CHARACTERIZATION OF PLATELET FUNCTION AND HEMOSTASIS IN THE *Cib1*$^{-/-}$ MOUSE

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ABSTRACT

JAN CATHERINE DENOFRIO: Characterization of Platelet Function and Hemostasis in the *Cib1*$^{-/-}$ Mouse
(Under the direction of Dr. Leslie Parise)

Platelet activation initiates platelet-platelet aggregation, platelet-extracellular matrix interactions and thrombus formation. Platelet aggregation requires activation of the αIIbβ3 integrin, an event regulated by the integrin cytoplasmic tails. CIB1 binds to the cytoplasmic tail of the integrin αIIb subunit. CIB1 has been demonstrated to regulate αIIbβ3 activation in an acute setting. We studied the *Cib1*$^{-/-}$ mouse to determine if the chronic loss of CIB1 causes a defect in hemostasis. Plasma analysis exposed several significant differences in protein levels between the *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ plasma. Most notable were the increases in von Willebrand factor and soluble P-selectin. To understand the effect of two pro-thrombotic proteins circulating at higher levels in the plasma, we examined bleeding time and arterial thrombus formation discovered no significant difference in thrombus formation or stability between the *Cib1*$^{+/+}$ and the *Cib1*$^{-/-}$ mice.

Previous overexpression and knockdown studies in murine megakaryocytes from our lab demonstrated that CIB1 inhibits platelet integrin αIIbβ3 activation. We analyzed *Cib1*$^{-/-}$ mice to determine the function of CIB1 in platelets *in vivo*. We found that although these mice had no overt platelet phenotype, mRNA level of CIB1 homolog CIB3 was increased in *Cib1*$^{-/-}$ megakaryocytes. *In vitro* binding studies

demonstrate that recombinant CIB1, -2 and -3 bound specifically to an αIIb cytoplasmic tail peptide. Subsequent protein modeling experiments indicated that CIBs 1-3 each have a highly conserved hydrophobic binding pocket.

Therefore, the potential exists for compensation by these CIB family members for loss of CIB1, thereby preventing pathologic thrombus formation in *Cib1*$^{-/-}$ mice. Interestingly, the *Cib1*$^{-/-}$ mouse does have a phenotype that may provide a model for studying critical levels of plasma proteins required for normal hemostasis.

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LIST OF ABBREVIATIONS AND SYMBOLS

α	Alpha
β	Beta
Δ	Delta
A or Ala	Alanine
ADP	Adenosine diphosphate
BSA	Bovine Serum Albumin
C or Cys	Cysteine
Ca2+	Calcium ion
calm-l3	Calmodulin-like3
CHO	Chinese hampster ovary
CIB	Calcium and Integrin Binding
CT	Cytoplasmic tail
C-term	Carboxyl terminal
D or Asp	Aspartic acid
DTT	Dithiothreitol
E or Glu	Glutamic acid
ECM	Extracelluar Matrix
ELISA	Enzyme-Linked ImmunoSorbent Assay
ES	Embryonic Stem
F or Phe	Phenylalanine
FERM	4.1 protein/ Ezrin/ Radixin/ Moesin

FITC	Fluorescein isothiocyanate
G or Gly	Glycine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GP	Glycoprotein
h	Hour
H or His	Histidine
HEK	Human Embryonic Kidney
HRP	Horseradish peroxidase
I or Ile	Isoleucine
Icln	Chloride channel regulatory protein
IgG	Immunoglobulin G
IL	Interleukin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITC	Isothermal Titration Calorimetry
ITF	Intrinsic tryptophan fluorescence
K or Lys	Lysine
L or Leu	Leucine
M	Molar
M or Met	Methionine
Mg2+	Magnesium ion
min	Minute
N or Asn	Asparagine
NMR	Nuclear magnetic resonance

N-term	Amino-terminus
OD	Optical density
OPD	o-Phenylenediamine
P or Pro	Proline
PAR4	Protease activated receptor 4
plts	Platelets
PP1c	Protein phosphatase 1 catalytic subunit
PPP	Platelet poor plasma
PRP	Platelet rich plasma
Q or Gln	Glutamine
qPCR	Quantitative polymerase chain reaction
R or Arg	Arginine
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain
S or Ser	Serine
scr	Scrambled
SEM	Standard Error of the Mean
T or Thr	Threonine
TPI	Triosephosphate isomerase
V	Valine
W or Trp	Tryptophan
WASP	Wiskott-Aldrich Syndrome Protein
Y or Tyr	Tyrosine

CHAPTER 1

INTRODUCTION

1.1 Introduction to CIB1

Hemostasis is the process of blood clotting in response vascular injury. Misregulation of this physiological process can lead to either blood loss, or thrombotic vascular disease, which in either case, can be fatal. Understanding the mechanisms involved in hemostasis provides insights for treating or preventing abnormal blood clotting (thrombus formation). Integrin αIIbβ3, a transmembrane receptor on platelets and megakaryocytes, is critical for hemostasis and thrombus formation. Activation of the integrin promotes soluble ligand binding and platelet aggregation. The mechanisms that regulate αIIbβ3 activation are not fully understood; therefore identification of potential regulatory molecules is necessary.

CIB1 (also known as calcium and integrin binding protein, CIB, KIP1, SIP and calmyrin) was identified in 1997 in our lab by a yeast two hybrid screen for proteins that bind to the cytoplasmic tail of the αIIb subunit of αIIbβ3 (Naik, Patel, & Parise, 1997). However, the exact role of CIB1 in regulating platelet integrin αIIbβ3 activation has been unclear. Our lab showed by direct genetic manipulation, that CIB1 is a negative regulator of agonist-induced αIIbβ3 activation in megakaryocytes (Yuan et al., 2006b). In another study, the

introduction of antibodies into platelets that block CIB1 function inhibited platelet spreading on immobilized fibrinogen (Naik & Naik, 2003a), suggesting that CIB1 is required for platelet spreading. Yet another group observed that introduction of a CIB1 binding peptide into platelets inhibited αIIbβ3 activation, leading this group to conclude that CIB1 induces αIIbβ3 activation. Therefore, acute change in CIB1 by genetic manipulations, antibody or peptide inhibition strongly suggests that CIB1 regulates integrin αIIbβ3 activation. To understand the potential function of CIB1 *in vivo*, we analyzed the *Cib1*$^{-/-}$ mouse to determine the effect of CIB1 loss on hemostasis and platelet activation.

1.1.1 Basic properties of CIB1

CIB1 is a 22 kDa calcium binding protein that is expressed in several tissues and cell types (Shock et al., 1999). Sequence and structural analysis demonstrates that CIB1 has 4 EF-hands and an N-terminal myristoylation site. CIB1 is homologous to other EF-hand containing proteins such as calmodulin and calcineurin B (Naik et al., 1997). CIB1 also shares homology with several Ca^{2+}-myristoyl switch neuronal calcium sensor proteins but is not solely expressed in neuronal cells (Gentry et al., 2005). Furthermore, the hydrophobic binding channel found in EF-hand containing proteins is also present in CIB1 (Gentry et al., 2005). Other protein homologs have been identified, providing evidence of a CIB family of 4 proteins (Gentry et al., 2005). CIB1 is expressed many cell types (Shock et al., 1999), this implies (and has repeatedly proven)

that CIB1 interacts with other proteins and that CIB1 function is much more complex than simply regulating integrin αIIbβ3 agonist-induced activation.

1.1.2 CIB1 binding partners

Since the initial yeast two hybrid screen, the CIB1/αIIb interaction has been detected by other assays including isothermal titration calorimetry (Shock et al., 1999; Yamniuk & Vogel, 2005), ELISA (Naik et al., 1997; Tsuboi, 2002; Yuan et al., 2006b), intrinsic tryptophan fluorescence (Barry et al., 2002; Yamniuk et al., 2005) and surface plasmon resonance (Vallar et al., 1999). However, CIB1 expression is not limited to αIIbβ3-expressing cells but is widely expressed in various cell types that do not express integrin αIIbβ3, thereby prompting the investigation of CIB1 interactions with other proteins (Naik et al., 1997; Shock et al., 1999).

Evidence has emerged demonstrating an important role of CIB1 as a kinase regulator. CIB1 binds polo-like kinases SNK and FNK and inhibits SNK activity (Ma, Liu, Yuan, & Erikson, 2003). CIB1 also binds focal adhesion kinase (FAK) and has been proposed to regulate FAK activation during platelet spreading on fibrinogen, through integrin αIIbβ3 (Naik et al., 2003a). The interaction of CIB1 and p21-activated kinase (PAK1) a serine/threonine kinase has been studied in our lab. CIB1 is required for PAK1 adhesion-induced activation (Leisner, Liu, Jaffer, Chernoff, & Parise, 2005) and mouse lung endothelial cells from the *Cib1*$^{-/-}$ mice have decreased levels of PAK1 activation after adhesion to fibronectin (Zayed et al., 2007). The importance of CIB1

regulation of PAK1 activation was demonstrated by changes in PAK1-dependent cell migration that correlates directly with expression levels of CIB1 (Leisner et al., 2005). Overexpression of CIB1 decreases cell migration 2.5 fold in REF52 cells, likewise knocking down endogenous CIB1 leads to increases in haptotactic migration of HeLaS3 and REF52 cells (Leisner et al., 2005). CIB1 can also bind and regulate the activity of 3-phosphoinositide-dependent protein kinase 1 (PDK1) (Zhao *in submission*, 2007). PDK1 is involved in cell survival and cell progression. Wildtype CIB1 overexpression protected cells from apoptotic stimuli, while CIB1 mutants that are either unable to bind PDK1 or do not localize to the membrane, lose this apoptotic protection (Zhao *in submission*, 2007). Conversely, knockdown of CIB1 leads to decreased cell survival in response to apoptotic stimuli, while the overexpression of PDK1 rescues this phenotype (Zhao *in submission*, 2007).

Studies in the brain have demonstrated that CIB1 can bind to presenilin-2 and the role of this interaction in Alzheimer's disease is currently under investigation (Bernstein et al., 2005; Blazejczyk et al., 2005). More recently, CIB1 was reported to bind the inositol 1,4,5-trisphosphate receptor ($InsP_3R$) Ca^{2+} release channel (White, Yang, Monteiro, & Foskett, 2006). The authors found that in single channel gating studies CIB1 could activate $InsP_3R$ (White et al., 2006). However, when CIB1 was overexpressed in PC-12 cells the amplitude of agonist-induced cytosolic calcium fluctuations decreased and *in vitro* pre-exposure of CIB1 to $InsP_3R$ reduced the number $InsP_3R$ available for stimulation by inositol 1,4,5-trisphopshate ($InsP_3$) (White et al., 2006).

1.1.3 CIB1 homology

1.1.3.1 Homology to other EF-hand containing proteins

EF-hands are helix-loop-helix structural motifs that often bind calcium (Gifford, Walsh, & Vogel, 2007). This calcium binding can induce a conformational change in the protein that enhances ligand affinity (Yap, Ames, Swindells, & Ikura, 1999; Shock et al., 1999; Tsuboi, 2002). CIB1 has four EF-hands, two C-terminal canonical Ca^{2+} binding EF-hands and two N-terminal non-classical EF-hands (Fig. 1.1). CIB1 is homologous to other EF-hand containing proteins, calcinerin B (58%) and calmodulin (55%) (Naik et al., 1997). CIB1 is also homologous to neuronal calcium sensor proteins KChIP1 (46%) and frequenin (43%). Like KChIP1 and frequenin, CIB1 has an N-terminal myristoylation site, which may target the protein to the membrane (Gentry et al., 2005). It has been demonstrated however, that CIB1 myristoylation is not required for binding to membrane bound αIIbβ3 (Shock et al., 1999; Tsuboi, 2002). EF-hand III binds a calcium ion with a dissociation constant (Kd) near 1.9 μM while EF-hand IV binds with a dissociation constant of 0.5 μM (Yamniuk, Nguyen, Hoang, & Vogel, 2004). CIB1 can also bind Mg^{2+} at EF-III with a K_d of 120 μM and this binding induces a similar conformational change as with Ca^{2+} binding (Yamniuk et al., 2004). It has been proposed that CIB1 is Mg^{2+}-bound in resting cells since the intercellular concentration of Mg^{2+} is higher than that of Ca^{2+} (Mg^{2+} concentration ~ 10^{-3} to 10^{-4} M, Ca^{2+} concentration ~ 10^{-7} to 10^{-8} M)

(Yamniuk et al., 2004; Yamniuk et al., 2005). CIB1 protein stability is greater when bound to Ca^{2+} than Mg^{2+} (Yamniuk et al., 2004).

1.1.3.2 CIB1 homology to other CIB family members

Though CIB1 was the first CIB to be identified, it is not the ancestral original CIB. Homology screening determined that the Cib family consists of four genes (Cib1-4) (Gentry et al., 2005). There was only one Cib ancestral gene present through *Drosophila* evolution. Cib1 diverged from the ancestral Cib between the evolution of *Drosophila* to *Danio*. A genetic duplication occurred during the evolution of *Danio* to *Xenopus* that led to the current Cib2 and Cib3 genes, Cib2 being more homologous to the ancestral Cib. The Cib4 sequence did not emerge until mammalian evolution. Homology to *Caernorhabditis* ancestral CIB as determine by BLAST searches are shown in (Fig. 1.2)

Previous studies of the sequences of CIB family members indicted that CIB2 shares 59% homology to CIB1 while CIB3 and CIB4 are 62% and 64% homologous to CIB1, respectively (Fig 1.3) (Gentry et al., 2005). CIB2 has been identified in HEK293 cells by mass spectrometry but currently there is no other literature on the protein (Mayhew et al., 2006). There is no published data on CIB3 and CIB4 protein expression. Using the Prosite database (http://ca.expasy.org/prosite/) (de Castro et al., 2006) and Prosite My Domains Image Creator (http://ca.expasy.org/tools/mydomains/), potential functional domains of CIBs1-4 can be mapped (Fig. 1.4). Based on Prosite analysis, EF-hands are found in all CIB proteins. There is one N-terminal incomplete EF-hand

predicted in CIBs1-3 but predicted calcium binding EF-hands at the C-terminus of all CIB proteins.CIB2 and CIB3 have more acidic residues between helices H4 and H5 (EF-hand 2 in CIB1) and have the potential to bind a third calcium (Gentry et al., 2005). Human CIB1, -2 and -3 each has an N-terminal myristolyation site (Fig 1.4). Other predicted post-translational modification sequences predicted by this program include an N-glycosylation site in CIB1 and CIB2, and casein kinase II phosphorylation sites in CIB1-4. In addition, CIB1, -3 and -4 have protein kinase C phosphorylation sites (de Castro et al., 2006).

1.1.4 Structure of CIB1

In 2004, the structure of CIB1 was solved by our lab (Gentry et al., 2005) and another (Blamey, Ceccarelli, Naik, & Bahnson, 2005). Both structures are based on human CIB1 with an N-terminal truncation of the first eight amino acids (CIB1Δ1-8); however, the two structures depict distinct differences in tertiary and quaternary structure. Gentry *et al.* describe CIB1 as a monomeric protein with a large hydrophobic binding pocket (Fig 1.5) (Gentry et al., 2005). In contrast, Blamey *et al.* report a structure that aligns head to toe to create a dimer and is potentially redox regulated (Blamey et al., 2005). The Gentry et al. CIB1 structure was resolved at 2.0 Å and the Blamey et al. structure was resolved at 2.3 Å. Currently, the monomeric form of CIB1 is more accepted as sedimentation equilibrium, gel filtration and diffusion nuclear magnetic resonance (NMR) spectroscopy all provide data supporting a single oligomeric state (Gentry

et al., 2005; Sobczak et al., 2005; Weljie, Yamniuk, Yoshino, Izumi, & Vogel, 2003).

1.1.5 Hydrophobic binding pocket of CIB1

The hydrophobic binding pocket of CIB1 is the putative αIIb binding site. Interestingly, the C-terminal extension of CIB1 partially blocks the hydrophobic binding pocket. Vogel and colleagues investigated if and how the C-terminus could be affecting ligand binding to the hydrophobic pocket (Fig. 1.6) (Yamniuk, Ishida, & Vogel, 2006). Using solution NMR spectrometry, optical spectrometry and isothermal titration calorimetry (ITC) they determined that αIIb peptide binding causes both large chemical shift perturbations and increased the flexibility of the C-terminus (Yamniuk et al., 2006). This implies that the C-terminal tail moves upon binding, expanding the depth and size of the hydrophobic pocket. To test this conclusion, Yamniuk et al. deleted the last 12 amino acids of CIB1, thereby removing the H10 helix (CIB1ΔH10). They identified the exposed hydrophobic surfaces of both full-length and mutant CIB1 by steady state fluorescence spectroscopy and found that CIB1ΔH10 displays a larger hydrophobic surface than full-length CIB1 (Yamniuk et al., 2006). Taken together the results of these experiments indicate that the C-terminal extension of CIB1 may act to enhance target specificity, meaning only specific sequences displace the C-terminal tail and bind CIB1 (Yamniuk et al., 2006).

1.1.6 *Cib1*$^{-/-}$ mouse model to study the effect of the chronic loss of CIB1

Cib1 is located on human chromosome 15 and mouse chromosome 7. Mouse *Cib1* contains seven exons and six introns. To generate the knockout mouse, Dr. Weiping Yuan from our lab replaced genomic DNA (all of exon 4 and most of exon 5) with the reversed neomycin gene (Yuan et al., 2006a). No other protein encoding genes align in the area covered by *Cib1* on the + or – strand. The Semaphorin 4b gene is adjacent to the *Cib1* gene, but does not overlap.

The general health and development of the *Cib1*$^{-/-}$ mice is normal; however, *Cib1*$^{-/-}$ male mice are sterile (Yuan et al., 2006a). This phenotype is not present in the male *Cib1*$^{+/-}$ mice and the female reproduction in both *Cib1*$^{+/-}$ and *Cib1*$^{-/-}$ mice is normal. The sterility defect in the *Cib1*$^{-/-}$ mouse occurs during the haploid stage of spermatogenesis (Yuan et al., 2006a). The exact mechanism causing this defect is currently unknown.

Upon further characterization of the *Cib1*$^{-/-}$ mice, we discovered a defect in angiogenesis (Zayed et al., 2007). More specifically, the loss of CIB1 results in endothelial cells that display decreased migration, PAK1 and ERK1/2 activation and matrix metalloproteinase 2 expression (Zayed et al., 2007). These decreases result in defects in ischemia-induced pathologic angiogenesis in the *Cib1*$^{-/-}$ mouse (Zayed et al., 2007).

1.2 BASICS OF HEMOSTASIS

Hemostasis is the physiologic process of blood clotting at areas of vascular injury. Upon damage to the endothelial cells lining a blood vessel,

adhesive extracellular matrix proteins (ECM) become exposed such as collagen and von Willebrand factor (vWF). Platelets are attracted to these matrix proteins as they express several adhesion receptors that bind to individual ECM proteins. Glycoprotein (GP) VI binds collagen (Massberg et al., 2003; Nieswandt et al., 2001; Nieswandt & Watson, 2003) and the GPIb/IX/V complex binds vWF (Ruggeri, De Marco, Gatti, Bader, & Montgomery, 1983; Ruggeri, 1988; Ruggeri, Zimmerman, Russell, Bader, & De Marco, 1992). These initial contacts between the platelets and the ECM result in platelet rolling along the damaged endothelium. These contacts are transient but serve to activate platelets and initiate thrombus formation. Once activated, platelets release soluble pro-coagulant proteins and agonists, which act as a signal to recruit more platelets. As platelets continue to roll along the endothelium, stronger adhesive reactions cause platelets to stop and spread to cover the exposed ECM. Integrins, which are heterodimeric adhesion receptors, change conformation and have increased ligand affinity upon agonist-induced activation. Active integrin $\alpha 2\beta 1$ binds collagen (Nieswandt et al., 2003) and integrin $\alpha IIb\beta 3$ binds vWF (Hato, Pampori, & Shattil, 1998; Kasirer-Friede et al., 2004). At this time, platelets also interact with each other to form aggregates. Platelets aggregate by binding fibrinogen, the major $\alpha IIb\beta 3$ ligand. One integrin $\alpha IIb\beta 3$ from one platelet adheres to the same fibrinogen molecule as another $\alpha IIb\beta 3$ on a different platelet. Since integrin $\alpha IIb\beta 3$ is highly populated on the platelet surface, these fibrinogen links result in stable aggregates (Parise, 1999; Phillips, Charo, & Scarborough, 1991). Aggregates build up from the ECM-bound platelets and the combination results

in stable thrombus formation, known commonly as a blood clot. Not surprising, the process of clot break-down is just as methodical.

A careful balance of pro- and anti-coagulant proteins is essential in hemostasis (Fig.1.7). A pathologic state can result when pro-coagulant proteins are unable to initiate and stabilize a thrombus, or if anticoagulant proteins are unable to disassemble the clot. Insufficient surface receptor expression on platelets or endothelial cells, or defective ECM proteins or coagulation clotting factors can lead to a hemorrhagic state. For example, patients with Glanzmann Thrombasthenia have a defect in integrin αIIbβ3 expression or function. These patients have prolonged bleeding because they cannot aggregate properly (Phillips, Jenkins, Luscher, & Larrieu, 1975). On the other side of the spectrum, patients with type 2B von Willebrand Disease have increased vWF binding to platelets, which results in spontaneous aggregation. The hyper-aggregation results from increased affinity of vWF to GP1b (Lillicrap, 2007).

Cardiovascular disease is the leading cause of death in the developed world (Nieswandt, Aktas, Moers, & Sachs, 2005). In the United States, cardiovascular disease is associated with almost 40% of all deaths (Rosamond et al., 2007). Pathologic thrombus formation can lead to cardiovascular disease when the thrombus size occludes blood flow. This usually occurs in vessels damaged by atherosclerotic plaques that have constricted vessel size and elasticity, and provide highly pro-thrombotic and pro-inflammatory environment. Thrombi can also embolize, causing a downstream blockage in smaller vessels.

Common thrombus-related cardiovascular diseases include myocardial infarctions and strokes.

1.2.1 Plasma proteins

Soluble proteins in plasma are also essential for aggregate formation and thrombus stabilization. Some plasma proteins such as vWF, P-selectin, fibrinogen and thrombospondin, are stored intracellular granules in cells and are released or exposed upon cellular activation; this could potentially provide a method of regulation to preventing unnecessary thrombus formation. Platelets and endothelial cells exocytose their storage granules (α-granules and Weibel Palade bodies respectively) and either release their contents into the plasma or onto the extracellular plasma membrane surface. High levels of pro-thrombotic proteins in plasma have been proposed to be markers of platelet or endothelial cell activation and could provide a measure of vascular health and integrity (Blann, Faragher, & McCollum, 1997; Blann & Lip, 1997; Guray et al., 2004). Increased plasma P-selectin levels alone correlate with myocardial infarction (Blann et al., 1997; Ikeda et al., 1994), thrombotic consumptive platelet disorders (Chong et al., 1994; Katayama et al., 1993), and peripheral arterial occlusive disease (Galkina & Ley, 2006). Furthermore, mice genetically engineered to express increased levels of soluble P-selectin have faster clotting times in a P-selectin concentration-dependent manner (Andre, Hartwell, Hrachovinova, Saffaripour, & Wagner, 2000).

1.2.2 Mouse models used in hemostatic research

Mouse models have provided great insight into the mechanisms of normal hemostasis and hematological disorders. Von Willebrand Disease (vWD) is the most common inherited bleeding disorder (Rodeghiero, Castaman, & Dini, 1987). The disease results from mutations in the *vWF* gene and can be classified into three types; 1, 2 and 3. Type 2 has four subtypes; 2A, 2B, 2M and 2N. As mentioned earlier, the physiological functions of vWF include tethering platelets to endothelial cells or exposed ECM and stabilizing platelet aggregates. Another important function of this protein is to bind Factor VIII and prevent its degradation. The *vWF*$^{-/-}$ mouse most closely mimics Type 3 vWD, displaying spontaneous bleeding and reduced Factor VIII levels (Denis et al., 1998). Research on the *vWF*$^{-/-}$ mouse demonstrated that vWF is necessary in thrombus stability and is vital in high shear stress (Ni et al., 2000). Studies using gene-transfer to rescue the phenotype of the *vWF*$^{-/-}$ mouse have shown that it may be possible to correct or at least ameliorate the bleeding disorders associated with vWD (Pergolizzi et al., 2006).

