TWO DISTINCT ROLES OF INTEGRIN $\alpha_{\text{IIb}}\beta_3$ ‘OUTSIDE-IN’ SIGNALING:

PLATELET SPREADING AND CLOT RETRACTION

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

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Fig. 4.1 Schematic model of regulation of platelet spreading and clot retraction during α₁β₃ mediated ‘outside-in’ signaling
Platelet activation is central, in the process of physiological hemostasis and pathological thrombosis. In quiescent platelets the megakaryocyte/platelet lineage specific integrin $\alpha_{\text{IIb}}\beta_3$ is found in a low-affinity ligand binding state. However, upon platelet activation there is a rapid switch in the conformation of the integrin to a high affinity ligand binding state (a.k.a. ‘inside-out’ signaling) ultimately facilitating platelet aggregate formation. Ligand occupied integrin then sends another signal into the platelet (a.k.a. ‘outside-in’ signaling) regulating processes of platelet spreading and clot retraction. Previously, we had identified Calcium and integrin binding protein – 1 (CIB1) as a binding partner of the cytoplasmic tail of integrin subunit $\alpha_{\text{IIb}}$. Former work on CIB1 done at other laboratories had implicated CIB1 as an activator as well as an inhibitor of integrin $\alpha_{\text{IIb}}\beta_3$ activation. Furthermore, experimental evidences from our lab indicated CIB1 as an effector molecule regulating integrin signaling. Thus, amidst contradictory literature, I sought to investigate the actual role of CIB1 in integrin ‘inside-out’ signaling. Using Cib1 knock-out murine platelets, my results show that Cib1 is not required for agonist induced integrin activation as well as dense granular ATP release.
Later in my study I went ahead to characterize the integrin ‘outside-in’ signaling pathway using pharmacological inhibitors and human platelet spreading and clot retraction as my read-outs. The results obtained show that the enzymes PI3K, PLC, PKC and FAK are all important regulators of integrin ‘outside-in’ signaling and control both platelet spreading as well as clot retraction. However, p38 MAPK and ROCK are only required for clot retraction and are dispensable for platelet spreading. Furthermore, Src tyrosine kinase on one hand is required for platelet spreading and FAK activation, whereas, its inhibition causes faster clot retraction. Thus, it becomes evident that integrin mediated outside-in signaling regulates two distinct processes viz., clot retraction and platelet spreading, and either of them cannot be used interchangeably while studying integrin ‘outside-in’ signaling.
Chapter 1

INTRODUCTION

1.1 Platelet Overview

Cardiovascular diseases (CVDs) are the leading cause of death in the United States of America amongst all ages and genders (Fig. 1.1.A) followed by cancer and other diseases\(^1\). About two-thirds of all the deaths due to CVDs are because of coronary heart disease and stroke\(^1\) (Fig. 1.1.B) which result from abnormal clot (or thrombus) formation in the blood vessel - a process known to involve the specialized cells in the blood stream called platelets.

Exposure of circulating platelets to the extra-cellular matrix (ECM) exposed from injured blood vessels triggers platelet activation and subsequent hemostatic plug (clot) formation preventing excessive blood loss from the incapacitated vessel (Fig. 1.2.A). This process is crucial for normal physiological hemostasis. However, in injured vessels, such as at the sites of ruptured atherosclerotic plaques, the platelets get activated forming a thrombus and occluding the blood vessel (Fig. 1.2.B). This results in serious adversities in downstream tissues like in heart attack and stroke\(^2\). Therefore, understanding the processes involved in platelet activation and clot
formation are extremely important for the discovery of novel mechanisms for therapeutic interventions.

Fig. 1.1. (A) Cardiovascular diseases (CVDs) and other major causes of death: all ages and both sexes (United States: 2008). Source: NCHS and NHLBI (figure modified from Roger et al., 2012). (B) Percentage breakdown of deaths due to CVDs (United States: 2008). Source: NCHS (figure reproduced from Roger et al., 2012).
**Fig. 1.2.** (A) Platelets form a haemostatic plug to stop bleeding from an injured blood vessel. (B) Atherosclerotic plaque rupture results in clot formation that blocks the blood flow, which may result in a heart attack or stroke (figure reproduced from Gregg, 2003).

### 1.2 Platelet Structure

Platelets are formed by fragmentation of a gigantic polyploid cell present in bone marrow **viz.** Megakaryocyte (MK). Structurally, platelets contain a membrane system, cytoplasm having structural and enzymatic proteins, granules as well as organelles, all derived from precursor MKs (Fig. 1.3.A). Normal resting human platelets are 2–5 μm in diameter and approximately 0.5μm in thickness. It is important to mention here that platelets are anucleate having a very little capability of expressing new proteins; hence, they are inherently equipped with pre-synthesized molecules needed to perform their physiological functions.
1.2.1 Membrane system

The surface of the resting platelet lacks any protrusions. However, a high-resolution scanning electron micrograph shows that the platelet plasma membrane (PM) has several small pleats as well as a huge network of membrane invaginations called the open canalicular system (OCS) both providing additional membrane required during platelet spreading over surfaces\textsuperscript{5}. In addition, the OCS serves as a means of increasing surface area between the cytoplasm and blood enabling exchange of small molecules and limiting entry of large circulating proteins like antibodies\textsuperscript{5}. Besides this, the PM of platelets has high levels of glycoprotein receptors and signaling proteins that allow the platelet to respond quickly to vascular damage. Apart from the outer PM and associated OCS, there is an internal smooth endoplasmic reticulum membrane system known as the dense-tubular system (DTS) which is the store of intracellular Ca\textsuperscript{2+} critical for platelet activation\textsuperscript{6}. Moreover, it had been reported that DTS is the site for thromboxane synthetase activity which leads to thromboxane A\textsubscript{2} generation in activated platelets\textsuperscript{6}.

1.2.2 Cytoskeletal system

The platelet cytoskeletal system is instrumental in maintaining the discoid (Fig. 1.3.B) shape of quiescent platelets. Moreover, the morphological changes in an activated platelet are also governed by the cytoskeletal system. The cytoskeletal
system of a platelet can be divided into three: (1) Spectrin-rich skeleton on the inner side of plasma membrane; (2) Actin-rich cytoplasmic cytoskeleton; and (3) Microtubular system that forms a coiled structure and lines the circumference of platelet\textsuperscript{5}. Platelet activation results in rearrangement of the cytoskeleton leading to formation of filopodia (Fig. 1.3.C) and lamellipodia (Fig. 1.3.D) like structures particularly observed during platelet spreading on surfaces. Following platelet aggregation and clot formation, platelets must generate contractile forces that act to stabilize the aggregate and mediate clot retraction. Non-muscle myosin IIA and IIB motor proteins reported to be expressed in platelets are important for generating the contractile forces together with the F-actin\textsuperscript{5}.

1.2.3 Granular system

Two types of granules specific to MK/platelet lineage, viz. Alpha (\(\alpha\)) - granules and Dense (\(\delta\)) - granules, have been reported (Fig. 1.4.). On an average there are about 80 \(\alpha\)-granules\textsuperscript{7} per human platelet, more abundant than \(\delta\)-granules, that are 10-fold fewer (6-10)\textsuperscript{8}. 
**Fig. 1.3.** (A) Cartoon showing platelet ultrastructure highlighting important components. *(Retrieved from http://www.platelet-research.org/3/morphology.htm).* (B-D) Scanning electron micrographs of murine platelets under different stages of activation\(^9\) – discoid (B), spiky (C) and fully spread (D) platelets.

**Fig. 1.4.** Transmission electron micrograph showing platelet \(\alpha\)- and \(\delta\)-granules (Image: *Courtesy of Meghna Naik*).
The content of α-granules is mainly proteinacious in nature which includes (A) **Adhesive proteins**: Fibrinogen, fibronectin, von Willebrand factor (vWF); (B) **Glycoprotein receptors**: P-selectin and a proportion of surface expressed glycoprotein GPIb-IX-V as well as integrin α₃β₃; (C) **Coagulation factors**: prothrombin, factors V and XIII; (D) **Fibrinolytic proteins**: Plasminogen, Plasminogen activator inhibitor-I etc.; and (E) **Growth factors**: Platelet factor-4, Platelet derived growth factor, Vascular endothelial growth factor etc. On the other hand, the δ-granules contain small molecules that are known to play a role in platelet activation including ADP, ATP, Ca²⁺ and serotonin. Apart from the plasma membrane and α-granular membrane, the protein P-selectin, integrin α₃β₃ and GPIb-IX-V complex are reported to be present in the membranes of δ-granules. Furthermore, the Ca²⁺ present in δ-granules imparts an electron dense property to these granules allowing them to be visualized by electron microscopy without further staining.

1.3 **Platelet Activation and Functions During Physiological Hemostasis**

The main function of platelets is to achieve hemostasis at times of injury in the blood vessel to prevent excessive blood loss. The circulating platelets are pushed towards the periphery of vessel by larger blood cells (primarily RBCs) in flowing blood, ideally placing them close to the endothelium, thereby facilitating a fast response to vascular damage. A vascular lesion results in denudation of the endothelium thereby exposing ECM proteins like collagen to the flowing blood.
platelets sense this injury and get activated, leading to formation of a thrombus to plug the hole in the injured vessel. In order to understand this complex process

![Fig. 1.5. Stages in the development of a platelet thrombus on collagen exposed at sites of injury (reproduced from Gibbins, 2004)](image)

The initial indirect interaction of platelets with subendothelial collagens via von Willebrand factor facilitates transient tethering and rolling leading to unstable weak adhesion. This is followed by more-stable binding to collagen by GPVI and integrin α2β1. Synergistic signaling from vWF/GP-Ib-IX-V and Collagen-GP-VI-FcRγ complexes result in shape change, spreading, secretion and release of multiple prothrombotic factors. Together with this, the integrin affinity modulation through inside-out signaling promotes fibrinogen-αIIbβ3 mediated platelet aggregation and stable adhesion by firm binding of collagen and von Willebrand factor to their respective integrins α2β1 and αIIbβ3.
better, it is divided into several steps (Fig. 1.5.) some of which can occur at the same time in the same platelet:

(1) **Tethering, rolling and adhesion:** Upon vascular injury, the exposed von Willebrand factor (vWF), synthesized and secreted by endothelial cells and found immobilized on subendothelial ECM protein collagen, interacts with the receptor GPIb-IX-V present on platelet surface. This vWF/GPIb-IX-V interaction results in tethering and rolling of circulating platelets eventually slowing them down and promoting weak adhesion of platelets to the exposed sub-endothelial matrix proteins via integrins like α₂β₁ (collagen) and α₄β₃ (vWF and fibrinogen). This initial unstable adhesion at the injured site occurs concomitantly with the interaction of collagen receptor GPVI-FcRγ with exposed collagen resulting in receptor clustering and initiation of a strong activating signals causing platelet activation\(^{12,13}\).

