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Interactions of the Platelet GP Ib-IX-V Complex with Immobilized von Willebrand Factor under Flow Conditions

by

Alicia J. Schade

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

Doctor of Philosophy

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ABSTRACT

Interactions of the Platelet GP Ib-IX-V Complex with Immobilized von Willebrand Factor under Flow Conditions

by

Alicia J. Schade

Platelets play key roles in physiology, such as the arrest of bleeding following vascular injury, and in pathology, such as mural arterial thrombosis. The initial step in both processes is platelet adhesion to von Willebrand factor. The adhesion molecule on the platelet surface responsible for this interaction is the platelet glycoprotein (GP) Ib-IX-V complex. This complex consists of four subunits, and the GP Ibα subunit contains the vWF binding site. The exact region or regions of GP Ibα that are involved in the GP Ib-IX-V – vWF interaction are unknown.

The purpose of this work was to investigate the importance of several structural domains of GP Ibα in the GP Ib-IX-V – vWF interaction under high fluid shear stress. This was done by several methods which all used a parallel-plate flow chamber to create wall shear stress: an antibody blocking study of cell rolling, mutational analysis of four different domains of GP Ibα, and dog/human chimeras of GP Ibα.

First, we evaluated the blocking effects of GP Ibα and vWF antibodies on rolling of CHO cells expressing the GP Ib-IX complex on immobilized vWF and then compared this data with the antibody blocking studies of modulator- and shear-induced platelet
aggregation. Our results suggest that the mechanism of the GP Ib-IX-V – vWF interaction in rolling is different than the mechanisms involved in shear- and modulator-induced platelet aggregation.

Next, we used mutational analysis to investigate the importance of the leucine-rich repeat (LRR) region, the disulfide loop region, the anionic sulfated region, and the cytoplasmic region of GP Ibα in the GP Ib-IX-V – vWF interaction under high fluid shear stress. By comparing the interaction of the mutant cells with immobilized vWF to the interaction of the wild-type cells with the same surface, we found that all four of these regions of GP Ibα are important in the receptor-ligand interaction under flow.

Finally, using dog/human chimeras of GP Ibα, we found that the N-terminal 59 residues containing the N-terminal flanking disulfide loop and the first LRR of GP Ibα, may not be ligand binding sites for vWF but rather may be regulatory elements for the GP Ib-IX-V – vWF interaction under high fluid shear stress. Using re-humanized dog/human chimeras of GP Ibα, we found evidence to support the involvement of the second, third, and fourth leucine-rich repeats in the receptor-ligand interaction.
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CHAPTER 1

Introduction

Coronary artery disease is a major cause of mortality and morbidity in the United States, and one possible clinical manifestation of this disease is complete vascular occlusion of the coronary arteries. Vascular occlusion often is the result of an arterial thrombus directly blocking blood flow leading to myocardial infarction.\textsuperscript{1} Current therapeutics and preventative measures for arterial thrombus formation are only moderately effective.\textsuperscript{2} To develop novel therapeutics for this disease, the detailed molecular mechanisms of arterial thrombus formation need to be investigated, especially under physiological flow conditions.

Two different mechanisms of arterial thrombus formation under the dynamic forces generated by blood flow have been identified: mural arterial thrombosis and bulk-fluid shear stress induced platelet aggregation. The first step in both processes is the adhesion of platelets to von Willebrand factor (vWf). The platelet glycoprotein (GP) Ib-IX-V complex contains the vWf binding site. This introductory chapter provides background information on the fluid dynamics of blood flow and on the model systems used to generate fluid shear forces. Next, a section on the structure and function of the platelet GP Ib-IX-V complex will be presented, followed by a detailed section on the structure and function of one of the subunits of the complex. This chapter concludes with a presentation of the thesis objectives.
1.1 Fluid Dynamics of Blood

Blood flow is a dynamic process that imposes several mechanical forces on the vessel wall and on the components of blood.\(^3\) One of the forces exerted on the vessel wall is caused by blood pressure, and this force acts normal (perpendicular) to the vessel wall. The vessel wall is also exposed to tensile stresses acting circumferentially caused by deformations in the vessel wall. Finally, the vessel wall and the components of blood (red blood cells, platelets, etc.) are constantly experiencing forces acting parallel to the vessel wall created by the velocity gradient of blood flow in the radial direction (Figure 1.1). At the vessel wall, this tangential force, known as wall shear stress, is defined as the force on the vessel wall or blood component surface per unit area imposed by the flow of a viscous fluid.

The properties of blood flow and the geometry of the vasculature are quite complicated. A simple model of blood flow through a blood vessel can be obtained by assuming rigid vessel walls of constant vessel diameter. If we model blood as an incompressible Newtonian fluid, the velocity profile of steady-state laminar blood flow in a long tubular vessel is described by\(^4\):

\[
v_z = 2\langle v_z \rangle \left[ 1 - \left( \frac{r}{R} \right)^2 \right]
\]

where \(v_z\) is the axial velocity, \(r\) is the radial position, and \(R\) is the radius of the tube. \(\langle v_z \rangle\) is the mass average velocity of the fluid, which is a function of the pressure gradient, described by:

\[
\langle v_z \rangle = \frac{-R^2}{8\mu} \left( \frac{\Delta P}{L} \right)
\]
Figure 1.1. Forces acting on a blood vessel. The compressive stress acts perpendicular to the vessel wall and is caused by blood pressure. Deformations in the vessel wall cause the tensile/circumferential stress. The tangential shear stress acts parallel to the vessel wall and is caused by the viscous flow of blood. Modified from Papadaki and Eskin.\textsuperscript{5}
where $\mu$ represents the fluid viscosity and $\Delta P/L$ is the pressure gradient (the change in pressure per length of the vessel, $L$). Equation 1 yields a parabolic velocity profile, where the axial velocity is maximal at the center of the vessel and zero at the vessel wall (Figure 1.2).

The fluid shear rate is the velocity gradient in the radial direction and describes the relative motion between adjacent fluid layers (laminae) that are moving at different velocities.$^4$ Shear rate ($\gamma$) is expressed in units of inverse seconds, s$^{-1}$. Taking the derivative of Equation 1 with respect to radial position yields the shear rate for a tubular vessel given by:

$$\gamma = -\frac{dv_z}{dr} = 4\langle v_z \rangle \frac{r}{R^2} \tag{3}$$

This equation shows a linear relationship between shear rate and radial position (Figure 1.2), where the shear rate is zero at the vessel center and maximal at the vessel wall. The shear rate at a stationary vessel wall can be calculated by setting $r = R$ as follows:

$$\gamma_w = \frac{4\langle v_z \rangle}{R} \tag{4}$$

where $\gamma_w$ represents the wall shear rate.

Another term often used to describe the forces created by flowing fluid is shear stress, which is the amount of force exerted by the flowing fluid per unit area.$^4$ Multiplying the shear rate by the fluid viscosity gives the shear stress ($\tau$), expressed in units of dyn/cm$^2$, as follows:

$$\tau = \mu \gamma = \mu 4\langle v_z \rangle \frac{r}{R^2} \tag{5}$$
Figure 1.2. Velocity and shear rate profiles in a tubular vessel. Assuming rigid vessel walls and Newtonian, laminar blood flow, the velocity profile $v_z(r)$ in a tubular vessel is parabolic with maximum velocity ($v_{\text{max}}$) at the center of the vessel and the velocity gradient equal to zero at the vessel wall. The shear rate profile is linear in this system, where the shear rate is zero at the center of the vessel and maximum at the vessel wall. Modified from Ross, Alevriadou, and McIntire.\textsuperscript{3}
In models of mural arterial thrombosis, it is important to know the shear stress acting at the vessel wall and this can be obtained by setting \( r = R \) in Equation 5 as follows:

\[
\tau_w = \mu \lambda_w = \mu \frac{4\langle v_z \rangle}{R} \tag{6}
\]

where \( \tau_w \) represents the wall shear stress. It is useful to express wall shear stress as a function of flow rate, \( Q \), where flow rate is defined as:

\[
Q = \pi R^2 \langle v_z \rangle \quad \text{and} \quad \langle v_z \rangle = \frac{Q}{\pi R^2} \tag{7a,b}
\]

Substituting the value of \( \langle v_z \rangle \) in Equation 7b into Equation 6 yields the wall shear stress for a tubular vessel in terms of flow rate as described by:

\[
\tau_w = \frac{\mu 4Q}{\pi R^3} \tag{8}
\]

Blood flow rates and vessel diameters vary greatly throughout the vasculature, and therefore the values of wall shear rate and wall shear stress show large variations. Table 1.1 lists some typical wall shear values found in the human vascular system assuming the viscosity of whole blood is approximately 0.038 poise.

<table>
<thead>
<tr>
<th>Vessel</th>
<th>Wall Shear Rate (s(^{-1}))</th>
<th>Wall Shear Stress (dyn/cm(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veins</td>
<td>20-200</td>
<td>0.76-7.6</td>
</tr>
<tr>
<td>Large arteries</td>
<td>300-800</td>
<td>11.4-30.4</td>
</tr>
<tr>
<td>Arterioles</td>
<td>500-1600</td>
<td>19.0-60.8</td>
</tr>
<tr>
<td>Stenotic Vessels</td>
<td>800-10,000</td>
<td>30.4-380</td>
</tr>
</tbody>
</table>
In a blood vessel, the pressure gradient is the driving force behind blood flow. The pressure gradient driving the flow of a fluid through a tubular vessel in terms of volumetric flow rate is described by:

$$\frac{\Delta P}{\Delta z} = \frac{8\mu Q}{\pi R^4}$$

(9)

where $\Delta P/\Delta z$ represents the pressure gradient in the vessel in the axial direction. This equation is known as the Hagen-Poiseuille equation and is valid for steady-state, laminar flow of incompressible Newtonian fluids.\(^4\)

The equations stated above assume Poiseuille flow which implies the flow is laminar, where the fluid moves in smooth layers.\(^4\)\(^6\) Laminar flow exists for blood when the Reynolds number is less than approximately 2300\(^3\) (the laminar-turbulent transition occurs at about 2300\(^4\)). The Reynolds number is described as:

$$N_{Re} = \frac{\text{inertial force}}{\text{viscous force}} = \frac{\rho \langle v_z \rangle^2}{\mu \left( \frac{\langle v_z \rangle}{2R} \right)} = \frac{2R \langle v_z \rangle \rho}{\mu}$$

(10)

where $\rho$ represents the fluid density. The Reynolds number is a dimensionless number that represents the relative contribution of inertial forces and viscous forces to fluid flow in a tubular vessel. If there is a greater inertial force present the Reynolds number will be large, and if there is a greater viscous force present, the Reynolds number will be small. Generally in the human vasculature, turbulent flow is uncommon with local Reynolds numbers being below 2300 almost everywhere.\(^3\)

The equations stated above are useful for gaining a general understanding of the dynamics of blood flow, but do not exactly represent the forces in circulation for several reasons.\(^3\)\(^6\)\(^7\) To begin, blood circulation is pulsatile and steady-state conditions do not
truly apply. Second, the vascular system does not consist of straight vessels, but contains branches and curvatures that can induce regions of secondary flow and recirculation. Third, regions of turbulent flow can exist in stenotic vessels and in vessels with severe atherosclerotic damage. Fourth, in capillaries and other very small diameter vessels, blood flow is not really Newtonian because the vessel diameters are about the size of some of the cells passing through them so that blood flow in these regions really exhibits multiphasic behavior. Finally, blood exhibits shear thinning (Figure 1.3) meaning the viscosity of blood is not constant at all shear rates. At low shear rates (below approximately 100 s\(^{-1}\)), blood viscosity increases. At higher shear rates, blood does exhibit Newtonian viscosity behavior. All these factors affect blood flow dynamics and have their greatest impact on the smaller vessels of the vascular system.

1.2 Experimental Models for Studying Blood Shear Forces

To better understand the mechanisms of arterial thrombosis, several devices have been developed to create shear forces \textit{in vitro}.\(^3\) These devices have been used to study and model platelet adhesion, activation, and aggregation. Two classes of systems have been developed: 1) devices for study of mural thrombosis that have a solid-liquid interface that is well defined and where the local fluid effects are well controlled such as parallel-plate, tubular, and annular flow chambers, and 2) devices that apply a uniform shear force to all of the fluid phase, such as viscometers, for study of bulk fluid shear-induced aggregation.

A parallel plate flow chamber consists of three parts held together by the application of a vacuum: a polycarbonate slab, a silicon gasket, and a glass coverslip
Figure 1.3. Non-Newtonian nature of blood below shear rates of 100 s$^{-1}$. In this Casson plot or double square root plot, the square root of shear rate ($\gamma$) is plotted on the abscissa and the square root of shear stress ($\tau$) is plotted on the ordinate. At shear rates above 100 s$^{-1}$, blood acts as a Newtonian fluid where the viscosity of blood is constant (0.0324 poise). At shear rates below 100 s$^{-1}$, blood viscosity varies with shear rate and therefore exhibits Non-Newtonian behavior. Modified from Merrill.$^7$
(Figure 1.4). The glass coverslip can be coated with a protein matrix or a cell monolayer. Fluid is often perfused through the chamber using a syringe pump. The flow rate of the perfused fluid, $Q$, and the thickness of the silicon gasket, which determines the height of the flow channel, are two of the parameters that affect wall shear stress. Wall shear stress in a parallel plate flow chamber can be described by a variant of Equation 8 that takes into account the differences in geometry as follows:

$$\tau_w = \mu \gamma_w = \frac{6Q\mu}{b^2 w}$$  \hspace{1cm} (11)

where $b$ is half the height of the flow channel and $w$ is the width of the flow channel.

Wall shear rate can be determined by dividing Equation 11 by the fluid viscosity.

Parallel plate flow chambers are useful for investigating the interaction between cells and a surface, such as a protein matrix or a cell monolayer, under shear stress generated by flowing fluid. Specifically relating to hemostasis and thrombosis, the parallel plate flow chamber system has been used to study the interaction of platelets with components of the subendothelial matrix, such as several types of collagen and von Willebrand factor (vWF), that are exposed by endothelial cell damage.\(^8\) To help define the molecular mechanisms involved in the interaction of platelets with vWF, for example, the parallel plate flow chamber has also been used to study the interactions between heterogeneous cells expressing platelet glycoproteins and immobilized vWF under arterial shear stresses.\(^9,10\)

The second type of device used to study the forces generated by shear stress is the cone-and-plate viscometer. A cone-and-plate viscometer consists of plate that is stationary and a cone that rotates (Figure 1.5) that are separated by variable distances, depending on the radius and cone angle. The shear rate generated by this viscometer is
Figure 1.4. Parallel-plate flow chamber. The parallel-plate flow chamber consists of three parts held together by a vacuum: A) polycarbonate slab, B) silicon gasket, and C) glass coverslip. The geometry of the chamber creates a well-defined wall shear stress at the surface of the coverslip (Equation 11). In these studies, the glass coverslip was coated with solutions of von Willebrand factor.
Figure 1.5. Cone-and-plate viscometer. This viscometer consists of a stationary plate and a rotating cone. The geometry of the viscometer allows for a constant, uniform shear rate to be applied to the entire fluid phase (Equation 12). The cone angle is represented by $\alpha$ and the rotational velocity by $\omega$. Modified from Kroll et al.\textsuperscript{8}
proportional to the rotational speed and is a function of the cone angle (usually small, about 0.3 to 1.0 degree) as described by:

$$\tau = \mu \gamma = \frac{\mu \omega}{\alpha}$$

(12)

where $\omega$ is the angular (rotational) velocity and $\alpha$ is the angle between the cone and the plate. The geometry of viscometers allows for a constant and uniform shear force to be applied to the entire fluid phase. The plate of a cone-and-plate viscometer can be coated with a silicon film rendering it nonthrombogenic so that the fluid-solid interface interactions are minimized ensuring that only the effects of fluid shear force on the fluid are evaluated. These properties make the cone-and-plate viscometer useful for studying the effects of shear force on platelet function, such as the mechanisms of shear-induced vWF binding to platelets, shear-induced platelet activation, and shear-induced platelet aggregation.

1.3 Molecular Mechanisms of Mural Thrombus Formation

Platelets play key roles in physiologic hemostasis (hemostatic plug formation in response to blood loss) and pathologic thrombosis (mural arterial thrombus formation in response to atherosclerotic plaque rupture or angioplasty). Pathologic mural arterial thrombosis occurs at higher shear levels, such as those found in stenotic or partially occluded vessels, compared to the shear levels at which the physiologic arrest of blood flow occurs. Mural arterial thrombosis consists of three basic steps: platelet adhesion, platelet activation, and platelet aggregation.
The first step in arterial mural thrombus formation is adhesion of platelets to the exposed subendothelial matrix. This initial step is mediated by the binding of the platelet glycoprotein (GP) Ib-IX-V complex to vWF in the subendothelial matrix (usually insolubilized on collagen) under high shear stress. Binding of vWF to the GP Ib-IX-V complex leads to platelet activation characterized by increases in intracellular concentrations of Ca^{2+} and phosphorylation of several signal proteins. The exact signal transduction pathways that lead to activation are unknown but studies have shown the involvement of phophatidylinositol 4,5 bisphosphate breakdown, phosphatidic acid generation, activation of protein kinase C, thromboxane A_2 synthesis, and cytoskeletal association of activated P13 kinase and pp60scr. Studies by Andrews et al. have suggested that the platelet activation occurs by receptor crosslinking, which could be achieved by multimeric vWF. Platelet activation results in platelet secretion of many platelet agonists, shape changes, and activation of the platelet integrin GP IIb/IIIa (α_{IIb}/β_3) leading to platelet aggregation. Platelet aggregation is mediated by the binding of activated GP IIb/IIIa to fibrinogen at low shear levels and vWF at high shear levels. These proteins serve as bridging molecules linking platelets together in the formation of a growing thrombus.