The model for Glanzmann Thrombasthenia has also proven to be invaluable for understanding and potentially treating the disease. The integrin subunit $\beta3^{-/-}$ mouse is used as a model for Glanzmann Thrombasthenia (GT) because platelets from these mice have a similar phenotype to those of GT patients (Hodivala-Dilke et al., 1999). Investigators were able to explore the possibility of gene therapy to treat the disease by utilizing the platelet-specific

αIIb promoter to express the β3 gene (Fang et al., 2005). The successful expression of β3 did, in fact, improve bleeding times (Fang et al., 2005).

Previous methods to investigate hemostasis in humans involved *ex vivo* experiments. Human platelets can be isolated from whole blood and studied by aggregation, flow cytometry and adhesion to immobilized ligand. Though blood volume can be an issue, these types of assays can also be easily performed with mouse models, but the principal motive to use mice to study hemostasis is to visualize a thrombus or blood clotting in real time.

Intravital microscopy has been an invaluable tool in hemostasis and thrombosis research. Studies on platelet aggregation, transient ("rolling") or firm adhesion along with thrombus stability have all been studied *in vivo* by intravital microscopy (Celi et al., 2003; Dubois, Panicot-Dubois, Gainor, Furie, & Furie, 2007; Falati et al., 2004; Falati et al., 2006; Furie & Furie, 2006; Furie & Furie, 2007; Sim, Flaumenhaft, Furie, & Furie, 2005). Other valuable methods of research are based on visual observations of vessel integrity such as measuring bleeding times or thrombus-dependent vessel occlusion at sites of inflicted injury. These experiments require larger blood vessels than those needed for intravital microscopy but still provide an excellent visual of thrombus formation and stability (Denis & Wagner, 2007).

Of course all results from knockout or transgenic mice must take into account the possibility of compensation by proteins with highly similar functions. In addition, the lack of a phenotypic change in a genetically altered mouse does not mean there is no function of the protein or it could indicate that the function of

the protein is non-essential to hemostasis (Hynes & Wagner, 1997). It should also be noted that mice are not the only valuable animal models of thrombosis. In fact, a pig model of vWD demonstrated that soluble vWF circulating in the plasma, opposed to platelet stored vWF, was essential in arterial thrombosis (Nichols et al., 1991; Nichols et al., 1995). Other important studies have used guinea pigs (Andre et al., 1996b; Andre et al., 1996a), rats (Kurz, Main, & Sandusky, 1990), dogs (Coller & Scudder, 1985), zebrafish (Jagadeeswaran, Gregory, Day, Cykowski, & Thattaliyath, 2005; Thattaliyath, Cykowski, & Jagadeeswaran, 2005) and primates (Gruber & Hanson, 2003; Wu et al., 2002; Wu, Meiring, Kotze, Deckmyn, & Cauwenberghs, 2002; Gruber et al., 2007).

1.2.3 Other methods of hemostatic research

(The following section, 1.2.3, includes excerpts from the review by Lui et al. 2007. I am the co-first author of this review and contributed significantly with both writing and editing.)

"Because circulating platelets are anucleate, direct and rapid genetic manipulation of platelets to study molecular mechanisms is not currently possible. In addition, cells other than platelets or primary megakaryocytes (platelet precursors) do not have the ability to perform platelet specific functions such as agonist-induced αIIbβ3 activation (Fig. 1.8). Thus, even megakaryocyte-like cell lines, which have many platelet-like properties, including αIIbβ3 expression, do not respond to common platelet agonists to activate the fibrinogen-binding capacity of αIIbβ3, perhaps due to a lack of proper activation machinery, which is still being defined (Shattil & Leavitt, 2001). Therefore, while

nucleated megakaryocyte-like cell lines can be genetically manipulated, they are not useful via this approach for determining whether any given target molecule plays a role in αIIbβ3 activation or platelet aggregation. Because of these limitations, some investigators have used alternative approaches such as expressing artificial, constitutively active versions of αIIbβ3 in CHO cells in order to explore mechanisms of turning integrins "off", but these have significant limitations because they do not mimic or involve natural pathways of αIIbβ3 activation in platelets (Han et al., 2006; Hughes et al., 1997; Shattil et al., 2001). Knockout and transgenic mice can be generated to allow study of target molecules in the platelets of these mice, but these approaches are neither rapid nor high-throughput. Finally, several labs have exploited the fact that megakaryocytes derived from bone marrow, fetal livers, or in culture from embryonic stem (ES) cells are nucleated and can therefore be directly genetically manipulated. These types of megakaryocytes can be matured in culture to the point that they express αIIbβ3 and respond to agonists to activate the fibrinogen-binding capacity of the integrin (Shattil et al., 2001). Using primary murine megakaryocytes we recently determined that a protein, CIB1, functions as a direct, endogenous inhibitor of αIIbβ3 activation (Yuan et al., 2006b). Thus, megakaryocytes provide a useful model system, sharing many properties with platelets and representing the best directly genetically manipulatable platelet-like system to date. Finally, ES or CD34+ progenitor cells in culture can generate platelet-like particles, offering the potential to genetically manipulate platelet

protein expression (Fujimoto, Kohata, Suzuki, Miyazaki, & Fujimura, 2003; Ungerer et al., 2004). (Liu et al., 2007)**"**

"Since integrin αIIbβ3 is only expressed on platelets and megakaryocytes, and as mentioned above **"**platelets are not amenable to direct genetic manipulation, megakaryocytes have been used to study integrin αIIbβ3 signaling. Proteins affecting αIIbβ3 signaling have been studied by overexpressing or knocking down their expression in bone marrow or ES cell-derived megakaryocytes via Sindbis virus, retrovirus and lentivirus-mediated transduction. Using a Sindbis virus expression system, our lab and others have successfully overexpressed many proteins and have determined the effects of these proteins on agonist-induced activation of αIIbβ3. Initially, the Shattil lab demonstrated that Sindbis virus-mediated expression of Tac-integrin β3 cytoplasmic tail selectively inhibited αIIbβ3 activation in response to PAR4 peptide, ADP, or epinephrine (Shiraga et al., 1999). Based on their pioneering work, our lab set up a similar primary megakaryocytes system to test the function of CIB1, H-Ras, and other molecules that have been implicated in αIIbβ3 activation. We found that overexpression of CIB1-EGFP completely inhibited agonist-induced fibrinogen binding when compared to either overexpression of EGFP alone or untransduced megakaryocytes (Yuan et al., 2006b). However, when we overexpressed a CIB1 mutant protein (CIB F173A-EGFP) containing a single Phe to Ala substitution that does not bind to αIIb cytoplasmic tail (Barry et al., 2002), these transduced megakaryocytes were unable to suppress PAR4 peptide-induced αIIbβ3 activation (Yuan et al., 2006b). These results suggest

that CIB1 is a potential negative regulator of αIIbβ3 activation upon agonist stimulation. As mentioned above, we also knocked down endogenous CIB1 via siRNA in bone marrow-derived megakaryocytes and observed a small but statistically significant increase in fibrinogen binding to megakaryocytes. These data complement the CIB1 overexpression studies and suggest a negative regulatory role for CIB1 in modulating αIIbβ3 activation (Yuan et al., 2006b).

Another molecule proposed to be a negative regulator of integrin activation is H-Ras (Fujimoto et al., 2003; Hughes et al., 1997; Hughes et al., 2002; Ramos, Kojima, Hughes, Fenczik, & Ginsberg, 1998; Sethi, Ginsberg, Downward, & Hughes, 1999). Ginsberg and colleagues, using a constitutively active chimeric integrin expressed in a CHO cell system, found that H-Ras suppressed integrin activation (Hughes et al., 1997; Hughes et al., 2002). Early studies demonstrated that H-Ras suppressed integrin activation via the Raf-MEK-ERK pathway (Fig 1.9) (Hughes et al., 1997). However, later studies showed ERK activity was not necessary for H-Ras suppression of integrin activation (Hughes et al., 2002). I sought to investigate the role of H-Ras on endogenous integrin αIIbβ3 activation using megakaryocytes overexpressing constitutively active H-Ras via retrovirus. Presumably, if H-Ras negatively regulated integrin function, agonist-induced fibrinogen binding would be decreased in cells overexpressing constitutively active H-Ras12V versus vector alone. Contrary to the hypothesized results, our data demonstrated that H-Ras did not suppress integrin activation. In fact, as shown in Figure 1.10, the cells overexpressing constitutively active H-Ras12V exhibited a trend of increased agonist-induced

αIIbβ3 activation. Furthermore, the presence of PMA, a known activator of H-Ras (Chang et al., 2005; Dower et al., 2000; Lorenzo et al., 2001), did not suppress αIIbβ3 activation in megakaryocytes overexpressing the vector alone. Therefore, these results do not support a negative regulatory role for H-Ras in agonist-induced αIIbβ3 activation. While both cells are valid systems, the differing results between the bone marrow-derived megakaryocyte system and a chimeric integrin-CHO cell system highlight the strong role of the intracellular environment in signaling studies. Therefore, it is important to understand the role of the endogenous proteins in their natural cellular environment whenever possible**"**(Liu et al., 2007).

1.3 CIB1 AND INTEGRIN αIIbβ3

(The following section section 1.3 contains excerpts from the review by Leisner et al., 2007. I contributed significantly in both the writing and editing of this review.)

"Integrin activation is essential for platelet function and as a result, the molecular mechanisms that regulate integrin activation have been the focus of intense study. In general, integrins are ubiquitous transmembrane α/β heterodimers that mediate cell–cell and cell–matrix interactions, which are vital for both normal and pathophysiological processes. At present, there are 18 α and 8 β subunits that noncovalently combine to form at least 24 distinct integrins. Structurally, integrin receptors are largely extracellular, with each subunit possessing a single-pass transmembrane domain and a relatively short cytoplasmic domain (from 20 to 70 amino acids). Integrin αIIbβ3 is the most well

characterized integrin family member and studies involving integrin αIIbβ3 have provided invaluable insight into mechanisms of integrin activation. Furthermore, many of the functional properties of αIIbβ3 have proved widely applicable to other integrin receptors. Like αIIbβ3, ligand binding to other integrins is regulated by intracellular signaling. The exact mechanisms by which intracellular signals culminate at the cytoplasmic face and lead to integrin activation and ligand binding still remain unclear.

Central to the propagation of inside-out and outside-in signaling events are the integrin transmembrane and cytoplasmic domains that are thought to play an important role in regulating integrin affinity states (Ginsberg, Partridge, & Shattil, 2005). Early mutational studies demonstrated that deletion of either the α or β cytoplasmic domain results in integrin activation (Hughes, O'Toole, Ylanne, Shattil, & Ginsberg, 1995; O'Toole et al., 1991), as do point mutations in the conserved GFFKR motif within the αIIb membrane proximal region (O'Toole et al., 1994). Additional studies have provided evidence for an interaction between the membrane-proximal regions of the α and β cytoplasmic tails through a salt bridge between a conserved Arg995 in the α tail and an Asp723 in the β tail, which is thought to maintain the integrin in an inactive state (Hughes et al., 1996). While the αIIb and β3 cytoplasmic tails were reported to form complexes in solution (Vinogradova et al., 2002) and to form homodimers in lipid micelles (Li et al., 2001), other studies failed to demonstrate any interaction between the cytoplasmic tails (Ulmer, Calderwood, Ginsberg, & Campbell, 2003). These differences may in part be due to the varying length of the isolated integrin

cytoplasmic domains used in each study. In subsequent nuclear magnetic resonance (NMR) studies, a low-affinity interaction was detected only with full-length cytoplasmic tails (Li et al., 2001; Vinogradova et al., 2002), suggesting additional sites of interaction between the integrin subunits. A recent study by Ma et al. (Ma et al., 2006) used mutational analysis to demonstrate that distinct regions within the α and β tails cooperate to regulate integrin activation. Specifically, mutation of αIIb residues V990F992R995 within the membrane-proximal KVGFFKR motif was shown to disrupt the electrostatic and hydrophobic bonds between the α and β tails, leading to extensive integrin activation. NMR spectroscopy also revealed that a naturally occurring S752P mutation (Chen et al., 1992) disrupted a C-terminal helix within the β3 tail and partially suppressed integrin activation.

Recent evidence indicates that the integrin α and β transmembrane domains also play an important role in the regulation of integrin activation. Mutation of various residues within the transmembrane helical domains results in integrin activation (Li et al., 2005; Luo, Springer, & Takagi, 2004; Luo, Carman, Takagi, & Springer, 2005; Partridge, Liu, Kim, Bowie, & Ginsberg, 2005) presumably by altering the length of the transmembrane domains or by disrupting the transmembrane helix–helix packing interface that maintains the integrin in an inactive state. In fact, platelet activation can be induced by the addition of a peptide corresponding to the αIIb transmembrane domain that presumably disrupts the inactive transmembrane heterodimer (Yin et al., 2006). Consistent with this, Litvinov et al. (Litvinov et al., 2006) recently demonstrated that

mutations within the transmembrane helix domain were sufficient to induce αIIbβ3 activation by using an interesting laser tweezer-based spectroscopy technique to compare the fibrinogen-binding strength of individual activated wildtype αIIbβ3 molecules to αIIbβ3 molecules containing transmembrane helix mutations.

Additional insight into integrin function has recently been gained from reports of high-resolution structures of the integrin extracellular domains. Crystal structures of αVβ3 (Xiong et al., 2001), αIIbβ3 (Xiao, Takagi, Coller, Wang, & Springer, 2004) and single-particle electron microscopy studies of α5β1 (Takagi, Strokovich, Springer, & Walz, 2003) reveal a bent or closed conformation in the absence of ligand binding, which has been suggested to represent an inactive integrin state. While large-scale shifts to a more open conformation have been associated with ligand binding to αIIbβ3 (Xiao et al., 2004) and α5β1 (Takagi et al., 2003), the subsequent structure of αVβ3 in complex with a ligand-mimetic peptide demonstrated minor structural rearrangements in the αV subunit relative to the β3 subunit (Xiong et al., 2002). Recently, the crystal structure of αVβ3 in the bent conformation was shown to stably bind fibronectin (Adair et al., 2005), suggesting that large global changes are not required for switching to a high-affinity binding state. While these structures may or may not reflect the true nature of intact integrins, they provide important evidence for dynamic molecular changes within the integrin that may be involved in regulating integrin activation".(Leisner, Yuan, Denofrio, Liu, & Parise, 2007)

1.3.1 Integrin αIIb cytoplasmic domain

"Currently, only a few αIIb cytoplasmic tail binding proteins have been identified and a number of these proteins bind to the conserved membrane-proximal KXVGFFRKR motif found in all α subunits. Early studies proposed that calreticulin may function in regulating integrin αIIbβ3 in platelets since it was found to bind to the conserved KXVGFFKR region within the α3 and α5 cytoplasmic tails (Rojiani, Finlay, Gray, & Dedhar, 1991) and regulate cell adhesion (Leung-Hagesteijn, Milankov, Michalak, Wilkins, & Dedhar, 1994). Recently, Reilly et al. (Reilly, Larkin, Devocelle, Fitzgerald, & Moran, 2004) revisited this question and were not able to detect calreticulin binding to the αIIb tail in platelets under a variety of platelet activation states and experimental conditions, suggesting an integrin independent role for calreticulin in platelet function. Interestingly, this same group (Larkin et al., 2004) identified a chloride channel regulatory protein, ICln, that bound to the KVGFFKR motif in αIIb and this interaction functionally regulated platelet activation (Larkin et al., 2004). Protein phosphatase 1 (PP1c) was also shown to bind to the αIIb tail via the KVGFFKR motif (Vijayan, Liu, Li, & Bray, 2004) and to constitutively co-immunoprecipitate with αIIbβ3 in resting platelets. Upon thrombin stimulation, PP1c was found to dissociate from αIIbβ3, resulting in PP1c activation and subsequent dephosphorylation of its substrate, myosin light chain. Recently, triosephosphate isomerase (TPI) was identified as an αIIb interacting protein via yeast two-hybrid analysis (Liu, Corjay, Feuerstein, & Nambi, 2006). Though the authors detected TPI mRNA in human platelets, current data limit TPI function to

its involvement in an energy production pathway specific to red blood cells and brain cells. Therefore, further studies are necessary to determine a functional role for TPI in platelet integrin function**”** (Leisner et al., 2007)

“Since CIB1 binds directly to the αIIb tail, we and others have hypothesized that this interaction plays a role in regulating αIIbβ3 function. Many early studies of integrin αIIbβ3 have relied on ectopic expression of αIIbβ3 in Chinese hamster ovary (CHO) cells or other nucleated cell types that fail to activate αIIbβ3 in response to agonist stimulation. Recent studies have utilized megakaryocytes, which, like platelets, have the capacity to activate αIIbβ3 in response to physiological agonists (Shattil et al., 2001; Shiraga et al., 1999). In addition, megakaryocytes are amenable to genetic manipulation, thereby providing an extremely useful system to dissect the molecular pathways leading to integrin activation. Overexpression of CIB1 in megakaryocytes completely inhibits agonist-induced fibrinogen binding, while overexpression of a CIB1 mutant (CIB1 F173A) that does not bind to the αIIb tail (Barry et al., 2002) fails to suppress agonist-induced αIIbβ3 activation. Conversely, depletion of CIB1 by RNA interference results in enhanced αIIbβ3 activation in response to agonist (Yuan et al., 2006b). These results suggest that the CIB1/αIIb interaction may negatively regulate αIIbβ3 activation, which may be important in preventing spontaneous or inappropriate activation of αIIbβ3.

In contrast to the inhibitory role of CIB1 in agonist-induced αIIbβ3 activation, Tsuboi (Tsuboi, 2002) proposed that CIB1 activates αIIbβ3. It was reported that introduction of a peptide encompassing the C-terminal residues

179–188 of CIB1 into platelets inhibited agonist-induced αIIbβ3 activation by blocking endogenous CIB1 binding to αIIb. The interpretation of these data, however, is questioned by recent findings of Yamniuk *et al*. (Yamniuk et al., 2006), who were unable to detect an interaction between the CIB1 peptide (residues 180–191 in this study) and a CIB1 mutant lacking these C-terminal residues or more importantly between an αIIb tail peptide and the C-terminus of CIB1 (residues 96–191).

Reports by Naik and Naik (Naik & Naik, 2003b) suggest a role for CIB1 in αIIbβ3-mediated outside-in signaling during the process of platelet spreading on fibrinogen. Interestingly, they find that CIB1 is required for lamellipodia, but not filipodia formation. Furthermore, they also propose that CIB1 regulates platelet spreading on fibrinogen by regulating focal adhesion kinase activity (Naik et al., 2003a)**”** (Leisner et al., 2007).

“Additional studies have further explored the role of CIB1 with regard to αIIbβ3 and platelets. An interaction between CIB1 and activated Rac3, but not Rac1 or Rac2, stimulated αIIb3-mediated adhesion and spreading of CHO cells expressing αIIbβ3 (Haataja, Kaartinen, Groffen, & Heisterkamp, 2002). CIB1 was also reported to interact with Wiskott–Aldrich syndrome protein (WASP) and this complex has been suggested to promote integrin αIIbβ3 activation and platelet aggregation (Tsuboi, Nonoyama, & Ochs, 2006)**”**. (Leisner et al., 2007)

1.3.2 Integrin β3 cytoplasmic domain

"In contrast to the integrin α subunit cytoplasmic tails, the cytoplasmic tails of integrin β subunits are highly conserved and possess defined motifs required for integrin function. In particular, the β3 cytoplasmic tail contains two well characterized tandem NPXY motifs, which serve as recognition sequences for phosphotyrosine binding (PTB) proteins (Calderwood et al., 2003) and sites of tyrosine phosphorylation (Phillips, Nannizzi-Alaimo, & Prasad, 2001). The wide variety of signaling, regulatory and cytoskeletal proteins interacting with the β3 cytoplasmic domain serve to link αIIbβ3 to cytoskeletal signaling complexes that appear to be important for both inside-out and outside-in signal transduction events (reviewed in (Liu, Calderwood, & Ginsberg, 2000)). (Excerpt from Leisner et al., 2007) (Leisner et al., 2007).**"**

"Recently, talin, which binds directly to most β cytoplasmic domains via a PTB-like interaction with the conserved β tail NPXY motifs, has been implicated as a crucial mediator of integrin activation (reviewed in (Ratnikov et al., 2005)). The head domain of talin possesses a band 4.1, Ezrin, Radixin, Moesin homology (FERM) domain that is folded into F1, F2 and F3 subdomains (Calderwood, 2004), of which F2 and F3 have been shown to interact with the membrane proximal residues of the β3 tail (Calderwood et al., 2003). The F3 domain exhibits a substantially greater binding affinity for the β3 tail relative to F1 and F2 and the recent crystal structure of the F3 domain in complex with a β3 membrane proximal peptide (residues 739–750) has identified crucial residues important for this interaction (Garcia-Alvarez et al., 2003). Talin is also proposed to disrupt the inhibitory salt bridge between the α and β cytoplasmic tails, leading

to integrin activation (Ratnikov, Partridge, & Ginsberg, 2005; Tadokoro et al., 2003). (Leisner et al., 2007).**"**

"Interestingly, an interaction between talin and the αIIb tail has been observed (Knezevic, Leisner, & Lam, 1996; Yuan et al., 2006b). Recently, CIB1 was shown to interfere with talin binding to both an αIIb tail peptide and purified αIIbβ3, apparently by competing with talin for the CIB1 binding site on the αIIb tail. CIB1 inhibition of talin binding may potentially act as a regulatory mechanism in platelets to limit or control the extent of ligand binding to αIIbβ3 (Fig. 1.11).**"** (Leisner et al., 2007).

1.4. OVERALL GOAL AND PURPOSE OF STUDY

The overall goal of my studies, described in this dissertation, is to characterize hemostasis in the *Cib1*$^{-/-}$ mouse. I hypothesized, for several reasons mentioned above, (i.e. *Cib1*$^{-/-}$ endothelial cell disfunction during the pathologic angiogenesis and earlier acute genetic studies with megakaryocytes), that the *Cib1*$^{-/-}$ mice would have hyper-activable platelets and a pro-coagulant phenotype. Over the next couple chapters, I will present data that support several conclusions. First, the *Cib1*$^{-/-}$ mouse could be a valuable murine model for elucidating the critical threshold of plasma protein levels and better defining the balance of physiologic versus pathologic hemostasis. Second, my research has expanded our knowledge of regulatory molecules involved agonist-induced integrin αIIbβ3 activation. My results clearly show a difference in CIB1 regulation

of agonist-induced αIIbβ3 activation under acute versus chronic genetic manipulation. Since I did not prove my hypothesis in *CIB1*$^{-/-}$ mice, there are at least two interpretations to my data. On interpretation is that CIB1 does not regulate agonist-induced αIIbβ3 activation. However, another interpretation is that compensatory mechanisms developed in the *Cib1*$^{-/-}$ mice to prevent increased agonist-induced activation. If so, either CIB1 itself in the absence of compensation or the potential compensatory mechanisms could provide drug targets to control platelet activation.

