RICE UNIVERSITY

Characterization of Platelet Glycoprotein Ib-IX-V – von Willebrand Factor Interaction under Shear Conditions

by

Anand Ramasubramanian

A THESIS SUBMITTED
IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE

Doctor of Philosophy

APPROVED, THESIS COMMITTEE:

Larry V. McIntire, Ph. D.,
E.D. Butcher Professor of Bioengineering

J. David Hellums, Ph. D., E.D. Butcher
E.D. Butcher Professor of Bioengineering
and Chair of Bioengineering

Kyriacos Zygarakis, Ph. D.,
A.J. Hartsook Professor of Chemical
Engineering and Bioengineering and
Chair of Chemical Engineering

José A. López, M.D., Scientific Director,
Thrombosis Research Section and
Professor of Medicine,
Baylor College of Medicine

Joel L. Moake, M.D., Associate Director,
J.W. Cox Laboratory for Biomedical
Engineering, Rice University and
Professor of Medicine,
Baylor College of Medicine

HOUSTON, TX
FEBRUARY 2004
INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

UMI®

UMI Microform 3122527
Copyright 2004 by ProQuest Information and Learning Company.
All rights reserved. This microform edition is protected against unauthorized copying under Title 17, United States Code.

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346
Characterization of Platelet GP Ib-IX-V – von Willebrand Factor Interaction under Shear Conditions

by

Anand Ramasubramanian

Abstract

Arterial thrombosis is one of the important pathophysiological mechanisms that lead to cardiovascular diseases. In this thesis, we have made an attempt to better characterize the kinetic and molecular mechanisms that underlie the critical first step in arterial thrombosis, namely, the interaction between platelet glycoprotein (GP) Ib and von Willebrand factor (VWF).

In the first part of the work, we evaluated the kinetics of interaction between platelet GP Ib-IX-V complex and VWF under arterial flow conditions. The GP Ibα subunit of GP Ib complex binds to VWF through the A1 domain of VWF. Impaired GP Ib–VWF interaction due to GP Ibα mutations can result in bleeding abnormalities including platelet-type von Willebrand disease (ptVWD). We measured the cellular on- and off-rate constants of CHO cells expressing wild-type or gain- or loss-of-function mutant GP Ibα interacting with VWF-A1–coated surfaces at different shear stresses. We found that the gain-of-function mutant, K237V, rolled very slowly and continuously on VWF-A1 surface while the loss-of-function mutant, Q232V, showed fast, saltatory movement compared to the wild-type (WT). The off-rate constants, calculated based on the analysis of lifetimes of transient tethers formed on surfaces coated with limiting densities of VWF-A1, revealed that the Q232V and K237V
dissociated 1.25-fold faster and 2.2-fold slower than the WT. The cellular on-rate constant of WT, measured in terms of tethering frequency was 3-fold more and 3-fold less than Q232V and K237V, respectively. Thus, the gain- and loss-of-function mutations in GP Ibα affect both the association and dissociation kinetics of the GP Ibα–VWF-A1 bond.

In the second part of the work, we compared the interaction of unusually large multimers of VWF (ULVWF) and that of the normal plasma multimers of VWF (P-VWF) platelets. ULVWF multimers are implicated in the pathology of a thrombotic disorder, thrombotic thrombocytopenic purpura (TTP) due to their increased affinity for platelets. We found that the ULVWF multimers are more effective than the normal P-VWF multimers in mediating (a) platelet aggregation in solution at high shear stress (b) ristocetin-modulated platelet agglutination and (c) platelet adhesion to immobilized VWF under arterial shear conditions.
To
my mentors
Shri L.V.M. & Shri J.-f.D.
ACKNOWLEDGEMENTS

I am extremely fortunate to have met so many wonderful people during my stay at Rice. Every one of them enriched my life in more ways than just one. In particular, I owe a deep sense of gratitude to the following persons:

- Dr. Larry V. McIntire, my thesis advisor, for his tremendous encouragement and support to realize my dream.

- Dr. Jing-fei Dong for teaching me all that I know and much more.

- Dr. Joel "von Willebrand" Moake for his infectious, boyish enthusiasm for research and for teaching a thing or two about thrombosis.

- Dr. José A. López for transfected CHO cells and for tips on good writing skills.

- Dr. Miguel A. Cruz for buckets of VWF-A1 and for the permission to use his lab.

- Dr. Shih-Hsin Kao for hours of enlightening discussions especially during the early stages of this work.

- Dr. J. David Hellums for encouraging me to apply to Rice and also for serving on my thesis committee.

- Dr. Kyriacos Zygourakis for serving on my thesis committee.

- Ms. Leticia Nolasco, Nancy Turner, Marcella Estrella, Carol Sun and Angie Bergeron for exceptional technical assistance.

- Members of McIntire and Dong labs for making my laboratory experience enjoyable.

- All my friends who had to endure my constant complaints about cells, proteins and images. Special thanks to Suni, Vicky, Balu, Shri for their camaraderie and Vatsan for his goodwill.

- My IMF: NIH, Welch foundation and Mary R Gibson foundation.

- And most of all, my family back home.
CONTENTS

Abstract ...................................................................................................................... ii
Acknowledgements ................................................................. ................................ v
List of Figures ...................................................................................... ................................ ix
List of Tables .......................................................................................... xi

1 Introduction .......................................................................................... 1
  1.1 Rheology of Blood ................................................................. 2
  1.2 Mural Thrombosis ................................................................. 4
  1.3 Structure and Function of GP Ib-IX-V Complex ......................... 9
  1.4 Structure and Function of VWF ................................................ 14
  1.5 Models for Cell Adhesion and Thrombosis .................. 18
    1.5.1 Mathematical models ................................................ 20
    1.5.2 Experimental methods ................................................ 26
  1.6 Summary and Thesis Objectives ............................................. 38

2 Kinetics of Platelet Glycoprotein GP Ibα – VWF-A1
Interactions ........................................................................................................ 40
  2.1 Introduction .................................................................................. 40
  2.2 Materials and Methods .......................................................... 45
    2.2.1 Cell lines ........................................................................ 45
    2.2.2 Flow cytometry ................................................................. 46
    2.2.3 Preparation of VWF-A1 coated coverslips .................. 47
    2.2.4 Measurement of VWF-A1 surface density .................. 47
    2.2.5 Parallel plate flow chamber and digital image processing .... 48
    2.2.6 Measurement of rolling velocities ......................... 49
2.2.7 Tethering rate and adhesion strength ........................................49
2.2.8 Pause time analysis ....................................................................50

2.3 Results ..........................................................................................52

2.3.1 Interaction of GP Ib-IX cells with VWF-A1 coated surfaces ........52
2.3.2 Detachment strength ...................................................................52
2.3.3 Kinetics of rolling of CHO cells expressing wild-type and mutant GP Ibα on VWF-A1 coated surfaces ............................................55
2.3.4 Kinetics of tethering of GP Ibα expressing cells on VWF-A1 coated surfaces .........................................................................59
2.3.5 Kinetics of dissociation and estimation of transient tether lifetimes ..................................................................................63
2.3.6 Kinetics of dissociation and mechanical strength of GP Ibα – VWF-A1 transient tethers .................................................................68

2.4 Discussion .......................................................................................71

3 Effect of Unusually Large Multimers of VWF on Platelet – VWF Interactions .................................................................................83

3.1 Introduction .....................................................................................83

3.2 Materials and Methods ...................................................................85

3.2.1 Preparation of washed platelets and reconstituted blood ...............85
3.2.2 Preparation of P-VWF and ULVWF .............................................86
3.2.3 Estimation of VWF multimer composition and antigen level ..........87
3.2.4 Shear-induced platelet aggregation ..............................................87
3.2.5 Ristocetin-induced platelet agglutination .....................................88
3.2.6 Measurement of VWF surface adsorption ...................................88
3.2.7 Perfusion studies .........................................................................89

3.3 Results ..........................................................................................89
3.3.1 VWF concentration in P-VWF and ULVWF preparations .......... 89
3.3.2 Shear-induced platelet aggregation .................................... 90
3.3.3 Ristocetin-induced platelet agglutination .............................. 94
3.3.4 Platelet deposition on VWF coated surfaces .......................... 94
3.3.5 VWF adsorption on glass coverslips ................................. 96
3.4 Discussion ........................................................................... 99

4 Future Work ............................................................................ 104
4.1 Kinetics of GP Ibα – VWF-A1 Interactions ............................... 104
4.2 Effect of Unusually Large VWF multimers on Platelet – VWF
  Interactions ............................................................................. 106

Reference .................................................................................. 108
LIST OF FIGURES

Fig. 1.1 Schematic of mural thrombosis 7
Fig. 1.2 Platelet activation mechanism 8
Fig. 1.3 Schematic of glycoprotein GP Ib-IX-V complex 10
Fig. 1.4 Different regions of GP Ibα subunit of GP Ib-IX-V complex 11
Fig. 1.5 Schematic of structure of VWF and VWF-A1 domain 16
Fig. 1.6 Energies of interaction in cell–cell recognition 21
Fig. 1.7 Cell–substrate contact region in peeling model 25
Fig. 1.8 Schematic of atomic force microscope in force–extension mode 27
Fig. 1.9 Schematic of real time SPR (BIAcore) 30
Fig. 1.10 Schematic of viscometers and flow chambers 33

Fig. 2.1 The GP Ib-IX complex with an exploded view of the disulfide loop 42
Fig. 2.2 Measurement of receptor and ligand densities 53
Fig. 2.3 Detachment strength of GP Ibα – VWF-A1 tethers 54
Fig. 2.4 Kinetics of rolling – Velocity trajectories 56
Fig. 2.5 Kinetics of rolling – Distribution of rolling durations 57
Fig. 2.6 Kinetics of rolling – Average rolling velocity and duration 58
Fig. 2.7 Kinetics of tethering – Tethering rate 60
Fig. 2.8 Kinetics of tethering – Tethering vs. rolling 62
Fig. 2.9 Cell tracking in a shear field to isolate transiently tethered cells 64
Fig. 2.10 Calculation of off-rate from transient tether time distribution 66
Fig. 2.11 Pause time analysis by velocity threshold 67
Fig. 2.12 Kinetics of dissociation – Off-rate calculation 69
Fig. 2.13 Kinetics of dissociation – Shear stress-induced dissociation 70
<table>
<thead>
<tr>
<th>Fig. 3.1</th>
<th>Auto-radiograph of ULVWF and P-VWF multimers on agarose gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fig. 3.2</td>
<td>Shear stress induced platelet aggregation</td>
</tr>
<tr>
<td>Fig. 3.3</td>
<td>Ristocetin induced platelet agglutination</td>
</tr>
<tr>
<td>Fig. 3.4</td>
<td>Platelet deposition on VWF-coated surfaces</td>
</tr>
<tr>
<td>Fig. 3.5</td>
<td>Equilibrium adsorption of ULVWF and P-VWF on glass coverslips</td>
</tr>
</tbody>
</table>
LIST OF TABLES

Table 1.1  Wall shear stress in different types of blood vessels  3
Table 1.2  Receptors involved in adhesion, activation and aggregation of platelets  5

Table 2.1  CHO cell lines used in this study  46
Table 2.2  Dissociation rate constants for different values of velocity thresholds  65
Table 2.3  Kinetic and mechanical properties of receptor–ligand bonds  74
Table 2.4  Some definitions for pause durations found in the literature  77

Table 3.1  Concentration of VWF in ULVWF and P-VWF preparations  92
Table 3.2  Estimation of total proteins in VWF preparations  92
CHAPTER 1

Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality in the western countries and is soon expected to become the most common cause for death in the rest of the world. More than 64 million Americans suffered from some form of this disease last year alone, of which nearly one million died (http://www.americanheart.org). CVD, as defined by the American Heart Association, includes coronary and rheumatic heart diseases, stroke and hypertension.

One of the important mechanisms that lead to CVD is arterial thrombosis, a process that results from an abnormal interaction between the coagulation proteins, platelets and/or the subendothelium in arteries. Currently available therapeutics provides only moderate protection against thrombosis. The effect of these drugs has to be tightly controlled since those that completely abolish the formation of clots are not desirable, as it would affect normal hemostasis leading to bleeding disorders (Loscalzo and Schafer, 2003). The platelet thrombus formation depends on several factors, including local hemodynamics that affects not only the transport of cells and proteins but also the mechanical forces experienced by the cells, availability of cell surface receptors and ligands, which may be serum or surface proteins or other cell surface molecules and the interaction between them (Ross et al., 1998). The introductory chapter throws light on some of these aspects – properties of blood and flow behavior and platelet transport mechanisms. Additionally, a review of the current status of cell-cell adhesion and cell-surface adhesion literature, relevant to vascular biology is
presented. The chapter concludes with an overview of the broad goals and objectives of the current research.

1.1 Rheology of Blood

Blood is a complex suspension comprising different cell types and proteins of varying sizes and shapes. As blood flows through the vessel, the vessel wall experiences different types of forces: tangential shear stress (acting in a plane parallel to the fluid flow) caused by blood flow over endothelial cells, a normal (perpendicular) stress caused by blood pressure and a tensile (longitudinal) stress caused by vessel deformation (Patrick and McIntire, 1996). The shear stress is of particular concern in affecting the gene expression in cells and in the transport of cells to the injured surface.

Blood is a thixotropic (shear-thinning) fluid with a nearly constant viscosity of 4 cP at shear rates higher than 100 s\(^{-1}\) and the viscosity increases rapidly as shear rate is lowered below 10 s\(^{-1}\) (Dintenfass, 1971). The differences in the size and compliance of blood vessel wall results in a range of shear stresses experienced by the vessel wall (Table 1.1). Consequently, there arise differences in the endothelial response to shear stress, differences in the transport rates of blood components to the blood vessel wall and the force experienced by the blood cells as they interact with each other. For instance, the thrombi formed under high flow rates (arteries and arterioles) are platelet-rich while those formed under low flow rates (veins and venules) are fibrin-rich (Loscalzo, 1995). Through most of the circulation system, blood flow is laminar with Reynolds' numbers less than 2100. However, complex vessel geometries can result in stagnant zones, formation of eddy currents and separation of streamlines thus resulting in an increase in the local deposition of the components necessary
Table 1.1: Wall shear stresses and rates in different types of blood vessels for a blood viscosity of 3.8 cP (Ross et al., 1998)

<table>
<thead>
<tr>
<th>Vessel type</th>
<th>Wall shear stress (dyn/cm²)</th>
<th>Wall shear rate (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veins</td>
<td>0.76 – 7.6</td>
<td>20 – 200</td>
</tr>
<tr>
<td>Large Arteries</td>
<td>11.4 – 30.4</td>
<td>300 – 800</td>
</tr>
<tr>
<td>Arterioles</td>
<td>19.0 – 60.8</td>
<td>500 – 1600</td>
</tr>
<tr>
<td>Stenosed Arteries</td>
<td>30.4 - 380</td>
<td>800 – 10000</td>
</tr>
</tbody>
</table>

for plaque and thrombus formation (Karino et al., 1987). Stenotic arteries and bifurcation regions, which have areas of both increased shear stress and low shear stress recirculation zones, are known to be the preferential sites for thrombus formation (Zairns et al., 1983; Badimon and Badimon, 1989). True turbulent flow is rare, except in severely stenotic vessels.

To maintain normal physiology, the blood cells and plasma proteins should be able to interact with the vessel wall in a controlled fashion. In the case of inflammation, the neutrophils adhere to the endothelium and invade the extra-cellular matrix in response to chemokines, while in hemostasis, platelets adhere to the sub-endothelial proteins and initiate the coagulation cascade in response to an injury (McEver, 2001). Both these processes require that the cells be transported, sufficiently rapidly, to the endothelial surface from flowing blood. Mass transport in flowing fluids occurs by both diffusion (Brownian motion) and also by convection (bulk flow). Diffusion is a molecular phenomenon as against convection, which is a bulk phenomenon and diffusional transport is orders of magnitude slower than convective transport. In fluids flowing through a tube, the axial mass transport is
dominated by convection while the radial transport is dominated by diffusion (Bird et al., 1960). For hemostatic and inflammatory responses, cellular and protein transport from flowing blood to the vessel wall by pure diffusion is not sufficiently rapid. Nature has designed ingenious ways to speed up the process: (a) the continued collisions between the cellular components, particularly erythrocytes, lead to an enhanced radial mixing of platelets and large protein molecules and radial dispersion of platelets towards the wall (b) the erythrocytes, because of their discoid shape, demonstrate a radial hematocrit gradient leading to a drift force on platelets and neutrophils toward the region of lower hematocrit at the wall (Wang and Keller, 1985). Thus the margination of leukocytes and radial dispersion of platelets help to maintain the normal physiologic functions in systemic circulation (Lipowsky, 1995). Once near the vessel wall, the surface receptors of leukocytes and platelets can interact with either the endothelial cell receptors or subendothelial proteins to effect inflammation and thrombosis.

1.2 Mural Thrombosis

Thrombosis can occur either by platelet aggregation through surface interaction (mural) or by bulk-fluid phase aggregation. There are several molecular mechanisms and pathways involved in platelet attachment to endothelial cells and subendothelial structures (adhesion), platelet activation and platelet cohesion to each other (aggregation, either mural or bulk). Table 1.2 lists some of the important receptors present on the platelet surface and their functions. The thrombogenic and non-thrombogenic mechanisms are exquisitely balanced to maintain normal physiology (Loscalzo and Schafer, 2003). Endothelial cells that
Table 1.2: Receptors and their most common ligands involved in adhesion activation and aggregation of platelets

<table>
<thead>
<tr>
<th>Platelet receptor</th>
<th>Ligand</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP Ib-IX-V</td>
<td>VWF</td>
<td>Adhesion</td>
</tr>
<tr>
<td>GP IIb/IIIa</td>
<td>VWF, Fibrinogen</td>
<td>Aggregation</td>
</tr>
<tr>
<td>GP Ia/IIa</td>
<td>Collagen</td>
<td>Aggregation</td>
</tr>
<tr>
<td>GP VI</td>
<td>Collagen</td>
<td>Aggregation</td>
</tr>
<tr>
<td>ADP receptor</td>
<td>ADP</td>
<td>Activation</td>
</tr>
<tr>
<td>TxA2 receptor</td>
<td>TxA2, PGH2</td>
<td>Activation</td>
</tr>
<tr>
<td>PAR-1</td>
<td>Thrombin</td>
<td>Activation</td>
</tr>
<tr>
<td>P-selectin</td>
<td>PSGL-1</td>
<td>Leukocyte adhesion</td>
</tr>
</tbody>
</table>

line the blood vessel wall provide a non-thrombogenic surface for blood flow through several redundant and interactive mechanisms: (i) synthesis and release of prostacyclin (prostaglandin I₂), which inhibits platelet activation and induces vasodilation by increasing cyclic AMP (Moncada and Vane, 1979), (ii) produces endothelium-derived relaxation factor, nitric oxide, which inhibits platelet activation and induces vasodilation by increasing cyclic GMP (Stamler et al., 1989), (iii) synthesizes tissue plasminogen activator (t-PA), which is a profibrinolytic molecule (Diamond et al., 1989) and (iv) expresses membrane bound ADPase (Marcus et al., 1991), that can cleave ADP, a potent platelet activator. Endothelial denudation results in a direct loss of these normal, protective antithrombotic mechanisms and prothrombotic properties – like expression of interleukin-1 and tumor necrosis factor, which activate coagulation cascade (Colucci et al., 1983), synthesis and release of VWF, exposure of subendothelial VWF to flowing blood (Ward and Berndt, 2000) and also adsorption of
plasma VWF to vessel surface (Savage et al., 2002). The VWF molecules that are exposed to
the blood stream play a very critical role in recruiting platelets, especially under high shear
conditions. VWF binds to platelet glycoprotein (GP) Ib receptor on the platelet surface and
the GP Ib – VWF interaction is weak enough to let the platelets translocate on the surface in
response to fluid force. The bound GP Ib initiates signaling events that lead to activation and
subsequently result in the firm adhesion and aggregation of platelets (Fig. 1.1). Although the
exact signaling pathways are not known, one recent model (Nesbitt et al., 2002; 2003)
proposes that the initial adhesion event involving GP Ib binding to VWF triggers an outside-
in signal causing an increase in intracellular calcium followed by a low upregulation of GP
IIb/IIIa. The binding of GP IIb/IIIa to VWF results in oscillatory calcium currents that drive
further upregulation of GP IIb/IIIa and ADP release (Fig. 1.2 A-B). An increase in local ADP
concentration activates neighboring platelets and stimulates platelet calcium signaling events
throughout the population. The activated platelets lump together and aggregate.

