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The Tetraspanin CD9 Localizes to Platelet-Platelet Contacts and Regulates Thrombus Stability

Sarah Kathleen Hill
University of Tennessee Health Science Center

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Document Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Program
Molecular Sciences

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DOI
10.21007/etd.cghs.2008.0138

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THE TETRASPANIN CD9 LOCALIZES TO PLATELET-PLATELET CONTACTS AND REGULATES THROMBUS STABILITY

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Sarah Kathleen Hill
December 2008
DEDICATION

I gratefully dedicate this manuscript to my family, especially my husband, Michael Hill, for his endless patience and encouragement; our daughter Norah, for her motivation; and my parents, Kieth and Dianne Norris, for raising me to value education and enjoy scientific discovery.
ACKNOWLEDGEMENTS

I would like to acknowledge the insights, direction, and support of my research advisor, Lisa K. Jennings, Ph.D. I also thank my committee members, Marshall Elam, MD, Ph.D., Eldon Geisert, Ph.D., Rod Hori, Ph.D., and Susan Senogles, Ph.D for their generosity with their talents and time.

I thank members of the Jennings laboratory, both past and present: Svetozar Grgurevich, Ph.D., Jayaprakash Kotha, Ph.D., Celia Longhurst, Ph.D., Melanie White, Shila Cholera, Henry Speich, Ph.D., Charlett Golden, and Mary Holmes for support and assistance.

I thank Michael Whitt, Ph.D. and Carolyn Matthews for their training and advice with using the confocal microscope and John Cox, Ph.D. for his knowledge and assistance in quantifying the confocal data.

I thank Patrick Ryan, Ph.D. for generously sharing his laboratory space and supplies while the Coleman Building was inaccessible.

Finally, thanks to Shirley Hancock, Deanna Delffs, Larry Tague, and David Armbruster, Ph.D., for formatting review; David M. Trayer, M.S., Myralin A. Trayer, M.A., Siobhan Pehrson, and Michael Hill for proofreading; and the staff at the UT Help Desk for technical assistance.

This research was funded through the NIH Training grant 5T32HL007641-19 from the National Heart, Lung and Blood Institute. The Zeiss LSM 510 was obtained through a Shared Instrumentation Grant (S10 RR13725) from the NIH.
ABSTRACT

CD9, a member of the tetraspanin family, is highly expressed on platelets (50,000-80,000 copies per platelet). Tetraspanins have been implicated in modulation of integrin function, and it is hypothesized that CD9 will modulate GPIIb-IIIa, the major platelet integrin.

The association between CD9 and GPIIb-IIIa was analyzed using immunoprecipitations and confocal microscopy. These two proteins were found to associate with each other, particularly at areas of platelet-platelet contact and at the periphery. Confocal analysis revealed CD9 localization was most intense at platelet-platelet contact, as well as in platelet filopodia and lamellipodia, but there is a lack of CD9 at areas of platelet-matrix contact. Co-localization with F-actin decreased as platelets progressed through the stages of spreading.

In order to analyze CD9 contributions to platelet function, a Fab fragment was generated from mAb7, an antibody which binds with high affinity to the large extracellular loop of CD9. Fab fragments were used to avoid complications of bound antibody crosslinking CD9 with the platelet FcyRII receptor. Confocal analysis of platelet spreading indicated that CD9 perturbation by mFab7 resulted in increased platelet spreading on a variety of matrices, although platelet adhesion was unaffected on all matrices except fibrin. Platelet-platelet interactions, including aggregation and disaggregation, were studied using light transmission aggregometry. CD9 perturbation increased the extent of platelet aggregation in response to threshold levels of agonist, with diminished effect with more potent agonists such as TRAP (Thrombin Receptor Agonist Peptide). CD9 perturbation also diminished, but did not completely inhibit, platelet disaggregation induced by eptifibatide. Interestingly, CD9 perturbation did not alter platelet-mediated clot retraction of platelets in PRP (Platelet rich plasma). In response to CD9 alteration by mFab7, both increased fibrinogen binding and platelet activation (as evidenced by CD63 and P-selectin expression) were elicited in the presence of threshold ADP levels. Both responses were dependent on presence of low-level agonist, as CD9 perturbation in the absence of agonist yielded no effect.

Cytoskeletal isolation revealed that CD9 perturbation results in increased incorporation of actin and other cytoskeletal molecules into the platelet core cytoskeleton. Immunoprecipitations results showed increased CD63 incorporation into the tetraspanin web, although the level of CD9 coprecipitation with GPIIb-IIIa was unaffected. A preliminary analysis of tyrosine phosphorylation, particularly Akt, did not reveal alterations on tyrosine phosphorylation of signaling molecules.

These results suggest that CD9 functions as a scaffold, organizing molecules into specific domains on the platelet surface. It appears to function as
a “gatekeeper” for GPIIb-IIIa activation, particularly modulating activation and aggregate stability at low levels of agonist.
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<tbody>
<tr>
<td>ABP</td>
<td>Actin binding protein</td>
</tr>
<tr>
<td>ACD</td>
<td>Acid-citrate-dextrose</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>Akt</td>
<td>Cellular signaling molecule</td>
</tr>
<tr>
<td>Arp</td>
<td>Actin-related protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BFU</td>
<td>Burst forming unit</td>
</tr>
<tr>
<td>BiP</td>
<td>Binding protein</td>
</tr>
<tr>
<td>BME</td>
<td>Beta-mercapto ethanol</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CapZ</td>
<td>Capping protein</td>
</tr>
<tr>
<td>CD</td>
<td>Clusters of differentiation</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CGS</td>
<td>Citrate-Glucose-Salt</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cell line</td>
</tr>
<tr>
<td>CLEC</td>
<td>C-type lectin receptor</td>
</tr>
<tr>
<td>CMP</td>
<td>Common myeloid progenitor</td>
</tr>
<tr>
<td>Csk</td>
<td>C-terminal Src kinase</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>EC</td>
<td>Extracellular</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>EWI-2</td>
<td>Glu-Trp-Ile (EWI) motif-containing protein 2</td>
</tr>
<tr>
<td>Fab</td>
<td>Antigen-binding fragment of an antibody</td>
</tr>
<tr>
<td>Fak</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>Fb</td>
<td>Fibrin</td>
</tr>
<tr>
<td>FbFN</td>
<td>Fibrin-fibronectin cross-linked matrix</td>
</tr>
<tr>
<td>Fc</td>
<td>Constant fragment of an antibody</td>
</tr>
<tr>
<td>FG</td>
<td>Fibrinogen</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FN</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>GEMM</td>
<td>Granulocyte, erythrocyte, megakaryocyte, monocyte</td>
</tr>
<tr>
<td>GP</td>
<td>Glycoprotein</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HBEGF</td>
<td>Heparin binding EGF-like growth factor</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic stem cell</td>
</tr>
<tr>
<td>HT-1080</td>
<td>Human fibrosarcoma tumor cells</td>
</tr>
<tr>
<td>ILK</td>
<td>Integrin-linked kinase</td>
</tr>
<tr>
<td>IBP</td>
<td>Integrin binding protein</td>
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IP3  Inositol 3-phosphate
ITAM  Immunoreceptor tyrosine-based activation motif
ITIM  Immunoreceptor tyrosine-based inhibitory motif
KAI1  Tetraspanin CD82
kDa  Kilodalton
KO  Knock-out
LIBS  Ligand induced binding site
LSM  Laser scanning microscope
mAb  Monoclonal murine antibody
MAPK  Mitogen-activated protein kinase
MEP  Megakaryocyte-erythroid progenitor
MFI  Mean fluorescence intensity
MK  Megakaryocyte
NR  Nonreduced
P2Y  Purinergic receptor
PAR  Protease activated receptor
PBS  Phosphate-buffered saline
PETA  Platelet-endothelial cell tetraspan antigen
PHEM  Buffer containing PIPES, HEPES, EGTA, MgCl₂
PI3K  Phosphoinositols 3-kinase
PIP2  Phosphatidylinositol bisphosphate
PLC  Phospholipase C
PKB  Protein kinase B, also known as Akt1
PKC  Protein kinase C
PP1c  Protein phosphatase 1
PPP  Platelet-poor plasma
PRP  Platelet-rich plasma
PSG17  Pregnancy specific glycoprotein 17
PSGL  P-selectin glycoprotein ligand
PVDF  Polyvinylidifluoride
Rac  A small guanosine triphosphate, member of Ras family
RANKL  Receptor Activator of Nuclear factor-κB Ligand
rds  Retinal degeneration slow
Rho  A small guanosine triphosphate, member of Ras family
ROM-1  Retinal outer segment membrane protein 1
SDS-PAGE  Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SLP-76  Adapter signaling protein
Src  A cytoplasmic tyrosine kinase
Syk  Spleen tyrosine kinase
TBS  Tris-buffered saline
TGF  Transforming growth factor
TM  Transmembrane
TM4SF  Tetraspanin super family
TP  Thromboxane A2 receptor
TRAP  Thrombin receptor agonist peptide
TRITC  Tetramethyl rhodamine isothiocyanate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>TSSC</td>
<td>Tumor-suppressing subchromosomal transferable fragment cDNA</td>
</tr>
<tr>
<td>VASP</td>
<td>Vasodilator-stimulated phosphoprotein</td>
</tr>
<tr>
<td>Vav</td>
<td>Signaling protein</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiskott-Aldrich Syndrome protein</td>
</tr>
<tr>
<td>% PA</td>
<td>Percent platelet aggregation</td>
</tr>
<tr>
<td>% PD</td>
<td>Percent platelet disaggregation</td>
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CHAPTER 1 INTRODUCTION

1.1 Overview

Platelets are anucleate, disc-shaped cell fragments generated from megakaryocytes. They circulate through the vasculature and play a dual role in that they are critical for regulating hemostasis and wound healing (constituting a primary defense against bleeding) and thrombosis (which can lead to thrombotic disorders under pathological conditions). Platelets express a wide array of adhesion receptors that mediate adhesion, spreading, and cell-cell interactions. Tetraspanins are one family of proteins that modulate cell adhesion events in concert with integrins. Platelets express several tetraspanins—most prominently CD9—at 50,000-80,000 copies per platelet.

Tetraspanins constitute a unique class of integral membrane-spanning proteins. Four transmembrane domains, intracellular carboxy and amino termini, two extracellular loops, and unique motifs in the large extracellular loop characterize this family of proteins. Tetraspanins have been implicated in regulation of platelet integrin function, such as platelet adhesion, activation, and aggregation, yet the exact role of CD9 in human platelets has remained unclear. Based upon data in other cell model systems, it is hypothesized that CD9 may regulate platelet adhesive functions by regulating integrin activation and post-receptor occupancy events, such as thrombus stability.

1.2 Purpose of the Study

The purpose of this study is to determine the role of CD9 in human platelets. For the first time, the functional activity of CD9 in platelets will be defined by using a well-characterized anti-CD9 Fab fragment generated from an antibody (mAb7) that has been shown in other studies to modulate cell adhesive events. These data are critical for understanding which aspects of platelet function tetraspanins most influence and how tetraspanins, specifically CD9, may regulate platelet response to vascular injury. It is also hypothesized that CD9 function likely occurs on the platelet surface—such as adhesive functions or interaction with the integrin GPIIb-IIIa—mediated through its extracellular loops. This is because specific motifs in its cytoplasmic domains known to interact with platelet signaling molecules or adaptor proteins have not been identified. Thus, CD9 through its extracellular loop regions may function at threshold levels of activation, with a role in regulating the extent of integrin activation.

Through understanding the function of CD9, the influences of platelet pathophysiological responses can be further appreciated. These findings may lead to advances in wound healing, hemostasis and thrombosis, and vascular
cardiovascular diseases such as acute coronary syndromes by targeting CD9 and its function for pharmacological intervention.

1.3 Aims and Research Questions

1.3.1 Specific Aim 1

Determine the localization and protein partners of CD9 in human platelets. Flow cytometry will be performed to analyze CD9 expression in resting and activated platelets, which will shed insight if CD9 is primarily important in platelet activation or activated platelets, or if it primarily regulates other proteins during platelet events. Flow cytometry will also reveal relative surface expression of other members of the tetraspanin web in resting and activated platelets.

Immunoprecipitation and confocal microscopy will be used to determine the association and localization of CD9 and GPIIb-IIIa, the primary platelet integrin. It is anticipated that CD9 might exert its effect through regulating the extent of GPIIb-IIIa activation. These results will yield insight into the effect of platelet activation and integrin ligand binding on the CD9-GPIIb-IIIa association by platelets spread on substrate and in suspension. Experiments with platelets expressing a mutant GPIIb-IIIa will provide preliminary insight into possible areas of GPIIb-IIIa critical to its association with CD9.

Confocal microscopy will also be used to analyze the localization of CD9 into platelet spreading structures such as filopodia, lamellipodia, and platelet-platelet contact sites to initially determine if CD9 is involved in platelet adhesive events. Co-localization with F-actin will yield insight into CD9’s linkage with the cytoskeleton, although this could occur directly or indirectly through other members of the tetraspanin web.

1.3.2 Specific Aim 2

Determine platelet functions affected by CD9 perturbation. The effect of CD9 on platelet events will be determined by creating a Fab fragment which binds to the extracellular region of CD9. The full-length antibody has been shown to have a functional effect in smooth muscle cells and Chinese hamster ovary cells. A Fab fragment is used here to ensure that results are due to CD9 ligation without involvement of the receptor FcγRII.

Major platelet functions involved in this study include platelet aggregation and disaggregation, platelet adhesion and spreading on four substrate matrices—all of which are ligands for GPIIb-IIIa, ligand binding and platelet activation (via flow cytometry), and platelet-mediated clot retraction. These
events are mediated by GPIIb-IIIa, and it is anticipated that CD9, by regulating this integrin, will alter these events.

Aggregation and disaggregation, ligand binding, and platelet activation will be analyzed using low concentrations of platelet agonists. It is anticipated that CD9 will function as a gatekeeper for integrin activation, and that its impact will be most pronounced at threshold levels of platelet activation. Stronger levels of agonist would be expected to overwhelmingly activate the integrin, regardless of CD9 signaling.

1.3.3 Specific Aim 3

Determine mechanisms involved in the contribution of CD9 to platelet function. This includes examination of the tetraspanin web, incorporation of cytoskeletal proteins into the core cytoskeleton, phosphorylation of key signaling molecules, and the effect of CD9 perturbation on each.

CD9 may function as a membrane protein scaffold, and may be critical in recruiting cell surface molecules and organizing signaling complexes in the tetraspanin web. In this role, CD9 may function to keep proteins in contact with or away from GPIIb-IIIa or to maintain a desirable (active or inactive) conformation of GPIIb-IIIa. Flow cytometry will analyze the impact of CD9 perturbation on association of CD9, GPIIb-IIIa, and CD63 in resting and activated platelets. Platelet core cytoskeletons will be isolated and analyzed to determine the role of CD9 on the incorporation of cytoskeletal proteins. Platelet signaling molecules will also be evaluated, primarily phosphorylation of Akt. Research in our lab has shown the Akt pathway to be the primary CD9 signaling pathway (Kotha 2008). However, total tyrosine phosphorylation will also be analyzed to examine a possible involvement of another pathway.

It is anticipated that the linkage of CD9 to the cytoskeleton or signaling molecules is indirect, through GPIIb-IIIa, due to the short intracellular regions of CD9, although a novel CD9 binding partner may be discovered. Since CD9 is likely involved in mediating activation of GPIIb-IIIa, its cytosolic partners could also be altered.

1.4 Significance

Genetic research shows a biological significance for tetraspanins in mammalian systems. Inactivation of the tetraspanin TALLA-1 causes X-linked mental retardation (Zemni 2000); CD9 and CD81 knock-out mice have reduced fertility, with double knock-out mice being completely infertile (Kaji 2000, LaNaour 2000, Miyado 2000, Rubinstein 2006); and CD37 knock-out mice have reduced
immune response. In the platelet system, CD151 or TSSC-6 knock-out mice have impaired integrin outside-in signaling (Goschnick 2006, Lau 2004).

Currently available studies of CD9 functions in platelets have yielded limited information. At present, there have been no published studies showing defects or abnormalities in platelet function in the CD9 knock-out mice; however, there is no information regarding expression levels of (and possible compensation by) other tetraspanins or integrins. Use of anti-CD9 antibodies have consistently activated signaling mechanisms through the receptor FcγRII, making interpretation of results difficult. In this study, Fab fragments will be used in place of antibodies; thus, CD9 ligation can still be achieved without the involvement of the FcγRII.

The results of this project will lead to better understanding of the basic biology of CD9 and its role in platelet function. This will add to the knowledge of platelet function that could lead to the development of pharmacological agents that target CD9 and arrest thrombosis.

These results will also lead to a broader understanding of cell adhesive function and better define a family of molecules that regulate integrins, key receptors in cell adhesion, differentiation, and proliferation of cells. The functional role of the CD9-GPIIb-IIIa interaction is, at present, poorly defined. The ability of tetraspanins to modulate integrin conformation, as well as membrane complexes, ligand binding, and integrin-associated cell signaling pathways, will be more clearly defined, and our understanding of communication through platelet-platelet and platelet-matrix interactions will be advanced.
CHAPTER 2 REVIEW OF LITERATURE

2.1 Platelets

2.1.1 Platelet Formation and Function

Platelets are small cell fragments released from megakaryocytes, composed of a membrane, cytoplasm, granules, and organelles. Approximately $10^{11}$ platelets are produced in the human body on a daily basis; once released from the bone marrow megakaryocytes, they circulate in the blood for seven to ten days (Harker 1969). Their function is to maintain vascular integrity by regulating hemostasis and thrombosis.

Megakaryocytes are derived from hematopoietic stem cells (HSCs), which evolve from the multipotent haemangioblast, the precursor for all blood and blood vessel cells. The HSC yields the early Common Myeloid Progenitor (CMP), which is cloned as the (Granulocyte, Erythrocyte, Megakaryocyte and Monocyte) Colony-Forming Unit (CFU-GEMM). The CMP differentiates into the Megakaryocyte-Erythroid Progenitor (MEP), which yields both the erythroid and megakaryocytic lineages, controlled by GATA-1 (which regulates granulocyte and monocyte precursors). Cytokines and chemokines, in response to environmental factors, allow differentiation of MEP into the highly proliferative BFU-MK (Megakaryocyte Burst-Forming Unit) or the more mature CFU-MK (megakaryocyte colony-forming unit). These cells progress into megakaryoblasts, which are incapable of cell division but able to replicate DNA (Deutsch 2006). This yields the immature, polyploid (up to 128 N) megakaryocyte (Tomer 1988), containing elevated RNA levels, prominent ribosomes and rough endoplasmic reticulum, alpha and dense granules, and a primitive demarcation membrane. The maturing megakaryocyte develops a horseshoe-shaped nucleus and an expanded cytoplasm and amplifies platelet organelles and the demarcation membrane, which is continuous with the plasma membrane (Breton-Gorius 1976). The cell surface expresses GPIIb-IIIa (αIIbβ3), GPIX, and GPIb, and the granules contain von Willebrand factor (vWF), platelet factor 4, β-thromboglobulin, fibrinogen, coagulation factor VIII, and factor V (Breton-Gorius 1976). Megakaryocytes occur in about one in 2000 nucleated bone marrow cells, although this number may increase ten-fold in response to thrombocytopenia (Branehog 1975).

The megakaryocyte can extend long proplatelet projections; the demarcation membrane serves primarily as a membrane reservoir for proplatelet formation. These are filled with the proper specific organelles and granule contents, which migrate from the cell body to the proplatelet ends, to give rise to new circulating platelets (Italiano 1999, Italiano 2003). A single megakaryocyte may give rise to 2000-5000 new platelets (Long 1988). Two models suggest how
platelets arise: platelets may arise and bud off the tip of proplatelet (Choi 1995, Patel 2005), or there may be preformed territories with internal membranes that are released by fragmenting from the membrane (Mori 1993, Zucker-Franklin 1984).

