Identification of the Regions in Factor V Mediating its Edocytosis by Megakaryocytes to Form the Unique Platelet-Derived Cofactor Molecule

Sarah Abdalla
University of Vermont

Follow this and additional works at: https://scholarworks.uvm.edu/graddis

Recommended Citation
Abdalla, Sarah, "Identification of the Regions in Factor V Mediating its Edocytosis by Megakaryocytes to Form the Unique Platelet-Derived Cofactor Molecule" (2013). Graduate College Dissertations and Theses. 6.
https://scholarworks.uvm.edu/graddis/6

This Thesis is brought to you for free and open access by the Dissertations and Theses at ScholarWorks @ UVM. It has been accepted for inclusion in Graduate College Dissertations and Theses by an authorized administrator of ScholarWorks @ UVM. For more information, please contact donna.omalley@uvm.edu.
IDENTIFICATION OF THE REGIONS IN FACTOR V MEDIATING ITS ENDOCYTOSIS BY MEGAKARYOCYTES TO FORM THE UNIQUE PLATELET-DERIVED COFACTOR MOLECULE

A Thesis Presented

By

Sarah Abdalla

To

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements for the Degree of Master of Science Specializing in Biochemistry

October, 2012
Accepted by the Faculty of the Graduate College, The University of Vermont, in partial fulfillment of the requirements for the degree of Master of Science, specializing in Biochemistry.

Thesis Examination Committee:

____________________________________ Advisor
Beth A. Bouchard, Ph.D.

____________________________________
Robert J. Hondal, Ph.D.

____________________________________
Paula B. Tracy, Ph.D.

____________________________________
Russell P. Tracy, Ph.D.

____________________________________ Chairperson
Mary Cushman, M.D., M.Sc.

____________________________________ Dean, Graduate College
Domenico Grasso, Ph.D.

Date: August 21, 2012
ABSTRACT

Factor Va is a plasma protein that plays an important role in the regulation of blood coagulation by serving as the essential cofactor in thrombin generation via the prothrombinase complex. The procofactor, factor V, exists in two whole blood pools with 75-80% found in plasma, and 20-25% stored in the α-granules of platelets. As compared to the plasma procofactor, platelet-derived factor V is physically and functionally distinct, and displays a more procoagulant phenotype. Despite these profound differences, platelet-derived factor V originates via endocytosis of the plasma-derived procofactor by megakaryocytes. Endocytosis is mediated by two receptors: an unidentified, specific factor V receptor, and low density lipoprotein (LDL) receptor related protein-1 (LRP-1), a ubiquitous receptor that plays a role in endocytosis of proteins targeted for lysosomal degradation. These observations represent a novel role for LRP-1 in endocytosis of a protein that is functionally modified, and not targeted for lysosomal degradation. The goal of this study is to define the factor V regions involved in its interactions with the unidentified factor V receptor and LRP-1 expressed on megakaryocytes to begin to elucidate the molecular mechanisms regulating formation of the unique platelet-derived cofactor. Epitope mapping studies were performed using anti-factor V monoclonal antibodies, E9 and anti-factor V #2. Previous observations indicated that these factor Va light chain antibodies inhibited endocytosis of factor V by megakaryocytes. However, subsequent analyses demonstrated that only E9 inhibited both factor V binding and endocytosis. Thus, it was used for these studies. Western blotting of factor V and Va suggested that E9 recognizes a conformation-dependent epitope, which precluded the use of conventional epitope mapping approaches used for linear epitopes. E9 had no effect on factor Va cofactor activity in a plasma-based clotting assay suggesting that it does not perturb factor Va’s interactions with the membrane surface or factor Xa. Cleavage of lipid-bound factor Va by factor Xa at Arg1765 was also not affected by the presence of E9 suggesting that the epitope is not directed against this cleavage site. When E9 was used to immunoprecipitate the factor Xa-generated light chain cleavage products, both the 48/46 and 30 kDa light chain fragments were captured. These observations were confirmed using a solid phase competition assay where factor Xa-cleaved factor Va inhibited binding of 125I-factor V to E9 as well as intact factor V or Va. Limited proteolysis of the factor Va light chain with trypsin or Asp-N, generated products that were no longer detectable in this assay. These combined observations suggest that the anti-factor V light chain antibody, E9, has an epitope that is conformation-dependent and extremely labile. Future directions and alternative approaches are discussed.
DEDICATION