Platelet activation is associated with an increase in cytosolic calcium, shape change,
upregulation and activation of GP IIb/IIIa, generation of arachidonic acid metabolites (e.g.,
thromboxane A₂), induction of platelet coagulant activity and release of α- and dense granule
contents (Ware and Coller, 1995). Pro-thrombogenic granule contents like epinephrine and
ADP that are released diffuse into the blood stream bind to their respective receptors on
neighboring platelets and results in their activation. When ADP or other agonists like
epinephrine bind to their corresponding receptors, G proteins are activated, which in turn
activate phospholipase C (Manning and Brass, 1991). Phospholipase C hydrolyzes
phosphatidylinositol bisphosphate (PIP₂), producing diacylglycerol (DG) and inositol 1,4,5-
triphosphate (IP₃) (Berridge, 1993). Both DG and IP₃ are second messengers: IP₃ causes
Figure 1.1: Schematic of mural arterial thrombosis. Platelets on capture from blood stream roll on immobilized subendothelial VWF mediated by the platelet GP Ib-IX-V complex. Following the binding of platelets to VWF, platelets become activated which leads to the formation of stable bonds with VWF mediated by GP IIb/IIIa. GP IIb/IIIa is also involved in platelet aggregation with fibrinogen.
Figure 1.2: Platelet activation mechanism (A) Initial adhesion to VWF-A1 domain of VWF through GP Ib triggers a spike in intracellular calcium and activates GP IIb/IIIa, which binds to VWF through RGD sequence (B) GP IIb/IIIa binding to VWF causes oscillations in intracellular calcium resulting in upregulation of GP IIb/IIIa and release of ADP (C) When ADP binds to a neighboring platelet surface, it activates GTP-binding proteins which in turn activate phospholipase-C (PLC). PLC hydrolyzes phosphatidylinositol 4,5-biphosphate (PIP$_2$), resulting in the formation of diacyl glycerol (DG) and inositol triphosphate (IP$_3$). IP$_3$ causes release of calcium from dense granules and enhances the activation of phospholipase A$_2$ (PLA$_2$). PLA$_2$ releases arachidonic acid from membrane phospholipids, ultimately leading to the generation of prostaglandins and thromboxane A$_2$ that can activate platelets.
mobilization of intracellular calcium while DG activates protein kinase C (PKC) activity (Fig. 1.2 C). Both calcium mobilization and PKC activation are essential for full activation of platelets. Once initiated, platelet activation is amplified by several endogenous mechanisms, including the formation of stimulatory eicosanoids (e.g., PG\(_2\), PGH\(_2\)), which cause further mobilization of second messengers, platelet aggregation and release of dense granule contents. The functional response is thus greatly amplified and aggregation, when measured as the loss of single platelets, is complete in 3s (Jones and Gear, 1988).

1.3 Structure and function of GP Ib-IX-V complex

GP Ib-IX-V is a multifunctional receptor and is shown to bind several ligands including VWF (Marchese et al., 1999), Mac-1 (Simon et al., 2000), P-selectin (Romo et al., 1999), thrombin (Greco et al., 1996), high molecular weight kininogen (Bradford et al., 1997) and Factor XII (Bradford et al., 2000). The GP Ib-IX-V complex is a unique plasma membrane glycoprotein complex unrelated in structure to other membrane receptors including those that are involved in mediating cell adhesion (Andrews et al., 2003). The complex is comprised of four transmembrane domains consisting of two units each of GP Ib\(\alpha\), GP Ib\(\beta\) and GP IX and one unit of GP V (Fig. 1.3) (Lopez and Dong, 1997). GP Ib\(\alpha\) is disulfide-linked to GP Ib\(\beta\) via cysteiny1 residues proximal to the extracellular face of the membrane (Lopez, 1994). GP Ib\(\beta\) is noncovalently associated with GP IX as a 1:1 complex (Lopez et al., 1994). GP V also associates with the complex noncovalently, but is present only in half the number as GP Ib\(\alpha\) (Modderman et al., 1992; Li et al., 1995). Using monoclonal antibody studies, Berndt et al., (1985) estimated that there are about 12,500 copies of the GP Ib-IX-V complex on the surface of the platelets.
Figure 1.3: Schematic of glycoprotein Ib-IX-V complex. The complex consists of two units each of GP Ibα, GP Ibβ and GP IX and one unit of GP V. Solid circles attached to stalks represent O-linked carbohydrate; diamonds on stalks represent N-linked carbohydrate. The regions denoted in the figure are distinct regions of GP Ibα – (A) Leucine-rich repeat (B) Disulfide loop (C) Anionic sulfated region (D) Macroglycopeptide region and (E) Cytoplasmic region (adapted from Lopez and Dong, 1997).
Figure 1.4: Different regions of GP Ibα subunit of GP Ib-IX-V complex. The numbers denoted are amino acid sequences spanned by the particular region. LRR is Leucine-rich repeat.
GP Ibα is 610 amino acids in length and has an apparent molecular weight of 135 kDa with carbohydrates making up 50% by weight (Lopez et al., 1987). Structurally, GP Ibα can be divided into following distinct regions (Fig. 1.4): (1) Leucine-rich Repeat Region (LRR): Following a short disulfide loop at the N-terminal of GP Ibα, spanning amino acids Leu^{36}–Ala^{200}, is globular ~9 nm in diameter containing seven tandem leucine-rich repeats, which makes this subunit a member of the leucine-rich motif family of proteins (Lopez et al., 1988). A leucine-rich repeat consists of 22-26 amino acids rich in leucine and contains a conserved asparagine in the sixth position of the repeat. (2) Disulfide loop region (Cys^{209}–Cys^{264}): C-terminal to the LRR, there are two disulfide loops connecting Cys^{211}–Cys^{248} and Cys^{248}–Cys^{264} (Hess et al., 1991). (3) Anionic sulfated region (Asp^{269}–Asp^{284}): is a 19-amino acid sequence rich in negatively charged aspartate and glutamate residues (the anionic sulfated region) (Lopez et al., 1994). The sequence also contains three fully sulfated tyrosine residues at positions 276, 278 and 279, shown to be important in binding VWF and thrombin (Dong et al., 1994). (4) Macroglycopeptide region: This region consists of a sialomucin core rich in serine, threonine and proline residues and is heavily O-glycosylated and is ~400 nm long (Korrel et al., 1984). (5) Cytoplasmic region: Connecting the macroglycopeptide to the cytoplasmic region is a transmembrane region of GP Ibα that contains approximately 30 residues and spans the lipid bilayer of the platelet plasma membrane once (Lopez et al., 1988). The cytoplasmic tail of GP Ibα contains 96 amino acid residues and binding sites for two proteins, actin-binding protein and 14-3-3ζ, implicated in regulating the functional activity of the GP Ib-IX-V complex and in signal transduction (Andrews and Fox, 1991; Du et al., 1994). GP Ibβ (25 kDa, 181 amino acids) and GP IX (22 kDa, 160 amino acids) both have a single leucine-rich repeat with conserved flanking sequences (Lopez et al., 1988;
Hickey et al., 1989, 1993). The GP Ibβ cytoplasmic sequence of 34 amino acids appears to regulate platelet actin polymerization in response to agonist stimulation, as well as providing an additional 14-3-3ζ binding site (Calverley et al., 1998). GP V contains 15 extracellular leucine-rich repeats, conserved flanking sequences, a transmembrane domain and a short cytoplasmic domain (15 residues) (Hickey et al., 1993; Calverley et al., 1995). However, it has been shown that the GP Ib-IX complex is necessary and sufficient for the binding of VWF in vitro (Lopez et al., 1992).

The VWF binding domain is mapped to the N-terminal region of GP Ibα, spanning amino acids His1 – Asp287. All the three regions within this sequence have been shown to be crucial for interaction with VWF: (1) Leucine-rich Repeat Region (LRR): The main evidence for LRR being involved in GP Ibα binding to VWF came from the observation that three mutations found in this region, Leu57 to Phe (Miller et al., 1992), Ala156 to Val (Ware et al., 1993) and deletion of Leu179 (de La Salle et al., 1995) makes GP Ibα completely non-functional and patients with the mutation Ala156 to Val suffer from the bleeding disorder, the Bernard-Soulier Syndrome. Later, Shen et al., (2000) showed that CHO cells expressing mutations in the LRR either not bind at all or bind weakly to VWF in in vitro assays. (2) C-terminal disulfide loop (Cys209 – Cys248): Of the two disulfide loops, Cys211 – Cys248 and Cys248 – Cys264, the first disulfide loop is believed to be involved in the interaction of GP Ibα with VWF, as supported by the presence of mutations Gly233 to Val or Met239 to Val, in patients with Platelet-type von Willebrand Disease (PtvWD). Further evidences about the importance of this region came from in vitro observation that several point mutations introduced in this region altered the affinity of GP Ibα for VWF (Dong et al., 2000). (3) Anionic-sulfated region: It is highly anionically charged and three tyrosines (276, 278 and
279) are fully sulfated. Several groups have shown the importance of tyrosine sulfation in GP Ib binding to VWF (Dong et al., 1994; Marchese et al., 1995; Ward et al., 1996). Further, mutagenesis of recombinant GP Ibα shows that loss of anionic residues between Asp\textsuperscript{252} – Asp\textsuperscript{277} greatly diminished VWF binding (Murata et al., 1991).

### 1.4 Structure and function of VWF

VWF is a multifunctional, multimeric protein found in plasma and in the subendothelium of blood vessels (except capillaries). Normally, VWF is present at a concentration of 10 µg/ml in plasma (Miletich, 1995). Depending upon the extent of multimer formation, plasma VWF is composed of a population of molecules that can range up to 50 individual subunits and the multivalency of larger multimers confers greater binding affinities and a greater thrombogenic capacity in terms of higher adhesion (Sadler, 1998). VWF is synthesized exclusively in megakaryocytes and endothelial cells and stored in Weibel-Palade bodies found in these cells (Wagner et al., 1993). The platelet α-granule is another major site for the storage of VWF, storing up to 20% of total VWF present in plasma (Gralnick et al., 1985). The newly released VWF is shown to be rich in very large molecular weight multimers, called unusually large VWF (ULVWF), but is normally prevented from entering plasma by a VWF-cleaving enzyme present in plasma (Moake et al., 1982). This enzyme is the 13\textsuperscript{th} member of ADAMTS (A Disintegrin and Metalloprotease with ThromboSpondin motif) metalloprotease family (ADAMTS-13) (Dong et al., 2002).

VWF has a typical extended multimeric structure of variable length, which depends on the number of subunits (Slayter et al., 1985). The repeating unit, the VWF protomer, has a molecular mass of 500 kDa and is actually a dimer made of two anti-parallel subunits linked
by disulfide bonds at the C-terminal ends (Chopek et al., 1986). Amino acids within the mature VWF subunit are numbered 1 to 2050 (Fig. 1.5). VWF can be divided into 10 different modules, from N to C terminal, D1-D2-D'-D3-A1-A2-A3-D4-B-C1-C2 (Ward and Berndt, 2000). The D and the smaller B and C domains are not homologous to other known molecules, with the exception of RGD sequence in domain C1 (Shelton-Inloes et al., 1986). In contrast, the three A domains contain a structural motif common to many adhesive proteins (Colombatti et al., 1991). Each A domain of ~200 residues contains only two Cys residues: in the first and third A domains these form intrachain disulfide bonds, whereas the second A domain has a disulfide bond between adjacent residues (Meyer et al., 1993). Regions homologous to the VWF-A domains have been described in a variety of extracellular matrix proteins, complement components and in several adhesion receptors of the integrin family (known as “I-domains”), with one difference being the I-domains contain a metal-ion dependent adhesion site (MIDAS) while A-domains do not (Ward and Berndt, 2000). The role of VWF in hemostasis and blood coagulation in multifaceted: it stabilizes and functions as a carrier of factor VIII (Foster et al., 1987) and protects factor VIII cleavage from inactivation by factor C (Koppelman et al., 1996); functions as a cofactor for thrombin-catalyzed cleavage of factor VIII (Hill-Eubanks et al., 1990); interacts with fibrin and helps in stabilizing thrombi (Parker and Gralnick, 1987) and binds reversibly to platelet GP Ib and subsequently results in platelet aggregation by binding firmly to activated GP IIb/IIIa (Hantgan et al., 1990).

The binding of GP Ib to VWF leads to the initial transient contact of platelets with the subendothelium, i.e., platelet adhesion is initiated, following which platelet GP IIb/IIIa interacts with the VWF, resulting in irreversible platelet adhesion. Soluble plasma VWF has
Figure 1.5: Schematic diagram of the structure of VWF and VWF-A1 domain. Different domains in the VWF molecule are represented in the figure. Exploded view of VWF-A1 is shown along with different regions that act as binding sites for different molecules (adapted from Ward and Berndt, 2000)

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Binding sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP Ib</td>
<td>514 – 542</td>
</tr>
<tr>
<td>Botrocetin</td>
<td>539 – 553</td>
</tr>
<tr>
<td></td>
<td>569 – 583</td>
</tr>
<tr>
<td></td>
<td>629 – 643</td>
</tr>
<tr>
<td>Ristocetin</td>
<td>474 – 488</td>
</tr>
<tr>
<td>Collagen</td>
<td>695 – 708</td>
</tr>
<tr>
<td>Heparin</td>
<td>542 – 622</td>
</tr>
<tr>
<td></td>
<td>565 - 587</td>
</tr>
</tbody>
</table>
a very low affinity for GP Ib, probably because the GP Ib-binding site is hidden in the resting conformation (Ward and Berndt, 2000). Exposure of the GP Ib-binding site of VWF can be regulated by a series of physiological agonist factors, of which immobilization onto collagen or other subendothelial structures (Bolhuys et al., 1981) and high shear stress (Moake et al., 1986) are the most relevant. Non-physiologic modulators like the removal of sialic acid from VWF (Gralnick et al., 1985), immobilization on solid surfaces (Savage et al., 1996), proteolytic degradation or the presence of exogenous modulators like ristocetin (Howard and Firkin, 1971) or botrocetin (Andrews et al., 1989a), similarly induces its binding to GP Ib.

The GP Ib binding site is contained within the A1 domain (Fujimura et al., 1987; Berndt et al., 1989b). The A1 domain contains an intramolecular disulfide bond, Cys\(^{509}\) – Cys\(^{695}\), defining a predominantly positively charged sequence within the loop, and two discontinuous anionic flanking sequence containing sialylated glycosylation sites both upstream and downstream of the disulfide loop (Fig. 1.5). The sequence Asp\(^{514}\) – Glu\(^{542}\) within the disulfide loop has been identified as a potential site involved in binding GP Ib-IX-V (Berndt et al., 1992). The VWF activating compounds botrocetin binds to one or more sequences within the disulfide loop (Sugimoto et al., 1991), while ristocetin binds to the anionic flanking sequences outside the loop (Girma et al., 1990). Based on these evidences, Berndt et al., (1992) suggested a model in which inactive A1 loop is maintained by electrostatic interactions between anionic sequences flanking the 509 – 695 bond and cationic sequences within the loop. The binding of modulators and loss sialic acid residues may expose a GP Ib-IX-V binding site by altering the conformation of the A1 domain.

Functional abnormalities in VWF binding to platelets are caused by mutations in the VWF gene and are collectively grouped as von Willebrand disease (VWD) (Sadler 1998). It
could be quantitative defects comprising of partial deficiencies (VWD type1) and severe
deficiencies (VWD type3) or qualitative defects (VWD type2). VWD type1 is characterized
by a concordant reduction in VWF antigen, ristocetin cofactor activity, and factor VIII
activity but VWF multimers are normal. VWD type2 is itself heterogeneous and is
characterized by a disproportionately low level of ristocetin cofactor activity relative to VWF
antigen and absence of high-molecular weight multimers. In VWD type3, there is virtually
no VWF present. Another important life-threatening thrombotic disorder called thrombotic
thrombocytopenic purpura (TTP) results due to excessive binding of VWF to platelets.
Plasma in TTP patients was found to have higher VWF antigen levels and also a
disproportionately large fraction of ULVWF, which account for the higher thrombogenecity
of VWF (Moake et al., 1982). The presence of ULVWF in plasma is attributed to the absence
or inactivation of a plasma protease, ADAMTS-13, which is known to proteolyse ULVWF
molecules to smaller fractions before they are released into plasma.

1.5 Models for Cell Adhesion and Thrombosis

In order to better interpret the data from the experimental models for thrombosis it is
very critical to understand the fundamental aspects interactions of platelets receptors with
their corresponding ligands. In this section, the general principles underlying cell adhesion
will be introduced followed by an outline on the current mathematical models and
experimental techniques. A few key examples in the context of inflammation and thrombosis
will be discussed. There has been more work in the past in modeling inflammation than
thrombosis, but the same paradigmatic principles learned from inflammation apply to
thrombosis. In response to inflammatory stimuli, leukocytes roll, then arrest on endothelial
cells, and finally migrate into the surrounding tissues. In response to hemorrhage, platelets adhere to exposed subendothelial tissues and then recruit additional platelets into aggregates that function as procoagulant surfaces (McEver, 2001). In addition, leukocytes roll and then arrest on activated platelets and platelets roll on activated endothelial cells, showing that coagulation and inflammation are intimately linked. Platelet-platelet and leukocyte-leukocyte adhesion in bulk fluid phase are critical systemic events. Adhesion receptors present on cell surfaces mediate these processes and have evolved to perform specialized functions. Apart from mediating adhesion, the receptors also trigger signal transduction processes that alter the activation state of the cell. Table 1.2 summarizes the important receptors involved in thrombosis.

