. Anti-human α2 integrin monoclonal antibody (6F1) was a generous gift from Dr. Barry S. Coller (The Rockefeller University). Anti-human α2 integrin I-domain monoclonal antibody (12F1) labeled with PE, anti-α2 monoclonal antibody (clone 2), and anti-β1 integrin monoclonal antibody (clone 18) were purchased from BD Pharmingen. Anti-actin polyclonal antibody (C11) and anti-P-selectin antibody was purchased from Santa Cruz Biotechnology. Anti-activated β1 integrin antibody (HUTS-4) was purchased from Millipore. Goat anti-mouse and Mouse anti-goat secondary antibodies conjugated to horseradish peroxidase and West-femto chemiluminescence substrate were purchased from Pierce. Protein G agarose (PGA) beads were purchased from CalBiochem. Hank's Balanced Salt Solution lacking divalent cations (HBSS-) was purchased from Invitrogen.

The α2 integrin subunit-deficient mice, originally generated on a C57Bl/6 X 129/SvJ background were backcrossed 8 times to the C57Bl/6 background using a microsatellite marker-assisted selection ("speed congenics"), as previously described (69). Knock-out mice for FcRγ on the C57Bl/6 background were purchased from Jackson Labs. Animals were housed in pathogen-free conditions at Vanderbilt University Medical Center in compliance with IACUC regulations. All animals were appropriately age and sex matched.

Methods

Platelet Isolation− Washed platelets from human and mouse blood were prepared from blood obtained on the day of the experiment according to protocols described in chapter III.

Platelet Adhesion Assay− Adhesion assays were carried out using washed, isolated human platelets (1×10^8 platelets/mL) as done previously in chapter III. When extracellular inhibitors (Apyrase, SQ29,548, mα2-CRP, or 6F1 antibody) were used, platelets were treated for 10 minutes at 21°C before agonist treatment. When PAR4-AP was used, platelets were treated for 3 minutes at 21°C. After treatments, platelets were allowed to adhere to the substrates (30 μg/mL unless otherwise denoted) for 60 minutes unless otherwise denoted at 37°C. Each data point was performed in triplicate measurements.

FACS analysis− Washed platelets were resuspended at a concentration of 2×10^7 platelets/mL in adhesion buffer (0.5 % BSA in HBSS-) containing 2 mM $MgCl_2$. Platelets were treated with inhibitors 1 U/mL Apyrase and 1 μM SQ29,548 followed by PAR4-AP, PAR1-AP, or α-thrombin stimulation of platelets for 3 minutes at 21°C.

Aliquots of 50 μL of platelets were added to polystyrene analysis tubes (5 mL). Additions of 1-5 μg/mL of PE-labeled anti-α2 integrin antibody (12F1) or anti-P-selectin antibody were made to the platelets followed by an incubation for 30 minutes at 37°C. A 1:10 dilution of the platelets in the analysis tubes was made using adhesion buffer followed by analysis on the 3-laser BD LSRII. Flow Cytometry experiments were performed in the VMC Flow Cytometry Shared Resource with help from David K. Flaherty and Brittany Matlock. The VMC Flow Cytometry Shared Resource is supported by the Vanderbilt Ingram Cancer Center (P30 CA68485) and the Vanderbilt Digestive Disease Research Center (DK058404).

HUTS-4 Immunoprecipitation and analysis− Platelets were resuspended at a concentration of 5×10^8 platelets/mL in HBSS- containing 2 mM $MgCl_2$ or 2 mM $MnCl_2$. Platelets were treated with 1 U/mL Apyrase and 1 μM SQ29,548 fot 10 minutes at 21°C. Where indicated platelets were stimulated with 50 μM or 200 μM PAR4-AP for 3 minutes at 21°C. HUTS-4 (2.5 μg/mL) was added to platelets and incubated for 30 minutes at 37°C. Platelets were lysed using a 1:1 addition of ice-cold 2X RIPA lysis buffer (2% Triton X-100, 2% sodium deoxycholate, 2% sodium dodecyl sulfate, 0.15 M sodium chloride, 0.01 M sodium phosphate; pH 7.2). Lysates were incubated at 4°C on rotor for 30 minutes. PGA beads were added to lysates and incubated on rotor at 4°C for 1.5 hours. Beads were pellet by centrifugation at 1,000 rpm for 1 minute at 4°C and an aliquot of the platelet lysate was saved (output lysate). Beads were washed 3 times in 1X RIPA lysis buffer by pelleting the beads and aspirating the supernatant. SDS-PAGE sample buffer was added to the beads, which were then boiled for 10 minutes and run on a reducing 8% SDS-PAGE gel. The proteins were transferred to a nitrocellulose

membrane followed by immunoblot analysis with anti-α2 integrin (1:2000), anti-β1 integrin (1:2000), or anti-actin (1:2000) antibodies. Appropriate secondary antibodies linked with horseradish peroxidase were used with a chemiluminescence substrate to image and quantitate the labeled protein bands using a BioRad ChemiDoc with Quantity One software.

Triton X-100 insoluble actin cytoskeleton precipitation– Platelets were resuspended at a concentration of 1×10^9 platelets/mL in HBSS- containing 2 mM $MgCl_2$. Platelets were treated with 1 U/mL Apyrase and 1 μM SQ29,548 fot 10 minutes at 21°C. Next, platelets were left untreated or stimulated with 50 μM PAR4-AP for 0.5, 1, and 3 minute intervals at 21°C. Platelets were lysed using a 1:1 addition of ice-cold 2X TX100 lysis buffer (2% Triton X-100, 0.15 M sodium chloride, 0.01 M sodium phosphate; pH 7.2). Lysates were incubated at 4°C on rotor for 15 minutes. The TX100 insoluble actin cytoskeleton was pelleted by centrifugation at 14,000 rpm for 30 minutes at 4°C and an aliquot of the platelet lysate was saved (output lysate). Pelleted actin cytoskeletons were washed once with 1X TX100 lysis buffer by pelleting and aspirating the supernatant. SDS-PAGE sample buffer was added to the pellets of actin cytoskeleton, which were then boiled for 10 minutes and run on a reducing 10% SDS-PAGE gel. The proteins were transferred to a nitrocellulose membrane followed by immunoblot analysis with anti-α2 integrin (1:2000) or anti-actin (1:2000) antibodies. Appropriate secondary antibodies linked with horseradish peroxidase were used with a chemiluminescence substrate to image and quantitate the labeled protein bands using a BioRad ChemiDoc with Quantity One software.

Statistical Analyses− Means, standard deviations (SD), standard error of the means (SEM), one-way and two-way ANOVA for column statistics, linear and nonlinear curve fits, IC_{50} and EC_{50} values were calculated using GraphPad Prism 4 software.

Results

We have previously shown that suboptimal activation of protease-activated receptors enhances human and mouse platelet adhesion to a collagen substrate through $\alpha_2\beta_1$ integrin (150). In this study, we wanted to determine the change that occurs at the level of the $\alpha_2\beta_1$ (a change in integrin affinity and/or avidity) to produce the enhanced adhesion stimulated by PAR4 in human platelets. We hypothesized that the PAR4-stimulated enhanced platelet adhesion to collagen is produced by an affinity change of surface $\alpha_2\beta_1$ for the collagen substrate.

PAR4-Stimulated Enhanced Platelet Adhesion to α2-CRP in the Presence of Mg^{2+} or Mn^{2+}.

To test whether or not suboptimal activation of PAR4 by 50 μM PAR4-AP stimulated an affinity change in $\alpha_2\beta_1$ and caused the enhanced platelet adhesion to α2-CRP, we analyzed the platelet adhesion in the presence Mn^{2+}, an inducer of activated-integrin conformations. The binding interaction between $\alpha_2\beta_1$ and collagens, as well as α2-CRP, is metal ion-dependent, and Mg^{2+} is thought to be the metal ion involved in this interaction. However, Mn^{2+} can replace Mg^{2+} in its function and, unlike Mg^{2+}, induce a higher affinity conformation of the integrin. We hypothesized that forcing $\alpha_2\beta_1$ into a

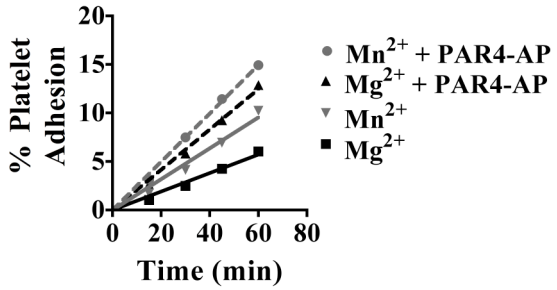

FIGURE 4-1: **PAR4-stimulated enhanced platelet adhesion to α2-CRP in the presence of Mg^{2+} or Mn^{2+}.** Time-courses for human platelet adhesion were done to analyze changes in human platelet adhesion to α2-CRP over time when platelets in the presence of 2 mM $MgCl_2$ (Mg^{2+}) or 2 mM $MnCl_2$ (Mn^{2+}) and apyrase (1 U/mL) + SQ29,548 (1 μM) were pretreated with or without 50 μM PAR4-AP. Results are percentages of adherent platelets (mean of 4 independent experiments done in triplicate measurements).

high affinity conformation using Mn^{2+} would cause an increase in platelet adhesion equal to the level stimulated by 50 μM PAR4-AP/Mg^{2+} and render the PAR4 activity obsolete.

As we have shown previously, suboptimal activation of PAR4 by 50 μM PAR4-AP stimulated enhanced platelet adhesion over time to α2-CRP in the presence of Mg^{2+} compared to Mg^{2+} alone (Figure 4-1). Interestingly, Mn^{2+} increased platelet adhesion over the level achieved with Mg^{2+} but was not to the level of 50 μM PAR4-AP/Mg^{2+}. Surprisingly, 50 μM PAR4-AP caused a similar enhancement of platelet adhesion as observed with Mg^{2+} but in the presence of Mn^{2+} where all $\alpha_2\beta_1$ are in the high affinity state. All adhesion assays were done in the presence of apyrase (1 U/mL) and SQ29,548 (1 μM) to inhibit potential platelet activation by secreted ADP and TXA_2. This data suggested that the enhanced platelet adhesion to α2-CRP stimulated by suboptimal activation of PAR4 is not due to an affinity change in $\alpha_2\beta_1$.

Suboptimal PAR4 Activation Does Not Increase the Affinity of $\alpha_2\beta_1$ for α2-CRP on Platelets.

To further support the evidence that suboptimal activation of PAR4 does not stimulate an increase in affinity of the $\alpha_2\beta_1$ for α2-CRP in human platelets, we carried out a series of experiments. First, we analyzed the ability of platelets pretreated with an inhibitory antibody (6F1; known to bind the I-domain of the α2 subunit and block the association of the integrin with collagen) to adhere to α2-CRP, and we hypothesized that more 6F1 would be required to inhibit adhesion of platelets treated with 50 μM PAR4-AP compared to untreated if the affinity of $\alpha_2\beta_1$ was increased. In these experiments, we observed the increased platelet adhesion over control stimulated by suboptimal PAR4-AP

(50 μM). However, we observed similar 6F1 dose response curves for control and PAR4-AP treated platelets, which had IC_{50} values of 0.054 ± 0.01 μg/mL and 0.067 ± 0.02 μg/mL, respectively (Figure 4-2A) suggesting that there is no increase in affinity of $\alpha_2\beta_1$ for α2-CRP. We calculated the IC_{50} values for each of the three independent experiments and determined that there was no significant difference between the mean IC_{50} values ($P > 0.05$) for control and PAR4-AP treated platelets (Figure 4-2A, inset).

We conducted further analyses to identify a change in the affinity of $\alpha_2\beta_1$ for α2-CRP upon stimulation of platelets with PAR4-AP by using soluble, monomeric α2-CRP (mα2-CRP) as a competitive inhibitor of platelet adhesion to the solid-phase α2-CRP (Figure 4-2B). We hypothesized that if suboptimal PAR4 activation caused an increase in the affinity of $\alpha_2\beta_1$ for α2-CRP, then PAR4-AP treated platelets would require less mα2-CRP to inhibit adhesion to the α2-CRP substrate. Human platelets treated with 50μM PAR4-AP showed enhanced adhesion to α2-CRP, but the adhesions were inhibited at similar concentrations of mα2-CRP (IC_{50} values for control = 17.41 ± 10.4 μg/mL and PAR4-AP = 26.44 ± 6.41 μg/mL). Like before, we calculated the IC_{50} values for each of the three independent experiments and plotted this data to determine if there was any significant change between control and PAR4-AP treated platelets (Figure 4-2B, inset). This data showed there was no significant difference between the mean IC_{50} values ($P > 0.05$).

Another approach we used to determine if PAR4 stimulates a change in affinity of $\alpha_2\beta_1$ for α2-CRP was to analyze platelet adhesion to varying concentrations of the α2-CRP substrate (Figure 4-2C). Here we hypothesized if PAR4 altered $\alpha_2\beta_1$ affinity, then platelets treated with 50 μM PAR4-AP would be able to adhere to a lower α2-CRP

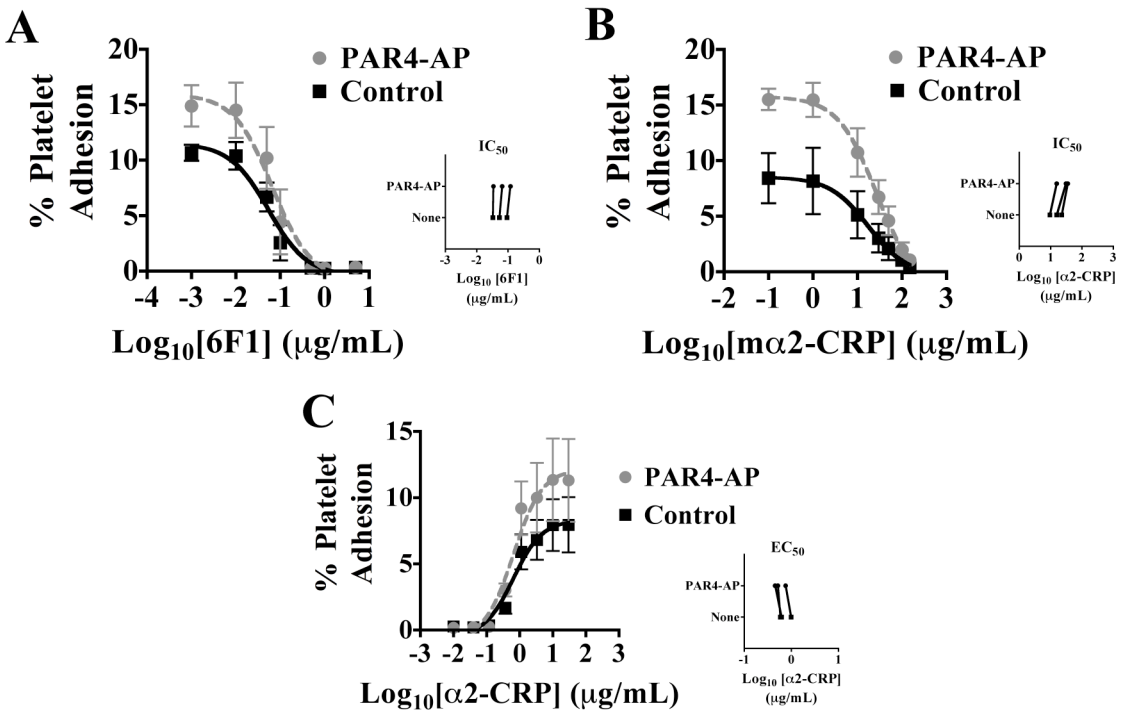

FIGURE 4-2: **Suboptimal PAR4 activation does not increase the affinity of $\alpha_2\beta_1$ for α2-CRP on human platelets.** *A*, Dose-response curves of the $\alpha_2\beta_1$ inhibitory antibody (6F1) on platelet adhesion to α2-CRP. Control platelets or platelets pretreated with 50 μM PAR4-AP in a range of concentrations of 6F1 and in the presence of apyrase (1 U) + SQ29,548 (1 μM) were allowed to adhere to α2-CRP. Results are percentages of adherent platelets (mean of 3 independent experiments performed in triplicate). *Inset*, Pairwise analysis of the IC_{50} values obtained from the 3 independent experiments. *B*, Dose-response curves of inhibition by soluble monomeric α2-CRP (mα2-CRP) on platelet adhesion to α2-CRP. Control platelets or platelets pretreated with 50 μM PAR4-AP in a range of concentrations of mα2-CRP and in the presence of apyrase (1 U) + SQ29,548 (1 μM) were allowed to adhere to α2-CRP. Results are percentages of adherent platelets (mean of 3 independent experiments performed in triplicate). *Inset*, Pairwise analysis of the IC_{50} values obtained from the 3 independent experiments. *C*, Dose-response curves of platelet adhesion to a range of α2-CRP substrate coating concentrations. Control platelets or platelets pretreated with 50 μM PAR4-AP in the presence of apyrase (1 U) + SQ29,548 (1 μM) were allowed to adhere to a range of α2-CRP substrate concentrations. Results are percentages of adherent platelets (mean of 3 independent experiments performed in triplicate). *Inset*, Pairwise analysis of the EC_{50} values obtained from the 3 independent experiments.

coating concentration when compared to untreated platelets. Platelets treated with or without 50 μM PAR4-AP showed similar dose response curves measuring platelet adhesion to varying α2-CRP coating concentrations. The mean EC_{50} values for control platelets adhering to α2-CRP was measured at 0.76 ± 0.44 μg/mL and at 0.61 ± 0.39 μg/mL for PAR4-AP treated platelets. We calculated the EC_{50} values for each of the three independent experiments and plotted this data to determine if there was any significant change between control and PAR4-AP treated platelets (Figure 4-2C, inset). This data showed no significant difference between the mean EC_{50} values ($P > 0.05$). Taken together, these data suggest the affinity of $\alpha_2\beta_1$ for α2-CRP is not altered by suboptimal PAR4 activation.

Suboptimal PAR4 Activation Does Not Induce the Activated Conformation of $\alpha_2\beta_1$ on Platelets.