This thesis is dedicated to my mother, Elizabeth Abdalla, without whom my academic journey in the United States would not be possible. Your unconditional love and support has contributed largely to the person that I am today. I am truly blessed to have you and words cannot describe my gratitude.
ACKNOWLEDGEMENTS

First and foremost, I would like to acknowledge my mentor, Dr. Beth Bouchard, who has been an encouraging and great teacher during my graduate career. I would like to thank you particularly for your support, guidance, and patience over the years. Overall, working with you has been an absolute joy. I would also like to thank Dr. Robert Hondal for introducing me to the Biochemistry department at the University of Vermont, for serving as a member of my graduate studies committee, and for providing assistance when needed. Additionally, I would like to thank the remainder of my graduate studies committee, Dr. Paula Tracy, Dr. Russ Tracy, and Dr. Barbara Grant for being an excellent team and for providing helpful criticism and feedback over the years. Your input on my project and overall performance in graduate school has contributed largely towards my academic growth. I would also like to extend my gratitude to the newest addition to my committee, Dr. Mary Cushman, for agreeing on an impromptu basis to serve as my chair, and for getting me involved in the annual American Heart Association Heart Walk.

Finally, I would like to thank my friends in the Biochemistry department and the College of Medicine, both current and past members, Francis Ayombil, Adam Lothrop, Jeremy Wood, Gregg Snider, Naiara Barbosa, Stell Patadji, Khine Win, and Makeda Semma. In particular, I would like to thank my best friend, Shaun Mascarenhas, who has always been there for me. I cherish our friendship, and simply cannot put into words how much it means, and hope that similar to old wine, it will continue to improve with age. My gratitude goes out to Lyndelle LeBruin, and Andra Telemacque, both whom have
served as my major support system in my home away from home. Out of all my friends Lyndelle LeBruin is one of the few who can relate to the graduate school journey. Your warm and compassionate personality never fails to shine through each time, and I’m grateful to have you as a friend. Despite being across the seas and several continents, Andra Telemacque has always been a phone call away. Your ever positive attitude, and humor has added a lot to our friendship, and I’m thankful to have you as a friend. I would also like to acknowledge my brother, Benedict Abdalla, for being my biggest supporter. Lastly, special thanks go to Nkem Aziken for always encouraging me to be fearless no matter what. Thank you for your friendship, support, and encouragement.
# TABLE OF CONTENTS

**DEDICATION** ..................................................................................................................... ii

**ACKNOWLEDGEMENTS** ....................................................................................................... iii

**LIST OF FIGURES** ................................................................................................................ viii

**CHAPTER ONE: Introduction** ................................................................................................. 1

1.1 Overview of Hemostasis ........................................................................................................... 2
1.2 The Prothrombinase Complex: Role of Factor V ................................................................. 6
1.3 Factor V: Biochemistry and Physiology ...................................................................................... 8
1.4 Platelet versus Plasma Factor V/Va .......................................................................................... 11
1.5 Structure/Function Relationships of Factor V/Va ................................................................. 16
   1.5.1 Factor Va Interaction with Membranes .............................................................................. 19
   1.5.2 Interaction of Factor Va with Factor Xa .......................................................................... 23
1.6 The Factor V Region Mediating its Endocytosis by Megakaryocytes ........................................ 26
1.7 Clinical Relevance of Characterizing the Megakaryocyte Receptor System Mediating Factor V Endocytosis ................................................................................................................. 28