Resting platelets circulate as small discs, approximately 0.5 x 3.0 μm in humans. An internal microtubule beneath the plasma membrane, composed of 13 stacks of tubulin subunits arranged head-to-tail in protofilaments, maintains the disc shape (Lecine 2000). It remains unclear whether the microtubule coil has any other function, as well as any advantage of a disc-shape (Hartwig 2006).

An internal actin filament cytoskeleton maintains platelet integrity. The two main components of the actin filament cytoskeleton are the membrane skeleton and the cytoplasmic actin scaffold.

Upon platelet activation, the microtubule reorganizes into multiple short microtubules that run from the platelet center toward outward protrusions, as well as a forming a compressed coil in the center of a spread platelet. Microtubule kinetics is controlled by dynein, a minus-end-directed motor, and kinesins, which are mainly plus-end-directed motors (Hartwig 2006).

2.1.2 Platelet Physiology

2.1.2.1 Activation by Agonists. Platelets express a number of agonist receptors: GPVI (collagen) (Clemetson 2001), PAR-1 and PAR-4 (thrombin) (Coughlin 2005), P2Y₁ and P2Y₁₂ (ADP) (Gachet 2006), and receptors for thromboxane A₂ (Thomas 1998) and epinephrine (Pozgajova 2006). A summary of platelet agonists can be found in Table 2.1.

Platelet agonists can be found in increased concentrations at sites of vascular injury. They lead to platelet activation, causing shape change, integrin activation via inside-out signaling, and granule secretion (see Table 2.2).

2.1.2.2 Adhesion and Spreading. Immediately upon vascular injury, platelets tether to the exposed subendothelium and become activated by the agonists present at the site of injury (collagen and thrombin). Once activated, platelets become firmly adherent and will release their storage granules, which contain ADP and other substances (see Table 2.2). This in turn promotes additional platelet recruitment and platelet aggregation (Denis 2007) (see Fig 2.1 and Fig 2.2).

Initial platelet tethering is mainly carried out by the GPIb-IX-IV complex binding to exposed von Willebrand factor (vWF) (see Fig 2.2), which undergoes a conformational change when bound to the matrix or exposed to high shear conditions (Bergmeier 2000, Berndt 2001, Ruggeri 2003). The interaction
Table 2.1  Comparison of Platelet Agonists

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Receptor</th>
<th>G Protein</th>
<th>Signaling Molecules</th>
<th>Effect</th>
<th>Importance</th>
<th>Medical Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>P2Y₁</td>
<td>Gq</td>
<td>PLCβ₂, RhoA, Rac, Src kinase, PKC</td>
<td>Weak, transient activation of GPIIb-IIIa</td>
<td>Shape change, initiation of aggregation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P2Y₁₂</td>
<td>Gi2</td>
<td>Inhibit adenylyl cyclase and cytosolic cAMP; activate PI3K, Akt/PKB, Rap1b, src family kinases</td>
<td>GPIIb-IIIa activation, dense granule release</td>
<td>Sustained aggregation response, leading to thrombus growth and stabilization</td>
<td>Ticlopidine Clopidigrel</td>
</tr>
<tr>
<td>Thrombin</td>
<td>PAR1</td>
<td>Gq, G₁₂/₁₃, Gi</td>
<td>PLCβ, IP₃, DAG, PKC, Rho/Rho kinase, inhibition of adenylyl cyclase</td>
<td>Strong activation of GPIIb-IIIa in response to low thrombin levels</td>
<td>Cytoskeletal rearrangement, shape change, degranulation, adhesion</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PAR4</td>
<td>Gq, G₁₂/₁₃</td>
<td>PLCβ, IP₃, DAG, PKC, Rho/Rho kinase,</td>
<td>Strong activation of GPIIb-IIIa in response to high thrombin levels</td>
<td>Cytoskeletal rearrangement, shape change, degranulation, adhesion</td>
<td></td>
</tr>
<tr>
<td>Collagen</td>
<td>α₂β₁</td>
<td>----</td>
<td>Syk, SLP-76, PLC</td>
<td>Activation of GPIIb-IIIa</td>
<td>Outside-in platelet activation</td>
<td></td>
</tr>
<tr>
<td>GPVI, GPIV</td>
<td>----</td>
<td>----</td>
<td>Src, Fyn, Lyn, Syk, PI3K</td>
<td>Activation of GPIIb-IIIa</td>
<td>Calcium release</td>
<td></td>
</tr>
</tbody>
</table>

(continued)
Table 2.1 (continued)

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Receptor</th>
<th>G Protein</th>
<th>Signaling Molecules</th>
<th>Effect</th>
<th>Importance</th>
<th>Medical Targets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epinephrine</td>
<td>α2-adrenergic receptors</td>
<td>----</td>
<td>IP3, DAG, PKC, PLC</td>
<td>Activation of GPIIb-IIIa</td>
<td>Granule secretion, aggregation</td>
<td>Aspirin, NSAIDS, others</td>
</tr>
</tbody>
</table>

Source:


<table>
<thead>
<tr>
<th>Granule</th>
<th>Alpha Granules</th>
<th>Dense Granules</th>
<th>Lysosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td># per platelet</td>
<td>80</td>
<td>7</td>
<td>2-4</td>
</tr>
<tr>
<td>Contents</td>
<td>Growth factors, coagulation proteins, adhesion molecules, cytokines, cell-activating agents, angiogenic factors, platelet-specific molecules (coagulation factor V, thrombospondin, P-selectin, von Willebrand factor), endocytosed molecules (FG); some contain exosomes</td>
<td>High concentrations of small molecules important to cell activation (ADP, serotonin, calcium, magnesium, pyrophosphate, nucleotides)</td>
<td>Components of endosomal-lysosomal degradative pathway (acid hydrolases, cathepsins D and E)</td>
</tr>
<tr>
<td>Development</td>
<td>Fusion of vesicles budding from trans-Golgi apparatus in megakaryocytes, or fusion of vesicles endocytosed from the plasma membrane</td>
<td>Endogenous synthesis in megakaryocytes; may develop from fusion with endocytic vesicles</td>
<td>Different ontogeny than alpha or dense granules, yet share common antecedents</td>
</tr>
<tr>
<td>Disorders</td>
<td>Gray platelet syndrome (variable bleeding diathesis; small abnormal vesicles; defect in formation or packing of alpha granule)</td>
<td>Dense Storage Pool Deficiency (decreased thrombus formation, varying hemostatic defect)</td>
<td></td>
</tr>
</tbody>
</table>
Platelets adhere to a matrix initially having a round morphology, but immediately extend fingerlike projections termed filopodia. Then lamellipodia are extended, which have been compared to webbing between a duck's toes. Finally, the membrane fully extends to allow the platelet to cover a large surface area.
Figure 2.2  Platelet Activation Leading to Adhesion and Spreading or Aggregation
Interaction of the platelet receptors GPIb and α2β1 with vWF or collagen on the exposed subendothelium leads to platelet activation and spreading on the subendothelium. Nearby platelets are recruited to form a thrombus and maintain hemostasis. Platelets can also be activated by soluble agonists (such as ADP), leading to platelet activation, granule secretion, and aggregation via binding and crosslinking GPIIb-IIIa on adjacent platelets.
between GPIb and vWF can withstand very high shear rates, yet is characterized by fast association and dissociation rates, allowing slow platelet translocation along the vessel wall (Savage 1998). In the absence of VWF, GPIb can still initiate platelet tethering through other ligands, possibly thrombospondin-1 (Bergmeier 2006, Jurk 2003). Under cases of low shear, a direct interaction between exposed collagen in the subendothelium and the platelet receptors α2β1 and GPVI can support adhesion (Clemetson 2001).

Collagen and thrombin at the site of injury lead to platelet activation and inside-out signaling. As platelets then become activated, platelet integrins—particularly GPIIb-IIIa—transition from their inactive conformation to an active conformation capable of binding agonists and transmitting intracellular signals. Activated integrins then bind their ligand on the vessel wall, develop filopodia and lamellipodia and become fully spread, and release the contents of their granules (see also Table 2.2). ADP and thromboxane A2 are among the granule contents released, promoting activation, and leading to the formation of a platelet plug and arrest of bleeding (Coleman 2004).

2.1.2.3 Aggregation. The major platelet protein involved in aggregation is GPIIb-IIIa (Fig 2.2), which is present in the platelet membrane and alpha granules, providing an extra pool of the integrin on the platelet surface upon platelet activation and granule release (Bennett 2005). Absent or dysfunctional GPIIb-IIIa leads to the bleeding defect Glanzmann thrombasthenia, characterized by platelets that are unable to form stable aggregates (Nurden 2005). Fibrinogen is the major GPIIb-IIIa ligand, capable of causing platelet aggregation by cross-linking GPIIb-IIIa on adjacent platelets. Fibrinogen can also be converted to fibrin by thrombin; the resulting fibrin can cross-link with fibronectin, contributing to thrombus stability by anchoring the thrombi to the vessel wall.

Thrombus formation is not abolished in fibrinogen-deficient mice, and other ligands for GPIIb-IIIa include vWF and FN (Ni 2000). vWF plays an essential role in occlusive thrombi formation at both arterial and venous shear rates (Nanda 2005, Ni 2000). Absence of vWF prevents additional thrombus growth and occlusion (Ni 2000). Additionally, in mice with reduced plasma FN levels, thrombus formation is highly defective, with increased emboli and delayed vessel occlusion (Ni 2003). However, this defect is specific to arteries, as no defect is found at venous shear (Matsukova 2006).

2.1.2.4 Cytoskeletal Rearrangement. In order for the platelet to be able to extend filopodia and lamellipodia, release its granules, and form stable aggregates, it must be able to rearrange its cytoskeleton.

Upon agonist exposure, platelets lose their discoid shape, becoming spherical and then rapidly extending filopodia and spreading on a surface. Shape change is driven by reorganization of the cytoskeleton and a doubling of the actin filament content (converted from globular actin) (Hartwig 2006).
The membrane skeleton is first disassembled. It is released from actin and becomes centered in spread platelets. This is partly mediated by adducin, which is dissociated from spectrin and the barbed ends of actin. Adducin is inactivated by PKC-mediated phosphorylation, phosphatidylinositides, or calcium-calmodulin-binding (Matsuoka 2000).

The cortical actin network is then disrupted. Gelsolin (20,000 per platelet) and cofilin (100,000 per platelet) function to fragment actin filaments, resulting in F-actin disassembly: gelsolin severs and caps barbed ends, while cofilin promotes the disassembly of actin from pointed ends. Increasing cytosolic concentrations of calcium triggers a conformational change in gelsolin, allowing it to bind and fragment actin. Gelsolin remains bound to the barbed end of actin until it is removed by polyphosphoinositides. Cofilin must be dephosphorylated to become active, requiring 15 to 60 seconds for maximal activation (Falet 2005). Gelsolin and cofilin, combined with the release of adducin from spectin-actin, remodel the membrane and actin cytoskeletons.

New actin filament assembly is initiated from the ends of actin filaments adjacent to the plasma membrane. Formation of lamellipodia, required for circumferential platelet spreading on the surface, requires a large amount of F-actin. This need is met as platelets double their cellular F-actin content as filaments form and grow through barbed-end growth, via uncapping and fragmentation of barbed filament ends and activation of actin-related protein 2/3 (Arp 2/3) (Hartwig 2006). Approximately 50% of gelsolin is dissociated from barbed ends of actin filaments, and the Arp 2/3 complex binds to actin near the barbed ends and nucleates a second filament. Upstream proteins and phospholipids, including Wiskott-Aldrich syndrome protein (WASP) family members and cortactin, regulate Arp 2/3 nucleation activity. Actin filament assembly is terminated via CapZ constitutively capping exposed barbed filaments; filopodial growth in cells can be promoted by modulating the activity of CapZ via proteins such as VASP/Mena (Gertler 1996, Mejillano 2004).

2.1.2.5 Clot Retraction. Platelet-mediated clot retraction is a necessary step for consolidation of a platelet thrombus. Retraction keeps the thrombus from falling apart by making it less susceptible to fibrinolysis (Denis 2007) and aids in wound closure, as the damaged edges are drawn together (Parise 1999).

Interactions between GPIIb-IIIa and fibrin (which is cleaved from fibrinogen by thrombin) results in outside-in signaling. Clot retraction is brought about as tyrosine residues on the cytoplasmic tail of GPIIb are phosphorylated (Phillips 2001, Shattil 2004), and cytoskeletal assembly and molecular translocations occur (Parise 1999). Clot retraction appears to be regulated through multiple pathways, including activation of PLCβ and Rho kinase, which activate MLC kinase and inhibit MLC phosphatase, and PLCγ2 downstream of Src kinase (Suzuki-Inoue 2007). This process is dependent on the contractile
protein thrombasthenin, as well as the interaction between actin and myosin (Suzuki-Inoue 2007).

2.1.3 Platelet Receptors

2.1.3.1 G Protein-Coupled Receptors. G-protein coupled receptors are one family of surface receptors involved in platelet activation (see Fig 2.3). These receptors contain seven membrane-spanning domains, and an intracellular and an extracellular terminus, and are also referred to as "seven transmembrane receptors" or serpine receptors. The intracellular domain of each of these receptors is coupled to a distinct heterotrimeric G protein, belonging to either the Gi, Gq, G12/13, or Gs families. Upon activation, the G protein triggers an intracellular signaling cascade resulting in platelet shape change, granule secretion, and platelet aggregation via integrin activation.

G-protein coupled receptors in human platelets include the ADP receptors P2Y₁ and P2Y₁₂, the thrombin receptors PAR-1 and PAR-4, and the thromboxane A2 receptors TPα and TPβ.

The P2Y₁ receptor is coupled to Gq and phospholipase Cβ, which leads to activation of the small G proteins RhoA and Rac and Src kinases (Kahner 2006). Activation of PLCβ leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to inositol triphosphate (IP3) and diacyl glycerol (DAG), leading to calcium release, PKC activation, and weak, transient activation of GPIIb-IIIa (Gachet 2006, Kahner 2006). P2Y₁ is important for platelet shape change, the initial wave of reversible aggregation, thromboxane A2 generation, procoagulant activity, adhesion to immobilized fibrinogen, and thrombus formation under shear (Cattaneo 2005, Murugappan 2006).

P2Y₁₂ is coupled to Gi2 (Gachet 2006). Activation leads to inhibition of adenyl cyclase and inhibition of cytosolic cAMP concentration via the Gα subunit, and activation of phosphoinositide 3-kinase (PI3K), Akt/PKB, Rap1b, and src family kinases (Gachet 2006, Kahner 2006). This receptor is important for stable platelet aggregation, in addition to sharing many of the same functions as P2Y₁ (Cattaneo 2005, Murugappan 2006). The combined action of both receptors is needed for full platelet aggregation in response to ADP (Cattaneo 2005). P2Y₁₂-blocking drugs such as clopidigrel and ticlopidine are clinically being used in the treatment of thrombotic disorders.

PAR1, which responds to low thrombin concentrations, and PAR4, which responds to higher thrombin concentrations, are activated by cleavage of an extracellular portion of the receptor. PAR4 is coupled to Gq and G12/13, and PAR1 is coupled to G1, G12/13, and Gi (Offermans 2006). Gq is coupled to PLCβ, especially β2 (leading to activation of PKC). G12/13 regulates the Rho/Rho kinase pathway, and Gi inhibits adenyl cyclase (Offermans 2006).
Figure 2.3  Platelet Receptors
A, G protein-coupled receptor; B, Integrin; C, Leucine-rich repeat protein; D, Immunoglobulin receptor; E, Tetraspanin.
These steps lead to platelet cytoskeletal rearrangement, resulting in platelet shape change and platelet degranulation and adhesion (Offermans 2006).

TPα and TPβ are coupled to G13 and Gq, resulting in activation of Rho-GEF and PLCβ. Both are important in platelet activation and shape change, and blockade of these receptors results in increased bleeding times and protection from thromboembolism (Murugappan 2004).

2.1.3.2 Integrins. Integrins are a family of integral cell-surface receptors made up of an alpha and a beta chain. Each chain consists of a large extracellular domain, a single transmembrane domain, and a short cytoplasmic domain (Fig 2.3). In mammals, 19 alpha and eight beta subunits combine to form 24 integrins (Humphries 2000). They mediate contact between a cell and its environment and transmit signals across the plasma membrane.

Integrins undergo a highly regulated structural change, providing a transition from a bent state with a low affinity for ligand binding (inactive) to an extended, open state with a high affinity for ligand binding (active) (Adair 2002, Takagi 2002, Vinogradova 2002). Conformational changes resulting from ligand binding may result in the unmasking of specific epitopes, separate from the ligand-binding domain, on the integrin. These “ligand-induced binding sites”, or LIBS, offer a means to study integrin activation and ligand binding without occupying the ligand-binding domain.

Integrin signaling can be grouped into “inside-out” signals, which control activation of the integrin, and “outside-in” signals, resulting from ligand binding. Inside-out signaling results from a stimulus from another cell receptor (such as G Protein-Coupled Receptors), which leads to a stimulus acting on the cytoplasmic portion of the integrin and modifying the contacts between the cytoplasmic tails, leading to a change in the transmembrane domains and altering integrin affinity for ligand and integrin avidity (via integrin clustering on the cell surface). Outside-in signals lead to recruitment of intracellular signaling molecules and cytoskeletal proteins, resulting in changes in platelet cytoskeleton and morphology (Arias-Salgado 2005; Han 2006; Tadokoro 2003).

Platelets express several integrins, including αIIbβ3, which binds fibrinogen, fibrin, fibronectin, and von Willebrand factor; αvβ3, which binds vitronectin; α2β1, which binds collagen and laminin; α5β1, which binds fibronectin; and α6β1, which binds laminin. Of these, αIIbβ3, or GPIIb-IIIa, has the highest expression level, at 50,000-80,000 per platelet, and has been the most extensively characterized.

αIIb is composed of 1008 amino acids. The extracellular domain is composed of an N-terminal β-propeller domain, thigh domain, and two calf domains. β3 is composed of 762 amino acids, with an extracellular domain consisting of an A domain, plexin/semaphorin/integrin domain, four EGF
domains, and a membrane-proximal βTD domain (Xiong 2001). The extracellular domains of the α and β subunits combine to form an ellipsoid "head" consisting of the β-propeller and A-domain, and two tails (Ma 2007). The transmembrane domains of each subunit form alpha helices that are believed to interact when the integrin is in a resting conformation (Adair 2002, Gottschalk 2005). The cytoplasmic tails form a complex between the membrane-proximal helices that is maintained by electrostatic and hydrophobic interactions; disruption of both these interactions leads to integrin activation (Ma 2006, Vinogradova 2002, Weljie 2002). The membrane-distal regions of the cytoplasmic tails include a divalent ion-binding site, although the significance of this feature is uncertain (Haas 1996). There is also a phosphotyrosine binding domain critical to integrin activation (Chen 1994, O’Toole 1995, Van der Geer 1995). More than 20 proteins have been identified as binding partners, most prominently talin (which links the integrin to the actin cytoskeleton and is critical in integrin-mediated focal adhesion formation) (Burr ridge 1996, Rees 1990). The cytoplasmic domains can also directly interact with the cytoskeletal proteins myosin, skelemin, filamin, α-actinin, and F-actin; the adaptor/signaling proteins paxillin, Shc, and Grb 2; the protein kinases/phosphatases Src, Csk, Syk, ILK, FAK, and PP1c; and BiP, Calreticulin, and β3-endonexin (Ma 2007). αIIbβ3 can also bind with the transmembrane proteins CD36, CD47/IAP, CD98, and CD31, as well as the tetraspanins CD9, CD63, and CD151, although these interactions likely involve interactions with the transmembrane and extracellular domains (Ma 2007).