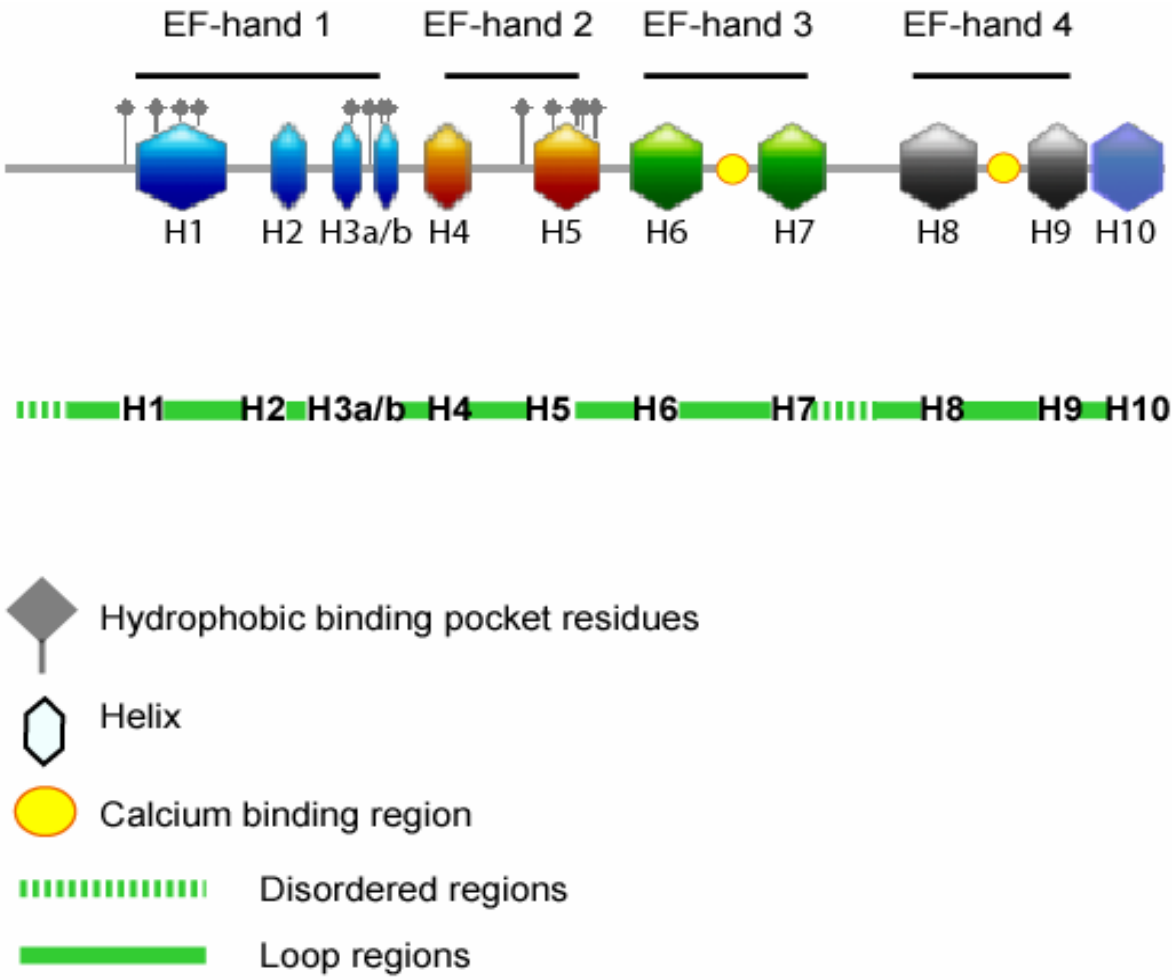

Figure 1.1 CIB1 structural motif and key residues. EF-hands and the correlating helices are diagramed. Key hydrophobic binding pocket residues are marked in grey and calcium binding regions in yellow. Loop regions are marked with a solid green line and disordered regions as marked by dotted green lines. Diagram was based on sequence alignment from Gentry et al. 2005.

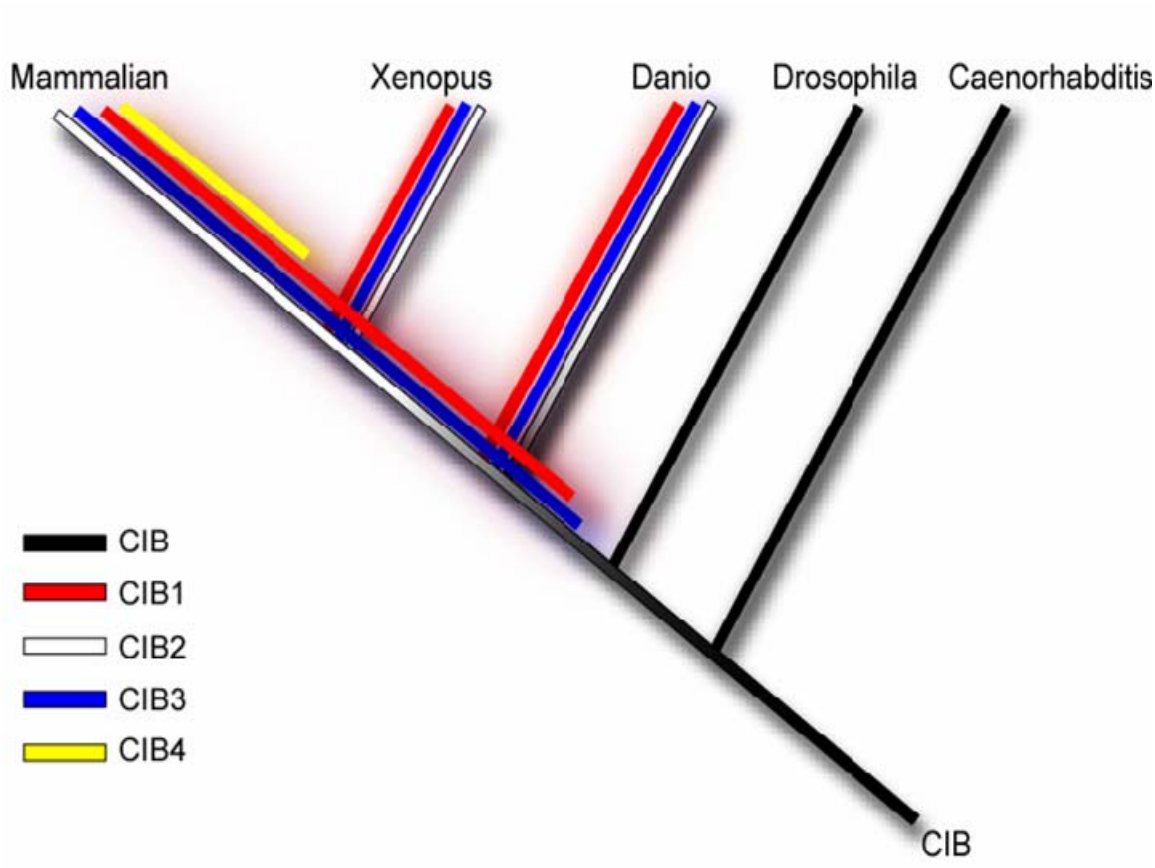

Figure 1.2 Evolution of CIB proteins. CIB family proteins can be identified through evolution as far back as *Caenorhabditis* via homology searches. Only a single ancestral CIB protein existed until the evolution of vertebrates. At this point in time, the CIB gene duplicated, leading to the existence of the current CIB2 and CIB3. CIB2 is the most homologous to ancestral CIB. CIB1 also evolved during the evolution of vertebrates. Only CIBs1-3 were in existence until mammalian evolution when the sequence CIB4 can be identified.

	CIB1	CIB2	CIB3	CIB4
L22	leucine	phenylalanine	phenylalanine	leucine
E26	glutamic acid	asparic acid	glutamic acid	glutamic acid
A30	alanine	leucine	leucine	isoleucine
R33	arginine	arginine	arginine	threonine
I58	isoleucine	isoleucine	isoleucine	valine
L61	leucine	methothionine	methothionine	leucine
E63	glutamic acid	glutamic acid	glutamic acid	--
L64	leucine	leucine	leucine	alanine
L86	leucine	leucine	methothionine	phenylalanine
F91	phenylalanine	phenylalanine	phenylalanine	valine
L94	leucine	methothionine	methothionine	methothionine
L95	leucine	phenylalanine	phenylalanine	alanine
F98	phenylalanine	leucine	methothionine	phenylalanine
I114	isoleucine	isoleucine	isoleucine	isoleucine
F115	phenylalanine	tyrosine	tyrosine	tyrosine
F117	phenylalanine	phenylalanine	phenylalanine	phenylalanine
L131	leucine	threonine	threonine	isoleucine
L132	leucine	leucine	valine	isoleucine
L152	leucine	valine	valine	leucine
I153	isoleucine	cysteine	cysteine	threonine
F173	phenylalanine	phenylalanine	phenylalanine	phenylalanine
I177	isoleucine	isoleucine	isoleucine	methothionine

Figure 1.3 Homology of critical residues in CIB1 involved in binding. (A) Residues important in the CIB1 hydrophobic binding pocket are listed and the corresponding amino acids of CIB2, CIB3 and CIB4 determined by sequence alignment (Gentry et al. 2005). Amino acids with the same or similar chemical properties are highlighted in yellow. (B) The critical residues involved in the CIB1/αIIb binding as identified by Barry et al. 2002. The high level of homolgous residues implies that CIBs 2-4 could potentially bind αIIb. The residues with different chemical properties allow variations in binding affinity and specificity between CIBs

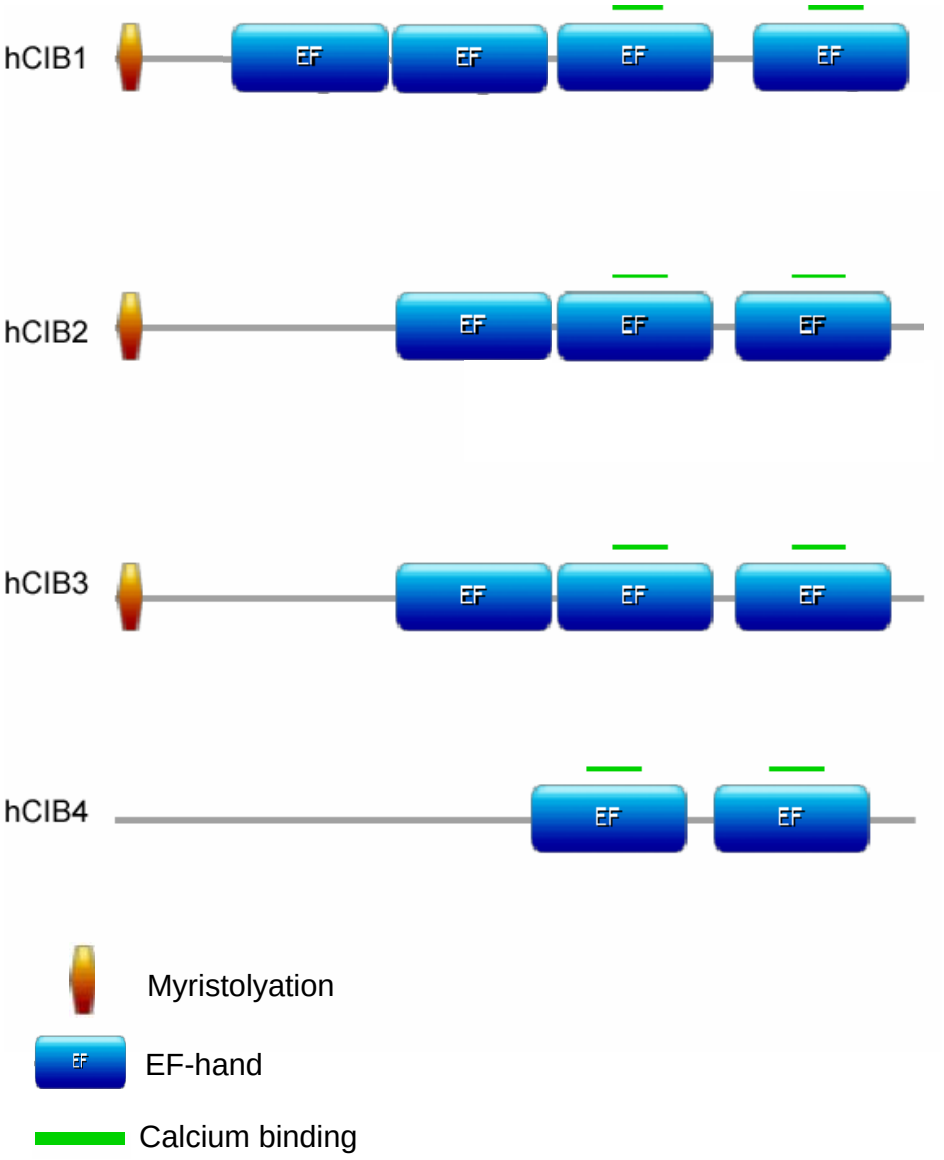

Figure 1.4 Potential EF-hands and myristoylation in CIBs1-4. EF-hands predicted by the Prosite protein motif database (www.expasy.ch/prosite/) and drawn in MyDomains Image Creator (www.expasy.ch/prosite/). All CIB proteins have at least 2 EF-hands that bind Ca^{2+}. An N-terminal myristoylation sequence is predicted for CIBs 1-3. Other EF-hands may exist in CIBs 2-4 but the protein sequence could be too degenerate for the stringency used by the program.

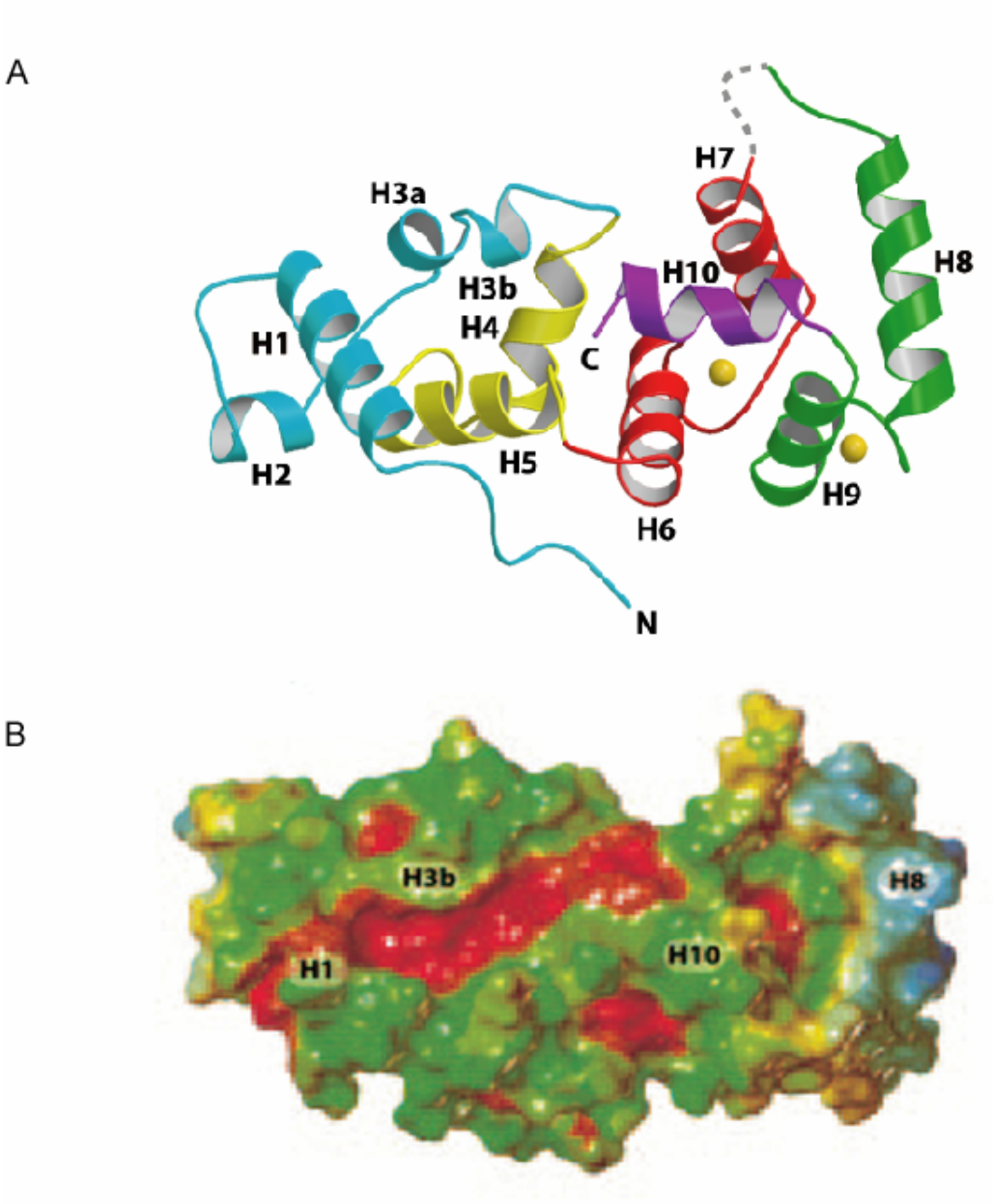

Figure 1.5 Overall EF-hand geometry and hydrophobic pocket of CIB1. (A) Ribbon diagram of the overall fold of CIB1 showing the positions of the high affinity Ca2_ ions (gold spheres) bound to EF-hands 3 and 4. Regions are color-coded: N-terminal region and EF1 are blue, EF2 is yellow, EF3 is red, EF4 is green, and the C-terminal helix is purple. Helices as well as the termini are labeled; a dotted line indicates the disordered loop between helices H7 and H8. (B) Coordinates for CIB1 (PDB code 1XO5) were read by the program SYBYL version 6.9.1(www.tripos.com), hydrogens were added, and MOLCAD molecular surfaces were generated and colored to represent the spectrum of hydrophobic potential (*red*, highly hydrophobic; *blue*, highly hydrophilic). The orientation of CIB1 in this molecular surface representation is very similar to that in (A). Reprinted from Gentry et al 2005, with permission from the Journal of Biological Chemistry 280(9), 8407-8415, 2005.

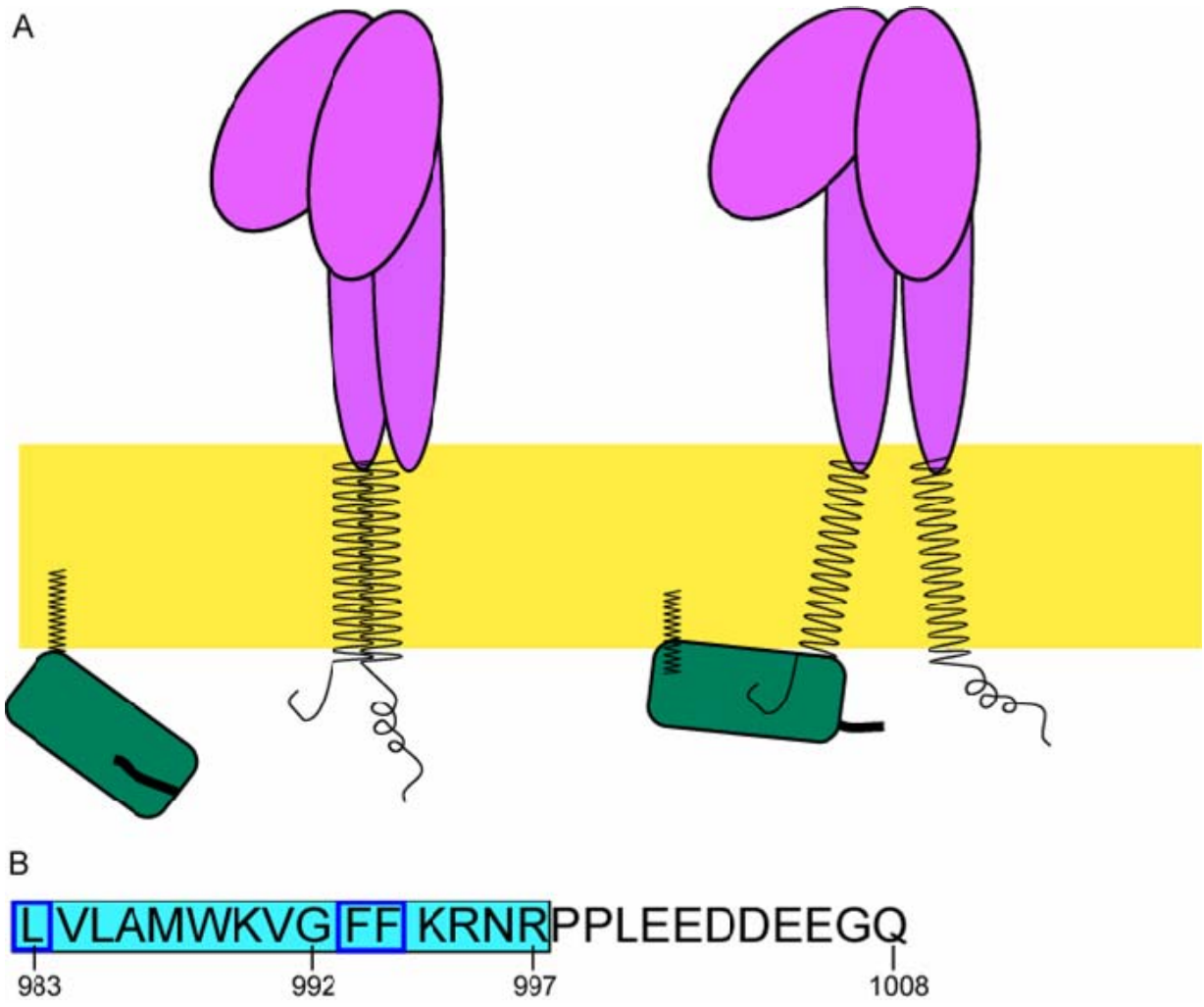

Figure 1.6 Model of CIB1 C-terminal displacement by αIIb. (A) The C-terminus of unbound CIB1 partially blocks the hydrophobic binding pocket. Upon binding to the cytoplasmic tail of the αIIb integrin subunit the C-terminal tail is displaced. This exposes more hydrophobic residues at the protein surface and increases pocket depth. The C-terminal tail likely acts as a screening mechanism to prevent non-specific interactions. Adapted from Yamniuk et al. 2006 (Yamniuk, Ishida, & Vogel, 2006). (B) Sequence in αIIb critical for CIB1 binding proposed by Barry et al. 2002 (Barry et al., 2002).

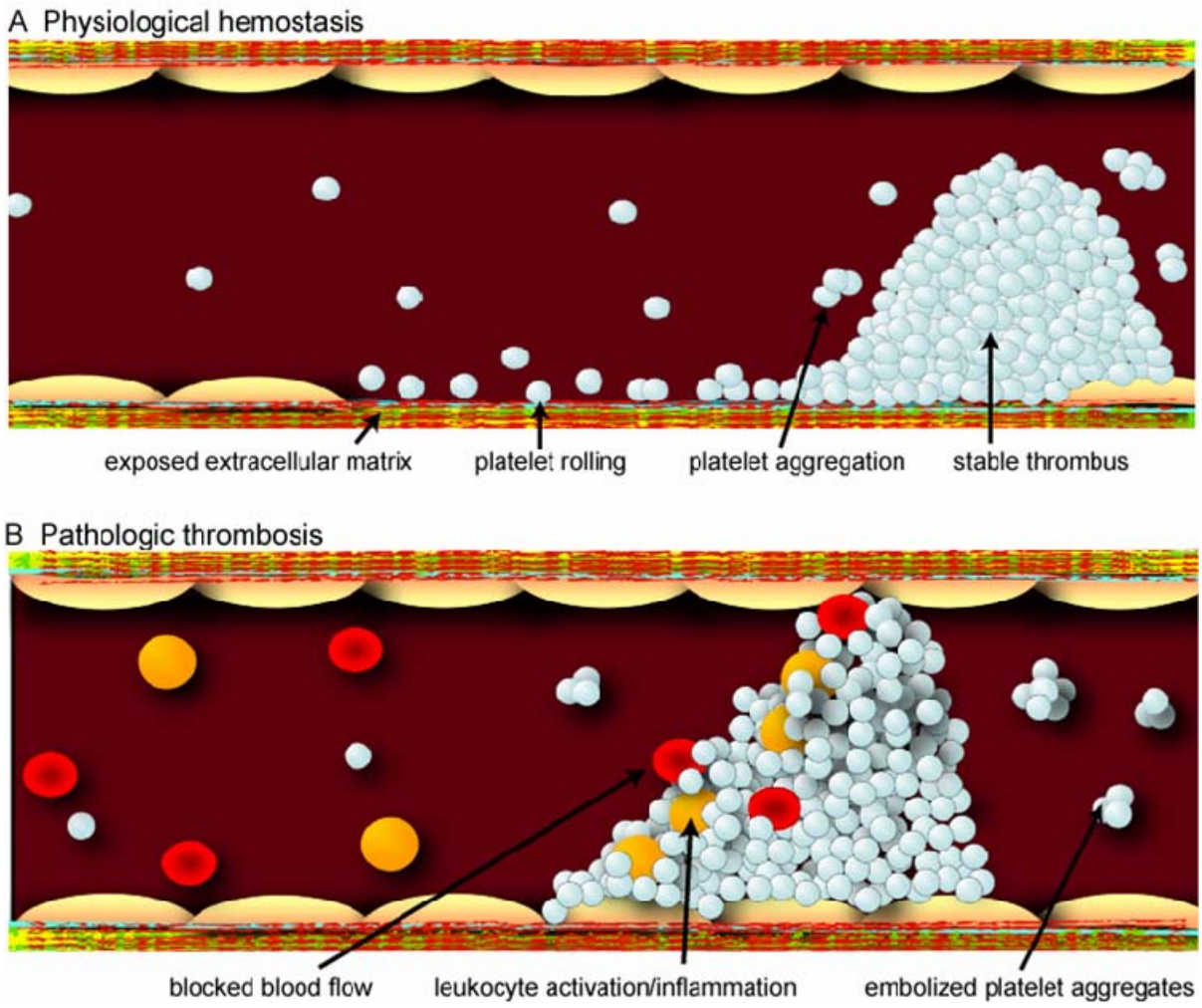

Figure 1.7 Diagram of physiologic hemostasis and pathological thrombus formation. (A) Upon vessel injury, the extracellular matrix proteins are exposed. This creates a highly adhesive surface that attracts platelets circulating in the blood stream. Platelets will form transient attachments initially and roll along the endothelium. This process promotes platelet activation. Activated platelets will release their own signaling molecules and recruit more platelets to the area. Soluble plasma proteins act as a bridge to allow platelet aggregation. Platelets continue to aggregate until a stable thrombus is formed, preventing blood loss and initiating wound repair. (B) If spontaneous platelet activation occurs or a clot cannot retract and dissolve, a thrombus can occlude a blood vessel inducing hypoxia. Thrombi can also embolize and block smaller downstream blood vessels. Cholesterol buildup and atherosclerotic plaque rupture often create a pro-thrombotic and inflammatory environment that promotes thrombus formation and contributes to cardiovascular disease.