**CHAPTER TWO: Introduction to Epitope Mapping** ............................................................... 33

2.1 Molecular Structure of Antibodies .......................................................................................... 34
2.2 Epitope Mapping Methods ....................................................................................................... 41
2.3 Structural Approaches ............................................................................................................. 42
   2.3.1 X-ray Crystalllography ....................................................................................................... 42
   2.3.2 Nuclear Magnetic Resonance ............................................................................................ 42
   2.3.3 Computational Methods ..................................................................................................... 43
2.4 Functional Approaches ............................................................................................................ 45
   2.4.1 Competitive Binding to the Antigen .................................................................................... 45
   2.4.2 Protein Modification ............................................................................................................ 48
      2.4.2.1 Site-Directed Mutagenesis .......................................................................................... 48
      2.4.2.2 Differential Chemical Modification ............................................................................. 49
   2.4.2.3 Hydrogen/Deuterium Exchange .................................................................................... 52
   2.4.3 Antigen Fragmentation Methods ....................................................................................... 55
      2.4.3.1 Limited Proteolysis ...................................................................................................... 55
      2.4.3.2 Chemical Fragmentation ............................................................................................... 60
      2.4.3.3 cDNA Fragmentation .................................................................................................... 60
CHAPTER THREE: Studies to Identify the Factor V Residues Mediating its Endocytosis to Form the Unique Platelet-derived Cofactor Molecule .......................... 65

3.1 MATERIALS AND METHODS ........................................................................................................... 66

3.1.1 Materials ....................................................................................................................................... 66

3.1.2 Proteins and Antibodies .............................................................................................................. 66

3.1.3 Cell Culture Reagents .................................................................................................................. 67

3.1.4 CMK Cell Culture .......................................................................................................................... 67

3.1.5 Factor V Iodination ....................................................................................................................... 67

3.1.6 SDS-PAGE and Phosphorimaging ............................................................................................... 68

3.1.7 Effect of Anti-factor V Antibodies on $^{125}$I-Factor V Binding to CMK Cells. ................... 68

3.1.8 Effects of E9 and Anti-factor V #2 on Factor V Cofactor Activity .............................................. 70

3.1.9 Western Blotting of Factor V ...................................................................................................... 71

3.1.10 Factor Xa Proteolysis of Factor Va ............................................................................................ 71

3.1.11 Immunoprecipitation of Factor Xa-Cleaved Factor Va by E9 ................................................... 72

3.1.12 Solid Phase Binding Assay .......................................................................................................... 72

3.1.13 Proteolysis of Factor V with Asp-N and Trypsin ........................................................................ 74

3.2 RESULTS ........................................................................................................................................... 75

3.2.1 The Effect of Various Factor V Light Chain Antibodies on $^{125}$I-factor V Binding to CMK Cells ................................................................. 75

3.2.2 E9 is a Potent Inhibitor of Factor V Binding to the CMK Cell Surface ....................................... 77

3.2.3 Western Blotting Analyses of Factor V using Various Anti-Factor V Light Chain Antibodies ................................................................................................. 77

3.2.4 Factor V Cofactor Activity is Not Perturbed by E9 ..................................................................... 81

3.2.5 E9 Does Not Affect Cleavage of Factor Va by Factor Xa at Arg1765 of the Light Chain ................ 81

3.2.6 Binding of Factor Xa-Cleaved Fragments of Factor V to E9 ...................................................... 88

3.2.7 Development of a Solid Phase Binding Assay to Probe Interactions Between E9 and Factor V .................................................................................................................. 90

3.2.8 Displacement of E9-bound $^{125}$I-factor V with Various Factor V Proteolytic Fragments ......................................................................................................................... 96

DISCUSSION .......................................................................................................................................... 103

FUTURE DIRECTIONS ............................................................................................................................ 106

REFERENCES .......................................................................................................................................... 111
LIST OF TABLES

Table 1. Inhibition of factor V endocytosis by various human and bovine factor V molecules and factor VIII. .............................................................................................................. 29
Table 2. Reagents for the chemical modification of proteins ........................................... 49
LIST OF FIGURES