Inside-out signaling of GPIIb-IIIa is initiated by various platelet agonists, including thrombin, ADP, and collagen; each is capable of initiating inside-out signaling events, but they likely act cooperatively in a physiological environment. Agonist signaling leads to activated PKC, which regulates serine/threonine phosphorylation of GPIIb-IIIa. DAG, calcium, and PI3K regulate GPIIb-IIIa activation through Rab1b (a small GTPase of the Ras family), thus affecting binding of proteins, particularly talin, to the cytoplasmic domain of GPIIb-IIIa (Focosi 2007). The head domain of talin activates GPIIb-IIIa by binding to actin directly or indirectly through vinculin and α-actinin. The binding of β3-endonexin can also activate GPIIb-IIIa by binding to the β3 NITY motif. Integrin binding protein (IBP) binds to αIIb and appears to be involved in integrin activation (Platelets 2007).

Following inside-out signaling, the integrin GPIIb-IIIa becomes competent to bind ligands (fibrinogen, fibronectin, fibrin, etc). Ligand binding alters the conformation of the integrin, as well as inducing integrin clustering. This further encourages binding of cytoskeletal proteins leading to outside-in signaling. Src kinases, which are constitutively bound to β3, become activated through autophosphorylation. This leads to recruitment of Syk and tyrosine phosphorylation of PLCγ2, which is necessary for lamellipodial formation. SLP-76 and Vav are also involved in regulation of PLCγ2. GPIIb-IIIa can also signal through Syk-independent cascades (Watson 2005).
αvβ3 is structurally similar to GPIIb-IIIa, although its alpha chain has a slightly longer cytoplasmic tail (Suzuki 1986). Vitronectin, not fibrinogen, is the preferred ligand, although it can bind several RGD-containing ligands (including osteopontin and adenovirus penton base) (DiGiovine 2001). ADP appears to induce the high-affinity conformation (Bennett 1997, Helluin 2000).

α2β1 is the only platelet integrin to contain an I domain, which usually constitutes the primary ligand-binding interface (Emsley 1997). This integrin binds collagen types I-VI and XI (Kehrel 1995), leading to ADP release (Atkinson 2003, Jung 2000) and phosphorylation of Src kinases, SLP-76, Syk, and PLC (Inoue 2003). Its absence leads to a bleeding disorder and reduced platelet response to collagen, although GPVI appears to be most important in collagen-mediated platelet activation (Kehrel 1995, Nieuwenhuis 1985).

α5β1 is important for platelet interaction with the fibronectin matrix. Unlike the β3 integrins, it can support adhesion under static conditions in the absence of platelet activation (McCarty 2004), although adhesion strength may be increased upon activation (Garcia 1998). Ligand interaction is unable to promote tyrosine phosphorylation, calcium release, or shape change (McCarty 2004), limiting the role of this integrin to initiating the interaction of resting platelets with the extracellular matrix (Kasirer-Friede 2007).

α6β1 is a receptor for laminins, a component of basement membranes and the extracellular matrix (Sonnenberg 1988). Platelet activation is not necessary for ligand binding (Sonnenberg 1991), and binding to laminin does not appear to induce granule release or aggregation (Nigatu 2006), although it does induce filopodial formation (Chang 2005).

2.1.3.3 Leucine-Rich Repeat Proteins. GPIb-IX-V is a platelet adhesion receptor in the Leucine-rich repeat family. Its major function is initiating thrombus formation at high shear stress in flowing blood (Andrews 2003). It binds to vWF in the subendothelial matrix or plasma, as well as Mac-1 on neutrophils and P-selectin on activated platelets and endothelial cells (Andrews 2003). Its absence or dysfunction leads to Bernard-Soulier syndrome, a rare but often severe bleeding disorder (Andrews 1997, Berndt 2001, Lopez 1998).

GPIb-IX-V consists of GPIbα disulfide-linked to GPIbβ, noncovalently complexed with GPIX and GPV in a 2:2:2:1 ratio (Fig 2.3) (Andrews 1997, Berndt 2001, Lopez 1998). All components are members of the leucine-rich repeat family. The N-terminal portion of GPIbα, consisting of eight leucine-rich repeats, contains binding sites for vWF, Mac-1, P-selectin, α-thrombin, clotting factors XI/XIIa, and high-molecular-weight kininogen (Berndt 2001).

High shear results in GPIb-IX-V binding von Willebrand factor, which is rapidly secreted following platelet activation. Following binding, platelets become activated, leading to shape change, spreading, and granule secretion caused by cytoskeletal rearrangement, recruiting additional platelets to the developing
thrombus. GPIb-IX-V signal transduction, involving PI-3-kinase, Syk, Src, and ERK-1/2, leads to activation of GPIIb-IIIa, mediating platelet aggregation (Andrews 2003, Berndt 2001).

In order to be competent to adhere and signal, GPIb-IX-V undergoes multiple post-translational modifications. GPIb sulfation within the ligand-binding domain regulates adhesion to vWF (Dong 2001, Huizinga 2002, Shen 2000, Tait 2002, Uff 2002) and thrombin (Berndt 2001); glycosylation of GPIb extends the ligand-binding domain and allows adhesion (Lopez 1998); cytoplasmic palmitoylation of GPIb and GPIX may regulate surface localization and association with other membrane proteins (Lopez 1998), and phosphorylation allows association with the signaling protein 14-3-3 (Bodnar 1999, Gu 1999).

2.1.3.4 Selectins. Selectins are proteins that bind glycoproteins and glycolipids in a calcium-dependent manner. They consist of an extracellular N-terminal lectin domain adjacent to a domain homologous to epidermal growth factor, a variable number of short consensus repeats, a single transmembrane domain, and a C-terminal cytoplasmic domain (Sperandio 2006, Thomas 2006).

Platelets express the P-selectin, also known as CD62P. P-selectin is found in alpha granules of platelets and is rapidly mobilized to the cell surface upon platelet activation, where it binds to P-selectin glycoprotein ligand 1 (PSGL-1) and Sialyl Lewis X-bearing glycoproteins (Smith 2007). The interaction between P-selectin and PSGL-1 allows platelet tethering to leukocytes under shear conditions and may contribute to leukocyte activation (Pitchford 2005, Zarbock 2006).

2.1.3.5 Immunoglobulin Receptors. Platelet Fc receptors mediate immune responses to IgG-containing complexes, implicating platelets in the adaptive immune response. These receptors contain immunoreceptor tyrosine-based activation motifs (ITAMS) (Fig 2.3) (Reth 1989), and upon ligand binding, Src-family kinases phosphorylate tyrosine residues within the ITAM sequence, leading to recruitment and activation of Syk or ZAP-70 tyrosine kinase, initiating downstream signaling events (Fodor 2006). These responses seem to be regulated by the phosphoinositides PI4,5P2 and PI3,4,5P3 (Booth 2006). The activation pathways are inhibited by immunoreceptor tyrosine-based inhibitory motifs (ITIMs), which bind to the downstream tyrosine phosphatases (Jackson 1997). In platelets, ITAM/ITIM signaling may be involved in hemostatic regulation as well as immune responses (Kasirer-Friede 2007).

Fc receptors are of particular importance in this study due to a localization and association of Fc receptors with tetraspanins. Anti-CD9 monoclonal antibodies crosslink CD9 to FcγRII, eliciting a potent activation response. While the functional relevance of this interaction has yet to be elucidated, tetraspanins have been shown to negatively regulate FcR signaling independently of co-ligation, possibly through interactions within the tetraspanin web (Moseley 2005).
Other receptors coupled to Fc receptors include GPVI, a collagen receptor (Berlanga 2000, Nieswandt 2000); GPIb-IX-V, a vWF receptor (Wu 2001, Arthur 2005); and potentially C-type lectin receptor-2 (CLEC-2), which has an as-yet unknown function in platelets (Gross 2006).

2.1.3.6 Tetraspanins. The structure and function of tetraspanins is discussed more fully in the following sections. Tetraspanin function in platelets is summarized in Table 2.3.

Platelets express several tetraspanins, among them CD9, CD63, CD151, and TSSC-6 (Fig 2.3, Fig 2.4, and Fig 2.5). They are believed to modulate integrin function, and indeed, both antibody-blocking and knockout studies yield platelets with incomplete GPIIb-IIIa functioning. Megakaryocytes have been shown to contain the transcripts for the tetraspanins NET5 (TSPAN9) and TSPAN33, and these may also be expressed at the protein level in platelets (Senis 2007).

The tetraspanin CD63, with an apparent molecular mass of 30-60 kDa, is expressed on platelet lysosomes in resting platelets and is expressed on the platelet surface upon exocytosis triggered by platelet activation. CD63 has been studied by blocking with a monoclonal antibody. These platelets retained normal adhesion to fibrinogen, but platelet spreading, F-actin reorganization, redistribution of vinculin, extensive tyrosine phosphorylation, and phosphorylation of Focal Adhesion Kinase (FAK) were diminished. CD63 was also shown to be linked to PI 4-kinase type II (Israels 2005).

The impacts of TSSC-6 and CD151 in platelet function have been studied using knock-out mice. It is important to note that expression levels of integrin GPIIb-IIIa is unaffected in these knock-outs (Goschnick 2006, Lau 2004).

TSSC-6 (Tumor-suppressing subchromosomal transferable fragment cDNA 6) is localized to the cell surface and in intracellular pools in murine platelets. Mice that are deficient for TSSC-6 exhibit increased tail bleeding time, increased blood loss, and more rebleeds in a tail-bleeding assay, all features of unstable hemostasis. They also exhibit secondary instability in a ferric chloride oxidative injury model, showing greater time to vessel occlusion and a greater number of emboli forming during the observation period. Impaired “outside-in” signaling of GPIIb-IIIa was evident, as these platelets had delayed clot retraction, defective spreading, and reduced aggregation at low concentrations of agonist (≤150 μM PAR-4, 5 μg/ml collagen). However, “inside-out” signaling was unaffected, as knockout mice showed normal fibrinogen binding, normal binding of an activation-dependent antibody to GPIIb-IIIa, and normal alpha granule release (Goschnick 2006).
### Table 2.3  Summary of Tetraspanins in Platelet Function

<table>
<thead>
<tr>
<th>Tetraspanin</th>
<th>CD9</th>
<th>CD9</th>
<th>CD151</th>
<th>CD63</th>
<th>TSSC-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method Studied</td>
<td>knock-out</td>
<td>antibody blocking</td>
<td>knock-out</td>
<td>antibody blocking</td>
<td>knock-out</td>
</tr>
<tr>
<td>Species</td>
<td>Mouse</td>
<td>human</td>
<td>mouse</td>
<td>human</td>
<td>mouse</td>
</tr>
<tr>
<td>Aggregation</td>
<td>Unknown</td>
<td>unknown</td>
<td>reduced in response to PAR4, collagen, ADP</td>
<td>unknown</td>
<td>reduced in response to PAR4 &lt;150 uM, Collagen 5 ug/ml.</td>
</tr>
<tr>
<td>Adhesion</td>
<td>Unknown</td>
<td>unknown</td>
<td>unaffected</td>
<td>unaffected</td>
<td>unaffected</td>
</tr>
<tr>
<td>Spreading</td>
<td>Unknown</td>
<td>unknown</td>
<td>impaired</td>
<td>Impaired</td>
<td>impaired</td>
</tr>
<tr>
<td>Ligand Binding</td>
<td>Unknown</td>
<td>unknown</td>
<td>normal soluble FG binding</td>
<td>unknown</td>
<td>normal soluble FG binding</td>
</tr>
<tr>
<td>Activation</td>
<td>Unknown</td>
<td>unknown</td>
<td>unaffected</td>
<td>unaffected</td>
<td>unaffected/ similar P-selectin expression</td>
</tr>
<tr>
<td>Cytoskeleton</td>
<td>Unknown</td>
<td>unknown</td>
<td>unknown</td>
<td>impaired F-actin reorganization, vinculin distribution</td>
<td>unknown</td>
</tr>
<tr>
<td>Signaling Molecules</td>
<td>Unknown</td>
<td>unknown</td>
<td>normal levels of calcium and IP3</td>
<td>reduced phosphorylation of FAK, IP’s PI4-kinase type II.</td>
<td>unknown</td>
</tr>
<tr>
<td>Clot Retraction</td>
<td>Unknown</td>
<td>unknown</td>
<td>impaired</td>
<td>unknown</td>
<td>impaired</td>
</tr>
<tr>
<td>Other insight</td>
<td>possible compensation by another tetraspanin or integrins is unknown</td>
<td>activation of FcγRII makes interpretation of data difficult</td>
<td>increased tail bleeding time/ rebleeding, unstable platelet thrombi/ more emboli</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.4  Schematic of Tetraspanin CD9

TM, Transmembrane domain; EC, extracellular loop. Numbers refer to amino acid residues. C-C, disulfide bond between cysteine residues. The area circled in red corresponds to the binding site for mAb7/mFab7.
Figure 2.5 Tetraspanin Web in Platelets
A, Resting platelet membrane, expressing FcR, CD9, a bent (inactive) GPIIb-IIIa, CD151, and TSCC6. B, Activated platelet membrane. GPIIb-IIIa now assumes an extended conformation, capable of ligand binding. CD63 is also brought to the surface, in association with CD9 and GPIIb-IIIa.
CD151, also known as PETA-3 (platelet endothelial tetraspan antigen-3), is physically and functionally associated with GPIIb-IIIa and modulates its “outside-in” signaling. CD151-deficient mice have delayed clot retraction; reduced aggregation to 500 μM PAR-4, 20 μg/ml collagen, and 10 μM ADP; and normal adhesion but incomplete spreading on fibrinogen, including reduced number of filopodia per platelet. Inside-out GPIIb-IIIa signaling was normal, as these platelets had normal FG binding, normal binding of an activation-dependent antibody to GPIIb-IIIa, normal alpha and dense granule secretion, and normal increases in cytosolic-free calcium and IP3 levels (Lau 2004).

In spite of CD9 expression on the platelet surface being greater than other tetraspanins, and being greatly studied, it is still poorly understood. It is localized to the platelet surface membrane and alpha granules (Cramer 1994). Studies with antibody ligation to CD9 have been inconclusive due to the linkage to the low-affinity immunoglobulin G receptor, FcγRIIa, in human platelets (Kuruda 1995, Qi 1996), although one study suggests that CD9 regulates platelet function independently of FcγRIIa (Wu 2000).

CD9 knock-out mice have not shown any critical platelet defects; however, expression levels of other tetraspanins, integrins, and surface proteins have not been characterized, allowing the possibility of compensation. Additionally, studies involving CD9 knock-out platelets have not utilized low concentrations of agonists, which would be suggested based on studies with TSSC-6 knockout platelets.

CD9 (see Fig 2.4 and Fig 2.5) is proposed to have roles in platelet aggregation and spreading. It may facilitate platelet functions via its association with GPIIb-IIIa or through direct ligand binding, as fibronectin has been shown to be a ligand for CD9 (Longhurst 2002).

### 2.2 Tetraspanins

#### 2.2.1 Tetraspanin Structure

Tetraspanins are integral membrane proteins containing four transmembrane alpha-helices (TM1, TM2, TM3, and TM4), two extracellular loops (EC1 and EC2), and short intracellular amino and carboxy termini (Berditchevski 2001, Boucheix 2001, Hemler 2001). EC1, also known as the small extracellular loop, is flanked by TM1 and TM2, while EC2, also known as the large extracellular loop, is flanked by TM3 and TM4.

Proteins are identified as tetraspanins based on a shared overall structure distinct from other proteins with four transmembrane domains and the conservation of specific amino acid residues in their transmembrane domains.
and the second extracellular loop (Hemler 2001, Maecker 1997). These characteristic motifs include the conserved cysteine-cysteine-glycine (CCG) sequence in EC2, which is involved in two of the two to four disulfide bridges observed in EC2 of all tetraspanins (Seigneuret, 2001). TM1, TM2, and TM3 consist of heptad repeat motifs, containing highly conserved asparagine and glycine in TM1 and TM2, and conserved leucine and glutamate/glutamine in TM3. Heptad repeat motifs are noticeably absent in TM4, although it contains conserved polar amino acids (Kovalenko 2005). There is a highly conserved asparagine-glycine-glycine motif in EC1 (Kovalenko 2005).

EC2, and rarely EC1, are glycosylated at one or more N-glycosylation sites, contributing to the overall tetraspanin size that ranges from 20 to 70 kDa (Yunta 2002).

Crystal structures of full-length tetraspanin proteins have not been generated, owing to inherent properties of membrane proteins; however, the crystal structure of the EC2 loop of CD81 has been solved (Kitadokoro 2001). This showed five alpha-helices arranged in “stalk” and “head” domains, and it is believed that these structural features are consistent among all tetraspanins (Kitadokoro 2001).

Tetraspanins are evolutionarily conserved. In addition to mammals, they have been found in Schistosomes (Wright 1990), early metazoans (Adell 2004), fungi (Gourgues 2002), and nematodes (Moribe 2004). Currently, more than 30 tetraspanins have been identified.

Tetraspanins are expressed in all human cell types, and most cells generally express multiple tetraspanins (Levy 2005). Tetraspanins may be expressed on both the cell surface and on granular membranes (Israels 2005). Some tetraspanins—such as CD9 and CD81—are expressed in almost all cell types, while others are more restricted—CD37 in B cells (Schwartz-Albeiz 1988), RDS/peripherin in retina (Travis 1991), uroplakins in bladder epithelium (Walz 1995), and CD53 in lymphoid-myeloid cells (Maeker 1997).

### 2.2.2 Tetraspanin Web

Tetraspanins have been implicated in a wide variety of cell processes. This is likely the result of interactions between tetraspanins and other associated proteins within tetraspanin webs (which is also known as tetraspanin microdomains, tetraspanin enriched microdomains, or tetraspanin complexes) (see also [Fig 2.5](#)).

Tetraspanins have the ability to associate with a wide variety of membrane proteins. Generally, the extracellular domains—mainly EC2—interact with laterally associated proteins or soluble ligands, the cytosolic regions associate
with cytoskeletal or signaling molecules, and the transmembrane domains
interact with other proteins necessary for maintenance of the tetraspanin web
(Levy 2005). Thus, the tetraspanin web acts as a molecular facilitator, organizing
membrane proteins and coupling receptors to signaling pathways (Levy 2005).

In differing cell types, the tetraspanin web is composed of different
tetraspanins and partner proteins. It is unclear whether all the tetraspanins in a
certain cell are associated with each other.

Within the tetraspanin web, specific tetraspanins directly associate with a
partner membrane protein (frequently a member of the integrin or
immunoglobulin superfamilies) and other tetraspanin proteins. These
tetraspanins also directly associate with a particular partner protein. Primary
interactions between tetraspanins and partner proteins are believed to be
mediated through extracellular domains, in particular EC2, due to resistance to
Triton-X 100 disruption. The majority of tetraspanin interactions with other
membrane proteins cannot withstand Triton-X 100 treatment, but can withstand
milder detergents and are termed secondary interactions (Serru 1999).

Palmitoylation of intracellular cysteine residues plays an important role in
tetraspanin-tetraspanin interactions; abolishment of this palmitoylation reduces a
tetraspanin’s interaction with other tetraspanins (Charrin 2002, Yang and Claas
2002). Palmitoylation of partner proteins also contributes to their incorporation
into the tetraspanin web (Yang and Kovalenko 2004). Palmitoylation provides a
regulatory mechanism to rearrange the tetraspanin web in response to
stimulation (Dempsey 1996).