Two of the most important physical phenomena that affect cellular adhesion are non-specific interactions and cellular deformation.

*Non-specific interactions*: are defined as interactions between a cell and surface or between two cells that do not involve specific receptors but they do increase or decrease the overall strength of the interaction (Hammer and Tirrell, 1996). The three relevant types of non-specific forces are electrostatic and electrodynamic forces and steric stabilization. The electrostatic forces arise due to the glycosylation of cell surface by sialic acid residues, which render an overall negative charge to the glycocalyx. Hence, for cell-cell adhesion, the electrostatic interactions are repulsive while for cell-surface adhesion it will depend on the charge on the external surface. The electrodynamic forces (van der Waals forces) are weak, attractive forces that result from the charge interactions of polarizable molecules. Steric stabilization is a repulsive force arising because of large molecules that make up the glycocalyx. The glycocalyx consists of polymers in a hydrated environment. As two polymer
coats approach each other, the layers overlap and some of the water of hydration is pushed out. A repulsive force termed steric stabilization results because of the osmotic tendency of water to return and the steric compression of polymer chains. The repulsive forces dominate the cell-cell interaction when the cell-cell separation distances are <20 nm and the van der Waals forces weakly dominate at a distance of 20 – 25 nm (Fig. 1.6).

*Cellular deformation:* Cells deform when external forces act on them. The deformability of blood cells is of interest because of its significant impact on flow resistance in the circulation. Adherent cells change their shape and elongate in the direction of flow with a resultant increase in the surface contact area and hence an increase in adhesion strength (Damino et al., 1999). Mechanical forces also induce biological responses in cells, which are regulated by cytoskeletal reorganization. The cytoskeletal properties affect not only cell shape, but also migration and adhesion (Lauffenburger and Linderman, 1993).

### 1.5.1 Mathematical Models

The importance of cell adhesion in maintaining normal physiological function has necessitated better understanding of these events, so as to be able to correct the pathological situations that lead to disease and also to engineer better drugs. There has been a tremendous effort, especially over the last two decades, in developing models that would quantify the various physical interactions underlying adhesion so that we can ask under what conditions cell adhesion will or will not occur. One may be interested in knowing the area of contact, number of bonds in the contact area as two cells adhere or the equilibrium and kinetic constants of the bonds that mediate adhesion. In this section, we shall trace the important milestones on the path in answering these questions.
Figure 1.6: Energies of interaction in cell-cell recognition: Include non-specific attraction specific attraction from intersurface bonding and electrosteric repulsion from the charged glycocalyx (adapted from Lauffenburger and Linderman, 1993)
In general the biophysical models for cell adhesion may be classified into three categories: equilibrium, mechanical and kinetic.

*Equilibrium Approach*

The equilibrium approach shows the bounds on conditions under which cell adhesion is feasible. Bell and co-workers (1984, 1986) developed the equilibrium view of cell adhesion by modeling the bonds as springs with the chemical potential defined by the following equation:

$$\mu^0(S) = \mu^0(L) + \frac{1}{2} \kappa (S - L)^2$$  \hspace{1cm} Eq. (1.1)

where, $\mu$ is the chemical potential of the bond, $\kappa$ is spring constant, $S$ is equilibrium distance and $L$ is the separation distance. They minimized the free energy change due to opposing forces of non-specific repulsion and adhesive attraction as two cells brought in contact, as a function of separation distance, contact area and the receptor density in the contact area. They delineated regions in parametric space where adhesion is favorable and where it is not. One of the serious drawbacks of this model is that it does not take into account the chemical potential associated with membrane deformation.

*Mechanical approach*

The energetics of membrane deformation known as the adhesive energy density is the basis for mechanical approach to cell adhesion. The adhesive energy density, $\psi$, is defined as the mechanical work required separating a unit area of adherent surface and is related to the work done by external forces in breaking the bonds. The adhesive energy density is a function of individual bond strength and the bond density. The model of Evans (1985)
showed that the relationship between the adhesive energy density and the tension generated on the cell membrane due to peeling \((T_\theta)\) has a form similar to Young’s equation for surface tension:

\[
\psi = T_\theta (1 - \cos \theta_0)
\]

Eq. (1.2)

where \(\theta_0\) is the cell-substrate contact angle. The model was used to predict the membrane contour in the contact area. The important parameter in the model is the elastic modulus of the membrane and other critical factors like receptor density, size of the contact area and the non-specific forces are not explicitly accounted for in the model. Though, the predictions of this model have only limited use in the sense that only the membrane contour and tension can be calculated, the abbreviated mechanical approach did show new ways for more sophisticated models that followed.

**Kinetic approach**

The dynamic models help investigate not only the equilibrium state of the cell but also the kinetics of formation of that state. In his seminal work, Bell (1978) noted two major differences between binding to cell surface receptors of hormonal ligands and of adhesive ligands. Firstly, the former reaction happens in solution and the molecules are free to diffuse, while in the latter, the molecules are constrained on the cell membrane and the association rate becomes heavily transport-limited. Secondly, after the hormonal ligand has bound to the receptor, there is no other force acting on the complex other than thermal agitation, while for a complex anchored to a surface, there is usually a dislodging force (like fluid force on an adherent cell) which can alter the rate of dissociation. Bell adopted the kinetic theory for
bond dissociation in solids and proposed a constitutive relation between dissociation rate and force:

\[ k_{\text{off}} = k^0_{\text{off}} \exp \left( \frac{\gamma F}{k_B T} \right) \]  

Eq. (1.3)

where, \( k_{\text{off}} \) is dissociation rate constant and \( k^0_{\text{off}} \) is zero-force dissociation rate constant, \( \gamma \) is reactive compliance of the bond, \( F \) is force applied to bond, \( k_B \) is Boltzmann constant and \( T \) is absolute temperature. Dembo et al., (1988) combined the receptor-ligand bond formation kinetics, fundamentally due to Bell (1978) with the mechanical approach for cell adhesion in the models described by Evans (1985). They proposed a set of constitutive laws relating the kinetics of bond formation and dissociation to the stress experienced by the bond. In essence, the model couples the equation for deformation of an elastic membrane with equations for the chemical kinetics of the adhesion molecules (Fig. 1.7). Using Arrhenius theory of reaction rates and modeling the bonds as Hookean springs in transition state and the bound state, the off-rates is related to the separation distance by:

\[ k_{\text{off}} = k^0_{\text{off}} \exp \left( \frac{(\kappa - \kappa_s)(S - L)^2}{2k_B T} \right) \]  

Eq. (1.4)

where \( \kappa \) and \( \kappa_s \) are transition constants at bound and transition state respectively, \( S \) is separation distance and \( L \) is equilibrium distance. Depending on the sign of \( (\kappa - \kappa_s) \), the bonds could be classified as slip bonds \((>0)\), catch bonds \((<0)\) or ideal bonds \( (=0)\). The rate of dissociation of slip bonds increase with an increase in force while that of catch bonds decrease with an increase in force. Apart from predicting the membrane profile in the contact area, an important outcome of this model is the prediction of the effect of kinetic constants and receptor densities on the membrane tension and on the rolling velocity of the cell.
Figure 1.7: Cell-substrate contact region in the peeling model used by Dembo et al., (1988). The model combines the mechanical and kinetic approaches. The cell is subjected to a pulling force, which results in membrane tension characterized by the mechanical properties of the membrane. The bonds are modeled as springs by the kinetic approach and they hold the membrane to the surface. Beyond an equilibrium length, the bonds experience tension characterized by spring constant.
1.5.2 Experimental Methods

Several different experimental methods have been developed over the past decade in obtaining the parameters important in the understanding of cell-cell and cell-surface adhesion. Recent technological advances have made it possible to design experiments that bring in direct information on individual interactions between surface-bound molecules, since data interpretation is greatly facilitated when single bonds are monitored, without a need to account for the mechanical properties of the surfaces and the topology of molecules. In this section, we shall describe these techniques and highlight a few experimental results.

Atomic Force Microscope

The atomic force microscope (AFM) in its force-measuring mode is capable of effecting displacements on an angstrom scale and measuring forces of the order of a few piconewtons (Fisher et al., 1999). These length and force scales measured by AFM are of the same order associated with formation and dissociation of bonds and are thus ideally suited for measuring bond kinetics. In AFM force-spectroscopy experiments, ligand molecules are immobilized on the tip of an AFM cantilever and their corresponding receptors on a counter surface (Fig. 1.8). The AFM sensor is approached toward the surface where the molecules can bind and is subsequently retracted at either a constant pulling velocity or constant force. By monitoring the cantilever deflection during such an approach-retraction cycle of constant pulling velocity, the binding, stretching and rupture of receptor-ligand complexes can be investigated in terms of forces, from which bond strength can be calculated. On the other hand, the length of time for which a bond lasts before it breaks during a constant force cycle gives estimates on the dissociation rate constant. Over the last decade, several investigators
Figure 1.8: Schematic diagram of AFM in its force-extension mode. Extension of a single molecule caused by retraction of the piezoelectric positioner results in deflection of the cantilever. This deflection changes the angle of reflection of a laser beam striking the cantilever, which is measured as the change in output from a photodetector.
have extensively used AFM to study biological molecules – mechanical properties of protein (Rief et al., 1997) or affinity constants for antibody-antigen interactions (Izrailev et al., 1997), to name a few. As far as hematology is concerned, some of the problems analyzed with AFM include studying the shear dependent shape change of VWF (Siedlecki et al., 1996), plasma protein adsorption on biomaterials (Bergkvist et al., 2003), platelet membrane elasticity (Radmacher et al., 1996), lysis kinetics of erythrocyte membrane (Hategan et al., 2003), integrin bond strength and kinetics (Zhang et al., 2002).

Fritz et al., (1998) studied the force-mediated kinetics of interaction between leukocyte adhesion molecule P-selectin and PSGL-1 by immobilizing recombinant forms of P-selectin and PSGL-1 on coverslips and AFM tips respectively. They measured the rupture force, lifetime and molecular elasticity for single bond unbinding events of P-selectin/PSGL-1 complexes. They found that the P-selectin/PSGL-1 complex (i) exhibits a persistence length of 97 nm, similar to that of single stranded DNA, (ii) has a rupture force of 165 pN is comparable to that of antibody/antigen binding and (iii) an off-rate constant of 0.022 s$^{-1}$, a value much lower than that measured by other methods (Alon et al., 1997). In a very interesting recent study, Zhu and co-workers (Marshall et al., 2003) followed the dissociation kinetics of P-selectin with monomeric and dimeric forms of PSGL-1, at different values of pulling forces. They observed that (i) the dimeric PSGL-1/P-selectin complex has twice the longevity of monomeric PSGL-1/P-selectin complex. (ii) PSGL-1/P-selectin bond breaks at a faster rate with an increase in force (slip-bond) at rupture forces higher than ~20 pN while at lower forces, the bond breaks at a slower rate with an increase in force (catch-bond).
The basic principle of BIAcore is the use of surface plasmon resonance (SPR) (Myszka, 1997; Glaser, 1999). SPR occurs when light is totally internally reflected at the interface between a medium of high refractive index, a thin layer with good electric conductivity (usually gold) and a medium of low refractive index (Fig. 1.9). The evanescent wave that develops at the interface interacts with free electrons in the conductive layer and gives rise to so called plasmons. The energy for this interaction is lost from the reflected light, resulting in a minimum of the reflected intensity at the resonance angle. The propagation of evanescent wave along the interface and thus the resonance angle depend on the refractive index in this thin layer adjacent to the interface. This principle can be used to measure refractive index changes due to adsorption of material to the surface of the sensor (high refractive index material coated with a metal layer) from a fluid or gas phase. In a typical experiment one of the components (referred to as the ligand) in a biospecific pair is immobilized on the sensor chip surface and the counterpart (the analyte) which interacts with the ligand is in solution and is passed over the gold film in a continuous flow. BIAcore detection systems use SPR to monitor the refractive index change as molecules interact at the sensor surface. The signal generated in arbitrary resonance units (RU) is approximately proportional to a change in mass (1 RU ~ 1 pg/mm² for protein).

Mehta et al., (1998) studied the affinity and binding kinetics of P-selectin binding to PSGL-1 using BIAcore. PSGL-1 was covalently coupled to BIAcore sensor chip and the analytes (P-selectin or mAbs) were perfused over the immobilized PSGL-1 ligand at low flow rates. The change in refractive index of the sensor chip as P-selectin molecules bind to PSGL-1 was recorded for about five minutes. Bound P-selectin was removed by perfusing
Figure 1.9: Schematic of Real-time SPR (BIAcore). The figure shows the optical configuration together with the sensor chip. The polarized light illuminates the chip at the point where the biospecific interaction takes place, and is reflected to the detector array. Through surface plasmon resonance a dark spot can be seen on the detector for one angle of incidence. This angle for extinction of light is changed when the analyte is adsorbed on the immobilized ligand on the sensor chip. By following the change in this signal with time, the association and dissociation rate constants can be determined.
EDTA and the change in refractive index because of molecular dissociation was followed. The equilibrium dissociation constant was obtained from a Scatchard analysis of the equilibrium binding of PSGL-1 to different concentrations of P-selectin. This value was determined to be 320 nM. The off-rate constant ($k_{off}$) was measured from the changes in refractive index during the dissociation phase. The specific response during different time points was fit to a mono-exponential decay function and the $k_{off}$ was estimated to be 1.4 s$^{-1}$. Nicholson et al., (1998) used SPR technology to study the kinetics of interaction between L-selectin and GlyCAM-1 and measured the equilibrium dissociation constant ($K_D$) to be 108 μM and the off-rate kinetic constant as 10 s$^{-1}$. Wild et al., (2001) reported the equilibrium dissociation constant for E-selectin binding to its ligand (ESL) to be 62 μM and the kinetic dissociation rate constant to be 4 s$^{-1}$. The lifetimes of selectins measured by this method compares well with those measured by flow chamber experiments (Alon et al., 1997).

**Viscometry**

Rotational viscometers produce constant and uniform shear stress on all cells and proteins in liquid suspensions, independent of the location of individual particle. Different kinds of viscometers – cone-and-plate (Jen and McIntire, 1984) or concentric cylinder (Cokelet and Smith, 1973) – are useful in studying the bulk shear-induced cell aggregation (Fig. 1.10 A-B). Cells in suspension are subjected to controlled shear between two closely separated surfaces for a definite period of time. The sheared suspension can then be analyzed for aggregates or for identity and activation of specific adhesion molecules using flow cytometer or coulter counter for aggregation and/or changes in receptor expression levels as measured by fluorescent antibodies (Taylor et al., 1996). The cone-and-plate viscometer has
been used extensively to explore the interactions between different types of blood cells including platelets (Huang and Hellums, 1993), white cells (Taylor et al., 1996) and red cells (Tees et al., 1993). The viscometer system is very useful in simulating the in vivo bulk aggregation processes. In a viscometer, the measurable properties are time-averaged values of interactions between several molecules and not real-time data as in a flow chamber or single molecular events as in an AFM. Hence, mathematical models are needed to extract molecular parameters like rate constants and bond strength from experimental data. Goldsmith and co-workers have performed pioneering work in this direction in interpreting viscometry data, primarily from antigen-antibody interactions (Tha and Goldsmith, 1986; Tees et al., 1996). Their models were based on the theory for interaction between neutrally buoyant spheres in shear flow. Tandon and Diamond (1998) applied these models to the experimental data of Taylor et al., (1996) to obtain the rate constants of selectin- and integrin-mediated aggregation. These authors fitted their model parameters to match their predictions to the published values on collision efficiency of neutrophils. Collision efficiency is defined as the fraction of the total number of collisions that result in the formation of aggregates. The association and dissociation rate constants are directly related to collision efficiency and the lifetime of the aggregates respectively. These authors estimated that, for L-selectin – PSGL-1 interaction, the on-rate and off-rate constants to be 1.6 x 10-12 cm²/s and 70 s⁻¹ respectively and the bond force at rupture to be 1 μdynes. For LFA-1 – ICAM-1 interaction, the on-rate constant was estimated as 1.6 x 10⁻¹² cm²/s and the bond force at rupture was 5 μdynes. The integrin binding was assumed to be irreversible in their model and hence the off-rate constant was zero.
Figure 1.10: Schematic of (A–B) Viscometers and (C–D) Flow chambers: (A) Concentric cylinder viscometer (B) Cone-and-plate viscometer (C) Parallel-plate flow chamber (D) Radial flow chamber. The dark dots represent cells or beads, which are subjected to shear.
Flow Chamber

The flow chamber consists of a detachable bottom surface that is coated with protein or cell monolayer and the cell suspension is flown over the surface as in a parallel plate flow chamber (Hubbell and McIntire, 1986) or radial flow chamber (Kuo and Lauffenburger, 1993) (Fig. 1.10 C-D). The force experienced by the cells can be controlled by varying the flow rate of the fluid or the chamber dimensions. The cell-surface interactions can be visualized in real time using a microscope coupled with a video camera and the data can be analyzed off-line. This arrangement very closely mimics the in vivo interactions between blood cells and vessel wall or endothelial ligands and hence has been a favorite model amongst several investigators over the last 15 years (Slack and Turitto, 1994). The determination of molecular kinetic constants in this system is inherently difficult due to both transport and reaction processes affecting the overall, observed interactions between cells with surfaces. In this section, we shall discuss some of the important steps taken toward obtaining the off-rate and on-rate constants of cellular interactions. It is not surprising that there are many more reports published on the measurement of $k_{off}$ than of $k_{on}$, since the latter is more difficult to calculate from the flow chamber data.