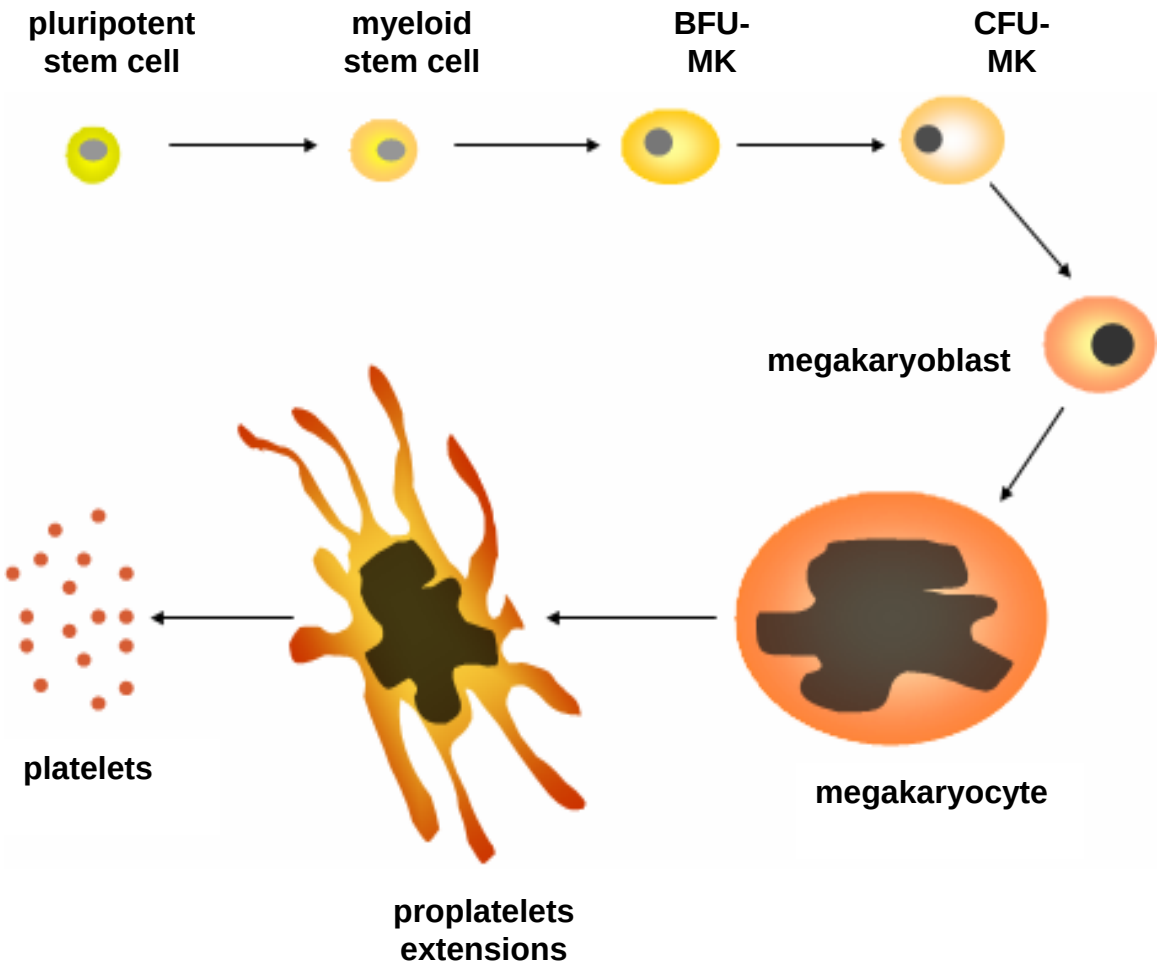

Figure 1.8 Megakaryocyte differentiation and platelet production. (A) Megakaryocyte differentiation and platelet production initiate when hematopoietic stem cells commit to the myeloid progenitor lineage. Differentiation continues through the megakaryocyte burst forming unit (BFU-MK) stage to megakaryocyte colony forming unit (CFU-MK) stage until the fully committed megakaryoblast stage. Afterward, cells stop dividing, become endomitotic and increase their nuclear and cytoplasmic volume and ploidy. Cells continue to grow in size until they become fully mature megakaryocytes. Megakaryocytes extend arm-like projections containing proplatelets, which eventually release to form the budding platelets. Reprinted from Liu et al 2007, with permission from Current Topics in Developmental Biology Vol 80, 2007. (Liu et al., 2007)

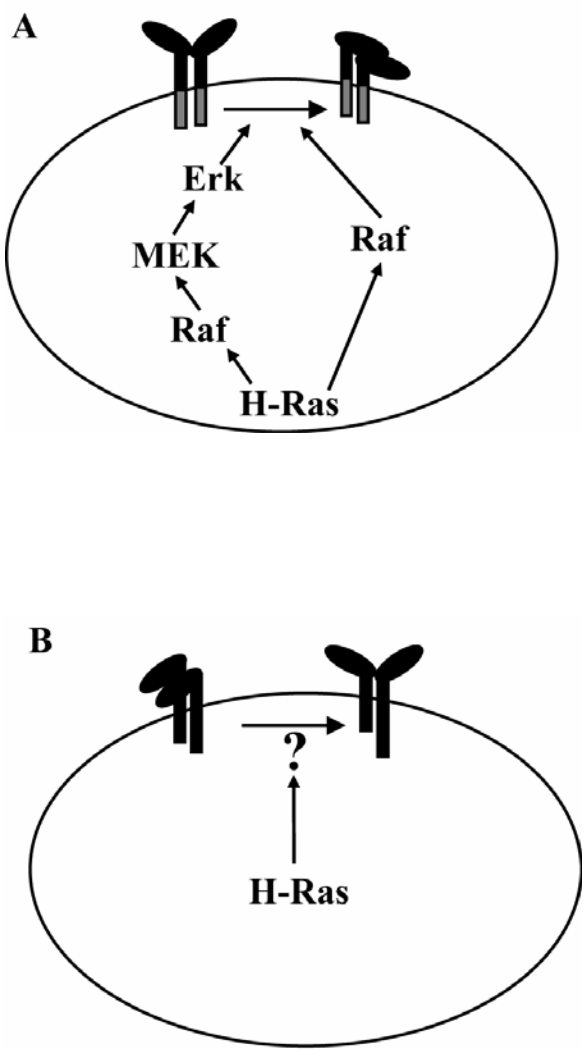

Figure 1.9 Effect of H-Ras on integrin activation. (A) Studies by Ginsberg and colleagues of integrin in CHO cell using a constitutively active H-Ras indicate that H-Ras suppressed integrin activation (Hughes et al., 1997, 2002). The signaling pathways involved in H-Ras -dependent suppression were determined to be both MEK/Erk dependent and independent. (B) The bone marrow-derived megakaryocyte system was used in our lab to determine the role of H-Ras on endogenous integrin activation. Reprinted from Liu et al 2007, with permission from Current Topics in Developmental Biology Vol 80, 2007. (Liu et al., 2007)

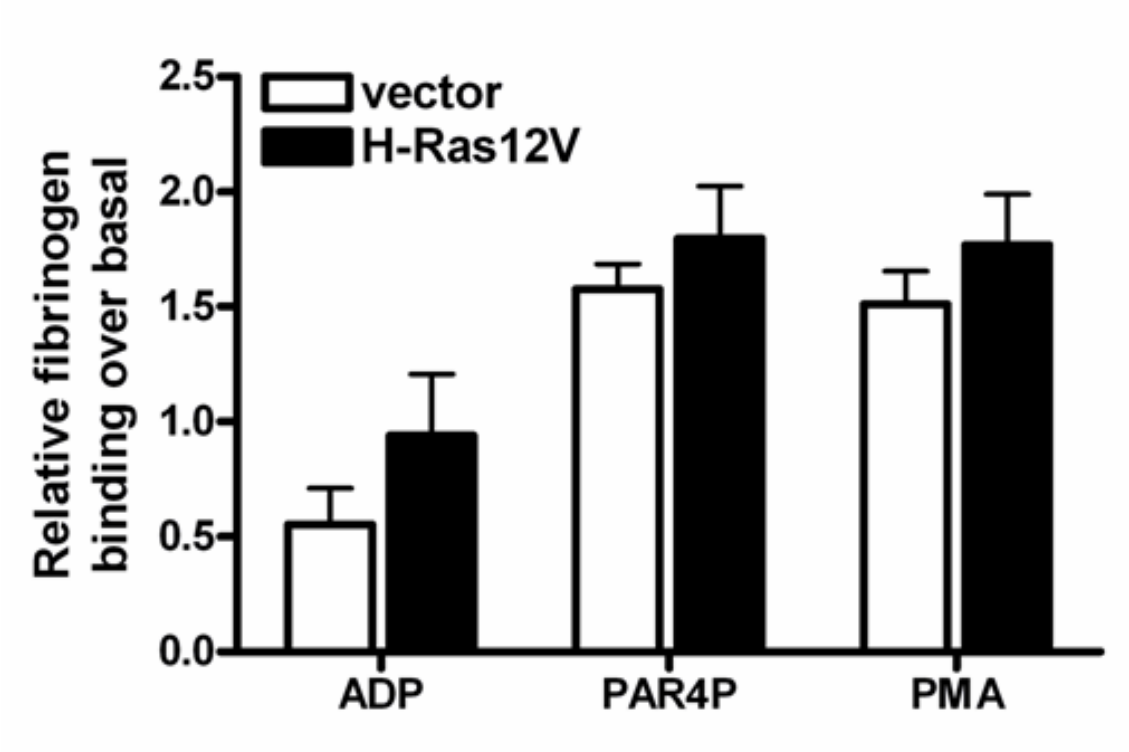

Figure 1.10 Effect of H-Ras on agonist-induced αIIbβ3 activation. Bone marrow-derived megakaryocytes were cultured as described in figure 2 and transduced with retrovirus to overexpress the constitutively active H-Ras mutant, H-Ras12V, or empty vector. Megakaryocytes were stimulated with 100 μM ADP, 10 mM PAR4 peptide or 1 μM PMA in the presence of fluorescently-labeled fibrinogen and analyzed by flow cytometry to assess αIIbβ3 activation as measured by fibrinogen binding. Data are presented as the fold increase of mean fluorescence over basal binding. Basal binding is defined as fibrinogen binding to unstimulated megakaryocytes. The results indicate no apparent inhibition of αIIbβ3 activation by activated H-Ras. Reprinted from Liu et al 2007, with permission from Current Topics in Developmental Biology Vol 80, 2007. (Liu et al., 2007)

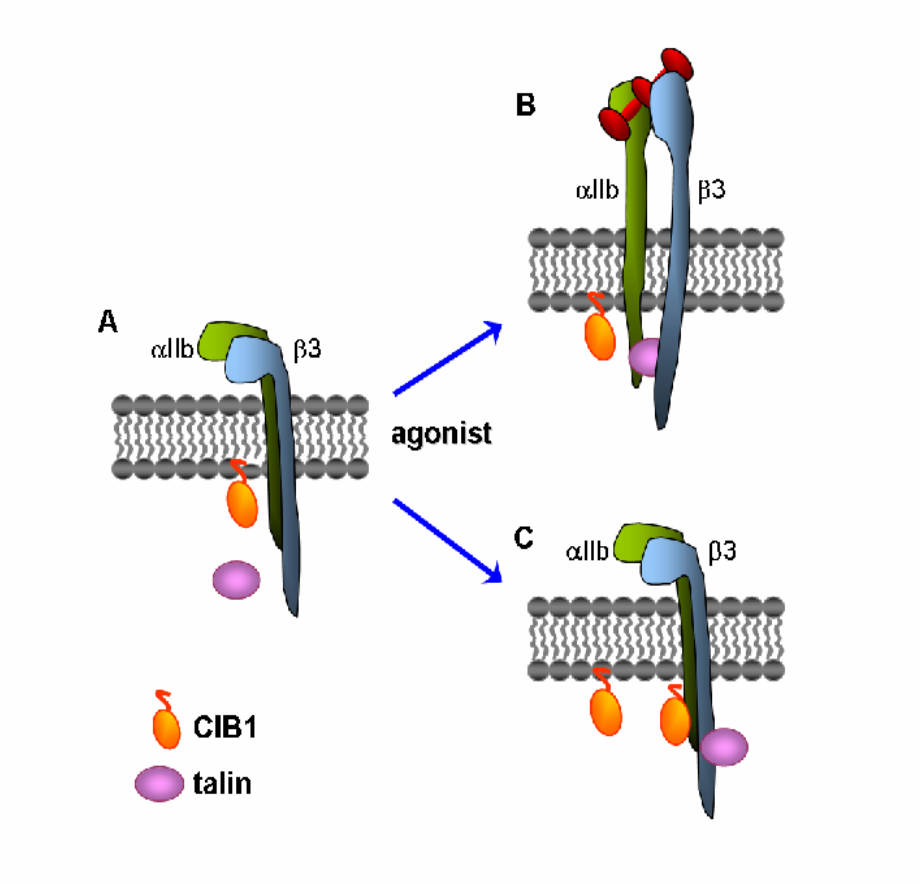

Figure 1.11 Model of CIB1/talin regulation of integrin αIIbβ3 activation. (A) In resting platelets, integrin αIIbβ3 exists in an inactive conformation, unbound to talin. A portion of CIB1 is localized at the platelet plasma membrane by a myristoyl modification of an N-terminal glycine. (B) Upon agonist stimulation, talin binds αIIbβ3 and induces a conformational change within the cytoplasmic face leading to integrin activation and ligand binding. (C) Agonist stimulation may also increase the level of CIB1 localization to the plasma membrane and enhance CIB1 binding to the αIIb cytoplasmic tail. The CIB1–αIIb interaction is thought to interfere with talin-integrin binding thereby attenuating full αIIbβ3 activation and ligand occupancy. Reprinted from Leisner et al 2007, with permission from Current Opinions in Hemotology 14(3), 2007. (Leisner, Yuan, Denofrio, Liu, & Parise, 2007)

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CHAPTER 2

INCREASED PLASMA P-SELECTIN AND vWF IN *CIB1*$^{-/-}$ MICE

PREFACE

This chapter is based on a manuscript soon to be submitted. The manuscript is authored by Jan C DeNofrio, Weiping Yuan, Mauricio Rojas, Mohamed Zayed and Leslie V Parise. Most of work and writing was done by Jan DeNofrio, with the help of Dr. Weiping Yuan for all mouse handling. Dr. Mauricio Rojas did the carotid artery thrombosis model and Dr. Mohamed Zayed provided the primary endothelial cells. Dr. Leslie Parise editing assistance.

2.1 INTRODUCTION

Blood coagulation and thrombus formation are complex processes essential to vessel integrity and the prevention of blood loss. Platelets and endothelial cells release both pro- and anti-thrombogenic and –angiogenic factors upon activation. The hemostatic molecules released into the plasma mediate adhesive interactions among the surrounding environment. Many plasma proteins have multiple functional roles in hemostasis and this physiologic process is carefully balanced by tightly controlled release of these molecules.

von Willebrand factor (vWF) is a hemostatic protein involved in platelet adhesion to the extracellular matrix (ECM), platelet aggregation, thrombus formation, and stabilization of coagulation factor VIII. Mutations in the vWF gene can result in mild to severe bleeding disorders. In fact, von Willebrand disease is the most commonly inherited bleeding disorder (Denis et al., 1998). von Willebrand factor is a large protein with a molecular mass 250 kDa that multimerizes to greater than 20,000 kDa (Ruggeri, 2000). von Willebrand factor has several binding domains that allow binding to collagen, the β3 integrin subunit, glycoprotein (GP) 1bα, coagulation factor VIII and heparin. von Willebrand factor is stored in both α-granules in platelets and Weibel-Palade bodies in endothelial cells (Matsushita et al., 2005; Ruggeri, 2003b; Wagner, Olmsted, & Marder, 1982; Wagner, 1990; Wagner, 1993) and is synthesized only in endothelial cells and platelet precursors, megakaryocytes (van Mourik, Romani, & Voorberg, 2002). Endothelial cells constitutively secrete nascent vWF molecules but store more biologically active ultra-large vWF multimers in Weibel-Palade bodies (Ruggeri, 1999; Handin & Wagner, 1989). This is thought

to be a control mechanism to restrict the highly adhesive and thrombogenic vWF multimers to areas of vessel damage (Ruggeri, 2003a). Ultra-large vWF can be observed transiently in normal plasma after endothelial cell stimulation and degranulation (Ruggeri, Mannucci, Lombardi, Federici, & Zimmerman, 1982); however, these circulating multimers are quickly cleaved by metalloproteinase ADAMTS13 (Fujikawa, Suzuki, McMullen, & Chung, 2001; Gerritsen, Robles, Lammle, & Furlan, 2001). von Willebrand factor stored in platelets is pre-synthesized by megakaryocytes (Jenkins & O'Donnell, 2006). Plasma vWF is proposed to originate mainly from endothelial cells (Bowie et al., 1986) since only endothelial cells can constitutively secrete vWF, although platelets also contribute to the vWF composition.

Another hemostatic protein stored only in platelet α-granules and endothelial cell Weibel-Palade bodies is P-selectin (McEver, 2001). P-selectin is expressed on the surface of these cells upon cellular activation. Once exposed on the membrane surface, P-selectin promotes leukocyte and platelet adhesion and rolling along the endothelium by binding its ligand PSGL-1 (Dole, Bergmeier, Mitchell, Eichenberger, & Wagner, 2005; Frenette et al., 2000; Johnson et al., 1995; Mayadas, Johnson, Rayburn, Hynes, & Wagner, 1993; Frenette & Wagner, 1997). The soluble form of P-selectin circulating in plasma results from proteolytic cleavage of the extracellular domain and is still fully functional and capable of binding PSGL-1 (Dunlop et al., 1992; Mehta, Patel, Laue, Erickson, & McEver, 1997). Soluble P-selectin in human plasma can also be produced from an alternatively spliced variant of P-selectin that lacks the transmembrane domain (Ishiwata et al., 1994). A high level of soluble P-

selectin is often associated with a pro-coagulant state, (Andre, Hartwell, Hrachovinova, Saffaripour, & Wagner, 2000; Ikeda et al., 1994; Blann, Faragher, & McCollum, 1997; Galkina & Ley, 2006) and thrombotic consumptive disorders such as disseminated intravascular coagulation and thrombotic thrombocytopenic purpura (Chong et al., 1994; Katayama et al., 1993).

CIB1 is a widely expressed protein that binds to platelet integrin αIIb subunit of the αIIbβ3 complex (Leisner, Yuan, Denofrio, Liu, & Parise, 2007) and interacts with several other proteins including presenilin 2 (Stabler, Ostrowski, Janicki, & Monteiro, 1999) and polo-like kinases Fnk and Snk (Kauselmann et al., 1999). CIB1 is required for PAK1 adhesion-induced activation and the loss of CIB1 leads to decreased cell migration (Leisner, Liu, Jaffer, Chernoff, & Parise, 2005). Studies of the *Cib1*$^{-/-}$ mouse demonstrated that CIB1 plays an important role in angiogenesis (Zayed et al., 2007). *Cib1*$^{-/-}$ endothelial cells have decreased PAK1 activation, ERK1/2 activation and matrix metalloproteinase 2 expression and the *Cib1*$^{-/-}$ mice have decreased pathologic angiogenesis compared to *Cib1*$^{+/+}$ mice (Zayed et al., 2007). Interestingly, male *Cib1*$^{-/-}$ mice are sterile as the complete loss of CIB1 disrupts the haploid stage of spermatogenesis (Yuan et al., 2006). Since CIB1 inhibits activation of αIIbβ3 in murine megakaryocytes and is required for outside in signaling (Naik & Naik, 2003) we investigated the hemostatic phenotype of *Cib1*$^{-/-}$ mice and found that both vWF and sP-selectin were significantly increased in the *Cib1*$^{-/-}$ plasma, while whole animal studies demonstrated normal thrombus formation and stability in *Cib1*$^{-/-}$ mice.

2.2 METHODS

2.2.1 Generation of *Cib1*$^{-/-}$ mice

Generation of *Cib1*$^{-/-}$ mice was previously described by Yuan et al. (Yuan et al., 2006). Briefly, Genomic *Cib1* DNA (all of exon 4 and most of exon 5) was replaced with the reversed neomycin gene (Yuan et al., 2006). All experiments performed with mice were in accordance with national guidelines and regulations and were approved by the UNC Institutional Animal Care and Use Committee (IACUC). Mice used in the experiments were backcrossed 10 generations onto a C57BL6 background.

2.2.2 Platelet isolation

Mice were euthanized by CO_2. Blood was drawn by cardiac puncture into an Eppendorf tube containing saline and ACD (85mM sodium citrate, 111 mM glucose and 71.4 citric acid)with 0.1 μM PGE_1 (Sigma). To separate blood cells, whole blood was centrifuged for 10 min at 250 x g and platelet rich plasma (PRP) was removed. Remaining blood was washed, centrifuged and PRP collected. PRP was pooled and centrifuged at 400 x g for 15 min. Platelet poor plasma was discarded and the platelet pellet was resuspended in warmed (37°C) CGS buffer (13mM sodium citrate, 30mM glucose and 120mM NaCl) containing 10 U/ml ayprase (Sigma). Resuspended platelets were incubated at 37°C for 20 min before being centrifuged at 500 x g for 15 min. The platelet pellet was resuspended in modified HEPES-Tyrodes buffer (12 mM $NaHCO_3$, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl,

10mM HEPES containing 0.1% BSA, 1mM $CaCl_2$, 1mM $MgCl_2$ at pH 7.4). Platelets were left to rest 1 h at room temperature (RT) before assaying.

2.2.3 Whole blood analysis

Whole blood was drawn into EDTA. Samples were analyzed by the Animal Clinical Chemistry and Gene Expression Laboratory at UNC Chapel Hill with the HESKA CBC Hematology System.

2.2.4 Luminex analysis

Blood was drawn into Eppendorf tubes containing EDTA and centrifuged at 2000 x g for 20 min. Plasma was separated into a new Eppendorf tube and 0.1 μM PGE_1 was added. To remove remaining platelets, the plasma was centrifuged at 1000 x g for 10 min, plasma collected and PGE_1 added. Platelet poor plasma (PPP) was centrifuged for 20 min at 2000 x g to remove contaminating microparticles. Protease inhibitor cocktail III (Calbiochem) was added and PPP was aliquoted and stored at -80°C until use. Plasma samples were analyzed via Luminex by Rules-Based Medicine Inc in Austin, Texas.

2.2.5 Plasma protein ELISA

Plasma was isolated as above. Soluble P-selectin concentration was measured by ELISA (R&D Systems) following manufacture's protocol with plasma diluted 1:50 in assay buffer. For the vWF ELISA, 10 μg/well of anti-human vWF antibody (DAKO) was incubated at 4°C overnight in Immunex 96-well plates

(DYNEX). The wells were washed three times with PBS. To block non-specific binding, 3% BSA in PBS was added and incubated for 1.5 h at RT. Wells were washed three times with PBS and plasma diluted 1:50 in PBS was added. Samples were incubated for 2 h at RT, washed three times and horseradish peroxidase-conjugated rabbit anti-human vWF antibody (DAKO) was added at a 1:1000 dilution. Antibody was incubated for 1 h at RT before the final three washes. Chromogenic SigmaFAST OPD tablets (Sigma) were used to detect protein binding. After 15 min the color reaction was stopped and O.D. values were read by a Medical Devices SpectraMax Plus plate reader at 490 nm. Graphed O.D. values were normalized per experiment by averaging the *Cib1*$^{+/+}$ values.