(2) **Cytoskeletal rearrangement and shape change:** One of the primary observations made during platelet activation is shape change, in which resting discoid platelets transiently attain spherical morphology and then start extending filopodia like structures. For these morphological changes to occur, the platelet cytoskeleton undergoes dynamic rearrangement where existing filamentous actin (F-actin) is depolymerized and new F-actin is synthesized.
(3) **Integrin activation, stable adhesion and platelet aggregation:** The activating signals originating from clustered GPVI-FcRγ as well as GPIb-IX-V propagate and modulate activation of integrins (e.g., αIIbβ3, α2β1 etc.). Active integrins can then bind to their ligands firmly (e.g., αIIbβ3/fibrinogen, αIIbβ3/vWF, α2β1/collagen) and lead to strong stable adhesion. Plasma and platelet derived soluble fibrinogen as well as vWF (bi- and multivalent adhesion proteins respectively) can bind to activated integrin αIIbβ3 present on different platelets and promote crosslinking of different platelets and facilitating platelet aggregation.

(4) **Spreading:** Firmly adherent platelets finally spread by extending filopodia and lamellipodia like structures to form a monolayer which covers a large area of exposed ECM at the site of vascular lesion.

(5) **Granular secretion:** Contents of α- and δ-granules are rapidly exocytosed from the activated platelet. The small molecule ADP released from the δ-granules of activated platelets potentiates maximal platelet activation (through platelet ADP receptors P2Y1 and P2Y12) as well as recruits more platelets to the growing thrombi. On the other hand, the released contents of α-granules, particularly adhesive protein like fibrinogen and coagulation factors (like factor V, VIII), augment clot formation.

(6) **Thromboxane A2 (TxA2) generation:** There is mobilization of a fatty acid called arachidonic acid in activated platelets leading to generation of TxA2.
with secreted ADP, newly synthesized TxA₂ is also released from the activated platelet which is another known potent platelet activator. Like ADP, released TxA₂ also potentiates platelet activation (through platelet TxA₂ receptor $T_{\alpha}$ and $T_{\beta}$) and participates in recruiting more platelets to growing thrombi.

(7) **Phospholipid scrambling and thrombin generation:** Phospholipid phosphatidylserine (PS) is present and maintained on the inner leaflet of the PM of quiescent platelets. However, upon activation of the platelet, PS flips and appears on the outer leaflet of the PM. This provides a highly pro-coagulant surface allowing assembly of coagulation factors (like factor V, X) enhancing local generation of the most thrombogenic compound known – thrombin, which fires-up the process of thrombus formation. Thrombin not only activates platelets (through platelet thrombin receptors, primarily PAR-1 and PAR-4 in human platelets and PAR-4 in murine platelets), but also results in conversion of plasma fibrinogen to fibrin thus, simultaneously regulating another arm of thrombus formation – the coagulation cascade (Fig. 1.6). Formation of the fibrin mesh holds together the primary platelet plug (stably adhered platelet aggregate) and makes it resistant to embolization due to the high shear forces of flowing blood.

(8) **Clot stabilization:** Clot formation and its subsequent retraction are important for providing stability to the thrombus. The clot so formed is consolidated by the integrin $\alpha_{IIb}\beta_3$ outside-in signaling (discussed later) during which the fibrinogen
receptors anchored to the actin cytoskeleton transmit the retractile forces to the extracellular fibrin polymers\textsuperscript{17}.

\textbf{Fig. 1.6.} Simplified illustration showing the key steps of the process of hemostatic plug formation\textsuperscript{18}. Thrombin plays an essential role in both the coagulation cascade and platelet activation (Fig. modified from Spahn and Rossaint, 2005).
1.4 **Integrin α_{IIb}β_{3}**

Integrins are transmembrane heterodimers of an α- and a β-subunit. The α_{IIb} subunit is megakaryocyte/platelet lineage specific. On the other hand, the β_{3} subunit is expressed in various cell types and is known to interact with two types of α-subunits, viz. α_{IIb} and α_{v}. In platelets, the β_{3} subunit predominantly interacts with the α_{IIb} subunit forming the integrin α_{IIb}β_{3} (or glycoprotein IIb/IIIa), which is the most abundant glycoprotein expressed on the platelet surface (~ 80,000 copies per resting human platelet)\(^{19}\). More α_{IIb}β_{3} integrins are found in the membranes of α- and δ-granules and OCS which are also expressed on the surface upon platelet activation\(^{19,20}\). The preferred ligand for α_{IIb}β_{3} is fibrinogen, though, it also binds to fibronectin and vWF\(^{21}\). In quiescent platelets, the integrin α_{IIb}β_{3} exists in a low-affinity state (Fig. 1.7.) which prevents it from binding to its ligand. Like other integrins, α_{IIb}β_{3} molecules are also capable of bi-directional signaling across it *viz.*, integrin ‘inside-out’ and ‘outside-in’ signaling\(^{21,23}\).
Fig. 1.7. Pictorial representation of integrin activation and bidirectional signaling across it (Fig. modified from Hynes, 2002).22

1.4.1 ‘Inside-out’ signaling and integrin α\textsubscript{IIb}β\textsubscript{3} activation

Engagement of various platelet adhesion receptors (e.g., GPIb-IX-V, GPVI-FcRγ etc.) or G-protein coupled receptors (e.g., P2Y1, P2Y12, PAR-1,PAR-4 etc.) by their ligands initiates a signaling cascade inside the cell which brings about modulation in integrin α\textsubscript{IIb}β\textsubscript{3} affinity (integrin activation). Such changes in the ability of integrin α\textsubscript{IIb}β\textsubscript{3} to bind to its ligand fibrinogen together with other events that begin with agonist stimulation of a platelet (e.g., secretion, shape change etc.) are collectively referred to as ‘inside-out’ signaling (Fig. 1.8.). Under physiological settings, the signal originating from different agonist receptors act cooperatively leading to rise of intracellular Ca\textsuperscript{2+}. This result in activation of the downstream effector CalDAGGEF1 which triggers the Rap1-
Fig. 1.8. Simplified schematic of agonist induced ‘inside-out’ signaling and integrin activation (modified from Li et al., 2010).  

RIAM axis leading to binding of talin, and possibly many other proteins like Kindlin, β3-endonexin, to the cytoplasmic tail of the β3 subunit. This in turn results in unclasping of the membrane proximal complex of αIIb and β3 cytoplasmic tails triggering a conformational switch of αIIbβ3 molecule from low-affinity state to high-affinity ligand binding state.
1.4.2 ‘Outside-in’ signaling by integrin $\alpha_{\text{IIb}}\beta_3$

Fibrinogen binding to the activated integrin molecule results in clustering of $\alpha_{\text{IIb}}\beta_3$ and triggers another signaling cascade across the activated $\alpha_{\text{IIb}}\beta_3$ (that goes into the platelet from outside) known as integrin ‘outside-in’ signaling$^{20}$. It has been reported that the $\alpha_{\text{IIb}}\beta_3$ ‘outside-in’ signaling regulates platelet spreading on fibrinogen, clot retraction, granular secretion$^{25}$ as well as ADP-induced TxA$_2$ generation$^{26}$. The dogma in the field of platelet biology is that the two major ‘outside-in’ signaling dependent platelet responses – spreading on immobilized fibrinogen and fibrin clot retraction – are regulated by a common signaling cascade and either of the two can be interchangeably used as an experimental readout for the purpose of studying $\alpha_{\text{IIb}}\beta_3$ ‘outside-in’ signaling cascade. Following this paradigm, several molecules (Fig. 1.9.) have been reported to participate in the signaling downstream of integrin $\alpha_{\text{IIb}}\beta_3$ which presumably form a complex in the integrin proximal region and thus, relay the downstream signal of $\alpha_{\text{IIb}}\beta_3$ into the cell. However, in the light of the ongoing work from our lab and from the reports$^{27,28}$ from X. Du’s lab it is becoming evident that platelet spreading on fibrinogen and clot retraction are two processes that are distinctly regulated during $\alpha_{\text{IIb}}\beta_3$ outside-in signaling.
1.5 Calcium- and Integrin-binding Protein 1 (CIB1)

Calcium- and integrin-binding protein 1 (CIB1) was first identified as a binding partner of the integrin subunit αIIb using yeast-two hybrid system\(^\text{29}\). It is a 22KDa, 191 amino acids long calcium binding polypeptide\(^\text{29}\) (Fig. 1.10.). Structural and sequence
analysis demonstrates that CIB1 has four EF-hand motifs of which only two toward the C-terminus are functional that can bind calcium ions\textsuperscript{30}. The N-terminus glycine residue (Gly2) can be myristoylated\textsuperscript{31} helping in localizing endogenous CIB1 to the inner side of PM positioning it proximal to integrin tails.

\textbf{Fig. 1.10.} (A) Illustration of Calcium and Integrin Binding Protein-1 showing N-terminal myristoylation site and two functional EF-hand motifs towards the C-terminus (\textit{Courtsey: Edward Bayley}). (B) Crystal structure of Ca\textsuperscript{2+}-CIB1 (reproduced from Blamey et al., 2005)\textsuperscript{30}.