A particle of radius $a$ bound to a macroscopic surface in a fluid of viscosity $\mu$ with a locally varying flow of shear rate $G$ is subjected to a disruptive force of order of $\mu a^2 G$ and when the bonds are ruptured, the particle would depart with a velocity of the order of $aG$ (Bongrand, 1999). Thus, if we consider a cell-size particle of 10 $\mu$m radius in a fluid of 0.001 Pa viscosity such as water, a shear rate of 10 s$^{-1}$ may generate a hydrodynamic drag of order of 0.1 pN and a relative velocity of 100 $\mu$m/s. Therefore, in principle, conventional microscopy may allow a detailed examination of single-bond rupture with a time resolution
of a few tens of milliseconds. Kaplaniski et al., (1993) studied the predominantly E-selectin mediated binding of human granulocytes to interleukin-1 activated endothelial cells in the presence of a low hydrodynamic drag (a few piconewtons) estimated to be much weaker than a standard receptor-ligand bond. They used an empirical model for binding frequency, which is defined as the rate at which a cell in flowing fluid stream would stop on interacting with the surface while in the field of view. For E-selectin interaction with endothelial cell surface, the binding frequency was estimated to be 0.04 s\(^{-1}\) at very low shear rate (5 s\(^{-1}\)). From the distribution of the duration of short-time arrests of rolling cells, the authors were able to calculate the off-rate constant to be \(-0.5\) s\(^{-1}\). Alon et al., (1995) made an elegant modification to this approach by observing that the probability of multiple bond formation is much lower for cells that tether briefly on the surface than that for cells that roll continuously. Using the pause durations of tethered cells and modeling the bond dissociation as first-order kinetics, they determined the off-rate constant of P-selectin–PSGL-1 interaction to be 0.95 s\(^{-1}\) and the exponent of the Bell equation (Eq. 1.3) to be 0.05 nm. Several authors have since then used this methodology to obtain the off-rate constant and mechanical properties of wild-type and mutant receptor interactions with their respective ligands when subjected to different shear stresses. Alon et al., (1997) determined the \(k_{off}\) values for L-, P- and E-selectin and these values compare very well with those determined by other techniques like SPR. de Chateau et al., (2001) determined the transient tether lifetime of integrin \(\alpha_\beta\)-MAdCAM-1 to be 20-fold longer and the bond mechanically weaker that the selectin bonds, which is consistent with the knowledge that the integrin bonds mediate firm adhesion of leukocytes unlike selectin bonds which mediate transient adhesion. Using similar technique, Ramachandran et al., (1999) showed that tyrosine replacement mutation of PSGL-1 affects both the kinetic and
mechanical properties of bonds with P- and L-selectin. Dwir et al., (2001) showed that cytoplasmic anchorage of L-selectin provides the necessary mechanical stability for selectin tethers to withstand fluid shear forces. In a very interesting recent work, Marshall et al., (2003) showed that increasing force first prolonged and then shortened the lifetimes of P-selectin complexes with PSGL-1, revealing both catch and slip bond behavior and was consistent with the binding data obtained from AFM. Very recently, Doggett et al., (2003) showed that the GP Ib–VWF bond, that helps to arrest platelets on injured surfaces, has kinetic and mechanical characteristics similar to that of selectin bonds.

While the off-rate constant can be defined and calculated in terms of the lifetime of the bonds, the on-rate constant between surface bound ligands and cell surface receptors are difficult to estimate. The observed rate of association of cells in solution on to ligand-coated surfaces is a resultant of both the rate of transport of cells to the surface and the intrinsic kinetic rate of association between the molecules. Depending on the hydrodynamic, cellular and kinetic parameters, the rate of association could be governed by either diffusion, convection or reaction rates. As a result, mathematical models become a necessity to delineate the controlling regime and to obtain the intrinsic molecular reaction rate from the flow chamber data. In one of the very few efforts in this direction, Chang and Hammer (1999) developed a model to predict the effect of relative motion between cell surface receptors and ligand-coated surfaces in determining the overall rate of binding. The lumped on-rate \( (k_{ad}) \) is experimentally obtained by fitting the number of bound cells in a given field of view at a given velocity using the following expression:

\[
\frac{N_b}{N_0} = 1 - \exp \left\{ - \frac{k_{ad} U}{L} \right\} \tag{1.5}
\]
where $N_o$ is the total number cells available to bind, $N_b$ is number of cells bound, $U$ is velocity of flow and $L$ is the length of the field of view. $k_{ad}$ so obtained is dependent on several factors like the ligand and receptor densities in the contact area, size of the contact area, distance between receptor and ligand, rate of transport of receptor to ligand surface and the intrinsic forward rate constant ($k_{in}$). By fitting published experimental data of Tempelman and Hammer (1994) to Eq. 1.5, $k_{ad}$ was obtained at different shear rates. To relate $k_{in}$ to $k_{ad}$, these authors used a mathematical model based on two-dimensional convection-diffusion equation and the theory of first passage times. Recently, Zhang and Neelamegham (2002) made further improvement to the model of Chang and Hammer (1999) by explicitly decoupling the physical features of the system that affect cell-substrate collision rates from the biological features that influence cellular adhesivity. The collision rates are expressed in terms of frequency parameters, which were obtained from transport models that describe the flux of cells from the fluid stream on to the ligand-coated surface (primary capture) or on to other cells, which are already tethered to the surface (secondary capture). The experimental data on cell rolling and adhesion is fit to the model to obtain the frequency and probability parameters. These parameters are then plugged into an expression to obtain lumped on-rate constant, which is dependent upon both intrinsic association rate and receptor densities.

Although the use of sophisticated mathematical models provides a better handle on quantifying the kinetics of cell adhesion, the association rate constants are still functions of a number of parameters that are not reaction-specific. These variables could be the local receptor and ligand densities on the cell surfaces or microvilli structure, which cannot be precisely measured. Another approach is to report the lumped on-rate in terms of tethering frequency. Tethering frequency is the rate at which cells tether for the first time to ligand-
coated surface from the fluid stream. The differences in the tethering frequencies will reflect the differences in intrinsic association rates when the physical parameters like ligand/receptor densities and hydrodynamic parameters are comparable. Though tethering frequency is dependent on the measurement method and can only provide a relative measure within the same set of experiments, it is still a useful and a physiologically relevant approach because of the inherent difficulties in obtaining the intrinsic rate constant (Ramachandran et al., 1999; 2001).

1.6 Summary and Thesis Objectives

Cell adhesion is critical in diverse biological processes including inflammation and thrombosis. In the cardiovascular system, platelet adhesion to vessel wall and platelet aggregation in bulk flow depend on the balance between dispersive hydrodynamic forces due to blood flow and attractive adhesive forces generated by the affinity of receptors for their ligands. Platelet interactions are highly specific and must be tightly regulated to maintain normal physiology. The high specificity can sometimes become compromised due to qualitative or quantitative deficiencies in the adhesion molecules resulting in severe morbidity and mortality.

The key objective of this thesis is to quantify the kinetic and molecular mechanisms that result in thrombotic disorders, using some of the in vitro experimental models described above. In the first part of this work, we evaluated the kinetics of interaction between platelet receptor GP Ibα and its ligand VWF-A1, under arterial flow conditions. The effect of gain- and loss-of-function mutations of GP Ibα on the GP Ib-VWF kinetics was evaluated along similar lines. These mutations were investigated not only for the better understanding of GP
Ib-VWF kinetics but also for their clinical relevance since equivalent gain-of-function mutations of GP Ibα are known to result in a bleeding disorder called platelet-type von Willebrand disease (ptVWD). In the second part of this work, we studied the molecular mechanisms leading to another thrombotic disorder called thrombotic thrombocytopenic purpura (TTP) was studied. TTP is a congenital bleeding disorder arising due to presence of unusually large forms of VWF (ULVWF) in systemic circulation. The ULVWF has higher affinity or avidity for platelets than normal plasma VWF (P-VWF), causing platelet aggregation followed by thrombocytopenia. The platelet interaction capabilities of ULVWF and P-VWF were compared in vitro using aggregometry, flow chamber and cone-and-plate viscometry studies. The effect of exogenous modulators in regulating platelet aggregation mediated by these molecules was also compared.
CHAPTER 2

Kinetics of Platelet Glycoprotein GP Ibα – VWF-A1 Interactions

2.1 Introduction

In response to a vascular injury, platelets adhere to the subendothelial matrix which sets off a sequence of signal events in the platelet that result in activation, spreading on the exposed matrix, granule release, and aggregation to form a hemostatic plug. The entire process is initiated by an interaction between the glycoprotein (GP) Ib-IX-V complex on the platelet surface and immobilized von Willebrand factor (VWF) on the exposed subendothelium (Andrews et al., 1997).

Soluble VWF circulates in blood plasma and immobilized VWF is found in the subendothelial matrix. VWF is a multimer made up of several monomers and each monomer is composed of eleven functional domains – D1, D2, D′, D3, A1, A2, A3, D4, B, C1 and C2 in the order spanning from N- to C- terminus (Sadler, 1998). The A1 domain of VWF which spans amino acids, Cys^{509} to Cys^{695}, has been identified as containing the binding site for the GP Ib-IX-V complex (Miyata and Ruggeri, 1999).

The GP Ib-IX-V complex contains four transmembrane polypeptide chains, GP Ibα, GP Ibβ, GP IX and GP V (Lopez and Dong, 1997). The complex associates with the platelet cytoskeleton and signal transduction proteins through which activation signals are processed. The ligand-binding site of VWF has been mapped to the GP Ibα subunit (Berndt et al., 2001). The GP Ibα subunit has three major structural features in its extracellular domain: a leucine-rich repeat motif, with conserved N- and C-terminal flanking disulfide loops (residues 1–268), an extremely anionic region containing three sulfated tyrosines and the
binding site for thrombin (269–287) and a highly O-glycosylated region between the leucine-rich repeat region and the transmembrane region that serves as a spacer (Fig. 2.1) (Lopez, 1994). The binding site for VWF is contained in the 45-kDa N-terminal region of approximately 300 amino acids. Mutations that increase the affinity of GP Ibα for VWF have been localized to the first of two C-terminal disulfide loops in the leucine-rich repeat region (see Fig. 2.1). These mutations produce a disease called platelet type von Willebrand disease (ptVWD) which is paradoxically associated with mild to moderate bleeding because the abnormally reactive receptor binds and clears the most hemostatically active large multimers of VWF from the circulation (Miller, 1996). Two cases of ptVWD result from a substitution of Val for Gly at residue 233 (G233V) and Val for Met at residue 239 (M239V). Both mutations are close to each other in the primary structure of the polypeptide within the Cys$^{209}$ – Cys$^{248}$ disulfide loop (Fig. 1). It has recently been shown from the co-crystal structure of GP Ibα with VWF-A1 that this region is directly involved in the binding to VWF (Huizinga et al., 2002).

It is of interest to note that, not only do these mutations affect the residues in close proximity to each other in the linear sequence of GP Ibα, but both mutations also convert existing non-valine amino acids to valine. Dong et al., (2000, 2001) have previously examined the effect of converting other non-valine residues in this region to valine on the function of GP Ib-IX-V complex under static and dynamic conditions. Two mutations — D235V and K237V — in addition to the naturally occurring G233V and M239V, displayed a gain-of-function phenotype. In contrast, some of the other valine mutations in this region resulted in a loss-of-function phenotype including N226V, K231V, Q232V and A238V. The gain-of-function mutants bound VWF spontaneously and had a heightened response to low
Figure 2.1: The GP Ib-IX complex. The drawing depicts the structural domains of the three subunits of the GP Ib-IX complex along with an exploded view of the disulfide loop region of GP Ibα. The mutations used in this study, K237V and Q232V, are highlighted. Solid circles and diamonds attached to the stalks represent O-linked and N-linked carbohydrates respectively.
concentrations of ristocetin or botrocetin, whereas the loss-of-function mutants bound VWF poorly compared to the wild-type GP Ib\(\alpha\). Also, on immobilized VWF, the gain-of-function mutants showed a decrease and the loss-of-function mutants showed an increase in the rolling velocities respectively, suggesting that these mutations affected the binding kinetics of GP Ib\(\alpha\) – VWF interaction. Among the gain-of-function mutants, K237V rolled the slowest and among the loss-of-function mutants, Q232V rolled the fastest compared to WT. For instance, at 5 dyn/cm\(^2\), the rolling velocities of Q232V (140 \(\mu\)m/s) and of K237 (20 \(\mu\)m/s) were respectively 2.5-fold more and 3-fold less than that of wild-type (60 \(\mu\)m/s) (Dong et al., 2000). These authors had speculated that the difference in rolling velocities could primarily be due to the differences in the dissociation binding kinetics of bonds that mediate the rolling process.

Recently, a few investigators have studied the kinetics of interaction of wild-type and gain-of-function mutants of GP Ib\(\alpha\) with VWF (Miura et al., 2000, Huizinga et al., 2002, Doggett et al., 2002; 2003). Miura et al., (2000) attributed the difference in affinities for mutant and wild-type GP Ib\(\alpha\) to the difference in the association rates of VWF and GP Ib\(\alpha\). Using a static biochemical assay, for wild-type GP Ib\(\alpha\) – VWF-A1 interaction, they measured the association rate constant \((k_{on})\) to be 1100 M\(^{-1}\) s\(^{-1}\) and dissociation rate constant \((k_{off})\) to be 0.0038 s\(^{-1}\). They also reported that the increase in affinity caused by type2B (mutation I546V in VWF-A1 domain that increases the affinity of VWF-A1 for GP Ib\(\alpha\)) and ptVWD mutations is due to a 4-fold increase in the \(k_{on}\) even though the \(k_{off}\) remains unchanged compared to the wild-type. In contrast to these studies, Doggett et al. (2002) reported a 1000-fold higher dissociation rate constant for wild-type GP Ib\(\alpha\) – VWF-A1 interaction than that reported by Miura et al., (2000) and also that the increase in affinity in
type2B VWD is due to a 5-fold decrease in the dissociation rate. Using surface plasmon resonance, Huizinga et al., (2002) reported a dissociation equilibrium constant \( (K_D) \) of the GP Ib\( \alpha \) – VWF-A1 interaction of 30 nM. This value is 100-fold lower than that reported by Miura et al., (2000). Very recently, Doggett et al. (2003) reported that alteration in the kinetics of GP Ib\( \alpha \) – VWF-A1 interaction because of ptVWD mutation is due to both enhanced cellular on-rate and decreased off-rate. Thus, considerable controversy exists in the measurement of the kinetic constants of this biologically important interaction and also as to whether the increase in affinity in type2B or ptVWD mutations is due to alterations in association or dissociation rate.

In this chapter, we report our studies on the kinetics of GP Ib\( \alpha \) – VWF-A1 interactions and quantification of the association and dissociation rate constants for both wild-type and mutant GP Ib\( \alpha \) interaction with VWF-A1. We have used Chinese Hamster Ovary (CHO) cells expressing wild-type and mutant GP Ib\( \alpha \) interacting with immobilized VWF-A1 as our model system. The GP Ib\( \alpha \) was expressed as part of the GP Ib-IX complex, which has been shown to behave functionally similar to the GP Ib-IX-V complex on platelets (Lopez et al., 1992). To study the effect of mutations on the kinetics of this interaction, we used cells expressing GP Ib\( \alpha \) carrying mutations K237V and Q232V as representatives of the gain- and loss-of-function phenotypes, because these two mutants showed the strongest and weakest interactions in both static and dynamic adhesion assays (Dong et al., 2000). These two mutations are located in the disulfide loop formed by a bond between Cys\textsuperscript{209} and Cys\textsuperscript{248} (Fig. 2.1). We used tethering frequency — the rate at which cells are captured to the VWF-A1 surface from free flow — as a measure of effective or cellular \( k_{on} \) and the lifetime of transiently tethered cells to measure \( k_{off} \). We demonstrate that the tethering frequency \( (k_{on}) \)}
for the WT is 3-fold lower and 3-fold higher than that of K237V and Q232V respectively, while the estimated unstressed dissociation rate constant ($k_{off}^0$) of the WT is 1.25-fold lower and 2.2-fold higher than that of Q232V and K237V respectively. Our results suggest that both the association and dissociation rates are important in contributing to the phenotypic differences. The kinetic analysis of the implications of gain-of-function mutation (K237V) will help us to better understand the functionally similar, naturally occurring ptVWD mutations and also might aid in the development of therapeutics based on a quantitative analysis. This is the first time that the kinetic effects of a loss-of-function mutation (Q232V) of GP Ib has ever been studied and our results could be important in understanding the fundamental mechanisms of cell rolling and adhesion.

2.2 Materials and Methods

2.2.1 Cell lines

Chinese hamster ovary (CHO) cells were transfected with either wild type or mutant DNA for GP Ibα, wild type DNA for GP Ibβ and GP IX as described elsewhere (Lopez et al. 1994). The cell lines used in this study and the receptor expression are given in Table 2.1. The cells were grown in α-MEM medium (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS). Selection drugs used for CHO βIX cells were 400 μg/ml G418 (Life Technologies) and 80 μM methotrexate (Sigma Chemical Co., St. Louis, MO). For the wild type and mutant cells, along with these two selection drugs, 400 μg/ml Hygromycin B (Sigma Chemical Co., St. Louis, MO) was used. All cells were maintained at 37 °C with 5% CO₂.
Table 2.1: Cell lines used in this study

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Abbreviation</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO βIX</td>
<td>B9</td>
<td>GP Ibβ, GP IX (Control)</td>
</tr>
<tr>
<td>CHO αβIX</td>
<td>WT</td>
<td>GP Ibα, GP Ibβ, GP IX</td>
</tr>
<tr>
<td>CHO βIX/α232</td>
<td>Q232V</td>
<td>GP Ibβ, GP IX, GP α232 mutant (Glu 232 to Val)</td>
</tr>
<tr>
<td>CHO βIX/α237</td>
<td>K237V</td>
<td>GP Ibβ, GP IX, GP α237 mutant (Lys 237 to Val)</td>
</tr>
</tbody>
</table>

2.2.2 Flow cytometry

Flow cytometry was used to determine the cell surface expression levels of the wild-type and mutant GP Ib-IX complex. CHO cells expressing the wild-type or mutant polypeptides were detached with 0.53 mM EDTA, washed with phosphate-buffered saline (PBS) and incubated with FITC-conjugated mouse monoclonal (AK2 clone) antibody to CD42b receptor (Research Diagnostics Inc., NJ) for 60 min at room temperature. This antibody binds subunits 1 - 59 of GP Ibα (Shen et al., 2000). The cells were then analyzed on a FACSscan flow cytometer (Becton Dickinson, CA) stimulating with 488-nm laser light and collecting light emitted at >530 nm. Nonspecific binding was determined by the background fluorescence from CHO βIX cells stained with the same antibody. The data were analyzed using Cellquest software from Becton Dickinson. The cells were repeatedly sorted for GP Ib-IX expression with antibody-coupled magnetic beads to maintain comparable expression levels between the wild-type and mutant GP Ibα cells. The GP Ibα surface density was estimated by measuring the number of binding sites available for FITC-
conjugated AK2 antibody. The number of binding sites was determined by comparing the fluorescent intensity of the sample against that of a set of calibrated beads carrying known number of antibody binding sites (Flow Cytometry Standard, Puerto Rico).

2.2.3 Preparation of VWF-A1 coated coverslips

The recombinant VWF-A1 was produced in *Escherichia coli* and purified as described previously (Cruz *et al.*, 2000). Glass coverslips (No. 1, 24 x 50 mm, Corning, NY) were coated with a 5 mm diameter, 20 μL spot of VWF-A1 solution (diluted to appropriate concentrations with PBS) and incubated for 60 min at room temperature in a humid chamber. The coverslips were then rinsed with 1 ml of PBS and coated with 200 μl of 5% HSA solution for 60 min at room temperature to block any nonspecific binding. Any excess HSA was rinsed off with 1 ml of 0.9% saline prior to assembly as the bottom of a parallel-plate flow chamber.

2.2.4 Measurement of VWF-A1 surface density

To measure the surface density of VWF-A1 on glass coverslips, small compartments were made on the coverslips using a rubber gasket (Secure-seal hybridization chamber, Sigma, MO). VWF-A1 was coated at different concentrations inside the compartments and allowed to equilibrate for 60 min. After blocking the surface for non-specific adhesion, the binding of VWF-A1 to glass coverslips was determined by an enzyme linked immunosorbent assay (ELISA) using horse radish peroxidase (HRP)-conjugated anti-6-His monoclonal antibody (Sigma, St. Louis, MO) that binds to the 6-His tag of the recombinant VWF-A1. The amount of VWF-A1 adsorbed on the surface at different VWF-A1 coating solution
concentrations was measured in terms of absorbance of o-phenylenediamine (OPD)/H₂O₂ solution at 490 nm (A₄₉₀). The number of binding sites was read from a calibration standard correlating the measured A₄₉₀ values at different known quantities of anti-6-His antibody in solution.