Inside-out signaling stimulated by GPCRs is known to regulate integrin conformation, so we determined whether or not suboptimal PAR4 activation induced the activated conformation of $\alpha_2\beta_1$ on the surface of platelets. To do this, we utilized a monoclonal antibody (HUTS-4) that recognizes an epitope exposed on the β1 subunit when β1 integrins are in activated conformations. We analyzed HUTS-4 binding to activated $\alpha_2\beta_1$ on the surface of human platelets that had been stimulated with 50 μM or 200 μM PAR4-AP by immunoprecipitation and western blot analysis (Figure 4-3). Platelets in the presence of Mn^{2+} instead of Mg^{2+} were used as a positive control for $\alpha_2\beta_1$ activation and showed the greater presence of α2 and β1 in the immunoprecipitation (IP) compared to the other treatments (Figure 4-3A). Suboptimal PAR4 activation (50 μM

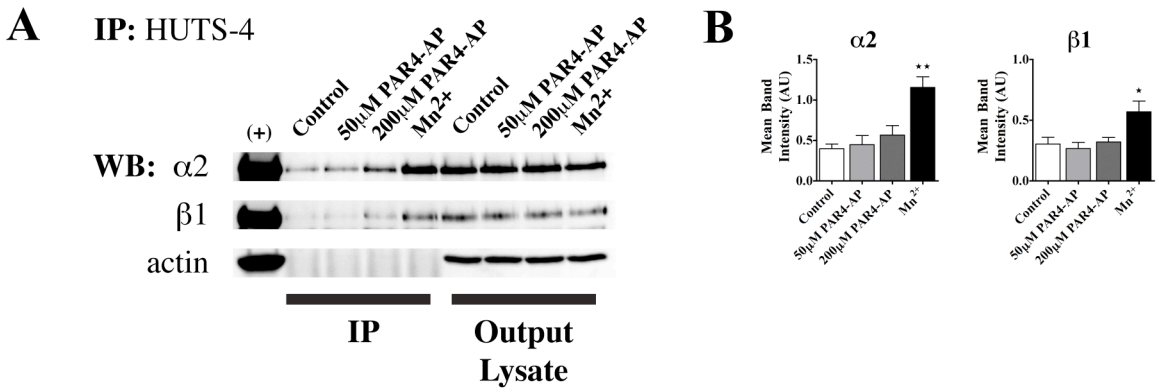

FIGURE 4-3: **Suboptimal PAR4 activation does not induce the activated conformation of α2β1 on platelets.** *A*, Western blot analysis of immunoprecipitates of activated β1-subunit using HUTS-4 antibody from control and PAR4 stimulated human platelets. In the presence of 2 mM $MgCl_2$, control platelets or platelets treated with 50 μM or 200 μM PAR4-AP for 3 minutes and lysed. Platelets in the presence of 2 mM $MnCl_2$ were also lysed and used as a positive control (Mn^{2+}) for the activated conformation of β1. Immunoprecipitation (IP) of activated β1 was done using 2.5 μg HUTS-4 and run on a gel with 1/20 volume of the IP output lysates, a whole cell platelet lysate as protein positive control (+). The gel was then transferred to a membrane for immunoblot analysis using antibodies for α2 and β1 subunits and actin. *B*, Quantification of α2 or β1 bands from the IP lanes of 5 independent experiments by measuring the mean band intensities and normalizing them to actin. Results are the mean band intensity in arbitrary units (AU) (average of 4 independent experiments performed in triplicate).

PAR4-AP) had a similar amount of activated $\alpha_2\beta_1$ as the untreated control, whereas 200 μM PAR4-AP had a small increase of activated $\alpha_2\beta_1$. When the band intensities of α2 or β1 proteins were normalized to actin and quantitated, 50 μM or 200 μM PAR4-AP did not significantly increase the amount of activated $\alpha_2\beta_1$ unlike the levels of activated $\alpha_2\beta_1$ observed with Mn^{2+} ($p < 0.001$ for α2 and $p < 0.05$ for β1; Figure 4-3B). This data shows that suboptimal PAR4 activation does not induce the activated conformation of $\alpha_2\beta_1$ and further rejects an affinity change of $\alpha_2\beta_1$ causing the enhanced adhesion.

Suboptimal PAR4 Stimulation Does Not Increase Surface $\alpha_2\beta_1$ on Platelets.

Another possible way PAR-activated platelets could increase their ability to adhere to α2-CRP is through modulating their avidity by increasing the surface expression of $\alpha_2\beta_1$. We tested this by analyzing the binding of a monoclonal antibody (12F1) to the α2 I-domain of PAR agonist treated platelets using fluorescence-activated cell sorting (FACS) analysis (Figure 4-4A). With suboptimal concentrations of PAR4-AP (50 μM), PAR1-AP (1 μM), and α-thrombin (1 nM) the levels of surface $\alpha_2\beta_1$ remained unchanged compared to untreated platelets. Interestingly, with optimal concentrations of PAR1-AP (20 μM) and α-thrombin (10 nM) there was a statistically significant increase (23.9% and 21.4%, respectively) in surface $\alpha_2\beta_1$ but not with 200 μM PAR4-AP. This increase of surface $\alpha_2\beta_1$ with optimal PAR activation correlated with α-granule secretion by the platelets (Figure 4-4B). We determined the surface expression of P-Selectin (a marker of α-granule secretion) on platelets under the same suboptimal and optimal treatments. With suboptimal concentrations of PAR4-AP (50 μM) or PAR1-

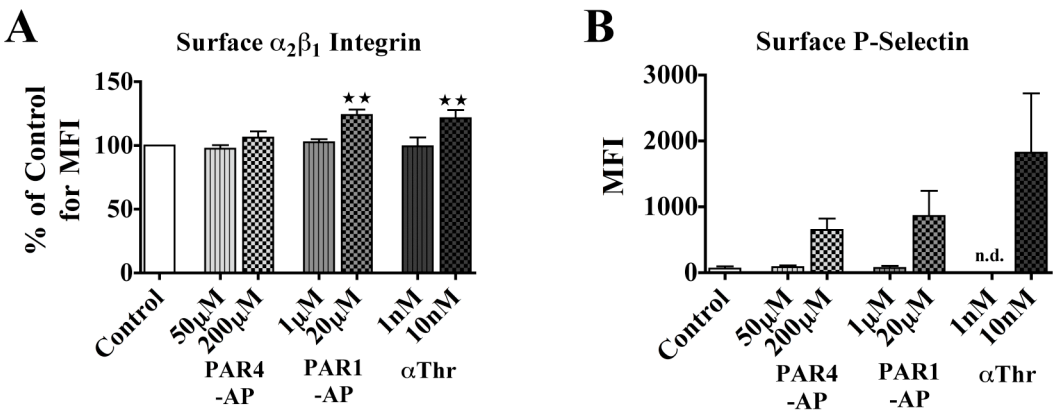

FIGURE 4-4: **Optimal but not suboptimal activation of PARs stimulated increased surface $\alpha_2\beta_1$ or P-selectin on human platelets.** *A*, Flow cytometric analysis of control platelets or platelets treated for 5 minutes with 50 μM or 200 μM PAR4-AP, 1 μM or 20 μM PAR1-AP, or 1 nM or 10 nM α-thrombin (αThr) and stained with the anti-α2 subunit monoclonal antibody (12F1) linked to phycoerythrin. Results are the percentages of control of the mean fluorescence intensity (MFI) (average of 4 independent experiments). *B*, Flow cytometric analysis of control platelets or platelets treated for 5 minutes with 50 μM or 200 μM PAR4-AP, 1 μM or 20 μM PAR1-AP, or 10 nM α-thrombin (αThr) and stained with the anti-P-selectin monoclonal antibody (CTB201) linked to phycoerythrin. Results are the mean fluorescence intensities (MFI) (average of 6 independent experiments).

AP (1 μM), the presence of P-Selectin on the platelet surface was not detected. However, with optimal concentrations of PAR4-AP (200 μM), PAR1-AP (20 μM), and α-thrombin (10 nM) there was a significant increase in surface P-Selectin evidence that α-granule secretion occurred. This data suggests that an increase in surface $\alpha_2\beta_1$ through secretion of α-granules is not the cause of enhanced platelet adhesion stimulated by suboptimal PAR4 activation.

The PAR4-Stimulated Enhanced Platelet Adhesion is Integrin Mediated and Specific for Collagen Substrates.

Suboptimal PAR4 activation stimulates platelet shape changes, and the different platelet morphologies may cause the enhanced adhesion through an increased sedimentation rate and/or an increased surface area involved in the platelet/substrate interaction. We tested this by measuring human platelet adhesion to other substrates (collagen I, poly-lysine, or laminin) when treated with or without 50 μM PAR4-AP (Figure 4-5A). Platelets did not adhere to the negative control substrate (BSA). On α2-CRP, as seen before, we observed a basal level of platelet adhesion that was significantly increased with 50 μM PAR4-AP. However, to poly-lysine (a nonspecific substrate) or laminin (an $\alpha_6\beta_1$-specific substrate) platelets adhered but the adhesion was not increased above basal levels with 50 μM PAR4-AP treatment. Platelet adhesion to collagen I (a substrate for $\alpha_2\beta_1$ and GPVI/FcRγ) had the highest platelet adhesion, and interestingly, this level was slightly reduced with suboptimal PAR4 activation. This PAR4-stimulated decrease was unexpected and suggested that the platelet interaction with collagen is more complex when GPVI/FcRγ is involved.

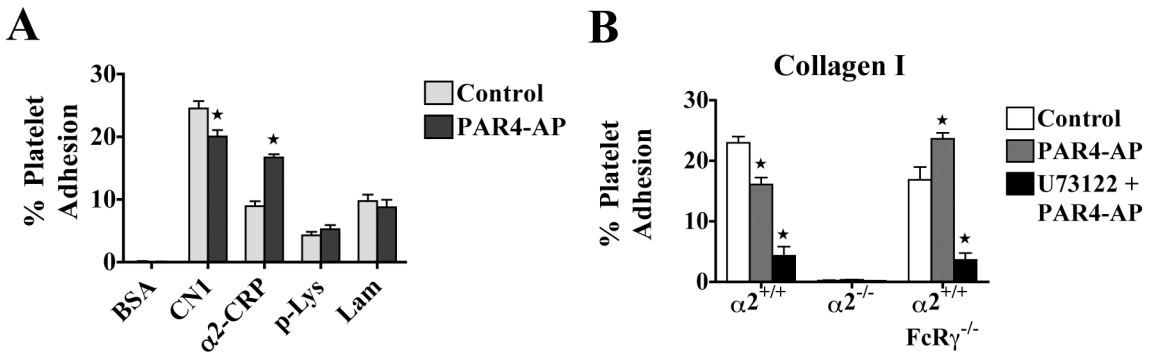

FIGURE 4-5: **PAR4-stimulated enhanced adhesion is $\alpha_2\beta_1$-mediated and specific for collagen substrates.** *A*, platelet adhesion assay to 30 μg/mL BSA, CNI, α2-CRP, poly-lysine (p-Lys), or laminin (Lam) using isolated human platelets with or without pretreatment with 50 μM PAR4-AP. Results are the mean percentages of adherent platelets (mean ± SEM of 4 independent experiments done with triplicate measurements). *B*, wild-type ($\alpha2^{+/+}$), α2-deficient ($\alpha2^{-/-}$), and FcRγ-deficient ($\alpha2^{+/+}$ $FcR\gamma^{-/-}$) mouse platelets were used in adhesion assays to analyze adhesion to collagen I when platelets in the presence of apyrase (1 U) + SQ29,548 (1 μM) were treated with 50 μM PAR4-AP. U73122 (10 μM) was used to inhibit PLC activity. Results are the mean percentages of adherent platelets (mean ± SEM of 4 independent experiments done with duplicate measurements).

To address this new complexity that GPVI/FcRγ contributes to PAR4-stimulated platelet adhesion to collagen, we used collagen receptor knockout mice for $\alpha_2\beta_1$ ($\alpha2^{-/-}$) or GPVI/FcRγ ($\alpha2^{+/+}$ FcRγ$^{-/-}$)(Figure 4-5B). We determined mouse platelet adhesion to collagen I with 50 μM PAR4-AP and observed a similar decrease in wildtype mouse platelet ($\alpha2^{+/+}$) adhesion that we saw with human platelets. When $\alpha_2\beta_1$ expression was lost, platelets did not adhere to collagen I under any of the treatments. However, when expression of GPVI/FcRγ was lost ($\alpha2^{+/+}$ FcRγ$^{-/-}$), we observed a statistically significant ($p < 0.001$) increase in platelet adhesion with suboptimal PAR4 activation, similar to that seen on α2-CRP. When PLC was inhibited with U73122, platelet adhesion decreased beyond the basal levels seen in the controls. Taken together, this data suggests suboptimal PAR4 activation stimulates an $\alpha_2\beta_1$-specific priming mechanism that increases platelet adhesion to collagen, and when the GPVI/FcRγ interaction is involved the platelet adhesion phenotype has an added complexity.

Suboptimal PAR4 Activation Causes a Temporary Increase of $\alpha_2\beta_1$ Association with the Actin Cytoskeleton.

Integrins are receptors that link the actin cytoskeleton of a cell with the extracellular environment, and this association has been linked to focal adhesion formation and clustering. We analyzed the amount of $\alpha_2\beta_1$ that is linked to the actin cytoskeleton during suboptimal PAR4 activation by western blot analysis of precipitated Triton X-100 insoluble actin cytoskeletons (Figure 4-6). With no PAR4-AP treatment we observed a small amount of $\alpha_2\beta_1$ associated with the insoluble actin cytoskeleton. After 0.5 minutes, human platelets treatment with 50 μM PAR4-AP showed a slight increase in

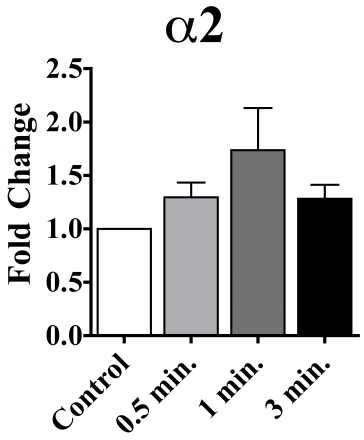

FIGURE 4-6: **Suboptimal PAR4 activation causes a temporary increase of $\alpha_2\beta_1$ association with the actin cytoskeleton of human platelets.** Quantification of α2 subunit protein bands detected by western blot analysis of Triton X-100 insoluble precipitates from control and PAR4 stimulated human platelets over time. In the presence of 2 mM $MgCl_2$, control platelets or platelets treated with 50 μM PAR4-AP for 0.5, 1, or 3 minutes were lysed with Triton X-100. The insoluble precipitates were pelleted, washed, and run on a gel. The gel was transferred for immunoblot analysis using antibodies for the α2 subunit and actin. Quantification of band intensities was done using the α2 proteins that precipitate with the actin cytoskeleton from 5 independent experiments. Results are the fold change over control for the mean band intensities that are normalized to actin (average of 4 independent experiments performed in triplicate).

the amount of $\alpha_2\beta_1$ associated with the actin cytoskeleton. At 1 minute, the amount of $\alpha_2\beta_1$ linked to the platelet cytoskeleton almost doubled, and by 3 minutes, the level had receded to almost basal levels. This data suggests that the increased adhesion stimulated by suboptimal PAR4 activation occurs temporally through $\alpha_2\beta_1$ association with the platelet actin cytoskeleton.

Discussion

In this study we provide further insight into the platelet priming mechanism of $\alpha_2\beta_1$ integrin for collagens that is initiated by suboptimal activation of PAR4 and increases adhesion of the platelets to collagen substrates. Our data show: (i) the $\alpha_2\beta_1$-specifc priming mechanism stimulated by 50 μM PAR4-AP is maintained when $\alpha_2\beta_1$ is activated by Mn^{2+}; (ii) suboptimal activation of PAR4 does not increase the affinity of $\alpha_2\beta_1$ for α2-CRP, induce the $\alpha_2\beta_1$ activated conformation detected by HUTS-4, or increase $\alpha_2\beta_1$ expression on the platelet surface; (iii) the PAR4-stimulated priming mechanism occurs on collagen I but not on poly-lysine or laminin; and (iv) suboptimal activation of PAR4 does transiently increase the amount of $\alpha_2\beta_1$ associated with the platelet actin cytoskeleton. Taken together, these results suggest that suboptimal PAR4 activation causes a change in $\alpha_2\beta_1$ avidity by increasing the association of $\alpha_2\beta_1$ to the actin cytoskeleton of the platelet without changing the affinity of $\alpha_2\beta_1$ for collagen.

The activation of integrins by extracellular stimuli (outside-in activation) has been shown to induce conformational changes in the integrins and cause a higher affinity state of the integrin for its ligand (106). The modulation of integrin affinity and conformation is a dynamic equilibrium between the low-affinity integrin conformation (closed/bent),

intermediate affinity confromation(s), and the high-affinity integrin conformation (open/extended) (44,106). The replacement of the physiological metal ion in the ligand binding domains of integrins ($\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$) with Mn^{2+} is known to activate and change the integrin conformation to the high affinity, open/extended conformer and Mn^{2+} is used as a positive control for integrin activation (141,142). This effect of Mn^{2+} on integrins has also been demonstrated for $\alpha_2\beta_1$ (132,151). We used Mn^{2+} as a positive control for $\alpha_2\beta_1$ activation in our analyses and show that Mn^{2+} can increase platelet adhesion to α2-CRP when compared to adhesion in the presence of the natural metal ion, Mg^{2+}, as well as induce the activated conformation detected by HUTS-4. Interestingly, the treatment of human platelets in Mn^{2+} with suboptimal concentrations of PAR4-AP (50 μM) produced an increase in platelet adhesion over the basal level (untreated platelets in Mn^{2+}). This increase is similar to what is seen in the presence of Mg^{2+} except both the basal and PAR4-AP levels in Mn^{2+} have an overall upwards shift in platelet adhesion produced by the activated $\alpha_2\beta_1$. This data suggested the enhanced $\alpha_2\beta_1$-mediated platelet adhesion stimulated by suboptimal activation of PAR4 was not caused by an increase in affinity of $\alpha_2\beta_1$ for α2-CRP.