Apart from MKs and platelets, CIB1 had been reported to be widely expressed in several other tissue types like pancreas, heart, liver\textsuperscript{32}. CIB1 shares sequence similarity to other EF-hand containing proteins such as calmodulin and calcineurin B\textsuperscript{29} as well as several Ca\textsuperscript{2+}-myristoyl switch neuronal calcium sensor proteins\textsuperscript{33}. So far 24 CIB1
interacting partners have been reported in literature and of these 11 are known to be expressed in platelets (Table 1.1.). Therefore, it is plausible that CIB1 may regulate multiple platelet functions spatio-temporally through its ability to interact with various proteins in a calcium-dependent manner.

Table 1.1: CIB1 binding partners (those in bold letters are expressed in platelets).

<table>
<thead>
<tr>
<th>S.No</th>
<th>Binding partner</th>
<th>Known function</th>
<th>Published report</th>
</tr>
</thead>
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<tr>
<td>1.</td>
<td>αIib</td>
<td>Receptor</td>
<td>Naik et al., 1997 J. Bio. Chem.</td>
</tr>
<tr>
<td>3.</td>
<td>Pik2</td>
<td>Kinase</td>
<td>Kausermann et al., 1999 EMBO</td>
</tr>
<tr>
<td>4.</td>
<td>Pik3</td>
<td>Kinase</td>
<td>Kausermann et al., 1999 EMBO</td>
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<tr>
<td>5.</td>
<td>DNA-PK</td>
<td>Kinase</td>
<td>Wu &amp; Lieber, 1997 Mutation Research</td>
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<tr>
<td>7.</td>
<td>FAK</td>
<td>Kinase</td>
<td>Naik &amp; Naik 2003 Blood</td>
</tr>
<tr>
<td>8.</td>
<td>ASK1</td>
<td>Kinase</td>
<td>Yoon et al., 2009 PNAS</td>
</tr>
<tr>
<td>10.</td>
<td>PDK1</td>
<td>Kinase</td>
<td>Zhao et al., 2007 The FASEB J.</td>
</tr>
<tr>
<td>13.</td>
<td>Rac3</td>
<td>GTPase</td>
<td>Haastaja et al., 2002 J. Bio. Chem</td>
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<td>Transcription Factor</td>
<td>Hollenbach et al., 2002 Biochimica et Biophysica Acta</td>
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<td>21.</td>
<td>G-16</td>
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<td>Tahara et al., 2005 Cancer, Immunology, Immunotherapy</td>
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<td>22.</td>
<td>AID</td>
<td>Deaminase</td>
<td>Demarest et al., 2010 PLoS One</td>
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<tr>
<td>24.</td>
<td>α-1c subunit</td>
<td>L-Type Ca²⁺ channel</td>
<td>Heineke et al., 2010 Nat. Med.</td>
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</tbody>
</table>

1.5.1 Role of CIB1 in αIibβ3 signaling

It has been reported previously that CIB1 may function as an endogenous inhibitor of integrin αIibβ3 activation³⁴. The authors demonstrate that overexpression
of Cib1 in MKs result in blockade of agonist-induced integrin activation. Furthermore, RNAi mediated knockdown of endogenous Cib1 resulted in activation of αIIbβ3. Contrary to above finding, another report suggests that CIB1 may function as an activator of αIIbβ3 via agonist-induced inside-out signaling in a calcium dependent manner. The author demonstrates that a CIB1-αIIb interaction under *in vitro* conditions result in increased 125I-Fibrinogen binding to αIIbβ3. Furthermore, a peptide corresponding to c-terminus of CIB1 was not only able to inhibit CIB1-αIIb interaction *in vitro*, but it reduced fibrinogen binding to αIIbβ3 in platelets activated by ADP.

In addition to this, the research in Naik lab demonstrates that CIB1 is a regulator of integrin outside-in signaling. It was shown that CIB1 localizes at the tip of transiently formed filopodia (Fig. 1.11.A) during platelet spreading on immobilized fibrinogen. Moreover, CIB1-αIIb association was essential for platelet spreading on fibrinogen since a function blocking antibody against CIB1 (UN7.79) blocked the spreading platelets in spiky morphology (Fig. 1.11.B) as opposed to control platelets which progressed to a fully spread morphology. Moreover, the observed spreading defects in human platelets in absence of CIB1-αIIb association were reproduced in platelets derived from Cib1−/− mouse (Fig. 1.12.). Also, addition of exogenous ADP to the platelets exhibiting defective spreading on immobilized fibrinogen (human
platelets treated with anti-CIB1 Ab. (UN7.79) or αIIb cytoplasmic tail peptide; murine platelets upon genetic deletion of Cib1 rescued the fully spread morphology (Fig. 1.13.)\textsuperscript{9,36} suggesting that CIB1 is important for integrin ‘outside-in’ signaling induced granular release of ADP which may potentiate full platelet activation and spreading.
Fig. 1.11. (A) Change in the localization of CIB1 (red) during human platelet spreading on immobilized fibrinogen\textsuperscript{36}. (B) Inhibition of human platelet spreading on immobilized fibrinogen upon incorporation of anti-CIB1 antibody UN7.79\textsuperscript{36}.

Fig. 1.12. Defective spreading of $Cib1^{-/-}$ platelets on immobilized fibrinogen (100µg/mL)\textsuperscript{9}.

Fig. 1.13. Rescue of $Cib1^{-/-}$ platelet spreading by addition of exogenous ADP\textsuperscript{9}.
Besides this, it has also been demonstrated that CIB1 associates with focal adhesion kinase (FAK) and regulates its kinase activity\(^{37}\) (Fig. 1.14.). Additionally, \textit{in-vivo} analysis including tail-bleeding times and FeCl\(_3\)-induced injury model of thrombosis demonstrated impaired hemostasis in \textit{Cib1}\(^{-/-}\) mice compared to \textit{Cib1}\(^{+/+}\) littermates (Fig. 1.15.). Moreover, \textit{Cib1}\(^{-/-}\) platelets also showed delayed clot retraction (Meghna Naik, Unpublished data).

**Fig. 1.14.** (A) CIB1 co-immunoprecipitates with FAK\(^{37}\). (B) \textit{Cib1} overexpression in CHO cells causes increased FAK-pY as well as enhanced associated kinase activity\(^{37}\).

**Fig. 1.15.** (A) \textit{Cib1}\(^{-/-}\) mice show higher mean tail bleeding time compared to \textit{Cib1}\(^{+/+}\) control mice suggesting defect in normal hemostasis\(^9\). (B) \textit{Cib1}\(^{-/-}\) mice show defect in stable \textit{in-vivo} thrombus formation compared to \textit{Cib1}\(^{+/+}\) litter mates as seen in respective representative flow patterns observed in response to 10\% FeCl\(_3\)-induced injury in carotid artery. Baseline carotid blood flow [1], duration of injury [2], and recording of flow post-injury [3]. Formation of unstable thrombus in \textit{Cib1}\(^{-/-}\) mice is shown by arrows\(^9\).
1.6 Aim of the Study

As mentioned previously, integrin $\alpha_{\text{IIb}}\beta_3$ ‘outside-in’ signaling is known to regulate platelet spreading, clot retraction and granular secretion\textsuperscript{25}. Moreover, the observed defect in platelet spreading\textsuperscript{9,36} as well as the delay in clot retraction (Meghna Naik, Unpublished data) upon inhibition of CIB1 interaction with $\alpha_{\text{IIb}}$ (either genetically using $\text{Cib1}^{-/-}$ platelets or by using function blocking antibody UN7.79) strongly suggest that the CIB1 serves as an integrin $\alpha_{\text{IIb}}\beta_3$ effector molecule and regulate integrin ‘outside-in’ signaling. However, it is also an established fact that proper integrin ‘inside-out’ signaling is a prerequisite for an efficient ‘outside-in’ signaling under normal conditions. Moreover, in light of controversial reports – indicating CIB1 as an inhibitor\textsuperscript{34} as well as an activator\textsuperscript{35} of integrin activation – it becomes increasingly important to clarify role of CIB1 in integrin ‘inside-out’ signaling. Thus, it was hypothesized that $\text{CIB1 regulates agonist induced integrin ‘inside-out’ signaling as well as granular secretion}$.

Secondly, amidst evidences of platelet spreading and clot retraction being distinctively regulated by integrin outside-in signaling, later part of my investigation was devoted to characterizing integrin $\alpha_{\text{IIb}}\beta_3$ ‘outside-in’ signaling using pharmacological approach. This was primarily done to establish the existence of separate arms of outside-in signaling cascade regulating platelet spreading on fibrinogen and clot retraction independently.
2.1 Materials

2.1.1 Reagents and Antibodies

All the reagents used were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise mentioned. PAR-4 peptide (AYPGKF) was purchased from AnaSpec (Fremont, CA). Collagen and The bioluminescence reagent Chrono-LUM was procured from Chrono-Log (Havertown, PA). Human fibrinogen and γ-thrombin were obtained from Enzyme Research Laboratories (South Bend, IN). Most of the pharmacological inhibitors used in this study were bought from CalBiochem (EMD Millipore, San Diego, CA). Focal adhesion kinase (FAK) inhibitor TAE-226 was from Novartis Pharma (AG, Switzerland). A polyclonal antibody against phospho-FAK (Tyr-397) was purchased from BioSource (Invitrogen, Carlsbad, CA) and the total-FAK antibody was from Cell Signaling (Beverly, MA). Chemiluminescence substrate LumiGlo® was obtained from New England Biolabs (Ipswich, MA). HyBlot CL autoradiography films were from Denville Scientific Inc. (Metuchen, NJ).
2.1.2 Animals

*Cib1⁻/⁻* mice were generously provided by Dr. W. Yuan and Dr. Leslie Parise (University of North Carolina, Chapel Hill, NC). Generation and characterization of *Cib1⁻/⁻* mice has been described elsewhere. Noteworthy here is that *Cib1⁻/⁻* male mice are sterile. Therefore, the colony was propagated by crossing *Cib1⁺/⁻* male with a *Cib1⁺/⁻* or a *Cib1⁻/⁻* female. Subsequent litters were tail snipped after weaning and genotyped by PCR method to determine their genotype. The mice used in this study were of the C57BL/6 genetic background (backcrossed 10 generations), and *Cib1⁺/⁺* littermates and/or sex- and age-matched wild type mice were used as controls in all the experiments. All the procedures performed were in accordance with a protocol approved by the University of Delaware Institutional Animal Care and Use Committee.