Figure 1.1 Protein-enzyme complexes of the coagulation cascade. ........................................ 5
Figure 1.2 The role of complex formation in thrombin generation. ........................................ 7
Figure 1.3 Megakaryocytes mediate the endocytosis of factor V ........................................ 10
Figure 1.4 Hypothetical model of the megakaryocyte cell surface events giving rise to platelet-derived factor V. ................................................................. 12
Figure 1.5 Factor V activation by thrombin ............................................................................ 18
Figure 1.6 Cleavage of factor Va by factor Xa ....................................................................... 25
Figure 1.7 Inhibition of factor V endocytosis by megakaryocytes with various anti-factor V antibodies. .......................................................................................... 30
Figure 2.1 Structural characteristics of antibodies ................................................................. 35
Figure 2.2 General approach for differential chemical modification of antigens coupled with mass spectrometry ............................................................................... 51
Figure 2.3 General approach for characterizing conformational and discontinuous epitopes by H/D exchange and mass spectrometry ................................................ 53
Figure 2.4 Epitope excision .................................................................................................... 56
Figure 2.5 Epitope extraction ................................................................................................. 58
Figure 3.1 Effect of monoclonal factor V light chain antibodies on factor V binding ... 76
Figure 3.2 Inhibition of factor V binding to the megakaryocyte cell surface by E9 ..... 78
Figure 3.3 Displacement of factor V from the megakaryocyte cell surface with E9 ..... 79
Figure 3.4 Western blotting of intact and thrombin-activated factor V with various anti-factor V light chain antibodies ............................................................................. 80
Figure 3.5 Factor Va cleavage by factor Xa on PC/PS vesicles ............................................. 83
Figure 3.6 Anti-factor V #2 does not detect factor Xa-catalyzed cleavage products of factor V by western blotting ............................................................................. 85
Figure 3.7 E9 does not affect cleavage of the factor Va light chain at Arg1765 ........ 86
Figure 3.8 Immunoprecipitation of factor Xa-cleaved factor Va with E9 ..................... 89
Figure 3.9 Titration of factor V and E9 in a solid phase binding assay ............................. 92
Figure 3.10 Displacement of E9-bound factor V with unlabeled single chain factor V .. 94
Figure 3.11 Displacement of E9-bound factor V by factor Va and factor Xa-cleaved factor Va ........................................................................................................ 97
Figure 3.12 Effect of Asp-N-derived factor V fragments on factor V binding to E9 .... 98
Figure 3.13 Effect of trypsin-derived factor V fragments on factor V binding to E9 .... 100
CHAPTER ONE: Introduction
1.1 Overview of Hemostasis

Blood is a heterogeneous mixture of red blood cells, leukocytes, and platelets suspended in a fluid known as plasma that contains lipids, carbohydrates, salts, hormones, proteins, and gases (Williams and Nelson 1990). The components of blood serve a host of different functions, including oxygen delivery to tissues, protection against infectious agents, and transport of vitamins and nutrients throughout the body (Alper 1990; Erslev and Lichtman 1990). These physiological processes occur in a dynamic environment and therefore necessitate the fluidity of blood. In order for blood to be maintained in a fluid state, humans have evolved a mechanism that is initiated upon tissue injury; this process is known as hemostasis. During hemostasis, blood coagulation is initiated and terminated in a rapid and highly regulated manner allowing for the equilibrium between a procoagulant and anticoagulant state to be restored, thus preventing the event of uncontrolled bleeding or disseminated thrombosis following vascular damage.

The sequence of events that occur during hemostasis following vascular damage can be divided into four separate but related stages: primary hemostasis, coagulation or secondary hemostasis, termination, and fibrinolysis. During primary hemostasis, blood vessel damage occurs disrupting the endothelial cells lining the blood vessel wall leading to the exposure of subendothelial proteins including collagen, fibronectin, fibrinogen, laminin and von Willebrand factor that are typically sequestered in the intact vasculature away from blood (Weiss, Baumgartner et al. 1978; Hindriks, Ijsseldijk et al. 1992; Beumer, MJ et al. 1994; Savage, Saldivar et al. 1996; Farndale, Sixma et al. 2004).
Platelets adhere to the damaged site through interactions with these subendothelial proteins via a number of integrins and glycoprotein (GP) receptors (Ruggeri, Bader et al. 1982; Santoro 1986; Moroi, Jung et al. 1989; Tandon, Kralisz et al. 1989; Savage, Almus-Jacobs et al. 1998; Shen, Romo et al. 2000; Inoue, Suzuki-Inoue et al. 2006). The adhesion of platelets to collagen via its specific receptor leads to platelet activation. Activated platelets undergo a dramatic shape change due to cytoskeletal changes and release hemostatically-active α and dense granule components that are comprised of a number of different coagulation factors, including fibrinogen and factor V/Va (Jandrot-Perrus, Lagrue et al. 1997; Polgar, Clemetson et al. 1997; Kehrel, Wierwille et al. 1998; Hartwig 2007). The activation of platelets also leads to a conformational change in GPIIb/IIIa (integrin αIIb/β3), which mediates platelet aggregation and platelet plug formation by bridging adjacent platelets to each other via fibrinogen. While platelet plug formation is sufficient to stop the loss of blood initially, the formed platelet plug must be stabilized by the formation of fibrin via blood coagulation to prevent rebleeding at the injury site.