The tetraspanin web also includes interactions with cytoskeletal and
signaling molecules. These signaling molecules include Rho GTP-binding
proteins (Schwartz 2000), G protein-coupled receptors and their intracellular
associated heterotrimeric G-proteins (Little 2004), phosphatidylinositol 4-kinase
(Berditchevski 1997, Yauch 1998), and PKC isozymes (Zhang 2001). The
tetraspanin web is linked to the actin cytoskeleton via EWI-2 and EWI-F (Sala-
Valdes 2006).

Tetraspanin-enriched membrane domains are physically and functionally
separate from lipid rafts, although they share some characteristics (Claas 2001,
Hemler 2003). Both can associate with cholesterol and gangliosides and can be
recovered from low-density membrane fractions of sucrose gradients. However,
tetraspanin webs and lipid rafts exhibit differences in membrane solubility and
have different protein content (lipid rafts often contain GPI-linked proteins and
caveolin, which are rarely found in tetraspanin webs) (Berditchevski 2002, Foster
2003, Hemler 2005, Yauch 2000). Lipid rafts may associate with the tetraspanin
web under certain conditions, thereby facilitating signal transduction (Israels
2007).
With specific regard to the tetraspanin CD9, interactions have been shown with the integrins α3β1 (Yanez-Mo 1998), α4β1, α6β1, α5β1, αIIbβ3 (GPIIb-IIIa), and precursor β1 (Yunta 2002); the growth factor receptors TGF-α and HB-EGF (Lagaudriere-Gesbert 1997); immunoglobulin superfamily members FRPP and EWI-2 (Stipp 2001); CD36 and CD9P-1 (Miao 2001), CD19 (Horvath 1998), and CD46 (Yunta 2002); the signaling molecules PKC and PI4K (Boucheix 2001); and the soluble ligands FN (Longhurst 2002), and PSG 17 (Waterhouse 2002). Many more protein partners will likely be identified in the future.

2.2.3 Tetraspanin Function

It is unknown whether tetraspanins alter integrin conformation or affinity for ligand. In addition, for the most part, tetraspanins do not affect static cell adhesion. However, other integrin-dependent events—migration, spreading, and cell morphology, among others—can be greatly affected by tetraspanins.

2.2.3.1 Cell Motility. Tetraspanins have been found to be expressed in motility-related structures, such as lamellipodia, and involved in cell motility.

In keratinocytes, the tetraspanins CD9 and CD81 are highly expressed in filopodia at lateral and apical surfaces, as well as in the footprints and rippings of motile keratinocytes, suggesting a role in keratinocyte motility. In a wound healing assay, cell migration was inhibited with antibodies to CD9 and CD81, and to a lesser extent, to CD151, perhaps via involvement in integrin recycling during keratinocyte migration (Penas 2000).

When HT-1080 cells are transfected with CD9, lamellipodia formation is significantly decreased, and this effect was reversed upon antibody ligation. CD9 was able to influence lamellipodial formation by downregulating WAVE2 (Huang 2006).

In Chinese hamster ovary cells, expression of CD9 enhances cell motility on fibronectin. This is likely via a direct interaction between CD9 EC2 and FN (Longhurst 2002).

2.2.3.2 Tumor Cell Metastasis. Tetraspanins CD9 and CD82 are considered metastasis suppressors in solid tumors (Wright 2004). These tetraspanins are downregulated in progression of several cancers, including non-small cell lung, breast, pancreas, colon, and prostate, and decreased expression of these tetraspanins result in poorer prognosis (Boucheix 2001, Saito 2006).

The tetraspanin CD82 (also known as KAI1) tumor metastasis suppressor activity is believed to be through the association of CD82 with the EGF receptor tyrosine kinase, which accelerates ligand-induced clearance of EGF receptor from the cell surface (Bienstock 2001).
Adenoviral transduction of CD9 or CD82 into lung tumor cells, whether via direct implantation or through intratracheal administration, dramatically reduced metastasis to mediastinal lymph nodes (Takeda 2007). However, overexpression of CD9 in human prostate cancer cell lines did not suppress metastatic properties, suggesting that CD9 partner proteins may be needed for full anti-metastatic effect (Zvieriev 2005).

2.2.3.3 Cell Proliferation and Differentiation. The tetraspanin TSSC-6 colocalizes into domains on T cells containing T cell molecules involved in T cell stimulation, such as CD4 and CD8. Deletion of TSSC-6 yields mice with normal lymphoid development, yet T cells showed elevated T cell receptor-dependent proliferation in vitro in response to concanavalin A, anti-CD3, and anti-CD28 (Tarrant 2002).

Proliferation of T lymphocytes from CD81-deleted mice is also enhanced in response to various stimuli. In B cells, deletion of the tetraspanin CD81 results in diminished numbers of a subset of B cells (Miyazaki 1997). CD81 is also involved in driving helper T cells toward polarized Th2 differentiation (Deng 2000, Maecker 1997).

In the brain, deletion of CD81 results in mice with increased numbers of astrocytes and microglia, resulting in greatly enlarged brains (Geisert 2002). CD81 may control astrocyte and microglia proliferation in a cell-cell contact-dependent manner; neurons inhibit proliferation by interacting with CD81 on cultured astrocytes (Kelic 2001).

Tetraspanins have been implicated in growth factor signaling as well. CD9 regulates pro-TGFα, pro-HB-EGF, and pro-amphiregulin, all of which are membrane-bound agonists for the EGF receptor (Inui 1997, Shi 2000). CD9 is necessary for HB-EGF mitogenic activity (Nakamura 2000).

During osteoclast differentiation, CD9 expression increases during RANKL-induced osteoclastogenesis. A CD9-neutralizing antibody suppressed RANKL-induced multinucleated osteoclast formation and mRNA expression of osteoclast differentiation marker genes, and this was shown to be through regulating long-term phosphorylation of p44/42 MAPK (Yi 2006).

CD9 is also involved in megakaryocyte differentiation. In hematopoietic stem cells, those with the highest expression of CD9 are committed to the B-lymphoid or megakaryocytic lineages, and expression of CD9 is found to precede expression of CD41 in megakaryocyte precursors. Antibody ligation to CD9 inhibited the in vitro terminal differentiation of human CD34+ cells into megakaryocytes. It was observed that antibody ligation deeply altered membrane structures (demarcation membranes and heterogeneous multivesicular body membranes) of these cells (Clay 2001).
2.2.3.4 Cellular Morphology. The tetraspanins peripherin/RDS and ROM-1 are expressed in the retina and have specialized roles in maintaining the outer segment discs in their characteristic flattened, parallel layers (Kohl 1998). Point mutations in the EC2 domain are associated with various human retinal dystrophies (Kohl 1998) and disruption of the peripherin/RDS tetraspanin gene causes retinal degeneration (Connell 1991).

Mutation of a CD151 site influencing integrin interaction resulted in mutant cells lacking integrin-dependent spreading and cellular cable morphology on Matrigel (Kasarov 2002). Another mutation on CD151, affecting palmitoylation, resulted in cells that could interact with integrins but not with other tetraspanins. This resulted in kidney epithelial cells transitioning from a fibroblastic morphology to an epithelial morphology (Yang 2002). A third mutation in CD151, in the C-terminal tail (abolishing a theoretical linkage to cytoskeletal or cytoplasmic signaling molecules), altered integrin-dependent cell spreading, cellular cable formation functions, and adhesion strengthening (Lammerding 2003, Zhang 2001).

In small cell lung cancer cells, CD9 expression is usually diminished. Increased expression of CD9 suppressed neurite-like process outgrowth and promoted apoptotic death. This was found to be via PI3K/Akt signaling (Saito 2006).

2.2.3.5 Virus-Induced Syncitia Formation. The tetraspanins CD9, CD81, and CD82 have been implicated in virus-induced syncitia formation (Fukudome 1992, Willet 1997).

In human T lymphoblasts, antibody ligation of either CD81 or CD9 enhanced syncitia formation induced by HIV-1 envelope proteins and viral entry into the lymphoblasts. In addition, decreased CD81 and CD9 expression increased syncitia formation and viral entry, while overexpression resulted in decreased susceptibility to syncitia formation (Gordon-Elonso 2006).

2.2.3.6 Intracellular Vesicles. The tetraspanin web may be adapted to facilitate vesicular fusion and fission with the cell membrane (Hemler 2003). Tetraspanin expression is enriched in the endocytic pathway. Exosomes from B cells are enriched for CD37, CD53, CD63, CD81, and CD82 by 7- to 124-fold (Escola 1998). Jurkat cell activation results in CD81 removal from the cell surface in parallel with release of CD81-containing exosomes (Fritzscheing 2002).

2.2.3.7 Cell-Cell Interactions. Investigations into megakaryocyte and proplatelet formation suggest that CD9 is involved in altering membrane structures, suggesting a role in membrane reorganization, especially cell fusion (Clay 2001).
Fluorescence microscopy has revealed that CD9, while ubiquitously expressed across the cell membrane, is increased at areas of cell-contact, suggesting a role in cell-cell interactions. This phenomena is observed both in cells natively expressing CD9, such as kidney epithelium and breast cancer cells, (Nakamura 1995, Yang 2006) and in cells that have been transfected with CD9 (Yang 2006). Additionally, CD9, CD81, and CD151, along with integrins, accumulate at keratinocyte cell junctions (Penas 2000).

One of the most striking phenotypes of the CD9 knock-out mouse model is the reduced fertility in females. CD9 has been shown to have a crucial role on oocytes during sperm-egg fusion—CD9 knock-out mice oocytes are deficient in their ability to fuse with sperm (Kaji 2000, La Naour 2000, Miyado 2000). Furthermore, anti-CD9 antibodies can inhibit sperm-egg fusion (Chen 1999, La Naour 2000, Miller 2000), and CD9 recombinant proteins corresponding to residues 173-175 in CD9 EC2 partially inhibit fusion when added to the oocyte prior to insemination but not when preincubated with sperm, showing that CD9 is probably not a sperm receptor, but more likely influencing other members of the tetraspanin web (Zhu 2002).

CD9 also appears to be involved in implantation, as CD9 is variably expressed in the murine uterus depending on the stage of implantation, and was upregulated in an ovarian steroid hormone-dependent manner (Weimin 2007).

In human ova, antibody ligation of CD9, prior to zona removal, prevented the clustering of $\alpha_6\beta_1$ integrin clustering and gamete fusion. Antibody ligation to CD151 is able to partially inhibit sperm-egg fusion. This suggests that these tetraspanins are able to control the redistribution of membrane proteins into the clusters necessary for gamete fusion (Ziyyat 2006).

### 2.2.4 Tetraspanin CD9

CD9 was shown to be localized to the p region of chromosome 12 (Benoit 1991). cDNA from a megakaryocyte library showed that CD9 is comprised of eight exons, producing a protein with 228 amino acids (Lanza 1991).

3.1 Materials

Nonspecific mouse IgG, Fab fragment, and goat anti-mouse Fc specific F(ab')2 fragment were obtained from Jackson Immunolaboratories (West Grove, PA). Adenosine 5'-diphosphate (ADP), tetramethyl rhodamine isothiocyanate (TRITC)-labeled phalloidin, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Fab, nonspecific mouse IgG1, β-octylglucopyranoside, and other general reagents were purchased from Sigma Chemical Company (St. Louis, MO). FITC-labeled anti-CD9 antibody, clone ML-13, was purchased from BD Biosciences (San Jose, CA). PE-labeled anti-CD63 and anti-P-selectin were from BD Pharmingen (San Jose, CA). Collagen was purchased from Chronolog Corp (Havertown, PA) and TRAP (thrombin receptor agonist peptide) was purchased from Peninsula Laboratories (San Carlos, CA). Eptifibatide was provided by Schering-Plough (Kenilworth, NJ). Fibrinogen was from Enzyme Research Laboratories (South Bend, IN) and fibronectin was from Gibco (Carlsbad, CA). Gelcode Blue Stain was from Pierce (Rockford, IL) and Coomassie blue was from BioRad (Hercules, CA). mAb7 was obtained from mouse ascites and purified by Affi-gel Protein A column (BioRad, Hercules, CA). mFab7 was generated and purified using Immunopure® Fab Preparation Kit (Pierce, Rockford, IL) and purified Fab underwent buffer exchange into phosphate-buffered saline (PBS), pH 7.4, using Zeba™ Desalt Spin Columns (Pierce, Rockford, IL). Human coagulation Factor XIII was from Calbiochem (San Diego, CA). Vectashield mounting media was purchased from Vector Laboratories (Burlingame, CA). Superfrost microscope slides and microscope cover glass were from Fisher Scientific (Pittsburgh, PA). Anti-fibrinogen antibody was obtained from The Binding Site (San Diego, CA). Protease inhibitor cocktail tablets were from Roche (Basel, Switzerland).

3.2 Tetraspanin Expression on Resting and Activated Platelets

Flow cytometry was used to assay the levels of the tetraspanins CD9, CD63, and CD151; the integrin GPIIb-IIIa, and the protein GPIb on the surface of resting and activated platelets (See Fig 3.1). Blood was obtained from healthy adult donors into 0.1 M buffered citrate as an anticoagulant. PRP was prepared (Table 3.1) and diluted with PPP to a final platelet count of 250,000 platelets/μl. Platelets were either allowed to remain resting or were activated with 10 μM ADP or 2 μM TRAP. Platelets were then incubated with primary antibody for 30 minutes at 37°C (R&D monoclonal anti-human CD151 antibody, catalog #MAB1884 for CD151; 10E5 and 7E3 for GPIIb-IIIa; 6D1 for GPIb; BD Pharmingen Purified mouse anti-human monoclonal antibody, catalog #3556019 for CD63; mAb7 for CD9; and mIgG, Sigma MOPC-21, as a negative control).
Figure 3.1 Measurement of Surface Proteins by Flow Cytometry
Platelets are incubated with fluorescently-labeled antibodies which bind a specific surface protein. Platelets are incubated with the antibody, and level of fluorescence is measured by a flow cytometer.
Table 3.1  Preparation of PRP and PPP

<table>
<thead>
<tr>
<th>Step</th>
<th>Instructions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Whole blood is obtained by venipuncture into 0.1 M buffered citrate. Other anticoagulants may be used as indicated. 10 ml total volume is used per 15 ml tube.</td>
</tr>
<tr>
<td>2</td>
<td>Tubes are centrifuged at 135 x g for 15 minutes. Platelet-rich plasma (PRP), the top layer, is removed and set into a clean 15 ml tube. PRP must be carefully removed to avoid contamination with white blood cells in the buffy coat.</td>
</tr>
<tr>
<td>3</td>
<td>The remaining blood is centrifuged at 2,500 x g for 15 minutes. Platelet-poor plasma (PPP), the top layer, is carefully removed and set into a clean 15 ml tube.</td>
</tr>
<tr>
<td>4</td>
<td>PRP is counted with a Coulter Counter, and diluted with PPP to the desired concentration.</td>
</tr>
</tbody>
</table>
Ten microliters of each sample were transferred to a microfuge tube with 500 μl filtered PBS+10 μl goat anti-mouse FITC-labeled antibody, and samples were incubated for ten minutes at room temperature prior to data collection using the flow cytometer (using the Platelet Green template). This was repeated using ten normal, healthy adult donors.

3.3 Association of CD9 and GPIIb-IIIa in Resting and Activated Platelets

3.3.1 Co-Immunoprecipitation

Whole blood was obtained by venipuncture and mixed with ACD as an anticoagulant (Table 3.2). Platelets were washed twice with CGS (Table 3.3), resuspended in platelet lysis buffer containing 1% CHAPS (Table 3.4), and lysed for 20 minutes on a rocker at 4ºC. Samples were clarified by centrifugation at 18,300 x g for 10 min at 4ºC, discarding the pellet. Protein concentration was measured using Biorad Detergent-Compatible Assay kit and adjusted to 1.5 mg/ml. One ml lysate and 75 μl Protein A/G beads were added to each of five eppendorf tubes, and samples were incubated for 60 minutes on a rocker at 4ºC and then precleared by centrifugation at 18,300 x g for one minute. Supernatants were collected and transferred to five new tubes, to which was added either 5 μg D3, 5 μg D3 + 3 mM eptifibatide, 5 μg AP3, 5 μg mAb7, or 5 μg mIgG (MOPC-21) as a negative control. Samples were incubated with primary antibody for one hour on a rocker at 4ºC. 75 μl Protein A/G beads were added to each tube and incubated for 60 minutes on a rocker at 4ºC. Samples were centrifuged three times at 18,300 x g with cold lysis buffer, discarding the supernatant after each wash. Samples were boiled with fresh non-reduced sample buffer (Table 3.5), separated on a 5%-20% gradient separating gel (Table 3.6 and Table 3.7), and transferred to a PVDF membrane. Membranes were blocked by rocking with 5% nonfat dry milk in 0.1% TBS-Tween at room temperature for 60 minutes then incubated with primary antibody in 5% nonfat dry milk in TBS-Tween overnight at 4ºC (mAb7 is used to detect CD9, and C3 is used to detect αIIb) (Table 3.8). Bound antibodies are labeled with HRP-labeled sheep anti-mouse antibody and detected with ECL-PLUS kit prior to development onto photo paper.