2.2 Methods

2.2.1 Platelet Preparation

2.2.1.1 Mouse platelet rich plasma

Mice (8-12 weeks old) were anesthetized by injecting a mixture of Ketamine/Xylazine into the abdominal cavity as per the approved protocol. Subsequently, blood was isolated from exposed inferior vena-cava into a heparinized 1mL syringe using 23 gauge heparinized needle. After removing the needle from the syringe, the isolated blood was immediately transferred to a microcentrifuge tube.
containing 9:1 (v/v) 3.8% sodium citrate. The anti-coagulated blood was transferred to a 5ml polypropylene snap-cap tube and then diluted 1:1 with Tyrode’s buffer (v/v; Appendix A.II). The tubes were centrifuged at 150 xg for 7 minutes using a swinging bucket rotor and the platelet rich plasma (PRP; cloudy suspension) was collected by carefully pipetting it out into a fresh tube without disturbing the packed white and red blood cells. After adjusting the platelet concentration using Tyrode’s buffer, a 300µl aliquot of diluted PRP was centrifuged at 16,100 xg in a microcentrifuge tube. The clear fluid devoid of platelets thus obtained served as platelet poor plasma (PPP) in my experiments.
2.2.1.2 Human PRP

Whole blood was drawn into 9:1 (v/v) 3.8% sodium citrate by venipuncture from healthy, drug-free adults under informed consent. Approval was obtained from the University of Delaware Institutional Review Board for these studies. A 25 mL aliquot of anti-coagulated blood was transferred to a 50 mL centrifuge tube and was spun at 200 xg for 10 minutes. Subsequently, the PRP (yellowish cloudy suspension) was isolated by carefully pipetting it out into a fresh tube without disturbing the packed white and red blood cells. Platelet poor plasma was prepared by centrifuging all of the residual blood, in the same tube with packed RBCs, at 800 xg for 10 minutes and isolating the pale colored plasma devoid of any cells.

2.2.1.3 Washed human platelets

Whole blood was drawn into 6:1 (v/v) Acid/Citrate/Dextrose solution (pH 4.5) by venipuncture from healthy, drug-free adults and PRP was obtained as described previously. After adding prostaglandin E₁ (100nM)\(^{32}\), which inhibits platelet activation, PRP was centrifuged at 800 xg for 10 minutes. Thereafter, the supernatant (plasma devoid of platelets) was aspirated and the platelet pellet was resuspended in Tyrode’s buffer – Tube-I (Appendix A. III). Resuspended platelets were incubated at 37 °C for 10 minutes prior to spinning them down again at 800 xg for 10 minutes. Next, the pellet was resuspended in Tyrode’s buffer – Tube-II (Appendix A.III). As before, the
resuspended platelets were rested once again at 37 °C for 10 minutes prior to subjecting them to a final spin at 800 xg for 10 minutes. The pellet was finally resuspended in Tyrode’s buffer – Tube-III (Appendix A - C) and the platelets were incubated at 37 °C for 30 minutes. This final incubation allows the platelets to recover from the stress that arose from sequential centrifuging steps.

2.2.2 Platelet Functional Assays

2.2.2.1 Platelet aggregation and ATP secretion

Murine platelet aggregation was performed in PRP containing 2 x 10^8 platelets / mL using lumi-aggregometer (Chrono-Log, Havertown, PA). An aliquot of 237.5µL of PRP was taken into a siliconized glass cuvette with a stir-bar in it. This platelet suspension in cuvette was incubated at 37 °C for 5 minutes. The cuvette was then placed in the well and 12.5µL of bioluminescence reagent Chrono-LUM was added. The mixture was allowed to warm-up to 37 °C for 2 minutes. During this time, attempts were made to record a stable base line. Following this, the platelets were stimulated by indicated agonist under continuous stirring (1000 rpm) conditions at 37 °C. Triplicate aggregation (percent light transmission) and secretion (bioluminescence) tracings were digitally recorded using AggroLink software (Chrono-Log Corporation) for each agonist and the experiments were repeated at least three times. All experiments were performed within 3-4 hours of blood isolation from the animal.
2.2.2.2 Platelet spreading on immobilized fibrinogen

Pre-washed coverslips (Appendix B) were coated with 250µL of fibrinogen (100µg/mL) or bovine serum albumin (BSA; 3%) dissolved in 1x phosphate-buffered saline (PBS) and were incubated at 37 °C for 60 minutes in a humid chamber. Following the incubation, the fluid was aspirated and the coverslips were blocked by putting 250µL of 0.5% heat-inactivated BSA dissolved in 1x PBS and incubating them at 37 °C for another 60 minutes in similar humid conditions. Simultaneously, platelets were prepared for the experiment. Briefly, human PRP containing PGE$_1$ (100nM) and aspirin (1mM) was incubated for 30 minutes at 37 °C prior to starting the washing procedure. Subsequently, the washed platelets were diluted to a final concentration of 1 x 10$^7$ platelets / ml using Tyrode’s buffer – Tube-III (Appendix A). Apyrase (final concentration: 1U/mL) was then added to the platelet suspension and incubated for 10 minutes at room temperature. It should be noted here that the ‘inside-out’ signaling was blocked by addition of aspirin (inhibits TxA2 synthesis) and apyrase (scavenges secreted ADP in order to mimic true ‘outside-in’ signaling scenario. Next, aspirin/apyrase treated platelets were aliquoted in pre-labeled microcentrifuge tubes and a pharmacological inhibitor dissolved in dimethyl-sulfoxide (DMSO) was added to the corresponding tubes and incubated for an additional 20 minutes at room temperature. Thereafter, the platelets were added to the respective coverslips after removing the blocking buffer.
Coverslips containing platelets were incubated for 45-60 minutes at 37 °C in the same humid chamber. The process of spreading on the fibrinogen coated control coverslips was monitored every 15 minutes microscopically using Nikon inverted light microscope equipped with 40x objective lens. At the end of spreading, excess fluid and unbound platelets were washed thrice with Tyrode’s buffer and then fixed with 250 µL of freshly prepared 4% paraformaldehyde for 20 minutes. Lastly, after washing twice with 1x PBS the coverslips were mounted on a clean glass slide. Differential interference contrast (DIC) images showing morphology of the platelets on coverslips were captured using Zeiss Axioskop II light microscope using Zeiss Plan-Apochromat 100x/1.4NA oil immersion lens. The images were digitally recorded using Zeiss AxioVision software. For quantification, the surface area (in pixels) of all platelets in a particular field of view was calculated using NIH-ImageJ software. Each spreading experiment was repeated at least three times.

2.2.2.3 Platelet mediated fibrin clot retraction

For clot retraction assays the protocol adopted was mentioned elsewhere\textsuperscript{28}. In short, the platelet concentration in human PRP was adjusted to 3.5 x 10\textsuperscript{8} platelets / mL using PPP. After that aspirin (1mM) was added and platelets were incubated at 37 °C for 30 minutes. Thereafter, the aspirinated platelet suspension was aliquoted in pre-labeled 5ml polypropylene tubes and a pharmacological inhibitor was added to corresponding
tubes and incubated for an additional 20 minutes at room temperature. Then treated or untreated platelets were transferred to a pre-labeled borosilicate glass tubes. Clot retraction was initiated by addition of γ-thrombin (0.2U/mL) to the platelet suspension and vortexing briefly at a very slow speed. Tubes were then placed in the imaging rack and left undisturbed at 37 °C. Images of these tubes were captured every 15 minutes using a point-and-shoot Nikon digital camera starting from time zero until 90-120 minutes. For quantification, two-dimensional surface area of retracting clot in each tube at the given time point was measured using NIH-ImageJ software and used to calculate percent clot retraction. Each experiment was repeated at least three times.

2.2.3 Platelet Biochemistry

2.2.3.1 Sample preparation from spread platelets

Non-tissue culture petri-dishes (35mm) were coated with 2-3 mL of fibrinogen (100µg/mL) or bovine serum albumin (BSA; 3%) solution and incubated at 37 °C for 60 minutes. Following incubation, the fluid was aspirated from all the dishes and then they were blocked by gently putting 2-3 mL of 0.5% heat-inactivated BSA solution along the sides and incubating the dishes again at 37 °C for another 60 minutes. Simultaneously, platelets were prepared for the experiment in a similar way as they were prepared for spreading on coverslip, except final platelet concentration was adjusted to 1 x 10⁸ platelets/mL. Finally after inhibitor treatment, 1 mL of platelet suspension was added
to the respective dish after removing the blocking buffer. The dishes containing platelets were incubated for 45 minutes at 37 °C. Following spreading, the fluid from each dish containing unattached platelets was collected in a labeled microcentrifuge tube and attached platelets on the dishes were lysed by addition of 100µL of ice-cold 1x lysis buffer (1% NP-40, 150 mM NaCl and 50 mM Tris–HCl pH 7.5, containing 10µg/mL each of leupeptin and aprotinin, and 1 mM each of NaF, sodium orthovanadate and PMSF). Fluid collected from each dish in the corresponding microcentrifuge tube was spun down briefly at 16,100 xg and the supernantant was discarded. The pellet in each tube was lysed using the platelet lysate prepared in each corresponding dish (this was done in order to normalize the total numbers of platelets being used) and added back to the dish. Next, 25µL of 5x Laemmli-sample buffer was added to each dish and mixed. The prepared samples were collected in a microcentrifuge tube and boiled for 5 minutes and stored at -20 °C until used.