We further ruled out an induced change in the affinity of $\alpha_2\beta_1$ for α2-CRP stimulated by 50 μM PAR4-AP by using inhibitors for blocking the $\alpha_2\beta_1$/collagen interaction. Both a non-competitive inhibitor (6F1 antibody) and a competitive inhibitor (mα2-CRP) were able to inhibit platelet adhesion to the α2-CRP substrate for both untreated and 50 μM PAR4-AP treated platelets with similar IC_{50} values. Similar results have been seen previously looking at inhibition with 6F1 or a GFOGER-containing CRP of unstimulated platelet adhesion to collagen (49). If the 50 μM PAR4-AP treatment

caused an affinity change in $\alpha_2\beta_1$, it would take more 6F1 antibody to inhibit the treated platelets than the control, but platelet adhesion for both was inhibited by similar amounts of antibody. For the inhibitor mα2-CRP, if PAR4 stimulated an affinity change in $\alpha_2\beta_1$, the treated platelets would be inhibited by a smaller concentration of mα2-CRP since they would have a higher affinity for the inhibitor as well; however, we observed inhibition by mα2-CRP at similar concentrations.

Another way we addressed the affinity of $\alpha_2\beta_1$ was to compare adhesion of 50 μM PAR4-AP treated platelets to controls on low substrate coating concentrations. If the affinity of $\alpha_2\beta_1$ increased with PAR4-AP treatment, then the treated platelets would be able to adhere to lower substrate coating concentrations of α2-CRP. We did not observe this, and treated and control platelets adhered to the α2-CRP with similar EC_{50} values. These data, taken together, show that the suboptimal activation of PAR4 does not increase the affinity of $\alpha_2\beta_1$ for the α2-CRP substrate on platelets to produce the enhanced cell adhesion.

Another tool used to determine the activation state of integrins are conformation-sensitive antibodies that bind to epitopes exposed on integrins in a particular activation state (115,152,153). We utilized the HUTS-4 antibody that binds to a site on the β1 subunit exposed upon integrin activation to immunoprecipitate activated β1 integrins from PAR4-AP treated platelets and untreated controls. PAR4-AP treatments did not induce the activated conformation of $\alpha_2\beta_1$ beyond basal levels like the Mn^{2+} positive control did. The implication that the enhanced $\alpha_2\beta_1$-mediated platelet adhesion triggered by suboptimal PAR4 activation is not caused by an affinity change of the integrin correlates with the fact that $\alpha_{IIb}\beta_3$ is not being activated on these platelets. It is

interesting that we did not see a significant amount of the activated $\alpha_2\beta_1$ conformer with the optimal concentration of PAR4-AP (200 μM), because this concentration of PAR4-AP does induce platelet α-granule secretion (P-selectin) as well as the activated conformation of $\alpha_{IIb}\beta_3$ on platelets as determined by PAC-1 binding (117). This may be due to an inherent difference between mechanisms of activation for $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ that will require further analysis.

Cells can modulate their adhesiveness to a substrate by varying the expression level of the receptor for that substrate. The importance of the available level of surface receptors in adhesion is demonstrated for $\alpha_2\beta_1$ in our inhibition data and has been shown previously with α2-deficient mouse platelets and induced expression of $\alpha_2\beta_1$ on K562 cells (69,154). An increase in the surface expression of $\alpha_2\beta_1$ on platelets stimulated by 50 μM PAR4-AP would promote increased platelet adhesion to α2-CRP, but we do not detect more surface $\alpha_2\beta_1$ with suboptimal treatments. However, we show that full activation of the platelet PARs can stimulate platelets to move more $\alpha_2\beta_1$ to their cell surface and this correlates with P-selectin mobilization to the surface through α-granule secretion. The increase in surface $\alpha_2\beta_1$ seems to be PAR1-mediated and is another means for platelets to adjust their avidity for a collagen substrate at a vascular injury site.

Another possible mechanism induced by suboptimal PAR4 activation to increase platelet adhesion could be a morphology change of the platelet that leads to an increase in the interacting surface area between the platelet membrane and substrate or to an increase in the rate of platelet sedimentation. If a platelet morphology change was the cause, we would predict that other receptor-binding substrates (laminin and collagen I) as well as a non-specific/ionic substrate (poly-lysine) would adhere more platelets stimulated with

50 μM PAR4-AP. We did not see an increase in platelet adhesion on laminin or poly-lysine with suboptimal PAR4 activation suggesting that a platelet shape change was not the cause. The lack of enhanced adhesion to laminin (an $\alpha_6\beta_1$-specific substrate) also suggested the effect was $\alpha_2\beta_1$-specific (155). Interestingly, we observed with the 50 μM PAR4-AP treated human platelets a small but significant attenuation to the basal level of platelet adhesion on collagen I and not an increase like that seen on α2-CRP.

We wanted to analyze this difference between collagen I and α2-CRP further to see whether or not the involvement of GPVI/FcRγ in the collagen I interaction was causing the attenuation. The use of $\alpha2^{-/-}$ and $FcR\gamma^{-/-}$ mouse platelets elucidated that the difference was caused by the interaction of GPVI/FcRγ with collagen I. Wild-type mouse platelets treated with 50 μM PAR4-AP showed a decrease in platelet adhesion similar to human platelets. Mouse platelets that lacked $\alpha_2\beta_1$ ($\alpha2^{-/-}$) completely lost the ability to adhere to collagen I supporting the fact that the interaction of GPVI/FcRγ with collagen I is dependent on $\alpha_2\beta_1$ binding to collagen I. When $\alpha_2\beta_1$ is the only interacting receptor with collagen through loss of expression of GPVI/FcRγ ($\alpha2^{+/+}$ $FcR\gamma^{-/-}$), the basal level of platelet adhesion to collagen I was decreased compared to wild-type, but with suboptimal PAR4 activation the level of platelet adhesion increased. An important thing to note is this analysis was done under static adhesion conditions and not under flow. The static adhesion conditions can promote GPVI/FcRγ interactions with collagen that are not present under flow unless $\alpha_2\beta_1$ has bound to collagen first (69). The decrease in adhesion to collagen I observed between control and PAR4-AP treated wild-type platelets could be because the PAR4-AP treated platelets are primed before their interaction with collagen whereas the control platelets are in a resting/unactivated state, which could

cause the activating effect of GPVI/FcRγ ligation to be processed differently. The lower level of basal platelet adhesion (control) seen with the FcRγ-deficient platelets compared to wild-type suggests that GPVI/FcRγ might be able to strengthen the interaction of $\alpha_2\beta_1$ with collagen similar to the PAR4-stimulated priming mechanism. GPVI/FcRγ signaling through PLCγ2 could induce a change in $\alpha_2\beta_1$ valency and promote $\alpha_2\beta_1$ binding to lower affinity binding sites (GQRGER and GASGER) (27). This supports the role of $\alpha_2\beta_1$ in platelet adhesion to collagens under flow conditions where GPVI/FcRγ plays a supportive role in platelet adhesion but an essential role in platelet activation and thrombus development (73). The role of GPVI/FcRγ in strengthening $\alpha_2\beta_1$-mediated binding to collagen will require further examination.

Another surprising observation was the degree to which platelet adhesion was attenuated by inhibition of PLC activity (U73122). One study showed that platelets adhering to a GFOGER-containing CRP could stimulate PLCγ2 activity and raise intracellular Ca^{2+} levels (85). It is interesting to postulate that $\alpha_2\beta_1$ can induce an increase in its own avidity to collagen through PLC signaling similar to $G\alpha_q$-linked GPCRs (PAR4). However, the level obtained with the activity stimulated by $\alpha_2\beta_1$ is not maximal since PAR4 can induce a further increase in platelet adhesion to collagen. This reduced effect could be caused by the variable type of Ca^{2+} mobilization stimulated by $\alpha_2\beta_1$ or by the variety of $\alpha_2\beta_1$ binding sites with different affinities (Gxx'GEx') that collagen I contains (27,85). These data suggest that suboptimal activation of PAR4 can cause enhanced platelet adhesion to collagen I through $\alpha_2\beta_1$, but in the presence of multiple types of receptor interactions, the platelet adhesion to collagen I becomes more complex.

The suboptimal activation of PAR4 induces a morphology change in platelets as shown previously (150). Platelet shape change involves actin cytoskeleton dynamics, and a purpose integrins serve is to link a cell's actin cytoskeleton with the extracellular environment (156). The α2 subunit's cytoplasmic tail has been shown to directly link to F-actin, and the β1 subunit is known to indirectly link to actin through talin (157,158). We show that platelets stimulated with 50 μM PAR4-AP have a transient increase in the amount of α2 subunits associated with the platelet's Triton X-100 insoluble actin cytoskeleton with a maximum acheived 1 minute after stimulation. This data suggests that the enhanced platelet adhesion induced by suboptimal PAR4 activation is a result of the modulation of $\alpha_2\beta_1$ avidity through its dynamic linkage to the actin cytoskeleton of the platelet. It is interesting to speculate that this avidity change is caused by a redistribution of surface $\alpha_2\beta_1$ that increases the number of adhesive bonds at the interaction site with collagen (106).

Taken together, these data show that the priming mechanism of $\alpha_2\beta_1$-mediated adhesion to collagen by suboptimal activation of PAR4 occurs through modulation of avidity through a transient association with the actin cytoskeleton that does not cause a change in affinity of $\alpha_2\beta_1$. The priming mechanism does not affect $\alpha_6\beta_1$ and seems to be $\alpha_2\beta_1$-specific. It is possible that GPVI/FcRγ might regulate $\alpha_2\beta_1$ avidity similar to PAR4. Further studies are necessary to determine the validity of these speculations, as well as to determine how the avidity of $\alpha_2\beta_1$ is changing.

CHAPTER V

IN VIVO AND *IN VITRO* ANALYSES OF GLYCOPROTEIN VI (GPVI)/ Fc RECEPTOR γ CHAIN (FcRγ) COMPLEX ON PLATELETS IN HEMOSTASIS AND THROMBOSIS

Hemostasis is a vital process in mammals that maintains vascular integrity. An important step in hemostasis and thrombosis is the interaction of platelets with exposed subendothelial fibrillar collagens at sites of vascular injury to induce adhesion, activation, and aggregation. Two receptors on platelets allow for direct binding of collagens, $\alpha_2\beta_1$ integrin ($\alpha_2\beta_1$) and Glycoprotein VI (GPVI)/Fc Receptor γ-chain (FcRγ) complex. These receptors and their signaling mechanisms have been heavily studied, but there is still more to learn about how these receptors cooperate in the hemostatic function of platelets. This study examined the collagen receptors in platelet adhesion by utilizing two collagen-related peptides: α2-CRP containing the $\alpha_2\beta_1$-specific binding motif (GFOGER) and GPVI-CRP containing the GPVI/FcRγ-specific binding sequence $(GPO)_5$. We show that under static adhesion conditions GPVI/FcRγ can serve as an adhesion receptor but it is dependent upon platelet aggregation. Activation of GPVI/FcRγ by convulxin can stimulate an increase in $\alpha_2\beta_1$-dependent platelet adhesion. In this study, we also conducted a comparative study of mice deficient in α2 subunit, GPVI, or FcRγ on pure (C57Bl/6J) or mixed (129×1/SvJ × C57Bl/6J) backgrounds using *in vivo* and *in vitro* assays to analyze platelet activation and thrombosis. Our data support the importance of $\alpha_2\beta_1$ under conditions where platelets experience shear stress. Unexpectedly, FcRγ-

deficient mice (FcRγ$^{-/-}$) differed from GPVI-deficient mice (GPVI$^{-/-}$) *in vivo* and *in vitro*. FcRγ$^{-/-}$ mice had carotid artery occlusion times similar to wild-type and fewer pulmonary emboli than GPVI$^{-/-}$. Scanning electron micrographs of GPVI$^{-/-}$ and FcRγ$^{-/-}$ platelets adhering to collagen substrates showed that there were not any morphology differences between genotypes. We determined that the phospho-tyrosine protein profile of resting GPVI$^{-/-}$ and FcR$^{-/-}$ platelets is different and this difference needs further examination. This data supports $\alpha_2\beta_1$ being the major collagen adhesive receptor on platelets under flow conditions where as GPVI/FcRγ is important in activating platelets to induce aggregation and support stable adhesion.

Introduction

Hemostasis is a highly regulated balance of prothrombotic and antithombotic components that prevent blood loss from the vasculature while at the same time maintaining its fluidity. The hemostatic balance found in mammals that maintain an intact blood circulation system within an organism involves multiple overlapping mechanisms. Platelets play a central part in this balance especially during arterial hemostasis and pathological thrombosis and serve as nodes that weigh all the prothrombotic and antithrombotic stimuli that guage their response. A potent prothrombotic stimulus that platelets are exposed to at vascular injury sites is subendothelial collagen.

Platelets express two receptors for collagens: the $\alpha_2\beta_1$ integrin ($\alpha_2\beta_1$) which serves as the primary adhesion receptor for collagen and glycoprotein VI (GPVI)/FcRγ chain complex which acts as the major signaling receptor for collagen (41,63,65). GPVI

is a single-span transmembrane protein and a member of the immunoglobulin superfamily (137). FcRγ is also a single-span membrane protein that contains a cytoplasmic signaling domain called an immunoreceptor tyrosine-based activation motif (ITAM) and exists as a covalently linked (extracellular disulfide bond) homodimer at the plasma membrane of cells (47,159). GPVI and FcRγ associate through a salt bridge between residues in the transmembrane domains, and this association is important to form the functional collagen signaling receptor (47,160). GPVI/FcRγ complex is thought to be composed of two covalently linked FcRγ proteins in ionic association with two GPVI molecules. This is supported by the facts that only the GPVI dimer shows a measurable affinity (K_D = 0.5 μM) for fibrillar collagen whereas monomeric GPVI does not, and the crystal structure of the collagen binding domain of GPVI was arranged as a back-to-back dimer (47,58,59).

Loss of GPVI/FcRγ complex on the surface of human platelets through mutations in the *Gp6* gene or caused by anti-GPVI antibodys results in mild bleeding phenotypes in these patients (63,161-163). Genetic deletion of either GPVI or FcRγ or antibody-mediated depletion of GPVI from mouse platelets causes loss of platelet activation stimulated by collagen as well as attenuated thrombus formation under flow conditions *in vivo* and *in vitro* (73,74,118,164,165). The loss of the ability of GPVI and FcRγ to associate through the salt bridge interaction causes the receptor to become nonfunctional, and even receptor density has a part in its ability to function as a collagen receptor (160,166). In this study we utilize the GPVI-deficient ($GPVI^{-/-}$) and FcRγ-deficient ($FcR\gamma^{-/-}$) mice in comparison to $\alpha_2\beta_1$-deficient ($\alpha2^{-/-}$) mice in *in vivo* and *in vitro* analyses.

GPVI/FcRγ utilizes the ITAM motif to conduct the majority of its signal transduction. Binding of collagen, CRP, or convulxin (a C-type lectin from the venom of *Crotalus durissus terrificus*) activates GPVI/FcRγ through clustering of the receptors, which causes the tyrosine phosphorylation of the ITAM motif by association and activation of the Src family tyrosin kinases Fyn and Lyn (61,107). The phosphorylation of the ITAM domain causes the association of another tyrosine kinase Syk which iduces the activation of downstream effectors like PLCγ2, PI3K, and small GTPases that all contribute to platelet activation and aggregation (107).

Receptor-specific collagen-related peptides (CRPs), short peptides that contain repetitive collagen-like sequences (GXX') capable of forming triple helices similar to native collagens, are useful tools. GPVI/FcRγ has been shown to bind to the amino acid sequence $(GPO)_n$ where $n \geq 2$, and chemical crosslinking of CRPs containing these motifs bestows the ability to activate platelets (119,120). We designed a 42 amino acid CRP (GPVI-CRP) that contains the GPVI binding motif found in collagen I $(GPO)_5$ within GXX' repeats to specifically analyze the interaction of platelets with collagen through GPVI/FcRγ.

In this study, we wanted to further analyze the role of GPVI/FcRγ on platelets during hemostasis and thrombosis. We utilized a GPVI-specific CRP (GPVI-CRP) and collagen receptor deficient mice in *in vivo* and *in vitro* assays to elucidate the functions of GPVI/FcRγ on platelets. We show data that further support the role of GPVI/FcRγ on platelets as a signaling receptor important in platelet activation. We also shed some new insight into the effect of convulxin on platelet adhesion to collagen, as well as identify a difference between $GPVI^{-/-}$ and $FcR\gamma^{-/-}$ platelets.

Experimental Procedures

Materials and Animals

Collagen I from rat-tail tendon was purchased from Upstate Cell Signaling Solutions. The α2-CRP, GPK$(GPP)_5$GFOGER$(GPP)_5$GPK, and the GPVI-CRP, GPK$(GPO)_5$GFOGDR$(GPP)_5$GPK were synthesized by Celtek Peptides. U73122, U73343, and RGDS peptide were purchased from Calbiochem. Convulxin was purchased from Enzo Life Sciences. Bovine serum albumin (BSA), DMSO, glutarahldehyde, EDTA, $MgCl_2$, PGE_1, p-nitrophenol-N-acetyl-β-D-glucosaminide, Apyrase, and other chemicals were purchased from Sigma Aldrich. The activating peptide for PAR4 (PAR4-AP; AYPGKF) was purchased from GL Biochem. SQ29,584 were purchased from Cayman Chemical. Anti-human α2 integrin monoclonal antibody (6F1) was a generous gift from Dr. Barry S. Coller (The Rockefeller University). Anti-phosphotyrosine monoclonal antibody (p-Tyr-100) was purchased from Cell Signaling Technology. Anti-actin polyclonal antibody (C11) was purchased from Santa Cruz Biotechnology. Goat anti-mouse and Mouse anti-goat secondary antibodies conjugated to horseradish peroxidase and West-femto chemiluminescence substrate were purchased from Pierce. Hank's Balanced Salt Solution lacking divalent cations (HBSS-) was purchased from Invitrogen.