Recent studies have suggested that platelets behave in a manner analogous to blood leukocytes in their translocation from the bloodstream to the vessel wall. Savage et al. showed that platelets translocate on immobilized vWF under high shear, indicating the GP Ib-IX-V – vWF interaction has fast on- and off-rates resembling selectin-ligand interactions. Recently, Fredrickson et al. also quantitatively verified this finding of fast on- and off-rates for the receptor-ligand interaction by demonstrating that heterogeneous
cells expressing the GP Ib-IX-V complex rolled on immobilized vWf.\textsuperscript{9} Savage et al. also showed that stable platelet adhesion to vWf requires GP IIb/IIIa (an integrin) binding to vWf.\textsuperscript{20} This scenario mimics the path taken by blood leukocytes, which initially roll on activated endothelium mediated by selectins, on the endothelial surface followed by firm adhesion mediated by β\textsubscript{2} integrins also expressed on the endothelial surface.\textsuperscript{21} Figure 1.6 is a schematic that summarizes the events that lead to mural thrombus formation and shear-induced platelet aggregation.

1.4 **Structure and Function of Platelet GP Ib-IX-V Complex**

The platelet GP Ib-IX-V complex functions as the platelet receptor for vWf and the platelet high affinity thrombin receptor. The complex is composed of four subunits: GP Ib\textalpha, GP Ibβ, GP IX, and GP V (Figure 1.7).\textsuperscript{11} Each subunit is encoded by its own gene, but the genes have similar structure.\textsuperscript{22-25} Together known as GP Ib, the GP Ib\textalpha and GP Ibβ subunits are covalently linked by a disulfide bond.\textsuperscript{26} GP IX is noncovalently linked to GP Ibβ,\textsuperscript{27,28} and GP V is noncovalently to GP Ib\textalpha.\textsuperscript{29,30} All four glycoproteins are structurally similar, being Type I transmembrane proteins and being members of the leucine-rich repeat family.\textsuperscript{26}

The structural arrangement of the glycoproteins that compose a GP Ib-IX-V complex is unknown. A set of clues came from the determination of the number of copies of each subunit on the platelet surface. Using a monoclonal antibody study, Berndt et al. estimated that there are about 25,000 copies of GP Ib\textalpha and GP IX on the surface of the platelet.\textsuperscript{31} Modderman et al. estimated that there are about 11,000 molecules of GP V on a platelet’s surface.\textsuperscript{29} Based on this knowledge, it is proposed that two GP Ib-IXs
Figure 1.6. Schematic of mural arterial thrombosis. Previous studies have suggested that platelets initially roll on immobilized vWf, mediated by the platelet GP Ib-IX-V complex, when translocating from the blood stream to the site of vessel injury. Following the binding of platelets to vWf, the platelets become activated which leads to the formation of stable bonds with vWf mediated by platelet GP IIb/IIIa. This glycoprotein also mediates platelet aggregation.
Figure 1.7. Schematic of the platelet GP Ib-IX-V complex. The platelet GP Ib-IX-V complex consists of four subunits: GP Ibα, GP Ibβ, GP IX, and GP V. The GP Ib subunits are covalently linked through a disulfide bond. Proteins that associate with the cytoplasmic domains of the complex are also shown. MGP – macroglycopeptide. ABP – antibody binding protein. Modified from López and Dong.11
associate with one GP V, with the GP V located in the middle. This study lead López to propose a \((\alpha_2, \beta_2, \gamma_2 \delta)_n\) stoichiometry, where \(\alpha\) is GP Ib\(\alpha\), \(\beta\) is GP Ib\(\beta\), \(\gamma\) is GP IX, and \(\delta\) is GP V. An investigation of the platelet high affinity thrombin receptor (GP Ib-IX-V) provided clues as to how many \(\alpha_2, \beta_2, \gamma_2 \delta\) might make up a complex. The high affinity thrombin receptor has been estimated to have a molecular weight of about 900 kDa which suggests that a thrombin receptor consists of more than one \(\alpha_2, \beta_2, \gamma_2 \delta\), with the most likely conformation being \(n = 2\). Figure 1.8 shows a schematic representation of the proposed arrangement of the GP Ib-IX-V complex proposed by López et al. (in press). This arrangement predicts the weight of the complex close to the molecular weight of a platelet high affinity thrombin receptor. The sum of the molecular weights of GP Ib\(\alpha\) (between 135-150 kDa) times four, GP Ib\(\beta\) (about 25 kDa) times four, GP IX (about 19 kDa) times four, and GP V (about 83 kDa) times two ranges from 882 to 942 kDa.

Several studies have shown that the GP Ib-IX-V complex associates with the platelet cytoskeleton and signal transduction proteins. Initial detergent studies by Solum et al. using Triton-X showed that the complex associates with the Triton-insoluble platelet fraction which contains the cytoskeleton. Studies by Fox and Okita et al. revealed that this cytoskeletal association is mediated by actin-binding protein 280 (APB-280). Next, Andrews et al. found that ABP-280 binds to the cytoplasmic region of GP Ib\(\alpha\). Later, they mapped the region of APB binding on GP Ib\(\alpha\) to residues Thr536-Phe568 (the middle of cytoplasmic region of GP Ib\(\alpha\)). Experiments by Du et al. found that the GP Ib-IX-V complex also associates with the \(\zeta\) isoform of 14-3-3 protein through the cytoplasmic domain of GP Ib\(\alpha\) (near the C-terminus). The proposed function of
Figure 1.8. Schematic of the proposed subunit arrangement in the GP Ib-IX-V complex. See text for details. Modified from López, Smith, and Dong.43
this 14-3-3 protein might be to transduce the signal initiated by the binding of the complex with its ligand. The phosphorylation of cytoplasmic domain of GP Ibβ by protein kinase A may also serve to transduce the vWF binding signal.42

vWF is one of the ligands of the GP Ib-IX-V complex, and it is a dimer of a monomer that contains eleven domains.44 The dimer multimerizes into very large multimeric forms which are functional active. It is produced in platelets and endothelial cells and stored in the α-granules of platelets and in the Weibel-Palade bodies of endothelial cells. Soluble vWF circulates in blood plasma, and immobilized vWF can be found in the subendothelial matrix. The A1 domain of vWF has been identified as containing the binding site for the complex (Figure 1.9).45-47 Platelets do not spontaneously bind soluble vWF under normal conditions.44 The receptor-ligand interaction will occur if vWF is immobilized,48 if there are high shear forces present,49 or if the modulators ristocetin or botrocetin are present.50-52 Both modulators induce vWF dependent platelet aggregation, where ristocetin is a peptide antibiotic and botrocetin is a protein isolated from snake venom.

The ligand binding sites of vWF and thrombin in the GP Ib-IX-V complex have been mapped to the GP Ibα subunit. The exact binding region or regions of vWF on GP Ibα is currently unknown. Studies have identified the vWF binding site in the N-terminal 45 kDa region of GP Ibα.53-58 Several studies have shown that several domains are important: the leucine-rich repeat region, the disulfide loop region, and the sulfation region (Figure 1.10).11 More details on the regions of GP Ibα involved in vWF binding can be found in subsequent sections of this introduction.
Domains of vWf

Figure 1.9. Schematic of the A1 domain of vWf. The A1 domain contains a disulfide loop linking Cys509-Cys 695. The disulfide loop has been purposed to be the location of the binding sites for GP Ib-IX-V complex, heparin, and sulfatide. Modified from Andrews, López, and Berndt.44
Figure 1.10. Schematic of three regions within the N-terminal 45 kDa region of GP Ibα believed to be important in the binding of vWF. Also indicated are the locations of three mutations found in Bernard Soulier Syndrome. Modified from López and Dong.⁹
1.5 Structure and Function of the GP Ibα Subunit

GP Ibα is the largest glycoprotein in the GP Ib-IX-V complex\textsuperscript{26} and it can be divided into six structural domains (Figure 1.7). They are the leucine-rich repeat region, the disulfide loop region, the anionic sulfated region, the macroglycopeptide region, the transmembrane region, and the cytoplasmic region, from the N-terminus to the C-terminus, respectively.\textsuperscript{26}

1.5.1 Leucine-rich Repeat Region

The N-terminus of GP Ibα contains a stretch of seven leucine-rich repeats, which makes this subunit a member of the leucine-rich motif family of proteins.\textsuperscript{23} A leucine-rich repeat (LRR) consists of 22-26 amino acids rich in leucine and contains a conserved asparagine in the sixth position of the repeat.\textsuperscript{26} The secondary and tertiary structure of this region in GP Ibα is not known, except for the disulfide binding pattern,\textsuperscript{59} and therefore its structure is predicted based on the structure of another member of the leucine-rich motif family, the porcine ribonuclease inhibitor.\textsuperscript{60} This protein consists of 15 leucine-rich repeats with each repeat containing a β-α structural unit. Together the repeats form a non-globular, horseshoe shaped structure with the α-helixes making up the outer edge of the horseshoe and the β-strands forming a β-sheet making up the inside edge. From this information, it is predicted that the seven leucine-rich repeats of GP Ibα form a semi-horseshoe shaped structure (Figure 1.10).

The proteins of the leucine-rich family perform many different cellular functions and are located in many different compartments within a cell. One common property of proteins containing leucine-rich repeats is that they are involved in protein-protein
interactions, including ligand-receptor interactions. Leucine-rich repeat domains may be directly involved in ligand binding as suggested by Thomas et al., where they found that LRRs 1-6 of the lutropin/choriogonadotropin receptor are required for the receptor to bind its ligand.

The leucine-rich repeat region is believed to be involved in the interaction of GP Ibα with vWF. Main support for this hypothesis came from the investigation of platelets from patients with Bernard Soulier Syndrome (BSS). Three mutations have been identified in the leucine-rich repeat region of GP Ibα which render the GP Ib-IX-V complex non-functional. The mutations are Leu57 to Phe, Ala156 to Val, and deletion of Leu179 (Figure 1.10). Patients with the Ala156 to Val mutation have BSS, Bolzano variant and De Marco et al. found that platelets with this mutation do not agglutinate to ristocetin, showing that this mutation eliminates the GP Ib-IX-V complex interactions with vWF. This evidence is in strong support of the fact that the leucine-rich repeat region of GP Ibα is important in the interaction of the GP Ib-IX-V complex with vWF either directly (binding vWF) or indirectly (structurally assisting the vWF binding of another region of GP Ibα).

1.5.2 Disulfide Loop Region

Between the leucine-rich repeat region and the anionic sulfated region of GP Ibα there are two loops linked by disulfide bonds: Cys211-Cys248 and Cys248-Cys264 (Figure 1.11). The first disulfide loop is believed to be involved in the interaction of GP Ibα with vWF. Analysis of the functional properties of platelets from patients with
Figure 1.11. Schematic of the disulfide loop region and the anionic sulfated region of GP Ibα. The locations of the two mutations identified in Platelet-type von Willebrand Disease are also indicated. Modified from López.26
Platelet-type von Willebrand Disease (PtvWD) provided some evidence in support of this hypothesis. PtvWD is a bleeding disorder that mimics Type 2B von Willebrand Disease in having a selective loss of the high molecular-weight multimers of vWF in the patient’s plasma. Two mutations have been identified in PtvWD that occur in the GP Ibα subunit: Gly233 to Val and Met239 to Val. Both single amino acid substitutions are to a valine, which is a more hydrophobic residue. An increased avidity between the platelets and vWF in this disease is shown by the fact that these platelets can bind vWF in the presence of lower concentrations of modulator compared to wild-type receptors, and these platelets can also spontaneously bind vWF without the presence of modulators. The fact that PtvWD is a bleeding disorder although the diseased platelets bind vWF with increased avidity appears paradoxical. The increased avidity of the platelet-vWF interaction causes an abnormal depletion of the high molecular-weight multimers of vWF. Therefore, pathologic bleeding can occur following vascular injury due to the decreased amounts of vWF in the patient’s plasma. PtvWD provides evidence that the disulfide loop region of GP Ibα is important in the binding of vWF with the GP Ib-IX-V complex. Whether this region directly binds this vWF or contributes in an indirect way as to modulate the strength of the GP Ibα-vWF interaction remains unknown.

1.5.3 Anionic Sulfated Region

The anionic sulfated region spans from residues Asp269 to Asp287 of GP Ibα and is located between the disulfide loop region and macroglycopeptide region (Figure 1.11). It is highly anionically charged, having ten acidic residues (either Asp or Glu) spanning this 19 amino acid region. This region has three tyrosines that are fully sulfated:
Tyr276, Tyr278, and Tyr279,\textsuperscript{69,70} creating three more anionic charges in this domain of GP Ibα. The importance of post-translational tyrosine sulfation of the anionic sulfated region of GP Ibα in ristocetin- and botrocetin-induced vWF binding was shown by three independent groups. First, Dong et al. showed that heterogeneous cells expressing GP Ib-IX cultured in sulfate-depleted media containing sodium chlorate bound decreased amounts of vWF in the presence of ristocetin and failed to aggregate in a vWF dependent manner at high shaking frequencies.\textsuperscript{69,71} Then Marchese et al. demonstrated that recombinant GP Ibα fragments (residues 1-302) synthesized in sulfate-free conditions had reduced vWF binding in the presence of both ristocetin and botrocetin.\textsuperscript{72} Later, Ward et al. found that in order for proteolytic fragments of GP Ibα to optimally block botrocetin-induced vWF binding to platelets, the fragments needed to contain residues Try276-Glu282 of GP Ibα which included the three sulfated tyrosines.\textsuperscript{70} Recently, the importance of post-translational tyrosine sulfation in dynamic conditions was shown by Fredrickson et al. by using heterogeneous cells expressing GP Ib-IX cultured in sulfate-depleted media containing sodium chlorate, where fewer of these sulfate depleted cells rolled on immobilized vWF compared to controls.\textsuperscript{9}

1.5.4 Macroglycopeptide Region

This region of GP Ibα is heavily glycosylated with mostly O-linked carbohydrates and with a few N-linked carbohydrates.\textsuperscript{26} The sugar content of this region has made crystallization of GP Ibα very difficult, and at this time X-ray crystallography of GP Ibα has not been successful. The sugar modifications make this domain very rigid, which may prevent secondary structure formation, and therefore it is hypothesized that the
structure of this region is linear. The long linear structure of the macroglycopeptide region is believed to serve as a ladder positioning the rest of the N-terminus of GP Ibα significantly out into the external environment of the platelet creating better availability for ligands to interact with the GP Ib-IX-V complex.

1.5.5 Transmembrane and Cytoplasmic Regions

The transmembrane region of GP Ibα contains approximately 30 residues that span the lipid bilayer of the platelet plasma membrane once. The C-terminus of GP Ibα extends into the cytoplasm of the platelet and consists of approximately 100 residues. The GP Ib-IX-V complex is associated with the platelet cytoskeleton through actin-binding protein 280 (ABP-280). The cytoplasmic region of GP Ibα binds to ABP-280 through residues Thr536-Phe586. ABP-280 is a dimer with each monomer having a GP Ibα binding site and recently this binding site was identified (residues 1850-2136). The GP Ib-IX-V complex also associates with proteins that are involved in signal transduction following vWF binding to GP Ibα. Du et al. identified the association of the ζ isoform of 14-3-3 protein with the C-terminal of GP Ibα. This protein may interact with SH2 domains of signal proteins.

Studies have shown that the association of the GP Ib-IX-V complex with the cytoskeleton is important in receptor function and signal transduction. Using CHO cells expressing GP Ibα with truncations of the cytoplasmic region that eliminated GP Ib-IX association with the cytoskeleton, Cunningham et al. found that truncating the cytoplasmic region of GP Ibα resulted in a different CHO cell spreading morphology compared to CHO cells expressing wild-type GP Ibα. Additionally, they found that
truncating the cytoplasmic region did not affect botrocetin-induced vWF binding. In contrast, Dong et al. found decreased vWF binding in the presence of ristocetin of CHO cells expressing truncated GP Ibα in comparison to CHO cells expressing wild-type GP Ibα. Using the technique of fluorescence recovery after photo-bleaching, Dong et al. also found that truncated cells had increased mobility of the GP Ib-IX-V complex on the plane of the plasma membrane compared to wild-type cells. The mobility of the complex was proportional to the length of cytoplasmic truncation. Recent studies using CHO cells expressing GP Ibα with deletion of the region that binds ABP-280 showed that these cells rolled faster on immobilized vWF as compared to CHO cells expressing wild-type GP Ibα. Overall, these studies show that the cytoplasmic region of GP Ibα is important in binding of vWF and in signal transduction leading to morphology changes following vWF binding.