2.2.6 Flow cytometry

P-selectin expressed on the platelet surface was measured by flow cytometry using a FITC-labeled P-selectin antibody (EMFRET, Germany). Whole blood samples were prepared as described by manufacture's instructions. P-selectin expression was induced by activation with 100 μM ADP or 0.25 mM PAR4 peptide (thrombin receptor activating peptide, AYPGKF, synthesized at the University of North Carolina Protein Synthesis Core). After a 15 min incubation with the antibody, samples were diluted with Tyrodes-HEPES and analyzed on a BD FACSCanto flow cytometer (BD Biosciences) at a low flow rate. Mean fluorescence values were normalized by agonist stimulated divided by unstimulated.

2.2.7 Western blot analysis

Washed platelets or endothelial cells were lysed in modified CHAPS buffer (20 mM HEPES, pH 7.4, 0.15 M NaCl, 10 mM CHAPS, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM of $CaCl_2$ and $MgCl_2$ and Protease Inhibitors Cocktail Set III on ice for 30 min. Lysates were collected after a 10 min centrifugation. Protein concentration was quantified by the BCA protein assay (Pierce). Protein samples were separated by SDS-PAGE, transferred to a PVDF membrane and subjected to Western blotting. Antibodies against mouse P-selectin and GAPDH were from Santa Cruz Biotechnology. Protein expression was quantified via densitometry with software Quantity One (BioRad Fluor-S Multimager) and adjusted using GAPDH as loading control.

2.2.8 Endothelial cell culture

Endothelial cells were isolated and cultured as described (Zayed et al., 2007). Cell lysates were prepared as described above.

2.2.9 Carotid artery thrombosis

Adult mice (24-34 g) were anesthetized with isoflurane and maintained under inhaled anesthesia at a temperature of 36-37.5°C. The carotid artery was exposed and rinsed with saline. To measure baseline flow rate, a Doppler flow probe (#0.5VB307, Transonic Systems Inc) connected to a flow meter (T106, Transonic Systems Inc) was placed on the blood vessel and both were covered with a lubricating jelly. After baseline was determined, the artery was rinsed with saline and a 1 x 2 mm piece of Whatman paper saturated with 20% ferric chloride was

placed on the vessel. After 2 min, the filter paper was removed from the artery and replaced with lubricating jelly. Blood flow was measured until the vessel occluded.

2.2.10 Bleeding time assay

Mice were anesthetized with isoflurane and given an I.P. injection of 10 μl/g mouse of ketamine hydrochloride (75μg/kg). Mouse tails were cut at 4mm from the tip and placed into PBS warmed to 37°C. Time was recorded until the stream of blood fully stopped. Mouse tails were cauterized after 15 min if bleeding did not stop.

2.2.11 Statistical analysis

All values are presented as the mean plus or minus standard error of the mean. A *t*-test was used to evaluate statistical significance. P values less than 0.05 were considered statistically significant.

2.3 RESULTS

2.3.1 Plasma protein analysis

Plasma proteins come from a variety of cellular sources. The concentration of proteins in plasma can be affected by changes in gene expression and protein synthesis or changes in exocytosis of storage granules. We asked whether the loss of CIB1 affects granule release, by altering platelet or endothelial cell activation and subsequently altering plasma protein levels. We used Luminex technology to

perform a general plasma protein analysis (Table 1). Of the initial 67 proteins screened, vWF concentration displayed the greatest difference between *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ plasma, with a 1.5 fold increase of vWF in *Cib1*$^{-/-}$ plasma. We confirmed the Luminex results by a vWF ELISA (Fig. 1A).

von Willebrand factor is released into plasma from intercellular granules of endothelial cells and platelets. We next determined if soluble P-selectin, a protein not in our initial screen, was also increased in *Cib1*$^{-/-}$ plasma, as they share cellular locations. We measured soluble P-selectin (sP-selectin) concentration by an ELISA. We found that *Cib1*$^{-/-}$ mice also have an increased concentration of sP-selectin in their plasma, like vWF, compared to *Cib1*$^{+/+}$ mice (Fig. 1B).

2.3.2 Whole blood analysis

Since there was an increase in plasma P-selectin and vWF in *Cib1*$^{-/-}$ mice, we examined hemostasis in the *Cib1*$^{-/-}$ mice on a macroscopic level. A complete blood cell count was preformed on whole blood samples and demonstrated there were no significant differences between the two genotypes (Table 2). Therefore changes in plasma protein levels were not due to variations in blood cell number or size.

We next used flow cytometry to investigate the activity of platelets in whole blood. By measuring platelet integrin αIIbβ3 activation with an activation specific antibody, we determined that basal activation (i.e. the activation of resting platelets) in *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ platelets are similar (Fig. 2A). Furthermore, platelet activation by agonists ADP or PAR4 induced integrin αIIbβ3 activation to the same degree in

both *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ platelets. Therefore, platelets circulating in the blood of the *Cib1*$^{-/-}$ mice are not more activated than those in the *Cib1*$^{+/+}$ mice.

2.3.3 *In vivo* experiments

We then employed whole animal models to determine the response of *Cib1*$^{-/-}$ mice to blood vessel injury, since altered plasma proteins could affect this response. A bleeding time assay measures the time necessary for bleeding to stop after the removal of a small portion of the mouse's tail (Subramaniam et al., 1996). We found that the average bleeding time for *Cib1*$^{-/-}$ mice tended to be prolonged to that of *Cib1*$^{+/+}$ *mice*; however, the time difference was not statistically significant (Fig. 2B). The ferric chloride injury model is frequently used for assessing *in vivo* responses to injury to the blood vessel endothelium (Denis & Wagner, 2007). Ferric chloride causes tissue damage by iron- mediated chemical oxidation, resulting in separation of endothelial cell junctions and exposure of type I collagen (Furie & Furie, 2005; Kurz, Main, & Sandusky, 1990). To determine the role of CIB1 in thrombus formation upon vascular injury, ferric chloride was placed on the carotid artery to expose the underlying ECM (Kurz et al., 1990). Time to full vessel occlusion by a stable thrombus was similar between the *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ mice (Fig. 2C). Thus *Cib1*$^{-/-}$ mice exhibit no differences in thrombus formation or stability leading to vessel occlusion and blood clotting *in vivo* as measured by these assays.

2.3.4 Protein expression of vWF and P-selectin in platelets and endothelial cells

To identify the cellular source of the increased plasma vWF and sP-selectin, we analyzed lysates of platelets and cultured endothelial cells isolated from *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ mice. von Willebrand factor expression in platelet lysate was determined by ELISA and Western blotting. There was no distinguishable difference in vWF expression between platelets from *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ mice (Fig. 3). An ELISA (Fig. 4A) and Western blotting (Fig. 4B) for P-selectin expression were also preformed with platelet lysates from the *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ mice. Again there was no detectable difference in P-selectin level in the platelets from the two genotypes. We also analyzed endothelial cell lysates for P-selectin expression by Western blotting and did not see differences in protein expression between the *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ samples (Fig. 4C).

Platelet activation leads to exposure of P-selectin on the platelet surface; therefore we used flow cytometry to examine any potential changes in P-selectin exposure. We found similar surface expression levels of P-selectin on resting platelets from *Cib1*$^{+/+}$ mice and the *Cib1*$^{-/-}$ mice, and on platelets activated by 0.25 mM PAR4 or 100 μM ADP (Fig. 4D). P-selectin surface expression and agonist-induced α-granule release are not affected by the loss of CIB1. These results imply that it is unlikely that *Cib1*$^{-/-}$ platelets have a higher concentration of P-selectin stored in their α-granules or that the *Cib1*$^{-/-}$ platelets release the constituents of their α-granules more readily than *Cib1*$^{+/+}$ platelets.

2.4 DISCUSSION

In this study we investigated hemostasis in the *Cib1*$^{-/-}$ mouse. Plasma analysis showed significant differences between *Cib1*$^{-/-}$ and *Cib1*$^{+/+}$ mice by Luminex analysis. Most interesting was the increase of vWF in the plasma of the *Cib1*$^{-/-}$ mice. To validate the results of our Luminex analysis, we verified that vWF concentration is increased in the plasma of *Cib1*$^{-/-}$ by our ELISA. VWF is stored in platelet α-granules and endothelial cell Weibel-Palade bodies. Since P-selectin is also stored in same subcellular locations, we asked if sP-selectin concentration in the plasma was also increased, as it was not part of our initial Luminex screen. Using an ELISA, we determined that *Cib1*$^{-/-}$ mice have an increased concentration of sP-selectin in their plasma than *Cib1*$^{+/+}$ mice.

We then asked if the increased plasma concentration of these two pro-coagulant proteins (Andre et al., 2000; Hartwell et al., 1998; Ruggeri, 2003b) caused increased thrombotic tendencies in the *Cib1*$^{-/-}$ mouse. However, platelets in whole blood from *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ mice displayed comparable basal αIIbβ3 binding and responded to agonists PAR4 and ADP stimulation similarly, when measured by an activation specific antibody binding to integrin αIIbβ3. This implies that *Cib1*$^{-/-}$ platelets in whole blood are not pre-activated by molecules found in their blood plasma. We then used whole animal models to obtain a global picture of *in vivo* hemostasis in the *Cib1*$^{+/+}$and *Cib1*$^{-/-}$ mice. Both the bleeding time assay and the carotid artery occlusion model showed no statistically significant difference between these two genotypes. Therefore, we can conclude that thrombus formation and stability are normal in *Cib1*$^{-/-}$ mice. Interestingly, bleeding times for both the vWF$^{-/-}$ and P-selectin$^{-/-}$ mouse are prolonged (Denis et al., 1998; Subramaniam et al.,

1996). Mice deficient in vWF also have a prolonged time until full blood vessel occlusion in the ferric chloride injury model (Denis et al., 1998), while in a similar thrombosis model using a laser to initiate vessel injury, P-selectin$^{-/-}$ mice have decreased thrombus stability (Falati et al., 2004). Since deficiency in vWF or P-selectin leads to defects in thrombus formation and stability, it is surprising that mice that have increased plasma concentrations of both proteins, have no thrombotic effect as measured by these assays. This implies that there are critical thresholds for both proteins that above or below will result in hemostatic defects.

To identify the source of sP-selectin and vWF in plasma, we analyzed platelets and endothelial cells from the *Cib1*$^{-/-}$ mice. Platelet lysates from *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ mice displayed no change in vWF or P-selectin concentration by ELISA or Western blot. Surface expression of P-selectin on platelets from *Cib1*$^{-/-}$ and *Cib1*$^{+/+}$ mice was similar at a resting state and upon agonist stimulation. This implies that the *Cib1*$^{-/-}$ platelets do not have increased P-selectin stored or released from their α-granules.

The primary endothelial cells used in this report were isolated and cultured from *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ mice. Therefore, there is the possibility that these cells may have different protein expression when removed from the mouse and grown in culture conditions. For this reason, we cannot exclude the possibility that endothelial cells *in vivo* are the source of the increase in plasma sP-selectin and vWF. Previous studies demonstrate that the loss of CIB1 affects cultured endothelial cell migration, proliferation and tubule formation (Zayed et al., 2007). These studies also demonstrated that CIB1 is involved in pathological angiogenesis *in vivo* (Zayed et

al., 2007). A defect in endothelial cell function upon the loss of CIB1 supports the hypothesis that these cells may also have difficulty regulating P-selectin and vWF levels. Furthermore, *Cib1*$^{-/-}$ platelets do not display any significant differences from *Cib1*$^{+/+}$ platelets (DeNofrio et al. *submitted*).

Many studies have demonstrated a strong functional interaction between P-selectin and vWF (Denis, Andre, Saffaripour, & Wagner, 2001a; Dole et al., 2005) and both of these proteins are involved in atherosclerosis (Denis et al., 2001a; Dole et al., 2005; Ley, 2003; Mendolicchio & Ruggeri, 2005; Andre et al., 2000; Guray et al., 2004). Elevated sP-selectin and vWF are often considered predictors of cardiovascular events (Galkina et al., 2006; Montalescot et al., 1998; Varughese et al., 2007; Blann et al., 1997; Blann & Lip, 1997). Interestingly, other procogaulant proteins involved in cardiovascular events such as C-reactive protein (Danenberg et al., 2007) and tissue factor (Mackman, 2004) were found to have similar levels in *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ plasma (Table1). The administration of interleukin-11 has been shown to increase plasma vWF levels in human clinical studies (Tepler et al., 1996) and both *vWF*$^{+/+}$ and *vWF*$^{+/-}$ mice (Denis et al., 2001b). Level of endogenous interleukin-11 is unchanged in the plasma from *Cib1*$^{-/-}$ mice. Though the mechanism promoting the increase of plasma P-selectin and vWF remains unclear, the *Cib1*$^{-/-}$ mouse can potentially be used to further our understanding of the physiologic roles of plasma proteins that are involved in hemostasis.

Plasma protein concentration higher in *Cib1*-/- mice (fold value over *Cib1*+/+)		**Plasma protein concentration lower in *Cib1*-/- mice** (fold value over *Cib1*+/+)	
GST-1 Yb	1.26	β-2 microglobulin	.87
SGOT	1.14	MDC	.81
TIMP-1	1.34	Eotaxin	.79
von Willebrand factor	1.48	TPO	.93
		Myoglobin	.40

Plasma protein concentrations with no significant difference			
Apolipoprotein	Growth Hormone	IL-12p70	MIP-2
Calbindin	GST-α	IL-17	MIP-3β
CD40	Haptoglobin	Insulin	MMP-9
CD40L	IFN-g	IP-40	NGAL
Clusterin	IgA	KC/GROα	Osteopontin
C-Reactive protein	IL-1α	Leptin	OSM
Cystatin C	IL-1β	LIF	RANTES
Endothelin-1	IL-2	Lymphotactin	SCF
EGF	IL-3	MCP-1	Serum Amyloid P
FactorVII	IL-4	MCP-3	Tissue Factor
FGF-basic	IL-5	MCP-5	TNF-α
FGF-9	IL-6	M-CSF	VCAM-1
Fibrinogen	IL-7	MIP-1α	VEGF
GCP-2	IL-10	MIP-1β	
GM-CSF	IL-11	MIP-1γ	

Table 2.1 Luminex analysis of plasma from *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ mice. Values in upper boxes are the fold difference of *Cib1*$^{-/-}$ protein concentration over the *Cib1*$^{+/+}$ protein concentration. Lower panels represent proteins that were screened demonstrated no significant difference in concentration between plasma from *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ mice.

Cell/protein	WT	KO
WBC (10^3/ul)	8.63+/-0.83	8.77+/-0.61
LYMF(10^3/ul)	5.85+/-0.60	6.77+/-0.49
GRAN(10^3/ul)	1.875+/-0.42	1.16+/-0.12
MONO(10^3/ul)	0.99+/-0.10	0.83+/-0.05
PLT (10^3/ul)	513.41+/-27.4	475.5+/-13.8
LYMF(%)	68.35+/-5.18	77.32+/-1.23
GRAN(%)	21.67+/-4.32	13.79+/-0.94
MONO(%)	10.87+/-0.93	8.88+/-0.36
HCT(%)	41.13+/-0.94	40.1+/-0.64
MCV (fl)	44.50+/-0.53	42.79+/-0.23
MPV (fl)	5.91+/-0.04	5.96+/-0.04
HGB (pg)	14.78+/-0.34	14.74+/-0.20
MCH (g/dl)	15.95+/-0.14	15.73+/-0.07
MCHC(%)	35.93+/-0.31	36.81+/-0.23
RDW(%)	20.44+/-0.34	18.31+/-0.38

Values are given as mean +/- SEM.

Table 2.2 Comprehensive blood cell analysis. The concentration of each blood cell type was determined along with other important parameters. The blood cell analysis did not display any significant differences between the *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ whole blood.

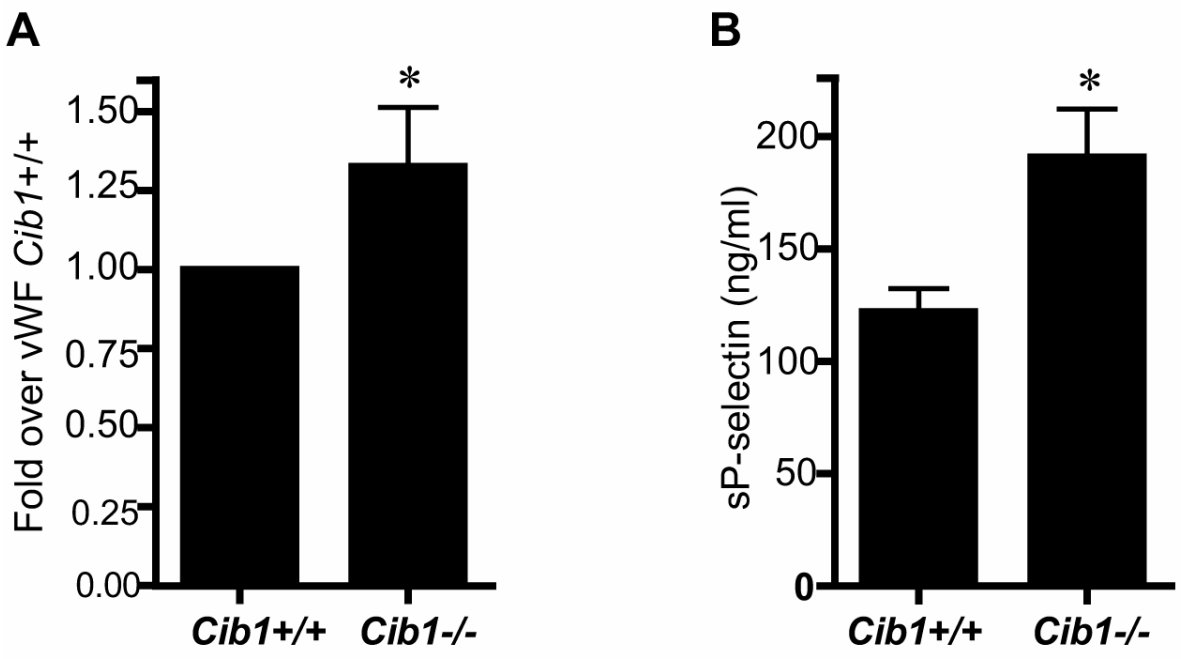

Figure 2.1 Plasma from *Cib1*$^{-/-}$ mice has increased levels of von Willebrand Factor and soluble P-selectin compared to *Cib1*$^{+/+}$ plasma. (A) Plasma samples analyzed by ELISA demonstrate that plasma from *Cib1*$^{-/-}$ has higher levels of von Willebrand factor than plasma from *Cib1*$^{+/+}$ as determined by ELISA. Data was normalized by setting the mean von Willebrand factor O.D. from *Cib1*$^{+/+}$ plasma to 1 (N ≥ 4). (B) *Cib1*$^{-/-}$ mice have more soluble P-selectin circulating in their plasma than *Cib1*$^{+/+}$ mice as measured by ELISA. Concentrations were determined from known mouse soluble P-selectin standards (N = 17).

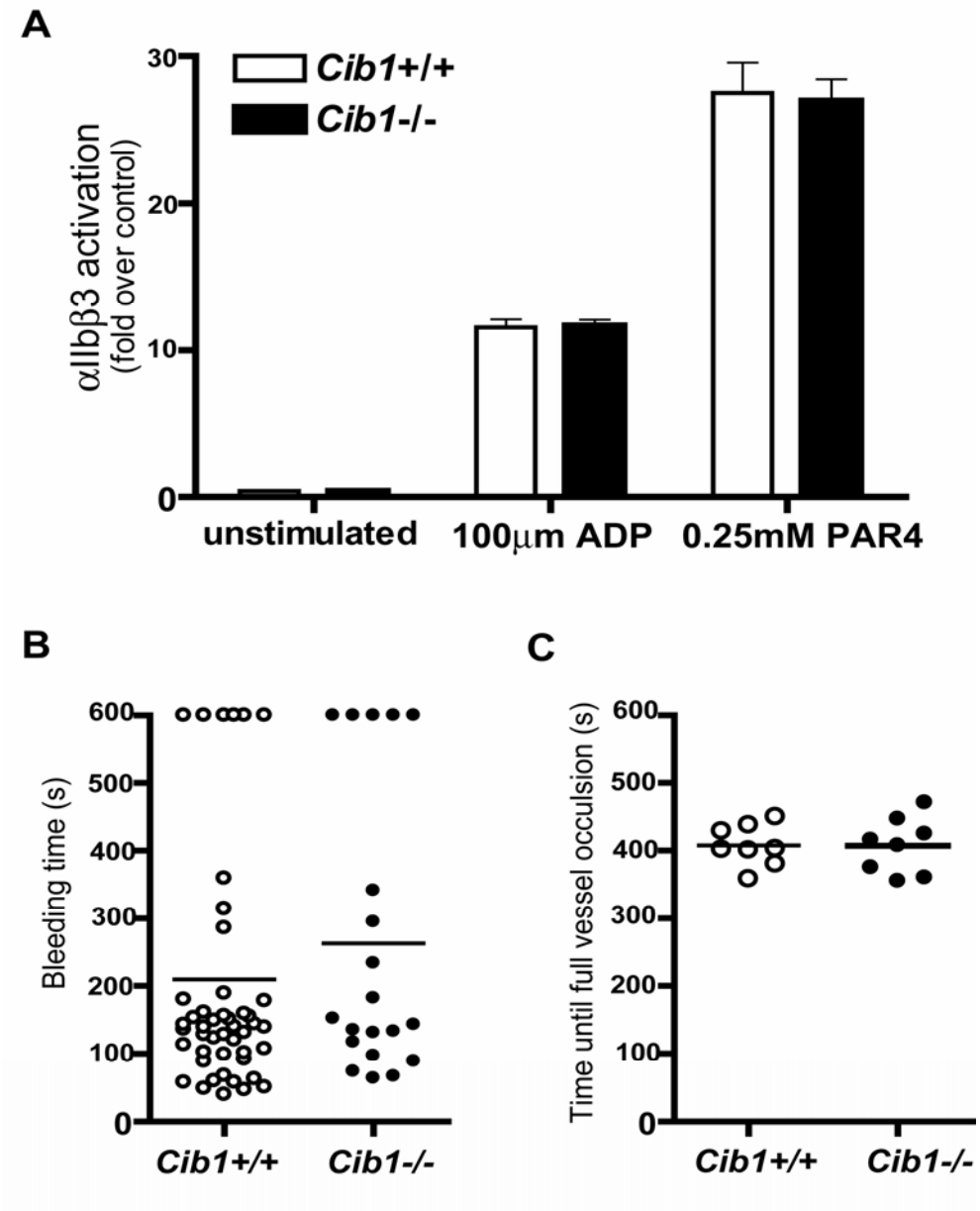

Figure 2.2 Platelet activation and thrombus formation are normal in *Cib1*$^{-/-}$ mice. (A) Platelet activation, as measured by integrin αIIbβ3 basal binding and agonist-induced activation, demonstrates that platelets from *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ mice are activated similarly. Integrin activation was determined by flow cytometry to detect binding of an αIIbβ3 activation-specific antibody (N = 6). (B) Bleeding time assay was used to measure thrombus formation and stability in *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ by cutting the distal 4 mm portion of the tail and recording clotting time. *Cib1*$^{-/-}$ mice had mildly prolonged bleeding time but the difference was not statistically significant from *Cib1*$^{+/+}$ (N ≥ 20). (C) A carotid artery thrombosis model was also used to detect differences in thrombus formation and stability. Ferric chloride (20%) saturated paper was placed on the carotid artery to induce injury. Time necessary for full vessel occlusion was recorded and graphed (N = 8).