3.3.2 Confocal Analysis

Coverslips, in individual wells of a 20-well tissue culture plate, were coated with 500 μg/ml FG, 50 μg/ml plasma FN, fibrin, or fibrin-FN overnight at 4ºC (Table 3.9). Coverslips were then blocked with 1 ml 20 mg/ml BSA in PBS for 2 hours at 37ºC and washed 3x with PBS (Table 3.8). 500 μl of washed platelets (Table 3.10), at 1x10⁷ platelets/ml in HEPES-Tyrode buffer (Table 3.11), were incubated 1 hour on the coverslips. Non-adherent platelets were removed by washing 3x with HEPES-Tyrode buffer. GPIIb-IIIa was stained with D3 for 30
### Table 3.2  Anticoagulants

<table>
<thead>
<tr>
<th>Material</th>
<th>ACD</th>
<th>0.1 M Buffered Citrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium citrate</td>
<td>0.085 M</td>
<td>0.06 M</td>
</tr>
<tr>
<td>Dextrose</td>
<td>0.1 M</td>
<td>---</td>
</tr>
<tr>
<td>Citric acid</td>
<td>0.07 M</td>
<td>0.04 M</td>
</tr>
</tbody>
</table>

### Table 3.3  CGS

<table>
<thead>
<tr>
<th>Material</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium Citrate</td>
<td>0.01 M</td>
</tr>
<tr>
<td>Dextrose</td>
<td>0.03 M</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.12 M</td>
</tr>
<tr>
<td>pH</td>
<td>6.5</td>
</tr>
<tr>
<td>Amount</td>
<td>Material</td>
</tr>
<tr>
<td>----------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>10 mM</td>
<td>HEPES</td>
</tr>
<tr>
<td>200 mM</td>
<td>NaCl</td>
</tr>
<tr>
<td>1%</td>
<td>CHAPS or BRIJ-98, as indicated</td>
</tr>
<tr>
<td>5 mM</td>
<td>MgCl$_2$</td>
</tr>
<tr>
<td>2 mM</td>
<td>NaF</td>
</tr>
<tr>
<td>100 mM</td>
<td>Na$_4$P$_2$O$_7$</td>
</tr>
<tr>
<td>1 per 10 ml</td>
<td>Protease inhibitor tablet</td>
</tr>
</tbody>
</table>
### Table 3.5 Formula for 1/3x NR Sample Buffer

<table>
<thead>
<tr>
<th>Amount</th>
<th>Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.8 ml</td>
<td>0.5 M Tris, pH 6.8</td>
</tr>
<tr>
<td>1.6 ml</td>
<td>Glycerol</td>
</tr>
<tr>
<td>0.32 g</td>
<td>SDS</td>
</tr>
<tr>
<td>0.32 ml</td>
<td>Bromophenol blue</td>
</tr>
<tr>
<td>Q.S. to 4 ml</td>
<td>dH2O</td>
</tr>
</tbody>
</table>

### Table 3.6 Formulas for SDS-PAGE Gels

<table>
<thead>
<tr>
<th>Material</th>
<th>5% Separating Gel</th>
<th>20% Separating Gel</th>
<th>Stacking Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled H_2O</td>
<td>22.5 ml</td>
<td>0.301 ml</td>
<td>6.1 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>334.4 μl</td>
<td>1.1 ml</td>
<td>----</td>
</tr>
<tr>
<td>1.5 M Tris, pH 8.8</td>
<td>10.0 ml</td>
<td>5 ml</td>
<td>----</td>
</tr>
<tr>
<td>0.5 M Tris, pH 6.8</td>
<td>----</td>
<td>----</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>401.2 μl</td>
<td>200.7 μl</td>
<td>100 μl</td>
</tr>
<tr>
<td>30% acrylamide/bis</td>
<td>6.7 ml</td>
<td>13.4 ml</td>
<td>1.3 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 μl</td>
<td>5 μl</td>
<td>10 μl</td>
</tr>
<tr>
<td>10% APS (fresh)</td>
<td>120.3 μl</td>
<td>61.5 μl</td>
<td>50 μl</td>
</tr>
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</table>
### Table 3.7  Formula for Western Blot Buffers

<table>
<thead>
<tr>
<th>Material</th>
<th>Running Buffer</th>
<th>Blot (Transfer) Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRIS</td>
<td>18 g</td>
<td>12.2 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>86.4 g</td>
<td>57.6 g</td>
</tr>
<tr>
<td>SDS</td>
<td>3.0 g</td>
<td>4.0 g</td>
</tr>
<tr>
<td>Methanol</td>
<td>----</td>
<td>800 ml</td>
</tr>
<tr>
<td>Total volume</td>
<td>3 L</td>
<td>4 L</td>
</tr>
</tbody>
</table>

### Table 3.8  Buffered Saline Recipes

<table>
<thead>
<tr>
<th>Material</th>
<th>Phosphate-buffered saline (PBS)</th>
<th>Tris-Buffered Saline (TBS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>0.14 M</td>
<td>----</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$</td>
<td>0.06 M</td>
<td>----</td>
</tr>
<tr>
<td>NaCl</td>
<td>2.87 M</td>
<td>0.15 M</td>
</tr>
<tr>
<td>Tris</td>
<td>----</td>
<td>0.01 M</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
<td>7.4</td>
</tr>
</tbody>
</table>
### Table 3.9  Fibrin and Fibrin-Fibronectin Matrix Components

<table>
<thead>
<tr>
<th>Material</th>
<th>Fb</th>
<th>FbFN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrinogen</td>
<td>2.4 mg/ml</td>
<td>2.4 mg/ml</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>0</td>
<td>240 μg/ml</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>0.02 M</td>
<td>0.02 M</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.15 M</td>
<td>0.15 M</td>
</tr>
<tr>
<td>Tris-HCl, pH 7.5</td>
<td>0.05 M</td>
<td>0.05 M</td>
</tr>
<tr>
<td>Human coagulation factor XIII</td>
<td>5 μg/ml</td>
<td>5 μg/ml</td>
</tr>
<tr>
<td>γ-Thrombin</td>
<td>2 units/ml</td>
<td>2 units/ml</td>
</tr>
</tbody>
</table>
Table 3.10  Preparation of Washed Platelets

<table>
<thead>
<tr>
<th>Step</th>
<th>Instructions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Whole blood is obtained by venipuncture into ACD, at a ratio of 1.4 ml ACD to 8.6 ml whole blood. 10 ml total volume is used per 15 ml tube.</td>
</tr>
<tr>
<td>2</td>
<td>Blood is centrifuged at 135 x g for 20 minutes. Platelet-rich plasma, the top layer, is removed and set into a clean 15 ml tube. PRP must be carefully removed to avoid contamination with white blood cells in the buffy coat. Red and white blood cells are discarded.</td>
</tr>
<tr>
<td>3</td>
<td>PRP is centrifuged at 800 x g for 10 minutes. The supernatant is removed and the platelet pellet is gently resuspended in 1 ml of CGS. This 1 ml is transferred to a fresh tube, avoiding transfer of any platelets remaining in clumps. CGS is added to the fresh tube to bring the volume up to 5 ml.</td>
</tr>
<tr>
<td>4</td>
<td>The platelet suspension is centrifuged at 800 x g for 10 minutes. The platelet pellet is washed with CGS as in step 3.</td>
</tr>
<tr>
<td>5</td>
<td>After 2 washes with CGS, the pellet is resuspended in the appropriate buffer, counted with the Coulter Counter, and diluted to the appropriate concentration.</td>
</tr>
</tbody>
</table>
Table 3.11  Modified Tyrode’s Buffer (HEPES-Tyrode Buffer)

<table>
<thead>
<tr>
<th>Material</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaCl$_2$</td>
<td>2 mM</td>
</tr>
<tr>
<td>BSA</td>
<td>0.1%</td>
</tr>
<tr>
<td>NaCl</td>
<td>137 mM</td>
</tr>
<tr>
<td>HEPES</td>
<td>20 mM</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.6 mM</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>1 mM</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$</td>
<td>3.3 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>2.7 mM</td>
</tr>
<tr>
<td>pH</td>
<td>7.4</td>
</tr>
</tbody>
</table>
42 minutes and with Sigma TRITC-labeled whole molecule goat-anti-mouse antibody, 1:128 in PBS, for 30 minutes at room temperature. After washing with PBS, CD9 was stained for 30 min with FITC-labeled CD9 antibody clone ML-13, diluted 1:40 in PBS. Platelets were fixed for 30 min with 4% paraformaldehyde in PBS and washed 3x with PBS. Coverslips were mounted with Vectashield mounting media and edges were sealed with clear nail polish. Samples were viewed with the confocal microscope using the 100x objective. Care was taken to observe samples using the same settings.

### 3.3.3 Impact of Mutant GPIIb-IIIa

Platelets from patients with Glanzmann Thrombasthenia, featuring a mutant GPIIb-IIIa expressed on the platelet surface, were compared with platelets from patients with normal GPIIb-IIIa. Platelets were allowed to spread on fibronectin or fibrin-fibronectin, and CD9 and GPIIb-IIIa were immunostained as above. Fibronectin and fibrin-fibronectin matrices were selected due to fibronectin being a ligand for CD9 as well as GPIIb-IIIa. It is unlikely that platelets from patients with Glanzmann Thrombasthenia would be able to adhere or spread on fibrinogen or fibrin matrices, as the mutated GPIIb-IIIa would likely not be able to bind to these ligands.

### 3.4 Localization of CD9 during Platelet Spreading

#### 3.4.1 Platelet-Matrix Contact

Platelets were allowed to spread as described previously. During viewing with the confocal microscope, the upper (apical—facing away from matrix) and lower (basal—in contact with matrix) boundaries of the platelet were selected by focusing the microscope just above and below the regions providing a focused view of the platelet. Usually this resulted in a 2-3 nm selection. The LSM 510 then provided slices corresponding to the apical, central, and basal sections of the platelet, which were analyzed for CD9 and GPIIb-IIIa localization and colocalization.

#### 3.4.2 Platelet-Platelet Contact

Platelets were allowed to spread on 500 µg/ml fibrinogen for 60 minutes in the absence of any Fab molecules to allow maximal spreading. Platelets were then fixed with 4.0% paraformaldehyde and permeabilized with 0.4% β-octylglucopyranoside in PHEM buffer (Table 3.12). CD9 was labeled with FITC-labeled anti-CD9 antibody (clone ML-13). Samples were mounted and viewed with a Zeiss Laser Scanning Microscope using the 100x objective.
Table 3.12  PHEM Buffer

<table>
<thead>
<tr>
<th>Material</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>PIPES</td>
<td>60 mM</td>
</tr>
<tr>
<td>HEPES</td>
<td>25 mM</td>
</tr>
<tr>
<td>EGTA</td>
<td>10 mM</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>2 mM</td>
</tr>
<tr>
<td>pH</td>
<td>6.9</td>
</tr>
</tbody>
</table>
Areas of platelet-platelet contact and corresponding areas of platelet periphery not in contact with adjacent platelets were selected and pixel intensity was quantified using the Zeiss LSM510 software.

3.4.3 Platelet Spreading Structures

To observe CD9 localization during the course of platelet spreading, platelets were allowed to spread on 500 μg/ml fibrinogen for 5-60 minutes in the absence of any Fab molecules. At selected time points, platelets were fixed with 4% paraformaldehyde and permeabilized with β-octylglucopyranoside in PHEM buffer, as described above.

Platelet F-actin was labeled with TRITC-Phalloidin. CD9 was labeled with FITC-labeled anti-CD9 antibody (clone ML-13). Samples were mounted and viewed with a Zeiss Laser Scanning Microscope using the 100x objective. Care was taken to visualize images using the same settings (See also Fig 2.1).

3.5 Generation and Purification of mAb7 Fab (mFab7) Fragments

3.5.1 Generation of mAb7

mAb7 was obtained from mouse ascites, purified using an Affi-gel Protein A column, and dialyzed into PBS. Antibody concentration was assessed using an Ultrospec 1000 UV/Visible Spectrophotometer (Pharmacia Biotech) and purity confirmed with both nonreducing and reducing SDS-PAGE.

3.5.2 Generation of mFab7

Papain digestion of mAb7 and Fab purification was carried out using Immunopure® Fab Preparation Kit (Pierce, Rockford, IL) (Fig 3.2). Briefly, mAb7 was incubated with immobilized papain for five hours in a 37° shaking waterbath. The resulting digest was added to a Protein A column, allowing separation of Fab fragments from Fc fragments and uncleaved, full-length antibody. The purified Fab underwent buffer exchange into phosphate-buffered saline (PBS), pH 7.4, using Zeba™ Desalt Spin Columns (Pierce, Rockford, IL). Protein concentration was determined as above.

3.5.3 Determination of Purity and Function of mFab7

3.5.3.1 Flow Cytometry. Binding ability of mFab7 was assessed by flow cytometry. Blood was collected by venipuncture through a 21-gauge butterfly
Figure 3.2  Antibody Cleavage and Purification of Fab Fragment
The antibody mAb7 was cleaved by papain, resulting in an Fc and 2 Fab fragments. The resulting digest was poured through a Protein A column, which bound to Fc fragments and any undigested mAb7. Fab fragments (mFab7), which did not interact with Protein A, pass through and are collected.
needle into 0.1 M buffered citrate. Samples, per established methods, [White 1999] were centrifuged at 135 x g for 15 minutes to obtain platelet-rich plasma (PRP). The remaining blood was centrifuged at 2500 x g for 15 minutes to obtain platelet-poor plasma (PPP). Platelet counts were performed using a Beckman Coulter Z2 Particle Count and Size Analyzer (Beckman Coulter, Miami, FL), and PRP was diluted with autologous PPP to adjust the platelet count to 2.5 x 10^8 platelets/mL. Increasing concentrations of nonspecific Fab, mAb7 whole molecule, or mFab7 were added to the adjusted PRP and incubated at 37° C for 30 minutes. FITC-conjugated goat-anti-mouse Fab was added and samples were incubated at room temperature for ten minutes. Fluorescence levels were measured using a BD FASCalibur flow cytometer with Cell Quest Pro software (Becton Dickinson, Mountain View, CA) (see Fig 3.1).

Purity of mFab7 samples were also confirmed via flow cytometry. PRP was prepared as above, and incubated with saturating amounts of mAb7 or mFab7, or corresponding amounts of nonspecific IgG or IgG-Fab at 37° C for 30 minutes. FITC-labeled goat anti-mouse Fc was incubated at room temperature for ten minutes to verify the absence of Fc fragments or intact antibody in the mFab7 preparation. Fluorescence levels were measured as above.

**3.5.3.2 SDS-PAGE.** 50 μg of mFab7, mAb7, mIgG, and nonspecific mIgG-Fab were run on both nonreducing and reducing 12% SDS-PAGE to determine purity of mAb7 and mFab7. Gels were evaluated for bands indicating the presence of intact antibody by Coomassie blue staining. An unknown protein of 60 kDa was verified by mass spectrometry to be murine albumin.

**3.5.3.3 Platelet Aggregation.** To evaluate the ability of antibodies to induce platelet aggregation, mAb7 (up to 20 μg/ml) or mFab7 (up to 100 μg/ml) were added to stirring suspensions of platelets and aggregation response was monitored for 15 minutes (Fig 3.3).

Aggregations were also performed using up to 100 μg/ml mFab7 without inducing aggregation even after 15 minutes, while mAb7 concentrations ≥ 0.2 μg/ml induced an aggregation response, confirming that mFab7 was functionally greater than 98% pure.

To further ensure that the Fc receptor was not participating in the enhanced aggregatory response, mFab7 and control Fab were pre-incubated with a goat F(ab′)2 anti-mouse-Fc before adding to platelets, at a rate of 10 F(ab′)2 per possible intact mAb7, as calculated above.

**3.6 CD9 Perturbation**

Unless otherwise indicated, CD9 perturbation was carried out by pre-incubating platelets with 20 μg/ml mFab7 for 15 minutes at room temperature.
Figure 3.3 Light Transmission Aggregometry
Upon addition of agonist, formation of platelet aggregates is measured by increased light transmission and tracings are recorded on a light transmission aggregometer.
prior to carrying out the experiment. 20 μg/ml nonspecific murine Fab was used as a control. The control Fab was isotype-matched to mFab7 and both Fab products were handled identically in all experiments.

### 3.6.1 Platelet Spreading

Fibrin-coated coverslips and fibrin-fibronectin matrix-coated coverslips were prepared as previously described [Corbett, 1996]. Additionally, coverslips were covered with 500 μg/ml fibrinogen or 50 μg/ml plasma fibronectin overnight. ACD-anticoagulated blood was washed twice with CGS (0.01 M sodium citrate, 0.03 M dextrose, 0.12 M NaCl), pH 6.5, and resuspended in a modified Tyrode’s buffer (2 mM CaCl₂, 0.1% bovine serum albumin, 137 mM NaCl, 02 mM HEPES, 5.6 mM glucose, 1mM MgCl₂, 2.7 mM KCl, 3.3 mM NaH₂PO₄), pH 7.4, at 2x10⁷ platelets/ml. Platelets were incubated with 20 μg/ml mFab7 or nonspecific control Fab and allowed to adhere and spread on coverslips for 30 minutes. Nonadherent platelets were removed by washing with Tyrode’s buffer, and adherent platelets were fixed with 4.0% paraformaldehyde for 30 minutes. Platelets were permeabilized with 0.4% β-ocytglucopyranoside in PHEM buffer (60 mM PIPES [piperazine-N,N’-bis (2-ethane sulfonic acid)], 25 mM HEPES, 10 mM EGTA, 2mM MgCl₂), pH 6.9, for 5 minutes. Platelet F-actin was labeled with TRITC-labeled phalloidin and CD9 was labeled with FITC-labeled anti-Fab antibody. Coverslips were mounted onto slides with Vectashield Mounting Media and edges were sealed with clear nail polish. Samples were visualized with a Zeiss laser scanning microscope (LSM 510) using the 100x objective. Care was taken to visualize samples using the same settings.

To quantitate pixel intensity, areas of platelet-platelet contact or corresponding areas of the platelet periphery were highlighted and pixel intensity (pixels per μm) was quantitated by the LSM 510 software.

### 3.6.2 Platelet Aggregation and Disaggregation

Platelet aggregation was measured by light transmission in a dual-channel lumiaggregometer (Payton Scientific, Buffalo, NY) (See Fig 3.3). PRP was preincubated with 20 μg/ml mFab7 or control Fab for 15 minutes at room temperature. Agonist concentration was calculated as follows: ADP—the highest concentration that would give a biphasic response in the absence of any Fabs (2-3 μM); Collagen—the concentration that would give a 50% response in the absence of Fabs (0.3-1 μg/ml); TRAP—the lowest concentration that would give a sustained response in the absence of Fabs (5-12 μM). Low concentrations of agonist were chosen as this was the expected range to see an impact of CD9. Following preincubation with mFab7 or control Fab, the agonist was added to the stirring suspension of platelets, and aggregation response was recorded for 15
minutes. The maximum aggregation response was determined for each donor, typically at 2.5 minutes after agonist addition.

Percent platelets aggregation (%PA) was calculated at 5, 10, and 15 minutes after agonist addition. To assess platelet disaggregation, 4000 nM eptifibatide was added to the platelet aggregates 2.5 min after agonist addition and percent platelet disaggregation (%PD) was calculated 5, 10, and 15 minutes after antagonist addition (Fig 3.4).

The following were used to calculate %PA and %PD:

\[
\%PA = \frac{\text{(number of chart divisions on the aggregometer tracing from baseline to aggregation response at given time point)}}{\text{(total number of chart divisions representing 0\% [PRP] to 100\% aggregation [PPP])}} \times 100;
\]

\[
\%PD = \frac{\%PA_{\text{max}} - \%PA_{\text{time point}}}{\%PA_{\text{max}}}
\]

### 3.6.3 Platelet Activation

PRP in citrate was prepared as described above, at 2.5x10^8 platelets/ml, and preincubated with 20 μg/ml mFab7 or control Fab. Selected platelet samples were activated with 2 μM ADP or 5 μM TRAP and incubated at room temperature for 40 minutes. PE-labeled anti-P-selectin, anti-CD63, or nonspecific mIgG antibodies were added to evaluate expression of P-selectin, a marker of alpha granule release, and CD63, a marker of lysosomal release, indicating platelet degranulation. Platelet fluorescence data, indicating surface protein expression, were acquired using a BD FASCalibur flow cytometer with Cell Quest Pro Software (Becton Dickinson, Mountain View, CA).

### 3.6.4 Fibrinogen Binding

Whole blood was drawn into buffered citrate and PRP was prepared by centrifugation at 135 x g for 15 minutes and supernatant discarded. Pelleted platelets were resuspended in Tyrode’s buffer (138 mM NaCl, 2.9 mM KCl, 12 mM NaHCO3, 0.4 mM MgCl2, 5.5 mM Dextrose, 0.36 mM NaH2PO4, 1.8 mM CaCl2, pH 7.4) at 2.5x10^8 platelets/ml. 100 μl of platelets were incubated with 20 μg/ml mFab7 or control Fab. Platelets were incubated with 20 μM ADP or saline control for 30 minutes at 37°C to allow binding of plasma fibrinogen. Anti-fibrinogen FITC antibody was added and mixed and data were acquired using a
Figure 3.4  Eptifibatide-Induced Platelet Disaggregation

A, A platelet thrombus is maintained by fibrinogen binding and crosslinking GPIIb-IIIa on adjacent platelets. B, Eptifibatide, a small peptide, interacts with GPIIb-IIIa and competitively inhibits fibrinogen binding, resulting in platelet disaggregation. The GPIIb-IIIa antagonists abciximab and tirofiban work similarly.
BD FASCAlibur flow cytometer with Cell Quest Pro software (Becton Dickinson, Mountain View, CA).

### 3.6.5 Cytoskeletal Incorporation

Triton X-100-insoluble cytoskeletons of control and ADP-activated platelets in plasma, pretreated with 20 μg/ml mFab7 or 20 μg/ml control Fab, were prepared according to the method of Kouns et al [Kouns WC, 1991]. Briefly, PRP in citrate were prepared at 3x10^8 platelets/mL. After pretreatment with Fab, 2 μM ADP was added to stirring platelet suspension at 37°C. After five minutes, samples were rapidly removed and lysed with ice-cold 2% Triton lysis buffer (100 mM Tris-HCl, 10 mM EGTA, 2% Triton X-100, 2 mM 2-mercaptoethanol, and protease inhibitor cocktail tablet; Table 3.13) and lysates were kept on ice.