Blood coagulation is initiated by the exposure of subendothelial cell-expressed tissue factor at the site of injury, which binds to plasma factor VIIa to form the extrinsic Xase complex, and in the presence of calcium activates factor X to factor Xa and factor IX to IXa (Nemerson 1988; Morrissey, Macik et al. 1993). Factor Xa, in turn, forms a complex with activated platelet released, membrane-bound platelet-derived factor V/Va to make a small amount of thrombin (Barton, Jackson et al. 1967; Walsh 1974; Mann, Nesheim et al. 1990). Thrombin activates factor VIII to VIIIa, which together with factor
IXa and calcium forms the intrinsic Xase complex to activate factor X to Xa thus further contributing to thrombin generation (Hemker and Kahn 1967; Hultin and Nemerson 1978). Not only does thrombin effect cleavage of fibrinogen to fibrin (Bailey, Bettelheim et al. 1951; Doolittle 1973), it further propagates the response by activating plasma and platelet factor V to Va, and recruits more activated platelets to the growing thrombus (Figure 1.1).

Subsequent to formation of the fibrin clot, the coagulation response must be terminated to prevent excessive thrombosis. Thrombin downregulates its own formation by the activation of protein C, which gives rise to activated protein C (APC) (Kisiel, Canfield et al. 1977; Esmon and Owen 1981; Comp, Jacocks et al. 1982). Numerous anticoagulant proteins including specific protease and protease inhibitors such as APC downregulate various components of the coagulation response to terminate thrombin formation and activity (Rau, Beaulieu et al. 2007). Furthermore, following repair of the damaged vessel wall, fibrin is cleaved by a specific protease to resolve the fibrin clot and prevent downstream embolism.
Figure 1. Protein-enzyme complexes of the coagulation cascade.

The extrinsic tenase complex, comprised of tissue factor (TF), factor VIIa, and Ca$^{2+}$ generates factor Xa, which is in complex with platelet factor Va, produces a small amount of thrombin. Thrombin activates factor VIIIa together with factor IXa, which is also formed by the extrinsic tenase complex, in the intrinsic tenase complex, generates additional factor Xa. The convergence of both pathways onto the prothrombinase complex, composed of the cofactor, factor Va and the serine protease factor Xa assembled on an activated platelet surface in the presence of calcium, allows for the explosive production of thrombin. As indicated, thrombin plays multiple roles in the propagation, as well as downregulation of blood coagulation.
1.2 The Prothrombinase Complex: Role of Factor V

The assembly of protein-enzyme complexes is crucial for hemostasis. The prothrombinase complex, in particular, is central to clot formation as it activates prothrombin to thrombin, which is involved in numerous procoagulant and anticoagulant events. This complex is comprised of the nonenzymatic cofactor, factor Va and the serine protease, factor Xa, assembled on an activated platelet membrane surface in the presence of Ca$^{2+}$ (Barton, Jackson et al. 1967; Walsh 1974; Mann, Nesheim et al. 1990) (Figure 1.2). While all the components of Prothrombinase are necessary for prothrombin activation (Nesheim, Taswell et al. 1979), the cofactor factor Va plays a critical role in the regulation of blood coagulation via this complex. The prothrombinase complex as a whole enhances the catalytic rate of thrombin generation by 300,000-fold as compared to factor Xa alone (Nesheim, Taswell et al. 1979). When the cofactor, factor Va, is excluded from prothrombinase assembly, the rate of thrombin generation is observed to decrease by 10,000-fold.