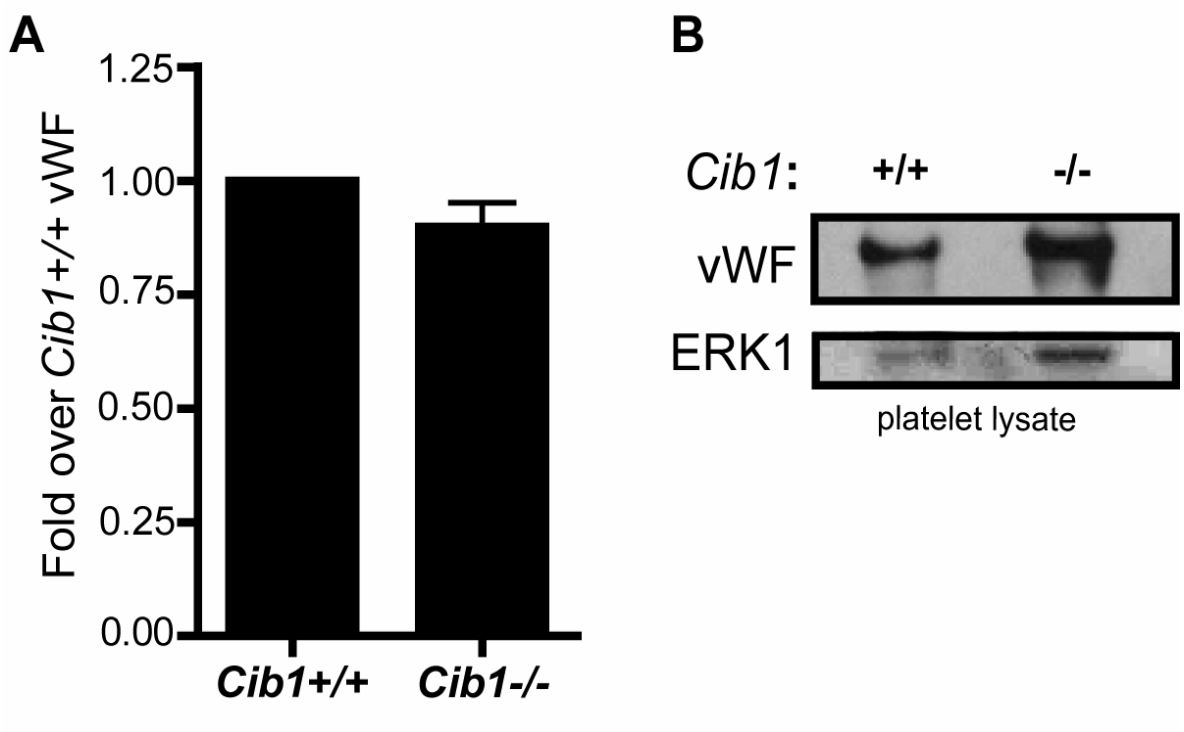

Figure 2.3 Platelets from *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ mice have similar levels of von Willebrand factor. Platelet lysates analyzed by a von Willebrand factor ELISA (A) or Western blotting (B) demonstrate *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ platelets have similar levels of intracellular von Willebrand Factor. Y-axis in (A) shows *Cib1*$^{-/-}$ OD divided the mean *Cib1*$^{+/+}$ mean O.D.. ERK1 was used as a loading control for the Western blots (N = 2 for ELISA experiments).

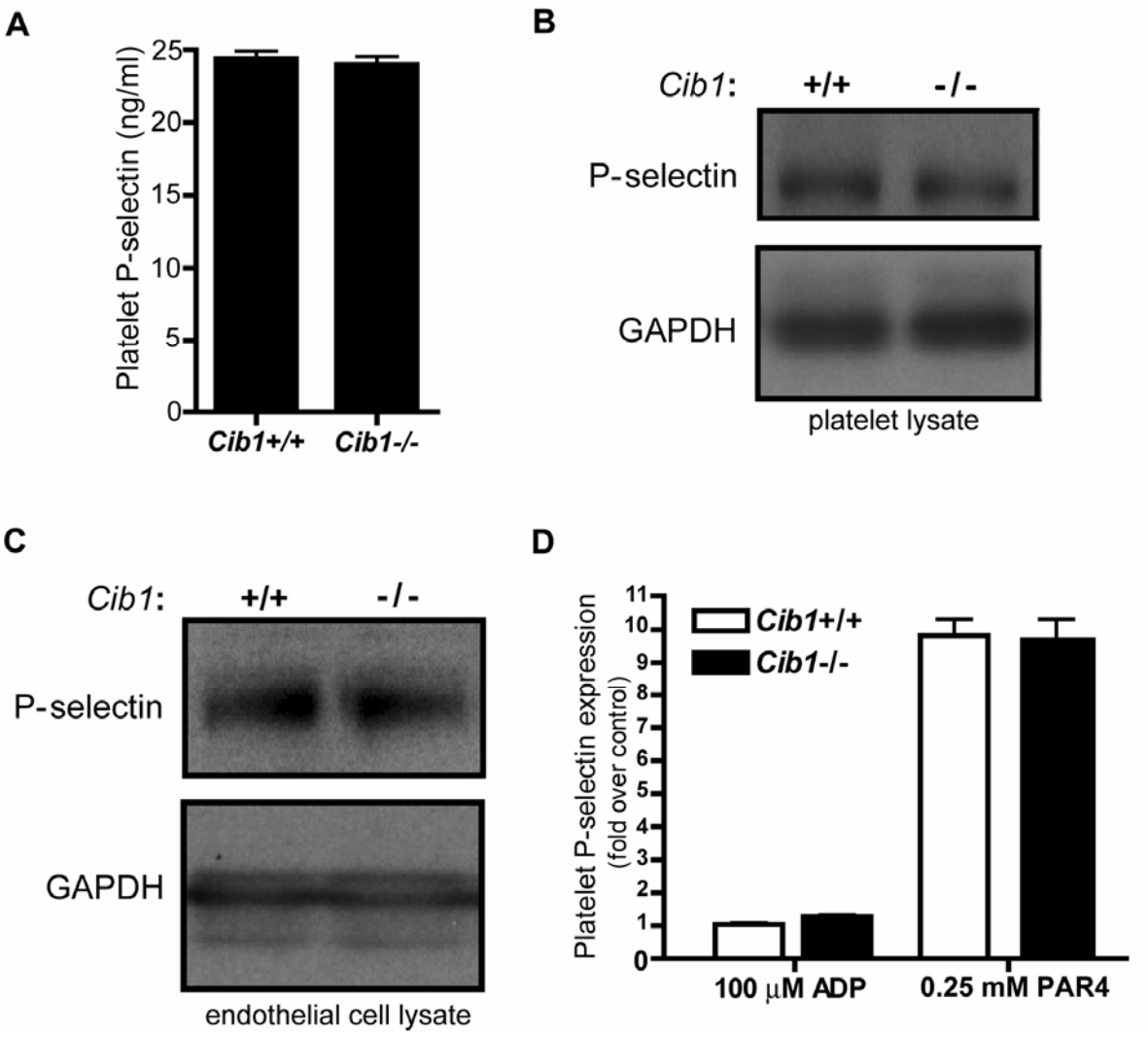

Figure 2.4 P-selectin levels are similar in *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ platelets and endothelial cells. Soluble P-selectin ELISA (A) and Western blotting (B) of platelet lysates demonstrated P-selectin expression is comparable in platelet lysates from *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ mice (N ≥ 2). Western blots of endothelial cell lysates also showed similar levels of P-selectin expression between the two genotypes. GAPDH was used as a loading control for both (B) and (C). (D) Platelet P-selectin surface expression is not affected by the loss of CIB1 as measured by flow cytometry. P-selectin was expressed on the platelet surface at similar levels after agonist stimulation with 100 μM ADP or 0.25 mM PAR4 peptide) (N ≥ 3).

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CHAPTER 3

CHARACTERIZATION OF CIB1 KNOCKOUT PLATELETS: POTENTIAL COMPENSATION BY CIB FAMILY MEMBERS

PREFACE

The work in this chapter has been submitted to the Journal of Biological Chemistry. This manuscript was authored by Jan DeNofrio, Weiping Yuan, Brenda Temple, Holly Gentry and Leslie Parise. Most of the experiments and writing were done by Jan DeNofrio and Dr. Weiping Yuan, who share co-first authorship. Dr. Brenda Temple did the CIB homology prediction model and Dr. Holly Gentry helped purify some of the recombinant proteins.

3.1 INTRODUCTION

Platelets respond to blood vessel injury to prevent further blood loss and restore vascular integrity. They become activated at the site of vascular injury by newly exposed extracellular matrix proteins (ECM) and secreted soluble agonists. Platelet activation promotes soluble and matrix adhesive protein binding to activated surface receptors. The interaction of platelets with soluble proteins such as fibrinogen initiates platelet aggregation, whereas platelet interaction with ECM proteins such as collagen allows platelet adhesion.

Integrins are heterodimeric adhesion receptors that undergo conformational changes upon agonist stimulation, which results in increased ligand affinity. This process is termed inside-out signaling (for review see Parise 1999) (Parise, 1999). The most abundant integrin on the platelet surface is αIIbβ3. Active integrin αIIbβ3 initiates and propagates the formation of platelet aggregates by binding to fibrinogen. Although fibrinogen is the primary ligand for αIIbβ3, the activated integrin also binds fibronectin, von Willebrand factor and vitronectin to support platelet adhesion to the extracellular matrix (Kieffer & Phillips, 1990; Phillips, Charo, & Scarborough, 1991). Since uncontrolled activation of integrin αIIbβ3 can cause pathologic thrombus formation, precise regulation of the integrin is critical for hemostasis (Andrews & Berndt, 2004).

CIB1 (CIB, calmyrin, and KIP1) was identified in a yeast-two hybridization screen to identify binding partners for the integrin αIIb cytoplasmic tail (Naik, Patel, & Parise, 1997). CIB1 is a widely expressed protein that contains two calcium binding and two non-calcium binding EF hands (Gentry et al., 2005; Yamniuk & Vogel,

2005). CIB1 interacts with a number of serine/threonine kinases such as DNA-dependent protein kinase (Wu & Lieber, 1997), polo-like kinases Fnk and Snk (Kauselmann et al., 1999), and PAK1 (Leisner, Liu, Jaffer, Chernoff, & Parise, 2005), as well as presenilin 2 (Stabler, Ostrowski, Janicki, & Monteiro, 1999), FAK(Naik & Naik, 2003a) and the InsP3 receptor (White, Yang, Monteiro, & Foskett, 2006). Recent *in vivo* studies demonstrate that CIB1 also plays a role in pathological forms of angiogenesis (Zayed et al., 2007) and is essential for spermatogenesis (Yuan et al., 2006a).

Previously, the function of CIB1 relative to αIIbβ3 was explored by acutely overexpressing and knocking down the protein in murine megakaryocytes. These studies implicated CIB1 as a negative regulator of agonist-induced αIIbβ3 activation (Yuan et al., 2006b). In separate studies where CIB1 function was also rapidly blocked by use of a CIB1 antibody, it was found that CIB1 is necessary for proper spreading on immobilized fibrinogen (Naik & Naik, 2003b). Taken together these studies imply that acute alterations in CIB1 protein, misregulates αIIbβ3 activation.

To fully characterize the role of CIB1 in αIIbβ3 activation, we asked if the chronic loss of CIB1, via study of a knockout mouse, would result the same phenotype as acutely altered CIB1. While we hypothesized that loss of CIB1 would result in increased agonist-induced activation of integrin αIIbβ3, and hyper-activatable platelets, the *Cib1*$^{-/-}$ mice (Yuan et al., 2006a) did not display any overt defect in platelet function. Taken together, these results suggest that either CIB1 does not regulate the platelet functions tested or that developmental compensation has occurred. Interestingly, mRNA expression for the CIB1 homolog CIB3 is

increased in cultured megakaryocytes isolated from the *Cib1*$^{-/-}$ mice. These proteins may have a redundant or overlapping function in αIIbβ3 regulation and may compensate for the lack of CIB1. To test this, we produced recombinant CIB1, -2 and -3 and compared their ability to bind to the αIIb cytoplasmic tail.

3.2 METHODS

3.2.1 Mice

Generation of *Cib1*$^{-/-}$ mice was previously described by Yuan et al. (Yuan et al., 2006a). All mouse experiments were performed in accordance with national guidelines and regulations and were approved by the UNC Institutional Animal Care and Use Committee (IACUC). Mice used in these experiments were backcrossed 10 generations to the C57BL6 background.

3.2.2 Platelet isolation

Mice were euthanized by CO_2. Blood was drawn by cardiac puncture into an Eppendorf tube containing saline and ACD (85 mM sodium citrate, 111 mM glucose and 71 mM citric acid) with 0.1 μM PGE_1 (Sigma). To separate blood cells, whole blood was centrifuged for 10 min at 250 x g. Platelet rich plasma (PRP) was removed and the remaining blood was washed with ACD/saline/PGE_1 solution, centrifuged and PRP collected. PRP was pooled for each mouse and centrifuged at 400 x g for 15 min. Platelet poor plasma (PPP) was discarded and the platelet pellet resuspended in warmed (37°C) CGS buffer (13 mM sodium citrate, 30 mM glucose

and 120 mM NaCl) containing 10 U/ml apyrase (Sigma). Resuspended platelets were incubated at 37°C for 20 min before being centrifuged at 500 x g for 15 min. The platelet pellet was resuspended in 0.5 ml modified HEPES-Tyrodes buffer (12 mM $NaHCO_3$, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, 10mM HEPES containing 0.1% BSA, 1mM $CaCl_2$, 1mM $MgCl_2$ at pH 7.4). Platelets were allowed to rest for 1 h at room temperature (RT) before assaying.

3.2.3 Electron Microscopy

PRP was isolated as above and centrifuged at 400 x g for 15 min. PPP was removed and the platelet pellet was fixed by the addition of 2% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4 (all buffers provided by UNC Microscopy Services Laboratory). After 2 h at RT, the glutaraldehyde solution was removed, the pellet washed with cacodylate buffer and then fixed in potassium ferrocyanide-reduced osmium tetroxide for 1 h at RT. The pellet was dried with ethanol and embedded in PolyBed 812 epoxy resin (Polysciences, Inc. PA). Sections of the pellet (70 nm thick) were mounted on copper grids and stained with 4.0% uranyl acetate and 0.4% lead citrate. Sections were observed with a LEO EM 910 transmission electron microscope (LEO Electron Microscopy, Inc. NY) using an accelerating voltage of 80 kV.

3.2.4 Platelet spreading

Glass coverslips were coated with 1% BSA (Serologicals Protein Inc) or 100 μg/ml fibrinogen (Calbiochem). Platelets were isolated and washed as above and

resuspended in Tyrodes-HEPES buffer to 1 x 10^7 plts/ml. Precoated coverslips were washed twice with Tyrodes-HEPES buffer and 100 μl platelets were added and incubated for 1 h at 37°C. Coverslips were aspirated to remove unattached cells and remaining cells were fixed with 1.0% paraformaldehyde for 15 min at RT. Coverslips were washed twice and permeablized with 0.2% Triton-PBS for 30 seconds and washed three times with Tyrodes-HEPES. For staining, coverslips were incubated in the dark with 1:1000 phallodin-488 (Molecular Probes) for 30 min. Coverslips were rinsed and adhered to slides with FlouroSave (Calbiochem).

3.2.5 Flow cytometry

Washed platelets were incubated with FITC-labeled antibody for 15 min (JON/A, glycoprotein (GP) 1b and respective IgG controls, EMFRET, Germany) or 30 min (αIIb or α2 and respective IgG controls, BD BIOSCIENCE) at RT. For αIIbβ3 activation experiments, agonist (0.1 U thrombin or 10 μM ADP final concentrations) was added simultaneously with the antibody. After incubating, samples were diluted with Tyrodes-HEPES and analyzed on a BD FACSCanto flow cytometer (BD Biosciences) at a low flow rate. Surface expression data were normalized by dividing the mean fluorescent intensity of the antibody by the IgG control intensity. Activation data were normalized by dividing the agonist stimulated mean fluorescence by that of the unstimulated cells.

3.2.6 Aggregation

Platelets were isolated as above and diluted into Tyrodes-HEPES buffer to 1.5 x 10^8 plts/ml. Platelets were aliquoted into aggregation tubes and incubated at 37° C for 15 min before the addition of agonist, 0.1 mM PAR4 peptide (AYPGKF, synthesized at UNC Protein Chemistry Core) or 5 μM ADP. Traces were recorded for 5-6 min in a CHRONO-LOG Corporation Model 700 Aggregometer. Aggregometry data were analyzed by Aggrolink8 software (CHRONO-LOG).

3.2.7 Western blot analysis

Washed platelets were lysed in modified CHAPS buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM CHAPS, 50 mM NaF, 10 mM β-glycerophosphate, 1:100 concentration of Protease Inhibitors Cocktail Set III (Calbiochem), 1 mM $CaCl_2$ and 1 mM $MgCl_2$) on ice for 30 min. Lysates were collected after a 10 min centrifugation and the protein concentration was quantified by the BCA protein assay (Pierce). Protein samples were separated by SDS-PAGE, transferred to a PVDF membrane and subjected to Western blotting. A CIB1 chicken polyclonal antibody was used to detect mouse CIB1. The antibody against GAPDH was from Santa Cruz Biotechnology.

3.2.8 Primary megakaryocyte culture

Murine bone marrow-derived megakaryocytes were cultured as previously described (Shiraga et al., 1999). Briefly, bone marrow cells were flushed and isolated from tibias and femurs of mice and cultured for three days in medium containing Interleukin (IL)-6, IL-11 and thrombopoietin (PeproTech).

3.2.9 Quantitative reverse transcriptase-PCR

Megakaryocytes derived from bone marrow stem cells were isolated as above. RNA was isolated with the Qiagen RNA Isolation kit. Isolated RNA was transcribed into cDNA using Applied Biosystems' High-Capacity Reverse Transcription Kit. Primers for *Cib1, Cib2, Cib3, Cib4* and *Gapdh* were purchased from SuperArray. Sequences were proprietary, reference positions for partial product sequence amplified by primers, relative to NCBI RefSeq entry provided by SuperArray; Cib1 260-278 (RefSeq Accession# NM_011870), Cib2 590-609 (RefSeq Accession# NM_019686), Cib3 326-344 (RefSeq Accession# XM_356089.4), Cib4 271-291 (RefSeq Accession# XM_131990.3) and Gapdh 962-983 (RefSeq Accession# NM_008084.2). SYBR Green PCR Mastermix was also from SuperArray and manufacturer's protocol was followed. Real time qPCR reactions were run on Applied Biosystems Instruments Prism 7900HT Sequence Detection System using the standard 1 cycle at 95° C for 10 min and 40 cycles of 95° C for 15 s then 60° C for 1 min. Fold change in gene expression was calculated using the $\Delta\Delta$Ct method(Livak & Schmittgen, 2001; Schefe, Lehmann, Buschmann, Unger, & Funke-Kaiser, 2006).

3.2.10 Protein purification

PCR primers were designed to amplify *Cib1-3* mRNAs isolated from murine megakaryocytes. PCR products of *Cib1*, *-2* and *-3* were ligated into the pEXP5-NT/TOPO vector (Invitrogen) and correct sequences were verified. BL21 bacteria

(Stratagene) were transformed with pEXP5-NT/TOPO vectors, grown at 37°C and induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h at 30°C to produce desired proteins. pEXT5-NT/TOPO calmodulin-like 3 from Invitrogen was used as a control. Bacteria producing CIB1, CIB2 and calmodulin-like 3 were lysed by sonication and centrifuged at 10,000 x g for 20 min. Supernatant was run on an FPLC-nickel column to purify the protein. Since CIB3 was insoluble, the bacteria producing CIB3 were lysed and denatured in buffer containing 8 M urea (Sigma), 100 mM NaH_2PO_4 (Mallinckrodt) and 10 mM Tris-Cl (Fisher) at pH of 8.0. Lysate was centrifuged to remove cellular debris and Ni-NTA slurry (Qiagen) was added to the supernatant and incubated at RT for 1 h with shaking. The lysate-resin mixture was loaded into a column and flow- through was removed. The column was washed twice with buffer containing 8 M urea, 100 mM NaH_2PO_4 and 10 mM Tris-Cl at pH 6.3. Protein was eluted with buffer containing 8 M urea, 100 mM NaH_2PO_4 and 10 mM Tris-Cl at pH 5.9. Eluate was dialyzed into 8 M urea, 200 mM NaCl and 50mM Tris pH 8.0 at 4° C. After 4 h, an equal volume of buffer containing 200 mM NaCl and 50 mM Tris pH 8.0 with 2 mM dithiothreitol (DTT, ROCHE) was added to dilute the urea and left to dialyze overnight. Buffer was then exchanged with 2 M urea, 200 mM NaCl, 50 mM Tris pH 8.0 and 2 mM DTT to continue dialyzing and allow protein refolding. This process continued every 8 h until the urea was fully removed. The DTT was then dialyzed out with 200 mM NaCl, 50 mM Tris pH 7.5. The protein was then dialyzed into a final buffer containing 20 mM Tris pH 7.5, 300 μM $CaCl_2$ and 85 mM NaCl.

3.2.11 ELISA

An αIIb cytoplasmic tail (CT) peptide (sequence LVLAMWKAGFFKRNR synthesized by Genscript Corporation) or a scrambled control peptide (sequence DKFGRPPKVENEELEDRGEF, synthesized by UNC Protein Chemistry Core Facility) was coated on a microtiter plate (NUNC) overnight at 4°C at a concentration of 75 μg/ml. BSA (3%) was used to block non-specific interactions in peptide-coated and non-coated wells. Proteins were diluted in 50 mM HEPES, 150 mM NaCl with 0.1 mM $CaCl_2$ and $MgCl_2$ pH 7.4 to a range of 0.5 μg/ml to 20 μg/ml and were allowed to adhere to the plate for 1 h at RT. The samples were removed and the plate was washed three times with 0.5% TBS-Tween, pH 7.4. A primary anti-HIS antibody was added and incubated for 1 h at RT. The antibody was removed and plate was washed as above. An anti-rabbit-HRP antibody was then added to the microtiter plate for 1 h at RT. The secondary antibody was removed and the plate was washed as above. Binding was visualized by a color reaction with chromogenic substrate OPD Sigma Fast Tablets (Sigma) for 10 min at RT in the dark. The reaction was stopped with 4N H_2SO_4 and the plate was read at 490 nm in Medical Devices SpectraMax Plus plate reader. For competitive binding assays, 5 μg/ml of CIB1, -2 and -3 were incubated with increasing concentrations (1.0-100 μg/ml) of soluble αIIb peptide. Each sample and concentration was performed in triplicate for all ELISAs.

3.2.12 CIB homology and model analysis

CIB homologs in the UniProt database (Bairoch et al., 2005) were identified using sequence similarity BLAST searches (Altschul & Lipman, 1990) of the human CIB1 protein. We used ClustalX (Chenna et al., 2003) to generate a multiple sequence alignment of the three human CIB homologs (CIB1 (Q99828), CIB2 (O75838), CIB3 (Q96Q77)), and homologs of the fruitfly and worm CIB gene (*D. melanogaster* (Q9W2Q5), *D. pseudoobscura* (Q28ZR4), *C. elegans* (Q93640)). We then mapped highly conserved residue positions onto the crystallographic structure of human CIB1 (PDB ID 1XO5 (Gentry et al., 2005)).

3.2.13 Statistical analysis

All values are presented as the mean ± standard error of the mean (SEM). A *t*-test was used to evaluate statistical significance. P values less than 0.05 were considered statistically significant. N ≥ 3 for all experiments.

3.3 RESULTS

3.3.1 Platelet function is normal in *Cib1*$^{-/-}$ mice

We first confirmed that CIB1 was indeed absent in the *Cib1*$^{-/-}$ platelets by Western blotting (Fig. 1*A*). The overall morphology of the platelets by transmission electron microscopy appeared normal (Fig. 1*B*); platelet shape and organelles were similar in both genotypes. Flow cytometry with antibodies specific to integrins αIIbβ3 and α2β1, and glycoprotein (GP) Ib (Fig. 1*C*) indicated that there were no statistically significant differences detected in the expression levels of these three

proteins on platelets isolated from the *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ mice. These results thereby assure that any difference displayed in our αIIbβ3 functional studies was not due to a change in surface expression of αIIbβ3.

To examine potential differences in αIIbβ3 activation between *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ mice, we measured binding of an activation-specific antibody binding (JON/A) to αIIbβ3 integrin in resting or agonist stimulated platelets by flow cytometry (Fig. 2*A*). Basal αIIbβ3 activity in resting platelets was comparable in *Cib1*$^{-/-}$ and *Cib1*$^{+/+}$ mice (measured by the binding of unstimulated cells to the JON/A antibody). We also found no significant difference in the ability of *Cib1*$^{-/-}$ platelets to respond to a relatively weak agonist (10 μM ADP), or strong agonist (0. 1 U thrombin) versus platelets isolated from *Cib1*$^{+/+}$ littermate controls. Platelets aggregation was also used to evaluate αIIbβ3 activation. We found that *Cib1*$^{-/-}$ platelets aggregate in a similar manner when stimulated by agonist 0.1 mM PAR4 peptide or 10 μM ADP as those from *Cib1*$^{+/+}$ *mice* (Fig. 2*B*). These results imply that the absence of CIB1 does not alter the integrin αIIbβ3 expression and activation in platelets.

Acute blockade of CIB1 in platelets inhibits platelet spreading on immobilized fibrinogen (Naik et al., 2003b; Naik et al., 2003a). We therefore tested platelets from the *Cib1*$^{-/-}$ mice to determine the effect of the loss of CIB1 on platelet spreading, but found no difference between size and overall cell number of platelets adhered to fibrinogen in the presence or absence of ADP (Fig. 3*A*-*C*). Platelet spreading was also similar between the two genotypes after PAR4 stimulation, or on collagen in the absence or presence of ADP (data not shown). Therefore, outside-in signaling as measured by platelet spreading appears to be normal in the *Cib1*$^{-/-}$ deficient mice.