Platelet lysates were centrifuged at 4°C at 15,600 x g to isolate the platelet core cytoskeleton. The resulting samples were denatured and electrophoresed on a 5-20% SDS-polyacrylamide exponential gradient gel overnight. Proteins were visualized by staining with Coomassie Blue. Densitometry was performed by photocopying each gel, and carefully removing and weighing each band with a Sartorius Research quantitative electric balance (Sartorius Research, Goettingen, Germany).

### 3.6.6 Akt Phosphorylation

#### 3.6.6.1 During Platelet Aggregation

PRP was prepared from whole blood and adjusted to 3x10^8 platelets/ml with PPP. Samples were pretreated with saline, control Fab, or mFab7 for 15 minutes at room temperature, then monitored with a lumiaggregometer for five minutes upon addition of 2 μM ADP or saline. Samples were then centrifuged, and pellets were collected, denatured, and subjected to electrophoresis on a 5-20% gradient SDS-PAGE. Proteins were transferred to a PVDF membrane, blocked with 5% BSA in TBS-Tween, and incubated with an anti-phospho-Akt antibody overnight on a rocker at 4°C. The primary antibody was labeled with a secondary donkey anti-rabbit-HRP antibody for 60 minutes in the presence of 5% BSA in TBS-Tween. Membranes were developed using the ECL-PLUS kit and stored in PBS at 4°C until continuing with stripping. To strip, membranes were completely submerged in Restore Western Blot Stripping Buffer and agitated for 15 minutes at 37°C. After washing with TBS-Tween, western blotting was continued with an anti-Akt (total) antibody. Alternatively, the antibody 4G10 was used in place of anti-phospho-Akt antibody, to measure total protein tyrosine phosphorylation.

#### 3.6.6.2 Without Stirring or Aggregation

PRP was prepared from whole blood and adjusted to 3x10^8 platelets/ml with PPP. 500 μl samples were treated with saline, control Fab, or mFab7 in eppendorf tubes for 15 minutes at
<table>
<thead>
<tr>
<th>Reagent</th>
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</table>
room temperature, then treated with saline or 2 μM ADP. After addition of each treatment, the suspension was carefully and slowly brought up and down twice in a pipette to gently mix. After a five minute incubation, the samples were centrifuged in a pre-cooled microcentrifuge and pellets were washed twice with ice-cold saline.

Samples were denatured with reducing sample buffer and electrophoresed on a 5-20% gradient SDS-PAGE. Samples were transferred to a PVDF membrane, which was incubated overnight on a rocker at 4ºC with anti-phospho-Akt antibody. After generous washing with TBS-Tween, Akt was labeled with donkey anti-rabbit HRP, and 4G10 was labeled with goat anti-mouse HRP. Membranes were developed with ECL-PLUS kit and stored in PBS until continuing with stripping. To strip, membranes were completely submerged in Restore Western Blot Stripping Buffer and agitated for 15 minutes at 37ºC. After washing with TBS-Tween, western blotting was continued with an anti-Akt (total) antibody.

3.6.7 Tetraspanin Web Organization

Whole blood was obtained by venipuncture using ACD as an anticoagulant. Platelets were washed twice with CGS and resuspended at 250,000/mm³ in ACD. After pretreatment with 20 μg/ml mFab7 or control Fab for 15 minutes at room temperature, platelets were activated with 2 μM ADP or allowed to remain in a resting state. Samples were removed for flow cytometric evaluation of CD63 and P-selectin on the platelet surface to verify platelet activation. Platelet samples were then resuspended in 1% Brij-98 lysis buffer and lysed overnight on a rocker at 4ºC. Samples were clarified by centrifugation at 18,300 x g for 10 minutes at 4ºC and the pellet was discarded. Protein concentration was detected using the Biorad Detergent-Compatible Assay kit and protein concentration adjusted to 1.5 mg/ml.

Samples were precleared with Protein A beads, centrifuged, and the supernatant transferred to a clean tube. Samples were then incubated for one hour at 4ºC with mlgG as a control or with 10E5, an antibody that binds the GPIIb-IIIa complex regardless of activation conformation. Samples were then incubated with Protein A beads overnight on a rocker at 4ºC.

Immunoprecipitation complexes were separated on a 5-20% reducing SDS-PAGE and transferred to a PVDF membrane. After the transfer, the membrane was cropped from 15 kDa to 35 kDa to probe for CD9 (mAb7); from 35 kDa to 75 kDa to probe for CD63 (BD anti-CD63); and from 75 kDa to above 160 kDa to probe for GPIIb-IIIa (C3). Probing occurred overnight at 4ºC. Primary antibodies were then labeled with HRP-conjugated Sheep anti-mouse antibody in TBS-Tween + 5% nonfat dry milk and images developed with ECL-PLUS kit.
3.6.8 Clot Retraction

Whole blood was obtained by venipuncture using 0.1 M buffered citrate as an anticoagulant. PRP was obtained and diluted with PPP to obtain platelets at either 250,000/mm$^3$ or 100,000/mm$^3$. After 15 minutes incubation in eppendorf tubes with 20 μg/ml mFab7, 20 μg/ml control Fab, corresponding volume of saline, or 20 μg/ml D3 (as a positive control) samples were transferred to 12 x 75 glass tubes. CaCl$_2$ was added to each tube, samples were mixed by flicking, and each tube was capped and incubated in a 37 °C waterbath. Tubes were observed every 15 minutes until retraction was complete.

3.7 Statistical Significance

Values are reported as mean ± standard deviation as calculated by Microsoft Excel (Microsoft Corporation, Seattle, WA). T-tests were performed using Microsoft Excel. Results of cytoskeletal incorporation and aggregation with recombinant proteins were evaluated using ANOVA (Boneferroni test) with Sigma stat software.

Results are expressed as percentages ± standard deviation. p value of ≤0.05 was accepted as statistically significant.
CHAPTER 4 RESULTS

4.1 Tetraspanin Expression on Resting and Activated Platelets

The surface expression levels of CD9, the tetraspanins CD63 and CD151, and the tetraspanin-associated proteins GPIb and GPIIb-IIIa (integrin αIIbβ3) was evaluated in platelets that were either allowed to remain resting or were activated with 10 μM ADP or 5 μM TRAP (Fig 4.1A). Although TRAP is a more potent agonist than ADP, these concentrations should induce platelet activation, including granule release.

Although CD9 has been characterized as a member of alpha granules (Brisson 1997, Cramer 1994), the surface expression of CD9 in resting platelets (mean fluorescence intensity (MFI) = 183.2 ± 28.7) versus ADP- (236.4 ± 25.8) or TRAP-activated (229.0 ± 30.3) platelets was not statistically significantly different. The lack of statistical significance may be due to the wide range of CD9 expression among individual donors, as each donor showed an increase in CD9 surface expression upon activation, particularly with ADP (Fig 4.1B).

As expected, resting platelets did not express CD63 (MFI = 8.2 ± 1.3, compared to background control mlgG (MFI = 8.0 ± 1.0). CD63 expression was detected upon ADP activation (16.5 ± 3.2) and TRAP activation (27.7 ± 5.9). The increase in CD63 expression was statistically significant in activated vs. resting platelets (p<0.05), but not between ADP and TRAP activation (p>0.05).

CD151 was detected on the surface of resting platelets (MFI = 24.6 ± 2.5), and was statistically significantly increased by ADP activation (MFI = 42.4 ± 2.8, p ≤ 0.01 compared to resting) or by TRAP activation (MFI = 46.2 ± 3.4, p ≤ 0.01).

GPIIb-IIIa surface expression was detected by the antibody 10E5. This antibody binds to the GPIIb-IIIa complex, and has an equal affinity for both the active and inactive conformation of GPIIb-IIIa, so integrin activation following platelet activation will not alter detection of surface expression. GPIIb-IIIa was detected on the surface of resting platelets (MFI = 129.6 ± 11.0), and was statistically significantly increased by activation by ADP (MFI = 218.5 ± 15.8, p<0.01) or TRAP (MFI = 214.4 ± 13.8, p<0.01).

GPIb, part of the GPIb-IX-V complex, was also detected on the surface of resting platelets (MFI = 84.5 ± 4.5). Its level was increased to 117.2 ± 7.7 by ADP activation and to 103.9 ± 7.3 by TRAP activation. The difference between resting and ADP-activated platelets was statistically significant (p<0.01), but the difference between resting and TRAP-activated platelets was not statistically significant (p>0.05). This slight decrease following TRAP activation, compared to ADP activation, may be due to this receptor shedding its alpha subunit following potent activation (Bergmeier 2004).
Figure 4.1  Expression of Tetraspanins on the Platelet Surface
A, Platelet surface expression of CD9, CD151, CD63, GPIIb-IIIa, and GPIb under resting conditions or activated with ADP or TRAP, as measured by flow cytometry. mIgG provides a negative background control. CD9 does not show a statistically significant increase in expression upon platelet activation. CD151, CD63, and GPIIb-IIIa display a statistically significant increase in expression upon platelet activation, whether by ADP or TRAP. GPIb displays an increase in surface expression upon mild activation by ADP, but not a statistically significant increase upon stronger activation by TRAP. B, mAb7 binding to resting or activated platelets of individual donors. *, p<0.05; **, p<0.001; n=5.
4.2 Association of CD9 and GPIIb-IIIa in Resting and Activated Platelets

4.2.1 Co-Immunoprecipitations

Immunoprecipitations were performed using 1% CHAPS, a mild detergent which does not disrupt the integrity of the tetraspanin web. The platelet lysate was either immunoprecipitated with mIgG, a nonspecific antibody; mAb7, a CD9 EC2 antibody; or D3, a GPIIb-IIIa antibody (Fig 4.2). D3 was used in the presence or absence of eptifibatide to evaluate the effect of receptor occupancy on CD9-GPIIb-IIIa interactions.

After protein separation by SDS-PAGE, a denaturing polyacrylamide gel electrophoresis, immunoprecipitates were probed for CD9 or GPIIb (a component of GPIIb-IIIa). D3 was found to immunoprecipitate GPIIb, with no difference upon inclusion of eptifibatide. D3 also co-immunoprecipitated a portion of CD9, with no difference upon inclusion of eptifibatide. Conversely, mAb7 was able to immunoprecipitate CD9 and co-immunoprecipitate a portion of GPIIb. The negative control, mIgG, was not able to immunoprecipitate either protein, confirming antibody specificity.

These results indicate that a portion of CD9 and GPIIb-IIIa associate with each other in the membrane, and that this interaction is maintained with 1% CHAPS. Integrin binding to eptifibatide does not appear to alter the association between CD9 and GPIIb-IIIa, suggesting that the association between CD9 and GPIIb-IIIa may be controlled by another mechanism besides ligand binding to the integrin.

4.2.2 Confocal Analysis

Platelets were allowed to spread on four matrix substrates: FG, FN, Fb-FN, or Fb (Fig 4.3). All of these matrix proteins are ligands for GPIIb-IIIa, and FN is additionally a ligand of CD9. After 60 minutes, most adherent platelets were fully spread, although there were some platelets just extending filopodia or lamellipodia. At this concentration, some platelets did not contact other platelets, while other platelets were in direct physical contact with adjacent platelets.

On all matrices, CD9 was most intense at platelet-platelet contacts. Additionally, CD9 was pronounced at the platelet periphery, within the extended filopodia and lamellipodia, and centralized at discrete points in the platelet body (potentially where granules were clustered).

The matrices displayed some modest differences in CD9 distribution. CD9 localization on fibrin or fibrin-FN was more diffuse, with increased signal across the entire surface of the platelet compared to FG or FN.
Figure 4.2  Co-Immunoprecipitation of CD9 and GPIIb-IIIa
A, GPIIb is detected in samples immunoprecipitated with the CD9 antibody mAb7, as well as the GPIIb-IIIa LIBS antibody D3 in the presence or absence of 4000 nM eptifibatide. B, CD9 is detected in samples immunoprecipitated with mAb7, and a fraction of C9 is co-immunoprecipitated with D3. Co-immunoprecipitation is not altered by the presence of 4000nM eptifibatide. mIgG is not able to precipitate GPIIb or CD9.
Figure 4.3  Localization of CD9 and GPIIb-IIIa in Spread Platelets

CD9 staining by ML13-FITC is similar in platelets spread on either FG or FN. CD9 can be found across the entire platelet surface, but is most intense at the platelet periphery or in filopodia. There is also an increased level of CD9 at platelet-platelet contact sites. GPIIb-IIIa staining by D3-TRITC on both matrices is concentrated on the periphery of the spread platelets. Staining is also present in platelet centers. Colocalization between CD9 and GPIIb-IIIa is found at the platelet periphery. Colocalization is also found at platelet-platelet contact sites in platelets spread on FG. In platelets spread on fibrin, CD9 localization is diffuse across the entire platelet surface. In platelets spread on fibrin, CD9 localization is diffuse across the entire platelet surface. In platelets spread on fibrin-FN, the CD9 localization appears as an intermediate between platelets spread on fibrin. On both matrices, colocalization is found at the platelet-platelet contact sites.
GPIIb-IIIa also tended to be most intense at the platelet periphery and platelet centers. GPIIb-IIIa was most diffuse in platelets spread on fibrin. There was extensive co-localization between CD9 and GPIIb-IIIa in spread platelets on all matrices. Co-localization was most pronounced at the platelet periphery and at platelet-platelet contacts. This suggests that CD9 plays an important role in modulating GPIIb-IIIa adhesive functions at platelet-platelet contacts and in platelet spreading. CD9 may alter the conformation of GPIIb-IIIa at these areas, or may be modulating the presentation of ligand to integrin.

4.2.3 Impact of Mutant GPIIb-IIIa

Platelets from patients with Glanzmann Thrombasthenia, expressing mutant GPIIb-IIIa on the surface that is incapable of binding ligand, were able to bind and spread on fibronectin and fibrin-fibronectin (Fig 4.4). The platelet adhesion and spreading ability may be due to other platelet integrins, to CD9, or to a possible unaffected ligand binding site on GPIIb-IIIa. Interestingly, a subset of the platelets expressing the mutant GPIIb-IIIa exhibited abnormal platelet spreading on both matrices—they spread over a larger surface area on fibrin, and over a smaller surface area on fibrin-fibronectin, compared to normal controls.

Although co-localization analysis is difficult to perform due to the diminished GPIIb-IIIa staining, it appears that the expressed GPIIb-IIIa exhibits localization and CD9 colocalization similar to normal platelets.

In the platelets expressing mutant GPIIb-IIIa, flow cytometric analysis demonstrated that CD9 expression was increased (data not shown). This could be due to attempted compensation, as CD9 is believed to be a receptor for fibronectin and could attenuate the impact of mutated GPIIb-IIIa. However, it is also possible that the mutation of GPIIb-IIIa alters the CD9-GPIIb-IIIa association to such an extent that there is better access for antibody binding.

4.3 Localization of CD9 during Platelet Spreading

4.3.1 Platelet-Matrix Contact

Slices of spread platelets were viewed independently of other slices above or below the desired plane using the confocal microscope (Fig 4.5). Platelets were sliced into three sections: the basal section included the segment of the platelet in contact with the matrix; the apical section was the upper third of the platelet facing away from the matrix; and the central section comprised the portion of the platelet between these two sections.
Figure 4.4  CD9 Localization in Glanzmann Thrombasthenia

A, Comparison of normal and GT platelets spread on FN. B, Comparison of normal and GT platelets spread on Fb-FN. Green, CD9; Red, GPIIb-IIIa; Yellow, merge. Note that GPIIb-IIIa mutation does not alter platelet adhesion nor the colocalization between CD9 and GPIIb-IIIa; however, extent of platelet spreading is altered, with platelets spreading more on Fb and less on Fb-FN compared to normal controls.
Figure 4.5  Analysis of CD9 Localization in Platelet Sections with or without Matrix Contact

GPIIb-IIIa (D3-TRITC, red) is localized in the basal portion of the platelet (in contact with the matrix). Much lower GPIIb-IIIa levels are observed in the apical sections of the platelet. CD9 (ML13-FITC, green) is found in the apical and central sections of the platelet, but only a low level of expression is observed in contact with matrix. Co-localization (yellow) between CD9 and GPIIb-IIIa is found mainly at the platelet-platelet contact sites. There is only a low level of CD9 and GPIIb-IIIa colocalization at platelet-matrix contact sites on the basal portion of the platelet. Additionally, only a low level of colocalization is observed at the apical sections of the platelets.
GPIIb-IIIa expression was found to be intense in the basal section, where platelets were in contact with the matrix. GPIIb-IIIa was also found at regions of platelet-platelet contact in the basal and central sections. Surprisingly, there was very little CD9 expression observed in the basal section of the platelets on any of the matrices. This indicates that CD9 does not likely have an important role in direct adhesion to the matrix—even to fibronectin, a known ligand—or to modulating GPIIb-IIIa adhesion to the matrix. However, the expression of CD9 in spreading structures such as filopodia and lamellipodia suggests a role for CD9 in platelet spreading events. CD9 expression was intense in the central region of the platelet, where it was strongly colocalized with GPIIb-IIIa, particularly at platelet-platelet contact sites. This further indicates a likely involvement of CD9 in modulating the role of GPIIb-IIIa in platelet-platelet interaction and thrombus stability.

Only low levels of CD9 or GPIIb-IIIa were observed in the apical sections of platelets. This is not unusual, as both proteins would be expected to be localized to sections of the platelet membrane involved in contact with platelets or matrix.

4.3.2 Platelet-Platelet Contact

When platelet spreading resulted in platelet-platelet contact, CD9 staining was greatly intensified at these contact points (pixel intensity = 141.5 ± 8.9) compared to the platelet periphery not involved in platelet-platelet contact (pixel intensity = 32.7 ± 16.2) (Fig 4.6). It is evident that CD9 is being actively localized into areas of platelet-platelet contact, and not merely an additive effect, as the intensity at platelet-platelet contact is more than double the intensity at non-contact periphery.

4.3.3 Platelet Spreading Structures

The localization of CD9 was initially characterized in platelets throughout the stages of adhesion and spreading on fibrinogen, as well as its colocalization with F-actin, using confocal microscopy (Fig 4.7). Immediately upon adhesion to fibrinogen, platelets developed a rounded shape, with CD9 distributed throughout the entire platelet surface. CD9 was colocalized with F-actin as evidenced by the yellow merge signal. As the platelet began extending filopodia and lamellipodia, CD9 was increasingly localized to these extensions. Co-localization with F-actin was still observed, although the degree of co-localization was decreased as platelet spreading proceeded. As the platelet progressed to a fully spread morphology, CD9 was distributed evenly across the platelet membrane; however, after 60 minutes, a large portion of CD9 localized in punctate clusters at the leading edge of the platelet. Co-localization of CD9 and F-actin was no longer evident.
Figure 4.6  Localization of CD9 at Platelet-Platelet Contacts

A, CD9 staining is intensified at areas of contact between spread platelets (red arrowheads).  
B, Quantitation of pixel intensity at platelet-platelet contacts vs. the platelet periphery not involved in platelet-platelet contacts. These data indicate that CD9 is actively localized at platelet-platelet contacts, and that the observation is not due to the additive effect of multiple platelet membranes in contact. **, p<0.01. n=4.
Figure 4.7  Localization of CD9 and Actin during Platelet Spreading
Washed platelets in progressive stages of platelet spreading. In newly adherent, round platelets, CD9 (ML13-FITC) covers the platelet surface. There is a high level of colocalization with F-actin (Phalloidin-TRITC). As the platelet extends filopodia and lamellipodia, CD9 is highly expressed in these areas. The level of CD9 and actin co-localization appears to diminish as the platelet proceeds through the spreading process. In a nascent, spread platelet, the CD9 is found across the platelet membrane but is most strongly expressed at the platelet periphery. As platelets are allowed to spread for 60 minutes, CD9 localizes to discrete, punctuate areas on the platelet periphery, although there is still CD9 localized across the entire platelet surface. n=4.
4.4 Generation and Purification of mAb7 Fab (mFab7) Fragments

To evaluate the role of CD9 independently from the platelet FcγRII receptor, Fab fragments were created from the monoclonal antibody mAb7, an antibody with a high affinity for CD9 EC2. This antibody was chosen due to its functional effects in smooth muscle cells (unpublished data) as well as its functional effects in CHO cells transfected with human GPIIb-IIIa (Cook 1999).