2.2.5 Parallel plate flow chamber and digital image processing

Cells were perfused through the parallel plate flow chamber placed on an inverted-stage phase-contrast microscope (DIAPHOT-TMD; Nikon; X-20 or X-10 phase objective and X-5 projection lens, Nikon, Garden City, NY). The parallel-plate flow chamber consisted of a polycarbonate slab, a silicone gasket creating a defined gap, and a glass coverslip coated with VWF-A1 held together by application of a vacuum (Slack and Turritto, 1994). The chamber was maintained at 37°C by an air curtain incubator attached to the microscope. The wall shear stress (τₜ) depends on the height of the gap (b), the width of the chamber (w), the fluid viscosity (μ) and the flow rate through the chamber (Q) as defined by the following equation (Ross et al., 1998):

\[ \tau_w = \frac{6Q\mu}{b^3w} \]  
Eq. (2.1)

Cells resuspended to the desired concentration were perfused through syringe pump (Harvard Apparatus, Hollison, MA) and the microscopic images of cells were videotaped at 30 fps using silicon-intensified target video camera (Model C2400; Hammatsu, Waltman, MA) attached to the microscope. The digitized images were collected off-line and analyzed using ISee software (Inovision Corp., Durham, NC).
2.2.6 Measurement of rolling velocities

Velocities of rolling cells were measured by tracking the displacements of individual cells frame-by-frame, every 0.033 s, in the direction of flow (Nanotrack, Inovision). For each experiment, at least 50 cells were tracked up to a maximum time period of 10 s. To differentiate cells rolling on the surface from the cells in free flow, we used a velocity threshold cut-off, below which the cells are believed to be in contact with the surface. The velocity threshold was determined based on the velocity at which tethered cells are released from the surface, back into the fluid stream. At 1.6 dyn/cm², the distribution of release velocities was approximately normally distributed \( n = 150 \) with a mean of 260 \( \mu m/s \) and a SD of 60 \( \mu m/s \). Hence > 97% of the cells traveling at a velocity < 80 \( \mu m/s \) are likely to be in contact with the surface. Hence 80 \( \mu m/s \) was chosen as the operational threshold to separate cells in contact with the surface from cells free in flow. Only cells that stayed in contact with the surface for at least 0.2 s were considered for rolling. The time spent by a cell during one continuous sequence of velocities below the velocity threshold is defined as the rolling duration and rolling velocity was calculated from the distance moved in this period.

2.2.7 Tethering rate and adhesion strength

CHO cells expressing GP Ib-IX complex were perfused into the flow chamber at concentration of 10⁶ cells/ml over VWF-A1 coated coverslips. The cell concentration at the inlet was maintained at similar levels and the fields of view were chosen at approximately the same distance from the inlet to minimize any effect of variations in the cell flux with distance from the inlet. The rate at which the free-flowing cells tethered to the surface was measured during the first 60 s of perfusion. The tethering rate at different shear stresses was normalized
by dividing the number of cells tethered by the number of cells transported across the field of view in the focal plane of the immobilized VWF-A1. The tethering rates for WT, Q232V and K237V cells were corrected for any non-specific interaction by subtracting out the background values obtained for CHO βIX cells (lacking GP Ibα) under identical conditions. To measure the strength of adhesion, detachment assays were performed as follows: cells were allowed to accumulate at low shear stress (0.5 dyn/cm²) for 3 min. Any non-adherent cells were washed off by perfusing cell-free buffer and the wall shear stress was doubled every 30 s and the number of cells remaining adherent on the surface was counted.

2.2.8 Pause time analysis

The interaction lifetime between CHO cells expressing the GP Ib-IX complex and VWF-A1 adsorbed surfaces was quantified by analyzing the transient tethering events. A transient tethering event is defined as the abrupt stoppage of a free flowing cell without evidence of its translocation on the surface before rejoining the bulk flow, to resume a velocity equivalent to that of a non-interacting cell. We used VWF-A1 concentrations lower than would support continuous rolling of CHO cells (15 - 55 µg/ml). This ensures that the cell does not roll and is probably tethered to the surface by the basic binding unit that mediates the interaction under such conditions (Alon et al., 1997, Ramachandran et al., 1999). We tried two different methods for spotting cells that transiently tether and to measure the pause duration of the transient tethers: (1) In Method 1, we used the time–distance trajectory of cells interacting with the surface. If the distance moved by a cell when tethered to the surface is less than a certain predetermined distance, the cell is transiently tethered and the length of time for which the tethering event lasts is the pause duration. The predetermined
distance was chosen based on the resolution of the camera and the imaging system, tether extension and cell deformation due to fluid force. We recorded about 150-250 such transient tethering events at each shear stress for each of the mutants at a spatial resolution of 1 μm and a temporal resolution of 1/30 s, using the software ISee (Inovision, Durham, NC). We used first-order dissociation kinetics, \( dN_b/dt = -k_{off} N_b \), to fit the tether duration distribution, where \( N_b \) is the number of bound cells. The resultant plot is a straight line with slope = \(-k_{off}\), the dissociation rate constant. At least three independent measurements of \( k_{off} \) were made with both the mutants and wild-type at each wall shear stress examined. (2) In Method 2, we used the instantaneous velocity plots of the cells interacting with the surface to calculate the pause duration. The transient tether events are defined based on an operational velocity threshold. If the instantaneous velocity of a cell is below this value, then the cell is in contact with the surface and if the velocity is above this value, then the cell is in free flow. Pause duration is the time spent by a cell in this velocity valley and is presumed to correlate to the lifetime of a tether bond. We recorded about ~ 80 events using the software Optimas (Media Cybernetics, CA) at two values of shear stresses using wild-type cells from two experiments each. After discarding the longest 10% of the tethering events, the rest of the tether time distribution was fit to the first-order dissociation model described above to obtain \( k_{off} \). For reasons that will be described in the “Discussion” section, we have mainly calculated off-rate constants by Method 1, unless otherwise mentioned explicitly that it was calculated by Method 2.
2.3 Results

2.3.1 Interaction of GP Ib-IX cells with VWF-A1 coated surfaces

The level of surface expression of GP Ibα in cells expressing wild-type GP Ibα or the mutants K237V and Q232V along with CHO βIX control is shown in Fig. 2.2 A. It can be seen from the fluorescence plots for the cell samples that the GP Ibα expression in the mutants and wild-type are equivalent. We maintained the expression at such comparable levels so that the functional differences observed in our experiments are intrinsic and not due to differences in the receptor densities. The GP Ibα receptor density was estimated as the number of AK2 binding sites to be ~100/μm². CHO cells expressing GP Ibαβ-IX complex were perfused over immobilized VWF-A1 coated surfaces at different shear stresses. The interaction of the cells with the surface was specific for GP Ibα–VWF-A1 interaction because neither did the CHO cells expressing GP Ibαβ-IX (WT, K237 and Q232V) interact with HSA coated surfaces nor did cells expressing only GP Ibβ-IX (without GP Ibα) interact with VWF-A1 coated surfaces. The adsorption of VWF-A1 as a function of VWF-A1 coating solution concentration was calculated in terms of absorbance at 490 nm (Fig. 2.2 B). The VWF-A1 site densities were estimated to be 72, 228, 351 and 415 sites/μm² for VWF-A1 coating solution concentrations of 10, 30, 70 and 100 μg/ml respectively.

2.3.2 Detachment strength

We performed a detachment assay by perfusing the WT or mutant cells on VWF-A1 surface at low shear stress and then increasing the shear stress progressively to detach adherent cells. The adherent cells were distinguished from the cells in free flow by the brightness of the adherent cells in the focal plane when observed through a phase contrast
Figure 2.2: (A) Cell surface expression of GP Ibα (WT, Q232V and K237V) on transfected CHO cells: The cells were incubated with FITC-conjugated mouse monoclonal antibody AK2 in 5% HSA solution for 60 min. The expression levels were detected using FACScan flow cytometry. CHO cells expressing GP Ibβ-IX (without α-subunit) were used as control. (B) Adsorption of VWF-A1 on glass coverslips at 37°C. The absorbance values are averaged from two experiments performed in triplicate.
Figure 2.3: Detachment strength of GP Ibα – VWF-A1 tethers: Cells expressing WT and mutant GP Ibα were allowed to accumulate at 0.5 dyn/cm² shear stress on surfaces coated with 100 μg/ml VWF-A1 solution for 3 min. Then cell-free buffer was perfused and the shear stress was doubled every 30 s. (A) Snapshot image as seen through a microscope. The cells adherent on the surface can be seen as bright spots compared to those that are not in focus by phase contrast. (B) The percentage of cells remaining adherent was determined. The data represent mean ± SD of three experiments. Q232V and WT did not form stable tethers even at low shear stresses: while most of the Q232V cells moved a fraction of cell diameter before detachment, majority of the WT cells moved a few cell diameters before spontaneously rejoining the flow.
microscope (Fig. 2.3 A). We found that even at 0.5 dyn/cm², Q232V formed only brief tethers with the surface while WT rolled for very short distances before detaching spontaneously. The K237V cells rolled on the surface and detached slowly when the shear stress was increased (Fig. 2.3 B). Only ~ 50% of the cells detached even at a shear stress of 16 dyn/cm², indicating that K237V – VWF-A1 interaction has either very low dissociation rate (very long lifetime) or high association rate which results in the formation of many new bonds as the cell moves thus strengthening the interaction.

2.3.3 Kinetics of rolling of CHO cells expressing wild-type and mutant GP Ibα on VWF-A1 coated surfaces

We studied the kinetics of rolling based on instantaneous velocities obtained by following the displacements of the GP Ib-expressing cells as they roll on VWF-A1 surface. Fig 2.4 A, B and C show, respectively, representative velocity profiles in each individual frame of Q232V, WT and K237V cells rolling on surfaces coated with 100 µg/ml VWF-A1 at a shear stress of 1.6 dyn/cm². The K237V cells roll more slowly and continuously while the Q232V cells show predominantly saltatory motion punctuated by brief rolling events compared to the WT. To examine the stability of rolling, we determined distribution of rolling durations for many Q232V, WT and K237V cells. Fig. 2.5 A, B and C illustrate the distribution of rolling durations for WT and K237V respectively; the contrast is evident. While the longest time for which the Q232V cells roll continuously on the surface is 0.6 s with > 90% of the cells lasting on the surface for less than half second, a significant fraction of K237V cells stayed for the entire duration monitored (10 s) with > 70% of the cells rolling for at least 1 s. The WT cells rolled for a maximum period of 2.2 s with > 85% of the cells
Figure 2.4: Kinetics of rolling of cells expressing wild-type or mutant GP Ib receptors at 1.6 dyn/cm² shear stress on surfaces coated with 100 μg/ml VWF-A1: Only cells that stayed in contact with the surface for at least 0.2 s were considered for rolling analysis. (A, B and C) represent the instantaneous velocity profiles of Q232V, WT and K237V cells. The dashed line denotes the velocity threshold value of 80 μm/s used to separate the cells free in flow from those that contact and roll on the surface (see Materials and Methods).
Figure 2.5: Kinetics of rolling of cells expressing wild-type or mutant GP Ib receptors at 1.6 dyn/cm² shear stress on surfaces coated with 100 μg/ml VWF-A1: (A, B and C) Distribution of rolling duration for the Q232V, WT and K237V cells. The durations are obtained based on the plots obtained in Fig. 2.4.
Figure 2.6: Kinetics of rolling of cells expressing wild-type or mutant GP Ib receptors at 1.6 dyn/cm² shear stress on surfaces coated with 100 μg/ml VWF-A1. (A) Distribution of rolling velocities for the Q232V, WT and K237V cells (B) Average rolling duration and average rolling velocity while in contact with the surface. The values are based on at least 150 cells for WT and K237V and 60 cells for Q232V, each followed for a maximum of 10 s, averaged from three to five independent experiments.
interacting with the surface for less than 1 s. The average rolling duration calculated based on these distributions is 5.3-fold more for K237V and 1.8-fold less for Q232V than for WT (Fig. 2.6 A). We also calculated the distribution of rolling velocities for the three cell types (Fig. 2.6 B). The K237V cells roll much slower than the WT cells, the mode of the distribution being 5-fold lower than that of WT. The Q232V rolling distribution indicates a tendency for Q232V cells to roll faster than WT cells. The mean rolling velocity of K237V is 2.15-fold lower and that of Q232V is 1.26-fold higher than that of WT, respectively (Fig. 2.6 A).

2.3.4 Kinetics of tethering of GP Ibα expressing cells on VWF-A1 coated surface

We measured the tethering frequency, which is proportional to the cellular association rate, by calculating the rate at which CHO cells tethered to VWF-A1 coated surfaces at different coating densities and shear stresses (Ramachandran et al., 1999; Dwir et al., 2000; Chateau et al., 2001). The initial tethering frequency on surfaces coated with 100 μg/ml VWF-A1 solution at different shear stresses is shown in Fig. 2.7 A. In general, the tethering frequency decreases with an increase in shear stress possibly due to the decreased time of contact. At any given shear stress, more K237V cells tethered than the Q232V or the WT. The tethering frequency of K237V was 3-fold greater than WT, which was again 3-fold greater than Q232V, implying that the rate of bond formation ($k_{on}$) for the gain-of-function mutant is nearly 10-fold more than that of the loss-of-function mutant. We also determined the effect of VWF-A1 coating concentration on the tethering rate. Fig. 2.7 B shows the results at a shear stress of 1.2 dyn/cm$^2$ and it can be seen that at low VWF-A1 coating concentration (10 μg/ml), while hardly any Q232V and WT cells interact with the VWF-A1
Figure 2.7: Kinetics of tethering of CHO cells expressing WT or mutant GP Ib to VWF-A1 surfaces: Initial rate of tethering of CHO cells expressing WT or Q232V or K237V mutants to (A) surfaces coated with 100 μg/ml VWF-A1 at the indicated shear stresses and (B) at a shear stress of 1.2 dyn/cm² on surfaces coated with different concentrations of VWF-A1.
coated surfaces, a significant number of K237V cells tether to the surface. An increase in VWF-A1 coating concentration to 100 μg/ml increases the tethering frequency of all the cell types.

Moreover, the examination of the videotapes indicated that the two mutants differed not just at the rate of capture from the flowing stream, but also on the ability to sustain interactions with the surface. The Q232V mutant detached after traveling very short distances for a short period of time while K237V stayed on the surface for longer duration traveling longer distances. This prompted us to analyze the fate of the cells once they have tethered to the surface by examining the distance traveled on the VWF-A1 surface before joining the fluid stream. The measured distances were grouped in bins of 5 μm and the number of cells in each group was expressed as a % of total number of cells tethered. A typical plot at a shear stress of 1.6 dyn/cm² on 100 μg/ml VWF-A1 is shown in Fig. 2.8 A. The Q232V mutants travel predominantly short distances (> 95% travel less than one-half cell diameter) and the K237V mutants travel predominantly long distances (> 70% travel at least one cell diameter) while the WT move distances intermediate between the two mutants.

While the short travel distances likely represent the stretching of the tethers or extension of cell membrane, longer travel distance are due to the breakage of old bonds in the rear edge and formation of new ones in the front. Hence we classified tethers that last less than 5 μm distances as transient tethers and those of more than 5 μm distance as rolling tethers. Fig. 2.8 B shows the % of transient and rolling tethers at 0.8, 1.2 and 1.6 dyn/cm² on surfaces coated with 100 μg/ml VWF-A1. Again, we observe that the K237V cells has much greater efficiency in converting transient tethers to rolling tethers than the WT or the Q232V
Figure 2.8: Kinetics of tethering of CHO cells expressing WT or mutant GP Ib to VWF-A1 surfaces: (A) The distance traveled by tethered cells at 1.6 dyn/cm² shear stress before detachment from surfaces coated with 100 μg/ml VWF-A1. The cell diameter is ~15 μm. (B) Fraction of the cells that are transiently tethered before detachment or converted to rolling adhesion. The data presented is a mean ± SD of three to five experiments.
cells. The fraction of cells that form rolling tethers increases with increase in shear stress for WT and K237V but is unaffected for Q232V.

2.3.5 *Kinetics of dissociation and estimation of transient tether lifetimes*

The alteration in the affinities between the wild-type, gain- and loss-of-function mutants represent differences in the association or dissociation rates of the GP Ibα – VWF-A1 bonds. The tethering rate, which reflects cellular $k_{on}$, seems to be significantly affected by the mutations. We also studied the effect of mutations on the tether dissociation rate ($k_{off}$) and hence we measured the lifetime of transient tether durations, which may represent single adhesive bonds (Alon *et al.*, 1997; Smith *et al.*, 1999; Ramachandran *et al.*, 1999). At VWF-A1 coating densities that would not support continuous rolling, the interaction of the cells with the surface was characterized by discrete ratchet-like steps that can be quantified using the time-distance trajectories. Fig. 2.9 *A* and *B* show the time-distance and time-instantaneous velocity trajectories respectively, of three cells – a rolling cell, a transiently tethered cell and a cell in free flow. It can be seen that the cell in free flow has a constant velocity as indicated by the straight line in the time-distance plot. The rolling cell, on capture from free flow, experiences a drastic reduction in the velocity. As the cell creeps in the direction of flow, the motion is characterized by jerky fluctuations in the velocity profile. On the other hand, though a transiently tethered cell interacts with the surface, it has no appreciable movement as seen from the distance and velocity trajectories. We calculated the lifetime of a transient tethers using two methods: (1) In Method 1, the pause duration was calculated from the time-distance trajectory of transiently tethered cells (denoted by ‘t’ in Fig. 2.9 *A*). Unlike rolling cells, transiently tethered cells show little or no movement on the
Figure 2.9: Cell-tracking in a shear field to isolate transiently tethered cells (A). Displacement in the direction of flow for three different cells – transient tether, rolling and in free flow. \( t \) represents the pause duration for a tethered cell (B). Corresponding instantaneous velocity profiles. The tethered cells have near zero velocity when in contact with the surface.
surface (< 3 μm). We calculated the pause durations for several cells and the distribution was fit to a first-order kinetics (Fig. 2.10). The tether lifetime appears to obey first-order kinetics and the slope of the line = -k_{off}. (2) In Method 2, the pause duration was calculated from the time spent below a certain velocity threshold by transiently tethered cells as seen in the instantaneous velocity profiles (Fig. 2.11). The length of time a cell spends in the velocity valley below a certain threshold gives the lifetime of the tether. It can be seen from Fig. 2.11 that depending on the choice of velocity threshold, the tether durations could be widely different (denoted as circles). For the same trajectory, the tether durations range from 1–2 to 4–10 frames depending on whether 10 and 60 μm threshold was used, respectively. The distribution of tether duration so calculated was fit to a first-order dissociation model as in Fig. 2.10 to obtain the off-rate constant. We used this method for calculating the off-rate constants for WT at two different shear stresses and the results are shown in Table 2.2.