The α2 integrin subunit-deficient mice, originally generated on a C57Bl/6 X 129/SvJ background were used as well as mice backcrossed 8 times to the C57Bl/6 background using a microsatellite marker-assisted selection ("speed congenics"), as previously described (69). Knock-out mice for FcRγ on the C57Bl/6 and C57Bl/6 X

129/SvJ background were purchased from Jackson Labs. GPVI deficient mice on a C57Bl/6 X 129/SvJ background were a kind gift from Dr. Thomas J. Kunicki (Scripps Research Institute). GPVI deficient mice were backcrossed 8 times to the C57Bl/6 background using a microsatellite marker-assisted selection ("speed congenics"), as previously described. Animals were housed in pathogen-free conditions at Vanderbilt University Medical Center in compliance with IACUC regulations. All animals were appropriately age and sex matched.

Methods

Platelet Isolation– PRP or washed platelets from human and mouse blood were prepared from blood obtained on the day of the experiment according to protocols described in chapter III.

Platelet Adhesion Assay– Adhesion assays were carried out using washed, isolated human platelets (1×10^8 platelets/mL) as done previously in chapter III. When extracellular inhibitors (Apyrase, SQ29,548, RGDS, U73122, U73343, or 6F1 antibody) were used, platelets were treated for 10 minutes at 21°C before agonist treatment. When PAR4-AP was used, platelets were treated for 3 minutes at 21°C. After treatments, platelets were allowed to adhere to the substrates (30 μg/mL unless otherwise denoted) for 60 minutes unless otherwise denoted at 37°C. Each data point was performed in triplicate measurements.

Platelet Aggregation– Aggregation assays using PRP were performed on a BIO/DATA Corporation PAP-4 aggregometer at 37°C with stirring (1200 rpm) as described (69). Agonists were added at designated final concentrations.

Cloning, expression and purification of α2 Integrin I Domains– The cloning and expression of the α2 integrin I domain was similar to our previously described methods (124) and as detailed in chapter III.

α2 I-domain Binding– Recombinant α2 I-domain binding was determined by a solid-phase binding assay as previously described (126) and as described in chapter III. Purified recombinant α2 E318A I-domain was used at 100 nM.

In vivo photochemical injury of the carotid artery of mice– Carotid artery thrombosis was induced as described previously (71). Briefly, male mice approximately 12 weeks of age were anesthetized with an intraperitoneal injection of sodium pentobarbital, secured in the supine position, and placed under a dissecting microscope. The right common carotid artery was isolated through a midline cervical incision, and an ultrasonic flow probe (Model 0.5 VB; Transonic Systems) was applied. A 1.5-mW, 540-nm laser beam (Melles Griot) was applied to the artery from a distance of 6 cm. Rose bengal dye (Fisher Scientific), 50 mg/kg body weight, was then injected into the tail vein, and flow in the vessel was monitored until complete occlusion occurred.

In vivo collagen-induced pulmonary thromboembolism in mice– Collagen-induced thrombosis was carried out as previously described (71). Wild-type, $\alpha_2\beta_1$ integrin-deficient, and FcRγ-deficient female mice were anesthetized by intraperitoneal injection of 100 to 150 μL of a mixture of ketamine and xylazine. Blood was collected into EDTA-coated microtainer tubes for determination of the baseline platelet count and hematocrit. 25 μg of collagen (equine tendon type I fibrillar collagen) along with 1 μg epinephrine (Sigma) in phosphate-buffered saline (PBS), or PBS alone, were injected into the right jugular vein; 1 minute after injection a second blood sample was taken and cell

counts were measured. Mice were humanely killed 3 minutes after injection and lungs were collected and placed in formalin. Quantitation of pulmonary thrombosis was done on lung sections stained with hematoxylin and eosin were digitally imaged using an Olympus Camedia C-3040 Zoom camera. Five random x 20 fields were photographed for each specimen. Analysis of thrombus number for each mouse was made using Olympus Camedia Master 2.5 software, and then expressed as thrombi per square millimeter ± SEM.

Scanning electron microscopy– Platelet adhesion assays were done similar to those described in chaprter II with some minor changes. Platelets at a concentration of 2×10^7 platelets/mL were adhered substrates (30 μg/mL) bound to round glass coverslips (Electron Microscopy Sciences; 22 mm diameter) for 1 hour at 37°C. Coverslips were washed 3 times with adhesion buffer. Platelets adhered to the coverslips were fixed using 2% glutaraldehyde for 30 minutes at 21°C and then washed 3 times with 0.1 M sodium cacodylate buffer and further processing (fixation, drying, and sputter coating) were performed in part through the use of the VUMC Cell Imaging Shared Resource and the EM Core. Imaging was done using a Hitachi S-4200 Scanning Electron Microscope.

Mouse platelet phosphotyrosine analysis– Mouse platelets were resuspended at a concentration of 5×10^8 platelets/mL in HBSS- containing 2 mM $MgCl_2$. Platelets were left untreated or stimulated with 10 μg/mL collagen I, 10 μg/mL α2-CRP, or 200 μM PAR4-AP for 1 minute at 21°C after which an excess of ice-cold HBSS- was added to stop the interaction. Platelets were pelleted at 4,000 rpm for 4 minutes at 4°C and the supernatant was aspirated off. Platelets were lysed using SDS-PAGE sample buffer containing protease and phosphatase inhibitors, which were then boiled for 10 minutes

and run on a reducing 10% SDS-PAGE gel. The proteins were transferred to a nitrocellulose membrane followed by immunoblot analysis with anti-phosphotyrosine (1:2000) or anti-actin (1:2000) antibodies. Appropriate secondary antibodies linked with horseradish peroxidase were used with a chemiluminescence substrate to image the labeled protein bands using a BioRad ChemiDoc with Quantity One software.

Statistical Analyses− Means, standard deviations (SD), standard error of the means (SEM), one-way and two-way ANOVA for column statistics, and nonlinear curve fits were calculated using GraphPad Prism 4 software.

Results

Design and Characterization of GPVI/FcRγ-Specific Collagen-Related Peptide (GPVI-CRP).

Similar to α2-CRP, we designed a GPVI/FcRγ-specific CRP (GPVI-CRP) that replaced one of the series of five GPP repeats with a series of five GPO repeats for GPVI/FcRγ binding and changed the amino acid E to D to eliminate $\alpha_2\beta_1$ binding (Figure 5-1A). We examined human platelet adhesion under static conditions to a range of coating concentrations of GPVI-CRP in comparison to α2-CRP and observed that an optimal coating concentration for both CRPs is 30 μg/mL (Figure 5-1B). To ensure $\alpha_2\beta_1$ was not involved in the platelet adhesion to GPVI-CRP, we analyzed platelet adhesion to these substrates in the presence of an $\alpha_2\beta_1$-specific inhibitory antibody (6F1). We observed a loss of platelet adhesion in the presence of 6F1 to collagen I and α2-CRP, but platelet adhesion to GPVI-CRP was not affected by 6F1 (Figure 5-1C). This data was supported by the observation that the recombinant activated-mutant (E318A) α2 I-

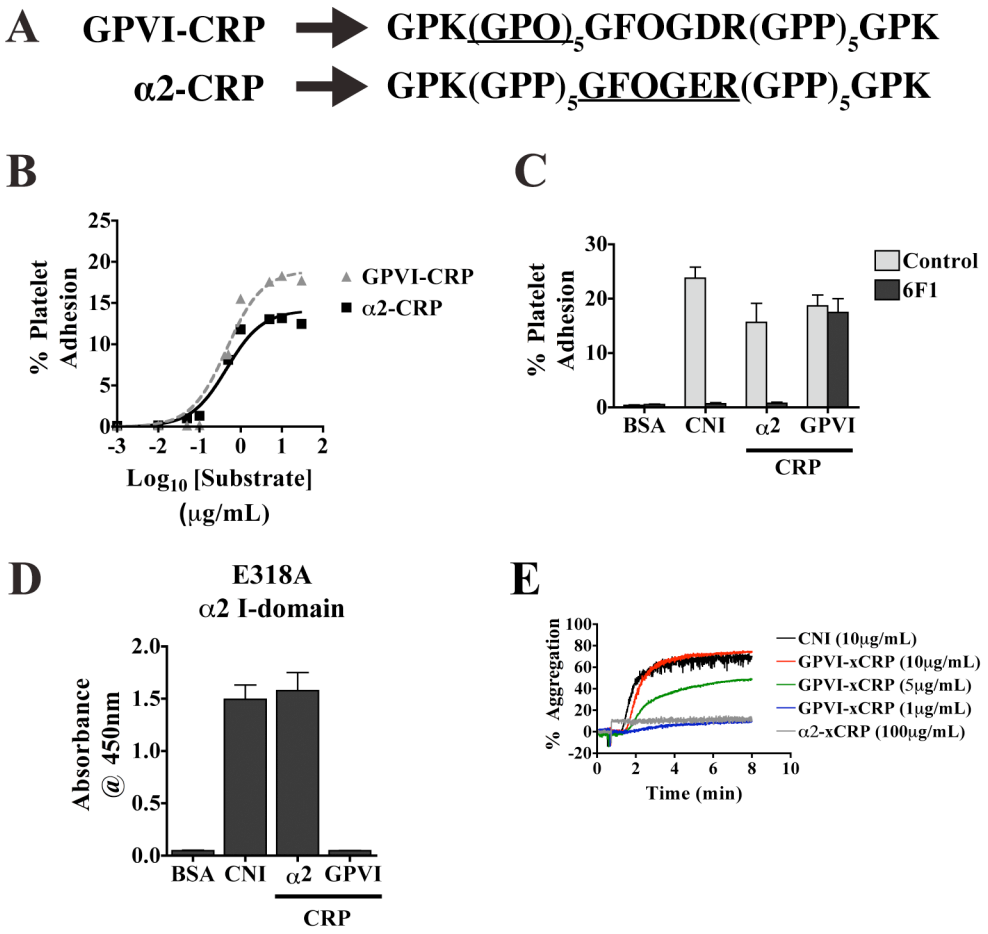

FIGURE 5-1: **Design and characterization of GPVI/FcRγ-specific collagen-related peptide (GPVI-CRP).** *A*, the amino acid sequences of the 42 mer CRPs designed to bind specifically to either GPVI/FcRγ (GPVI-CRP; GPVI binding motif is underlined) or $\alpha_2\beta_1$ integrin (α2-CRP; $\alpha_2\beta_1$ binding motif is underlined). *B*, Dose-response curves of platelet adhesion to a range of substrate coating concentrations for GPVI-CRP or α2-CRP. Results are percentages of adherent platelets (mean of 3 independent experiments performed in triplicate). *C*, platelet adhesion assay to BSA, CNI, α2-CRP, or GPVI-CRP using isolated human platelets in the presence of 2 mM $MgCl_2$ with or without 10μg/mL inhibitory α2 integrin antibody (6F1). Results are percentages of adherent platelets (mean of 6 independent experiments performed in triplicate). *D*, α2 integrin I-domain binding assay using BSA, CNI, α2-CRP, or GPVI-CRP as substrates for the recombinant activated mutant (E318A) α2 I-domain in 2 mM $MgCl_2$. Results are absorbances at 450 nm (mean of 3 independent experiments performed in triplicate). *E*, Platelet aggregation assays were done using human PRP to determine concentrations of GPVI-xCRP and α2-xCRP. Shown are representative data of three independent experiments. *CNI*, collagen I; *BSA*, bovine serum albumin.

domain did not bind to GPVI-CRP but did bind to collagen I and α2-CRP (Figure 5-1D). CRPs that contain GPVI/FcRγ recognition motifs have been shown to require chemical cross-linking into polymeric complexes to stimulate GPVI/FcRγ-specific platelet activation (120). We chemically cross-linked GPVI-CRP (GPVI-xCRP) or α2-CRP (α2-xCRP) and tested their abilities to stimulate platelet aggregation (Figure 5-1E). GPVI-xCRP was able to induce dose-dependent platelet aggregation similar to collagen I, whereas a very high concentration of α2-xCRP did not. Taken together, this data shows that the GPVI-CRP functions as a GPVI/FcRγ-specific ligand that mimics the receptor's interaction with collagen I.

GPVI-Mediated Platelet Adhesion Under Static Conditions is Dependent on Aggregation.

We further investigated platelet adhesion through GPVI/FcRγ because we were surprised to see platelet adhesion to GPVI-CRP when there was no platelet adhesion seen on collagen I when the $\alpha_2\beta_1$ interaction was blocked (6F1; Figure 5-1C). We also noticed that adhesion of platelets to GPVI-CRP under static conditions could be highly variable. We analyzed platelet adhesion to these collagen substrates as well as to a 1:1 mixture of α2-CRP and GPVI-CRP in the presence of 2 mM $MgCl_2$ or 5 mM EDTA (Figure 5-2A). All substrates showed platelets adhering in the presence of Mg^{2+} with collagen I and the 1:1 CRP mix having the highest levels of adhesion. When platelet adhesion was observed microscopically, we saw individual platelets adhering and spreading on collagen I with some aggregation, and the platelets adhered to the 1:1 CRP mix closely resembled those on collagen I. On α2-CRP, we saw individual platelets

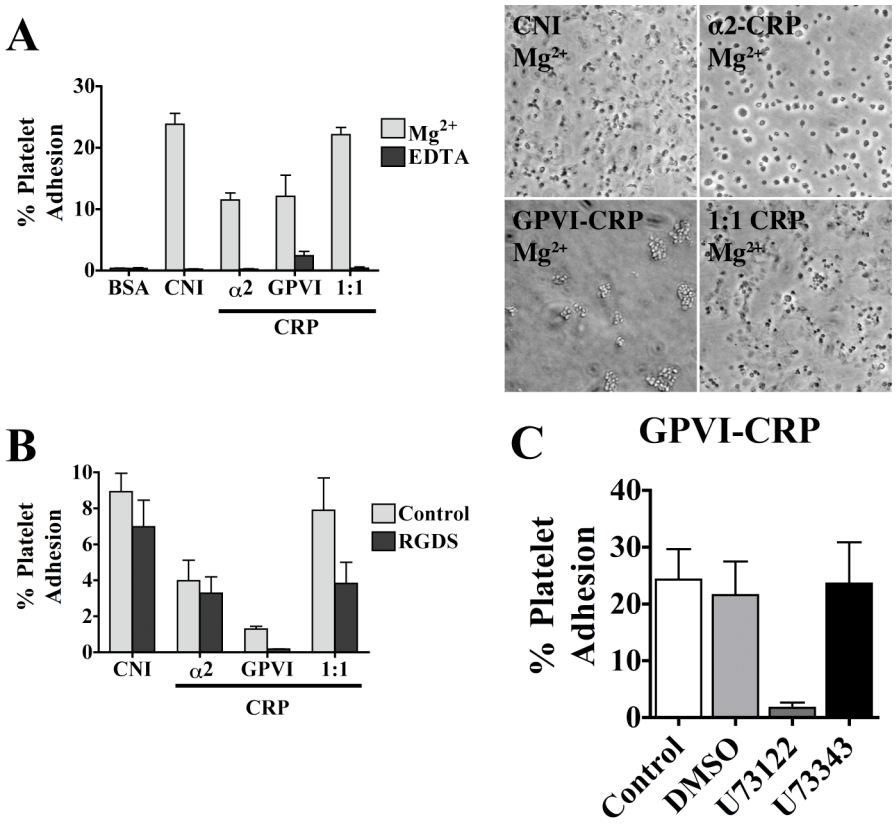

FIGURE 5-2: **GPVI-mediated platelet adhesion under static conditions is dependent on aggregation.** *A*, Platelet adhesion assay to BSA, CNI, α2-CRP, GPVI-CRP, or a 1:1 mix of α2- and GPVI-CRP using isolated human platelets in the presence of 2 mM $MgCl_2$ or 5 mM EDTA; on the right, representative microscopic images (40X) of adherent platelets from the adhesion assay. Results are percentages of adherent platelets (mean of 5 independent experiments performed in triplicate). *B*, Platelet adhesion assay to BSA, CNI, α2-CRP, GPVI-CRP, or a 1:1 mix of α2- and GPVI-CRP using isolated human platelets with or without 1 μM RGDS to inhibit $\alpha_{IIb}\beta_3$ integrin. Results are percentages of adherent platelets (mean of 3 independent experiments performed in triplicate). *C*, Platelet adhesion assay to GPVI-CRP using isolated human platelets treated with DMSO, 10 μM U73122 (PLC inhibitor), or 10 μM U73343 (negative control). Results are percentages of adherent platelets (mean of 5 independent experiments performed in triplicate).

adhering with less spreading and no aggregation. On GPVI-CRP, most of the platelet adhesion observed was platelet aggregates, but we did see a few individual spread platelets adhering. This was further supported by SEM analysis of adherent mouse platelets (Figure 5-6). In the presence of EDTA, adhesion was lost on collagen I, α2-CRP, and the 1:1 CRP mix, and adhesion on the GPVI-CRP was significantly attenuated.

We wanted to determine what role $\alpha_{IIb}\beta_3$ was playing in platelet adhesion to GPVI-CRP since chelation of metal ions with EDTA had an effect on adhesion to GPVI-CRP as well as the presence of platelet aggregates on GPVI-CRP. We analyzed platelet adhesion to these same substrates in the presence or absence of 1 mM RGDS (a peptide that binds to $\alpha_{IIb}\beta_3$ and inhibits platelet aggregation)(Figure 5-2B). In the presence of RGDS, a small decrease was seen on collagen I and no decrease was seen on α2-CRP. However, a significant decrease in platelet adhesion was seen on the 1:1 CRP mix, and a loss of platelet adhesion was seen on GPVI-CRP. This data suggested that platelet adhesion to GPVI-CRP under static conditions was dependent on platelets forming aggregates through inside-out activation of $\alpha_{IIb}\beta_3$. This was further supported by analyzing platelet adhesion to GPVI-CRP during inhibition of PLC, an important downstream signaling molecule in the GPVI/FcRγ pathway (Figure 5-2C). Platelets were pretreated with the PLC inhibitor (U73122), the negative control molecule (U73343), or DMSO and allowed to adhere to GPVI-CRP. Almost a complete loss of platelet adhesion was seen when PLC was inhibited by U73122 compared to the other controls. This data supports the fact that GPVI/FcRγ does not serve as an adhesion receptor but does support its role in platelet activation and aggregation.

Convulxin Stimulates Enhanced Platelet Adhesion to α2-CRP.