Given the essential role of factor Va in thrombin generation, as expected, a deficiency in the procofactor (factor V) results in a bleeding diathesis. Factor V deficiency or parahemophilia is a rare autosomal recessive disorder with an incidence rate of 1:1,000,000 (Asselta, Tenchini et al. 2006; Segers, Dahlback et al. 2007). Parahemophilia is characterized by low or undetectable amounts of factor V that result by way of multiple mechanisms, including mutations (missense and nonsense mutations, insertions, and deletions) in the factor V gene (F5), mutations in the genes (LMAN1 or
Figure 1.2 The role of complex formation in thrombin generation.

The prothrombinase complex, comprised of the serine protease factor Xa (FXa), the nonenzymatic cofactor, factor Va (FVa), and Ca\(^{2+}\) assembled on the platelet membrane surface is crucial in the generation of thrombin. Relative to the activity of factor Xa alone, the inclusion of all the Prothrombinase constituents results in a 300,000-fold increase in the rate of prothrombin activation. The presence of factor Va in the complex is most critical, as its removal results in a 10,000-fold decrease in the rate prothrombin activation.

1.3 Factor V: Biochemistry and Physiology

In whole blood, the procofactor, factor V, exists in two pools with approximately 75-80% circulating in plasma and 20-25% found within platelets (Breederveld, Giddings et al. 1975; Osterud, Rapaport et al. 1977; Vicic, Lages et al. 1980; Tracy, Eide et al. 1982). The platelet-derived factor V pool is stored in platelet α-granules (Chesney, Pifer et al. 1981; Wencel-Drake, Dahlback et al. 1986) and is released upon platelet activation. Factor V is synthesized by the liver and circulates in plasma as a large molecular weight (330 kDa) protein (Rapaport, Ames et al. 1960; Kupfer, Gee et al. 1964). The factor V gene (F5), isolated in 1992, is located on human chromosome 1q23 (Jenny, Pittman et al. 1987; Cripe, Moore et al. 1992), spans 80 kb of DNA, and is composed of 25 exons and 24 introns. The exons are 72 to 287 base pairs in size, and the introns range from 448 base pairs to 11.4 kb. Following transcription, the factor V mRNA is 6.9 kb and has a 6672 bp coding region (Cripe, Moore et al. 1992). Currently, the mechanism underlying factor V transcription and translation are unknown. However, it is known that following translocation into the endoplasmic reticulum, factor V undergoes several post-translational modifications, including phosphorylation (Kalafatis, Rand et al. 1993; Rand, Kalafatis et al. 1994; Kalafatis 1998; Gould, Silveira et al. 2004), N-linked and O-linked
glycosylation (Jenny, Pittman et al. 1987; Fernandez, Hackeng et al. 1997; Silveira, Kalafatis et al. 2002), and sulfation (Hortin 1990; Pittman, Tomkinson et al. 1994). Additionally, the encoded factor V protein undergoes processing to remove a 28 residue signaling peptide resulting in the secretion of a mature human factor V molecule of 2196 amino acids (Kane and Davie 1986). Factor V circulates in plasma at a concentration of 21-30 nM (Tracy, Eide et al. 1982) and has a half life of 12-15 hours (Borchgrevink and Owren 1961).

Early studies in animal models suggested that megakaryocytes synthesize platelet-derived factor V; and while mouse megakaryocytes do synthesize factor V (Chiu, Schick et al. 1985; Gewirtz, Keefer et al. 1986; Yang, Pipe et al. 2003; Rowley, Schwertz et al. 2012), it is clear that human platelet-derived factor V originates exclusively via endocytosis of the plasma-derived procofactor by megakaryocytes (Camire, Pollak et al. 1998; Bouchard, Williams et al. 2005; Gould, Simioni et al. 2005; Suehiro, Veljkovic et al. 2005; Bouchard, Meisler et al. 2008). Megakaryocytes are platelet-progenitor cells that reside in the bone marrow. As platelets possess no biosynthetic capability, the majority of the proteins that are found in the platelet granules are synthesized by megakaryocytes [thrombospondin, plasminogen activator inhibitor-1, and von Willebrand factor (Sporn, Chavin et al. 1985; Sitar, Borroni et al. 1986; Konkle, Schick et al. 1993; Roussi, Drouet et al. 1995)] or endocytosed from plasma [fibrinogen (Handagama, Scarborough et al. 1993), albumin (Handagama, Shuman et al. 1989), IgG (Handagama, Rappolee et al. 1990), and factor V (Bouchard, Williams et al. 2005; Gould, Simioni et al. 2005)] and subsequently packaged into the α-granules (Figure 1.3). While both
Figure 1. 3 Megakaryocytes mediate the endocytosis of factor V.