2.2.3.2 Western blotting

Aliquots of 25-30 µL of protein samples were separated by using 8% sodium dodecyl sulfate – poly-acrylamide gel electrophoresis (SDS-PAGE; reducing conditions) at 120 volts. Subsequently, the separated proteins were transferred to a PVDF membrane by applying a voltage of 30 volts for 90 minutes. The membranes were blocked with 3% BSA (for the use of membrane with anti-phospho-FAK \textsuperscript{397} specific antibody) or 5% non-fat milk (for reprobing later with total-FAK antibody) dissolved in 1x Tris-buffered saline–
tween-20 (TBS-T). Blocked membrane was incubated overnight at 4 °C with primary antibody (1:1,000 dilution) diluted in blocking buffer. Next, the membrane was thoroughly washed 3-5 times, 10 minutes each, with 1x TBS-T. Following washing, the membrane was incubated with HRP-conjugated secondary antibody (1:2,000 dilution for probing phospho-FAK<sup>397</sup> and 1:10,000 dilution for probing total-FAK) in blocking buffer for 60 minutes at room temperature. Later, the membrane was washed again 3 times with 1x TBS-T. The bands were visualized by treating membrane with LumiGlo® substrate and exposing them to autoradiography film that was developed using Kodak X-OMAT 1000A processor.

### 2.2.4 Statistical Analysis

Statistical analysis of the data was performed using Student’s t-test. P ≤ 0.05 was regarded as statistically significant. Each experiment was repeated at least three times.
Chapter 3

RESULTS

3.1 CIB1 is not Required for Integrin \( \alpha_{\text{IIb}}\beta_3 \) ‘Inside-out’ Signaling

Integrin \( \alpha_{\text{IIb}}\beta_3 \) activation is a pre-requisite for its interaction with the soluble ligand fibrinogen. This interaction serves two purposes: (a) It promotes platelet aggregation by crosslinking different platelets together, and (b) It triggers integrin ‘outside-in’ signaling. In light of contradictory literature stating CIB1 as an inhibitor\(^{34}\) as well as an activator\(^{35}\) of \( \alpha_{\text{IIb}}\beta_3 \), it was crucial to clarify the actual role of CIB1 in agonist-induced integrin activation in order to be convinced that the defects seen in ‘outside-in’ signaling-dependent process – platelet spreading on fibrinogen\(^9,36\) and clot retraction (Unpublished data: Naik and Naik) – are not due to a defect in upstream ‘inside-out’ signaling at the level of integrin activation.

Therefore, to test the effect of ablation of Cib1 on integrin activation, I analyzed the murine platelet aggregation under ex-vivo settings. When I stimulated the Cib1\(^{-/-}\) and Cib1\(^{+/+}\) platelets with various concentrations of agonists, 2-Me-S-ADP and PAR-4 peptide, no difference in agonist-induced platelet aggregation was seen\(^9\) (Fig. 3.1.).
Similarly, when different doses of agonists, collagen or U46619 (lipid analog of TxA₂), was used no difference in platelet aggregation between $Cib1^{-/-}$ and $Cib1^{+/+}$ platelets was seen (Fig. 3.2.). Upon quantification of more than three independent experiments, no statistically significant difference in the extent

![Graphs showing platelet aggregation](image)

**Fig. 3.1.** Platelet aggregation in absence of CIB1 is normal upon stimulation with 2-MeS-ADP and PAR-4 Peptide (AYPGKF).
Fig. 3.2. Deletion of Cib1 does not affect platelet aggregation upon stimulation with collagen and low dose of TxA₂ mimetic – U46619.

of platelet aggregation was observed between Cib1⁻/⁻ and Cib1⁺/⁺ platelets. Therefore, the results from these experiments indicate that the inside-out signaling (or integrin activation) is normal in the absence of Cib1 ruling-out the possibility of a defective inside-out signaling.

3.2 ATP Secretion is Normal in Cib1 Null Platelets

Activation of platelet results in exocytosis of its α- and δ-granular contents. Moreover, rescue of Cib1⁻/⁻ platelet spreading on immobilized fibrinogen upon addition
of exogenous ADP suggests that CIB1 may be a potential regulator of dense granular secretion. Therefore, in order to test this, I looked at the release of ATP, which is believed to be stored in the δ-granules along with ADP, using a bioluminescence assay. Upon stimulation of Cib1−/− and Cib1+/+ platelets with varying concentrations of agonists, PAR-4 peptide and collagen, no statistically significant difference in the release of ATP was seen (Fig. 3.3.). Thus, the results from these experiments indicate that CIB1 is not required for ATP release.

**Fig. 3.3.** ATP release from the δ-granules is unaffected in the Cib1−/− platelets compared to the Cib1+/+ platelets upon stimulation with varying concentrations of the agonist PAR-4 peptide and collagen.
3.3 PI3K, PLC, PKC and FAK are Essential for Both Platelet Spreading and Clot Retraction

Next, I sought to characterize the αIIbβ3 ‘outside-in’ signaling, where Naik lab had observed the defect in Cib1−/− platelets. Here, using pharmacological inhibitors and human platelets, platelet spreading on immobilized fibrinogen as well as platelet mediated fibrin clot retraction were studied as a readout for the αIIbβ3 ‘outside-in’ signaling. When human platelets were treated with the specific inhibitors of enzymes, phosphoinositide 3-kinases (PI3K; LY294002), phospholipase C (PLC; U73221), protein kinase C (PKC; bisindolylmaleimide I (Bis. I) and Rö-31-8220) and focal adhesion kinase (FAK; TAE-226) marked inhibition in platelet spreading was observed (Fig. 3.4.). When PI3K was inhibited (10µM LY294002), platelets mainly maintained their discoid shape on immobilized fibrinogen with several platelets showing few filopodial projections (Fig. 3.4.C). Similarly, 10µM U73221, which inhibits PLC-β and -γ isoforms, resulted in failure of filopodia and lamellipodia formation and maintained platelets in discoid morphology over fibrinogen (Fig. 3.4.D). However, pre-treatment with PKC inhibitors (10µM Bis. I or Rö-31-8220) resulted in spiky platelet morphology with several filopodia like extensions. Interestingly, some of the filopodia of PKC inhibitor treated platelets were found to be split at the tip (Fig. 3.4.E and 3.4.F). Furthermore, treatment with FAK inhibitor also resulted in spiky morphology of platelets with several filopodia (Fig. 3.4.G). Additionally, when I looked at platelet mediated fibrin clot retraction using same concentration of
inhibitors as in platelet spreading experiments, a similar trend was observed. Inhibitors of PI3K, PKC and FAK resulted in delayed clot retraction as compared to control (Fig. 3.5.). Furthermore, the inhibitory effect of U73221 was even more pronounced in the clot retraction assays, compared to other inhibitors, as it completely abolished the retraction process (Fig. 3.5.). Quantification of more than three independent experiments yielded statistically significant differences (as indicated) in the surface area of spreading platelets (Fig. 3.4.H) and percent clot retraction at a given time point (Fig. 3.5.) when compared to appropriate controls. Thus, it can be concluded that PI3K, PLC, PKC and FAK, all are essential for both platelet spreading and clot retraction.
**Fig 3.4.** PI3K, PLC, PKC and FAK are essential for spreading of platelet over immobilized fibrinogen. Illustrated in this figure is platelet spreading over immobilized BSA (A, control) and over 100µg/ml of immobilized fibrinogen in the absence (B) or the presence of indicated pharmacological inhibitor (C-G). Surface area of more than 300 platelets from more than three independent experiments were quantitated using NIH-ImageJ software and presented as the average surface area (in pixels). Error bars represents SEM (H).
Fig 3.5. Pharmacological inhibition of enzymes, PI3K, PLC, PKC and FAK resulted in delayed clot retraction. Two dimensional images of retracted clot shown above are representative images at 45 minutes time point. Quantitation was performed as mentioned in materials and methods section. Error bars represent SEM. (* P < 0.05 and **P < 0.01).
3.4  **p38 MAPK and ROCK are Required for the Process of Clot Retraction, but, are Dispensable for Platelet Spreading on Fibrinogen**

Contrary to above results, when platelets were treated with inhibitors of enzymes Rho-associated kinase (ROCK; 10 µM Y-27632) and p38 Mitogen activated protein kinase (p38-MAPK; 20 µM SB203580), an inhibition in platelet mediated clot retraction was observed. As indicated, at the mentioned time points the difference in percent clot retraction upon treatment with a particular inhibitor compared to control at the same time point was found to be statistically significant (Fig. 3.6.). Furthermore, although very modest, a slight delayed trend and inhibition of clot retraction at an early time point was observed upon inhibition of Rac1-GTPase (Rac1; 10 µM NSC23766) (Fig. 3.6.). Contrariwise, platelet spreading on immobilized fibrinogen was unaffected upon treatment with these inhibitors and no statistically significant difference in the surface area of inhibitor treated platelets was found when compared to vehicle controls (Fig. 3.7.).

3.5  **Src is Indispensable for Platelet Spreading on Fibrinogen; However, its Inhibition Causes Enhanced Clot Retraction**

In the process, I also looked at the effect of inhibition of Src tyrosine kinase, an integrin proximal enzyme that gets activated early on in integrin ‘outside-in’ signaling. Platelets treated with Src-family kinase inhibitor (10 µM PP2) failed to spread on
immobilized fibrinogen and remained stalled in discoid morphology (Fig. 3.8.A). However, the effect of PP2 on the process of clot retraction was remarkably different. The inhibitor treated platelets resulted in faster clot retraction compared to controls. This experiment was done only two times (n = 2) and therefore, further statistical analysis wasn’t feasible. (Fig. 3.8.B).