The ability of GPCRs to prime $\alpha_2\beta_1$ through a PLC-dependent mechanism and increase platelet adhesion to collagen suggested to us that the activation of GPVI/FcRγ might be capable of stimulating a similar effect. Snake venoms contain toxins that affect platelet activities; convulxin (Cvx) is a C-type lectin from venom that binds to and activates GPVI/FcRγ (167). We hypothesized that activation of GPVI/FcRγ prior to contacting collagen could enhance platelet adhesion to collagen by priming $\alpha_2\beta_1$. First, we determined what concentrations of Cvx stimulated human platelet aggregation and found 0.1 and 0.5 nM Cvx to be suboptimal and 1 and 10 nM Cvx to be optimal concentrations (Figure 5-3A). Using this range of Cvx concentrations, we tested this hypothesis by pretreating platelets with the different Cvx concentrations in the presence or absence of 1 mM RGDS and measured platelet adhesion to α2-CRP (Figure 5-3B). We observed an increase in platelet adhesion that was independent of platelet aggregation and was largest at 10 nM Cvx. We next compared the GPVI/FcRγ-stimulated enhanced adhesion to what we see with suboptimal PAR4 activation (Figure 5-3C). With 5 nM Cvx, we saw a significant increase in platelet adhesion over basal. This platelet adhesion was similar in level to the increase stimulated by 50 μM PAR4-AP and independent of platelet aggregation as shown in the platelet images. This data suggests that optimal activation of GPVI/FcRγ by Cvx can prime the platelet for $\alpha_2\beta_1$-mediated adhesion to collagen.

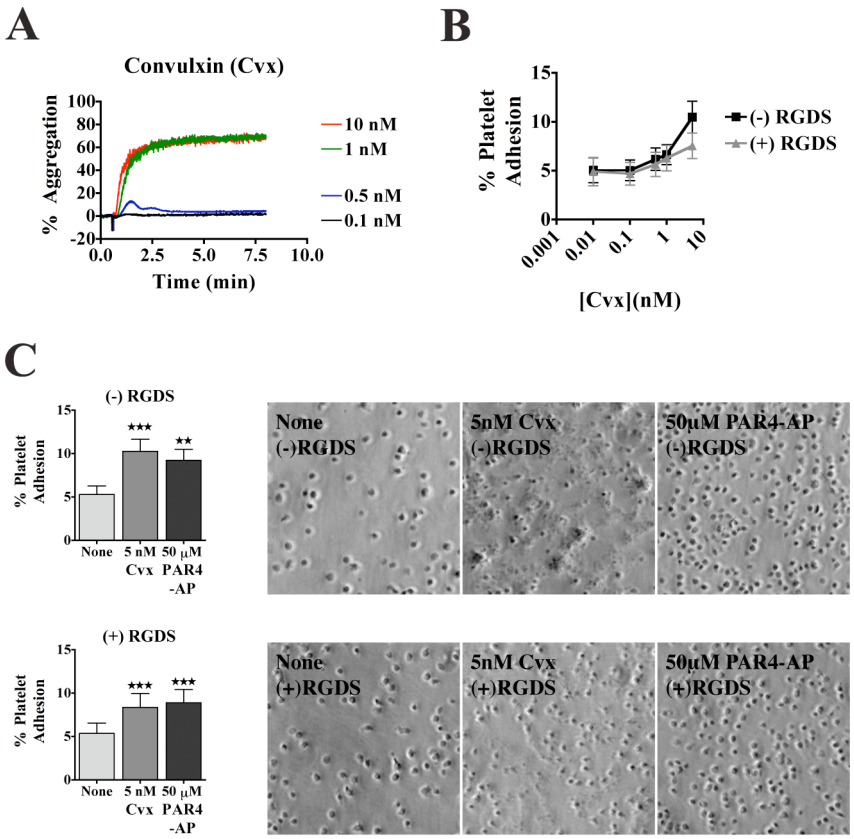

FIGURE 5-3: **Convulxin (Cvx) stimulates enhanced platelet adhesion to α2-CRP.** *A*, Platelet aggregation assays were done using human PRP to determine effective concentrations of convulxin (Cvx). Shown are representative data of three independent experiments. *B*, Dose-response curve for a range of Cvx concentrations in relation to human platelet adhesion to α2-CRP (30 μg/mL) in the presence of apyrase (1 U/mL) + SQ29,548 (1 μM) with or without 1 μM RGDS. Results are percentages of adherent platelets (mean ± SEM of 3 independent experiments each done in triplicate). *C*, Platelet adhesion assay to α2-CRP using isolated human platelets in the presence of 2 mM $MgCl_2$, apyrase (1 U/mL), and SQ29,548 (1 μM) and treated with 5 nM Cvx or 50 μM PAR4-AP with or without 1 μM RGDS; on the right, representative microscopic images (40X) of adherent platelets from the adhesion assay. Results are percentages of adherent platelets (mean of 6 independent experiments performed in triplicate).

Carotid Artery Thrombosis in $\alpha_2\beta_1$ or GPVI/FcRγ Deficient Mice.

To analyze the individual roles the collagen receptors have *in vivo*, we utilized mice deficient in $\alpha_2\beta_1$ or GPVI/FcRγ. We compared $\alpha2^{-/-}$, FcRγ$^{-/-}$, and GPVI$^{-/-}$ mice on a 129/SvJ × C57BL/6J (mixed) genetic background in an *in vivo* model of arterial thrombosis that involves shear stresses to look at the roles of the platelet collagen receptors. This assay uses laser-activated Rose Bengal dye to produce photochemical injury of the carotid artery in order to measure the time to complete vessel occlusion (Figure 5-4A). We hypothesized that the $\alpha2^{-/-}$, FcRγ$^{-/-}$, and GPVI$^{-/-}$ mice would have delayed times to arterial occlusion compared to wild-type mice. However, the carotid artery occlusion time for the FcRγ$^{-/-}$ mice was 39.3 ± 15.9 minutes (mean ± SD) and was similar to wild-type mice (44.4 ± 7.8) in comparison to the $\alpha2^{-/-}$ (74.5 ± 19.8) and GPVI$^{-/-}$ (74.6 ± 28.3) occlusion times. FcRγ$^{-/-}$ occlusion times were not significantly different than wild-type ($p > 0.05$), whereas $\alpha2^{-/-}$ and GPVI$^{-/-}$ occlusion times were statistically significant ($p < 0.05$) compared to wild-type and FcRγ$^{-/-}$. This was a surprising result and could be an effect of the mixed genetic background, so we measured the time to carotid artery occlusion in $\alpha2^{-/-}$, FcRγ$^{-/-}$ and wild-type mice on a pure genetic background (C57BL/6J) (Figure 5-4B). The FcRγ$^{-/-}$ mice on the pure background had an occlusion time of 54.8 ± 13.7 minutes (mean ± SD), which was similar to wild-type (52.4 ± 7.4) when compared to $\alpha2^{-/-}$ (66.4 ± 21.7), although the $\alpha2^{-/-}$ difference was not statistically significant. These data show that mice deficient in FcRγ do not have a defect in carotid artery thrombosis induced by photochemical injury like GPVI$^{-/-}$ and $\alpha2^{-/-}$.

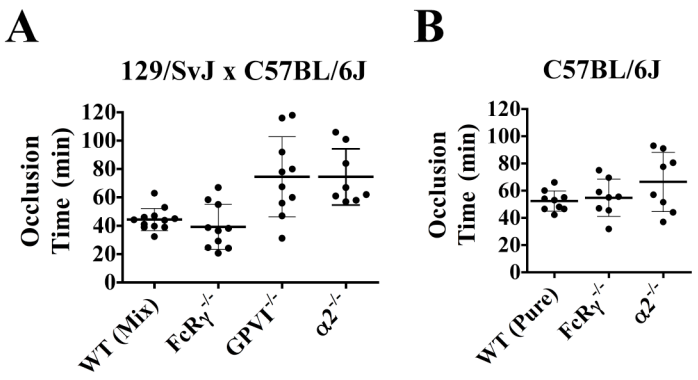

FIGURE 5-4: **Carotid artery thrombosis in $\alpha_2\beta_1$ or GPVI/FcRγ deficient mice.** *A*, *In vivo* analysis of arterial thrombosis using laser-activated Rose Bengal dye to produce a photochemical injury of the carotid artery. Measurements of the time to complete vessel occlusion were recorded in wild-type (WT), FcRγ$^{-/-}$, GPVI$^{-/-}$, and α2$^{-/-}$ mice on the mixed genetic background (129/SvJ × C57BL/6J). *B*, *In vivo* analysis of arterial thrombosis using laser-activated Rose Bengal dye to produce a photochemical injury of the carotid artery. Measurements of the time to complete vessel occlusion were recorded in wild-type (WT), FcRγ$^{-/-}$, and α2$^{-/-}$ mice on the pure genetic background (C57BL/6J).

Collagen Induced Pulmonary Thromboembolism in $\alpha_2\beta_1$ or GPVI/FcRγ Deficient Mice.

To further analyze the roles of the collagen receptors *in vivo*, we tested $\alpha2^{-/-}$, $FcR\gamma^{-/-}$, $GPVI^{-/-}$, and wild-type mice on the 129/SvJ × C57BL/6J (mixed) background or the pure C57BL/6J background using a model of pulmonary thromboembolism. This assay initiates intravascular thrombosis independent of shear stress by the intravenous injection of collagen I. Since the $FcR\gamma^{-/-}$ mice differed from the $GPVI^{-/-}$ mice in the carotid artery thrombosis assay, we postulated that the $FcR\gamma^{-/-}$ mice would have more incidences of thromboembolisms than $GPVI^{-/-}$ mice. One readout for this assay is to count the number of thrombi/mm^2 present in the lungs (Figure 5-5). On the mixed background, wild-type mice had 28.4 ± 5.3 thrombi (mean ± SD) and $\alpha2^{-/-}$ mice had 29.5 ± 6.8 thrombi in their lungs (Figure 5-5A). The $FcR\gamma^{-/-}$ mice had a slightly higher amount of thrombi (8.4 ± 1.9) compared to the $GPVI^{-/-}$ mice (1.5 ± 0.5) and this was statistically significant ($p = 0.0002$). Both thrombi counts for $FcR\gamma^{-/-}$ and $GPVI^{-/-}$ mice were significant when compared to wild-type and $\alpha2^{-/-}$ mice. The loss of $\alpha_2\beta_1$ expression on the mouse platelets did not affect thrombi formation when compared to wild-type mice. A similar trend was seen with the thrombi counts from $\alpha2^{-/-}$, $FcR\gamma^{-/-}$, $GPVI^{-/-}$, and wild-type mice on a pure genetic background (Figure 5-5B) where $FcR\gamma^{-/-}$ mice had 7.0 ± 1.7 thrombi and $GPVI^{-/-}$ mice had 2.7 ± 0.7, which were statistically significant from $\alpha2^{-/-}$ and wild-type mice. Interestingly, the thrombi counts between $FcR\gamma^{-/-}$ and $GPVI^{-/-}$ were also significantly different ($p = 0.0002$). There was no significant difference between the numbers of thrombi in $\alpha2^{-/-}$ and wild-type mice.

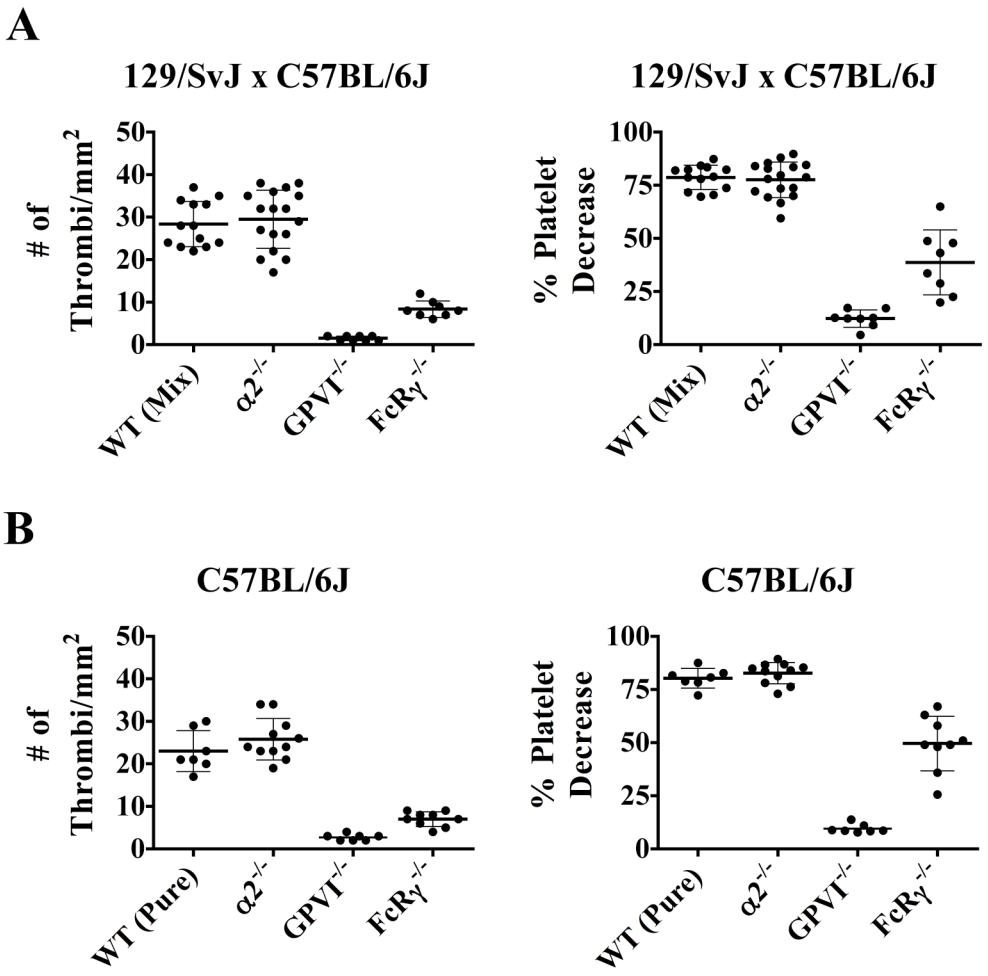

FIGURE 5-5: **Collagen induced pulmonary thromboembolism in $\alpha_2\beta_1$ or GPVI/FcRγ deficient mice.** *A*, *In vivo* analysis of pulmonary thromboembolism induced by injection of 25 μg collagen and 1 μg epinephrine. Measurements of the number of thrombi observed per mm^2 in 5 random 20X fields (left side) and the percent decrease in platelet number caused by the collagen injection (right side) were determined for wild-type (WT), $FcR\gamma^{-/-}$, $GPVI^{-/-}$, and $\alpha2^{-/-}$ mice on the mixed genetic background (129/SvJ × C57BL/6J). *B*, *In vivo* analysis of pulmonary thromboembolism induced by injection of 25 μg collagen and 1 μg epinephrine. Measurements of the number of thrombi observed per mm^2 in 5 random 20X fields (left side) and the percent decrease in platelet number caused by the collagen injection (right side) were determined for wild-type (WT), $FcR\gamma^{-/-}$, $GPVI^{-/-}$, and $\alpha2^{-/-}$ mice on the pure genetic background (C57BL/6J).

Another readout for the pulmonary thromboembolism assay is to measure the platelet decrement in the blood before and after collagen treatment (Figure 5-5). On the mixed background, the wild-type mice had an average platelet decrease of 78.7% ± 5.7 (mean ± SD) and the $\alpha 2^{-/-}$ mice had 77.6% ± 8.3 and the difference between these groups was not significant (Figure 5-5A). However, $GPVI^{-/-}$ mice had the lowest decrease in circulating platelets (12.3% ± 4.1) and FcRγ showed an intermediate decrease (38.7% ± 15.3). The difference in platelet decrement between $FcR\gamma^{-/-}$ and $GPVI^{-/-}$ again was significant ($p = 0.0002$). A similar pattern was observed with $\alpha 2^{-/-}$, $FcR\gamma^{-/-}$, $GPVI^{-/-}$, and wild-type mice on the pure background (Figure 5-5B). $GPVI^{-/-}$ mice had the most significant decrease in circulating platelets (9.6% ± 2.1) and $FcR\gamma^{-/-}$ showed an intermediate decrease (49.6% ± 12.8), and the difference in platelet decrement between $FcR\gamma^{-/-}$ and $GPVI^{-/-}$ was significant ($p = 0.0002$). These data further support the role of GPVI/FcRγ in *in vivo* thrombosis, but also highlight an innate difference between platelet activities in $FcR\gamma^{-/-}$ and $GPVI^{-/-}$ mice.

Morphologies of Wild-Type, $\alpha 2^{-/-}$, $GPVI^{-/-}$, and $FcR\gamma^{-/-}$ Mouse Platelets Adhering to Collagen Substrates.