Upon cleavage with papain and passage through a Protein A column, the isolated Fab fragments, henceforth designated mFab7, along with intact mAb7 and control mlgG and control mlgG Fab fragments, were electrophoresed reduced and nonreduced through SDS 5-20% polyacrylamide gradient gels. Protein staining by Gelcode Blue showed a homogeneous preparation with no detectable intact mAb7 even with > 50 μg Fab protein loaded onto the gel (Fig 4.8A). The contaminant band of approximately 60 kDa in mAb7 and mFab7 lanes was excised and subjected to mass spectrometry. This protein was identified as murine albumin (data not shown).

As intact mAb7 can induce platelet aggregation, aggregation tracings were recorded with mAb7 and mFab7 to verify that mFab7 was purified, without any traces of intact antibody. Aggregations performed using up to 100 μg/ml mFab7 did not induce aggregation even after 15 minutes, while mAb7 concentrations > 0.2 μg/ml induced an aggregation response (Fig 4.8B). Therefore, from a functional perspective, the mFab7 was virtually free of intact, functional mAb7.

A FITC-labeled, goat anti-mouse-Fab antibody was used to verify binding competency of mFab7 under flow cytometry. Mean fluorescence intensity (MFI), measuring binding of anti-Fab to mAb7 and mFab7, was 92.45 and 112.84, respectively, indicating Fab7 binding was similar to that of the intact mAb7 antibody (Fig 4.8C). When a FITC-labeled goat anti-mouse-Fc antibody was used, the MFI for the mAb7 sample was 108.58 as expected, while the MFI of the mFab7 sample was reduced to control mouse levels, further indicating that the mFab7 sample was free of intact mAb7 (Fig 4.8D).

4.5 CD9 Perturbation

4.5.1 Platelet Spreading

Human platelets were pre-incubated with either control isotype-matched Fab (control) or mFab7 then allowed to spread on one of four matrices—fibronectin (FN), fibrin (Fb), Fb-FN cross-linked matrix, or fibrinogen (FG)—all four of which are ligands for GPIIb-IIIa, the major platelet integrin, and are...
Figure 4.8  Purification of mFab7 from mAb7
A, mAb7 digests on a non-reducing or reducing SDS-PAGE. Lane 1, undigested control mIgG; lane 2, control Fab; lane 3 undigested mAb7; lane 4, mFab7; 50 μg protein per lane.  B, mAb7 causes platelet aggregation when added to a stirring suspension of platelets, but mFab7 does not cause platelet aggregation.  C, Flow cytometric analysis indicates mFab7 binding ability on the platelet surface.  D, Flow cytometric analysis reveals that an anti-Fc antibody does not bind to platelets with mFab7, further indicating purity.
A. 

1. Albumin
2. Heavy chain
3. Light chain; Fab fragments
4. Reduced

B. 

1. Intact Ab.
2. Intact Fab
3. Albumin

20 μg/ml mAb7 (full length)

20 μg/ml mFab7
C. 

![Bar chart C](image)

D. 

![Bar chart D](image)

Figure 4.8 (continued)
exposed to platelets as components of plasma, atherosclerotic plaques, or at sites of vascular injury.

Pre-incubation with mFab7 did not appear to alter the localization or staining pattern of CD9 or F-actin, but it increased the stage of spreading of platelets on all four matrices (Fig 4.9). When platelets on Fb were quantified based on stage of spreading, it was found that platelets were in the early stages of platelet spreading—either newly adherent or extending filopodia—when they were preincubated with control Fab (p<0.05), whereas platelets treated with the mFab7 were more likely to be fully spread. Similarly, platelets on FN, Fb-FN, or FG were adherent or extending filopodia when pretreated with control Fab (p<0.05), but were extending lamellipodia or fully spread when pretreated with mFab7.

Interestingly, treatment with mFab7 increased the total number of adherent platelets on Fb-FN per frame (p<0.05), but this treatment led to no difference in the total number of adherent platelets per frame on the other matrices.

Additionally, mFab7 decreased the relative amount of actin co-localizing with CD9 on FbFN (62 ± 12% with control Fab vs 30 ± 6% with mFab7) and on FG (45 ± 7% with control Fab vs 24 ± 7% with mFab7) (Fig 4.10), but a change was not statistically significant on FN or Fb. A decrease in colocalization is likely because mFab7 treatment enhances platelet spreading, and a normal decrease in CD9-actin colocalization is observed as platelets spread more fully.

4.5.2 Platelet Aggregation and Disaggregation

MFab7 also increased the aggregation of platelets in response to low concentrations of ADP (2-3 μM) or collagen (0.6-1.0 μg/ml) (Fig 4.11 and Table 4.1). In response to threshold ADP, the control sample exhibited a mean maximal aggregation of 54.3 ± 5.0%, which then decreased to 43.7 ± 4.7%, 26.7 ± 4.0%, and 20.0 ± 2.0% after 5, 10, and 15 minutes, respectively. When mFab7 was used, the maximal aggregation is 73.7 ± 7.5%, and the aggregate is more stable, with 70.7 ± 12.7%, 64.7 ± 16.3%, and 59.3 ± 15.5% aggregation for 5, 10, or 15 minutes after addition of agonist. When threshold levels of collagen were used, the results were similar: for control-treated platelets, the percent aggregation is 62.0 ± 10.4%, 60.3 ± 13.3%, 52.0 ± 14.9%, and 44.7 ± 14.1%, respectively, for maximal aggregation, and 5, 10, or 15 minutes after addition of agonist. When threshold levels of collagen were used, the results were similar: for control-treated platelets, the percent aggregation is 62.0 ± 10.4%, 60.3 ± 13.3%, 52.0 ± 14.9%, and 44.7 ± 14.1%, respectively, for maximal aggregation, and 5, 10, or 15 minutes after addition of agonist. For mFab7-treated platelets, this increased to 72.3 ± 3.1%, 72.3 ± 3.1%, 68.0 ± 2.6%, and 62.3 ± 2.1%, respectively. When platelets were activated by threshold levels of Thrombin Receptor Agonist Peptide (TRAP, 5-10 μM), mFab7 did not increase the aggregation: 72.3 ± 7.6%, 71.3 ± 8.7%, 65.3 ± 13.6%, and 58.7 ± 15.3% for control vs. 72.7 ± 9.7%, 71.7 ± 11.7%, 66.7 ± 14.0%, and 61.0 ± 14.9% for mFab7 (Fig 4.11C and Table 4.1).
Figure 4.9 CD9 Affects Platelet Spreading on Fibronectin (FN), Fibrin (Fb), Fibrin-Fibronectin Matrix (Fb-FN), or Fibrinogen (FG)

A, Quantification of platelets throughout stages of platelet spreading. B, Confocal microscopy images show platelets that are incubated with 20 μg/ml mFab7 (right) exhibit greater spreading on all matrices than platelets that are incubated with 20 μg/ml of a nonspecific control Fab. Localization of CD9 does not appear to be altered by mFab7 binding. C, Zoom of Merge view from B. *, p<0.05. **, p<0.01. n=5.
Figure 4.9 (continued)
Figure 4.9 (continued)
Figure 4.10  Impact of CD9 Perturbation on Colocalization with F-actin
CD9 perturbation with mFab7 results in decreased levels of F-actin (as measured by TRITC-phalloidin) co-localizing with CD9 (as measured by ML13-FITC) in platelet spreading on FbFN and on FG. Perturbation does not lead to statistically significant changes in colocalization in platelets spread on Fb or on FN. n=5. **, p<0.01.
Figure 4.11  CD9 Affects Platelet Aggregation

A, Platelets activated by ADP. Preincubation with mFab7 results in a significant increase in percent aggregation at all time points compared to preincubation with control Fab. 

B, Platelets activated by collagen. Preincubation with mFab7 results in a significant increase in percent aggregation at maximum aggregation and at 10 and 15 minutes after addition of collagen. 

C, Platelets activated by thrombin receptor agonist peptide (TRAP). Preincubation with mFab7 does not alter platelet aggregation. max, maximum aggregation after addition of agonist.

*, p<0.05. **, p<0.01. n=4.
Figure 4.11 (continued)
Table 4.1. Perturbation of CD9 Affects Percent Platelet Aggregation

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<td>71.3 ± 8.7</td>
<td>65.3 ± 13.6</td>
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</table>

1 Percent platelet aggregation, showing the maximum aggregation achieved, and percent aggregation at 5, 10, and 15 minutes after addition of agonist, ± standard deviation.
Eptifibatide, a GPIIb-IIIa antagonist, is also used in conjunction with these agonists to evaluate platelet disaggregation. The addition of eptifibatide to platelet aggregates enhances the rate and extent of disaggregation of platelet aggregates by displacing bound fibrinogen to the activated GPIIb-IIIa receptor. The ability of eptifibatide to increase the rate and extent of platelet disaggregation was decreased by treatment with mFab7 with all three agonists studied (Fig 4.12 and Table 4.2); however, the effect of eptifibatide was not totally blocked. When threshold ADP was the agonist, percent aggregation at 5, 10, and 15 minutes after addition of eptifibatide was 38.3 ± 2.1%, 23.7 ± 2.5%, and 19.3 ± 2.3%, respectively, for control-treated platelets, and 57.3 ± 5.1%, 44.7 ± 6.1%, and 38.7 ± 6.7%, respectively, for mFab7-treated platelets. When threshold collagen was the agonist, the percent aggregation for control-treated platelets was 31.0 ± 19.1%, 27.0 ± 17.4%, and 24.6 ± 16.7%, respectively, while for mFab7-treated platelets it was 62.3 ± 8.1%, 57.0 ± 9.8%, and 53.3 ± 11.2%, respectively. mFab7 even exerted an effect on disaggregation when TRAP was used as the agonist: percent aggregation was 45 ± 18.1%, 33.7 ± 15.3%, and 29.0 ± 13.0% with control-treated platelets, and 62.3 ± 8.1%, 57.0 ± 9.8%, and 53.3 ± 11.2% for mFab7-treated platelets.

Additionally, mFab7 and control Fab were pre-incubated with a goat F(ab’)2 anti-mouse-Fc before adding to platelets, at a rate of 10 F(ab’)2 per possible intact mAb7, as calculated above. Although the F(ab’)2 slightly decreased aggregation, the mFab7 still exhibited increased aggregation and thrombus stability compared to the control Fab.

The results from these experiments led to the hypothesis that CD9 may indeed serve as a link to integrin activation and its function may serve as a tipping point for platelet reactivity. Further examination of GPIIb-IIIa function, such as fibrinogen binding, was measured with or without Fab treatments.

### 4.5.3 Platelet Activation

Platelet activation was assessed by detection of CD63 and P-selectin (CD62) via flow cytometric analyses (Fig 4.13). Resting platelets, in the absence of agonist, did not have a statistically significant difference in the measurement of either CD63 or P-selectin between control- and mFab7-treated platelets. However, when platelets were activated by threshold levels of ADP (2 μM), mFab7 treatment increased the expression of both CD63 and P-selectin: the MFI for control-treated platelets was 33.3 ± 0.2 for P-selectin and 14.1 ± 3.0 for CD63, while for mFab7-treated platelets the MFI was increased to 91.1 ± 14.8 for P-selectin and 25.6 ± 3.3 for CD63. Interestingly, mFab7 did not increase the expression of either protein when platelets were activated with threshold TRAP (10 μM). Future studies can be performed to determine the optimal concentration of TRAP for mFab7 to amplify the signal.
Figure 4.12  CD9 Affects Eptifibatide-Induced Platelet Disaggregation

4000 nM eptifibatide is added to agonist-activated platelets at the point of maximum aggregation (2.5 min after addition of agonist). Incubation with mFab7 results in more stable aggregates with all agonists tested. A, ADP; B, Collagen; C, TRAP. *, p<0.05; **, p<0.01. n=4.
Figure 4.12 (continued)
Table 4.2. Perturbation of CD9 Affects Eptifibatide-Induced Platelet Disaggregation\(^2\)

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Fab</th>
<th>Max aggregation</th>
<th>5 min</th>
<th>10 min</th>
<th>15 min</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>ADP</td>
<td>mFab7</td>
<td>69.4±4.0</td>
<td>57.3±5.1</td>
<td>44.7±6.1</td>
<td>38.7±6.7</td>
</tr>
<tr>
<td></td>
<td>control Fab</td>
<td>63.3±4.5</td>
<td>38.3±2.1</td>
<td>23.7±2.5</td>
<td>19.3±2.3</td>
</tr>
<tr>
<td>Collagen</td>
<td>mFab7</td>
<td>72.7±3.7</td>
<td>62.3±8.1</td>
<td>57.0±9.8</td>
<td>53.3±11.2</td>
</tr>
<tr>
<td></td>
<td>control Fab</td>
<td>59.3±8.0</td>
<td>31.0±19.1</td>
<td>27.0±17.4</td>
<td>24.6±16.7</td>
</tr>
<tr>
<td>TRAP</td>
<td>mFab7</td>
<td>74.7±0.6</td>
<td>63.3±1.5</td>
<td>51.3±3.1</td>
<td>46.3±2.1</td>
</tr>
<tr>
<td></td>
<td>control Fab</td>
<td>68.7±4.5</td>
<td>45±18.1</td>
<td>33.7±15.3</td>
<td>29.0±13.0</td>
</tr>
</tbody>
</table>

\(^2\) Percent platelet aggregation, showing the maximum aggregation achieved, and percent aggregation at 5, 10, and 15 minutes after addition of eptifibatide, ± standard deviation.
Figure 4.13 CD9 Affects Platelet Activation
Pre-incubation with 20 μg/ml mFab7 increases P-selectin (CD62P) and CD63 expression on platelets activated with 2 μM ADP, compared to platelets pre-incubated with 20 μg/ml nonspecific control Fab. mFab7 did not affect P-selectin or CD63 expression on platelets that were activated with 10 μM TRAP or on platelets that were not activated by agonist. **, p<0.01. n=5.
4.5.4 Fibrinogen Binding

In the absence of agonist stimulation, platelets bound a low level of fibrinogen, with no difference between control Fab-treated platelets and mFab7-treated platelets (mean MFI = 48.06 ± 22.3 and 48.13 ± 22.5, respectively) (Fig 4.14). When platelets were activated without stirring by 2 μM ADP, the binding of fibrinogen increased slightly (MFI = 70.13 ± 5.4). The most notable increase in fibrinogen binding occurred when platelets treated with mFab7 and activated by ADP (MFI = 122.1 ± 15.9). The increase in fibrinogen binding of ADP-activated platelets that are treated by mFab7 versus treated by control Fab is significant as determined by ANOVA.

4.5.5 Cytoskeletal Incorporation

In order to explore CD9 effects on the integrin-cytoskeleton linkage, core cytoskeletons were prepared from mFab7 treated platelets. Platelet samples were lysed at five min after addition of agonist. mFab7 increased the incorporation of the cytoskeletal proteins actin binding protein (ABP), α-actinin, and actin into the core cytoskeleton compared to control-treated platelets (Fig 4.15A). Platelets activated by 2 μM ADP, pre-incubated with control Fab or without Fab, did not form stable aggregates, and thus the incorporation of these proteins was similar to the level of incorporation in resting platelets. Similarly, incubation with mFab7 or control Fab in the absence of ADP did not increase cytoskeletal protein incorporation. However, when platelets were incubated with mFab7 and activated with 2 μM ADP, the incorporation of ABP, α-actinin, and actin into the core cytoskeleton increased to levels higher than seen with mFab7 or ADP alone. The level of incorporation was similar to levels observed when platelets were activated with 5 μM ADP, a concentration which allows formation of more stable aggregates. The relative density of actin incorporated into the platelet cytoskeletons is shown (Fig 4.15B). Statistical significance is designated by asterisks.

4.5.6 Akt Phosphorylation

In order to examine possible signaling molecules involved in CD9-mediated platelet functions, platelets were pretreated with mFab7, control Fab, or saline control and stirred in the aggregometer, with or without threshold ADP, for 5 minutes. Total platelet lysates were separated and transferred to a PVDF membrane, which was blotted with the antibody 4G10. This antibody is capable of binding to phosphorylated tyrosine residues without preference for a particular protein. This resulted in the appearance of several bands, most prominently Akt. However, CD9 ligation by mFab7 did not appear to alter the intensity of any of the bands (4G10 data not shown).
Figure 4.14 CD9 Affects Ligand Binding
Platelet incubation with mFab7 results in increased fibrinogen binding in the presence of ADP as indicated by increased PAC-1 binding. mFab7 does not affect fibrinogen binding in resting platelets. **, p<0.01. n=4.
Figure 4.15 CD9 Alters the Platelet Core Cytoskeleton

A. Coomassie-blue-stained reduced gel showing increase in ABP, myosin, α-actinin, and actin in the presence of ADP and mFab7. Lanes: A, total platelet lysates, at 50% platelet count compared to other lanes; B, resting platelets; C, treated with 2 μM ADP; D, treated with mFab7; E, treated with control Fab; F, treated with 2 μM ADP+mFab7; G, treated with 2μM ADP+control Fab; H, treated with 5 μM ADP; I, molecular weight standards. B, Quantification of actin incorporated into core cytoskeleton. *, p<0.05; **, p<0.01. n=3.
Akt has been shown to be involved in CD9-related signaling cascades (Kotha 2008, Saito 2006), and its phosphorylation has been shown to be reduced by the tetraspanin CD151 in rat fibroblasts (Sawada 2003). For these reasons, Akt phosphorylation was specifically studied. However, it did not appear that mFab7 ligation to CD9 altered the level of phosphorylated Akt compared to control Fab or saline treatments (Fig 4.16). Although it appears that Akt phosphorylation is not involved in mediating the platelet activation through CD9 perturbation, it must be noted that the levels of Akt phosphorylation were very weak even in the positive controls.

Examination of Akt phosphorylation in the absence of stirring or incubation also showed that there was no effect induced by mFab7 ligation to CD9 (data not shown).

Future work may determine specific conditions under which CD9 modulates the Akt pathway, or another signaling pathway in platelets may be identified as crucial to the impact of CD9 on platelet phenotypes.

4.5.7 Tetraspanin Web Organization

Immunoprecipitations were carried out to observe the interactions between GPIIb-IIIa, CD9, and CD63, key members of the tetraspanin web. This web may be a critical scaffolding feature in the cell membrane, as well as having key roles in regulating GPIIb-IIIa regulation.

GPIIb-IIIa was immunoprecipitated with the antibody 10E5, which is capable of binding both active and inactive GPIIb-IIIa with equal affinity. It was capable of immunoprecipitating equivalent levels of GPIIb-IIIa when platelets were treated with mFab7 or control Fab, in the presence or absence of 2 μM ADP. An equal level of CD9 was immunoprecipitated in all samples (Fig 4.17). This indicates that although CD9 ligation via mFab7 may have a subtle impact on CD9-GPIIb-IIIa interactions, it is not capable of abolishing this interaction. As previous reports indicate that the CD9-GPIIb-IIIa interaction is not dependent on platelet activation or integrin activation state, it is expected that mFab7 treatment would be capable of encouraging activation without altering the tetraspanin-integrin association.

When platelets were treated with control Fab, CD63 was not co-precipitated with GPIIb-IIIa, even in the presence of 2 μM ADP. This concentration of ADP is at the threshold of platelet activation and capable of inducing a low level of CD63 surface expression among a portion of the platelets.