Table 2.2: Dissociation rate constants for different values of velocity thresholds

<table>
<thead>
<tr>
<th>Velocity cut-off (μm/s)</th>
<th>k_{off} (s^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.8 dyn/cm²</td>
</tr>
<tr>
<td>10</td>
<td>7.6 ± 2.5</td>
</tr>
<tr>
<td>20</td>
<td>5.6 ± 0.2</td>
</tr>
<tr>
<td>30</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>40</td>
<td>4.2 ± 0.2</td>
</tr>
<tr>
<td>50</td>
<td>4.4 ± 0.1</td>
</tr>
<tr>
<td>60</td>
<td>3.4 ± 0.05</td>
</tr>
</tbody>
</table>
Figure 2.10: Calculation of $k_{off}$ from the distribution of tether duration for wild-type at 0.8 dyn/cm$^2$. The tether durations were calculated from the trajectory of transiently tethered CHO cells.
Figure 2.11: Pause time analysis by velocity threshold. The tracings show the instantaneous velocity profile of a cell interacting with the surface. The dotted lines show the velocity threshold used to separate pauses from free-flow. The circles represent the pause durations. The velocity thresholds used are (A) 10 μm/s and (B) 60 μm/s, both for the same tracing.
2.3.6 **Kinetics of dissociation and mechanical strength of GP Ibα–VWF-A1 transient tethers**

We used Method 1 described above to calculate the transient tether lifetimes of wild-type, gain- and loss-of-function mutant GPIbα–VWF-A1 bond at different shear stresses. Fig. 2.12 A shows a representative semi-log plot of pause time distribution at different shear stresses for WT. The tether lifetime appears to obey first-order kinetics and the slope of the line = \( -k_{off} \). The \( k_{off} \) values increase with an increase in shear stress indicating that the tether lifetime decreases as the force on the bonds increase. Fig. 2.12 B is a similar plot at 0.8 dyn/cm\(^2\) and it can be seen that the \( k_{off} \) of Q232V is 2-fold and WT is 1.5-fold greater than that of K237V respectively. The good linear fit may suggest, though not prove, the measurement of single bond or a quantal unit that mediates the interaction. We do not know the exact number of molecular bonds involved in the tether structure. Nevertheless, these \( k_{off} \) values represent the lifetimes of the smallest functional unit of adhesion that permits the cell interaction with the surface and is a useful estimate of the intrinsic kinetic and mechanical properties of the receptor-ligand pairs.

To relate the effect of fluid force on the \( k_{off} \), we used the Bell model (1978): \( k_{off} = k_{off}^0 \exp(\sigma F_b/kT) \) to fit the \( k_{off} \) values measured at different shear stresses. In this equation, \( F_b \) is the force on the tether bond, \( k \) is Boltzmann constant and \( T \) is absolute temperature. The Bell model parameters, \( k_{off}^0 \) and \( \sigma \) are zero-force dissociation constant and reactive compliance respectively. The higher the \( k_{off}^0 \) value, the lower is the bond lifetime and higher the \( \sigma \) value, greater is the sensitivity to force-induced dissociation. The net force acting on the tether, \( F_b \) because of fluid shear force \( (F'_s) \) and torque \( (\tau) \) can be related to the wall shear using the
Figure 2.12: Kinetics of dissociation of transiently tethered CHO cells expressing WT or mutant GP Ib on VWF-A1 coated surfaces (A) The natural logarithm of the number of cells that remain tethered is plotted as a function of time after initiation of the tether. The negative of the slope is $k_{off}$. The $k_{off}$ decreases with an increase in shear stress for WT (B) A plot of first-order dissociation kinetics for WT, Q232V and K237V at 0.8 dyn/cm$^2$. The $k_{off}$ of Q232V is twice as much as that of K237V.
Figure 2.13: Kinetics of dissociation of transiently tethered CHO cells expressing WT or mutant GP Ib on VWF-A1 coated surfaces. (A) Estimation of force on the GP Ibα – vWF-A1 tether bond. The Goldman equation was used to calculate the hydrodynamic torque, τ, and force, F_s, on the cell. The force and torque balances on a tethered cell in shear flow are F_s = F_b cos θ and F_b sin θ = τ_s + R F_s, where R is cell radius and l is lever arm. Calculation with R = 8.5 μm and θ = 60° yielded the force on the tether bond, F_b ~ 450 pN/(dyn/cm²). (B) We used Bell model (see text) to fit the experimental data. At each shear stress, 150 – 250 events were collected and each point represents mean from three different experiments. Error bars show SD. The data were fit to the Bell equation, k_{off} = k_b^0 \exp (\sigma F_s/kT).
lubrication theory of Goldman et al., (1967) (Fig. 2.13 A). The equations describing the fluid shear rate to the shear force and torque acting on a cell in contact with a wall are given by:

\[ F_s = (1.7005)6\pi \mu a^2 S \quad \text{Eq. (2.2)} \]

\[ \tau = (0.94399)4\pi \mu a^3 S \quad \text{Eq. (2.3)} \]

where \( \mu \) is the viscosity of cell suspension, \( a \) is the radius of the cell and \( S \) is shear rate. The force on the tether bond (\( F_b \)) at a given shear rate is calculated from the above equations and from geometric arguments (Fig. 2.13 A). We have fit the \( k_{\text{off}} \) values measured at various shear stresses, to the Bell model (1978), in terms of \( F_b \) and the plot is shown in Fig. 2.13 B (Chateau et al., 2001; Ramachandran et al., 2001). It can be seen that the tether bonds of both the mutants and of the wild-type are more prone to dissociation with an increase in force. We estimated the \( k_{\text{off}}^0 \) values (in s\(^{-1}\)) for WT, K237V and Q232V to be 5.66 ± 0.55, 2.56 ± 0.62 and 7.15 ± 1.18 and the corresponding \( \sigma \) values (in Å) to be 0.058 ± 0.007, 0.089 ± 0.013 and 0.06 ± 0.01 respectively. It can be seen that the \( k_{\text{off}}^0 \) value of WT is 1.25-fold lower and 2.2-fold greater than Q232V and K237V respectively. The \( \sigma \) value of Q232V is comparable to WT but that of K237V is ~1.5-fold greater than the WT.

2.4 Discussion

Quantification of the interactions of platelet GP Ib\( \alpha \) with VWF-A1 domain can help us better understand the mechanisms that result in altered phenotypes caused by mutations in the receptor or the ligand. Dong et al., (2000, 2001) have shown earlier that the gain- and loss-of-function mutants of GP Ib\( \alpha \) have altered affinities for VWF by modulator titration and in flow chamber assays. Here, we characterized these differences by analyzing the basic kinetics of the interaction between VWF-A1 and wild-type GP Ib\( \alpha \), and gain- and loss-of-
function GP Ibα mutants under controlled flow conditions. We calculated the cellular association rate constants (tethering frequencies) and dissociation rate constants (tether lifetimes) at different shear stresses and coating densities. Wild-type GP Ibα has a 1.25-fold higher and 2.2-fold lower unstressed dissociation rate constant than the loss- and gain-of-function mutants, respectively. On the other hand, the tethering frequency of WT GP Ibα is 3-fold higher and 3-fold lower than that of loss- and gain-of-function mutants, respectively. These changes in both the on- and off-rates likely account for the phenotypic manifestations of these mutations.

We made several observations that highlight the remarkable changes in the GP Ib – VWF-A1 interaction caused by the GP Ibα mutations K237V and Q232V. First, very few K237V cells could be seen to roll continuously at VWF-A1 coating concentrations as low as 5 μg/ml while many of the Q232V cells did not roll continuously even at a VWF-A1 concentration as high as 150 μg/ml. The WT showed predominantly saltatory motion in this concentration range (data not shown). Second, the K237V cells converted transient tethers to rolling tethers (at 1.6 dyn/cm² on surfaces coated with 100 μg/ml VWF-A1) much more efficiently (50 – 75%) than Q232V cells (10 – 20%) and WT cells (25 – 40%) (Fig. 2.8). Third, the average rolling velocity of Q232V cells was 1.26-fold faster and that of K237V cells 2.15-fold slower than that of the WT, respectively (Fig. 2.6 B). Fourth, the average rolling duration (the time spent by a rolling cell in continuous contact with the surface), was ~2-fold less for Q232V and ~5-fold more for K237V cells compared to the WT.

Rolling adhesion is an inherently unstable transition state, delicately poised between firm adhesion and lack of adhesion. Maintenance of stable rolling requires that the average number of bonds that are formed and broken be nearly equal. The rate of bond formation is
related to the cellular association rate constant, $k_{on}$ and the rate of bond dissociation is quantified by dissociation rate constant, $k_{off}$. We calculated the $k_{off}$ values for the wild-type and mutant GP Ibα interactions with VWF-A1 from measures of the lifetime of transient tethering events. We used low VWF-A1 coating densities that would support tethering but not continuous rolling of the CHO cells so as to minimize the number of bonds that mediate the transient tethering events. By doing so, we have measured the lifetime of the smallest functional unit of the interaction that could be observed at the spatial and temporal resolution employed in this study. The tether time distribution followed first-order dissociation kinetics and has properties that suggest the smallest functional units of interaction behave as single bonds. Although such properties do not prove the absence of multiple bonds, these events are of physiological relevance as they represent the smallest functional unit of adhesion that permits cell interactions in flow. From Table 2.3, it can be seen that the $k_{off}^0$ values are affected to different degrees by Q232V and K237V mutations. While the K237V mutation results in a 2.2-fold decrease in $k_{off}^0$, the Q232V mutation results in a 1.25-fold increase.

The lifetime (1/$k_{off}^0$) of the GP Ibα – VWF-A1 bond we have measured compares very well with the lifetime of selectin-carbohydrate bonds (Table 2.3) measured using various techniques including flow chamber assays and surface plasmon resonance (Alon et al., 1997; Mehta et al., 1998, Ramachandran et al., 1999). The selectin-carbohydrate bond performs the function of recruiting leukocytes from the flowing blood stream to the site of inflammation in much the same way that the GP Ibα – VWF-A1 bond recruits platelets from flowing blood in hemostasis and thrombosis. Hence, one would expect the kinetic properties of the two classes of bonds to be similar (Konstantaopoulos et al., 1998, Doggett et al., 2002; 2003). Our results (Table 2.3) indicate that this is indeed the case. These bonds
Table 2.3: Kinetic and mechanical properties of receptor–ligand bonds

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>$k_{off}^0$ (s$^{-1}$)</th>
<th>$\sigma$ (Å)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP Ibα (WT)</td>
<td>vWF-A1(WT)</td>
<td>5.66</td>
<td>0.058</td>
<td>this work</td>
</tr>
<tr>
<td>GP Ibα (Q232V)</td>
<td>vWF-A1(WT)</td>
<td>7.15</td>
<td>0.06</td>
<td>this work</td>
</tr>
<tr>
<td>GP Ibα (K237V)</td>
<td>vWF-A1(WT)</td>
<td>2.56</td>
<td>0.089</td>
<td>this work</td>
</tr>
<tr>
<td>GP Ibα (WT)</td>
<td>vWF-A1(WT)</td>
<td>0.0038</td>
<td>—</td>
<td>Miura et al., (2000)</td>
</tr>
<tr>
<td>GP Ibα (WT)</td>
<td>vWF-A1(type2B)</td>
<td>0.0036</td>
<td>—</td>
<td>Miura et al., (2000)</td>
</tr>
<tr>
<td>GP Ibα (WT)</td>
<td>vWF-A1(WT)</td>
<td>3.21</td>
<td>0.18</td>
<td>Doggett et al., (2002)</td>
</tr>
<tr>
<td>GP Ibα (WT)</td>
<td>vWF-A1(type2B)</td>
<td>0.56</td>
<td>0.26</td>
<td>Doggett et al., (2002)</td>
</tr>
<tr>
<td>GP Ibα (G233V)</td>
<td>VWF-A1 (WT)</td>
<td>0.55</td>
<td>0.29</td>
<td>Doggett et al., (2003)</td>
</tr>
<tr>
<td>L-selectin</td>
<td>PNAd</td>
<td>6.80</td>
<td>0.2</td>
<td>Alon et al., (1997)</td>
</tr>
<tr>
<td>P-selectin</td>
<td>PSGL-1</td>
<td>1.1</td>
<td>0.29</td>
<td>Ramachandran et al., (1999)</td>
</tr>
<tr>
<td>α4β7/Ca</td>
<td>MAdCAM-1</td>
<td>0.046</td>
<td>0.91</td>
<td>de Chateau et al., (2001)</td>
</tr>
</tbody>
</table>
lifetimes are shorter than those of integrins estimated to be > 20 s, which mediate firm adhesion of neutrophils to endothelium (de Chateau et al., 2001). The short tether lifetimes of transient interactions like selectin and GP Ib bonds are crucial for promoting rolling adhesions (Chang et al., 2000).

Interestingly, the zero-force dissociation constant ($k_{off}^d$) value for the GP Ibα–VWF-A1 interaction obtained by us is significantly higher (~1500-fold) than that reported by Miura et al., (2000). This discrepancy could be due to the use of solution-phase biochemical assay as opposed to our measurements under dynamic flow conditions. The static assay is likely to underestimate the impact the mechanical forces on the bond lifetime. This possibility is supported by the recent work of Doggett et al., (2002). They reported the dissociation rate constant of GP Ibα on immobilized platelets and VWF-A1 coated beads using a flow chamber assay to be 3.21 s$^{-1}$, a value close to that obtained in this work (Table 2.3). It should be noted that the force on the GP Ib tether bond on the CHO cell surface at a shear stress of 1 dyn/cm$^2$ used in our experiments compares roughly to that of 20 dyn/cm$^2$ experienced by the platelet tether bond, given the 5 to 10-fold size difference between platelets and CHO cells (Fredrickson et al., 1998).

It is instructive in this context to discuss the usage of two different plots of cellular trajectory, namely time–distance plots and instantaneous velocity plots, reported in the literature for the measurement of transient tether lifetime and $k_{off}$. We calculated the tether lifetime based on the time–distance trajectory of transiently tethered cells (described as Method 1 in “Materials and Methods” section). The critical issue is to differentiate transiently tethered cells from rolling cells. The reason being, while rolling
is necessarily mediated by multiple tether bonds, transient tethering events can be mediated by single tether bonds. Ideally, cells held on the surface mediated by single tether bonds will not move on the surface before rejoining the fluid stream. However, in reality there might be some motion observed due to the spatial and temporal resolution of the microscope, cell deformability and extension of microvilli tether under the action of fluid force. To account for these effects we have used a distance threshold of 3 \( \mu \text{m} \). If a tethered cell moves less than this threshold distance before detaching from the surface, then a single tether bond probably mediates the interaction. While some investigators have employed this method with different values for the distance threshold (Table 2.4), a few others have used a different method based on instantaneous velocity profiles for distinguishing transitory tethered cells and calculating the tether lifetime (described Method 2 in "Materials and Method" section). When a cell gets captured from free flow, its velocity drops to a value lower than that in a free flow. A rolling cell exhibits a non-zero velocity due to the formation of bonds in the front edge and breakage of bonds in the rear, as it crawls in the direction of flow (Fig. 2.9). On the other hand, a transiently tethered cell that is held on the surface by a single tether bond shows movement only due to cell deformation and tether extension. Hence, such a tethered cell will have a near-zero velocity that is even lower than that of a rolling cell. By defining a suitable threshold to separate the bigger fluctuations due to bond breakage (rolling) from the smaller fluctuations due to tether extension (transient tethering), it should theoretically be possible to separate transient tethering events mediated by single tether bonds from rolling events mediated by multiple tether bonds. Therefore, the time spent by a transiently tethered cell in the velocity valley below the threshold is the pause duration
(Fig. 2.11). A few investigators have analyzed the instantaneous velocity profiles from flow chamber data by this method and they have all used different choices for the operational velocity threshold (Table 2.4). When

<table>
<thead>
<tr>
<th>Reference</th>
<th>Receptor – Ligand</th>
<th>Pause time definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaplanski et al., 1993</td>
<td>E-selectin–ESL</td>
<td>2 µm distance</td>
</tr>
<tr>
<td>Alon et al., 1997</td>
<td>L-selectin–PNAd</td>
<td>2 µm distance</td>
</tr>
<tr>
<td>Chen and Springer, 1999</td>
<td>L-selectin–PNAd</td>
<td>90 µm/s change in velocity</td>
</tr>
<tr>
<td>Ramachandran et al., 1999</td>
<td>P-selectin–PSGL-1</td>
<td>Did not report</td>
</tr>
<tr>
<td>Chen and Springer, 2001</td>
<td>P-Selectin–PSGL-1</td>
<td>Minimum velocity + 30 µm/s</td>
</tr>
<tr>
<td>Rinker et al., 2001</td>
<td>Monocyte–Endothelium</td>
<td>15% of free-stream velocity</td>
</tr>
<tr>
<td>Doggett et al., 2003</td>
<td>GP Ibα–VWF-A1</td>
<td>Did not report</td>
</tr>
</tbody>
</table>

we obtained tether time distributions using different values of velocity threshold and calculated the $k_{off}$, we found that the $k_{off}$ values varied tremendously depending on the value of the velocity threshold (Table 2.2). This threshold is difficult to estimate since the actual number of tethers in the contact area, the rate of tether extension and the rate of bond dissociation are unknown quantities. In addition, the error spatial and temporal resolution of the microscope gets amplified as we calculate the instantaneous velocities from the position of the cell in individual frames and the fluctuations in velocity due to experimental artifacts like the mechanical error in the image acquisition system can result in false positives. Further, we observed that the method using velocity threshold has a strong bias towards large pause durations while neglecting brief, short pauses. For
instance, using a threshold of 20 μm/s will eliminate a brief tether lasting one frame with a movement of 1 μ but will include a rolling event lasting 100 frames with a movement 10 μ, though in reality, the former is much more likely represent a single tether bond than the latter. This could be a reason for the majority of $k_{\text{off}}$ values calculated by Method 2 (Table 2.2) being lower than when calculated by Method 1 (Fig. 2.12 A).

In addition to $k_{\text{off}}^0$, we also measured the tethering frequency, the rate at which cells tether to the VWF-A1 surface from free flow. The tethering frequency is proportional to the rate of formation of the initial bond, provided the bond is sufficiently strong and long-lived to be observed and if the fraction of cells that is tethered to the surface for durations shorter than the sampling rate is small. The tethering frequency can be viewed as a lumped cellular association rate constant ($k_{\text{on}}$), being dependent on hydrodynamic parameters like shear rate and relative velocity of the approaching surfaces, physical parameters like the distribution and orientation of receptor and ligand moieties as well as the intrinsic association rate constant. At a given shear stress (assuming comparable receptor and ligand densities), the differences in the tethering frequency will be a surrogate measure of the intrinsic differences in the $k_{\text{on}}$ due to mutations (Ramachandran et al., 1999; Dwir et al., 2000; de Chateau et al., 2001). In general, the tethering frequency decreases with increasing shear stress (Fig. 2.7 A) due to shorter contact time between the reacting surfaces. At any given shear stress, the tethering frequency of WT was higher than Q232V and lower than K237V. In fact, the mutations resulted in a 3-fold lower tethering rate for Q232V and a 3-fold increase for K237V. Also, the tethering frequency of K237V at VWF-A1 coating concentrations as
low as 10 μg/ml is more than that of Q232V at a concentration as high as 100 μg/ml (Fig. 2.7 B).