We analyzed $\alpha 2^{-/-}$, $GPVI^{-/-}$ and $FcR\gamma^{-/-}$ mouse platelets using scanning electron microscopy to determine if there were variances in platelet morphology during adhesion and interaction with collagen substrates that was producing the difference in the *in vivo* analyses (Figure 5-6). Individual platelets with filopodial extensions were observed on collagen I and α2-CRP for wild-type, $GPVI^{-/-}$, and $FcR\gamma^{-/-}$ but not for $\alpha 2^{-/-}$. On the GPVI-CRP, only aggregates of platelets were seen for wild-type and $\alpha 2^{-/-}$ genotypes but were

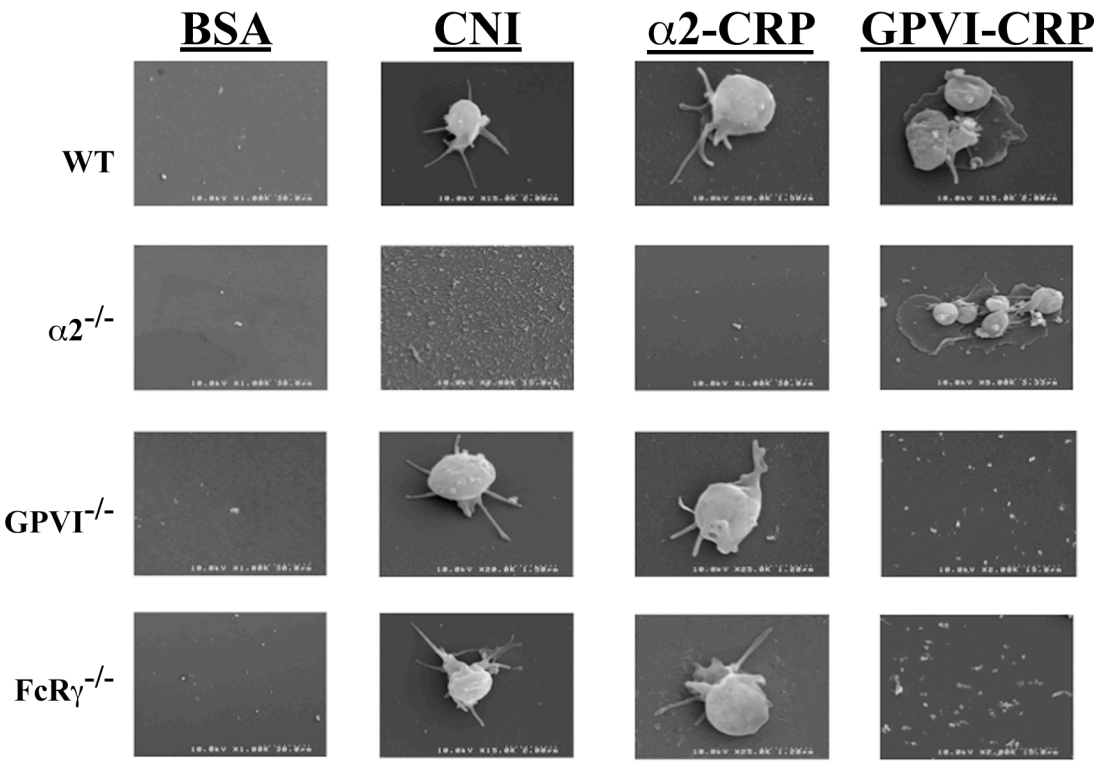

FIGURE 5-6: **Morphologies of wild-type, $\alpha 2^{-/-}$, $GPVI^{-/-}$, and $FcR\gamma^{-/-}$ mouse platelets adhering to collagen substrates.** Scanning electron micrographs of wild-type, $\alpha 2^{-/-}$, $GPVI^{-/-}$, or $FcR\gamma^{-/-}$ mouse platelets adhering to BSA, CNI, α2-CRP, or GPVI-CRP in 2 mM $MgCl_2$ adhesion buffer.

not detected for GPVI$^{-/-}$ or FcRγ$^{-/-}$. This data suggests the differences seen between GPVI$^{-/-}$ and FcRγ$^{-/-}$ *in vivo* are not caused by differences in their ability to interact with collagen.

Protein Phospho-Tyrosine Analysis of Wild-Type, GPVI$^{-/-}$, and FcRγ$^{-/-}$ Mouse Platelets.

The difference between the *in vivo* activities of GPVI$^{-/-}$ and FcRγ$^{-/-}$ platelets was further analyzed through *in vitro* analysis of phophorylation of tyrosines on platelet proteins to determine levels of platelet activation (Figure 5-7). Mouse platelets were left untreated or stimulated with 10 μg/mL collagen I, 10 μg/mL α2-CRP, or 200 μM PAR4-AP for 5 minutes and then lysed and proteins containing phosphorylated tyrosines were detected through immunoblot analysis. Interestingly, untreated platelets showed a differential phospho-tyrosine profile when comparing wild-type, GPVI$^{-/-}$, and FcRγ$^{-/-}$ platelets that was maintained through treatments. Protein bands of interest were at molecular weights of about 25, 50, and 65 kDa. This data further supports an innate difference between platelet activities in FcRγ$^{-/-}$ and GPVI$^{-/-}$ mice.

Discussion

In this study, we provide further insight into the role of GPVI/FcRγ on platelets during hemostasis and thrombosis. Our data show: (i) in static adhesion assays, platelets adhered to GPVI-CRP in an $\alpha_2\beta_1$-independent manner, however, the adhesion is dependent on inside-out signaling through PLC and platelet aggregation mediated by $\alpha_{IIb}\beta_3$.; (ii) chemical crosslinking of GPVI-CRP (GPVI-xCRP) was able to stimulate

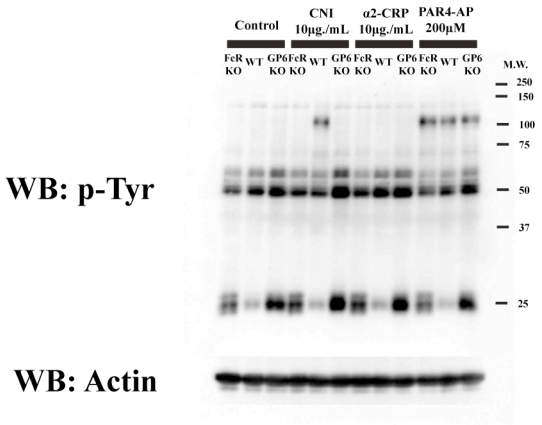

FIGURE 5-7: **Protein phospho-tyrosine analysis of wild-type, GPVI$^{-/-}$, and FcRγ$^{-/-}$ mouse platelets.** Western blot analysis of wild-type (WT), GPVI$^{-/-}$ (GPVI KO), or FcRγ$^{-/-}$ (FcR KO) mouse platelets. In the presence of 2 mM $MgCl_2$, control platelets or platelets treated with 10 μg/mL CNI, 10 μg/mL α2-CRP, or 200 μM PAR4-AP for 5 minutes and then lysed. Platelet lysates were run on a gel that was then transferred to a membrane for immunoblot analysis using antibodies for phospho-tyrosine and actin. Protein bands of interest were at molecular weights of about 25, 50, and 65 kDa.

platelet aggregation; (iii) activation of GPVI/FcRγ by Cvx stimulated an enhancement of $\alpha_2\beta_1$-mediated platelet adhesion to collagen; and (iv) GPVI$^{-/-}$ and FcRγ$^{-/-}$ mice show differences in thrombosis. Taken together, these results further support GPVI/FcRγ being an important platelet signaling receptor during hemostasis and thrombosis and identify a caveat with the use of genetic knockout mice for GPVI/FcRγ in thrombotic studies.

CRPs are important tools in studying the individual contributions of the platelet collagen receptors, $\alpha_2\beta_1$ and GPVI/FcRγ. The GPVI-CRP we utilized in this study confirms what others have seen with similar GPVI-specific CRPs when compared to collagen (60,120). Smethwurst *et al*. showed the amount of GPO repeats has an effect on the adhesiveness of the CRP as a substrate, where increasing the number of repeats (2 to 10 repeats) increases the platelet adhesion (119). It is worth noting that the GPVI-specific CRP that has been the model CRP in the field contains the sequence $(GPO)_{10}$ which is not physiologically relevant; GPVI-specific CRPs like GPVI-CRP that contain GPO sequences found in native collagens are less effective than $(GPO)_{10}$ but mimic platelet activities observed with collagen to a higher degree.

We further analyzed platelet adhesion to GPVI-CRP and compared it to collagen I, α2-CRP ($\alpha_2\beta_1$-specific substrate), or a 1:1 mix of GPVI-CRP and α2-CRP. Interestingly, the majority of platelet adhesion to the GPVI-CRP was dependent on platelet aggregation mediated by $\alpha_{IIb}\beta_3$. Verkleij *et al.* also observed platelet aggregates predominantly adhering to another GPVI-specific CRP (168). The 1:1 mix of CRPs resembled collagen I the best and further supported the fact that GPVI/FcRγ does not serve as an adhesive receptor but is an important platelet activating receptor. When GPVI/FcRγ signaling through PLC is inhibited platelet adhesion to GPVI-CRP is lost.

This data supports the dependence of GPVI/FcRγ on integrin ligation, whether it is through $\alpha_2\beta_1$ or $\alpha_{IIb}\beta_3$, for its interaction with collagen and suggests caution be used in the interpretation of data obtained using GPVI-specific CRPs that contain $(GPO)_n$ where $n \geq 6$.

The fact that we did not observe an increase in $\alpha_2\beta_1$-mediated platelet adhesion to the 1:1 mix of CRPs suggested further that $\alpha_2\beta_1$ must bind to collagen first followed by GPVI/FcRγ. In our previous studies, we identified a GPCR-mediated PLC-dependent priming mechanism of $\alpha_2\beta_1$-specific platelet adhesion to collagen (150). In this study, we show that optimal activation of GPVI/FcRγ by Cvx can stimulate an increase in $\alpha_2\beta_1$-mediated platelet adhesion similar to what PAR4 can induce. This data shows that GPVI/FcRγ activation can prime $\alpha_2\beta_1$ prior to the receptor binding the collagen substrate. This suggests that GPVI/FcRγ can stimulate stabilization of an already adherent platelet, but since it engages collagen after $\alpha_2\beta_1$, GPVI/FcRγ cannot prime $\alpha_2\beta_1$ but could induce strengthening of the $\alpha_2\beta_1$-collagen interaction. However, a GPVI/FcRγ agonist like Cvx that has its influence prior to the platelet contacting collagen would be able to prime $\alpha_2\beta_1$ and enhance the initial platelet adhesion to collagen.

The role of GPVI/FcRγ in hemostasis and thrombosis was further explored using collagen receptor deficient mice in *in vivo* assays. We utilized a laser-induced photochemical injury of the carotid artery of mice to model arterial thrombosis. This injury model supported an important role for $\alpha_2\beta_1$ in stable thrombus formation under conditions of high shear stress, but surprisingly the two types of GPVI/FcRγ deficient mice ($GPVI^{-/-}$ or $FcR\gamma^{-/-}$) gave conflicting results. Where $GPVI^{-/-}$ mice had an attenuated thrombus formation similar to $\alpha2^{-/-}$, $FcR\gamma^{-/-}$ mice were able to form occlusive thrombi

similar to wild-type. We checked to see if the mouse genetic background (129/SvJ × C57BL/6J) was causing this unexpected result by comparing it with mice on a pure imbred background (C57BL/6J). Previously, a study by Cheli *et al*. determined that the C57BL/6J genetic background contained a modifier of hemostasis that modulated the effect that loss of GPVI had on the bleeding phenotype of mice during *in vivo* thrombosis assays (169). We determined if this modifier of hemostasis was having a similar effect in the FcRγ$^{-/-}$ mice by analyzing laser-induced photochemical injury of the carotid artery in FcRγ$^{-/-}$ mice on the pure background (C57BL/6J). The FcRγ$^{-/-}$ mice had the same phenotype as wild-type on both pure (C57BL/6J) and mixed (129/SvJ × C57BL/6J) genetic backgrounds suggesting the modifier is not regulating thrombus formation in these mice. This meant that there was some other difference between the hemostatic functions of GPVI$^{-/-}$ or FcRγ$^{-/-}$ mice.

We further tested the *in vivo* thrombotic capabilities of GPVI$^{-/-}$ or FcRγ$^{-/-}$ mouse platelets using a collagen-induced pulmonary thromboembolism assay. The data from this assay supported what had been seen in the carotid artery thombosis model. GPVI$^{-/-}$ mice showed complete protection from collage-induced pulmonary thromboembolism, whereas FcRγ$^{-/-}$ mice showed an intermediate level of protection when compared to α2$^{-/-}$ and wild-type mice. This difference was observed in mice of both pure and mixed genetic backgrounds. Taken together, these data confirm an innate difference in the *in vivo* hemostatic functions of platelets in GPVI$^{-/-}$ and FcRγ$^{-/-}$ mice and suggests a dominant-negative effect caused from the deletion of GPVI.

For *in vitro* platelet aggregation assays, neither GPVI$^{-/-}$ nor FcRγ$^{-/-}$ platelets respond to collagen stimulation. However, the collagen-induced pulmonary

thromboembolism assay is an *in vivo* aggregation assay, but FcRγ$^{-/-}$ platelets are able to aggregate and produce a small amount of thromboembolism compared to GPVI$^{-/-}$. We wanted to determine why FcRγ$^{-/-}$ platelets were responding differently to collagen compared to GPVI$^{-/-}$ platelets, so we determined if their interaction with collagen was different. We show that GPVI$^{-/-}$ and FcRγ$^{-/-}$ platelets interact with collagen substrates in a similar fashion when comparing platelet morphologies by scanning electron microscopy. We next determined if GPVI$^{-/-}$ and FcRγ$^{-/-}$ platelets had a different phosphotyrosine protein profile upon activation or ligation of surface receptors. Interestingly, we found that GPVI$^{-/-}$ and FcRγ$^{-/-}$ platelets had different phosphotyrosine protein profiles when at rest and this difference was maintained during activation. Phosphotyrosine protein profile bands were not able to be identified but were of molecular weights of approximately 25, 50, and 65 kDa. These protein bands show more phosphorylation in GPVI$^{-/-}$ platelets, an intermediate level in FcRγ$^{-/-}$ platelets, and a low level in wild-type platelets. This data suggests that resting GPVI$^{-/-}$ and FcRγ$^{-/-}$ platelets have different levels of proteins with tyrosine phosphorylations, which may explain the differences observed with their hemostatic functions. However, more work needs to be done to identify the proteins in these bands whose phosphotyrosine statuses are varied and determine if that correlates with the level of GPVI$^{-/-}$ or FcRγ$^{-/-}$ platelet activity.

In this study, we further analyzed the role of GPVI/FcRγ on platelets during hemostasis and thrombosis. We utilized a GPVI-specific CRP (GPVI-CRP) and collagen receptor deficient mice in *in vivo* and *in vitro* assays to elucidate the functions of GPVI/FcRγ on platelets. These data further support the role of GPVI/FcRγ on platelets as a signaling receptor important in platelet activation and not as an adhesive receptor.

We also shed new insight on the effect of Cvx on platelet adhesion to collagen, as well as identified a difference between GPVI$^{-/-}$ and FcRγ$^{-/-}$ platelets. Further understanding of the functions of GPVI/FcRγ on platelets is important as this receptor is a potential target for antithrombotic therapy.

CHAPTER VI

DISCUSSION AND FUTURE DIRECTIONS

The studies presented in this dissertation examine the complex process of hemostasis in vertebrates and focus on the interaction of platelets with collagens at vascular wound sites. The mechanisms that contribute to normal hemostasis are also involved in pathological conditions of the cardiovascular system. Thrombosis is the production of a pathological blood clot, that can give rise to cardiovascular disease (CVD) and stroke, the leading causes of morbidity and mortality in the United States and the developed world (18,170). Platelets in the blood have a central role in cardiovascular thrombosis since they adhere to sites of vascular injury (e.g. a ruptured atherosclerotic plaque) where the subendothelial matrix composed of collagens is exposed to blood and leads to platelet aggregation and occlusion of the blood vessel.

A lot of research has been conducted on understanding platelet activation processes involved in hemostasis and thrombosis. From this research, pharmaceutical companies have been able to identify and develop some useful antiplatelet drugs to treat and prevent thrombosis such as aspirin, clopidogrel, and $\alpha_{IIb}\beta_3$ antagonists. However, these therapies still have limitations: aspirin is a weak inhibitor of platelet activation; clopidgrel has a slow onset of action because it has to be metabolized first and the metabolic rate is variable between patients; $\alpha_{IIb}\beta_3$ antagonists must be delivered intravenously; and overall, antiplatelet treatments cannot be separated from bleeding events (170). Development of more effective antiplatelet drugs for these and other

targets ($\alpha_2\beta_1$, GPVI/FcRγ, PARs, ect.) can improve upon therapies for thrombosis and further reduce mortality and morbidity caused by CVD and stroke.

For this reason, research has been conducted on the platelet collagen receptors ($\alpha_2\beta_1$ and GPVI/FcRγ) to understand the individual and cooperative functions they serve on platelets during hemostasis and thrombosis. Both collagen receptors are being pursued as pharmacological targets for antiplatelet treatments (170-172). However, there still remains some debate as to the roles $\alpha_2\beta_1$ and GPVI/FcRγ play (4,82). Currently, the consensus in the field seems to be that $\alpha_2\beta_1$ serves as the primary adhesive receptor contributing some intracellular signaling, whereas GPVI/FcRγ serves as the primary signaling receptor for activation with a secondary role in adhesion (27,62).

Our goal for these studies was to determine the contributions that the two platelet surface receptors, $\alpha_2\beta_1$ and GPVI/FcRγ, make toward the platelet interactions with collagens at vascular wound sites. The studies presented in this dissertation on $\alpha_2\beta_1$ and GPVI/FcRγ have led to some novel observations on the interactions of platelets with collagens. In chapter III, we show that $\alpha_2\beta_1$-mediated platelet adhesion to collagens can be increased through suboptimal activation (levels where $\alpha_{IIb}\beta_3$ is not activated) of $G\alpha_q$-linked GPCRs (specifically PAR4) utilizing a PLC-dependent signaling pathway. We further analyze this PAR4-induced $\alpha_2\beta_1$ priming mechanism of platelet adhesion to collagen in chapter IV and show that PAR4 induces a modulation of $\alpha_2\beta_1$ avidity that is not caused by a change in $\alpha_2\beta_1$ conformation or its affinity for collagen but does seem to be linked with a possible change in $\alpha_2\beta_1$ valency through a transient association with the platelet actin cytoskeleton. In chapter V, we analyzed aspects of GPVI/FcRγ-mediated platelet processes involved in hemostasis and thrombosis and show the importance of

GPVI/FcRγ as the primary signaling receptor for collagen as well as reveal a surprising difference between GPVI$^{-/-}$ and FcRγ$^{-/-}$ mice in platelet function. In this chapter, we will summarize some of our key findings and propose future research directions.

Downstream Signaling Molecules of GPCRs Involved in Priming of $\alpha_2\beta_1$ on Platelets.