1.6 Summary and Thesis Objectives

Hemostasis and thrombosis are both dynamic processes that occur in the presence of forces generated by blood flow. For example, wall shear stress is important in the formation of mural arterial thrombi and bulk shear forces are important in shear-induced platelet aggregation. For this reason, blood flow dynamics need to be taken into account during the development of novel therapeutic agents for the prevention of mural arterial thrombosis. Additionally, the details of the molecular mechanisms of thrombus formation need to be investigated to aid in development.

In thrombosis, platelets are involved in three sequential steps: adhesion to vWF, activation, and aggregation. Platelet adhesion to vWF is mediated by the platelet receptor,
GP Ib-IX-V complex. The GP Ibα subunit of the complex contains the vWF binding site in its 45 kDa N-terminal domain. This domain of GP Ibα contains approximately 300 residues and consists of three structural regions: the leucine-rich repeat region, the disulfide loop region, and the anionic sulfated region. Previous studies have suggested that each of these regions might be important in the receptor-ligand interaction.

The main objective of this thesis was to investigate the involvement of several domains of GP Ibα in the interaction between the GP Ib-IX-V complex and vWF under conditions of high fluid shear stress. The parallel-plate flow chamber was used to monitor the receptor-ligand interaction under wall shear stress.

First, as described in Chapter 3, we evaluated the blocking effects of GP Ibα and vWF antibodies on the rolling of CHO cells expressing the GP Ib-IX complex on immobilized vWF. The collected data was then compared with the blocking effects of the same antibodies on modulator- and shear-induced platelet aggregation to determine if different mechanisms of the GP Ib-IX-V – vWF interaction were involved.

Second, as presented in Chapter 4, the importance of the leucine-rich repeat region of GP Ibα in the GP Ib-IX-V – vWF interaction under high fluid shear stress was evaluated using mutational analysis. First, we evaluated the importance of the conserved sixth asparagine residue of the leucine-rich repeats in receptor-ligand binding. Next, we investigated the effect of deleting leucine-rich repeats 1-6 of GP Ibα on the interaction of the GP Ib-IX-V complex with vWF. Finally, we evaluated if the A156V mutation in GP Ibα in BSS, Bolzano variant is responsible for the defective vWF binding of the GP Ib-IX-V complex.
Third, as described in Chapter 5, the importance of the disulfide loop region of GP Ibα in the receptor - ligand interaction under high fluid shear stress was investigated. Mutational analysis was used to evaluate if this region (specifically the residues spanning from Asn226 to Ala244) of GP Ibα regulates the strength of the GP Ib-IX-V – vWF interaction as suggested by analysis of platelets from patients with Platelet-type von Willebrand Disease.

Fourth, as presented in Chapter 6, the importance of the charge distribution of the anionic sulfated region of GP Ibα in the GP Ib-IX-V – vWF interaction under high fluid shear stress was evaluated using mutational analysis. Specifically, we investigated the importance of the anionic charges at residues Y276, Y278, and Y279 of GP Ibα, created by post-translational tyrosine sulfation, in the ligand-receptor interaction under dynamic conditions.

Fifth, as described in Chapter 7, the regions of GP Ibα involved in vWF binding were mapped using dog/human chimeras of GP Ibα. Evaluations of the importance of the N-terminal 35 residues, the leucine-rich repeats 1-7, the disulfide loop region, and the anionic sulfated region of GP Ibα were performed.

Sixth, as presented in Chapter 8, the importance of the cytoplasmic domain of GP Ibα in the GP Ib-IX-V – vWF interaction under high fluid shear stress was evaluated using mutational analysis. This study was based on previous studies that have suggested that the association of the complex with the platelet cytoskeleton is important in the receptor-ligand interaction.
CHAPTER 2

Materials and Methods

2.1 Cell Lines.

The cell lines used in this study were generously provided by José López, M.D., Baylor College of Medicine, Houston, TX. The cell lines were created by transfecting Chinese hamster ovary cells (CHO) cells with wild-type or mutant DNA for GP Ibα, wild-type DNA for GP Ibβ, and wild-type DNA for GP IX as previously described.28,30 Briefly, using a commercially available kit (Transformer, Clontech, Palo Alto, CA), the technique of Deng and Nickoloff76 was used to mutate the cDNA of GP Ibα. The cDNA was then placed in the expression vector pDX77 by ligation into the EcoRI site. Finally, the vector was inserted into the CHO cells using liposomes as DNA carriers. DNA sequencing was used to verify all the mutations.

A summary of the CHO cell lines used in this study is listed in Table 2.1. CHO cells expressing wild-type GP Ib-IX-V and expressing only GP Ibβ and GP IX (CHO BIX cells) were used as controls. 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 BIX 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, 400 μg/ml G418 was used. All cells were maintained at 37°C with 5% CO2.
Table 2.1. Cell lines used in these studies.

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* Controls. ** The first letter in the mutation abbreviation is the single letter amino acid code that represents the amino acid found in wild-type GP Ibα at the position indicated by the numerals that follow it. The second letter in the mutation abbreviation is the single amino acid code that represents the amino acid that replaced the wild-type amino acid at the mentioned residue location. ◆ The LRR del abbreviation stands for deletion of LRRs 1-6 of GP Ibα.
Table 2.1 continued.

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<td>GP Ibβ, GP IX and GP α(1-152 human)/(153-282 dog)/(283-610 human) chimera</td>
</tr>
<tr>
<td>CHO βIX/αRH176</td>
<td>RH176</td>
<td>GP Ibβ, GP IX and GP α(1-176 human)/(177-282 dog)/(283-610 human) chimera</td>
</tr>
</tbody>
</table>

* Anionic sulfated region mutants - all three tyrosines were simultaneously replaced by either glutamate or phenylalanine. ** The number in the abbreviation represents the length of dog GP Ibα replacing the N-terminal residues of human GP Ibα. ♦ The number in the abbreviation represents the length of human GP Ibα replacing the N-terminal residues of dog/human GP Ibα.
Table 2.1 continued.

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Abbreviation</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO βIX/α604</td>
<td>α604</td>
<td>GP Ibβ, GP IX and GP α604 mutant</td>
</tr>
<tr>
<td>CHO βIX/α594</td>
<td>α594</td>
<td>GP Ibβ, GP IX and GP α594 mutant</td>
</tr>
<tr>
<td>CHO βIX/α582</td>
<td>α582</td>
<td>GP Ibβ, GP IX and GP α582 mutant</td>
</tr>
<tr>
<td>CHO βIX/α556</td>
<td>α556</td>
<td>GP Ibβ, GP IX and GP α556 mutant</td>
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<td>GP Ibβ, GP IX and GP α544 mutant</td>
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<td>CHO βIX/α533</td>
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<tr>
<td>CHO βIX/α518</td>
<td>α518</td>
<td>GP Ibβ, GP IX and GP α518 mutant</td>
</tr>
</tbody>
</table>

* The number in the abbreviation represents the last residue expressed in the truncated GP Ibα. The rest of the cytoplasmic region was deleted.
2.2 Antibodies.

The GP Ibα antibodies (IgG) used in this study were AK2 (RDI, Flanders, NJ), AN51 (DAKO, Carpinteria, CA), SZ2 (RDI), C34 (kindly provided by Michael Berndt, Baker Medical Research Institute, Melbourne, Australia), and WM23 (kindly provided by Michael Berndt) with the first four antibodies being found to block modulator-induced vWF binding. The binding epitopes on GP Ibα of these antibodies are: AK2 - residues 1-59 (Shen et al., unpublished data), AN51 - residues 1-35 (Shen et al., unpublished data), SZ2 - residues 276-282, C34 - residues 1-35 (Shen et al., unpublished data), and WM23 binds to the macroglycopeptide region.

The vWF antibodies (IgG) used in this study were 5D2, 6G1, CR2, CR3, and CR7 (all kindly provided by Michael Berndt). These vWF antibodies all have binding epitopes in the A1 domain of vWF (Shen et al., unpublished data).

2.3 Flow Cytometry.

Flow cytometry was used for two purposes in these studies. The first purpose was to determine if mutating GP Ibα affected the conformation of the subunit. We evaluated this effect by investigating the binding of a panel of GP Ibα antibodies to the mutated receptor. The second purpose was to determine the surface level of the complex on the CHO cells each time the cells were used in rolling experiments. Cell surface expression and antibody binding to the GP Ib-IX complex was determined by flow cytometry as described previously. Briefly, cells were detached with 0.53 mM EDTA and washed with phosphate-buffered saline (PBS). The cells were then incubated with the
monoclonal GP Ibα antibodies for 60 min at room temperature followed by an additional 30 min incubation with fluorescein isothiocyanate (FITC)–conjugated rabbit anti-mouse IgG (Zymed, South San Francisco, CA). After unbound antibody was removed, cell-surface expression of wild-type or mutated GP Ibα was measured by flow cytometry on a FACScan® flow cytometer (Becton Dickinson, San Jose, CA). Nonspecific binding was determined by the background fluorescence from CHO βIX cells stained with the same antibody. The data was analyzed using Cellquest® software from Becton Dickinson.

2.4 Preparation of vWF Coated Coverslips.

Human vWF was purified from human cyroprecipitate using a glycine and NaCl precipitation\cite{80,81} followed by separation using an agarose gel (2.5 x 50 cm with a 3,000 ml bed volume Sepharose 4B column, Pharmacia, Inc., Piscataway NJ). A Spectro vWF kit (Ramco Laboratories, Inc., Houston, TX) was used to quantify the amount of vWF collected using an enzyme-linked immunoassay where the concentration of vWF was determined relative to a single standard of known vWF concentration. For the flow experiments, the desired vWF concentration (2, 4, 5, 10, 50 or 100 µg/ml) was obtained by diluting the stock vWF solution of known concentration with Dulbecco’s PBS (Sigma Chemical Co.). Next, the diluted vWF solution was coated onto glass coverslips (No. 1, 24 x 50 mm; Corning, NY). Previous studies have shown that within this range of vWF concentration, the amount of protein absorbed on the glass surface increases non-linearly as the concentration of the protein in the solution increases.\cite{82} The coverslips were incubated with vWF solution for 45 min at room temperature. Prior to use, the coverslips were rinsed with 10 ml of 0.9% saline. When low dose vWF was used, the coverslips
were then coated with 400 ml of a 2% BSA solution for 2 hrs at room temperature to 
block any nonspecific binding. Any excess BSA was removed with 10 ml of 0.9 % saline 
prior to assembling the parallel-plate flow chamber where the coverslips formed the 
bottom of the chamber.

2.5 Parallel Plate Flow Chamber.

The interaction between vWF and GP Ib-IX-V complex was monitored in a 
parallel-plate flow chamber placed on an inverted-stage phase-contrast microscope 
(DIAPHOT-TMD; Nikon; X-20 phase objective and X-5 projection lens, Nikon, Garden 
City, NY). The parallel-plate flow chamber consisted of a polycarbonate slab, a silicon 
gasket (0.015 in) creating a defined gap, and a glass coverslip coated with vWF held 
together by application of a vacuum. The chamber was maintained at 37°C by an air 
curtain incubator attached to the microscope. Fluid was drawn through the gap in the 
chamber by a syringe pump (Harvard Apparatus, Holliston, MA). The wall shear stress 
created in the chamber is proportional to the fluid viscosity and flow rate and inversely 
proportional to the width of the chamber and height of the gap squared.483 Cells were 
either injected (500,000/ml) into the chamber and allowed to incubate with the 
immobilized vWF for one minute or perfused (100,000/ml) over immobilized vWF 
without preincubation. To increase the probability of cell-protein interactions, the cells 
were allowed to settle on the vWF surface in the injection method. When the rate of 
association of the receptor-ligand interaction was investigated, the cells were perfused 
through the chamber. A lower cell density was used in the perfusion method because of 
limitations in CHO cell quantity.
2.6 Digital Image Processing.

Images were collected using a silicon-intensified target video camera (Model C2400; Hammatsu, Waltman, MA) attached to the microscope. Subsequently, the images were recorded onto videocassette. Analysis of the collected images to determine cell rolling velocity was performed off-line on a Sparc Workstation utilizing Inovision digital imaging software (IC-300 Modular Image Processing Workstation, Inovision Corp., Durham, NC). Overlapping a set number of images for a determined amount of time (i.e. 60 frames/2 sec), resulted in an image that showed the path traveled by the CHO cells during this time period. The path distance was measured, and the average rolling velocity of each cell was obtained by dividing this distance by the amount of overlap time. Only cells that fit the definition of rolling were used in tabulating mean rolling velocity results. Rolling was defined for these experiments as when a CHO cell moves along the fluid flow direction while maintaining continuous contact with the immobilized vWf surface for at least two seconds. If the cell rolled a short distance, returned to the flowing stream and traveled at the fluid velocity for a distance of at least two cell diameters, and then rolled on the surface a short distance again, the cell was considered to be showing saltatory translocation.

2.7 Statistics.

A Student's t-test or an ANOVA f-test was used whenever required. Details on the individual statistical analyses can be found in the corresponding chapters. Results were reported as means +/- SEM.
CHAPTER 3

Inhibition of Rolling of Cells Expressing the GP Ib-IX Complex on Immobilized vWF by GP Ibα and vWF Antibodies: A Comparison of Shear- and Modulator-Induced vWF Binding

3.1 Introduction

The platelet GP Ib-IX-V – vWF interaction is important in both hemostasis and thrombosis. There are several means of examining the interaction between the receptor and ligand: rolling in the presence of fluid shear stress, shear-induced platelet aggregation, and modulator-induced platelet aggregation. Each of these means can be used to study different aspects of the interaction of GP Ib-IX-V with vWF.

Rolling in the presence of fluid shear stress is used to investigate the GP Ib-IX-V – vWF interaction and model the initial interaction of platelets with the subendothelium. This system uses a parallel-plate flow chamber to create a well defined wall shear stress. For example, coating the coverslip of the chamber with immobilized vWF leads to platelet rolling on the vWF surface. 20:82 Recent studies using this model have also shown that CHO and murine L cells expressing the GP Ib-IX-V complex also roll on an immobilized vWF matrix. 9:10

Another way to examine the interaction between the GP Ib-IX-V complex and vWF is shear-induced platelet aggregation. This system uses a viscometer to create a
uniform shear stress throughout the test fluid. Using this geometry, it was discovered that platelets can be activated and aggregated in the presence of high shear stress and that this aggregation depends on the GP Ib-IX-V complex and the integrin, GP IIb/IIIa. Like the flow chamber, this system is used to investigate the receptor-ligand interaction under shear stress, but in this system the interaction occurs between the GP Ib-IX-V complex and soluble vWF which is an important distinction, because soluble vWF has a different conformation and different binding characteristics than immobilized vWF.

A third mean of investigating the interaction between vWF and the GP Ib-IX-V complex is modulator-induced platelet aggregation. It has been shown that platelets aggregate in the presence of ristocetin or botrocetin. Ristocetin and botrocetin are often used to induce platelet aggregation because they provide an easy method of initially analyzing platelet function. However, it is still unknown as to what extent modulator-induced platelet aggregation mimics platelet aggregation in vivo.

In this study, we have examined the blocking effects of GP Ibα and vWF antibodies that block modulator-induced platelet aggregation on rolling of CHO cells expressing the GP Ib-IX complex on immobilized vWF. This data was compared with the data from antibody blocking studies of modulator- and shear-induced platelet aggregation. Our studies suggest that the mechanism of the GP Ib-IX-V – vWF interaction in rolling is different than the mechanisms involved in shear- and modulator-induced platelet aggregation.
3.2 Materials and Methods

Cell lines. Two stable cell lines were used in the rolling experiments, CHO αβIX and CHO βIX, which were described in Section 2.1.

Antibodies. The antibodies used in this study were the GP Ibα antibodies, AK2, AN51, C34, SZ2, and WM23, and the vWF antibodies, 5D2, 6G1, CR2, CR3, and CR7. More details about each antibody can be found in Section 2.2.

Flow chamber experiments. In the rolling experiments, 0.6 ml of CHO αβIX or CHO βIX cells in PBS at a cell density of 500,000/ml were injected into the chamber and incubated with coverslips precoated with a solution of 50 μg/ml of vWF (Section 2.4). To determine the effects of the GP Ibα antibodies on cell rolling, 25 μg/ml of antibody was incubated with the CHO αβIX cells for 15 min at room temperature. To determine the effects of the vWF antibodies on cell rolling, 25 μg/ml of antibody was incubated on the rinsed vWF pre-coated coverslip for 15 min at room temperature. The cells or cell-antibody solution were injected into the parallel-plate flow chamber, incubated for one minute, and then flow was initiated creating a wall shear stress of 10 dyn/cm² according to Section 2.5. Subsequent rolling velocity calculations were performed as described in Section 2.6.

Modulator- and shear-induced aggregation. The modulator- and shear-induced aggregation studies have been previously described. Briefly, in the modulator-induced platelet aggregation assay, platelet-rich-plasma was first incubated with 25 μg/ml of GP Ibα antibody for 10 min at room temperature and then stirred at 1000 rpm in the presence of either 1.0 mg/ml of ristocetin or 20 μg/ml of botrocon in an aggregometer. In the shear-induced aggregation study, 0.5 ml of platelet-rich-plasma was incubated with 25
μg/ml of GP Ibα antibody for 10 min before being placed in the viscometer. A shear stress of 90 dyn/cm² was applied for one minute. Platelet aggregation was defined as the binding of platelets to one another through the bridging molecule vWF and was determined in a Coulter counter by measuring the reduction in the number of particles.