Cell rolling depends on the efficiency of converting transient tethers to stable tethers by forming new tether bonds in the front edge as the old tether bonds in the rear edge dissociate. We have shown that the gain-of-function mutation leads to an increase in the cellular association rate (quantified by tethering frequency) by 3-fold and a decrease in dissociation rate (quantified by tether lifetime) by 2.2-fold. In contrast, the loss-of-function mutation results in a 3-fold decrease in the cellular association rate and a 1.25-fold increase in the dissociation rate. The composite effect of these kinetic changes for K237V is manifested by a ~5-fold increase in the rolling duration and by a ~2-fold decrease for Q232V. The close agreement in fold differences in the rolling velocities and off-rate constants — 1.26-fold increase for Q232V and 2.15-fold decrease for K237V compared to WT in rolling velocity — indicate that the rolling velocities are dependent more on the rate at which bonds break than the rate at which bonds form.

The clinical implications of changes in off- and on-rate due to the K237V mutation can be extrapolated to the naturally occurring G233V mutations associated with ptVWD. An increase in the rate of formation of GP Ibα – VWF-A1 bond results in an increase in the rate of fruitful ptVWD platelet – VWF encounters and the prolongation in the lifetime of the existing bonds greatly enhances the probability of spontaneous aggregation of ptVWD platelets compared to normal platelets. The characteristics of this ligand–receptor bond that has evolved in humans thus represents a tight balance between allowing adequate platelet tethering at the site of vessel injury and preventing the
spontaneous binding and clearance of hemostatically active VWF multimers present in the circulating blood.

Other investigators have attributed the increase in affinity associated with the gain-of-function mutations of either GP Ibα or VWF-A1 to both increases in the association rate and decreases in the dissociation rate. Miura et al., (2000) reported a 4-fold increase in $k_{on}$ as the sole reason for increased affinity of G233V GP Ibα mutant over wild-type while Doggett et al., (2002) reported a 5-fold decrease only in $k_{off}^0$ as a reason for increased affinity for the I546V gain-of-function mutant of VWF-A1. The discrepancy between our work and that of Miura et al., (2000) could be attributed to the differences between the static and dynamic adhesion assays used. Although, Doggett et al., (2002) also used a dynamic flow adhesion assay, they did not determine the role of on-rate in observed mutant and wild-type phenotype. Very recently, Doggett et al., (2003) have reported the alteration in the kinetics of GP Ib – VWF-A1 bond associated with the G233V mutation in GP Ibα. By perfusing VWF-A1 coated beads over a surface of immobilized platelets, they observed an enhancement in the tethering rate and also a decrease in the dissociation rate constant of the GP Ibα – VWF-A1 tether bond in the gain-of-function mutant. As shown in Table 2.3, the $k_{off}^0$ value estimated in their study compares very well with this work. The discrepancy in the reactive compliance $\sigma$ between the present study and that of Doggett et al., (2003), could be attributed to several possibilities including: (a) differences in the number, orientation and distribution of receptors and ligands on the immobilized platelet – bead system of Doggett et al., (2003) vs. immobilized ligand – free-flowing cell system used in the present study, (b) differences in the size and mechanical properties of cells vs. those of the beads and hence
the force experienced by the tether bond (c) possible extension of tethers from immobilized platelet surface or from the cell surface, which can alter the estimates on the force experienced by the tether bond or (d) though very unlikely, under-sampling of short-lived events at 30 fps resulting in the underestimation of off-rate constant at the highest shear stress, in the present work.

Our results also indicate that mutations altering the GP Ibα – VWF-A1 kinetics could be fundamentally different from some of the mutations altering selectin bond kinetics, despite the many similarities. Single tyrosine mutations in PSGL-1 have been shown to affect the affinity of neutrophil P- and L-selectins as manifested by differences in rolling behavior. These differences were due primarily to the alterations in dissociation rate as association rate remains unaffected (Ramachandran et al., 1999). Further, of the two components that determine the measured dissociation rate — the reactive compliance, σ and the zero-force dissociation rate constant, \( k_{off}^0 \) — these authors found that PSGL-1 mutations alter P-selectin – PSGL-1 interaction by altering the σ but \( k_{off}^0 \) is unaffected while the same mutation alters the L-selectin – PSGL-1 interaction by altering the \( k_{off}^0 \) without affecting σ. In contrast, our findings with the GP Ibα – VWF-A1 bond indicate that the σ value is unchanged by the Q232V mutation by increases by 1.5-fold for the K237V mutation (Table 2.3). Since the σ value represents the mechanical flexibility of the bond, it is probable that the moderate increase in the reactive compliance of the K237V GP Ibα – VWF-A1 bonds may compensate for an already low \( k_{off}^0 \).

In summary, we have characterized the kinetics of a physiologically and medically very important interaction between platelet glycoprotein (GP) Ibα and VWF-A1. Our findings show that these bonds have a rapid dissociation kinetics, similar to
selectin bonds, ideally suited for surveillance by platelet rolling on immobilized VWF at sites of vessel injury before adhering firmly to form thrombi. Comparison of the association and dissociation rates of gain- and loss-of-function mutations with wild-type showed an alteration in both of these quantities which might provide some mechanistic explanation to the observed differences in rolling behavior and affinities for these mutants with plasma VWF. Our results shed some light on understanding and possibly treating clinical abnormalities like ptVWD disease, which has a functional phenotype similar to the K237V mutant.
CHAPTER 3
Effect of Unusually Large Multimers of VWF on Platelet – VWF Interactions

3.1 Introduction

Platelet adhesion is the first step of hemostatic and thrombotic process and is mediated by the interaction between platelet surface receptor, platelet glycoprotein (GP) Ib and von Willebrand factor (VWF), which is a large multimeric protein found in plasma and in the subendothelium (Berndt et al., 2001). Following this step, a series of signal transduction events are initiated resulting in the firm adhesion and aggregation of platelets mediated by the interaction between platelet receptor GP IIb/IIIa with VWF and fibrinogen. These interactions also occur in systemic circulation leading to pathological platelet aggregation and thrombus formation, but it occurs only under high fluid shear stress as in stenotic vessels. VWF also performs several other important functions like protecting factor VIII cleavage from inactivation and interacting with fibrin and in stabilizing thrombi (Sadler, 1998).

VWF is synthesized and stored in two places: in the α-granules of platelets/megakaryocytes (Nachman et al., 1977) and the Weibel-Palade bodies of endothelial cells (Jaffe et al., 1974), from which VWF is released either constitutively or upon stimulation with histamine and other agonists. On synthesis, VWF undergoes a number of post-translational modifications including: extensive N- and O-linked glycosylation (Cruz et al., 1993), sulfation of asparagine (Carew et al., 1990), proteolytic cleavage of propeptide segment (Wagner, 1991) and multimerization (Verweij et al., 1988; Wise, 1988). It is not
known as to what determines the growth and size of the multimers. The newly secreted VWF is rich in unusually large forms of VWF (ULVWF), which are rapidly cleaved on entering the blood stream by a plasma metalloprotease called ADAMTS-13 into smaller normal plasma multimers (P-VWF) (Zheng et al., 2001). ADAMTS-13 reduces the size of large and unusually large forms of VWF by specifically cleaving the Y842/M843 peptide bond in the A2 domain of VWF, generating 176-kDa and 140-kDa fragments that are found in the normal circulation (Furlan 1996; Furlan et al., 1996). The importance of this proteolytic cleavage is emphasized from the severity of the thrombotic disorder, Thrombotic Thrombocytopenic Purpura (TTP), associated with ADAMTS-13 deficiency (Moake, 2004).

TTP is characterized by microvascular thrombosis, consumptive thrombocytopenia, microangiopathic hemolytic anemia, neurologic abnormalities, renal failure and fever (Moake, 2002). Episodes of TTP are associated with the excessive presence of large or unusually large VWF multimeric forms and the systemic clumping of platelets results in platelet counts below 20,000 /μl (Chow et al., 1998). Immunohistochemical studies of TTP thrombi reveal an abundance of VWF with little fibrinogen/fibrin. The disorder is rapidly progressive and if left untreated, is uniformly fatal (George, 2000). The presence of unusually large molecular weight multimers of VWF in plasma is believed to play an important role in inducing platelet aggregation and subsequent thrombocytopenia.

In this chapter, we discuss our studies to compare directly the ability of unusually large multimers (ULVWF) and normal plasma multimers of VWF (P-VWF) in mediating platelet adhesion in a parallel-plate flow chamber and in a viscometer that mimic adhesion to sub-endothelium and shear-induced aggregation, respectively. We observed that the ULVWF is more efficient than P-VWF in mediating platelet aggregation at shear rates found in
stenotic arteries, ristocetin-induced platelet agglutination and platelet adhesion at high arterial shear stress.

3.2 Materials and Methods

3.2.1 Preparation of washed platelets and reconstituted blood

Freshly drawn blood from a pool of 20 healthy volunteers, aged 20–35, was the source of platelets. All donors signed consent forms before the blood was drawn. Whole blood was drawn into 10% acid-citrate dextrose buffer (ACD, 85 mM sodium citrate, 111 mM glucose and 65 mM citric acid) at 37 °C. The citrated-blood was centrifuged at 150g for 10 min to obtain platelet-rich plasma (PRP), over a cushion of red- and white-blood cells. After separating the supernatant PRP, the residual cell suspension was centrifuged at 900g to isolate red cells. The red cells were washed twice with Ca^{2+} and Mg^{2+}-free HEPES (10 mM N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid, 145 mM NaCl, 5mM KCl, 0.5 mM Na_{2}HPO_{4}, 1mM MgSO_{4}, 5.5 mM glucose) buffer at pH 7.0.

To isolate platelets from PRP, 10 ng/ml of prostaglandin I_{2} (PGI_{2}) was added to PRP and the PRP was centrifuged at 900g for 15 min. The platelet pellet was separated from platelet-poor plasma and suspended in Ca^{2+} and Mg^{2+}-free HEPES buffer at pH 7.0. The suspension was centrifuged at 750g for 15 min. The supernatant was discarded and the platelet pellet was re-suspended in HEPES buffer containing 2 mM Ca^{2+}, 1 mM Mg^{2+} and 3.5 g/L BSA at pH 7.4. The number of platelets was determined using a Coulter counter. A suitable volume of washed platelet suspension was mixed with RBC to reconstitute blood with a platelet count of 300,000/μl and 50% hematocrit.
3.2.2 Preparation of P-VWF and ULVWF

Human plasma VWF (P-VWF) was purified from the cryoprecipitate fraction of normal plasma. 5 units of cryoprecipitate was mixed with 45 ml of citrate buffer (55 mM sodium citrate with pH adjusted to 7.4 with 55 mM citric acid) for 30 min. To the mixture was 575 ml glycine buffer (2.6 M glycine, 0.3 M NaCl, 25 mM Tris-base at pH 6.8) was added and stirred for another 30 min. The suspension was then centrifuged at 9000g for 20 min to separate fibrinogen. To every 1 L of supernatant, 90.6 g solid NaCl was added and stirred for 2 h to salt-out the VWF. The solution was centrifuged at 15000g for 30 min and the supernatant discarded. The white precipitate containing VWF was suspended in a minimum amount of VWF elution buffer (25 mM anhydrous citric acid, 50 mM NaCl at pH 6.15) and the solution was refrigerated at 4°C over night. VWF was then separated from other proteins by size exclusion chromatography using Sepharose 4B column (2.5 X 50 cm, a bed volume of 3000 ml, Amersham Biosciences), and eluted from the column at a flow rate of 50 ml/h. All the precipitation and separation processes were performed at 37 °C. The VWF collected was aliquoted and stored at 4 °C.

ULVWF multimers were produced from human umbilical vein endothelial cells (HUVECs). Confluent HUVECs (either primary or one-passage) were washed with PBS and incubated with a serum-free medium (M199, insulin 5–10 μg/ml, transferrin 5 μg/ml, 1% glutamine) for 48–72 h. The cultured cells were then treated with 100μM histamine (1 ml for every confluent T-75 flask) for 30 min at 37 °C to induce the release of ULVWF. After incubation, the conditioned medium containing ULVWF was centrifuged at 150g for 10 min to remove cell debris, and the supernatant was used as the source of ULVWF multimers. ULVWF was aliquoted and stored at −80 °C for use.
3.2.3 Estimation of VWF multimer composition and antigen level

VWF multimers in purified VWF preparations and endothelial supernatants were separated by sodium dodecyl sulfate (SDS)-agarose gel electrophoresis, overlaid with rabbit anti-human VWF antibody and analyzed by chemiluminescence using 1% agarose and a continuous buffer system (Dong et al., 2003). The amount of VWF antigen level was assayed using a commercial ELISA kit called Spectro VWF (Ramco laboratories, Houston). The assay kit is designed for VWF concentrations in the 0–100% range with 100% corresponding to the concentration of VWF in normal plasma, which is 10 μg/ml. Since the P-VWF preparation has a high concentration of VWF, we used a 1:20 dilution before following the assay protocol. ULVWF was assayed directly without prior dilution. The total amount of protein in the preparations was quantified using the Micro BCA protein assay kit (Pierce Biotechnology, IL). The experiments were performed on at least two samples, each measured in duplicate.

3.2.4 Shear-induced platelet aggregation

250 μl of washed platelets were adjusted with either ULVWF or P-VWF (at a final concentration of 10% VWF) to a volume of 500 μl. The platelet count was maintained at ~100,000/μl. The suspension was sheared at 5, 20 and 100 dyn/cm² for 60s on a cone-and-plate viscometer (RS1, HAAKE Instrument Inc., Paramus, NJ). At the end of shearing, 10 μl of the sheared sample was immediately fixed with 10 ml of Isoton II solution (Beckman-Coulter) containing 0.5% glutaraldehyde. The number of platelets was then counted using a Z2 Coulter Counter. Shear-induced platelet aggregation was defined as the percentage
reduction in single platelets compared to the unsheared controls from the same donors (Moake et al., 1986; Zhang et al., 2002).

3.2.5 Ristocetin-induced platelet agglutination

125 μl of washed platelets solution was mixed with suitable volumes of either P-VWF or ULVWF (10% final VWF concentration), 2 mg/ml fibrinogen and ristocetin at concentrations varying from 0–0.5 mg/ml, and the final volume was adjusted to 250 μl. The suspension was agitated for 20 min in an aggregometer at 37 °C. The increase in light transmission was used to measure of agglutination.

3.2.6 Measurement of VWF surface adsorption

Glass coverslips (No. 1, 24 x 50 mm, Corning, NY) were coated with a 5 mm diameter 25 μL spot of P-VWF or ULVWF solution (diluted to appropriate concentrations with PBS), and incubated for 60 min at room temperature in a humid chamber at 37 °C. The coverslips were then rinsed with 1 ml of PBS and coated with 200 μl of 3% BSA solution for 60 min at room temperature in order to block any nonspecific binding. The binding of VWF to glass coverslips was determined by an enzyme linked immunosorbent assay (ELISA) using horse radish peroxidase (HRP)-conjugated Dako polyclonal antibody (Sigma, St. Louis, MO). The amount of VWF adsorbed on the surface at different VWF coating solution concentrations was measured in terms of absorbance of o-phenylenediamine (OPD)/H₂O₂ solution at 490 nm (A₄₉₀).
3.2.7 Perfusion studies

Glass coverslips were coated with either P-VWF or ULVWF solution and blocked with 3% BSA as described above. Any excess BSA was removed with 1 ml of 0.9% saline prior to assembly as the bottom of a parallel-plate flow chamber. The parallel-plate flow chamber consisted of a polycarbonate slab, a silicone gasket creating a defined gap, and a glass coverslip coated with the either P-VWF or ULVWF held together by application of a vacuum (Slack and Turritto, 1994). The chamber was maintained at 37°C by an air curtain incubator attached to the microscope. Reconstituted blood was perfused through the parallel plate flow chamber placed on an inverted-stage phase-contrast microscope (DIAPHOT-TMD; Nikon; X-20 or X-10 phase objective and X-5 projection lens, Nikon, Garden City, NY), using a syringe pump (Harvard Apparatus, Hollison, MA). The wall shear stress depends on the height of the gap, the width of the chamber, the fluid viscosity and the flow rate through the chamber (Ross et al., 1998). Platelets were visualized and quantified by the fluorescent intensity of mepacrine (quinacrine dihydrochloride, 10 μM; Sigma) incorporated in their dense granules. Platelet adhesion events were videotaped at 30 fps using silicon-intensified target video camera (Model C2400; Hammatsu, Waltman, MA) attached to the microscope. The digitized images were collected off-line and analyzed using ISee software (Inovision Corp., Durham, NC).

3.3 Results

3.3.1 VWF concentration in P-VWF and ULVWF preparations

The composition of VWF multimers in P-VWF and ULVWF preparations was analyzed by autoradiography (Fig. 3.1). It can be seen that the multimeric composition of P-
VWF prepared within 3 months is comparable to that of normal plasma (lanes 2 and 3). The P-VWF multimers are degraded to smaller forms by about 5 months after preparation (lane 1 vs. lane 2). A major component of the ULVWF solution is very high molecular weight multimers not found in normal pooled plasma or in the P-VWF preparation (lane 4).

The VWF concentration in the preparations was measured using ELISA (Table 3.1). The results are expressed as percent VWF antigen with 100% corresponding to 10 μg/ml, the concentration of VWF present in normal plasma. While the ULVWF preparation contained relatively small amounts of VWF (20% or 2 μg/ml), P-VWF preparation is rich in VWF molecules (800% or 80 μg/ml). The VWF concentration in P-VWF preparation decreased to 400% (40 μg/ml) in about five months after preparation, which is compatible with the loss of multimers as measured by gel electrophoresis (lane 1 in Fig. 3.1).

We also measured the total amount of protein present in the two preparations (Table 3.2). The total amount of protein in the ULVWF preparation is higher than the amount of VWF measured by antigen assay indicating that the preparation has proteins other than VWF. In contrast, the P-VWF preparation contains almost exclusively VWF.