In chapter III, we identified a GPCR priming mechanism of $\alpha_2\beta_1$ on platelets that could increase platelet adhesion to collagen by approximately twofold. We showed that suboptimal activation of GPCRs on platelets with the agonists: thrombin, PAR-APs, ADP, or TXA_2 could induce this enhanced adhesion. The GPCRs for these agonists are a part of a large, versatile family of membrane receptors that are commonly used as targets for therapeutic drugs and share the ability to transduce their signals by coupling to one or more of the four subtypes of G proteins: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12/13}$ (66). The platelet GPCRs activated by thrombin and PAR-APs (PAR1 and PAR4), ADP ($P2Y_1$), or TXA_2 (TPα and TPβ) all share the ability to associate with $G\alpha_q$ (67). Upon ligation, $G\alpha_q$-linked GPCRs activate PLCβ isoforms which hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 3,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Figure 5-4). IP_3 and DAG further propagate and amplify the signal through mobilization of intracellular Ca^{2+} stores and activation of other effectors (e.g. protein kinase C (PKC)) that induce a multitude of cellular processes many of which involve integrins (173).

A study by Lian *et al.* suggested that the deletion of both PLCβ2 and β3 (PLCβ2/β3$^{-/-}$) from mouse platelets caused a rescue in an *in vivo* carotid artery injury model using $FeCl_3$, as well as partial attenuation in thrombin- or ADP-stimulated platelet

aggregation and Ca^{2+} mobilization (174). They demonstrated the importance of PLCβ2/β3 in the signaling pathways of thrombin and ADP receptors on platelets. We determined in chapter III that the suboptimal activation of the thrombin receptors (PAR1 and PAR4) induced intracellular Ca^{2+} mobilization, and inhibition of PLC signaling (U73122) during suboptimal activation of PAR1 or PAR4 blocks the enhanced platelet adhesion. Interestingly, the level of Ca^{2+} mobilization stimulated by suboptimal activation of PAR1 and PAR4 correlated with the effectiveness at increasing platelet adhesion to α2-CRP (chapter III); whereas, suboptimal PAR4 activation had a greater, more sustained Ca^{2+} mobilization that coincides with a greater increase in platelet adhesion. Similar effects in blocking enhanced platelet adhesion were seen with PLC inhibition during suboptimal activation of $P2Y_1$ or TPα/β. This suggested that PLCβ signaling in platelets regulates the priming of $\alpha_2\beta_1$ downstream of $G\alpha_q$-linked GPCRs. Future studies will further analyze known downstream effectors of PLCβ signaling.

We hypothesize that both of the PLC signal propagators (IP_3 and DAG) serve to trigger the enhanced platelet adhesion. This is supported by the fact that a DAG analog phorbol-12-myristate-13-acetate (PMA) can induce a partial increase in $\alpha_2\beta_1$-mediated platelet adhesion compared to 50 μM PAR4-AP (Figure 6-1). When IP_3-stimulated intracellular Ca^{2+} mobilization is inhibited by chelation to BAPTA-AM, $\alpha_2\beta_1$-mediated platelet adhesion (both basal and enhanced) is completely lost (data not shown). These results suggest that both IP_3 and DAG have a signaling role in the priming of $\alpha_2\beta_1$. Signaling molecules (PKC isoforms (α and β) or CalDAG-GEF), which are activated downstream of both IP_3 and DAG, will be studied since mouse platelets deficient in these molecules have hemostatic defects (175-177).

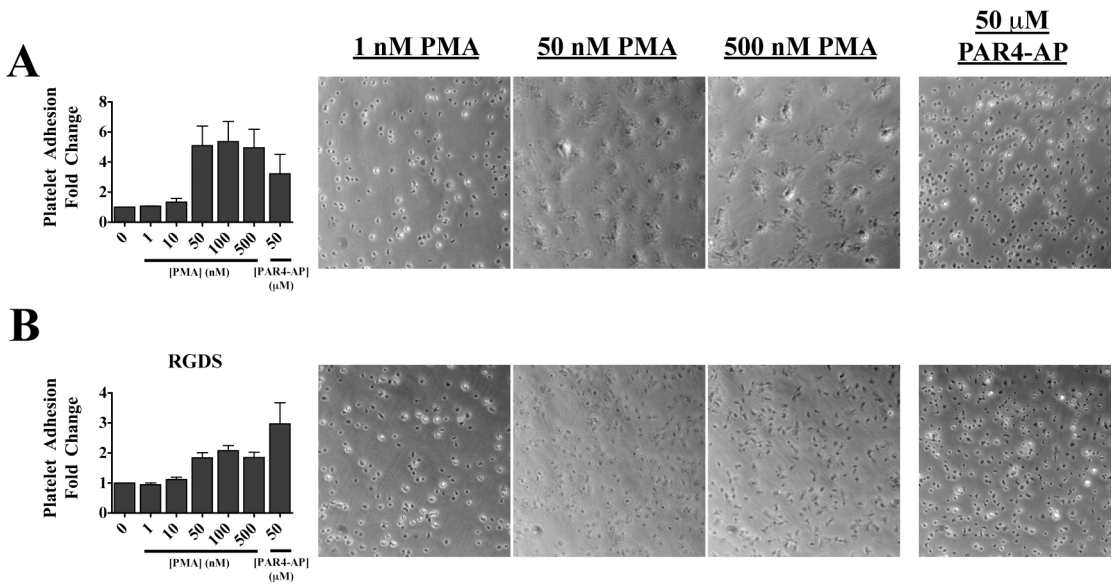

FIGURE 6-1: **PMA treatment stimulates an intermediate level of enhanced $\alpha_2\beta_1$-mediated platelet adhesion.** *A*, Platelet adhesion assay to α2-CRP using isolated human platelets in the presence of 2 mM $MgCl_2$, apyrase (1 U/mL), and SQ29,548 (1 μM) and treated with 0, 1, 10, 50, 100, 500 nM PMA or 50 μM PAR4-AP; on the right, representative microscopic images (40X) of adherent platelets from the adhesion assay. Results are the fold change in adherent platelets over basal (mean of 6 independent experiments performed in triplicate). *B*, Platelet adhesion assay to α2-CRP using isolated human platelets in the presence of 1 mM RGDS, 2 mM $MgCl_2$, apyrase (1 U/mL), and SQ29,548 (1 μM) and treated with 0, 1, 10, 50, 100, 500 nM PMA or 50 μM PAR4-AP; on the right, representative microscopic images (40X) of adherent platelets from the adhesion assay. Results are the fold change in adherent platelets over basal (mean of 6 independent experiments performed in triplicate).

Rap1 is another cytoplasmic molecule that is downstream of PLCβ and CalDAG-GEF signaling. Rap1 is a member of the Ras family of small GTPases and has been shown to be involved in cellular adhesion, polarity, differentiation, and growth (178). Rap1b is highly expressed in human and mouse platelets, and loss of Rap1b in mouse platelets disrupts platelet hemostatic functions (179). Rap1 has been shown to activate β1 and β3 integrins as well as regulate actin and microtubule cytoskeletal dynamics (178). Interestingly, we have obtained preliminary data that shows the activation of Rap1 in human platelets increases within 30 seconds of suboptimal PAR4 activation (Figure 6-2). Further studies will need to be performed to determine if the Rap1 activity produced by suboptimal PAR4 activation contributes to $\alpha_2\beta_1$ priming.

Lian *et al.* also showed that PLCβ2/β3$^{-/-}$ platelets failed to assemble filamentous actin upon thrombin stimulation when compared to wild-type and PI3K$^{-/-}$ platelets, and that PLCβ2/β3$^{-/-}$ platelets did not spread on a fibrinogen substrate (174). This data supports our findings that upon treatment of platelets with 50 μM PAR4-AP, $\alpha_2\beta_1$ transiently increases its association with filamentous actin within three minutes of stimulation (chapter IV) and that inhibition of PI3K with wortmanin had no effect on PAR4-stimulated enhancement of platelet adhesion (chapter III). A study by Calaminus *et al*. identified actin-rich, punctate structures in platelets (actin nodules) that formed during the early stages of adhesion to various substrates and were dependent on actin polymerization (180). Another study showed that actin polymerization and cdc42 activity were necessary for the interaction of $\alpha_2\beta_1$ with collagen during platelet adhesion through redistribution of $\alpha_2\beta_1$ on the platelet surface, whereas $\alpha_{IIb}\beta_3$ activation was not associated with actin cytoskeleton dynamics (149). Contrary to this $\alpha_{IIb}\beta_3$ data, other

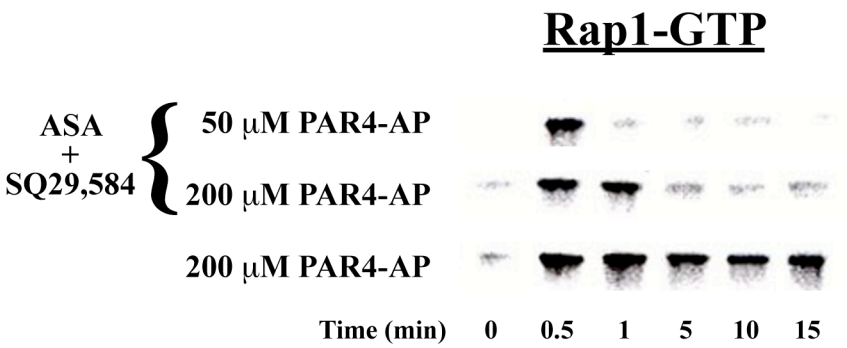

FIGURE 6-2: **Rap1 activation in PAR4-stimulated human platelets.** Western blot analysis of Ral GDS-RBD-agarose pull-down of Rap1-GTP (the activated form of Rap1) from PAR4 stimulated human platelets after 0, 0.5, 1, 5, 10, and 15 minutes. In the presence of 2 mM $MgCl_2$, platelets were treated with 50 μM or 200 μM PAR4-AP for in the presence or absence of 100 μM aspirin (ASA) and 1 μM SQ29,584. Platelets were stimulated for the indicated times and lysed. Ral GDS-RBD-agarose was used to pull-down activated Rap1 and run on an SDS-PAGE gel. The gel was then transferred to a membrane for immunoblot analysis using an antibody for Rap1.

studies have shown that $\alpha_{IIb}\beta_3$ does increase its association with the actin cytoskeleton in platelets stimulated by thrombin and is important in the signal transduction and stabilization of the $\alpha_{IIb}\beta_3$-ligand interaction (181,182). A study by Kovascovics *et al*. showed that upon thrombin activation of platelets, GPIb-V-IX associates with the actin cytoskeleton and is redistributed through a myosin II-dependent process (183). This evidence points to the actin cytoskeletal dynamics as being a possible connection between $G\alpha_q$-linked GPCR signaling and $\alpha_2\beta_1$-mediated platelet adhesion. Future studies will focus on the dynamics of the actin cytoskeleton and early morphologies of platelets stimulated with suboptimal concentrations of PAR4 agonists. It will be important to address the specificity of the $\alpha_2\beta_1$-priming mechanism in relation to other platelet integrins ($\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$).

Actin cytoskeletal dynamics have been shown to be important in cell adhesion and integrin function as well as in cooperation with GPCR signaling during directed cell migration (184-186). The reorganization of the actin cytoskeleton is crucial for the functions of the platelet in hemostasis and the amount of filamentous actin contributing to the cytoskeleton can double upon activation (187). Several intracellular proteins (Talin, ILK, and FAK) that are associated with focal adhesions and their formation are known to serve as indirect links of integrins to the actin filaments that form the cytoskeleton of cells. We have looked at the activation state of Talin, ILK, and FAK (detection of phosphorylation at activation sites) or their association with $\alpha_2\beta_1$ (co-immunoprecipitation with $\alpha_2\beta_1$) in platelets treated with 50 μM PAR4-AP and did not observe any changes in activation or $\alpha_2\beta_1$ association compared to control (data not shown). Other integrin-associated cytoplasmic proteins (filamin, α-actinin, vinculin, and

Kindlin-3) that have direct or indirect connections with the actin cytoskeleton have been shown to be involved in platelet hemostatic functions (188-191). Future studies will look at filamin, α-actinin, vinculin, and Kindlin-3 in PAR4-stimulated priming of $\alpha_2\beta_1$ on platelets.

Another future direction will be to determine the relevance of the $\alpha_2\beta_1$-priming mechanism triggered by suboptimal GPCR (PAR1, PAR4, $P2Y_1$, TPα, or TPβ) activation on platelet adhesion to α2-CPR under flow conditions. We hypothesize that suboptimal GPCR stimulation can increase platelet adhesion above basal levels under normal and pathological flow conditions. This hypothesis is supported by two studies that show a role for PAR activation in the platelet's interaction with collagen under flow conditions either *in vivo* or *in vitro* (136,139). It will also be interesting to determine if VWF binding to GPIb/V/IX is also capable of priming $\alpha_2\beta_1$ and increasing the adhesiveness of platelets to collagen (Figure 6-4) since GPIb/V/IX has been shown to stimulate PLCγ2 and regulate actin cytoskeletal dynamics (192).

PAR4-Stimulated Modulation of $\alpha_2\beta_1$ Avidity on Platelets.

We further analyzed the $\alpha_2\beta_1$ priming mechanism induced by suboptimal activation of PAR4 on platelets in chapter IV to determine what changes to $\alpha_2\beta_1$ were mediating the increased platelet adhesion to collagen. Cell adhesion that utilizes integrins to bind to a substrate is regulated through two mechanisms that increase the avidity (overall adhesiveness) of the cell for the substrate: 1) modulation of the strength of the individual integrin-ligand bond (affinity) and 2) modulation of the number and spatial organization of the integrin-ligand bonds (valency) (106). Both of these

mechanisms seem to be important in cell adhesion and migration, but the contribution of valency regulation especially in leukocyte and platelet adhesion is still unclear (106,144,186). Both affinity and valency modulation have been shown to have complimentary roles in cell adhesion mediated through $\alpha_L\beta_2$ and $\alpha_{IIb}\beta_3$ (193,194). Interestingly, there is also evidence of differential regulation of affinity and valency between β1 and β2 integrins by chemoattractant activation of GPCRs on eosinophils (195). Another study showed that chemokine activation of GPCRs on monocytes differentially and sequentially regulated the adhesiveness through $\alpha_4\beta_1$ and $\alpha_5\beta_1$ (196). Taken together, these data reveal the complexity of integrin avidity modulation on a cell and suggest differences in regulation between integrin types on the same cell.

Integrins are surface receptors that undergo long-range conformational changes upon either ligation to an extracellular substrate (collagen) or association of intracellular molecules (Talin) to the cytoplasmic tails of integrins (44). The conformation of the integrin determines the affinity of the integrin for its ligand; such as when the integrin is in a bent, inactivated conformation it has a low affinity for its ligand, but when the integrin is transformed to an extended, activated conformation it has a high affinity for its ligand (45). Generally, integrins are believed to be in the inactivated, low affinity conformation until they are induced into the activated conformation. This is evident for circulating platelets where an inactivated state for $\alpha_{IIb}\beta_3$ is maintained to avoid it binding to the abundant fibrinogen in the plasma and causing pathological thrombus formation (Figure 6-4). However, when the platelet is stimulated by thrombin, TXA_2, or ADP, $\alpha_{IIb}\beta_3$ is converted into the activated conformation through inside-out signaling from the GPCRs of these agonists and now binds to the plasma fibrinogen with high affinity

causing platelet aggregation. This degree of inactivation does not seem to apply to all integrins since $\alpha_2\beta_1$ and $\alpha_6\beta_1$ do not require an activating inside-out signal in order to bind to their ligands (chapter IV) (155). This highlights the idea that there is a dynamic equilibrium between the conformations of each integrin and the possibility that intermediate conformations and affinities are plausible as well as their regulation being variable between different types of integrins (45).

This equilibrium concept is further supported by structural studies of the α2 I-domain complexed with or without a GFOGER-containing CRP, which indicate the α2 I-domain is in a closed conformation that changes to an open conformation when the I-domain is bound to GFOGER (39,197). Emsley *et al.* suggest that the conformational rearrangement induced by ligation of the α2 I-domain to the GFOGER-containing CRP can be transmitted through the rest of the integrin and the transmittance can be bidirectional. While there is not a full protein structure of $\alpha_2\beta_1$, the x-ray crystal structure of an integrin with an αI-domain was recently determined for $\alpha_X\beta_2$ in the bent conformation with a closed αI-domain (198). Xie *et al*. showed that the $\alpha_X\beta_2$ inactivated structure was different than the structures obtained for the integrins that lack αI-domains: $\alpha_V\beta_3$ (199) and $\alpha_{IIb}\beta_3$ (200). The bent structure of $\alpha_X\beta_2$ has a different orientation of its α and β legs when compared to $\alpha_{IIb}\beta_3$ and $\alpha_V\beta_3$, and the αI-domain is highly flexible allowing for three conformational states and greater ligand accessibility. These data suggest that $\alpha_2\beta_1$ might be able to have several conformations each with different affinities for collagen.

In chapter IV, we analyzed whether or not suboptimal PAR4 stimulation was causing a change in the conformation of $\alpha_2\beta_1$ to the activated state and increasing the

integrin's affinity for the ligand. We show by several methods that suboptimal activation of PAR4 is not changing the affinity or inducing the activated conformation of $\alpha_2\beta_1$. Also when the activated conformation of $\alpha_2\beta_1$ is induced by Mn^{2+}, the PAR4-stimulated enhancement of platelet adhesion to collagen is still observed. The fact that $\alpha_2\beta_1$ is not being activated and having its affinity increased also correlates with $\alpha_{IIb}\beta_3$ not being activated by the suboptimal activation of PAR4. However, some cellular process is acting on $\alpha_2\beta_1$ to increase platelet adhesion to collagen, and our data suggests that the process is linked to actin cytoskeletal dynamics and is specific to $\alpha_2\beta_1$ since enhancement of platelet adhesion is not seen for another substrate/integrin interaction (e.g. laminin/$\alpha_6\beta_1$). Future studies will further test the specificity of the PAR4-stimulated priming mechanism for $\alpha_2\beta_1$ as well as determine what mechanism is modulating $\alpha_2\beta_1$ avidity on the platelet surface.