3.3 Results

Antibody blocking of rolling of CHO cells expressing the GP Ib-IX complex. To study the ability of GP Ibα and vWF antibodies to block rolling, the interaction of CHO cells expressing the GP Ib-IX complex with immobilized vWF in the presence of various antibodies was monitored in a parallel-plate flow chamber. CHO αβIX cells rolled on the vWF surface in the absence of antibodies at a velocity of about 70 ± 4 μm/sec, whereas the CHO βIX cells failed to adhere (Figure 3.1). CHO αβIX cell rolling was completely inhibited by AK2, AN51, and C34. These antibodies bind in the N-terminal sequence of amino acids 1-275. SZ2, which binds the anionic sulfated region of GP Ibα, did not completely inhibit the rolling of the CHO αβIX cells, but the rolling velocity of the SZ2 treated cells was significantly greater than the velocity of the untreated cells. CHO αβIX cell rolling was not affected by WM23, an antibody that binds the macroglycopeptide region of GP Ibα. Although a number of vWF antibodies can block shear-induced platelet aggregation (5D2, 6G1, and CR2), only 5D2 completely inhibited CHO αβIX rolling. The vWF antibodies (6G1, CR2, CR3) and the mouse IgG control antibody rolled at a velocity which was about 50% of the velocity of the wild-type cells. The decrease in the velocity in the presence of the mouse IgG antibody suggests that
Figure 3.1. Effects of the GP Ibα and vWf antibodies on rolling of CHO cells expressing GP Ib-IX on immobilized vWf. Cells were injected into the parallel-plate flow chamber and incubated for one minute after which flow was initiated at a wall shear stress of 10 dyn/cm². The coverslips were coated with a solution of 50 μg/ml of vWf. Values are the mean of 159-523 cells from three experiments for each data point with SEM error bars. *p < 0.001, Student's t-test, with respect to the velocity in the presence of WM23 for the GP Ibα antibodies, and with respect to the velocity in the presence of mouse IgG for the vWf antibodies.
another receptor-ligand interaction was possibly occurring, maybe an interaction between the antibodies on the precoated vWF coverslips and the CHO Fc receptors.

In addition to rolling of CHO αβIX cells on vWF, shear- and modulator-induced platelet aggregation also depends on the interaction of the GP Ib-IX complex with vWF. Tables 3.1 and 3.2 depict the differences in molecular mechanisms of shear- and modulator-induced GP Ibα-vWF binding. Table 3.1 contains the results of GP Ibα antibody blocking of shear-, ristocetin-, and botrocetin-induced platelet aggregation and a summary of the antibody blocking study on rolling. AK2 completely inhibited all three forms of induced platelet aggregation. AN51 moderately inhibited ristocetin-induced and but completely inhibited shear-induced platelet aggregation and rolling. C34 completely inhibited modulator-induced aggregation and cell rolling but only moderately inhibited shear-induced aggregation. SZ2 minimally blocked ristocetin-induced aggregation, but completely inhibited botrocetin-induced aggregation. SZ2 had no effect on shear-induced platelet aggregation. Again WM23 was used as a control antibody, and none of the forms of induced platelet aggregation were affected. Table 3.2 contains the results of vWF antibody blocking of rolling and shear-induced platelet aggregation. 5D2 was the only vWF antibody that completely inhibited cell rolling. 5D2, 6G1, and CR2 completely inhibited shear-induced platelet aggregation. The vWF antibodies, CR3 and CR7, did not affect rolling or shear-induced platelet aggregation.
Table 3.1. Inhibition of rolling or shear- or modulator- induced aggregation by antibodies against GP Ibα.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Rolling</th>
<th>Shear-Induced Aggregation*</th>
<th>Platelet Aggregation with Ristocetin*</th>
<th>Platelet Aggregation with Botroecetin*</th>
<th>Epitope in GP Ibα**</th>
</tr>
</thead>
<tbody>
<tr>
<td>AK2</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
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</tr>
<tr>
<td>AN51</td>
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<td>+++</td>
<td>+</td>
<td>N/A</td>
<td>1-35</td>
</tr>
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<td>SZ2</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>276-282</td>
</tr>
<tr>
<td>C34</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
<td>1-35</td>
</tr>
<tr>
<td>WM23</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>MGP</td>
</tr>
</tbody>
</table>

- no effect, + marginal, ++ moderate, +++ complete inhibition. *Worked performed by J.-F. Dong, S. Krause, and J.A. López. ** See Section 2.2. N/A, not performed. MGP, macroglycopeptide region.

Table 3.2. Inhibition of rolling or shear- induced aggregation by antibodies against vWF.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Rolling</th>
<th>Shear-Induced Aggregation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>5D2</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>6G1</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>CR2</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>CR3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CR7</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

- no effect, + marginal, ++ moderate, +++ complete inhibition. *Worked performed by J.-F. Dong, S. Krause, and J.A. López.

3.4 Discussion

Recently, two groups demonstrated platelet rolling on immobilized vWF and demonstrated that this rolling is dependent on interaction of the platelet GP Ib-IX-V complex with vWF.\textsuperscript{20,82} Fredrickson et al. further supported this finding by showing that CHO and murine L cells expressing the GP Ib-IX-V complex rolled on immobilized vWF.\textsuperscript{9} Using the same system utilized by Fredrickson et al., we have tested the blocking effects of GP Ibα and vWF antibodies on the rolling of CHO cells expressing GP Ib-IX
on immobilized vWF. By doing so, we have created a data set to compare with studies of antibody blocking of shear-, ristocetin-, and botrocetin-induced platelet aggregation.

We found that the GP Ibα antibodies AK2, AN51, and C34 completely inhibited the rolling of CHO αβIX cells on immobilized vWF. Inhibition of rolling by these antibodies was expected because their binding epitopes on GP Ibα are located in the N-terminal 45 kDa domain, the domain known to bind vWF.53-58

We compared the blocking effects of the GP Ibα antibodies on rolling and shear-, ristocetin-, and botrocetin-induced platelet aggregation to determine the GP Ib-IX-V – vWF interaction occurs by different mechanisms in these systems. Depending on the antibody used, the blocking effects of the GP Ibα antibodies on the receptor-ligand interaction differed among the four systems. For example, AK2 completely inhibited all four means of investigating the GP Ib-IX-V – vWF interaction. In comparison, SZ2 did not affect shear-induced platelet aggregation, but completely inhibited botrocetin-induced platelet aggregation. Additionally, we found that SZ2 did not completely inhibit rolling, but it did affect the rolling velocity. SZ2 caused a significant increase in velocity compared to the WM23 control suggesting that the antibody increases the rolling off-rate, which implies the antibody decreases the strength of the GP Ib-IX-V – vWF interaction. This finding is consistent with the blocking effects of SZ2 on ristocetin-induced platelet aggregation, where SZ2 marginally affected the receptor-ligand interaction. This comparison of the blocking effects of the GP Ibα antibodies, especially SZ2, reveals that different mechanisms are involved in the interaction of vWF with the GP Ib-IX-V complex in rolling, in shear-induced platelet aggregation, and in the presence of ristocetin or botrocetin. Additionally, this comparison reveals that the receptor-ligand interaction
in the presence of ristocetin more closely resembles the complex-vWF interactions in rolling than does botrocetin.

Next, we compared the blocking effects of the vWF antibodies on rolling and shear-induced platelet aggregation to determine if the GP Ib-IX-V – vWF interaction occurs by different mechanisms in the two systems. The blocking effects of the vWF antibodies differed between rolling and shear-induced platelet aggregation depending on the antibody used. For example, 5D2 completely inhibited rolling on immobilized vWF and shear-induced platelet aggregation. On the other hand, 6G1 and CR2 completely blocked shear-induced aggregation, but had no effect on rolling. This comparison of the blocking effects of vWF antibodies reveals that GP Ib-IX-V – vWF interaction in rolling and shear-induced platelet aggregation occurs by dramatically different mechanisms. This finding of differences in the interaction mechanisms of rolling and shear-induced aggregation was much pronounced in this comparison than in the previous comparison of the blocking effects of the GP Ibα antibodies. Differences in the conformation of immobilized vWF and soluble vWF under shear may provide clues into these different mechanisms.

In conclusion, modulators have been used for decades to investigate platelet function but they should be used with caution. Modulator-induced platelet aggregation may not be physiologically relevant, whereas rolling appears to be relevant to hemostasis and provides a good way of investigating the initial interaction between platelet receptors and immobilized vWF. More importantly, differences between the antibody blocking of ristocetin- and botrocetin-induced platelet aggregation and rolling were observed. Our study emphasizes the point that these systems do not evaluate platelet function the same
way. Based on these conclusions, we have chosen to use rolling to evaluate all of our future investigations of the platelet GP Ib-IX-V interaction with immobilized vWF.
CHAPTER 4

The Leucine-rich Repeat Region of GP Ibα Is Important in the Interaction of the GP Ib-IX-V Complex with vWF under High Fluid Shear Stress

4.1 Introduction

Polypeptide subunits of the GP Ib-IX-V complex contain leucine-rich repeat (LRR) motifs. GP Ibα has seven tandem repeats,\(^{22}\) GP Ibβ and GP IX both have one,\(^{23,31}\) and GP V has fifteen.\(^{25,34}\) The proteins of the leucine-rich family perform many different cellular functions and are located in many different compartments within a cell. One common property of proteins containing leucine-rich repeats is that they are involved in protein-protein interactions, including ligand-receptor interactions.\(^{61}\) Leucine-rich repeat domains may be directly involved in ligand binding as suggested by Thomas et al., where they found that LRRs 1-6 of the lutropin/chriogonadotropin receptor are required for the receptor to bind its ligand.\(^{62}\)

Direct evidence that suggested the leucine-rich repeat region of GP Ibα is important in the GP Ib-IX-V – vWF interaction came from the investigation of platelets from patients with Bernard Soulier Syndrome (BSS). Three mutations have been identified in the leucine-rich repeat region of GP Ibα which result in decreased amounts of complex surface expression and render the GP Ib-IX-V complex non-functional. One mutation is located in the first LRR, Leu57 to Phe,\(^{63}\) another is located in the sixth LRR,
Ala156 to Val, and a third is a deletion located in the seventh LRR, del Leu179. All three of these mutations result in platelets with defective abilities to agglutinate with ristocetin, indicating that these mutations affect the GP Ib-IX-V complex interaction with vWF in addition to preventing normal receptor expression.

The purpose of this study was to investigate the importance of leucine-rich repeat region of GP Ibα in the interaction of the GP Ib-IX-V complex with vWF under high fluid shear stress using CHO cells expressing GP Ibα with mutations in the LRR region (Figure 4.1). First, we evaluated the importance of the structure of this region by investigating the role of the conserved sixth asparagine residue of the LRRs in receptor-ligand binding. Previous studies (Dong et al., unpublished data) showed that changing the charge and shape of the amino acid in the conserved sixth position in the first and sixth LRRs resulted in decreased binding of several GP Ibα antibodies (Figure 4.2). Next, we investigated the function of the LRR region in the interaction of the GP Ib-IX-V complex with vWF by deleting LRRs 1-6 of GP Ibα. Shown previously (Dong et al., unpublished data), deleting the first six LRR resulted in elimination of binding of several GP Ibα antibodies (AK2, AN51, CLB-MB45) that block the modulator-induced vWF binding. The binding epitopes for these antibodies were previously mapped to a region within the N-terminal 282 amino acid residues (Figure 4.3). Finally, we evaluated whether the A156V mutation demonstrated in GP Ibα in BSS, Bolzano variant is responsible for the elimination of vWF binding to the GP Ib-IX-V complex.
Figure 4.1. Schematic of the leucine-rich repeat region of GP Ibα. This image contains the location of the five single amino acid substitutions in the mutants used in this study.
Figure 4.2. Antibody binding to cells expressing wild-type or N41S, N41K, N158S, or N158K mutated GP Ibα. The binding of the GP Ibα antibodies to these cells was determined by measuring the fluorescence of a secondary antibody using a flow cytometer as described in Section 2.3. Specific antibody binding was calculated by dividing the binding of the test antibody by the binding of WM23. WM23 binds to the macroglycopeptide region of GP Ibα and therefore serves as an internal control. Values are the mean of three experiments for each data point with SEM error bars. *p < 0.001, Student’s t-test, with respect to the specific antibody binding to the wild-type cells for each antibody. (Performed by Dong et al., unpublished data)
Figure 4.3. Antibody binding to cells expressing wild-type GP Ib\(\alpha\) or GP Ib\(\alpha\) with LRRs 1-6 deleted. The binding of the GP Ib\(\alpha\) antibodies to the cells was determined by measuring the fluorescence of a secondary antibody using a flow cytometer as described in Section 2.3. WM23 binds to the macroglycopeptide region of GP Ib\(\alpha\) and therefore serves as an internal control. Values are the mean of three experiments for each data point with SEM error bars. *\(p < 0.001\), Student's t-test, with respect to the geometrical mean fluorescence of the wild-type cells for each antibody. (Performed by Dong et al., unpublished data)
4.2 Materials and Methods

The cell lines used in this study were created by transfecting CHO βIX cells with mutant cDNA for GP Ibα as described in Section 2.1. The first mutation introduced to GP Ibα was a substitution to a valine at residue A156 in order to replicate the mutation that may be the cause of the Bolzano variant of BSS. This cell line does not contain the identical cDNA of the Bolzano propositus because the Thr145 to Met dimorphic substitution is not expressed in the mutant GP Ibα. Four other mutations were created in GP Ibα by substituting either a lysine or a serine at positions N41 and N158. Another mutated GP Ibα subunit was created by deleting LRR 1-6. We also created a cell line expressing both wild-type GP Ibα and GP Ibα with deletion of LRR 1-6. CHO αβIX cells were used as a positive control and CHO βIX cells were used as a negative control. The general location of the mutations used in this study is depicted in Figure 4.1. Details of all the CHO cells used in this study can be found in Table 2.1.

Two types of rolling experiments were performed in this study: injection and perfusion. In the injection rolling experiments, 0.6 ml of CHO cells in PBS at a density of 500,000/ml were injected into the chamber containing coverslips coated with a solution of 50 μg/ml of vWF (Section 2.4). Following an incubation period of one minute, flow was initiated creating a shear stress of 10 dyn/cm² according to Section 2.5. In the perfusion experiments, CHO cells in PBS at a density of 100,000/ml were perfused through the flow chamber for four min at flow rates that generated shear stresses of 5, 10, and 15 dyn/cm² according to Section 2.5. Subsequent rolling velocity calculations were performed according to Section 2.6. The surface level of GP Ibα on the CHO cells was determined by flow cytometry as described in Section 2.3.
4.3 Results

Rolling of CHO cells expressing the A156V mutation of GP Ibα on immobilized vWF. The dysfunctionality of platelets from patients with BSS, Bolzano variant suggests that the LRR region of GP Ibα is important in the GP Ib-IX-V – vWF interaction. To test this hypothesis, we investigated the effect of the valine mutation on the GP Ib-IX-V – vWF interaction under high fluid shear stress generated in a parallel-plate flow chamber. After creating a CHO cell line that expressed the valine mutation at residue 156 of GP Ibα (A156V), we injected the cells into the chamber. Then after one minute of incubation, flow was initiated generating a shear stress of 10 dyn/cm². Under these conditions, we found that the CHO αβIX cells and the A156V mutants rolled on a surface coated with a solution of 50 µg/ml of vWF (Figure 4.4). However, the rolling velocity of the valine mutant cells was significantly higher than the rolling velocity of the wild-type cells. Next, we investigated the effects of changes in shear stress on the rolling velocity of the valine mutant by perfusing the cells through the chamber at two different shear stresses. We chose to perfuse the valine mutant cells through the flow chamber without pre-incubation versus injecting them into the chamber with one minute of incubation so that we could investigate the effect of changes in shear stress on quantities related to both the on- and off-rate of the rolling interaction. The injection method eliminates the importance of the initial tethering of the cells to the surface from the flow stream, which is a function of on-rate of the interaction. At shear stresses of 5 and 10 dyn/cm², the wild-type and valine mutant cells were both able to tether and roll on the vWF surface (Figure 4.5). As in the injection experiment, A156V rolled significantly faster than the wild-type cells at both shear levels. Increasing the shear stress from 5 to
Figure 4.4. Shear-dependent rolling of CHO cells expressing wild-type or A156V GP Ibα on immobilized vWF. Cells were injected into the parallel plate flow chamber and incubated for one minute after which flow was initiated at a wall shear stress of 10 dyn/cm². The coverslips were coated with a solution of 50 μg/ml of human vWF. Values are the mean of 75-529 cells from three experiments for each data point with SEM error bars. *p < 0.001, Student's t-test, with respect to the velocity of the wild-type cells.
Figure 4.5. Rolling velocities of cells expressing wild-type or A156V GP Ibα on a coverslip coated with a solution of 50 μg/ml of vWF. Cells were perfused through the parallel-plate flow chamber at flow rates that generated wall shear stresses of 5 and 10 dyn/cm². Values are the mean of 97-176 cells from three experiments for each data point with SEM error bars. *p < 0.001, Student’s t-test, with respect to the velocity of the wild-type cells for each shear level and between the A156V velocities at 5 and 10 dyn/cm².
10 dyn/cm² resulted in a significant increase in the rolling velocity of the cells expressing the A156V mutant.