3.3.2 Shear-induced platelet aggregation

Washed platelets were exposed to constant shear stresses of 5, 20 or 100 dyn/cm² for 60s at room temperature. The number of platelet singlets increased at the lowest shear stress, due to disaggregation of pre-formed platelet aggregates (Fig. 3.2). The degree of disaggregation was comparable for both P-VWF and ULVWF preparations. As the shear stress was increased to the arterial value of 20 dyn/cm², the platelet aggregation in P-VWF and ULVWF solutions increased moderately to ~15%. But as the shear stress was increased
Figure 3.1: Autoradiograph of P-VWF and ULVWF multimers displayed by gel electrophoresis on SDS-agarose gel. Lane 1: P-VWF 5 months after preparation; Lane 2: P-VWF within 3 months of preparation; Lane 3: Normal plasma; Lane 4: ULVWF. ULVWF contains ultra-large multimers, compared to normal plasma and P-VWF. There is a loss in large multimer composition of P-VWF after 5 months.
Table 3.1: Concentration of VWF in the P-VWF and ULVWF preparations

<table>
<thead>
<tr>
<th>VWF</th>
<th>VWF antigen (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-VWF (3 months)</td>
<td>835 ± 10</td>
</tr>
<tr>
<td>P-VWF (&gt;5 months)</td>
<td>420 ± 98</td>
</tr>
<tr>
<td>ULVWF</td>
<td>21 ± 2</td>
</tr>
</tbody>
</table>

Table 3.2: Estimation of total protein in the VWF preparations.

<table>
<thead>
<tr>
<th>VWF</th>
<th>Total protein (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-VWF</td>
<td>100</td>
</tr>
<tr>
<td>ULVWF</td>
<td>1000</td>
</tr>
</tbody>
</table>
Figure 3.2: Shear-induced platelet aggregation: Platelets were exposed to indicated shear stress for 60s in a viscometer and the percent aggregation was calculated by counting the decrease in platelet singlets. The values are the average of 9 experiments.
further to the stenotic range of 100 dyn/cm², the ULVWF preparation (70%) was significantly more potent in aggregating platelets than P-VWF preparation (35%).

3.3.3 *Ristocetin-induced platelet agglutination (RIPA)*

Next, we studied the differences between the normal plasma multimers in P-VWF preparation and unusually large multimers of VWF in ULVWF preparation by RIPA, which is a standard VWF functional assay. We added various concentrations of ristocetin ranging between 0–0.5 mg/ml to washed platelet suspension containing either P-VWF or ULVWF at 10% final VWF concentration and followed agglutination (light transmission) in an aggregometer for 20 min (Fig. 3.3). At concentrations below 0.2 mg/ml, ristocetin did not agglutinate platelets, but as the concentration was increased over 0.3 mg/ml, the ULVWF preparation was more effective in agglutinating platelets than the P-VWF preparation. This trend continued for ristocetin concentrations up to 1.5 mg/ml and was consistent for all donors. In the absence of ristocetin, we did not observe any spontaneous aggregation if: (1) either P-VWF or ULVWF solution was added to washed platelets with or without physiological amounts of fibrinogen; or (2) either P-VWF or ULVWF solution at 10% final concentration was added to platelet-rich plasma (PRP). We also did not observe microaggregates in (1) or (2) using a microscope at 60X magnification.

3.3.4 *Platelet deposition on VWF-coated surfaces*

Platelet deposition patterns on immobilized normal plasma multimers and unusually large multimers of VWF were compared by perfusing reconstituted blood over P-VWF or ULVWF solution coated surfaces at 800 s⁻¹. Only platelet singlets and very small aggregates
Figure 3.3: Ristocetin-induced platelet agglutination: Washed platelets were mixed with ULVWF or P-VWF in an aggregometer with or without the addition of ristocetin. There was no spontaneous aggregation in the absence of ristocetin. The curves are representative tracings of eight aggregation assays with similar results.
were seen on both P-VWF and ULVWF surfaces (Fig. 3.4 A–B). Fig. 3.4 C shows the ratio of the number of platelets deposited on a P-VWF coated surface to that on a ULVWF coated surface. When both P-VWF and ULVWF were coated on the surface at the same VWF concentration (10%), only half as many platelets deposited on the P-VWF-coated surface compared to ULVWF-coated surface. As the coating concentration of VWF in P-VWF solution was doubled to 20%, however, the number of platelets deposited on the surface also doubled and became nearly equal to the number of platelets deposited on the surface coated with ULVWF solution at a VWF coating concentration of 10% (Fig. 3.4 C).

3.3.5 VWF adsorption on glass coverslips

We used ELISA to quantify the amount of VWF adsorbed on glass coverslips at different concentrations of VWF in either P-VWF or ULVWF solution. The VWF solution concentration is expressed in terms of percent antigen level with 100% corresponding to 10 μg/ml VWF, the normal plasma level. As shown in Fig. 3.5, the adsorption curves for the two forms of VWF are qualitatively different. The adsorption curve for ULVWF saturates beyond 5–10% and is nearly linear below 5%. In contrast, the adsorption curve for P-VWF is linear over the entire 0–40% range used in this study. When surface adsorption is expressed in terms of absorbance of a tagged antibody, the amount of ULVWF adsorbed at saturation (10% VWF) corresponds to the amount of P-VWF adsorbed on a surface that is coated with P-VWF solution with a concentration of 20% VWF (dotted lines in Fig. 3.5).
Figure 3.4: Platelet deposition on VWF coated surfaces: reconstituted blood was perfused over P-VWF or ULVWF coated surfaces at 800 s\(^{-1}\) for 120s and platelet deposition was visualized using fluorescence microscopy. (A) Platelets deposited on surface coated with 10% P-VWF. (B) Platelets deposited on surface coated with 10% ULVWF. (C) Ratio of platelets deposited on P-VWF and ULVWF coated surfaces at the indicated times after the initiation of flow. The values represent an average of 5 experiments performed with blood from 4 donors.
Figure 3.5: Equilibrium adsorption of ULVWF (A) and P-VWF (B) on glass coverslips. The figure is representative of three experiments, each performed in triplicate. The dotted arrows represent the concentrations of ULVWF and P-VWF in solution that give the same surface adsorption as measured by OD at 490 nm.
3.4 Discussion

The interaction between platelet glycoprotein GP Ib-IX-V complex and VWF is the crucial initiating event in thrombosis and hemostasis. VWF immobilized on the subendothelium binds platelets spontaneously but normal VWF in plasma (P-VWF) requires the presence of modulators, i.e., botrocetin, ristocetin or high shear stress (Ward and Berndt, 2000). In contrast, the VWF fraction containing unusually large multimers (ULVWF) is believed to bind platelets spontaneously with higher affinity than the plasma VWF and has been implicated in the pathophysiology of the thrombotic disorder called thrombotic thrombocytopenic purpura (TTP) (Moake 1997).

TTP is characterized by microvascular occlusion with thrombi composed entirely of platelets and VWF. The shear stress in the microvasculature is high and the plasma of TTP patients contains ULVWF multimers (Moake et al., 1982). In accord with this in vivo condition, we found that at a high shear stress of 100 dyn/cm², similar to levels in stenotic vessels, the ULVWF causes the aggregation of about twice as many platelets as P-VWF. Our findings are in agreement with some of the earlier studies by Moake et al. (1986, 1988) who showed that at a shear stress of 60 dyn/cm², ULVWF (but not P-VWF) aggregated platelets at a low VWF antigen level (2.5 U/dL). We found that as shear stress was lowered to the values found in large arteries (5–20 dyn/cm²) both ULVWF and P-VWF equally caused moderate platelet aggregation. In the absence of any shear stress, neither ULVWF nor P-VWF mediated any aggregation (Fig. 3.2). Our data on P-VWF is consistent with published reports that P-VWF requires shear stress to mediate platelet aggregation (Moake et al., 1982; Ikeda et al., 1991).
The addition of a low concentration of ristocetin, an exogenous modulator of platelet aggregation, did not induce aggregation in platelet suspensions containing either ULVWF or P-VWF. If the concentration of ristocetin was increased above 0.3 mg/ml, however, ULVWF was more effective than P-VWF in aggregating platelets. This phenomenon was characteristic of all ristocetin concentrations tested, including the maximum (1.5 mg/ml). Ristocetin is a glycopeptide that flocculates VWF in solution and causes agglutination of platelets (Scott et al., 1991). Flocculation of VWF occurs when bifunctional ristocetin dimers cross-link multiple copies of VWF by binding to a common recognition site on the VWF-A1 domain. The binding of ristocetin to VWF-A1 domains alters the VWF conformation so that GP Ib receptors on platelet surfaces can bind to VWF (Berndt et al., 1992). The multimeric nature of VWF molecules is critical for cross-linking by ristocetin, and hence for platelet agglutination. Thus, the increased agglutination by ULVWF than P-VWF could be due to larger size or more favorable conformation of ULVWF multimers than P-VWF multimers or both.

In addition to studying the effect of unusually large multimers of VWF in solution, we also evaluated the effect of immobilizing these multimers in mediating platelet adhesion under high arterial shear stress conditions. When glass surfaces were coated with ULVWF and P-VWF preparations containing the same VWF antigen level (10%), only about half the number of platelets adhered on the surface coated with P-VWF compared to the surface coated with ULVWF (Fig. 3.4). But as the VWF antigen concentration in the P-VWF coating solution was increased to 20%, the number of platelets adherent on the surface increased to the same level as on the surface coated with ULVWF solution of 10% VWF concentration. That is, immobilization of ULVWF at 10% VWF concentration was as effective as
immobilization of P-VWF at 20% VWF concentration. This observation is consistent with recent results using optical tweezers demonstrating that immobilized ULVWF multimers had greater affinity for GP Ibbα transfected CHO cells than immobilized P-VWF multimers (Arya et al., 2002).

We investigated the possibility that the difference in the VWF concentration in ULVWF and P-VWF solution reflects a difference in surface density. When we measured the VWF site density using ELISA, we observed that an equivalent number of binding sites are available for the antibody on a surface that is coated with either a ULVWF solution containing 10% VWF or a P-VWF solution containing 20% VWF (shown by dotted lines in Fig. 3.5). An equivalent number of binding sites could mean: (a) more effective exposure of epitopes of unusually large VWF multimers compared to normal plasma VWF multimers, although the actual number of ULVWF molecules is less; or (b) similar exposure of the epitopes of unusually large VWF multimers and normal plasma VWF multimers from equal numbers of VWF molecules. We have used a polyclonal antibody and equivalence in the number of binding sites for the antibody does not necessarily translate to equivalence in the adsorbed number of molecules since a polyclonal antibody has multiple epitopes, which can bind to different sites on the same molecule. Hence, it is not possible to distinguish between the above two possibilities based on this data.

The adsorption pattern of VWF on glass surface for the P-VWF and ULVWF preparations are different. At low VWF antigen concentration (< 10%), the amount adsorbed on the surface is more using ULVWF than P-VWF solution. This implies that the unusually large multimers are more "adsorbent" than the smaller multimers of P-VWF either because of differences in molecular size or conformation or both. ULVWF adsorption shows a
Langmuir-type pattern, attaining saturation at a VWF solution concentration of \( \sim 5-10\% \) (0.5–1 \( \mu \)g/ml), the adsorption of P-VWF is linear at least until a VWF solution concentration of 40\% (4 \( \mu \)g/ml). This difference could be because: (a) the larger ULVWF molecules saturate the surface more easily than the smaller P-VWF molecules; or (b) there are different concentrations of other proteins in the two preparations that compete for adsorption sites on glass. The latter possibility is enhanced by total protein estimations for ULVWF and P-VWF solutions that demonstrate (Table 3.2) different values for the two preparations.

The total protein assay indicates the presence of proteins other than VWF, especially in the ULVWF preparation. The ULVWF containing endothelial cell supernatant is obtained by stimulating endothelial cells with histamine. It has recently been found to contain a considerable amount of cytokines. It is, however, unlikely that these proteins affected the results of functional assays based on the control studies demonstrating that endothelial cell supernatant devoid of VWF did not elicit any platelet functional response (Dong and co-workers, unpublished observations).

With regard to VWF storage and assay, the processes are not straightforward since VWF is the largest protein found in circulation. Plasma VWF (P-VWF) includes multimers ranging up to millions of daltons that are obtained by the cleavage of even bigger unusually large VWF (ULVWF) multimers. We found that the largest P-VWF multimers are proteolysed over a period of months (Fig. 3.1). ULVWF multimers are stable at -80 \( ^\circ \)C, but also break down into smaller fragments if subjected to temperature fluctuations. The VWF preparations are best preserved as aliquots stored at low temperature until further use (4 \( ^\circ \)C for P-VWF and -80 \( ^\circ \)C for ULVWF). The aliquots should be thawed at 37 \( ^\circ \)C and centrifuged to separate any aggregates before use. Re-freezeing the aliquots results in visible
flocculation and loss of large VWF multimers. As shown in Table 3.1, concurrent with the breakdown of VWF multimers, the VWF antigen level as measured by the Spectro VWF assay declines. This indicates that the anti-VWF antibody used in the spectro VWF assay may recognize or bind large VWF multimers more effectively, perhaps because the antibody was raised in rabbits immunized with the full range of human VWF multimers purified from plasma.

In summary, we have compared the unusually large and normal plasma forms of VWF in mediating platelet adhesion and aggregation. Our studies show that the ULVWF multimers are more effective than the P-VWF multimers in mediating high shear stress induced aggregation, ristocetin-modulated platelet agglutination and the adhesion of platelets onto immobilized VWF under high arterial shear stress conditions. Our findings might shed some light on the pathophysiology of thrombotic microangiopathies.
CHAPTER 4

Future Work

4.1 Kinetics of GP Iβα – VWF-A1 Interactions

The parallel plate flow chamber employing VWF-A1 coated glass coverslips and GP Iβα transfected CHO cells permits us the detailed study of the initial adhesion of platelets to subendothelial surfaces. The cellular on-rate and off-rates estimated in this work are useful parameters in comparing receptor-ligand interactions in physiologically relevant situations. The methodology developed in this work to measure the kinetic rate constants can be used to study other important mutations in either GP Iβα or VWF-A1. Drs Jose Lopez and Jing-fei Dong of Baylor College of Medicine have developed a library of GP Iβα mutations, some of which are shown to be critical for the normal binding of GP Ib to VWF. Applying the techniques for determining the on- and off-rate constants used in this thesis to different GP Iβα mutations will provide insights into the structure–function relationship for the GP Iβα molecule. For instance, the cytoplasmic truncation mutations are shown to roll faster than the wild-type on VWF matrix (Schade et al., 2003). Since the cytoplasmic truncations result in the loss of anchor for GP Iβα molecule, a reasonable hypothesis would be that the GP Ib–VWF bond in cytoplasmic truncates will be mechanically weaker than the wild-type and will have a lower value of reactive compliance.

The kinetic rate constants can also be used to differentiate between states of the adhesion molecules – between active and inactive states. Simon et al., (2000) showed recently that, undifferentiated monocytic cells rolled on glyccocalcin (extracellular GP Iβα) coated surface, but differentiated monocytic cells adhered firmly on the same surface. Both
of these interactions are shown to be mediated by GP Ibα–Mac-1 bond and it has been proposed that the difference arises because of the change in the activation state of Mac-1. It will be instructive to apply our procedure for determining the rate constants to this system and determine the change in the off-rate constant and mechanical compliance of the GP Ibα–Mac-1 bond, as Mac-1 changes its activation state.

In this work, we have expressed on-rate in terms of tethering frequency. While tethering frequency is a useful parameter, it is still subjective in the sense that it can be used to compare different types interactions from experiments done only under similar set of conditions. A more useful quantity would be the intrinsic on-rate itself, but obtaining intrinsic on-rate from flow chamber data requires the use of an elaborate transport model to decouple the kinetic and transport rate processes. In a recent study, Zhang and Neelamegham (2003) provide a framework for extracting the lumped on-rate from rolling and adhesion data. The videotapes from our experiments can be used in conjunction with the suitable form of their model to obtain different interaction parameters. However, this framework has at least three fitting parameters and as these authors rightly point out, it will be possible to obtain only a lumped, cellular association parameter because of the inherent difficulties in estimating the precise number of receptor–ligand bonds in the contact area.

In order to estimate the “molecular” off-rate, as against the “cellular” off-rate measured in this thesis, it is necessary to isolate single molecular interactions. We can use atomic force microscope (AFM) to study the interactions between GP Ibα and VWF-A1 (see Marshall et al., 2003 for work on selectins). The extracellular domain of GP Ibα called glycocalcin (Simon et al., 2000) can be adsorbed on to the tips of the cantilever and VWF-A1 can be adsorbed on the piezo-electric positioner of the AFM. The cantilever is brought in
close proximity to the positioner to facilitate the formation of GP Ibα–VWF-A1 bond. The lifetime and the force required to break the bond will be used to obtain estimates on dissociation rate constant and the bond strength. This is a useful method to calculate the molecular properties with greater precision.

4.2 Effect of Unusually Large Multimers on Platelet – VWF Interactions

The unusually large multimers of VWF (ULVWF) are implicated in the thrombotic disorder (TTP) due to their increased affinity for platelets, than the normal plasma multimeric forms of VWF (P-VWF). In this thesis, we have shown by direct experimental observations that ULVWF is more efficient than P-VWF in mediating platelet aggregation in the presence of exogenous modulators and in high shear stress conditions, but do not aggregate platelets spontaneously. However, this is not the whole story. To compare with the in vivo observations in TTP patients, we need to use concentrations of ULVWF closer to what is found in the plasma of these patients. We were limited by the low concentration of ULVWF obtainable using the current experimental methods. On the other hand, though the VWF concentration is very high in the P-VWF preparation, the preparation contains other molecules like P-selectin and possibly other plasma proteins. Hence, it would a useful endeavor to increase the concentration and purity of VWF in ULVWF and P-VWF preparations, respectively. Purification of VWF, especially that of ULVWF by standard protein purification techniques is tricky because of the size, fragility and hyper-adhesive nature of the molecule. We could use an affinity chromatography column employing a polyclonal VWF antibody, which will selectively bind VWF molecules while other proteins
get washed out. VWF can then be eluted out of the column by using solvent with different pH value at which VWF–antibody binding is low.

In some of the experiments that are not documented in this thesis, we observed that immobilized ULVWF and not P-VWF, binds to untransfected CHO called CHO-K1 (ATCC, Manassas, VA) cells under both static and dynamic conditions. The CHO-K1 cells are known to contain several integrins, including $\alpha_v\beta_1$, $\alpha_v\beta_3$ and $\alpha_5\beta_1$ all of which bind to RGD sequences (Gu et al., 1999). Hence, it is possible that that the unusually large multimers and not the normal plasma multimers bind to the integrin receptors. However, we are not able to ascertain whether the ULVWF–CHO-K1 interaction is a specific, receptor-mediated interaction or is a non-specific interaction between the phospholipids on the CHO cell surface and the some other proteins (like cytokines and immunoglobulins) present in the ULVWF preparation. Again, the answer lies in the obtaining more pure and concentrated VWF.
REFERENCE


de Chateau, M., S. Chen, A. Salas and T.A. Springer. 2001. Kinetic and mechanical basis of rolling through an integrin and novel Ca$^{2+}$-dependent rolling and Mg$^{2+}$-dependent firm adhesion modalities for the $\alpha$4$\beta$7-MAdCAM-1 interaction. Biochemistry. 40:13972–13979


Moake, J.L., N.A. Turner, N.A. Stathopoulos, L. Nolasco and J.D. Hellums. 1988. Shear-induced platelet aggregation can be mediated by vWF released from platelets, as well as by exogenous large or unusually large vWF multimers, requires adenosine diphosphate, and is resistant to aspirin. *Blood.* 71:1366–1374