3.3.2 *Cib1* homolog *Cib3* is overexpressed in *Cib1*$^{-/-}$ primary megakaryocytes.

Our previous studies involving the acute modulation of CIB1 levels in megakaryocytes showed that CIB1 is an endogenous inhibitor of αIIbβ3 activation (Yuan et al., 2006b). Here we tested αIIbβ3 activation in megakaryocytes from the *Cib1*$^{-/-}$ mice to determine whether chronic loss of CIB1 has a similar phenotype to the acute megakaryocyte studies (Yuan et al., 2006b). Megakaryocytes differentiated in culture from the bone marrow stem cells of *Cib1*$^{-/-}$ mice bound fibrinogen in a similar manner as those from *Cib1*$^{+/+}$ mice. Specifically, both genotypes had similar levels of basal fibrinogen binding, and fibrinogen binding upon stimulation with agonist PAR4 (Fig. 2*C*). Therefore, activation of αIIbβ3 on megakaryocytes from the *Cib1*$^{-/-}$ mice is similar to that of megakaryocytes derived from *Cib1*$^{+/+}$ mice. This result models our *Cib1*$^{-/-}$ and *Cib1*$^{+/+}$ platelet data, and are in contrast to results obtained with short term direct protein or genetic manipulations (Naik et al., 2003a; Yuan et al., 2006b; Naik et al., 2003b; Tsuboi, 2002).

Since we did not see a change in αIIbβ3 activation in *Cib1*$^{-/-}$ mouse platelets or megakaryocytes, we considered the possibility of functional compensation by other CIB family members, as the chronic loss of CIB1 could alter the expression of other genes. Genes of human homologs *Cib2*, *Cib3* and *Cib4* have been reported (Gentry et al., 2005) and genes of mouse *Cib2-4* can be identified in the mouse gene database. Therefore, we examined the expression of these mouse genes using real-time qPCR with mRNA derived from cultured murine megakaryocytes. I found increased *Cib3* mRNA expression in the *Cib1*$^{-/-}$ bone marrow derived

megakaryocytes while *Cib2* message levels remained constant between *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ megakaryocytes (Fig. 3*A*). *Cib4* was excluded from further studies because only a very low level of *Cib4* cDNA (slightly above background) was detected from megakaryocytes and other tissue by reverse transcriptase-PCR and qPCR in either genotype (data not shown).

Since mRNA from the *Cib1*$^{-/-}$ megakaryocytes displayed a different expression pattern for CIB3 relative to *Cib1*$^{+/+}$ megakaryocytes, I further investigated the possibility of functional compensation. We produced and purified the recombinant CIB1, -2 and -3 proteins from bacterial cultures to test the binding properties of CIB family proteins with integrin αIIb. It is currently unknown whether CIB2 or CIB3 is expressed in platelets. CIB2 protein expression has been identified in HEK cells (Mayhew et al., 2006) but CIB3 protein remains to be examined or reported and antibodies that recognize mouse CIB2, -3, or -4 are not currently available. Nonetheless, I asked if recombinant murine CIB2 and CIB3 could bind to αIIb. We immobilized an αIIb-CT peptide in microtiter wells, added increasing concentrations of CIB1, -2, and -3 proteins and measured binding by ELISA (Fig. 4*B*). Interestingly, all three CIB proteins bound to immobilized αIIb-CT peptide. However, none of the proteins bound to a scrambled control peptide, implicating a specific interaction between these CIB proteins and αIIb (Fig. 4*B*). To further ensure the specificity of binding of these proteins to the αIIb-CT peptide, I added increasing concentrations of soluble αIIb peptide to compete with CIB1 protein binding to immobilized peptide. Addition of this soluble peptide decreased the binding of CIB1, -2 or -3 to immobilized peptide (Fig. 4*C*). , reaffirming that there is a specific

interaction between the αIIb-CT peptide and CIB proteins, whereas the soluble scrambled peptide did not interfere with the CIB protein binding to immobilized αIIb peptide (data not shown). These findings demonstrate that CIB2 and CIB3 can bind to the cytoplasmic tail of αIIb *in vitro* and therefore have the potential to compensate for the loss of CIB1.

3.3.3 CIB family computer modeling

For insight into the potential mechanism of how CIB2 and/or CIB3 might compensate for the loss of CIB1 in αIIbβ3 regulation, we performed computer modeling studies. We evaluated CIB family homology by aligning the ancestral CIB protein from worm (*Caenorhabditis elegans*) and fruit fly (*Drosophila melanogaster*) with the human CIB1-3 proteins. Highly conserved residues are represented in purple and yellow while non-conserved residues are represented in grey and orange (Fig. 5). The conservation is indicative of potential functionality that existed in the CIB ancestor and that has been maintained in the CIB1-3 homologs (purple and yellow residues Fig. 5).

Residues in CIB1 that are proposed to interact with αIIb based on previous studies and on the crystal structure (Barry et al., 2002; Gentry et al., 2005; Naik et al., 1997; Shock et al., 1999; Yamniuk et al., 2005; Yamniuk, Ishida, & Vogel, 2006) of CIB1 were specifically compared to the corresponding residues in CIB2 and CIB3. CIB residues hypothesized to form the hydrophobic binding pocket for the αIIb tail are designated by yellow for conserved and orange for non-conserved (Fig 5*A*). Almost all of the residues in the hydrophobic binding pocket are highly conserved

between the three human CIB homologs and the worm and fruit fly CIB homolog, indicating a potentially shared functional domain.

The C-terminus of CIB1 has been proposed to be displaced upon αIIb binding (Yamniuk et al., 2006) therefore we also performed a modeling analysis with the C-terminus of CIB2 and CIB3 removed (Fig. 5*B*). The additional residues exposed in CIB2 and CIB3 were highly homologous to those exposed in CIB1 upon C-terminal deletion (Fig. 5*B*). In addition, the length and depth of the hydrophobic binding pocket was enlarged similarly between each CIB protein. This suggests that all three CIB proteins may use the C-terminus to control binding specificity as proposed by Yamniuk et al. 2006 (Yamniuk et al., 2006) for CIB1.

3.4 DISCUSSION

Activation and regulation of platelet integrin αIIbβ3 has been extensively studied since identification of the protein in 1980 (Phillips, Jennings, & Edwards, 1980; Phillips, 1980), yet the precise mechanism of integrin activation and molecules involved in this process are still under investigation. Several intracellular binding partners of the integrin are known to regulate αIIbβ3 activation (Leisner, Yuan, Denofrio, Liu, & Parise, 2007). Interestingly, most of these regulatory proteins bind to the β3 cytoplasmic tail and not to αIIb (Leisner et al., 2007). The αIIb subunit currently has only a handful of binding partners and there has been conflicting reports of the function these proteins have in αIIbβ3 activation (Larkin et al., 2004; Leung-Hagesteijn, Milankov, Michalak, Wilkins, & Dedhar, 1994; Liu, Corjay,

Feuerstein, & Nambi, 2006; Reilly, Larkin, Devocelle, Fitzgerald, & Moran, 2004; Vijayan, Liu, Li, & Bray, 2004). Previously we identified CIB1 in a screen for binding partners, and potential regulators, of the αIIb cytoplasmic tail. Initial genetic studies of CIB1 function required a megakaryocyte model because only platelets and megakaryocytes express the required signaling machinery involved in integrin αIIbβ3 inside-out activation and platelets are not amenable to direct genetic manipulation (Liu et al., 2007). For a more comprehensive understanding of the role of CIB1 in the regulation of integrin αIIbβ3 activation, we developed the *Cib1*$^{-/-}$ mouse model and studied the function of platelets and megakaryocytes derived from these mice. We found no significant difference in the protein expression, basal activation or agonist-induced activation of αIIbβ3 measured by flow cytometry. There was also no significant difference in aggregation or spreading between platelets from *Cib1*$^{+/+}$ or *Cib1*$^{-/-}$ mice.

The lack of a phenotype in αIIbβ3 activation in *Cib1*$^{-/-}$ mice was unexpected because our megakaryocytes studies and other independent studies in platelets (Naik et al., 2003a; Yuan et al., 2006b; Naik et al., 2003b; Tsuboi, 2002; Vallar et al., 1999; Yamniuk et al., 2005) demonstrated that CIB1 regulates αIIbβ3 activation and platelet spreading. We suspected that this lack of phenotype in the *Cib1*$^{-/-}$ platelets may be due to a compensatory mechanism that occurred during mouse development. The most obvious possibility would be compensation by another CIB family member. The idea of functional compensation resulting in a limited phenotype is not novel. For example, Vav1 and Vav3 are functionally redundant in their role of activating PLC gamma2 (Pearce et al., 2004) and other proteins when

knocked down, partially or fully, did not display the platelet phenotype predicted by previous short term genetic manipulations (Schraw et al., 2004; Pearce et al., 2004; Pearce et al., 2002). The discrepancies in our previous megakaryocyte results and present *in vivo* results reiterate how fundamentally different chronic genetic mutations (knock-out or knock-in) are compared to acute genetic mutations (knock-down or overexpression). Both short and long term genetic manipulations provide valuable information but the results should be only interpreted under each experimental condition.

Real time PCR experiments detected an increase in the message of *Cib3* while the message of *Cib2* did not change between $Cib1^{+/+}$ and $Cib1^{-/-}$ in megakaryocytes. Therefore the potential binding of CIB2 and CIB3 to the αIIb tail was investigated. ELISA data indicated that CIBs1-3 can all bind to the cytoplasmic tail of αIIb. The apparent CIB2/αIIb interaction was unexpected since CIB2 was demonstrated to not bind αIIb in a yeast two-hybridization assay (Yuan et al., 2006b).

Sequence homology shows that human CIB2, CIB3 and CIB4 are 59%, 62% and 64%, homologous to CIB1, respectively (Gentry et al., 2005). Human CIBs show equivalent residues involved in the structure of the hydrophobic binding pocket, i.e. the canonical αIIb binding site by sequence alignment (Gentry et al., 2005). Currently, only human CIB2 has been reported as a protein in the literature (Mayhew et al., 2006); endogenous human or murine CIB3 and CIB4 proteins have not been identified to date or studied. The computer homology modeling analyses of CIB proteins provide insights into potential conserved αIIb binding domains in

CIB1-3 that support our protein binding data. We observed a highly conserved hydrophobic binding pocket in all CIBs1-3 protein. CIB1 is reported to undergo displacement of its C-terminus upon αIIb binding, which increases the size of the hydrophobic binding pocket (Yamniuk et al., 2006). Removal of the corresponding C-terminal residues in CIB2 and CIB3 also led to an increase in the size of the hydrophobic binding pocket. The residues exposed by displacement of the tail were also highly conserved between CIB1, -2 and -3. This theoretical structural change could potentially allow CIB2 and CIB3 to share binding partners with CIB1, e.g. integrin αIIbβ3.

It will be interesting to determine if any other tissues or cells types in the *Cib1*$^{-/-}$ mice have altered CIB family protein expression. It is known that the chronic loss of CIB1 affects spermatogenesis (Yuan et al., 2006a) and angiogenesis (Zayed et al., 2007) in the *Cib1*$^{-/-}$ mice. This begs the question of why some cell types or processes may have developed compensatory mechanisms, while other cells did not. For example, endothelial cells isolated from the *Cib1*$^{-/-}$ mice do not have fully functional compensatory mechanisms, as they have decreased migration in culture and *in vivo* these mice have defects in ischemia-induced pathologic angiogenesis (Zayed et al., 2007). Also of interest is the question of how many binding partners are shared by CIB1, -2 and -3? Since the functional role of the CIB family *in vivo* has not been established, the development of investigational tools such as expression plasmids and antibodies to other CIB family proteins will aid the investigation of CIB protein function and regulation.

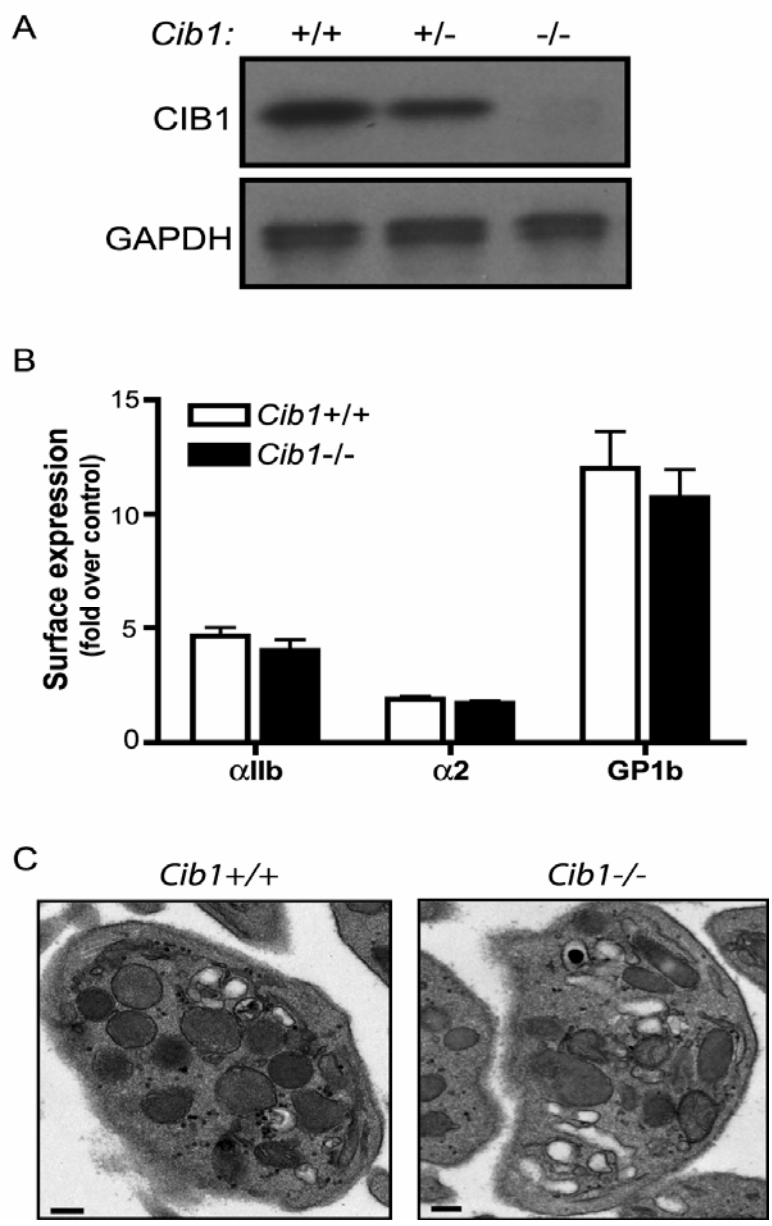

Figure 3.1 Platelet morphology of *Cib1*$^{-/-}$ mice. *(A)* CIB1 is absent in platelets isolated from the *Cib1*$^{-/-}$ mice. Platelet lysates from *Cib1*$^{+/+}$, *Cib1*$^{+/-}$ and *Cib1*$^{-/-}$ mice were subjected to Western blotting and CIB1 expression was compared. GAPDH was used as a loading control. *(B)* Surface receptor expression on *Cib1*$^{-/-}$ platelets is similar to expression on *Cib1*$^{+/+}$ platelets. To detect surface receptor expression, washed platelets were incubated with FITC-αIIb antibody, FITC-α2 antibody or FITC-IgG control for 30 min, or FITC-GP1b antibody or FITC-IgG for 15 min at RT. Flow cytometry was used to determine mean fluorescence. Values were normalized and presented as fold over IgG control (antibody/control) (N ≥ 9). *(C)* Resting platelet morphology is normal when examined by electron microscopy. There was no significant difference in size, shape and granule content between *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ platelets. Scale bar equals 0.2 μM.

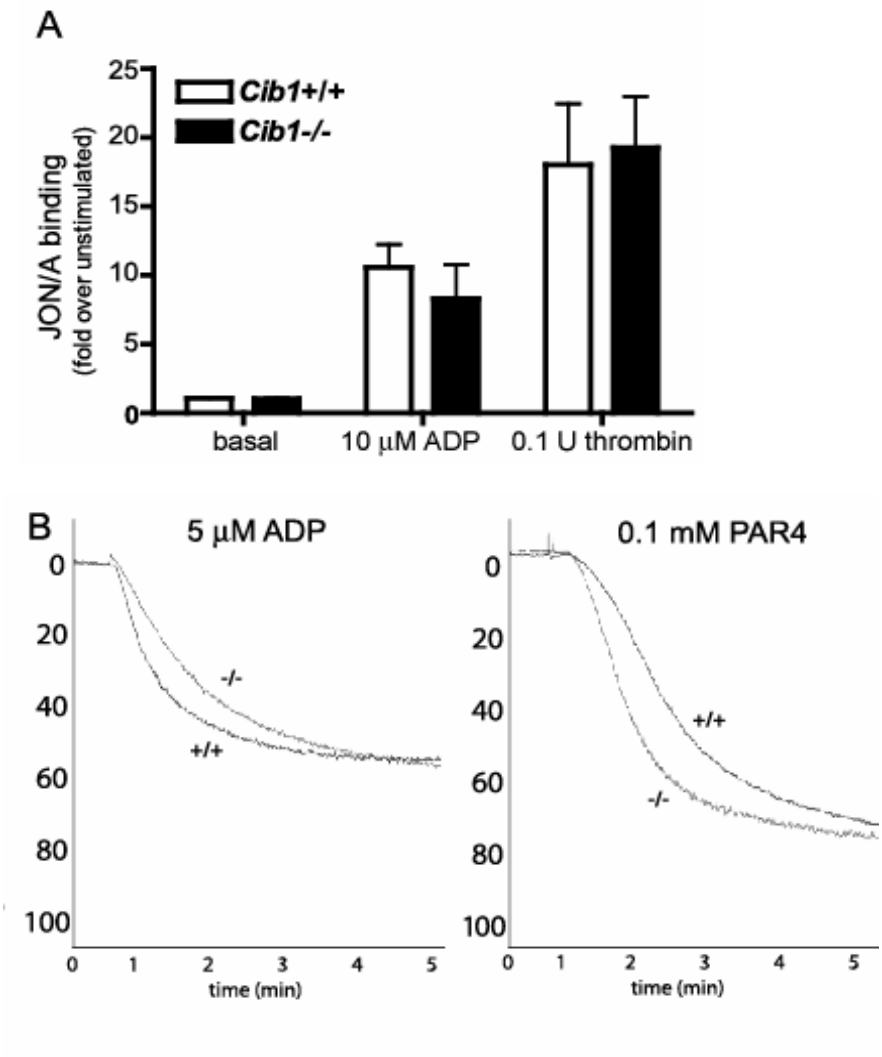

Figure 3.2 Platelet function is unaffected by the loss of CIB1. *(A)* Integrin αIIbβ3 activation is normal when assessed by flow cytometry. Washed platelets were incubated for 15 min with an αIIbβ3 active conformation specific antibody (JON/A) or IgG control. Basal expression was defined as JON/A binding over IgG control binding (JON/A antibody/control antibody). Agonist-induced activation was measured by stimulating platelets with either 10 μM ADP or 0.1 U thrombin and expressed as the increase in antibody binding in stimulated divided by nonstimulated platelets (stimulated/nonstimulated platelets) (N ≥ 4) . *(B)* Platelet aggregation is normal in washed *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ platelets. Washed platelets were stimulated by 5 μm ADP (left) or 0.1 mM PAR4 peptide (right) and platelet aggregation traces were recorded for 5 min.). (For Figure 2*C* see following page.)

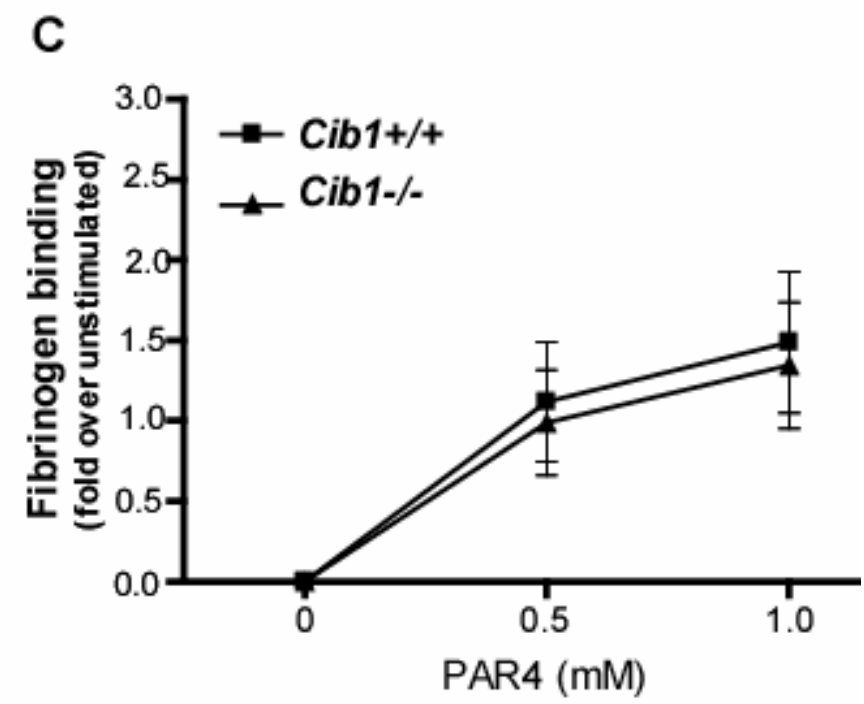

Figure 3.2 Platelet function is unaffected by the loss of CIB1. *(C)* Activation of integrin αIIbβ3 on cultured bone marrow derived megakaryocytes was similar between *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ mice when measured by flow cytometry. Megakaryocytes were stimulated with increasing concentrations of PAR4 peptide (0.5 mM and 1 mM) and binding of Alexa-546 labeled fibrinogen was determined. Mean fluorescent intensities were normalized by dividing the stimulated value by the unstimulated control (N = 3). (For Figure 2*A-B* see previous page.)

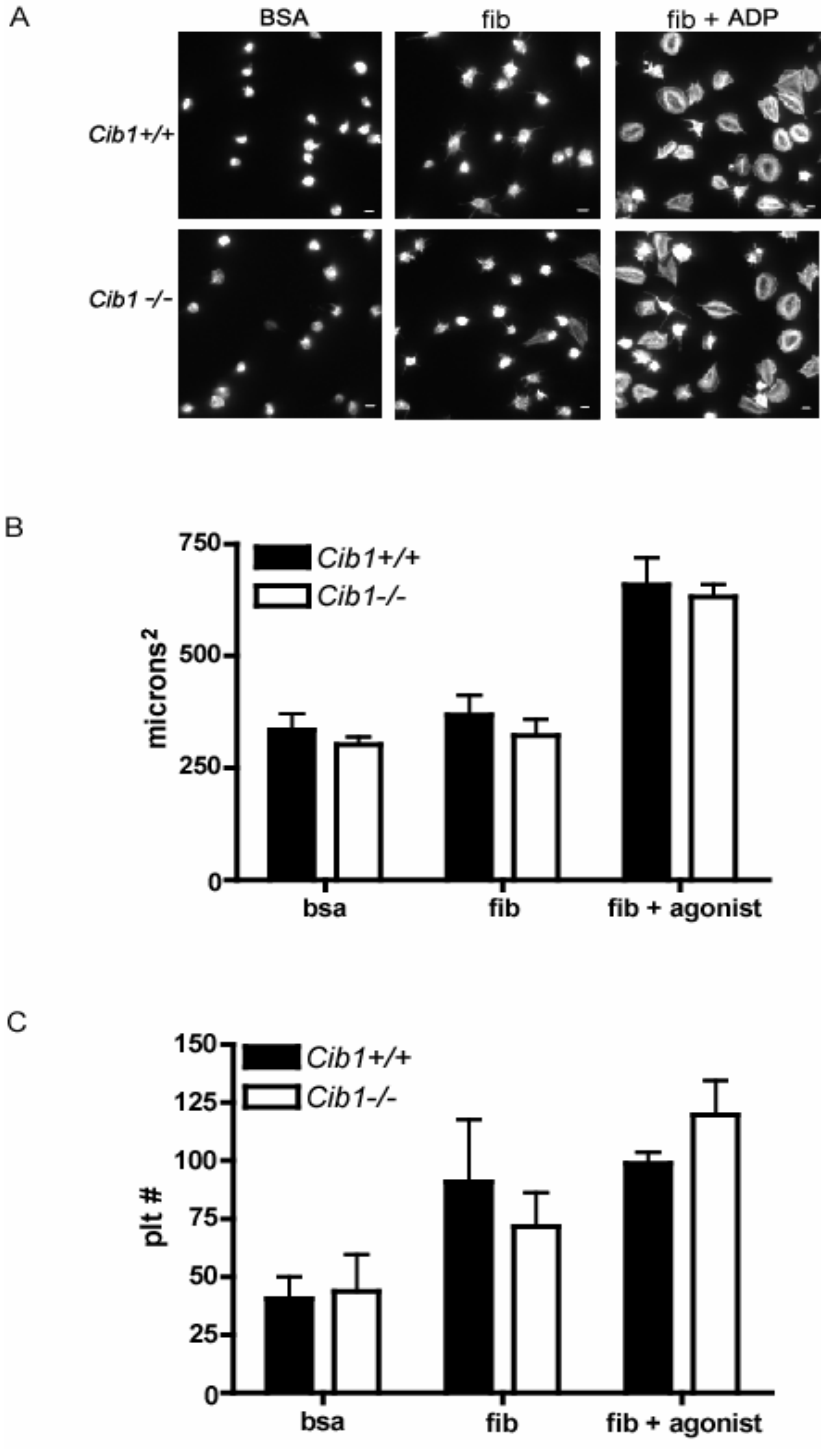

Figure 3.3 Platelet spreading is normal in the chronic absence of CIB1. *(A)* Platelets from *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ mice adhered and spread similarly on immobilized BSA or fibrinogen. *Cib1*$^{-/-}$ platelets did not respond differently in the presence or absence 100 μM ADP compared to *Cib1*$^{+/+}$ platelets. Platelets were allowed to spread for 60 min at 37°C. Scale bar = 1 μm. Platelet size *(B)* and count *(C)* were analyzed by ImagePro software and compared between *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$. There was no significant difference between the two genotypes.