Fig. 3.6. Pharmacological inhibition of p38-MAPK, and ROCK resulted in delayed clot retraction. However, only modest inhibition was observed upon Rac1 inhibition. Two-dimensional images of retracted clot shown above are representative images at 45 min. time point (for p38 and ROCK inhibitor treatment) except the Rac1 inhibitor treatment which is at 15 minute time point. Quantitation was performed as mentioned in materials and methods section. (* P < 0.05 and **P < 0.01).
**Fig. 3.7.** p38-MAPK and ROCK are not required for platelet spreading over immobilized fibrinogen. Platelet spreading over immobilized fibrinogen (100 µg/ml) in the absence or the presence of indicated pharmacological inhibitor. Quantitation was performed as mentioned in legend of figure 3.4.

**Fig. 3.8.** (A) Inhibition of Src blocks platelet spreading. (B, C) Inhibition of Src family kinases results in enhanced rate of clot retraction (N = 2).
3.6 FAK Functions Downstream of Src, PI3K, PLC and PKC in Integrin $\alpha_{\text{IIb}}\beta_3$ ‘Outside-in’ Signaling

As mentioned before, Naik lab identified a defect in the $\alpha_{\text{IIb}}\beta_3$ ‘outside-in’ signaling in the absence of CIB1 and established that CIB1 is a regulator of FAK activity. Therefore, considering this, I next sought to determine the relative position of FAK in the integrin ‘outside-in’ signaling cascade using pharmacological inhibitors and immunoblotting approach. My results suggest that FAK functions downstream of Src tyrosine kinase, PI3K, PLC and PKC, because treatment of platelets with inhibitors of these enzymes not only inhibited platelet spreading (on fibrinogen coated dishes), but also inhibited FAK activation as evidenced by reduced FAK-$\gamma^{397}$ phosphorylation compared to the vehicle control (Fig. 3.9.).

![Western blot showing FAK-$\gamma^{397}$ phosphorylation in the presence of various inhibitors. Results suggest that FAK activation is downstream of activation of Src tyrosine kinase, PI3-kinase, Phospholipase C and Protein kinase C during integrin $\alpha_{\text{IIb}}\beta_3$ ‘outside-in’ signaling. Blot shown here is representative of more than six independent experiments.](image)

**Fig. 3.9.** Western blot showing FAK-$\gamma^{397}$ phosphorylation in the presence of various inhibitors. Results suggest that FAK activation is downstream of activation of Src tyrosine kinase, PI3-kinase, Phospholipase C and Protein kinase C during integrin $\alpha_{\text{IIb}}\beta_3$ ‘outside-in’ signaling. Blot shown here is representative of more than six independent experiments.
Chapter 4

DISCUSSION

CIB1 had been previously identified as a calcium binding protein which is expressed abundantly in platelets and binds to the cytoplasmic tail of the α_{IIb} subunit of fibrinogen receptor, the integrin α_{IIb}β_{3}. CIB1 had been shown to be expressed in several tissues and can regulate diverse biological functions through its ability to interact with several other proteins (Table 1). In platelets CIB1 had been reported to function as an endogenous inhibitor of α_{IIb}β_{3} activation, as an activator of α_{IIb}β_{3}, as well as a downstream effector of integrin α_{IIb}β_{3} ‘outside-in’ signaling.

Though, our lab consistently observed integrin α_{IIb}β_{3} outside-in signaling defect in the Cib1/ platelets, the impairment in hemostasis seen in Cib1/ mice cannot be solely attributed to an altered ‘outside-in’ signaling at this point. This is primarily because under physiological conditions for a fully effective integrin ‘outside-in’ signaling to occur, a normal integrin activation process is required. Furthermore, a defect in integrin activation (affinity modulation) or its altered avidity (strength of adhesion) can also result in delayed time to blood vessel occlusion manifesting the impaired thrombosis.
phenotype seen in the *Cib1* knock-out mice. Secondly, rescue of defective platelet spreading in the absence of CIB1 interaction with α\textsubscript{IIb} upon addition of exogenous ADP\textsuperscript{9,36} indicates that CIB1 might regulate δ-granular release which stores endogenous ADP that upon release can function as a positive-feedback in the mechanism of thrombus formation\textsuperscript{40}. Thus, it was hypothesized that CIB1 regulates agonist-induced integrin α\textsubscript{IIb}β\textsubscript{3} activation as well as δ-granular secretion.

To test this hypothesis, I first looked at platelet aggregation under *ex-vivo* conditions. One of the pre-requisite of platelet aggregation is integrin α\textsubscript{IIb}β\textsubscript{3} activation, which allows it to bind to soluble fibrinogen. Thus, platelet aggregation assay can be extrapolated as a test for integrin activation. In response to various agonists I used (2-MeS-ADP, PAR-4 peptide, collagen and TxA\textsubscript{2} mimetic U46619) platelet aggregation, and therefore integrin activation, was found to be normal in *Cib1*\textsuperscript{-/-} platelets as compared to *Cib1*\textsuperscript{+/-} platelets which is consistent with the published results of DeNofrio *et al.*\textsuperscript{41}. Thus, it can be concluded from here that in platelets, Cib1 doesn’t function as an endogenous activator\textsuperscript{35} of integrin α\textsubscript{IIb}β\textsubscript{3} because even in the absence of Cib1 normal integrin activation and platelet aggregation was seen. Furthermore, since no platelet hyper-aggregation phenotype in response to low-doses of agonist was observed in the absence of Cib1, therefore, it may be possible that another *Cib* family member (like Cib3, whose mRNA was found to be upregulated in *Cib1*\textsuperscript{-/-} murine MKs\textsuperscript{41}) may play a compensatory
role. Moreover, it is also plausible that CIB1 doesn’t function as endogenous inhibitor of integrin αIIbβ3 activation and may be involved only in post-fibrinogen occupied integrin signaling.

In platelets, both ATP and ADP are stored in the δ-granules. Therefore, measuring ATP release serves as an indirect assay for ADP release from the δ-granules. The results from these experiments showed no significant difference in the amount of ATP released from Cib1−/− platelets compared to Cib1+/+ platelets in response to high- as well as low-doses of different agonists. Furthermore, we had previously published no difference in P-selectin exposure (found on membrane of α- as well as δ-granules in quiescent platelets, but becomes exposed on outer side of the plasma membrane of activated platelets following granular exocytosis) in Cib1−/− platelets compared to Cib1+/+ platelets upon activation with PAR-4 peptide. Overall taken together the results so far confirm that CIB1 is not required for agonist-induced integrin activation and δ-granular secretion.

After having these results in hand, it was ascertained that the impaired hemostasis identified in Cib1 knock-out mouse was due to consistent defect seen in integrin ‘outside-in’ signaling where delayed clot retraction (Unpublished data – Naik and Naik) and failure of full platelet spreading in the absence of CIB1 was observed.
This has a reason that the retractile forces generated during the clot retraction phase of thrombosis aids in providing stability to thrombus which prevents it from embolization\textsuperscript{44}. Hence, having a defect in generation of these contractile forces can also result in unstable thrombus formation and re-bleeding phenotype as seen in \textit{Cib1} knockout mice. Therefore, I next sought to investigate the integrin $\alpha_{\text{IIb}}\beta_3$ ‘outside-in’ signaling pathway.

In the field of thrombocyte biology, it is widely believed that the integrin ‘outside-in’ signaling regulates two process platelet spreading and clot retraction besides, having some contribution in granular secretion\textsuperscript{25,45} and thromboxane A\textsubscript{2} generation\textsuperscript{26,45}. The results from this part of my study interjects the paradigm (refer section 1.4.2) and establishes that the two processes, \textit{i.e.}, platelet spreading on fibrinogen and clot retraction, are distinctly regulated in the integrin ‘outside-in’ signaling pathway which is supported by the findings of Flevaris \textit{et al.}\textsuperscript{27}.

When I used a panel of inhibitors against the enzymes \textit{viz.} PI3K, PLC, PKC and FAK, a marked reduction in lamellipodia formation was seen compared to the vehicle treated control platelets. In particular, PI3K inhibition with 10$\mu$M LY294002 maintained the platelets in discoid morphology with several platelets showing 1-2 filopodia like structures compared to fully spread pancake like morphology of the vehicle treated control platelets. This data is consistent with a previously published report where the
authors also found inhibition in platelet spreading with 25µM LY294002 compound\textsuperscript{46}. It is important to note here that Heraud \textit{et al.}\textsuperscript{46} observed several filopodia like extensions from fibrinogen adherent platelets as opposed to 1-2 filopodia like structure seen in my hands with a lesser concentration (10µM) of inhibitor LY294002. This can be attributed to the differences in experimental methodologies adopted. As opposed to Heraud \textit{et al.}\textsuperscript{46}, I used apyrase (1U/ml; that scavenges secreted ADP) and aspirin (1mM; known to inhibit cyclooxygenases essential for TxA\textsubscript{2} generation) to mimic conditions of true integrin ‘outside-in’ signaling without any positive feedback from the ADP or TxA\textsubscript{2} signaling because it had been reported that the integrin ‘outside-in’ signaling also regulates granular secretion\textsuperscript{25} and TxA\textsubscript{2} generation\textsuperscript{26}. Furthermore, treatment with pan-PLC inhibitor (10µM U73122) also resulted in the inability of platelets to form filopodia or lamellipodia. Wonerow \textit{et al.}\textsuperscript{47} also saw similar inhibition in platelet spreading in the presence of 10µM PLC inhibitor U73122.

On the other hand, treatment of platelets with inhibitors of PKC and FAK resulted in spiky morphology of platelets with limited to no lamellipodia formation which is consistent with results observed by Haimovich \textit{et al.}\textsuperscript{48} and Wonerow \textit{et al.}\textsuperscript{47} upon treatment with PKC inhibitors Bis. I and Rö-31-8220, respectively, as well as by Jones \textit{et al.}\textsuperscript{49} who used structurally distinct FAK inhibitor PF-573,228. Besides such pharmacological approaches, reports from Buensuceso \textit{et al.}\textsuperscript{50} and Soriani \textit{et al.}\textsuperscript{51}, using
genetic approaches demonstrated that the β and the θ isoforms of PKC are of particular importance in integrin ‘outside-in’ signaling mediated murine platelet spreading on immobilized fibrinogen. Also, Hitchcock et al. also found significantly less surface coverage in FAK- platelets as compared to wild-type platelets upon spreading on fibrinogen.