Rolling of CHO cells expressing the 41 and 158 lysine and serine mutations of GP Ibα on immobilized vWF. We used the parallel-plate flow chamber to evaluate the importance of the conserved asparagine residue in the LRRs of GP Ibα on the GP Ib-IX-V – vWF interaction under high fluid shear stress. At a wall shear stress of 10 dyn/cm², the CHO αβIX cells rolled on the surface coated with a solution of 50 μg/ml of vWF, whereas the N41K cells did not (Figure 4.6). Both cells expressing the serine mutations (N41S and N158S) rolled significantly faster than the wild-type cells and the N41S cells rolled more than twice as fast as the N158S cells. Next, we used the perfusion system to compare the effects of changes in shear stress on the rolling velocity between the wild-type cells and the mutant cells. The perfused CHO αβIX cells showed a step-wise increase in rolling velocity, on coverslips coated with a 50 μg/ml vWF solution, in response to increases in shear stress from 5 to 15 dyn/cm² (Figure 4.7A). On the other hand, the number of wild-type cells that rolled decreased in a step-wise manner in response to the increase in shear stress (Figure 4.7B). After defining the effects of changes in shear stress on the rolling velocity of the wild-type cells, we proceeded to test the effects on the serine mutants. The N41K cells were not tested in the perfusion study because they did not roll in the injection study. The N158S mutant cells did not exhibit a change in rolling velocity in response to the increase in shear stress from 5 to 15 dyn/cm² (Figure 4.7A). The other serine mutant N41S did exhibit an increase in rolling velocity in response to the increase in shear stress from 5 to 10 dyn/cm², but failed to roll altogether on the vWF surface at a shear stress of 15 dyn/cm². The rolling velocities of
Figure 4.6. Rolling of CHO cells expressing wild-type or mutated GP Ibα on immobilized vWf. The same procedure was followed as in Figure 4.4. Values are the mean of 193-529 cells from three experiments for each data point with SEM error bars. *p < 0.001, Student’s t-test, with respect to the velocity of the wild-type cells.
Figure 4.7. A. Rolling velocities of cells expressing wild-type, N41S, and N158S GP Ibα on coverslips coated with a solution of 50 μg/ml of vWF. Cells were perfused through the parallel-plate flow chamber at flow rates that generated wall shear stresses of 5, 10, and 15 dyn/cm². Values are the mean of 75-350 cells from three experiments for each data point with SEM error bars. B. The number of wild-type cells and serine mutants that rolled on immobilized vWF. The cells that were rolling were counted starting from the flow of cells for a total of three minutes. Values are the mean of three experiments for each data point with SEM error bars. *p < 0.001, Student’s t-test, with respect to the number of rolling wild-type cells at each shear level.
N41S at wall shear stresses of 5 and 10 dyn/cm² were significantly higher than the rolling
velocities of the wild-type cells at the same shear levels. Similar to the wild-type cells,
the serine mutants did show a decrease in the number of rolling cells in response to
increased shear stress (Figure 4.7B).

Rolling of CHO cells expressing GP Ibα with deletion of LRRs 1-6 on
immobilized vWF. We further evaluated the involvement of the leucine-rich repeat region
of GP Ibα in the GP Ib-IX-V – vWF interaction under high fluid shear conditions by
injecting the cells expressing the deletion of LRRs 1-6 of GP Ibα into the parallel-plate
flow chamber. At a shear stress of 10 dyn/cm², no interaction between the deletion
mutant and the immobilized vWF surface was observed (Figure 4.8). The cells
expressing both wild-type GP Ibα and GP Ibα with LRRs 1-6 deleted rolled on the vWF
surface at a similar velocity as the CHO αβIX cells (Figure 4.8).

4.4 Discussion

First, we determined that the structure of the leucine-rich repeat region of GP Ibα
is important in the interaction of the GP Ib-IX-V complex with vWF under fluid shear
stress. Specifically, we evaluated the importance of the conserved asparagine in the sixth
position of the LRRs in the structure of the LRR region of GP Ibα. Altering the charge
and shape of the residue (asparagine to lysine) resulted in a severe functional defect in the
GP Ib-IX-V complex exhibited by either the failure of the mutant to be expressed on the
surface (N158K) or the complete inhibition of the receptor-ligand interaction under shear
stress (N41K). Rapid proteolysis of the mutant glycoprotein following translation (Dong
et al., unpublished data) resulted in the failure of the N158K mutant to be expressed on
Figure 4.8. Shear-dependent rolling of CHO cells expressing wild-type GP Ibα, GP Ibα with deletion of LRRs 1-6 (LRR del), or both wild-type GP Ibα and GP Ibα with deletion of LRRs 1-6 (LRR del/αwt) on immobilized vWF. The same procedure was followed as in Figure 4.4. Values are the mean of 191-529 cells from three experiments for each data point with SEM error bars.
the surface, indicating that this mutation caused a profound conformational change of the LRR region. The inhibition of rolling of the N41K cell, even though the complex was expressed at similar levels as the wild-type complex, is an indication that the mutation induced a dramatic, but less severe, conformational change of the LRR region. This proposed conformational change is supported by the finding that AK2, AN51, and CLB-MB45, antibodies with binding epitopes within the N-terminal 1-59 residues of GP Ibα, bound significantly less N41K compared to the binding to the wild-type cells.

On the other hand, substitution to a serine at the sixth position of the LRRs was a more conservative change (same charge as asparagine and similar in size). We found that cells expressing serine mutations at residue N41 or N158 of GP Ibα tethered and rolled on immobilized vWF. The N158S mutants generally rolled at velocities similar to the wild-type cells. The N41S mutants rolled at least twice as fast as the wild-type cells, which is consistent with an increase in off-rate. This change in off-rate suggests that the N41K mutation caused a decrease in the strength of the interaction between GP Ib-IX-V and vWF. The conformation of GP Ibα is probably not severely altered by the serine mutations as suggested by the fact only the binding of AN51 was significantly decreased.

Comparing the effects of replacing the conserved asparagine in the first and sixth LRR, our results suggest that the first LRR is probably more important in ligand binding and the sixth LRR is probably more important structurally. Substitution to a serine had a greater negative effect on the first LRR compared to the sixth, as demonstrated by faster rolling (an effect on vWF binding). On the other hand, substitution to a lysine had a larger negative effect on the sixth LRR compared to the first, as shown by a lack of receptor expression (an effect on conformation).
Second, we determined that the LRRs of GP Ibα are important in the function of vWf binding to the GP Ib-IX-V complex. From our study of the cells expressing GP Ibα with deletion of LRRs 1-6, we found that deleting LRRs 1-6 completely abolished the receptor-ligand interaction under fluid shear stress, indicating that the vWf binding site is located within these LRRs. This hypothesis is consistent with the finding that the GP Ibα antibodies, with epitopes within the first 268 residues of N-terminal of GP Ibα, failed to bind the LRR deletion mutants. The studies by Thomas et al., where they found that ligand binding of the lutropin/chriogonadotropin receptor requires LRRs 1-6 of the receptor, further support this hypothesis.

Third, we determined that the A156V mutation found in BSS Bolzano is partially responsible for the dysfunctional vWf binding of the diseased platelets. We found that CHO cells expressing the BSS Bolzano mutation in GP Ibα would tether and roll on immobilized vWf. The valine mutant rolled at a velocity significantly higher than the rolling velocity of the CHO αβIX cells. As mentioned above, this is consistent with an increase in rolling off-rate and thus a decrease in the strength of the GP Ib-IX-V – vWf interaction. This finding is in partial agreement with the studies of platelets from patients with BSS Bolzano, where De Marco et al. found that these platelets fail to bind vWf in the presence of ristocetin. A possible explanation for this difference could be that the GP Ibα of the Bolzano propositus also contained the Thr145 to Met dimorphic substitution. Maybe the introduction of this polymorphism into our A156V mutant would eliminate the GP Ib-IX-V – vWf interaction under fluid shear.

In summary, we have demonstrated that the structure of the leucine-rich repeat region of GP Ibα is important in the interaction of GP Ib-IX-V with vWf, specifically the
importance of the conserved sixth asparagine. We have also shown that the N-terminal
LRRs of GP Ibα may contain the vWF binding site. Additionally, this region of GP Ibα
may be important in protein-protein interactions among the glycoprotein subunits that
make up a vWF receptor.
CHAPTER 5

A Disulfide Loop in the N-terminus of GP Ibα Modulates the Strength of the

GP Ib-IX-V – vWF Interaction under High Fluid Shear Stress

5.1 Introduction

The N-terminus of GP Ibα contains three disulfide loops, seven tandem leucine-rich repeats, and an anionic sulfated region. The second disulfide loop (Cys211-Cys248) from the N-terminus of GP Ibα is located between the leucine-rich repeat region and third disulfide loop. Studies of platelets from patients with the bleeding disorder Platelet-type von Willebrand Disease (PtvWD) have suggested that the second disulfide loop is important in the interaction of platelet GP Ib-IX-V with vWF. In this disease, the function of vWF is normal but the function of the GP Ib-IX-V complex is not normal. Two mutations have been identified in PtvWD, and they are located on the GP Ibα subunit: Gly233 to a Val and Met239 to a Val. Interestingly, both mutations are single amino acid substitutions to a Val and both are located in the N-terminal second disulfide loop of GP Ibα. These mutations cause the GP Ibα subunit to have a higher binding affinity for vWF, which leads to a selective loss of the high molecular weight vWF multimers in the patient’s plasma. These mutations are gain-of-function mutations and ultimately result in pathological bleeding. For more details on PtvWD, see Section 1.5.2.
To better understand the molecular mechanism of the increased affinity of the interaction between PtvWD platelets and vWf, CHO cells expressing mutated GP Ibα were made. The mutations introduced into the GP Ibα subunit were single amino acid substitutions to a valine and were inserted into the second disulfide loop from residue N226 to residue A244 in an attempt to determine if mutating the other residues in the same region to valine would also result in the gain-of-function phenotype (Figure 5.1). Previous studies using these cell lines revealed that the second disulfide loop of GP Ibα modulates the strength of the GP Ib-IX-V – vWf interaction in the presence of ristocetin (Dong et al., unpublished data). Specifically, they found that CHO cells expressing GP Ibα with valine substitutions bound vWf in the presence of ristocetin in two patterns. The cells with the first phenotype (G233V, D235V, K237V, and M239V) bound vWf in low levels in the absence of ristocetin and in the presence of very little modulator indicating that these mutations caused a gain-of-function and hence the members of this group were called gain-of-function mutants. Mutation resulted in a second phenotype (N226V, K231V, Q232V, A238V, T240V, and A244V), called loss-of-function mutants, which bound decreased amounts of vWf in the presence of ristocetin. In the current study, we refer to the GP Ibα mutants according to these phenotypic divisions.

In this study, we investigated the effect of these valine mutations of GP Ibα on the interaction of GP Ib-IX-V with vWf under high fluid shear stress. We found that CHO cells expressing mutated GP Ibα rolled on immobilized vWf under fluid shear stress and that the velocity of rolling depended on the particular mutation. Our data suggests that the second disulfide loop of GP Ibα modulates the strength of the GP Ib-IX-V – vWf interaction under high fluid shear stress.
Figure 5.1. A schematic of the disulfide loop region of GP Ibα. This image contains the location of the eleven single amino acid substitutions to valine in the mutants used in this study. Also indicated are the positions of the two known mutations (V*) found in Platelet-type von Willebrand Disease.
5.2 Materials and Methods

The cell lines used in this study were created by transfecting CHO βIX cells with mutant cDNA for GP Ibα as described in Section 2.1. Single amino acid substitutions to a valine were introduced to the amino acid residues N226 to A244 of the second disulfide loop of GP Ibα (Figure 5.1). CHO αβIX cells were used as the positive control and CHO βIX cells were used as the negative control. Details of all the CHO cells used in this study can be found in Table 2.1.

Two types of rolling experiments were performed in this study: injection and perfusion. In the injection experiments, 0.6 ml of CHO cells in PBS at a density of 500,000/ml were injected into the chamber and incubated with coverslips coated with solutions of 2, 4, 10, or 100 μg/ml of vWf (Section 2.3). Following an incubation period of one minute, flow was initiated creating a wall shear stress of 10 dyn/cm² according to Section 2.6. In the perfusion experiments, CHO cells in PBS at a density of 100,000/ml were perfused through the flow chamber for four minutes at flow rates that generated wall shear stresses of 5, 10, and 15 dyn/cm² according to Section 2.4. Subsequent rolling velocity calculations were performed according to Section 2.5. The surface level of GP Ibα on the CHO cells was determined by flow cytometry as described in Section 2.3.

5.3 Results

*CHO cells expressing valine mutants of GP Ibα rolled on immobilized vWf.* To investigate the effect of mutating the residues in the second disulfide loop of GP Ibα on the interaction between the GP Ib-IX-V complex and vWf under conditions of high shear
stress, we injected the transfected cells into a parallel plate flow chamber with coverslips coated with a vWf solution of 100 µg/ml. At a wall shear stress of 10 dyn/cm², we found that cells expressing the valine mutations rolled on the immobilized vWf (Figure 5.2). The rolling velocities varied greatly among the mutants (Figure 5.3). The gain-of-function mutants (G233V, D235V, K237V, and M239V), defined previously as mutants that bound vWf spontaneously and in the presence of low amounts of ristocetin, rolled at 25-30% of the velocity of the wild-type cells. This study further divided the loss-of-function mutants (N226V, K231V, Q232V, A238V, T240V, and A244V), defined previously as mutants that bound less vWf compared to wild-type cells in the presence of ristocetin, into two groups. The first group of loss-of-function mutants, which consists of N226V, K231V, Q232V, A238V, rolled at significantly higher velocities compared to the velocity of wild-type cells. The second group of loss-of-function mutants, which consists of T240V and A244V, rolled at velocities similar to wild-type cells even though these cells presented as loss-of-function mutants in the ristocetin-induced vWf binding study.

Rolling velocities of the gain-of-function mutants did not change in response to changes in shear stress. We investigated the effect of changes in wall shear stress on the rolling velocity of the gain-of-function mutants by perfusing the mutants through the chamber at three different shear stresses. The perfused CHO αβIX cells showed an increase in rolling velocity, on coverslips coated with a 50 µg/ml vWf solution, in response to increases in wall shear stress from 5 to 15 dyn/cm² (Figure 5.4A). On the other hand, the number of wild-type cells that rolled decreased in response to the increase in wall shear stress (Figure 5.4B). After defining the effects of changes in shear stress on the rolling velocity of the wild-type cells, we proceeded to test the effects on the gain-of
Figure 5.2. Video images of cells expressing wild-type GP Ibα or valine mutated GP Ibα rolling on immobilized vWF (coverslips coated with a solution of 100 μg/ml of vWF). Cells were injected into the parallel-plate flow chamber and incubated for one minute after which flow was initiated at a wall shear stress of 10 dyn/cm². Cell rolling was recorded real-time using a VCR and later analyzed off-line using digital imaging software. These video images were created by overlapping 60 frames for 2 seconds that resulted in an image that shows the path traveled by the CHO cells during this duration. The top row of images was created from overlapping recorded video images during the incubation of the cells in the chamber prior to the initiation of flow. The bottom row of images was created from overlapping recorded video images during flow (one minute after initiating flow). The path distance was measured and the average rolling velocity of each cell was obtained by dividing this distance by the elapsed time (2 sec).
Figure 5.3. Shear-dependent rolling of CHO cells expressing wild-type or valine mutated GP Iboα on immobilized vWF. Cells were injected into the parallel plate flow chamber and incubated for one minute after which flow was initiated at a wall shear stress of 10 dyn/cm². The coverslips were coated with a solution of 100 µg/ml of human vWF. Values are the mean of 300-700 cells from three experiments for each data point with SEM error bars. *p < 0.001, Student's t-test, with respect to the velocity of the wild-type cells.
Figure 5.4. **A.** Rolling velocities of cells expressing wild-type or gain-of-function mutations of GP Ibα on coverslips coated with a solution of 50 μg/ml of vWF. Cells were perfused through the parallel-plate flow at flow rates that generated wall shear stresses of 5, 10, and 15 dyn/cm². Values are the mean of 35-530 cells from three or four experiments for each data point with SEM error bars. *p < 0.005, Student's t-test, with respect to the velocity of the wild-type cells at the same shear level. **B.** The number of wild-type cells and gain-of-function mutants that rolled on immobilized vWF. The cells that were rolling were counted starting from the flow of cells for a total of three minutes. Values are the mean of three or four experiments for each data point with SEM error bars. *p < 0.001, Student's t-test, with respect to the number of rolling wild-type cells at the same shear level.
-function mutants. None of the gain-of-function mutants exhibited a change in rolling velocity in response to the increase in wall shear stress from 5 to 15 dyn/cm² (Figure 5.4A). The mutants all rolled at similar velocities, which were 25-30% of the velocity of the wild-type cells. The gain-of-function mutants did show a decrease in the number of rolling cells in response to increased shear stress (Figure 5.4B). In the next part of the perfusion study, we compared the effect of receptor density on the number of rolling cells between the wild-type cells and the gain-of-function mutants at each shear level. For the wild-type cells, a correlation between the receptor density and the number of rolling cells was found at all the shear stress levels (Figure 5.5, top row). On the other hand, there was no correlation between the pooled receptor densities and number of rolling cells for all of the gain-of-function mutants (Figure 5.5, bottom row).