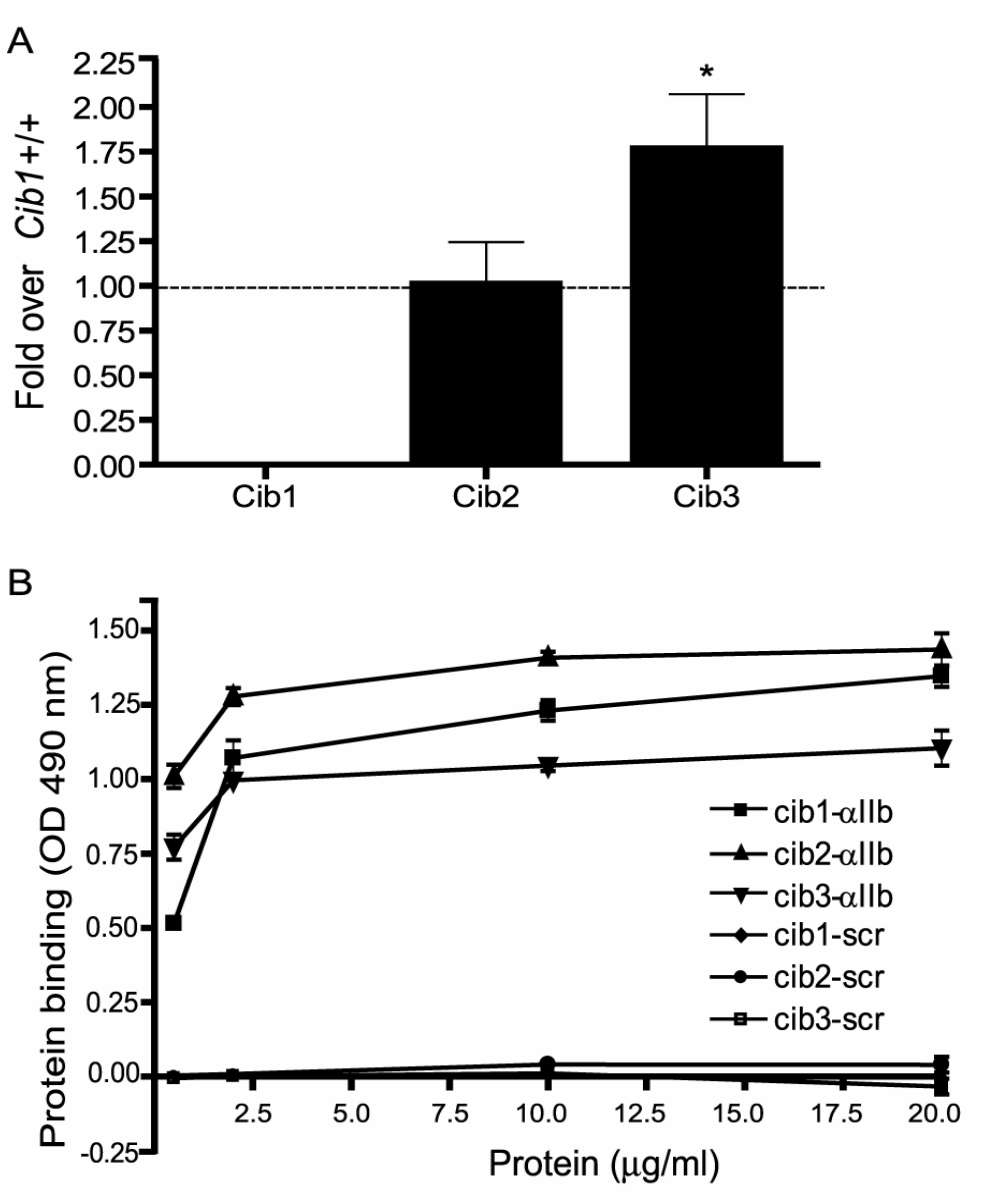

Figure 3.4 CIB family members interact with integrin αIIbβ3. *(A) Cib1*$^{-/-}$ mice have significantly increased CIB3 mRNA in megakaryocytes derived from bone marrow stem cells as measured by quantitative PCR, while CIB2 retains the same level of expression as *Cib1*$^{+/+}$. *Gapdh* was the housekeeping gene used to normalize CT values. Fold values were defined using the ΔΔCt calculation (Livak et al., 2001; Schefe et al., 2006). Fold value of 1 equates to no change in expression, while values above 1 equals increase in message and values below 1 equal decrease in message expression (N ≥ 5). *(B)* CIB1-3 proteins bind to integrin αIIb peptide as measured by ELISA. Peptides of the cytoplasmic tail of integrin subunit αIIb (αIIb) or control scrambled (scr) peptide were immobilized and increasing concentrations of CIB1,-2 or -3 were added to peptide or BSA coated wells. Data graphed is a representative experiment of OD 490 (nm) values of peptide coated wells minus non-specific binding (N = 3). (For Figure 3*C* see following page.)

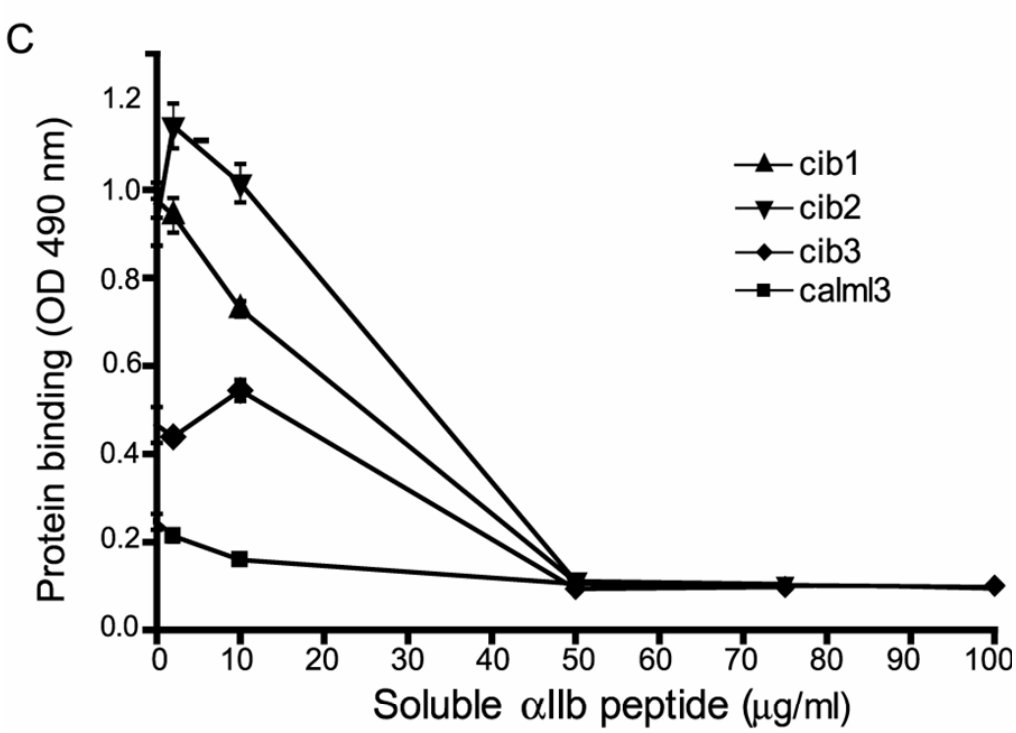

Figure 3.4 CIB family members interact with integrin αIIbβ3. *(C)* Soluble peptide competition ELISAs demonstrate that the CIB1-3 interaction with αIIb peptide is specific. Increasing concentrations of soluble αIIb peptide were incubated with CIB1, -2, -3 or control protein, calmodulin-like 3, for 30 min. Samples were added to immobilized αIIb peptide or BSA coated wells. Data is presented as in (*B*) (N = 3). (For Figure 3*A*-*B* see previous page.)

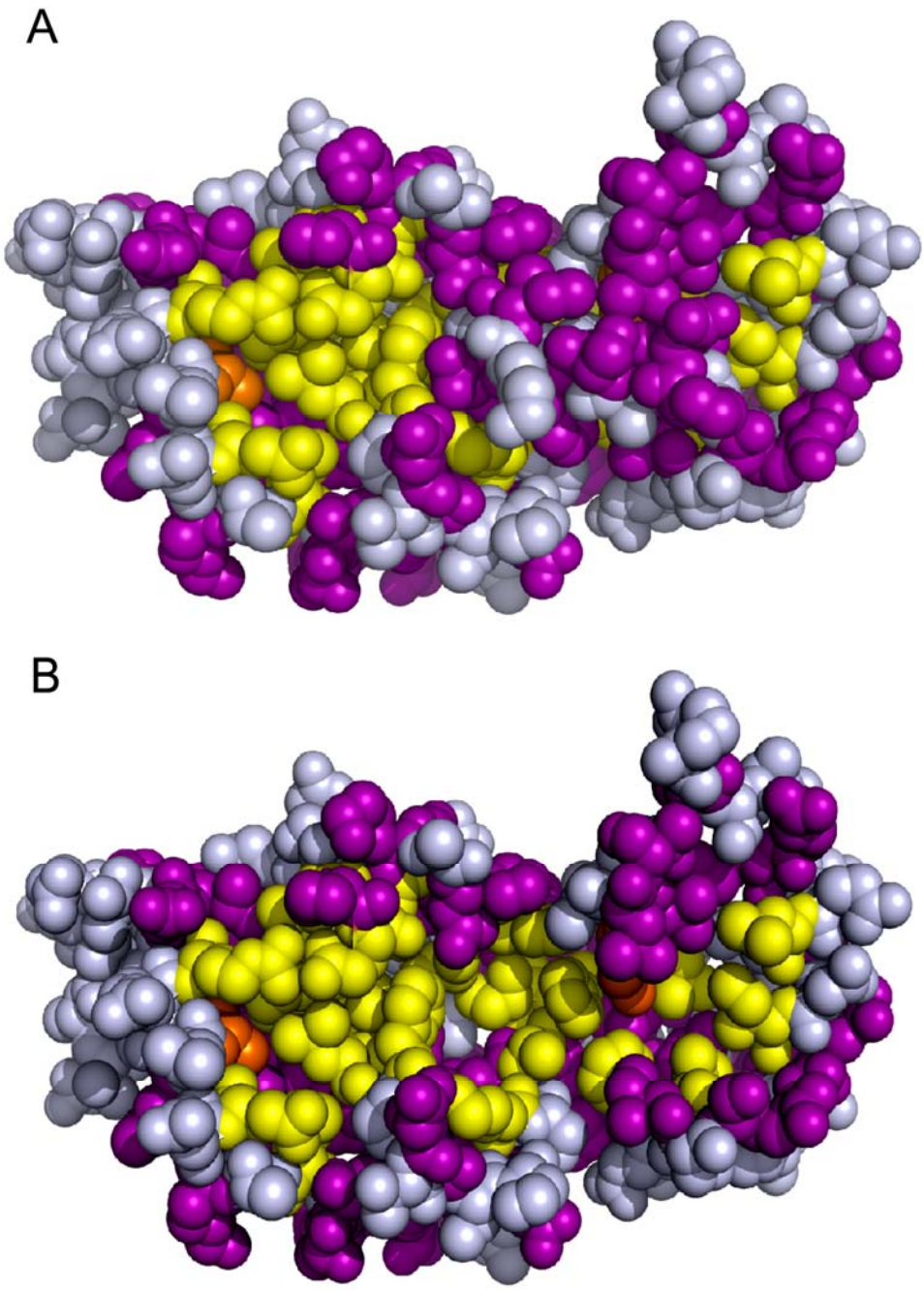

Figure 3.5 Computer modeling of CIB family proteins reveals a highly conserved hydrophobic binding pocket. *(A)* Homology map of CIB proteins. Grey represents non-conserved residues, purple represents conserved, yellow represents conserved residues in the hydrophobic binding pocket and orange represents non-conserved residue in the hydrophobic binding pocket. *(B)* Model of CIB after displacement of the C-terminal tail. Molecular surfaces are represented as in *(A)*.

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CHAPTER 4

CONCLUDING REMARKS

When I started working with the *Cib1*$^{-/-}$ mice, studies had been previously published proposing that CIB1 was a negative regulator of agonist-induced αIIbβ3 activation in megakaryocytes (Yuan et al., 2006). These earlier experiments required the use of genetically altered murine megakaryocytes because direct genetic manipulation of anucleate platelets was impossible and the knockout was proving to be trickier than expected to generate. Once the *Cib1*$^{-/-}$ mouse colony was backcrossed 9-10 generations, Dr. Weiping Yuan (who made the knockout with the help of Dr. Nobuyo Maeda) and I began to study the *Cib1*$^{-/-}$ mice to determine if the chronic loss of CIB1 altered hemostasis. We wanted to compare the phenotype of megakaryocytes with an acute knockdown of CIB1 to the phenotype of megakaryocytes from *Cib1*$^{-/-}$ mice cultured under the same conditions, but forced to mature and adapt while chronically deprived of CIB1 (and the phenotype of platelets from knockout mice) relative to agonist-induced activation of αIIbβ3. We also interested in exploring beyond the platelet and megakaryocyte and understand how the chronic loss of CIB1affects hemostasis. As it turned out, this led to a very serendipitous expansion of my research project.

4.1 CIB1 and hemostasis

Plasma analysis of the *Cib1*$^{-/-}$ mice exposed several significant differences in protein levels between the *Cib1*$^{+/+}$ and *Cib1*$^{-/-}$ plasma. The most notable were the increases in von Willebrand factor and soluble P-selectin concentration. To understand the effects of these two pro-thrombotic proteins circulating at higher levels in the plasma, we examined bleeding time and arterial thrombus formation in both genotypes. These studies demonstrated no significant difference in thrombus formation or stability between the *Cib1*$^{+/+}$ and the *Cib1*$^{-/-}$ mice. Therefore, the *Cib1*$^{-/-}$ mouse may provide a model for studying the critical range of plasma proteins required for normal hemostasis.

The *Cib1*$^{-/-}$ mice because of their increased vWF, yet normal hemostasis, provide an excellent research tool for studying von Willebrand Disease. von Willebrand Factor is synthesized in endothelial cells and megakaryocytes. Endothelial cells constitutively secrete the smaller less active multimers of vWF, while both platelets and endothelial cells store the more active ultra-large vWF multimers in granules until cellular activation. von Willebrand Factor multimerization occurs in the Golgi by disulfide bond formation (Purvis et al., 2007). When large multimers of vWF are exocytosed from the cell, they are highly adhesive to platelets (Ruggeri, 2007). Normally, metalloprotease ADAMTS-13 cleaves large vWF as a regulatory method to control thrombus formation, because the smaller multimers are less adhesive. Interestingly, high fluid shear stress will also promote platelet adhesion to smaller vWF multimers (Choi, Aboulfatova, Pownall, Cook, & Dong, 2007). Therefore, the lack of an increase of platelet aggregates that should have been visible in my flow cytometry experiments (Chapter 3) implies that the *Cib1*$^{-/-}$

mice do not have a defect in ADAMTS-13 and do not have high fluid shear stress. However, von Willebrand Disease researchers could use these mice to investigate the effect of changing ADAMTS-13 activity or arterial shear stress on the plasma with above average vWF levels.

To fully utilize the *Cib1*$^{-/-}$ mouse as a vWF research tool, the multimeric size of the circulating vWF would need to be determined. Smaller multimers would suggest a change in the constitutively-secreted vWF pathway. Alternatively, smaller vWF multimers could result from an increase in ADAMTS-13 activity. Conversely, if the vWF circulating in the *Cib1*$^{-/-}$ plasma is larger in multimeric size, this would provide information about the critical level of large vWF level that will still enable physiologic hemostasis. The *Cib1*$^{-/-}$ mouse is valuable to anyone investigating the pathologic levels of multimer vWF size and this research is tool towards understanding and treating vWD.

The fact that *Cib1*$^{-/-}$ mice have increased levels of soluble P-selectin and yet appear to have normal hemostasis is also very intriguing. The main ligand for P-selectin, PSGL-1 is expressed mainly on leukocytes but is also found on the platelet surface (Frenette et al., 2000; Mayadas, Johnson, Rayburn, Hynes, & Wagner, 1993). The soluble form of P-selectin still binds PSGL-1 (Frenette et al., 2000; Hrachovinova et al., 2003; Mehta, Patel, Laue, Erickson, & McEver, 1997) and could therefore compete with the cell surface-bound P-selectin for PSGL-1. Exploring leukocyte interactions with the endothelium or as part of platelet aggregate formation may provide additional answers to the potential effect(s) of increased sP-selectin.

Several studies have investigated the role of sP-selectin and vWF, in sync, to promote both physiologic and pathologic thrombus formation by involving leukocytes (Denis, Andre, Saffaripour, & Wagner, 2001; Dole, Bergmeier, Mitchell, Eichenberger, & Wagner, 2005; Mayadas et al., 1993; McEver, 1992; Pendu et al., 2006; Wagner, 2005). For example, experiments by Denis et al., 2001, demonstrate that *vWF*$^{-/-}$ mice had defective P-selectin storage into endothelial cell Weibel-Palade bodies and this ultimately resulted in to decreased leukocyte recruitment (Denis et al., 2001). It is quite possible that the role of these increased but still non-pathological concentrations of vWF and sP-selectin may have a greater effect on inflammation than hemostasis. Leukocyte rolling on the endothelium and platelet rolling on vessel-wall-bound leukocytes and endothelium are believed to be important components of the inflammatory response to allow these cells to home in on sites of infection (Denis et al., 2001; Dole et al., 2005; Hartwell et al., 1998; Mayadas et al., 1993; Pendu et al., 2006). Investigating *in vivo* leukocyte and platelet rolling in *Cib1*$^{-/-}$ mice could yield some more “CIB surprises”. Since the levels of vWF and sP-selectin are increased, one would hypothesize there would be a decrease in leukocyte rolling, and to a lesser degree platelet, rolling. This would result from less available unoccupied PSGL1. The transient interactions that enable platelet rolling are strongly based on vWF-GP1b/IX/V, and collagen-GPVI (see Chapter 1 section 2 for additional information). Increased plasma vWF may inhibit GP1b/IX/V binding to ECM-vWF. Given the increase in both vWF and sP-selectin, *in vivo* rolling experiments would be extremely interesting and informative for the hemostasis field.

Another fascinating question is how the *Cib1*$^{-/-}$ mice respond to physiological stress. It has already been shown that physiologic stress to *Cib1*$^{-/-}$ mice affects angiogenesis (Zayed et al., 2007). Inducing physiologic stress in these mice may even expose a hemostatic phenotype. For example, an interesting and logical extension to the study of inflammation in these mice would be to challenge them by injecting the endotoxin lipopolysaccharide (LPS). This would cause an increase in several plasma proteins and activate the endothelium. Hartwell et al. showed decreased neutrophil influx into inflamed areas in mice genetically engineered to express higher levels of sP-selectin (Hartwell et al., 1998).

4.2 CIB and integrin αIIbβ3

As mentioned earlier, CIB1 binds to the cytoplasmic tail of the integrin αIIb subunit and previous studies in murine megakaryocytes demonstrated that CIB1 is a negative regulator of agonist-induced αIIbβ3 activation (Yuan et al., 2006). Others have reported that introducing an inhibitory CIB1 antibody into platelets blocked platelet spreading on immobilized fibrinogen (Naik & Naik, 2003). Therefore, combining the effects of our acute changes in megakaryocytes with data showing that acute changes affect αIIbβ3 in platelets, along with several *in vitro* studies confirming the interaction between CIB1 and αIIb (Naik, Patel, & Parise, 1997; Naik et al., 2003; Shock et al., 1999; Yamniuk & Vogel, 2005), we logically hypothesized that the *Cib1*$^{-/-}$ platelets would behave accordingly. We analyzed *Cib1*$^{-/-}$ platelets by several different methods; however, no significant difference in basal or agonist-induced activation or platelet spreading was exhibited by platelets from *Cib1*$^{-/-}$ mice.

This lack of phenotype of *Cib1*$^{-/-}$ platelets mice was quite a surprise. And though plausible, and cannot be dismissed, it seems unrealistic to conclude that CIB1 when present has no function in regulating αIIbβ3 activation. The possibility that these mice adapted to the deprivation of CIB1 by compensatory mechanism(s) during development is more likely.

We explored the possibility of functional compensation by investigating the most homologous CIB1 proteins, CIBs2-4. Currently, there is minimal information on CIBs2-4, which did present a challenge. However, there were mRNA sequences identified that I used to perform quantitative real time PCR experiments. I discovered that mRNA level of CIB1 homologs CIB3 and CIB4 were increased in *Cib1*$^{-/-}$ megakaryocytes compared to *Cib1*$^{+/+}$ megakaryocytes. However, for reasons mentioned in Chapter 3, I did not pursue CIB4 for the remaining studies. I made the recombinant proteins of CIBs1-3 and measured *in vitro* binding to αIIb by ELISA. My results provided evidence that recombinant CIB1, -2 and -3 all bound specifically to an αIIb cytoplasmic tail peptide. Subsequent protein modeling experiments validated my data by indicating that CIBs1-3 each have a highly homologous hydrophobic binding pocket and residues critical to αIIb binding (Barry et al., 2002; Gentry et al., 2005). Therefore, the potential exists for compensation by these CIB family members for loss of CIB1 such that hyper-activatable platelets do not occur, which could thereby prevent pathologic thrombus formation in *Cib1*$^{-/-}$ mice.

An interesting side note is that when studying the evolution of CIB for my homology searches and with the computer modeling experiments done by Dr. Brenda Temple, it became clear that the dominant role of the “ancestral” CIB protein

would not be to regulate integrin αIIbβ3. In fact, a CIB sequence can be identified as far back evolutionarily as the worm *(Caernorhabditis),* while the sequence for the integrin subunit αIIb could only be identified as far back as zebrafish *(Danio),* which have both platelets and αIIbβ3. These observations provide further motivation to characterize the *Cib1*$^{-/-}$ mouse to discover other surprising functions of CIB1 and other family members unrelated to integrin αIIbβ3.

4.3 Final thoughts

In conclusion, my research provides the scientific community with new evidence that establishes the *Cib1*$^{-/-}$ mouse as a useful model for understanding the patho-mechanisms involved in not only hemostasis but also inflammation. Also, I have further demonstrated how acute and chronic experiments may provide differing results, which needs to be taken into consideration when evaluating data. Lastly, I have discovered the potential for CIB family proteins to share functions and binding partners, which could lead to prospective compensatory mechanisms. Further research is necessary to completely understand the roles of CIB1 and other CIB family proteins, but it appears likely that this protein family will have an increasing role in hemostasis that will be revealed with additional in depth investigations.

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