In parallel clot retraction studies, effect of pharmacological inhibition of PI3K, PLC, PKC and FAK enzymes was studied at the same concentration of inhibitor, as used for platelet spreading experiments. PI3K inhibition using LY294002 resulted in delayed clot retraction. Schoenwaelder et al. also reported similar initial lag in the initiation of clot retraction upon inhibition of PI3K using 25µM LY294002. Moreover, in their report they went ahead to demonstrate that it is the Type Ia p110-β isoform of PI3K that plays the major role in regulating thrombin-stimulated fibrin clot retraction in ex-vivo conditions. Similar trend of delayed clot retraction was observed when PKC inhibitors (Bis. I and Rö-31-8220) and FAK inhibitor (TAE-226) were used. On the other hand, inhibition of PLC with pan-PLC inhibitor U73122, known to inhibit both PLC-β and -γ isoforms, abolished completely the process of clot retraction. This complete blockade of clot retraction can be explained by inhibition of PLC-β which functions downstream of thrombin receptor and is responsible for the agonist-induced intracellular Ca²⁺ rise and the inside-out signaling which are important for the integrin outside-in signaling as well.
Suzuki-Inoue et al.\textsuperscript{54}, using PLC\textsubscript{γ2\textsuperscript{-/-}} platelets demonstrated that deletion of this isoform of PLC, which is reported to play a role downstream of the integrin α\textsubscript{IIb}β\textsubscript{3} outside-in signaling, resulted in delayed clot retraction but, did not prevented it from reaching completion. It is important to note that in these studies PLC-β isoform remained intact normally relaying thrombin induced signaling which was inhibited under my experimental conditions as a result of pan-PLC pharmacological inhibitor.

Upon using a different panel of inhibitors, for inhibiting p38-MAPK (20\textmu M SB203280) and Rho-associated kinase (ROCK) (10\textmu M Y-27632), very different results were obtained. It was observed that both p38-MAPK and ROCK inhibitors resulted in delayed clot retraction. Contrastingly, when same concentration of inhibitors was used for platelet spreading assays, no decrease in average surface area of spread platelets was observed compared to control. These results are consistent with those published by Flevaris et al.\textsuperscript{27} and Gong et al.\textsuperscript{55}.

However, when Rac1 GTPase was inhibited (10\textmu M NSC23766) only a very modest inhibition in clot retraction at an earlier time point was seen and there wasn’t any inhibition of platelet spreading at this concentration of inhibitor. The clot retraction data is contradictory to the published report of Flevaris et al.\textsuperscript{27} who saw delayed clot retraction upon pharmacological inhibition of Rac1 (20\textmu M NSC23766) as well as a
complete blockade of clot retraction process when Rac1<sup>−/−</sup> platelets were used. The apparent discrepancy can be attributed to difference in the concentration of inhibitor used under my experimental conditions. Similar to my results, Flevaris et al.<sup>27</sup> also reported no inhibition in platelet spreading upon pharmacological inhibition of Rac1 GTPase (20µM NSC23766), which is contrary to results published recently by Aslan et al.<sup>56</sup> who saw a marked reduction in platelet spreading upon inhibition of Rac1 with two structurally distinct Rac1 inhibitors (300µM NSC23766 and 50µM EHT1864). Thus, considering the range of concentration of inhibitor NSC23766 used in different studies, it becomes important to revisit clot retraction and platelet spreading experiments together with dose-dependent inhibition of Rac1 in order to conclude upon the relative role of Rac1 in either of the processes.

Src tyrosine kinase is one of the most abundant non-receptor tyrosine kinases expressed in platelets and is reported to be activated early on during integrin α<sub>IIb</sub>β<sub>3</sub> ‘outside-in’ signaling. Platelets treated with Src family kinase inhibitor (10µM PP2) failed completely to spread on fibrinogen immobilized on glass coverslips which is consistent with reported results of Wonerow et al.<sup>47</sup> and Aslan et al.<sup>56</sup>. In parallel, same concentration of Src inhibitor resulted in enhanced platelet mediated clot retraction which is in accordance with results of Flevaris et al.<sup>28</sup>, but contradictory to the observations of Suzuki-Inoue et al.<sup>54</sup>. The apparent discrepancy seen here can be
attributed to the experimental methodology adopted. Suzuki-Inoue et al.\textsuperscript{54} used washed platelet system supplemented with purified fibrinogen for their clot retraction assays as opposed to PRP used in my studies. Nonetheless, a consistent result of enhanced clot retraction upon Src tyrosine kinase inhibition adds ambiguity in our understanding which is discussed later in this section.

Recently, our lab has shown that overexpression of \textit{Cib1} in CHO cells augments integrin mediated cell migration on fibronectin\textsuperscript{57} and this migration was reduced upon inhibition of Src family kinases, PI3K and PKC\textsuperscript{57} by ultimately down-regulating MAPK activation. Moreover, our lab had also shown previously that CIB1 associates with FAK and regulates its activity\textsuperscript{37}. In addition, based on my results, FAK activation during integrin $\alpha_{IIb}\beta_3$ ‘outside-in’ signaling cascade is downstream of Src, PI3K, PLC and PKC. Haimovich \textit{et al.}\textsuperscript{48} and Ji et al.\textsuperscript{58} also demonstrated that FAK activation is downstream of PKC and PI3K respectively. Thus, taking all these results together it is plausible to speculate that CIB1 might regulate a very upstream event such as Src activation during integrin $\alpha_{IIb}\beta_3$ ‘outside-in’ signaling leading to activation of downstream effectors including FAK, thus, positioning CIB1 on the arm of integrin ‘outside-in’ signaling which regulates both spreading and clot retraction.
Towards the end, it is worth pointing out that the disparate observations: (1) reduced FAK activation (less Y397 phosphorylation) upon Src inhibition (using 10µM PP2) qualitatively shown by western blotting (Fig. 3.9); (2) delayed clot retraction upon FAK inhibition (using 10µM TAE-226); and (3) Enhanced clot retraction upon Src inhibition (using 10µM PP2); taken together adds ambiguity to my understanding. It is important to note here that both the results obtained (reduced FAK Y397 phosphorylation and enhanced clot retraction) upon inhibition of Src tyrosine kinase are qualitative in nature and no statistical analysis had been performed due to various limitations. Thus, though not certain, but, in the light of the results of Flevaris et al.\textsuperscript{28} it is conceivable that CIB1 induced Src-dependent FAK activation results in a late event of cleavage of integrin β3 tail by Ca\textsuperscript{2+}-dependent non-lysosomal cysteine protease, calpain, resulting in Src inhibition\textsuperscript{28,55} which results in augmentation of retraction process.

With all this data in hand, it becomes apparent that one arm (Fig. 4.1.) of this signaling regulates both spreading and clot retraction, whereas, the other arm appears to regulate only clot retraction and not spreading. In light of the results obtained in our lab – defective platelet spreading and delayed clot retraction in absence of CIB1 – it is very likely that CIB1 lies on the arm which regulates both spreading and clot retraction. Furthermore, additional data from our lab also implicate CIB1 in regulating Src activation\textsuperscript{59}. Thus, taking all this together, it appears that CIB1 association with integrin
subunit α_{IIb} cytoplasmic tail somehow modulates activation of Src tyrosine kinase relaying the outside-in signaling into the platelet. It has been reported that during

Fig. 4.1 Schematic model of regulation of platelet spreading and clot retraction during α_{IIb}β_3 mediated ‘outside-in’ signaling.
platelet spreading on fibrinogen, inhibition of SFKs abolishes Syk phosphorylation and its subsequent activation\cite{60}. Moreover, recently it has been reported that during integrin $\alpha_{IIb}\beta_3$ ‘outside-in’ signaling, Src and Syk kinases are responsible for phosphorylation of tyrosine residues ($Y^{700}$, $Y^{731}$ and $Y^{774}$) on adaptor protein c-Cbl\cite{61}. $Y^{731}$ phosphorylated c-Cbl provides a docking site for SH2-domain containing p85 subunit of PI3K\cite{62}. It is reported that PI3K functions upstream of PLC$\gamma_2$\cite{63} which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP$_2$) generating inositol 1,4,5-triphosphate (IP$_3$) and diacylglycerol (DAG). Release of calcium ions from internal stores upon binding of IP$_3$ to its receptors together with DAG aids in activation of PKCs. Active PKCs had been implicated in phosphorylation of various cytoskeletal proteins and regulation of cytoskeletal rearrangement. Recently our lab had shown that dynamic cytoskeletal rearrangement is important for FAK phosphorylation at $Y^{397}$ and its activation\cite{64}. Thus, it is plausible that the orchestration of spatio-temporal activation of FAK by CIB1 during integrin ‘outside-in’ signaling may involve the aforementioned key regulators of this signaling cascade. However to conclude upon this postulation requires further investigation.
Chapter V

FUTURE PERSPECTIVE

With all the data accumulated so far, the idea of Naik’s Lab that CIB1 regulates integrin ‘outside-in’ signaling gets reinforced. Furthermore, it also becomes apparent that the ‘outside-in’ signaling dependent processes – platelet spreading on immobilized fibrinogen and platelet mediated fibrin clot retraction – are distinctly regulated during integrin α\textsubscript{IIb}β\textsubscript{3} ‘outside-in’ signaling. In light of the results obtained in our lab – defective platelet spreading and delayed clot retraction in the absence of CIB1 together with my results from this work – it is very likely that CIB1 lies on the arm which regulates both spreading and clot retraction. Furthermore, additional data from our lab also implicate CIB1 in regulating Src activation\textsuperscript{59}. Thus, taking all this together, it appears that CIB1 association with integrin subunit α\textsubscript{IIb} cytoplasmic tail somehow modulates activation of Src tyrosine kinase relaying the outside-in signaling into the platelet.