Cells expressing the gain-of-function mutants rolled on a low density of vWF. To investigate if the gain-of-function mutants would roll on lower concentrations of vWF compared to wild-type cells, we reduced the concentration of the vWF solution used to coat the coverslips to 2, 4, or 10 µg/ml. In this part of the study, a shear stress of 10 dyn/cm² was used. The CHO αβIX cells rolled on the coverslips coated with solutions of 4 and 10 µg/ml of vWF, but reducing to 2 µg/ml did not support cell rolling (Figure 5.6A). The rolling velocity of the wild-type cells increased by 98% (48±7.79 vs. 95±11.47 µm/sec, Student’s t-test, n = 120-400 cells, p < 0.001) when the vWF coating solution was reduced 25-fold (100 to 4 µg/ml) (Figures 5.3 and 5.6A). On the other hand, all three coating concentrations of vWF supported the rolling of the four gain-of-function mutants (G233V, D235V, K237V, and M239V). Reducing the vWF coating concentration 50-fold (100 to 2 µg/ml) did not affect the rolling velocity of any of the gain-of-function mutants
Figure 5.5. Correlation of the GP Ibα receptor density with the number of wild-type cells and gain-of-function mutants that rolled at shear stresses of 5, 10, and 15 dyn/cm². The receptor densities and cell counts were pooled for the four gain-of-function mutants (G233V, D235V, K237V, and M239V). GP Ibα receptor densities were determined using flow cytometry as described in Section 2.3. The cells that were rolling were counted starting from the flow of cells for a total of three minutes.
Figure 5.6. A. Rolling velocities of cells expressing wild-type and gain-of-function mutations of GP Iba on coverslips coated with solutions of 2, 4, or 10 µg/ml of vWF. Cells were injected into the parallel-plate flow chamber and incubated for one minute after which flow was initiated at a wall shear stress of 10 dyn/cm². Values are the mean of 116-509 cells from three to five experiments for each data point with SEM error bars. B. The number of wild-type cells and gain-of-function mutants that rolled on immobilized vWF. The cells that were rolling were counted starting from the flow of cells for a total of three minutes. Values are the mean of three to five experiments for each data point with SEM error bars.
(Figures 5.3 and 5.6A). Next, we compared the effect of reducing the vWf density on the number of rolling cells between the wild-type cells and the gain-of-function mutants. We found that a significantly larger number of wild-type cells rolled on the surface coated with 10 μg/ml of vWf than on the surface coated with 4μg/ml (Figure 5.6) (78±12 vs. 208±36 μm/sec, Student’s t-test, n = 120-360 cells, p < 0.001). In contrast, only the gain-of-function mutant G233V showed a similar pattern as the wild-type cells, with an increase in the number of rolling cells in response to an increase in the vWf coating solution. The number of rolling cells remained constant for the other gain-of-function mutants in response to the vWf coating solution increasing from 2 to 10 μg/ml.

5.4 Discussion

In this study, we characterized the involvement of the disulfide loop region of GP Ibα in the interaction of the GP Ib-IX-V complex with immobilized vWf under high fluid shear stress. We found that the gain-of-function mutants (G233V, D235V, K237V, and M239V) rolled on immobilized vWf at velocities 2-3 times slower than the wild-type cells. The decreased rolling velocity of the gain-of-function mutants is consistent with a decrease in the rolling off-rate compared to the rolling off-rate of the wild-type cells. This decrease in off-rate suggests that the gain-of-function mutants bind vWf with a higher strength/avidity compared to the normal GP Ib-IX-V – vWf interaction. This result correlates well with the outcomes of the ristocetin-induced vWf binding study performed previously (Dong et al., unpublished data), where the gain-of-function mutants bound vWf in the absence and in the presence of low concentrations of ristocetin, which also suggests that the gain-of-function mutants bind vWf with a higher avidity. It is
important to note that the CHO cells expressing the mutations found in PtWD (G233V and M239V) exhibited an increase in the avidity of the interaction between the receptor and vWF similar to the increase in avidity between the diseased platelets and vWF.

We found that the loss-of-function mutants rolled on immobilized vWF in two patterns, resulting in the subdivision of these mutants into two groups. The first group of loss-of-function mutants (N226V, K231V, Q232V, A238V) rolled on the vWF surface but at rolling velocities 2 to 3 times greater than the velocity of the wild-type cells. The second group of loss-of-function mutants (T240V and A244V) rolled at velocities similar to the velocity of the wild-type cells. The increased rolling velocity of the first group of the loss-of-function mutants is consistent with an increase in the rolling off-rate compared to the rolling off-rate of the wild-type cells. The increased off-rate suggests that these loss-of-function mutants bind immobilized vWF with a decreased strength/avidity compared to the normal interaction between the GP Ib-IX-V complex and vWF. The rolling result of the first group of loss-of-function mutants correlates well with the data obtained in the ristocetin-induced vWF binding study (Dong et al., unpublished data), where in the presence of ristocetin, the loss-of-function mutants bound less vWF, which also supports the suggestion that the loss-of-function cells bind vWF with a lower avidity.

Taken together, our results suggest that the disulfide loop region of GP Ibα modulates the strength of the GP Ib-IX-V – vWF interaction. Although the mechanism through which the disulfide loop region of GP Ibα modulates the interaction strength remains to be determined, there are several possibilities. First, this region might contain the primary vWF binding site and therefore an increase in the valine content in certain
locations within the loop would directly affect ligand binding. Second, vWF binding might be bifocal and a secondary binding site for vWF might be located in the disulfide loop region. Altering the conformation of this region through substitutions to a valine would then either eliminate or enhance its contribution to vWF binding. Third, the disulfide loop region of GP Ibα may be indirectly involved in vWF binding. Perhaps the conformation of this region affects the conformation of the vWF binding site. In this case, increasing the valine content would alter the conformation of the disulfide loop region and result in a conformation alteration of the ligand binding site, a modification that could either reduce or enhance the receptor ligand interaction. Regardless of the mechanism, the disulfide loop region of GP Ibα is important in the interaction of the GP Ib-IX-V complex with immobilized vWF under fluid shear stress.
CHAPTER 6

The Anionic Charges Created by Post-translational Tyrosine Sulfation GP Ibα are Important in the Interaction of the GP Ib-IX-V Complex with von Willebrand Factor under High Shear Conditions

6.1 Introduction

The GP Ibα subunit of the GP Ib-IX-V complex contains a region with many anionic residues and three tyrosines that are sulfated. This anionic sulfated region (Asp269 to Asp287) of GP Ibα is located between the disulfide loop region and the macroglycopeptide region.26 The importance of the post-translational tyrosine sulfation of this region in the interaction of the GP Ib-IX-V complex with vWF in the presence of ristocetin and botrocetin was shown by several independent studies.69-70,72 Recently, the importance of post-translational tyrosine sulfation in dynamic conditions was shown by Fredrickson et al. using heterogeneous cells expressing GP Ib-IX cultured in sulfate-depleted media containing sodium chlorate, where fewer of these sulfate depleted cells rolled on immobilized vWF compared to controls.9

In this study, we further demonstrated the importance of the anionic sulfated region of GP Ibα in the GP Ib-IX-V – vWF interaction under high fluid shear stress. We used CHO cells expressing triple mutations in the anionic sulfated region of GP Ibα.
Specifically, either glutamate (E) or phenylalanine (F) was substituted for tyrosine at positions Y276, Y278, and Y279, creating 3Y-E and 3Y-F cells respectively. It has been shown previously that the GP Ibα antibody SZ2 failed to bind the 3Y-E and 3Y-F cells while binding of the other GP Ibα antibodies was normal (Figure 6.1) (Dong et al., unpublished data). In addition, it has also been shown that the 3Y-F cells failed to bind vWF in the presence of ristocetin and botrocetin and the 3Y-E cells bound vWF normally in the presence of ristocetin but failed to bind vWF in the presence of botrocetin (Figure 6.2); (Dong et al., unpublished data).

We found that the 3Y-E and 3Y-F cells exhibit rolling and saltatory translocation on immobilized vWF under high fluid shear conditions. If the cell rolled a short distance, returned to the flowing stream, and then rolled a short distance again, it was considered to be showing saltatory translocation. The 3Y-E cells had similar rolling properties as the wild-type cells. However, the 3Y-F cells exhibited a significantly higher percentage of saltatory translocation compared to wild-type cells. Our results demonstrate that charge distribution in the anionic sulfated region of GP Ibα is important in the binding of the GP Ib-IX-V complex with vWF under high shear conditions. Specifically, the anionic charges at residues Y276, Y278, and Y279, which in platelets are created by post-translational tyrosine sulfation, are necessary for optimal binding between the receptor and ligand.

6.2 Materials and Methods

The cell lines used in this study were created by transfecting CHO BIIX cells with mutant cDNA for GP Ibα as described in Section 2.1. Two mutants were created where
Figure 6.1. Antibody binding to cells expressing tyrosine mutated GP Ibα. The specific binding the GP Ibα antibodies AK2, AN51, C34, SZ2, TM60, and WM23 to cells expressing tyrosine mutated GP Ibα and wild-type GP Ibα was determined by measuring the fluorescence intensity of a FITC-conjugated secondary antibody using a flow cytometer. WM23 binds to the macroglycopeptide region of GP Ibα and therefore serves as an internal control. Values are the mean of three experiments for each data point with SEM error bars. *p < 0.001, Student's t-test, with respect to the specific antibody binding to the wild-type cells for each antibody. (Performed by Dong et al., unpublished data)
Figure 6.2. A. Ristocetin-induced $^{125}$I-vWF binding to CHO cells expressing wild-type and tyrosine mutated GP Ibα. In the presence of ristocetin (1.0 mg/ml), cells were incubated with various amounts of $^{125}$I-vWF for 30 min at room temperature followed by spinning through a sucrose cushion. Membrane bound radioactivity was counted and corrected for non-specific binding and receptor density differences. Values are from a representative of three independent experiments. B. Botrocetin-induced $^{125}$I-vWF binding to CHO cells expressing wild-type type and tyrosine mutated GP Ibα. The same procedure was followed as described in A except the experiments were carried out in the presence of botrocetin (20 μg/ml). Values are from a representative of three independent experiments. (Performed by Dong et al., unpublished data)
the three sulfated tyrosines of GP Ibα, located at residues Y276, Y278, and Y279, were replaced with either glutamate (E) or phenylalanine (F) by site-directed mutagenesis. CHO αβIX cells were used as a positive control and CHO βIX cells were used as a negative control. Details of all the CHO cells used in this study can be found in Table 2.1.

In the rolling experiments, 0.6 ml of CHO cells in PBS at a density of 500,000/ml were injected into the chamber and incubated with coverslips coated with a solution of 50 μg/ml of vWF (Section 2.4). Following an incubation period of one minute, flow was initiated creating a wall shear stress of 10 dyn/cm² (Section 2.5). Subsequent rolling velocity calculations were performed according to procedures in Section 2.6. The surface level of GP Ibα on the CHO cells was simultaneously determined by flow cytometry as described in Section 2.3.

6.3 Results

Rolling of CHO cells expressing 3Y-E and 3Y-F mutations of GP Ibα on immobilized human vWF. We evaluated the effects of altering the charge distribution of the anionic sulfated region of GP Ibα on the interaction of GP Ib-IX-V with immobilized vWF under high flow conditions. At a shear stress of 10 dyn/cm² and similar levels of GP Ibα expression, we found that the CHO αβIX and CHO βIX/α3Y-E cells rolled on the vWF surface, whereas the CHO βIX/α3Y-F (L, lower receptor density) cells did not (Figure 6.3A, left). Interestingly, the level of surface expression of GP Ibα on the 3Y-F cells had to be about twice as high (H, higher receptor density) as the level of receptor
Figure 6.3. A. Rolling velocities and receptor densities of cells expressing wild-type or mutated tyrosine residues of GP Ibα on 50 μg/ml of vWF. Only cells that exhibited continuous rolling were included in velocity measurements. GP Ibα receptor densities were determined using flow cytometry as described in Section 2.3 and designated with a L or H for the 3Y-F cells for lower or higher receptor levels, respectively. Values are the mean of 88-295 cells from three to five experiments for each data point with SEM error bars. *p < 0.001, Student’s t-test, with respect to the receptor density of the wild-type cells. B. Percentage of cells interacting with the 50 μg/ml vWF coated surface that were exhibiting rolling or saltatory translocation. The percentage was calculated by dividing the number of saltatory translocating cells or rolling cells by the total number of cells interacting with the coated surface (rolling and saltatory translocation). Values are the mean of three to five experiments for each data point with SEM error bars. *p < 0.001, ANOVA f-test, with respect to the percentage of rolling and saltation of the wild-type cells.
expression on the wild-type and 3Y-E cells in order to see any cell rolling of the 3Y-F cells (Figure 6.3A, right). Any receptor densities lower than this failed to support 3Y-F rolling. The 3Y-F(H), 3Y-E, and wild-type cells all rolled on the vWf surface at similar velocities. We also noted that a portion of the cell types exhibited saltatory translocation reported as a percentage of the total number of cells exhibiting rolling and saltatory translocation (Figures 6.3B). No significant difference in the percentage of cells exhibiting saltatory translocation was observed between the wild-type cells and the 3Y-E mutants (both about 25%). On the other hand, a significantly greater number of cells exhibited saltatory translocation for the 3Y-F(H) mutants (about 80%) compared to the other cell types.

6.4 Discussion

The anionic sulfated region of GP Ibα has been shown previously to be important in the interaction of the GP Ib-IX-V complex with vWf in the presence of ristocetin and botrocetin.\textsuperscript{69,70,72} Additionally, it has been shown that this region is important in the interaction under dynamic conditions.\textsuperscript{9}

The primary focus of our current study was to determine if the primary role of post-translational tyrosine sulfation of residues Y276, Y278, and Y279 GP Ibα was to create three additional anionic charges in the anionic sulfated region of GP Ibα, which may be important in the GP Ib-IX-V – vWf interaction under high fluid shear stress. We found that both the 3Y-E and 3Y-F cells exhibited rolling and saltatory translocation on immobilized vWf at a wall shear stress of 10 dyn/cm\textsuperscript{2}. The mean rolling velocities of the mutant cells were not significantly different from the rolling velocity of the wild-type
cells. A difference was observed in the relative amount of rolling versus saltatory translocation between the two mutants, expressed as a percentage of all the cells interacting with the vWF surface. The 3Y-F cells exhibited at least twice as much saltatory translocation as the 3Y-E and wild-type cells. This decrease in the percentage of rolling cells for 3Y-F indicates that the three phenylalanine substitutions affected the ligand binding function of the GP Ib-IX-V complex. This finding correlates well with previous modulator-induced vWF binding studies, where it was shown that the 3Y-F cells failed to bind vWF in the presence of ristocetin or botrocetin (Dong et al., unpublished data).

When the GP Ibα surface expression of the 3Y-F cells was at similar levels as the wild-type cells, the 3Y-F cells failed to interact with immobilized vWF under fluid shear stress. This is consistent with a decrease in rolling on-rate (the rate of association between GP Ibα and vWF) that was large enough to prohibit 3Y-F cell tethering or rolling. When the receptor density of the mutant cells was approximately twice that of the wild-type cells, we observed both cell rolling and saltatory translocation of the 3Y-F cells.

An increase in the percentage of saltatory translocating cells, compared to wild-type, could be caused by alterations in the rolling on-rate, rolling off-rate, or both. For the 3Y-F cells with high levels of surface GP Ibα expression, the increase in the percentage of cells exhibiting saltatory translocation is consistent with an increase in the rolling off-rate (the rate of dissociation between GP Ibα and vWF). The off-rate could be too high, which would prohibit the majority of the cells from exhibiting continuous rolling. The rolling on-rate of the higher GP Ibα expressing 3Y-F cells was adequate in
supporting the initial tethering of the mutant cells to the vWF surface from the flow stream.

There are two possible causes for the decrease in decreased function (as exemplified by the requirement of higher levels of receptor expression and also by the increase in rolling off-rate) of the cells expressing the phenylalanine mutated GP Ibα. First, mutating the tyrosine residues to phenylalanine residues could alter the confirmation of the vWF binding site of GP Ibα. This is unlikely, though, because the GP Ibα antibodies AK2, AN51, C34, and TM60 can still bind to the 3Y-F cells (Dong et al., unpublished data). Only SZ2 binding to 3Y-F was inhibited by the mutations (Dong et al., unpublished data), which is expected because this antibody has been shown to bind to the anionic sulfated region (Try276-Glu282) of GP Ibα. Second, amino acid substitutions at residues Y276, Y278, and Y279 to phenylalanine eliminates the possibility of post-translational sulfation and results in the removal of three anionic charges within the anionic sulfated region of GP Ibα. In addition, the substitutions to phenylalanine replace polar, hydrophilic residues with non-polar, hydrophobic residues. This change in charge distribution and polarity could directly affect the GP Ib-IX-V complex interaction with vWF. On the other hand, mutating the tyrosine residues to glutamate residues maintained the net negative charges at residues 276, 278, and 279. The anionic charge retention could explain why the 3Y-E cells rolled at a similar velocity and exhibited similar percentage of saltatory translocation as wild-type cells. Our results suggest that the primary role of post-translational tyrosine sulfation of GP Ibα in vWF binding is to provide three additional anionic charges to the anionic sulfated region of GP Ibα.
In summary, we have demonstrated that charge distribution in the anionic sulfated region of GP Ibα is important in the binding of the GP Ib-IX-V complex with vWF under high shear conditions. Specifically, the anionic charges at residues Y276, Y278, and Y279, which in platelets are created by post-translational tyrosine sulfation, are necessary for optimal binding between the receptor and ligand.
CHAPTER 7

Mapping of the vWF Binding Region of GP Ibα

7.1 Introduction

Several studies have provided results that suggest that the leucine-rich repeat region, the disulfide loop region, and the anionic sulfated region of GP Ibα are important in the receptor ligand interaction. First, platelets from patients with BSS with mutations in the leucine-rich repeat region of GP Ibα are dysfunctional in binding vWF, which suggests that the leucine-rich repeat region of GP Ibα is important in the interaction of GP Ib-IX-V with vWF. Second, platelets from patients with Platelet-type von Willebrand Disease, that contain mutations in the first disulfide loop of the disulfide loop region, have suggested this region is important in the interaction because the mutated platelets bind vWF with increased affinity. Finally, three independent studies have demonstrated that the anionic sulfated region is important by showing that the three tyrosines within the region have to be sulfated in order for vWF to optimally bind GP Ibα in the presence of ristocetin.