Megakaryocytes are derived from hematopoietic stem cells and undergo multiple differentiation and maturation stages during development in the bone marrow to ultimately produce platelets. **Panel A)** Mature megakaryocyte (Robert, Cortin et al. 2012). **Panel B)** Bone marrow-derived CD34+ cells were induced to differentiate into megakaryocytes (Bouchard, Williams et al. 2005). Following incubation with 100 nM factor V-AlexaFluor488 (green staining) and 100 nM fibrinogen-AlexaFluor633 (red staining) (17 hr, 37°C), the cells were visualized by confocal microscopy using a Zeiss 510 Meta confocal microscope. In the last panel, the individual images were merged using the Zeiss 510 software (yellow staining) (Bouchard et al., unpublished observations). Original magnification = 630x. In panel A, the scale bar = 2 µm, and in Panel B, the scale bars = 10 µm.
platelets and megakaryocytes are capable of endocytosis of fibrinogen, IgG and albumin (Sporn, Chavin et al. 1985; Sitar, Borroni et al. 1986; Konkle, Schick et al. 1993; Roussi, Drouet et al. 1995), factor V is endocytosed by only megakaryocytes (Camire, Pollak et al. 1998; Bouchard, Williams et al. 2005).

Endocytosis of factor V is clathrin-dependent and mediated by two receptors including an unidentified specific factor V receptor and low-density lipoprotein (LDL) receptor related protein (LRP-1) (Figure 1.4) (Bouchard, Meisler et al. 2008). LRP-1 belongs to a family of proteins related to the LDL receptor of endocytic receptors (AJ 1998; Gliemann 1998). LRP-1 is a widely distributed membrane receptor that is known to bind multiple, structurally unrelated ligands, targeting most but not all to lysosomes for degradation (Hussain, Strickland et al. 1999; Argraves 2001; Herz and Strickland 2001; Li, Cam et al. 2001; Bouchard, Meisler et al. 2008).

1.4 Platelet versus Plasma Factor V/Va

Despite their identical biosynthetic origin, plasma- and platelet-derived factor V(a) are structurally and functionally distinct. Studies have shown that the factor V that is found in platelets is stored and released as a mixture of intact single chain and partially proteolytically-activated factor V (Viskup, Tracy et al. 1987; Monkovic and Tracy 1990). This is in sharp contrast to the plasma-derived counterpart, which exists solely in a single chain form that does not express cofactor activity. The partially proteolytically active form of factor V exhibits significant activity that is ~5-50% the activity of fully thrombin-activated factor V (Kane, Lindhout et al. 1980; Vicic, Lages et al. 1980; Kane,
Figure 1. 4 Hypothetical model of the megakaryocyte cell surface events giving rise to platelet-derived factor V.

Two receptors, including the well-characterized LRP-1 molecule and the as of yet unidentified factor V receptor (FV Rc), mediate factor V (FV) endocytosis by megakaryocytes. The initial binding of factor V to the FV Rc facilitates subsequent binding of factor V to LRP-1 after which factor V is endocytosed in Ca$^{2+}$- and clathrin-dependent manners, phenotypically-modified, and trafficked to the α-granules. LRP-1 is believed to be trafficked back to the cell surface; however, the fate of the FV Rc is unknown. Additionally, it is unknown whether endocytosis is mediated by LRP-1 in complex with the FV Rc or by LRP-1 alone.
(Kane, Lindhout et al. 1980; Vicic, Lages et al. 1980; Kane, Mruk et al. 1982; Viskup, Tracy et al. 1987; Monkovic and Tracy 1990), and expresses a minimal increase (~2-3-fold) in activity upon activation with thrombin or factor Xa (Monkovic and Tracy 1990). In contrast, plasma factor V cofactor activity increases by 400-fold when activated by thrombin (Nesheim, Taswell et al. 1979). Furthermore, factor Xa can activate the platelet-derived factor V more effectively (50-100 times greater) than thrombin (Monkovic and Tracy 1990), the known potent activator of plasma factor V.