Interestingly, mFab7 treatment increased the amount of CD63 co-precipitated with GPIIb-IIIa. While this effect is most pronounced—as expected—in conjunction with low-level ADP, CD63 is also co-precipitated in the absence of
Figure 4.16  CD9 Perturbation Does Not Appear to Alter the Phosphorylation of Akt

Western blot showing level of phosphorylated Akt from platelet lysates. Treatments: mFab7+ADP; mFab7 without agonist; control Fab+ADP; control Fab without agonist; Saline control+ADP; saline control without agonist. While it appears that platelet activation via ADP increases Akt phosphorylation compared to resting samples, there is not an appreciable difference in Akt phosphorylation in samples treated with mFab7 versus controls. Similar results were seen in non-stirred platelets. n=2
Figure 4.17 CD9 Perturbation Alters Tetraspanin Web Associations

A, GPIIb-IIIa immunoprecipitated by 10E5. B, CD63 co-immunoprecipitates with GPIIb-IIIa in the presence of mFab7, even in the absence of agonist stimulation, but does not co-immunoprecipitate with GPIIb-IIIa in the absence of mFab7, even in when activated by weak agonist (2 μM ADP). C, CD9 co-immunoprecipitates with GPIIb-IIIa equally, regardless of presence of mFab7 or ADP.

Lanes: 1,2: mFab7; 3,4: mFab7 + 2 μM ADP; 5,6: control Fab; 7,8: control Fab + 2 μM ADP.
agonist. It is possible that a subset of platelets are mildly activated and thus expressing CD63 on the surface. It is also possible that, post-lysis, Fab7 enables association between CD63 from lysosomal membranes and GPIIb-IIIa/CD9 from the cell surface or alpha granules. Future research on the importance of CD9 in organization of the platelet tetraspanin web will likely yield exciting results.

4.5.8 Clot Retraction

Surprisingly, although CD9 perturbation was shown to modulate a wide variety of platelet functions, treatment with mFab7 did not alter clot retraction of platelets in PRP (Fig 4.18). Samples treated with mFab7, control Fab, saline, and D3 had formed a clot by 5 minutes. Between 15 minutes and extending through to overnight incubation when retraction was complete, the samples treated with mFab7, control Fab, and saline exhibited a similar extent of platelet-mediated clot retraction and at a similar rate. This was true whether platelets were at 250,000/mm$^3$ or at 100,000/mm$^3$. However, D3 samples were consistently resistant to clot retraction.
Figure 4.18  mFab7 Interaction with CD9 Does Not Alter Clot Retraction
Left to right, samples are: saline; 40 μg/ml control Fab; 40 μg/ml mFab7; 80 μg/ml D3. A, 5 minutes after addition of CaCl$_2$, clots are formed in all samples. B-F: Samples at 15 min, 30 min, 60 min, 120 min, and overnight. Samples treated with D3 resist clot retraction. There is no observable difference in clot retraction between samples treated with saline, control Fab, or mFab7. n=4.
CHAPTER 5  CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Tetraspanins have been implicated in a wide range of cell functions. These include cell motility (Huang 2006), tumor cell metastasis (Takeda 2007), cell proliferation (Ko 2006), differentiation (Unternaehrer 2007, Saito 2006), sperm-egg fusion (Ziyyat 2006), and virus-induced syncytia formation (Gordon-Elonso 2006).

5.1.1 Localization and Protein Partners of CD9

The content of the tetraspanin web was studied under conditions of resting and activated platelets. As shown in Fig 4.1, the surface expression of CD9 was found to be quite high on platelets, and was not found to be altered when platelets were resting or activated with ADP or TRAP. However, there was a wide variability in CD9 surface expression, which could contribute to the lack of statistical significance. These observations suggest a role for CD9 in maintaining the scaffolding of the tetraspanin web or modulating the function of proteins, such as GPIIb-IIIa, in the tetraspanin web, and thus modulating the activation state of platelets, rather than having a role directly involving or requiring platelet activation. GPIIb-IIIa and CD151 were also found on the surface of resting platelets, although the surface expression of these proteins was increased upon platelet activation. The increase in expression is likely due to the fusion of granules with the surface membrane upon activation. CD63 is not expressed on the surface of resting platelets. It is mildly expressed under weak activation by ADP, and surface expression is further increased under stronger activation by TRAP, when platelet lysosomal membranes fuse with the platelet surface membrane. GPIb is found on the surface of platelets, and surface expression increases under ADP activation, but the surface expression is not increased following activation by TRAP. This may be due to the receptor becoming shed or endocytosed following potent activation (Bergmeier 2004).

Immunoprecipitations confirmed that CD9 and GPIIb-IIIa associate with each other, as each was able to co-precipitate a portion of the other (Fig 4.2). Eptifibatide, a small molecule which mimics RGD-containing proteins and binds to GPIIb-IIIa, did not alter the level of co-precipitation, indicating that ligand binding does not alter the association between the integrin GPIIb-IIIa and the tetraspanin CD9. This further suggests a role for CD9 in regulating GPIIb-IIIa in both a resting and activated state.

The co-localization of CD9 and GPIIb-IIIa was also studied in spread platelets. A portion of the two proteins were found to be co-localized (Fig 4.3), consistent with immunoprecipitation data. Co-localization was observed at the
platelet periphery and platelet-platelet contacts, suggesting that modulation of GPIIb-IIIa by CD9 is most critical in forming or maintaining platelet-platelet interactions and in platelet spreading (Fig 4.6). Both molecules were frequently localized to platelet centers, as expected, since both have been reported as members of alpha granules (Brisson 1997).

Platelets from patients with Glanzmann Thrombasthenia, who express a disfunctional GPIIb-IIIa, were studied to analyze if integrin function is crucial to association with CD9. These platelets were still able to adhere and spread on fibrin and on fibrin-fibronectin, possibly through a contribution of CD9 or on other platelet integrins. These platelets exhibited greater spreading on fibrin, and decreased spreading on fibrin-fibronectin compared to normal controls (Fig 4.4). It is possible that these platelets exhibit an altered spreading ability, or that the alteration in spreading is due to an alteration in cytoskeletal linkages due to a mutant GPIIb-IIIa. The spreading alterations could also be due to the activation of different signaling cascades than normal due to activation of alternative integrins. Co-localization of CD9 and GPIIb-IIIa did not appear to be impacted by mutation of the integrin. It is interesting to note that flow cytometry confirmed a 10% increase in CD9 expression (data not shown), suggesting that CD9 may be partly compensating for the defective GPIIb-IIIa. However, it is possible that the increased signal could be due to better access for antibody binding to CD9.

CD9 was found to localize to filopodia, lamellipodia, and platelet-platelet contacts in spread platelets, leading to the hypothesis that CD9 was involved in platelet spreading and platelet interactions, including thrombus stability (Fig 4.6 and Fig 4.7). Interestingly, multiple studies indicate CD9 clusters at cell-cell contacts in a variety of cell types (Gordon-Elonso 2006, Nakamura 1995, Yang 2006).

It is interesting that CD9 was not found to localize to platelet-matrix contact sites on any of the matrices studied (Fig 4.5). This indicates that CD9 may not have a direct involvement in platelet adhesion or in modulating GPIIb-IIIa during matrix adhesion, but rather modulates GPIIb-IIIa activation during platelet-platelet interactions. These findings are consistent with other published reports, showing that tetraspanins do not localize to focal adhesions (Berditchevski 1999) and that modulation of integrins by tetraspanins can regulate adhesion strengthening but does not lead to cell adhesion to the extracellular matrix itself (Hemler 2001, Berditchevski 2001, Lammerding 2003).

5.1.2 Platelet Functions Affected by CD9 Perturbation

Platelet spreading has been shown to be affected by tetraspanins. CD151 knock-out platelets exhibit impaired cell spreading on fibrinogen (Lau 2004), as do platelets treated with CD63-blocking antibodies (Israels 2005). Additionally, transfection of CD9 into Chinese Hamster Ovary (CHO cells) has been
previously reported to increase CHO cell spreading on collagen and fibronectin (Cook, 1999). We here show that CD9 is involved in platelet spreading on four matrices: fibrin, cross-linked fibrin-fibronectin matrix, fibronectin, and fibrinogen (Fig 4.9). These four matrices are all ligands for GPIIb-IIIa and are involved in platelet functions. Fibronectin, an extracellular matrix protein, is exposed upon vascular damage; fibronectin is critical to platelet aggregation; fibrin is involved in clot retraction; and fibrin-fibronectin is important to wound healing. On all these matrices, spreading is enhanced by the presence of mFab7, an anti-CD9 Fab fragment, although CD9 localization is not affected. Moreover, on fibrin-fibronectin matrix, the presence of mFab7 increases the number of platelets adherent to the coverslip.

Platelet aggregation has been shown to be impaired in studies using CD151 knockout (Lau 2004) or TSSC-6 knockouts (Goschnick 2006). This, combined with the results that CD9 localizes to platelet-platelet contacts, led to the hypothesis that CD9 would be involved in thrombus stability. As shown in Fig 4.11, when platelets were activated by threshold levels of ADP or collagen, preincubation with mFab7 increased thrombus formation and inhibited spontaneous disaggregation. This effect was not seen with the potent agonist TRAP, even at low levels. This could be due to the fact that TRAP seems to act as an "all-or-nothing" signal; i.e., at increasingly lower concentrations it still induces a >70% aggregation, until the concentration is insufficient to elicit any aggregatory response. However, in the absence of agonist, mFab7 cannot evoke an aggregation response, even at concentrations as high as 100 μg/ml. Interestingly, platelet aggregations employing recombinant CD9 EC2, tagged with GST, show a decreased level of aggregation in response to low-dose ADP compared with GST-VAMP (Kotha, unpublished data). Reports also indicate that reduced CD9 surface expression correlate with increased platelet aggregation, although no causal relationship has been established yet (Vrelust 2007). It is possible that mFab7 treatment is inhibiting the ability of CD9 to restrain GPIIb-IIIa to its inactive conformation.

Eptifibatide, a cyclic RGD heptapeptide which acts as a GPIIb-IIIa antagonist, was used to examine platelet disaggregation responses. At threshold levels of ADP, collagen, and TRAP, platelet aggregates were more stable over fifteen minutes when they were pretreated with mFab7 compared to a nonspecific fab control (Fig 4.12). This leads us to believe that CD9 activation or blockade, via mFab7 treatment, can help stabilize the active conformation of GPIIb-IIIa, in the presence of agonist. This effect is seen at low levels of agonist, whereas at higher levels, the activation of GPIIb-IIIa is so sturdy as to overshadow the effect of CD9.

To examine the methods by which CD9 is activating GPIIb-IIIa, ligand binding assays were performed (Fig 4.14). mFab7, in the absence of agonist, did not have any effect, while in the presence of 2 μM ADP, there is a significant increase in fibrinogen binding. This shows that CD9 does encourage GPIIb-IIIa to
adopt a ligand-binding-competent conformation in the presence of agonist. In Fig 4.13, flow cytometric analysis revealed that P-selectin and CD63, markers of platelet activation, were increased by mFab7 pretreatment combined with ADP activation but not in the absence of platelet activation, revealing that in addition to modulating GPIIb-IIIa, CD9 perturbation also encourages platelet activation and release of platelet granules.

It is interesting that despite being strongly involved in other platelet functions, CD9 perturbation did not alter clot retraction of platelets in PRP (Fig 4.18). It is possible that the fibrin binding to GPIIb-IIIa initiating clot retraction leads to such a potent signal cascade, that any involvement of CD9 in clot retraction is overshadowed. This is particularly pertinent, as the effects of CD9 modulation are particularly evident when platelets are mildly activated.

5.1.3 Mechanisms Involved in Contribution of CD9 to Platelet Function

As the platelet cytoskeleton plays a key role in platelet shape change, including filopodial and lamellipodial formation and granule release, a critical step in platelet spreading and aggregatory responses, any changes in cytoskeletal proteins induced by mFab7 were assayed. It was found, in Fig 4.15, that mFab7 treatment in the absence of aggregation did not impact incorporation of key cytoskeletal proteins compared to nonspecific control Fab. While low-dose (2 \( \mu \text{M} \)) ADP induced aggregation, this aggregatory response was transient and insufficient to cause increased incorporation of cytoskeletal proteins in the absence of agonist stimulation. However, low-dose ADP combined with mFab7 treatment resulted in increased incorporation of cytoskeletal proteins, particularly actin binding protein (ABP), \( \alpha \)-actinin, and actin. This increased incorporation was statistically greater than either ADP treatment or mFab7 treatment alone, and was similar to the more potent changes resulting from activation by 5 \( \mu \text{M} \) ADP.

Phosphorylation of signaling molecules were analyzed in an attempt to clarify the signaling cascade in platelets pertinent to CD9. Total tyrosine phosphorylation by the antibody 4G10 (not shown) indicated several proteins altered by platelet activation, but did not elucidate any alterations induced by CD9 ligation.

Akt phosphorylation in particular was examined (Fig 4.16), as Akt is involved in CD9-related signaling cascades (Kotha 2008, Saito 2006), and 4G10 results indicated that Akt was particularly important in platelet activation. However, CD9 binding did not alter the level of Akt phosphorylation in stirred or non-stirred platelets. Further work may elucidate specific conditions under which CD9 modulates the Akt pathway. It is also possible that another signaling pathway in platelets may be identified as crucial to the impact of CD9 on platelet phenotypes. There are no known cytosolic binding partners for CD9 in human
platelets, but as platelets have a putative PDZ binding domain on their cytosolic tail, the possibility exists that an interaction will yet be defined.

The importance of CD9 to the interaction of the membrane proteins GPIIb-IIIa, CD9, and CD63 in the tetraspanin web was also studied (Fig 4.17). CD9 and GPIIb-IIIa association was consistent whether platelets were resting or activated, and was not impacted by mFab7 binding to CD9. This does not rule out the possibility that Fab treatment could be gently altering the association between CD9 and GPIIb-IIIa, but it certainly shows that it neither prohibits nor encourages the association. It is interesting that mFab7 treatment increased the association of CD63 into the tetraspanin web. This could be an indirect effect, as mFab7 treatment increases platelet activation, and thus there is more CD63 on the membrane to associate in the tetraspanin web. It is possible that events resulting from mFab7 ligation to CD9 preferentially causes an increase in CD63 inclusion into the tetraspanin web, and that CD9 modulates GPIIb-IIIa via CD63. It is worth noting that CD9 perturbation increased CD63 association with GPIIb-IIIa even in the absence of agonist, and thus it is possible that mFab7 treatment causes an increased association in trans post-lysis. The importance of CD9 in organization of the platelet tetraspanin web is worthy of further research.

5.2 Proposed Model

In the absence of CD9 perturbation via mFab7 or platelet agonist, CD9 exists in the tetraspanin web in association with GPIIb-IIIa, which assumes a bent, inactivated conformation (Fig 5.1A).

The addition of mFab7, which binds to CD9 EC2, may modulate the association between CD9 and GPIIb-IIIa, but this impact on CD9-GPIIb-IIIa association is not sufficient to cause integrin activation (Fig 5.1B). mFab7 does not interact with the receptor FcγRII, nor does it induce signaling through this receptor.

In the presence of agonist, the alteration in CD9-GPIIb-IIIa interaction is sufficient to stabilize the extended, active conformation of GPIIb-IIIa (Fig 5.1C). This applies even to low, threshold levels of agonist, which would cause only a weak, transient activation of GPIIb-IIIa in the absence of CD9 perturbation via mFab7. Although mFab7 treatment does not completely abolish CD9-GPIIb-IIIa interactions, it is possible that the mFab7 reduces the association of CD9 EC2 with the extracellular domains of GPIIb-IIIa, causing a phenotype associated with reduced CD9 expression (Vrelust 2007). It is anticipated that the impact of CD9 perturbation would be diminished with increasing levels of agonist, as GPIIb-IIIa would become more strongly activated.
Figure 5.1  Proposed Model of CD9 Function in Platelets
A, Resting platelet membrane. B, Binding of mFab7 does not activate GPIIb-IIIa. C, ADP in conjunction of mFab7 strongly activates GPIIb-IIIa. Black arrow, inside-out signaling from the ADP receptors P2Y1 or P2Y12 leading to activation and ligand binding of GPIIb-IIIa. Black circle, ligand bound to active GPIIb-IIIa. D, activation leads to increased linkage to the cytoskeleton and increased thrombus stability.
A.

B.
The increase in GPIIb-IIIa activation caused by agonist in conjunction with CD9 alteration then leads to increased linkage of integrin to the cytoskeleton and increased stability of platelet agonists (Fig 5.1D).

5.3 Recommendations for Future Study

Further characterization of the mFab7-CD9 interaction will need to be carried out. It is unknown at this time whether mFab7 ligation is performing an activating or blocking role. It is possible that mFab7 ligation is activating a signal cascade, which when combined with low-dose agonist, serves to encourage the active conformation of GPIIb-IIIa. It is also possible that mFab7 is shifting the conformation of CD9, leading to a direct change in the CD9-GPIIbIIIa interaction, whether through large extracellular loop or the fourth transmembrane domain. It could also be altering the association of CD9 with other tetraspanins (such as CD151 or CD63) or with a ligand (such as FN). It is even plausible that mFab7 is affecting CD9 function in trans, altering a possible extracellular interaction between CD9 and GPIIb-IIIa on nearby platelets. In light of other experiments involving CD9 function in platelets—such as the finding that platelet incubation with CD9 EC2 recombinant peptides inhibit platelet aggregation (J Kotha, unpublished data), or that decreased expression of CD9 is correlated with increased platelet aggregation in human patients (Vrelust 2007)—it is possible that CD9 encourages GPIIb-IIIa to maintain an inactive conformation, in balance with the tetraspanins CD151 and CD63 which seem to support activation. Treatment with mFab7 would then be blocking CD9 inhibition on GPIIb-IIIa, resulting in platelet activation in response to agonist, similarly to patients with reduced CD9 expression. Further characterization will shed light on this issue.

Further research using the mFab7 here created can also yield additional insight. It remains unclear what signaling cascades may be involved, and under what circumstances they may be modulated. Further characterization of the tetraspanin web, and the impact of CD9 modulation by mFab7, will also be valuable. This will provide vital information for characterizing the role of CD9 in modulating platelet function. More specific analysis of the exact sequence of CD9 involved in mFab7 binding, as well as an analysis of the impact on its association with GPIIb-IIIa, will be insightful. Studies involving CD9 EC2 recombinant peptides may be a useful tool in characterizing the extracellular association between CD9 and GPIIb-IIIa, although the possibility that mFab7 treatment could also alter the transmembrane associations between CD9 and GPIIb-IIIa cannot be ruled out at this point.

The CD9 knock-out mouse will also provide additional insight. At present, it is unknown what effect disruption of CD9 expression has on the expression levels of other tetraspanins and integrins on the platelet surface. Increased availability of antibodies against these murine proteins will allow expression levels to be quantified. Additionally, while the most obvious phenotype of these
mice is reduced fertility in the females (LaNaour 2000), careful studies of platelet function may reveal differences—which may involve increased aggregation and activation, rather than the inhibition seen with other tetraspanin knock-out mice. It is most likely that any change in platelet function will be seen using low concentrations of agonists.

I propose that CD9 is involved in modulating the activation state of GPIIb-IIIa. The addition of mFab7 to platelets, in the presence of ADP, reduces the CD9 EC2 interaction with GPIIb-IIIa, allowing GPIIb-IIIa to retain an active, extended conformation rather than a bent, inactive conformation. It will be interesting to see if patients expressing reduced levels of CD9 are more at risk for thrombotic events; therapies targeting increased surface expression of CD9 may become clinically relevant in the future.
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