In this study, we mapped specific spans of residues on GP Ibα that are involved in the GP Ib-IX-V – vWF interaction under high fluid shear stress using CHO cells that express chimeras of dog/human GP Ibα. Canine GP Ibα was chosen because it has been shown that dog platelets do not aggregate in the presence of human vWF and ristocetin.
7.2 Materials and Methods

Cell lines used in this study were created by Shen et al. (unpublished data) by transfecting CHO βIX cells with chimeric cDNA for GP Ibα as described in Section 2.1. The first group of chimeras used are called dog/human (DH) chimeras and consist of human GP Ibα with dog GP Ibα replacing part of the N-terminus of human GP Ibα. The dog/human chimeras range from having as little as 35 dog residues (~5% canine) up to 282 dog residues (~45% canine) (Figure 7.1). The second group of chimeras used are called re-humanized (RH) chimeras and consist of dog/human chimera 282 (DH282) with human GP Ibα replacing part of the N-terminus of DH282. The re-humanized chimeras consist of human GP Ibα, dog GP Ibα, and human GP Ibα from the N-terminus toward the C-terminus, respectively. As little as 59 residues of human GP Ibα and up to 176 residues of human GP Ibα are replaced in the N-terminal of DH282 (Figure 7.2). CHO αβIX cells were used as a positive control and CHO βIX and CHO α(dog)βIX cells were used as negative controls. Details of all the CHO cells used in this study can be found in Table 2.1.

In the rolling experiments, 0.6 ml of CHO cells in PBS at a density of 500,000/ml were injected into the chamber and incubated with coverslips coated with a solution of 50 μg/ml of vWf (Section 2.4). Following an incubation period of one minute, flow was initiated creating a wall shear stress of 10 dyn/cm² (Section 2.5). Subsequent rolling velocity calculations were performed according to procedures in Section 2.6. The surface level of GP Ibα on the CHO cells was determined by flow cytometry as described in Section 2.3.
Figure 7.1. Schematic of the N-terminal 45 kDa region of the cells expressing dog/human chimeras of GP Ibα. The dog/human (DH) chimeras were created by sequentially replacing the structural segments within the N terminal 45 kDa region of human GP Ibα with the same residues of dog GP Ibα. LRR = leucine-rich repeat. ASR = anionic sulfated region.
Figure 7.2. Schematic of the N-terminal 45 kDa region of the cells expressing re-humanized dog/human chimeras of GP Ibα. The re-humanized chimeras were created by sequentially removing dog sequences of DH282 and replacing with the N-terminal structures of human GP Ibα. LRR = leucine-rich repeat.
7.3 Results

Rolling of CHO cells expressing dog/human chimeras of GP Ibα on immobilized human vWF. To evaluate the effects of replacing the N-terminus of human GP Ibα with the N-terminus of dog GP Ibα on cell rolling on immobilized vWF, Shen et al. (unpublished data) created CHO cell lines expressing dog/human chimeras of GP Ibα (Figure 7.1). Before using the chimeras, we investigated the ability of CHO βIX cells expressing the entire dog GP Ibα subunit to roll on human vWF. We found that the CHO α(dog)βIX cells and CHO βIX cells did not roll on human vWF whereas the CHO αβIX cells did. With this knowledge, we proceeded to test the ability of the dog/human chimeras to roll on human vWF. We observed rolling only for the DH35 and DH59 chimeras with rolling velocities more than twice the rolling velocity of the CHO αβIX cells (Figures 7.3 and 7.4A). We also noted that a portion of the DH35 and DH59 cells exhibited saltatory translocation given as a percentage of the total number of cells exhibiting rolling and saltatory translocation (Figures 7.3 and 7.4B). Additionally, the DH200 cells exhibited 100% saltatory translocation without any cell rolling. The percentage of saltatory translocation is significantly greater for the DH35, DH59, and DH200 chimeras compared to the CHO αβIX cells.

Effects of re-humanizing the dog/human chimeras of GP Ibα on cell rolling on immobilized human vWF. To investigate if the rolling interaction between human vWF and the cells expressing dog/human chimeras of GP Ibα could be reestablished by replacing some of the dog GP Ibα residues with human GP Ibα residues, CHO cells expressing re-humanized chimeras of GP Ibα were created (Figure 7.2). Of these
Figure 7.3. Video images of cells expressing human GP Ibα or chimeras of dog/human GP Ibα rolling on immobilized vWF (coverslips coated with a solution of 50 μg/ml of vWF). Cells were injected into the parallel-plate flow chamber and incubated for one minute after which flow was initiated at a wall shear stress of 10 dyn/cm². Cell rolling was recorded real-time using a VCR and later analyzed off-line using digital imaging software. These video images were created by overlapping 60 frames for 2 seconds that resulted in an image that shows the path traveled by the CHO cells during this duration. The top row of images was created from overlapping recorded video images during the incubation of the cells in the chamber prior to the initiation of flow. The bottom row of images was created from overlapping recorded video images during flow (one minute after initiating flow). The path distance was measured and the average rolling velocity of each cell was obtained by dividing this distance by the elapsed time (2 sec). Arrows indicate positions of temporary rolling of cells exhibiting saltatory translocation between intervals of returning to the flow stream.
Figure 7.4. A. Rolling velocities of cells expressing wild-type GP Ibα or chimeras of dog/human GP Ibα on a surface coated with a solution of 50 μg/ml of vWF. Cells were injected into the parallel-plate flow chamber and incubated for one minute after which flow was initiated at a wall shear stress of 10 dyn/cm². Only cells that exhibited continuous rolling were included in velocity measurements. Values are the mean of 83-412 cells from three experiments for each data point with SEM error bars. *p < 0.001, Student’s t-test, with respect to the velocity of the wild-type cells. B. Percentage of cells interacting with the vWF coated surface that were exhibiting saltatory translocation. The percentage was calculated by dividing the number of saltatory translocating cells by the total number of cells interacting with the coated surface (rolling and saltatory translocation). Values are the mean of three experiments for each data point with SEM error bars. *p < 0.001, ANOVA f-test, with respect to the percentage of saltatory translocation of the wild-type cells.
mutants, we observed cell rolling in only the RH152 cells, which rolled approximately
twice as fast as the CHO αβIX cells. Interestingly, more than 80% of all the RH152 cells
that were interacting with vWF exhibited saltatory translocation instead of cell rolling
(Table 7.1). Saltatory translocation was also exhibited by the RH128 and RH176 cells
but the increase in salutation compared to wild-type was only significant for the RH128
chimeras (100% vs. <10%).

7.4 Discussion

The purpose of this study was to map vWF-binding sequences on GP Ibα because
currently the vWF-binding site is known to bind the N terminal 45 kDa region of GP Ibα,
a region that is very large containing about 300 residues. To achieve this goal, we have
created CHO cell lines that express dog/human chimeras of GP Ibα. The lengths of dog
GP Ibα replacing human GP Ibα were chosen based on the strategy of sequentially
replacing the structural segments within the N terminal 45 kDa region of human GP Ibα
with the same residues of dog GP Ibα. Table 7.2 lists the structures in the N terminal 45
kDa region of human GP Ibα.

In order to map vWF-binding sites on GP Ibα, we investigated the effects of
replacing the N-terminal of human GP Ibα with dog GP Ibα on cell rolling on
immobilized human vWF under high fluid shear stress. We found that DH35 and DH59
rolled on the human vWF surface suggesting that the human N-terminal disulfide loop
and the first LRR may not be ligand binding sites for vWF but rather may be regulatory
elements for the GP Ib-IX-V - vWF interaction. Although these structures may not be
Table 7.1. Interaction of cells expressing the re-humanized dog-human chimeric GP Ibα with immobilized human vWF under high fluid shear stress.*

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>CHO βIX</th>
<th>CHO αβIX</th>
<th>RH59</th>
<th>RH81</th>
<th>RH104</th>
<th>RH128</th>
<th>RH152</th>
<th>RH176</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell rolling</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Rolling velocity (μm/s)</td>
<td>-</td>
<td>68±1.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>126±6.7**</td>
<td>-</td>
</tr>
<tr>
<td>Saltatory translocating cells</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

*Grades were defined as follows; +, <10% cells, ++, 11 - 79% cells, and ++++, more than 80% cells. The percentage was among cells that interacted with immobilized vWF by either tethering, saltatory translocating, or rolling and only velocity for rolling cells were presented here.

** p < 0.001, Student’s t-test, with respect to the velocity of the wild-type cells.
Table 7.2. Structures in the N-terminal 45 kDa Region of GP Ibα.

<table>
<thead>
<tr>
<th>Structures in the N-terminal 45 kDa Region of GP Ibα</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disulfide loop</td>
</tr>
<tr>
<td>1st leucine-rich repeat</td>
</tr>
<tr>
<td>2nd leucine-rich repeat</td>
</tr>
<tr>
<td>3rd leucine-rich repeat</td>
</tr>
<tr>
<td>4th leucine-rich repeat</td>
</tr>
<tr>
<td>5th leucine-rich repeat</td>
</tr>
<tr>
<td>6th leucine-rich repeat</td>
</tr>
<tr>
<td>7th leucine-rich repeat</td>
</tr>
<tr>
<td>Disulfide loop</td>
</tr>
<tr>
<td>Disulfide loop</td>
</tr>
<tr>
<td>Anionic sulfated region</td>
</tr>
</tbody>
</table>

ligand binding sites for vWF, replacing these structures with dog GP Ibα did affect the properties of the interaction. First, both the DH35 and DH59 cells rolled at a velocity more than twice the velocity of the CHO αβIX cells indicating that the presence of the dog sequences caused an increase in the off-rate of rolling, exemplifying a decrease in the interaction strength between GP Ibα and vWF. Second, a significant portion of the DH35 and DH59 cells also exhibited saltatory translocation. This type of receptor-ligand interaction under fluid shear stress could be the result of altering the off-rate of the interaction to an extent that can no longer support continuous rolling. The other dog/human chimeras (DH81 through DH282) did not roll on immobilized vWF. This suggests that the second LRR of GP Ibα is critical for the receptor-ligand interaction because all the dog/human chimeras missing this repeat failed to roll.

The only other dog/human chimera besides DH35 and DH59 to interact with the human vWF matrix is DH200. These cells did not roll, but rather exhibited 100% saltatory translocation. The reason for this minimal regain of function for DH200 could
be that DH200 contains a long stretch of the N-terminal sequence of dog GP Ibα that could provide a tertiary structure similar to that of human GP Ibα.

Next, we tried to regain the receptor-ligand interaction between the dog/human chimeric GP Ibα of DH282 and human vWF under fluid shear stress by re-humanizing the DH282 chimera. This was done by sequentially removing dog sequences of DH282 and replacing with the N-terminal structures of human GP Ibα. We observed an interaction between human vWF and the re-humanized chimeras, RH128, RH152, and RH176. All three cell types exhibited saltatory translocation with RH128 and RH176 exhibiting 100% and RH152 exhibiting more than 80%. This indicates that some function, but not all, was regained by replacing the human second, third, and fourth LRRs (residues 59-128). The lack of complete recovery of function could be due to the fact that all the re-humanized chimeras still contain dog sequences in place of the residues of the human disulfide loop region and anionic sulfated region. The only re-humanized chimera that rolled was RH152, but it rolled at a velocity more than twice the velocity of the CHO αβIX cells (consistent with an increased off-rate, decreased interaction strength), indicating that replacing the fifth LRR did not regain the interaction properties of the CHO αβIX – human vWF interaction.

In conclusion, we have found that essentially all the residues in the N-terminal 282 residues of human GP Ibα have some importance in the GP Ib-IX-V complex interaction with human vWF. Using the dog/human chimera, we found the N-terminal 35 residues and the residues of the first LRR of human GP Ibα may not be ligand binding sites for vWF but rather may be regulatory elements for the GP Ib-IX-V – vWF interaction. Using the re-humanized chimeras, we found evidence to further support the
importance of the second, third, and fourth LRRs in the receptor-ligand interaction. Additional studies using different sets of dog/human chimeras of GP Ibα will provide more insight into identifying key structures in the N-terminal 45 kDa region of GP Ibα that are involved in the interaction of GP Ibα with vWF.
CHAPTER 8

Truncation of the Cytoplasmic Domain of GP Ibα Decreases the Strength of Interaction of Glycoprotein Ib-IX-V with Immobilized von Willebrand Factor under High Fluid Shear Stress

8.1 Introduction

The last 100 amino acids of the carboxyl-terminus constitute the cytoplasmic domain of GP Ibα, through which the complex interacts with the platelet cytoskeleton and other proteins. Previous studies have examined the possibility that the cytoplasmic region of GP Ibα may modulate vWF binding. Using a fluorescence recovery after photobleaching technique, Dong et al. found that truncating the cytoplasmic region of GP Ibα allowed the GP Ib-IX complex, which is anchored to the plasma membrane, to become mobile. Increasing the mobility of the GP Ib-IX-V complex on the plane of the plasma membrane led to decreased ristocetin-induced vWF binding, indicating that the anchorage is critical for optimal binding of vWF to the complex. This observation was further supported by a recent study showing CHO cells expressing a mutated GP Ibα with its ABP binding region deleted rolled faster on immobilized vWF under high fluid shear-stress conditions compared to cells expressing wild-type GP Ibα. Although Cunningham et al. found no difference in botrocetin-
induced vWF binding to CHO and melanoma cells expressing the cytoplasmic truncated GP Ibα, they observed a change in cell spreading pattern on immobilized vWF.74

We have investigated the effect of the GP Ibα cytoplasmic truncations on the interaction of the GP Ib-IX complex with immobilized vWF under high fluid shear stress. Previous studies have shown that the GP Ibα antibodies, AK2 and SZ2, bind to CHO cells expressing GP Ibα with cytoplasmic truncations at similar levels as CHO cells expressing wild-type GP Ibα (Figure 8.1) (Dong et al., unpublished data).

8.2 Materials and Methods

The cell lines used in this study were created by transfecting CHO βIX cells with truncated cDNA for GP Ibα as described in Section 2.1. Seven CHO cell lines were used in this study expressing GP Ibα with its cytoplasmic domain truncated to various lengths. For example, CHO βIX/α604 cells express GP Ibα consisting of residues 1-604 with the rest of the C-terminus of GP Ibα deleted (truncated). CHO αβIX cells were used as a positive control and CHO βIX cells were used as a negative control. Details of all the CHO cells used in this study can be found in Table 2.1.

Two types of rolling experiments were performed in this study: injection and perfusion. In the injection rolling experiments, 0.6 ml of CHO cells at a density of 500,000/ml were injected into the chamber and incubated with coverslips coated with solutions of 5 or 50 μg/ml of vWF (Section 2.4). Following an incubation period of one minute, flow was initiated creating a wall shear stress of 10 dyn/cm² according to Section 2.5. In the perfusion experiments, CHO cells at a density of 100,000/ml were perfused
Figure 8.1. Antibody binding to cells expressing truncated GP Ibα. The binding of the GP Ibα antibodies, AK2, SZ2, and WM23, to cells expressing truncated and wild-type GP Ibα was determined by measuring the fluorescence using a flow cytometer as described in Section 2.3. The ratio of specificity antibody binding was calculated by dividing the binding fluorescence level of the antibody being tested by the binding fluorescence level of the reference antibody WM23. Values are from a representative of three independent experiments. (Performed by Dong et al., unpublished data)
through the flow chamber for four min at flow rates that generated wall shear stresses of 5, 10, and 15 dyn/cm² according to Section 2.5. Subsequent rolling velocity calculations were performed according to Section 2.6. The surface level of GP Ibα on the CHO cells was determined by flow cytometry as described in Section 2.3.