Src activation requires dephosphorylation of a tyrosine residue on Src (Y\textsuperscript{529}) and phosphorylation of another tyrosine residue (Y\textsuperscript{418}). This dephosphorylation event is reported to be carried out by a phosphatase \textit{viz.} PTP-1B. Recently, Dr. Vijayan’s lab has reported that another phosphatase PP2A regulates PTP-1B activation and therefore, Src
activation\textsuperscript{65}. Furthermore, it has been shown that PP2A shares the same membrane proximal binding site on α\textsubscript{IIb} tail with CIB1\textsuperscript{66,67}. Thus, it is plausible that in fibrinogen bound activated platelets, Cib1 association with α\textsubscript{IIb} tail results in displacement of PP2A leading to its inactivation and consequently activation of PTP-1B and Src. This displacement of PP2A bound to α\textsubscript{IIb} by calcium bound Cib1 can be analyzed by employing immuno-precipitation assay. Inactivated human platelets maintained in suspension or the ones allowed to adhere on BSA would have PP2A bound to α\textsubscript{IIb} tails. Under such conditions, Cib1 should be minimally detected in immuno-complex pulled by anti-α\textsubscript{IIb} antibody. Furthermore, BAPATA-AM treated platelets (that chelates intracellular calcium) allowed to spread on immobilized fibrinogen would also show more PP2A and less Cib1 in immuno-complex pulled by anti-α\textsubscript{IIb} antibody. However, in the absence of BAPTA-AM, when platelets would be allowed to spread on immobilized fibrinogen then Cib1 would associate with α\textsubscript{IIb} tail displacing PP2A. Thus, there should be a decrease in the amount of PP2A present in such immuno-complex precipitated with anti-α\textsubscript{IIb} antibody.

Furthermore, determining activation level of PI3K in Cib1\textsuperscript{-/-} platelets during outside-in signaling would be another interesting thing to look at. This is primarily because PI3K had been implicated in integrin α\textsubscript{IIb}β\textsubscript{3} ‘outside-in’ signaling and my results also indicate that PI3K activation is important for FAK Y\textsuperscript{397} phosphorylation and its
subsequent activation. However, precise estimation of cellular PI3K activity had been a challenging task in past. But, recent technical advances have now made direct estimation of cellular PI3K activity easier than ever before. Fluorescent PIP2 derivatives, which functions as substrates for PI3K, can be used in *in-vitro* assays as reporters of PI3K activity\(^{68}\). By employing thin-layer chromatography the reporters can be effectively separated from their corresponding PI3K enzymatic products (fluorophore-conjugated PIP3), which can then be detected with high sensitivity by fluorescence. Making use of such an approach it is possible to study the PI3K activity in *Cib1*\(^{-/-}\) platelets compared to *Cib1*\(^{+/+}\) platelets that are allowed to spread on immobilized fibrinogen. Thus, the data accumulated from this work would be instrumental in providing insights on yet unanswered question of how CIB1 regulates spatio-temporal FAK activation during integrin \(\alpha_{IIb}\beta_3\) ‘outside-in’ signaling.
REFERENCES


64. Naik UP and Naik MU. (2008). Cib1 is required for FAK activation during outside-in signaling through platelet integrin αIIbβ3 [Abstract # 2873]. *Blood;* **112**.


A.I. Making Tyrode’s buffer stock solutions

- **Tyrode’s Buffer Stock I (100ml)**
  - Sodium Chloride (NaCl) - 16.00 g
  - Potassium Chloride (KCl) - 00.40 g
  - Sodium Phosphate, Monobasic (NaH$_2$PO$_4$) - 00.10 g
  - HEPES - 00.24 g
  Dissolve all the above mentioned ingredients one by one in ~ 80 ml of double distilled deionized water. After dissolving HEPES, adjust the pH of the solution to 7.2.
  - Sodium Bi-carbonate (NaHCO$_3$) - 02.00 g
  Add sodium bi-carbonate little-by-little. If it doesn’t dissolve, then increase the volume of water a little bit and then dissolve the remaining sodium bi-carbonate. Finally make-up the volume of the solution to 100ml using a volumetric flask and store at room temperature.

- **Tyrode’s Buffer Stock II (100ml)**
  - Magnesium Chloride, (MgCl$_2$.6H$_2$O) - 02.03 g
  Dissolve it in ~ 80 ml of double distilled deionized water. Finally make-up the volume of the solution to 100ml using a volumetric flask and store at room temperature.

- **Tyrode’s Buffer Stock III (100ml)**
  - Calcium Chloride, (CaCl$_2$.2H$_2$O) - 01.471 g
  Dissolve it in ~ 80 ml of double distilled deionized water. Finally, make-up the volume of the solution to 100ml using a volumetric flask and store at room temperature.
A.II. Making Tyrode’s buffer working solution for making murine PRP (~ 10 ml)

- Double distilled water: 9.0 ml
- Tyrode’s Buffer Stock I: 500 µl
- Tyrode’s Buffer Stock Stock II: 100 µl
- Glucose (C₆H₁₂O₆): 0.010 g
- HEPES: 0.024 g

Dissolve all the above mentioned ingredients. After dissolving HEPES, adjust the pH of the solution to 7.35.

- Tyrode’s Buffer Stock Stock III: 200 µl
- Bovine Serum Abumin (BSA): 0.035 g

In order to avoid foam formation, just add BSA on top of the solution and let the tube sit in water bath at 37 °C.

A.III. Making Tyrode’s buffer for washing human platelets (for 50ml blood)

**Master solution**

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<td>ddH₂O</td>
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<tr>
<td>Tyrode’s Buffer Stock II</td>
<td>1 ml</td>
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<tr>
<td>Glucose (C₆H₁₂O₆)</td>
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<td>HEPES</td>
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Adjust the pH to 7.2

**Tyrode’s with BSA**

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<tr>
<td>Tyrode’s without BSA with calcium</td>
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Split the master solution:

- Tyrode’s Buffer Stock III: 48 ml
- ddH₂O: 1 ml

Make-up the volume to:

- 50 ml
- 0.35 g

Divide the Tyrode’s buffer (TB) made above into three 50 ml tubes as follows:

**Tube I**

- 20 ml TB + BSA – Ca²⁺
- 20 µl 1000x PGE₁
- 20 µl 1000x Heparin

**Tube II**

- 20 ml TB + BSA – Ca²⁺
- 20 µl 1000x PGE₁

**Tube III**

- 10 ml TB – BSA + Ca²⁺
Appendix – B

• Washing glass coverslips for platelet spreading experiments
  
o Take 22x40 glass coverslips.  
o Make 0.2% Triton X-100 from 10% stock.  
o Gently scrub the coverslips both sides with a soft brush using 0.2% Triton X-100.  
o Rinse the coverslips with Tap water.  
o Then rinse them with DI water.  
o Wash them with Isopropyl alcohol.  
o Then rinse them thoroughly with ddH₂O.  
o Carefully place them in slide holder using tweezers and let them dry in oven at 37 °C for 15 – 20 min.
Appendix – C

- PERMISSION LETTER

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<tr>
<td>C</td>
<td>Procedure involving momentary or no pain or distress</td>
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<tr>
<td>D</td>
<td>Procedure where pain or distress is alleviated by appropriate means (analgesics, tranquilizers, euthanasia etc.)</td>
</tr>
<tr>
<td>X E</td>
<td>Procedure where pain or distress cannot be alleviated, as this would adversely affect the procedures, results or interpretation</td>
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Official Use Only

IACUC Approval Signature: [Signature]

Date of Approval: 2 May 2011
Principal Investigator Assurance

1. I agree to abide by all applicable federal, state, and local laws and regulations, and UD policies and procedures.

2. I understand that deviations from an approved protocol or violations of applicable policies, guidelines, or laws could result in immediate suspension of the protocol and may be reportable to the Office of Laboratory Animal Welfare (OLAW).

3. I understand that the Attending Veterinarian or his/her designee must be consulted in the planning of any research or procedural changes that may cause more than momentary or slight pain or distress to the animals.

4. I declare that all experiments involving live animals will be performed under my supervision or that of another qualified scientist. All listed personnel will be trained and certified in the proper humane methods of animal care and use prior to conducting experimentation.

5. I understand that emergency veterinary care will be administered to animals showing evidence of discomfort, ailment, or illness.

6. I declare that the information provided in this application is accurate to the best of my knowledge. If this project is funded by an extramural source, I certify that this application accurately reflects all currently planned procedures involving animals described in the proposal to the funding agency.

7. I assure that any modifications to the protocol will be submitted to by the UD-IACUC and I understand that they must be approved by the IACUC prior to initiation of such changes.

8. I understand that the approval of this project is for a maximum of one year from the date of UD-IACUC approval and that I must re-apply to continue the project beyond that period.

9. I understand that any unanticipated adverse events, morbidity, or mortality must be reported to the UD-IACUC immediately.

10. I assure that the experimental design has been developed with consideration of the three Rs: reduction, refinement, and replacement, to reduce animal pain and/or distress and the number of animals used in the laboratory.

11. I assure that the proposed research does not unnecessarily duplicate previous experiments. *(Teaching Protocols Exempt)*

12. I understand that by signing, I agree to these assurances.

<table>
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<th>2-22-11 Date</th>
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Rev 8/10  2  #1094-2011-0
## Names of All Persons Working on This Protocol

I certify that I have read this protocol, accept my responsibility and will perform only those procedures that have been approved by the IACUC.

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<tr>
<td>1. Ulhas P. Naik</td>
<td>Uhas P. Naik</td>
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<td>3. Sharmila Chatterjee</td>
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<td>5. Anil Nigam</td>
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<td>6. Brendan Bachman</td>
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