We hypothesize that PAR4 is modulating the avidity of $\alpha_2\beta_1$ on the platelet surface through actin cytoskeletal dynamics that cause a reorganization of $\alpha_2\beta_1$ to increase the number of $\alpha_2\beta_1$ molecules interacting with collagen. The concept of integrin valency modulation (clustering) on platelets is controversial, because it has been very difficult to analyze clustering due to the small size of platelets and this cell type's inaccessibility to genetic manipulation. However, a few studies on platelets support a role of $\alpha_{IIb}\beta_3$ clustering on activated platelets (109,182,201). Bunch used a monovalent Fab of the PAC-1 IgM (an antibody that binds to the activated conformation of $\alpha_{IIb}\beta_3$) to show thrombin stimulation of platelets induced clustering of $\alpha_{IIb}\beta_3$ and not its higher affinity conformation. Fox *et al*. showed that $\alpha_{IIb}\beta_3$ on platelets upon ligation becomes associated with the actin cytoskeleton and is redistributed on the surface in order to

stabilize the ligand interaction. A study by Isenberg *et al*. showed that clustering of $\alpha_{IIb}\beta_3$ on thrombin- or ADP-activated platelets occurs upon $\alpha_{IIb}\beta_3$ binding to fibrinogen or RGDS. Clustering of β2 and β3 integrins has also been demonstrated in other cell types such as leukocytes and genetically manipulated CHO cells (145,202,203). These data suggest that valency modulation of $\alpha_2\beta_1$ on platelets is a plausible mechanism for avidity regulation.

An area of contention in the analyses of $\alpha_2\beta_1$ changes in affinity is found in how the affinity changes are measured. Reports on $\alpha_2\beta_1$ affinity changes have used multivalent ligands in their assays. This means that the proposed affinity change cannot be separated from the interaction possibly being a valency change or a combination of the two processes. The multivalent ligands that have been used to study $\alpha_2\beta_1$ affinity changes on activated platelets are soluble and fibrillar collagens that contain multiple $\alpha_2\beta_1$ binding sites of varying affinities for $\alpha_2\beta_1$ (132,204). We sought to produce a monovalent $\alpha_2\beta_1$-specific ligand with a fluorescent tag for use in affinity studies by using the α2-CRP, but upon labeling the α2-CRP with hydrophobic fluorophores, the ligand acquires non-specific binding capabilities towards platelets. We have generated an α2-CRP that is biotinylated and plan to utilize this tool in future studies addressing $\alpha_2\beta_1$ affinity/valency modulation on platelets.

One way we are probing the effect of suboptimal PAR4 activation on platelets in modulating the avidity of $\alpha_2\beta_1$ for α2-CRP is by utilizing an optical trap-based force measurement assay (Figure 6-3A) (205). Optical trap-based assays have been widely used to measure forces in biological systems (206), for a wide range of applications from measuring the extensions of single DNA (207,208) or RNA (209) molecules, to pulling

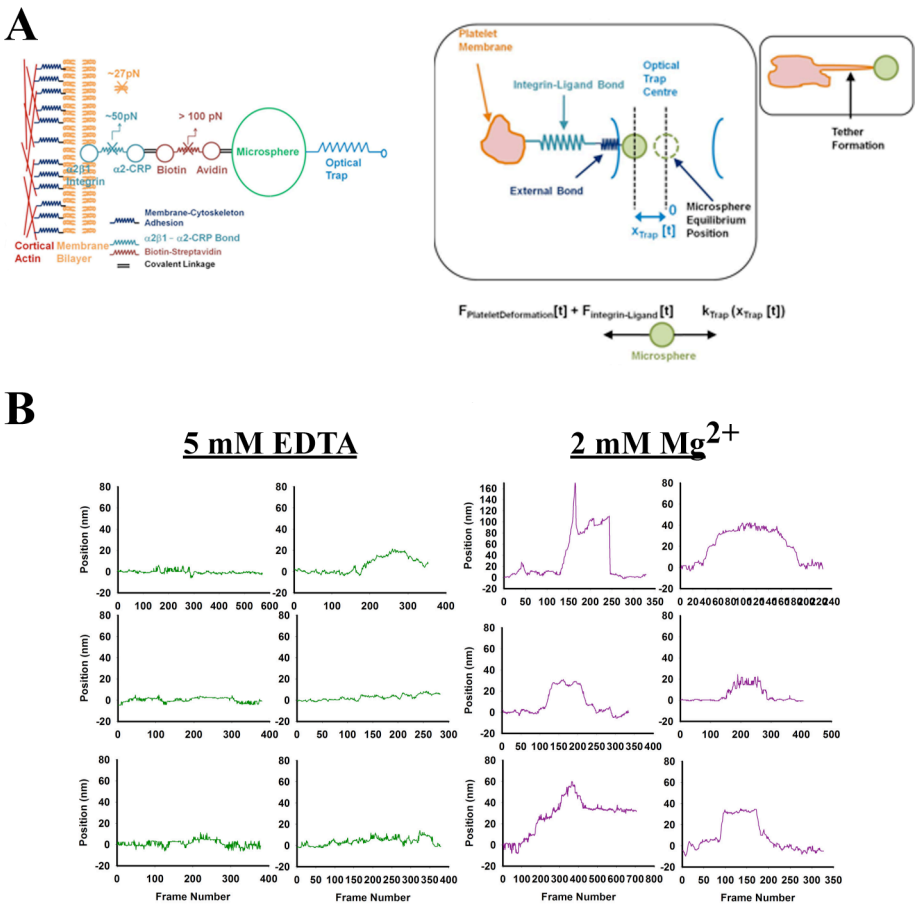

FIGURE 6-3: **Optical trap-based force measurements of bond ruptures between platelet $\alpha_2\beta_1$ and α2-CRP coated beads.** *A*, The conjugation of external, anchoring bonds utilized in our assays is depicted (Left). The different compliances within the system are depicted in order to ensure that in the analysis of our data we account for all the different bonds that might stretch. This is particularly important due to the fact that upon application of a point pulling force to a plasma membrane, it is possibly to deform the conjugating bonds or membrane preferentially instead of the integrin molecule. In order to avoid this we used biotin-streptavidin bonds as well as covalent bonds to produce the α2-CRP coated 1 μm microspheres. A typical platelet-integrin-microsphere force extension assay is shown (Right). The balance of forces over the microsphere in our assay is depicted. Tether formation in extreme cases of membrane deformation is also depicted. We incorporate membrane deformation into our analysis since it cannot be discounted. *B*, Interaction forces between an optically trapped microsphere and platelets in the presence of 5 mM EDTA (green traces) or 2 mM Mg^{2+} (purple traces) are depicted. The first inset for the Mg^{2+} experiments indicates tether formation rather than pure force extension of the integrin-receptor bond. We indented the microsphere by the exact same amount into each platelet, and the probe and the platelet were allowed to interact for a few seconds and then the stage was then translated away at a constant velocity (200nm/s and 1000nm/s). The stiffness of the optical trap was ~0.8pN/nm in all our assays, resulting in force loading rates of 160-800 pN/s. The result was extension of the integrin molecule super-imposed on top of the platelet membrane deformation. Sharp drops in force indicated the rupturing of the integrin molecules. The optical trap was calibrated by applying known viscous forces and observing how xTrap[t], the displacement of an optically trapped particle from the centre of the optical trap, changed with force. The slope of the graph of xTrap, Max, the maximum displacement from the centre of the trap, versus FApplied, the applied force gives us kTrap, the stiffness of the optical trap.

membrane tethers (210,211) from live cells and giant unilamellar vesicles (GUVs) (212), to measuring the force extension curves of integrin molecules *in vitro* (213) as well as in the context of platelets (214-216).

In order to test the feasibility of these experiments, we have developed control assays to probe whether we can detect a change in the affinity of integrin molecules for their substrate in the presence and absence of metal ions. We used an optical trap to measure the force required to extend and rupture the bond between $\alpha_2\beta_1$ and α2-CRP in the presence of metal ions (Mg^{2+}) or their absence (EDTA)(Figure 6-3B). Similar measurements on integrins using optical trap assays have revealed that the integrin bond behaves like a catch bond, i.e. over certain regimes of applied force the molecules affinity increases for its substrate (217). We observed similar behavior in our assays, as indicated by the force plateaus seen in distance-time records of the platelet interaction with α2-CRP in the presence of Mg^{2+} (Figure 6-3B). Since a lot of different information (binding kinetics, force of a single integrin bond, number of bond ruptures, surface area of interaction) can be captured from optical trap analysis, we plan to use the optical trap-based approach in future studies on the platelet's interaction with α2-CRP after suboptimal PAR4 activation. Using different metal ions (Mg^{2+} or Mn^{2+}) in the analyses of platelet $\alpha_2\beta_1$ binding forces, as well as analyzing the interactions of recombinant α2 I-domains (wild-type and E318A) with α2-CRP, we plan to determine specifically if suboptimal PAR4 activation is causing a modulation of $\alpha_2\beta_1$ valency on platelets.

GPVI/FcRγ Cooperation with $\alpha_2\beta_1$ in the Platelet-Collagen Interaction.

In chapter V, we determined how GPVI/FcRγ-mediated platelet processes are involved in hemostasis and thrombosis. We further support the importance of GPVI/FcRγ as the primary signaling receptor for platelet activation by collagen as GPVI-CRP stimulates platelet aggregation unlike α2-CRP. Our data does not support GPVI/FcRγ serving as a primary adhesive receptor as platelet adhesion to GPVI-CRP requires platelet aggregation. However, our data suggests that GPVI/FcRγ may strengthen $\alpha_2\beta_1$-mediated adhesion to collagen by triggering a similar clustering mechanism induced by $G\alpha_q$-linked GPCRs since Cvx can stimulate an increase in $\alpha_2\beta_1$-dependent adhesiveness in platelets.

Future studies will further analyze the cooperative mechanism of GPVI/FcRγ supporting $\alpha_2\beta_1$-mediated binding to collagen. We hypothesize that GPVI/FcRγ can induce clustering of $\alpha_2\beta_1$ on the platelet surface. Similar to the optical trap based assay using α2-CRP, we have generated a biotinylated GPVI-CRP and will measure bond forces between the GPVI-CRP and GPVI/FcRγ on platelets. We will then analyze the interaction of beads coated with a 1:1 mix of α2-CRP and GPVI-CRP with platelets and determine bond rupture forces. Another future direction will be to determine if GPVI/FcRγ uses the PLCγ2 signaling pathway to induce the increase in $\alpha_2\beta_1$-mediated platelet adhesion stimulated by covulxin. The $GPVI^{-/-}$ or $FcR\gamma^{-/-}$ mice will be useful tools in analyzing the cooperation of GPVI/FcRγ with $\alpha_2\beta_1$, but first it will be important to resolve the discrepancy between hemostasis observed between $GPVI^{-/-}$ or $FcR\gamma^{-/-}$ mice. Determining if the unaltered expression of FcRγ in the $GPVI^{-/-}$ platelets is producing a

dominant-negative phenotype will be important. Other studies using GPVI$^{-/-}$ or FcR$\gamma^{-/-}$ mice show differences in platelet functions between these two genotypes (218,219).

Summary

The studies presented in this dissertation examined the complex process of hemostasis in vertebrates and focused on the interaction of platelets with collagens at vascular wound sites. We identified a priming mechanism of $\alpha_2\beta_1$ through stimulation of Gα_q-linked GPCRs or GPVI/FcRγ that increases resting platelets' adhesiveness towards collagens through PLC signaling pathways (Figure 6-4). This priming mechanism of $\alpha_2\beta_1$ is triggered by suboptimal concentrations of GPCR agonists, a condition that would temporarily exist after the initial injury to the blood vessel wall due in part to the diluting factor of blood flow. Suboptimal activation of GPCRs increases $\alpha_2\beta_1$-mediated platelet adhesion to collagen while maintaining $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ in low affinity, inactivated conformations thus enhancing the ability of individual platelets to adhere to an arterial wound site under shear stresses and promote stable adhesion before platelet aggregation. We postulate that this priming mechanism modulates the receptor valency through actin cytoskeletal dynamics that redistributes $\alpha_2\beta_1$ into clusters. Optimal activation of platelets by Gα_q-linked GPCRs and GPVI/FcRγ induces the $\alpha_2\beta_1$ priming mechanism but also modulates integrin affinity and increases avidity through platelet aggregation mediated by $\alpha_{IIb}\beta_3$ binding to fibrinogen. These processes all cooperate to promote stable adhesion of platelets to collagen at vascular wound sites. Our studies shed new light onto the roles of $\alpha_2\beta_1$ and GPVI/FcRγ in hemostasis and thrombosis.

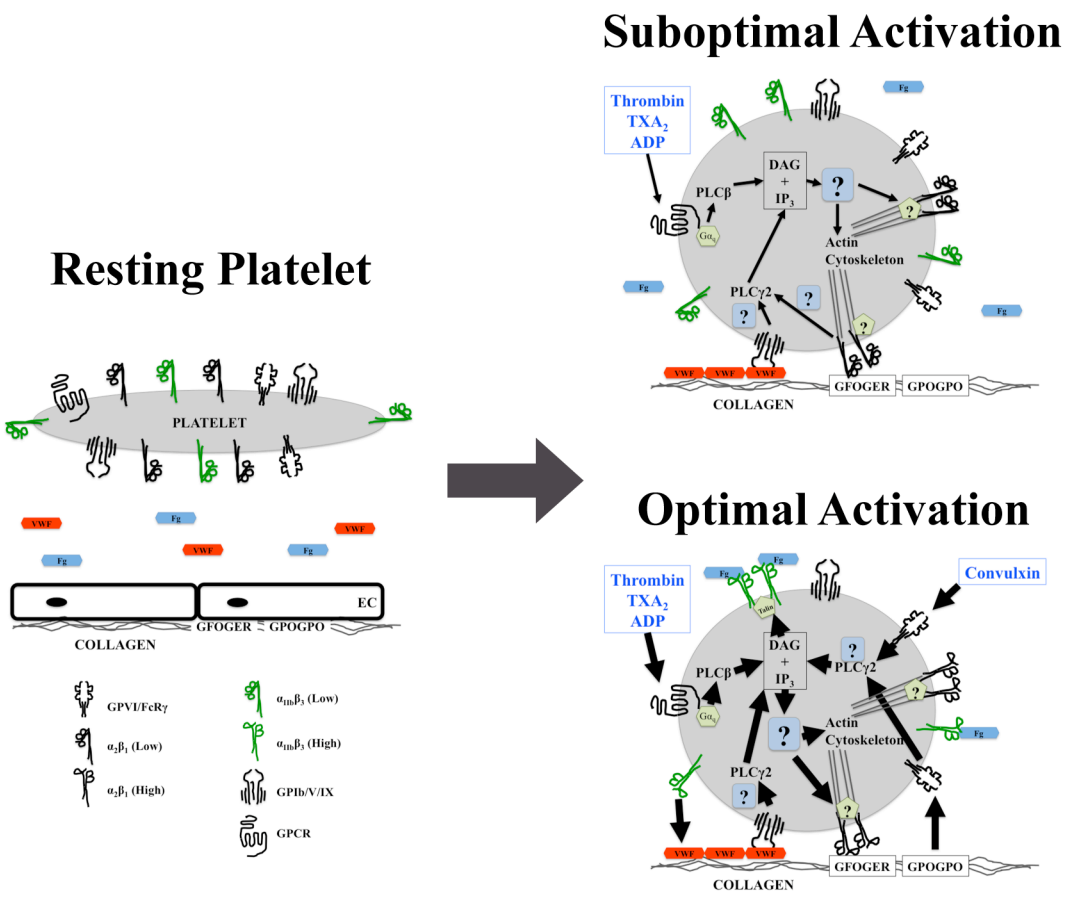

FIGURE 6-4: **A mechanism for platelet adhesion to collagen.** Platelets circulate through the vasculature in a resting state where integrins are in low affinity conformations and unable to bind to ligands due to their low affinity for the ligand (fibrinogen; Fg), masked binding epitopes (von Willebrand Factor; VWF), or blockade by endothelial cells (EC) (collagens). However when the platelet experiences an arterial vascular wound site, the platelet encounters activating stimuli that promote platelet adhesion, activation, and aggregation to form a stable thrombus. Under flow conditions, soluble activators require time to accumulate to effective concentrations while working against the diluting factor of blood flow. We identified a priming mechanism of $\alpha_2\beta_1$-mediated platelet adhesion to collagen that is triggered by suboptimal concentrations of agonists (those that do not activate $\alpha_{IIb}\beta_3$ causing platelet aggregation) for $G\alpha_q$-linked GPCRs (receptors for thrombin, ADP, and TXA_2). This priming mechanism works through the PLCβ signaling pathway (DAG and IP_3) and other downstream effectors causing an increase in $\alpha_2\beta_1$ avidity not through an affinity change in $\alpha_2\beta_1$ but through an association with the actin cytoskeleton possibly clustering $\alpha_2\beta_1$ that leads to an enhanced adhesiveness to collagen. This priming mechanism of $\alpha_2\beta_1$ would promote stable platelet adhesion to the wound site at the early stages of thrombus formation where collagen remains exposed and activator concentrations are low. Optimal concentrations of activators can also trigger the $\alpha_2\beta_1$-priming mechanism as well as induce the high affinity conformations of $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$, which all further increase the adhesiveness of the platelet to collagen as well as initiate platelet aggregation. Full activation of GPVI/FcRγ by convulxin can prime $\alpha_2\beta_1$-mediated platelet adhesion, and it is interesting to speculate that PLCγ2 signaling stimulated by GPVI/FcRγ binding to collagen could induce $\alpha_2\beta_1$ clustering to further strengthen platelet adhesion to collagen. It is also interesting to speculate that $\alpha_2\beta_1$ might be able to positively reinforce its own interaction with collagen through PLCγ2 activation. Question marks denote areas of future studies.

Cardiovascular disease and stroke are the leading causes of morbidity and mortality in the United States and the developed world, and blood platelets play a central role in cardiovascular thrombosis. Platelet adhesion to sites of vascular injury such as a ruptured atherosclerotic plaque, where the subendothelial matrix composed of collagens is exposed to blood, can lead to platelet aggregation and occlusion of the blood vessel. Understanding how platelets interact with collagens through the receptors $\alpha_2\beta_1$ and GPVI/FcRγ as well as how the collagen receptors' activities integrate into the overall hemostatic function of the platelet will be important in development of new and more effective antiplatelet drugs.

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