8.3 Results

Rolling of CHO cells on immobilized vWf under shear conditions. The effect of truncating the cytoplasmic region of GP Ibα on receptor-ligand interactions under high fluid shear stress was evaluated by using a parallel-plate flow chamber system described previously.9 Before incubating the CHO cells in the chamber, flow cytometry was performed to assure that all the cells expressed the same amount of wild-type GP Ibα or mutated GP Ibα. After one minute of incubation in the chamber with coverslips incubated with solution of 50 μg/ml of vWf, flow was introduced to generate a wall shear stress of 10 dyn/cm². Under these conditions, the CHO cells expressing wild-type GP Ibα and all of the CHO cells expressing truncated GP Ibα rolled on the immobilized vWf surface (Figure 8.2). Comparing the mean rolling velocity of the CHO αβIX cells with the truncated cells revealed an significant increase in velocity for all the truncated cell types except CHO βIX/α518 cells. Based on their rolling velocities, the truncated cells can be divided into two groups; truncations that preserved its association with the cytoskeleton (CHO βIX/α604, α594, α582) or eliminated it (CHO βIX/α556, α544, α533).39 The mean rolling velocity of the second group of cells was significantly higher
Figure 8.2. Rolling comparison of CHO cells expressing wild-type or truncated GP Ibα on immobilized vWF (coverslips coated with a solution of 50 µg/ml of vWF). Cells were injected into the parallel-plate flow chamber and incubated for one minute after which flow was initiated at a wall shear stress of 10 dyn/cm². Values are the mean of 201-634 cells from three experiments for each data point with SEM error bars. *p < 0.05, Student’s t-test, with respect to the velocity of the wild-type cells.
than that of the former group with the exception of α518 cells that rolled at a velocity similar to that of wild-type.

To investigate the role of immobilized vWF concentration in the rolling velocity of CHO cells with and without truncations of GP Ibα, we again injected the cells into the flow chamber but the concentration of the vWF solution incubated on the coverslip surface was reduced to 5 µg/ml. The lower concentration of vWF supported continuous cell rolling of all the cell types at a wall shear stress of 10 dyn/cm² (Figure 8.3) with the rolling velocities of mutant cells again being higher than the velocity of wild-type cells. Compared to rolling on the high concentration of vWF, two different features of cell rolling were observed with the low concentration of immobilized vWF. First, the rolling velocity of all the cell types increased when the vWF concentration decreased from 50 to 5 µg/ml (Figure 8.4A). The most dramatic increase in velocity occurred in cells expressing the α518 mutant with an increased of more than 80% (56 ± 5 vs. 92 ± 3 µm/sec). Excluding CHO βIX/α518 cells, the amount of velocity increase was greater in the cells expressing truncated GP Ibα that preserved its association with the cytoskeleton so that overall there was no significant difference in velocity among the truncations except for the CHO βIX/α556 cells which rolled significantly faster. Second, reducing the vWF concentration resulted in a percentage of cells showing saltatory translocation (Figure 8.4B). If the cell rolled a short distance, returned to the flowing stream, and then rolled a short distance again, it was considered to be saltatory translocation. The percentage of cells interacting with vWF that showed saltatory translocation was greater for all the cells expressing truncated GP Ibα compared to cells expressing wild-type GP
Figure 8.3. Video images of cells expressing truncated GP Ibα rolling on immobilized vWF (coverslips coated with a solution of 5 μg/ml of vWF). Cells were injected into the parallel-plate flow chamber and incubated for one minute after which flow was initiated at a wall shear stress of 10 dyn/cm². Cell rolling was recorded real-time using a VCR and later analyzed off-line using digital imaging software. These video images were created by overlapping 60 frames for 2 seconds that resulted in an image that shows the path traveled by the CHO cells during this duration. The top row of images was created from overlapping recorded video images during the incubation of the cells in the chamber prior to the initiation of flow. The bottom row of images was created from overlapping recorded video images during flow (snapped one minute after initiating flow). The path distance was measured and the average rolling velocity of each cell was obtained by dividing this distance by the elapsed time (2 sec). Arrows indicate positions of temporary rolling of cells exhibiting saltatory translocation between intervals of returning to the flow stream.
Figure 8.4. A. Rolling velocity comparison of CHO cells expressing wild-type or truncated GP Ibα on coverslips coated with a solution of 5 μg/ml of vWF. Cells were injected into the parallel-plate flow chamber and incubated for one minute after which flow was initiated at a wall shear stress of 10 dyn/cm². Only cells that exhibited continuous rolling were included in velocity measurements. Values are the mean of 22-376 cells from three to five experiments for each data point with SEM error bars. *p < 0.001, Student’s t-test, with respect to the velocity of the wild-type cells. B. Percentage of cells interacting with the 5 μg/ml vWF coated surface that showed saltatory translocation. A rolling cell was defined as cell that maintained continuous contact with the vWF coverslip. The percentage was calculated by dividing the number of saltatory translocation cells by the total number of cells interacting with the coated surface (rolling and saltatory translocation). Values are the mean of three to five experiments for each data point with SEM error bars. *p < 0.001, ANOVA f-test, with respect to the percentage of saltatory translocation of the wild-type cells.
Ibα. The cells expressing GP Ibα truncations that eliminated its cytoskeleton association had the highest percentage of cells showing saltatory translocation.

**Effects of wall shear stress on rolling velocity of cells expressing truncated GP Ibα.** To investigate the ability of CHO cells expressing truncated GP Ibα to tether to the vWF coated coverslip from the flow stream, the cells were perfused over immobilized vWF (50 μg/ml). At a wall shear-stress of 5 dyn/cm², the wild-type and truncated GP Ibα cells tethered to the vWF surface and then exhibited continuous rolling on the surface (Figure 8.5). All the cells expressing truncated GP Ibα rolled faster than cells expressing wild-type GP Ibα with CHO βIX/α556 cells rolling significantly faster than the other truncation cells.

We also examined the effect of wall shear stress on the rolling velocity of cells expressing truncated GP Ibα by performing a shear titration study. Varying the perfusion flow rate of the cell suspension through the flow chamber created wall shear stresses of 5, 10, or 15 dyn/cm². For CHO αβIX cells, no significant change in rolling velocity occurred from 5 to 10 dyn/cm² (Figure 8.6), but increasing to 15 dyn/cm² resulted in a significant almost 2-fold increase in velocity. The rolling velocity of cells expressing truncated GP Ibα fell into two patterns. The rolling velocity of cells expressing truncations that preserved the cytoskeleton association of the GP Ib-IX-V complex (CHO βIX/α604 and CHO βIX/α582) increased dramatically when shear stress increased from 5 to 10 dyn/cm² and remained unchanged when shear-stress was increased to 15 dyn/cm² (Figure 8.6). In contrast, cells expressing truncations that eliminated the cytoskeleton association of GP Ibα (CHO βIX/α544 and CHO βIX/α533) showed no significant change when wall shear stress increased from 5-15 dyn/cm² (Figure 8.6).
Figure 8.5. Comparison of perfusion rolling velocities of CHO cells expressing wild-type or truncated GP Ibα. CHO cells (100,000 cells/ml) were perfused over coverslips coated with a solution of 50 μg/ml of vWF at a wall shear stress of 5 dyn/cm². Values are the mean of 100-300 cells from three experiments for each data point with SEM error bars. *p < 0.05, Student’s t-test, with respect to the velocity of the wild-type cells.
Figure 8.6. Effect of shear stress on mean rolling velocity of cells expressing truncated GP Iβα. CHO cells (100,000 cells/ml) were perfused through the parallel-plate flow chamber at flow rates of 0.57, 1.14, and 1.71 ml/min generating wall shear stresses of 5, 10, and 15 dyn/cm², respectively. Values are the mean of 100-300 cells from three experiments for each data point with SEM error bars.
8.4 Discussion

The platelet GP Ib-IX-V – vWF interaction plays a critical role in arresting bleeding following injury to the blood vessel wall and in pathologic mural arterial thrombosis. Previous studies have shown that truncating the cytoplasmic domain of GP Ibα decreased ristocetin-induced vWF binding, but not botrocetin-induced vWF binding. Furthermore, cells expressing a GP Ibα mutant with a 41 amino acid deletion adhered and spread on immobilized vWF with thin and multi-branched protrusions as compared to a few short protrusions extended by wild-type cells.

The primary focus of our current study was to determine if truncating the cytoplasmic domain of GP Ibα also affects the interaction between the GP Ib-IX-V complex and vWF under high fluid shear-stress. We found that all the CHO cells expressing truncated GP Ibα rolled on immobilized vWF at two different densities (5 and 50 μg/ml) under three different wall shear-stresses (5, 10, and 15 dyn/cm²). The mean rolling velocities of the mutant cells were significantly higher than that of wild-type cells (Figures 8.2, 8.4A, and 8.5). Increases in rolling velocity are consistent with an increase in off-rate of the bonds between GP Ibα and vWF, one of two critical components in defining cell rolling.

Next we examined if truncating the cytoplasmic domain of GP Ibα altered the efficiency of cells to tether to the vWF matrix (related to on-rate) against opposing forces generated by fluid shear stress. Cells were directly perfused over immobilized vWF without initial incubation of the receptor and ligand. Perfusion studies revealed that the truncated GP Ibα cells retained the ability to tether and roll on immobilized vWF. At 5 dyn/cm², the same pattern of increased rolling velocity of the cells expressing truncated
GP Ibα compared to wild-type cells was followed, consistent with the hypothesis that the on-rate of the bond between GP Ibα and vWF was not affected by the cytoplasmic truncation of GP Ibα. Therefore the increases in rolling velocity seen in the cells expressing the cytoplasmic truncations of GP Ibα are consistent with increases in bond off-rate (the rate of dissociation between GP Ibα and vWF). Recently, Cranmer et al. showed that CHO cells expressing GP Ibα with cytoplasmic deletion of 535-586 rolled 50% faster than CHO cells expressing wild-type GP Ibα at a shear rate of 3000 s⁻¹.¹⁰

Savage et al. observed that platelets can roll on immobilized vWF in vitro as defined as a movement of platelets along the flow direction while maintaining constant contact with the immobilized matrix under constant fluid shear-stress.²⁰ The phenomenon was reproduced in CHO cells expressing the GP Ib-IX-V complex,⁹ demonstrating that rolling can be induced specifically by the interaction between GP Ibα and vWF. This new phenomenon suggests some unique features of the interaction between the GP Ib-IX-V complex and vWF. First, the interaction has to be initiated quickly (fast on-rate) to allow the cells to form tethering bonds with the matrix at very short contact times against the forces created by the fast flowing fluid. Second, a rate of dissociation (off-rate) has to be balanced with fluid shear stress so the ligand-receptor bond can be constantly broken by flowing fluid. Mutating the ligand or the receptor can alter both the on- and off-rate.

Although the mechanisms through which the cytoplasmic domain of GP Ibα affects the ligand binding function of the GP Ib-IX-V complex remain to be determined, previous studies have provided clues as to what happens when the cytoplasmic domain of GP Ibα is deleted partially or completely. As shown by Andrews et al.,³⁸ the GP Ib-IX-V
complex interacts with platelet's cytoskeleton through GP Ibα and the sequence through which the interaction occurs was later defined\textsuperscript{39} (Thr536-Phe568). One direct consequence of such an association may be to anchor the complex to the plane of plasma membrane as suggested by a fluorescence recovery after photobleaching technique.\textsuperscript{75} When the association is disrupted by truncating the cytoplasmic region of GP Ibα, the complex becomes mobile on the plasma membrane.\textsuperscript{75} The change in lateral mobility of the complex could affect its ligand binding function in at least three ways. First, the conformation of the vWF binding site of GP Ibα could be altered. This is unlikely because the antibodies AK2 and SZ2 can still bind truncated GP Ibα. These antibodies have been shown to block modulator-induced vWF binding\textsuperscript{69,78} and AK2 has also been shown to block the rolling of cells expressing the GP Ib-IX-V complex on immobilized vWF.\textsuperscript{9} Second, the mobility change could affect the formation of a functional receptor. Previous studies have shown that a functional receptor for vWF may consist of more than one GP Ib-IX-V complex\textsuperscript{11} and receptor formation may depend on the association of the complex with the cytoskeleton. A functional receptor may be less likely to form among GP Ib-IX-V complexes that are highly mobile. Third, the ligand of the GP Ib-IX-V complex, vWF, is unique in its multimeric structure and such a structure of domain repeats could allow vWF to bind multiple complexes simultaneously. Since the multiple A1 domains (the GP Ibα binding domain of vWF) in a vWF polymer are likely spaced equally, they may require the GP Ib-IX-V complex to be arranged in such a geometrical array on the cell surface that it can receive the ligand optimally. Once the fixed array of the receptor is disoriented, optimal ligand binding could be affected. Our hypothesis is
that the change in lateral mobility of the complex affects its ligand binding function by altering in the arrangement of the subunits of the receptor.

One interesting observation was that CHO βIX/α518 cells rolled at a velocity similar to wild-type cells on a vWF concentration of 50 mg/ml but rolled faster on lower vWF density (5 μg/ml) when the cells were allowed to incubate with the immobilize vWF (Figures 8.2 and 8.4A). The cells also rolled at a higher velocity when they were perfused over the vWF matrix (Figure 8.5), which eliminated cell incubation with the vWF. Coincidentally, this mutant has the greatest lateral mobility of all the truncated mutants.75 A logical explanation could be because when the α518 mutant is completely mobile on the cell surface, the complex can aggregate when it interacts with vWF. Complex aggregation may lead to locally concentrated complexes that can compensate for the lack of proper receptor formation and proper geometrical array of the receptor for polymeric vWF. However, when the ligand density is decreased, complex aggregation reduces to such an extent that it can no longer compensate for the lack of proper receptor formation and arrangement. Similarly, when the α518 mutant was perfused over the lower concentration vWF matrix, it was deprived its chance to aggregate the complex and thus rolled faster than the wild-type cells. Another possible reason why the α518 mutant rolled at a similar velocity as the wild-type cells could be that truncating almost the entire cytoplasmic domain of GP Ibα eliminated the association of another, currently unknown, signaling protein.

In summary, the cytoplasmic domain of GP Ibα is important in GP Ib-IX-V – vWF interaction under both static and flow conditions. Information on the detailed
molecular mechanisms by which this occurs will help with our understanding of
pathologic thrombosis and with the development of novel therapeutics to help prevent it.
CHAPTER 9

Overall Conclusions and Future Work

9.1 Overall Conclusions

Previous studies have shown that one or more vWF binding sites are located in the N-terminal 45 kDa region of GP Ibα.\textsuperscript{53-58} Our data suggests that one the primary binding site within this N-terminal region is located within leucine-rich repeats 1-6 of GP Ibα. Also, we showed that the more N-terminal LRRs are more likely involved in ligand binding and the more C-terminal LRRs are probable important in forming the tertiary structure of the entire LRR region. Additional studies using dog/human chimeras of GP Ibα narrowed the possible binding region even further, where we found that leucine-rich repeats 2-4 are critical for the GP Ib-IX-V – vWF interaction.

But this is not the whole story, we also found that all the regions within the 45 kDa region of GP Ibα are important in the receptor-ligand interaction including the disulfide loop region and the anionic sulfated region. We could not conclude that these other regions are critical for the interaction of GP Ib-IX-V with vWF, but mutational analysis of both of these regions did result in altered ligand binding. This finding suggests that the conformation of the ligand binding site is dependent on the overall conformation of the N-terminal domain of the GP Ibα subunit. Taking this concept one step further, our findings also suggest that the formation of an optimal vWF binding site...
depends on the overall subunit arrangement within a receptor, as supported by the fact that the association of the cytoplasmic region of GP Ib\(\alpha\) with the cytoskeleton is also important in the GP Ib-IX-V – vWF interaction under fluid shear stress.

In summary, the interaction of the GP Ib-IX-V complex with vWF under fluid shear stress probably occurs through a primary binding site within the 2-4 LRRs of GP Ib\(\alpha\) with additional contributions from surrounding structures, which might be required for the optimal conformation of the ligand binding site. The detailed mechanisms of binding and the mechanisms behind how certain domains of GP Ib\(\alpha\) modulate the receptor-ligand interaction remain unknown. In order to develop novel therapeutics for the prevention and treatment of mural arterial thrombosis, the study of the interaction between GP Ib-IX-V and immobilized vWF needs to be continued under fluid flow conditions.

9.2 Future Work

Listed below are a few ideas for future exploration of the interaction between the GP Ib-IX-V complex and vWF under flow conditions based on our current findings.

In our studies using dog/human chimeras and mutational analysis of the leucine-rich repeat region, we found that the LRRs of GP Ib\(\alpha\) are important in the GP Ib-IX-V – vWF interaction under fluid shear stress. The specific repeat or combination of repeats that are involved in the direct binding of vWF remains unknown. Future studies could include transfecting CHO cells with GP Ib\(\alpha\) that contains individual and combinations of deletions of entire leucine-rich repeats and then study the ability of these mutants to interact with vWF under flow.
Also using mutational analysis, we found that the disulfide loop region of GP Ibα modulates the strength of the receptor-ligand interaction under flow. Future work could investigate how the valine content of this region may be involved in this mechanism of modulation. Studying the interaction between CHO cells expressing GP Ibα with multiple simultaneous valine mutations in the disulfide loop region, and immobilized vWF may provide insight into this hypothesis.

We have shown that the post-translational tyrosine sulfation of the anionic sulfated region of GP Ibα is important in the GP Ib-IX-V – vWF interaction under fluid shear stress because it adds three additional anionic charges to the region which are necessary for optimal binding. The importance of the many other anionic charges in the region in the interaction under flow could be investigated using mutation analysis to eliminate individual or combinations of anionic charges from the region.
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