Compared to thrombin-activated factor Va, characterization of purified platelet-derived factor V have indicated that while the heavy chain is intact, the platelet-derived factor V light chain has a unique cleavage site at Tyr1543 that also leads to functional cofactor activity (Gould, Silveira et al. 2004). With respect to the proteolytic state of the partially activated platelet factor V molecule, species consistent with thrombin-activated factor V were observed in addition to unidentified peptide fragments that are most likely generated by a specific unknown protease(s).

In addition to the aforementioned differences between platelet and plasma factor V, platelet-derived factor V displays other unique physical and functional characteristics. A unique O-linked glycoform at Thr402 has been identified on the heavy chain of platelet factor V, however it is unknown as to whether this feature exists in all individuals as the finding was made using platelet-derived factor V isolated from a platelet pool (Gould, Silveira et al. 2004). The Ser692 residue of factor V is known to be phosphorylatable by a casein-like kinase enzyme, and casein kinase-2; however, this phosphorylation is only observable in plasma factor V and not platelet factor V implying that this form is resistant
to phosphorylation by these enzymes (Rand, Kalafatis et al. 1994; Kalafatis 1998; Gould, Silveira et al. 2004). The platelet-derived factor Va pool is also partially resistant to inactivation by APC (Camire, Kalafatis et al. 1995; Camire, Kalafatis et al. 1998). The serine protease, plasmin, which is known to inactivate plasma factor V in the presence of a membrane surface (Lee and Mann 1989) leads to increased and sustained activity in platelet-derived factor V (Conlon 1997). In the absence of the platelet membrane, no increase in activity is observed. Additionally, studies by Wood et al. have indicated that a percentage of platelet factor V is non-dissociable and bound to the membrane of the α-granule via a glycophasphatidylinositol (GPI) anchor (Wood and Fager 2008).

Several clinical observations support the notion that the platelet-derived factor V is more procoagulantly relevant in hemostasis compared to its plasma counterpart (Borchgrevink and Owren 1961; Tracy, Giles et al. 1984; Nesheim, Nichols et al. 1986; Weiss, Lages et al. 2001; Duckers, Simioni et al. 2010). At sites of vascular damage, the concentration of platelet-derived factor Va exceeds its plasma counterpart by approximately 100, as platelets contain 20% of the total pool of factor V in whole blood and are contained in such a small volume at sites of vascular injury that their concentration of factor V surpasses that of plasma (Nesheim, Nichols et al. 1986). The clinical importance of platelet-derived factor V is evident in individuals with Quebec platelet disorder, which is a hemorrhagic disorder in which platelet factor V levels are decreased (Tracy, Giles et al. 1984) due to enhanced proteolysis of factor V by the overexpression of urokinase-type plasminogen activator in α-granules (Kahr, Zheng et al. 2001). Also, in an individual with an inhibitor to plasma-derived factor V, but whose
platelet-derived factor V is unaffected, no adverse bleeding was observed and adequate hemostasis was supported during surgery (Nesheim, Nichols et al. 1986). Hemostatic improvement has also been observed in a factor V deficient patient receiving platelet transfusions (Borchgrevink and Owren 1961). The pivotal role of platelet factor V is further supported by the severe bleeding problems presented in factor V New York, a disorder characterized by defective platelet-derived factor V and normal plasma factor V levels (Weiss, Lages et al. 2001).

Though the significance of platelet factor V in hemostasis has been known for decades, its protective role in bleeding pathologies has only recently been closely investigated. Individuals with congenital factor V deficiency often present with hemorrhagic diatheses of variable severity that correlate poorly with plasma factor V levels (Lak, Sharifian et al. 1998). This variation in phenotype may be explained by two different studies that have shown patients deficient in plasma factor V with residual factor V in their platelets are protected against major bleeding events (Bouchard, Brummel Ziedins et al.; Bouchard, Chapin et al.; Duckers, Simioni et al. 2010; Castoldi, Duckers et al. 2011). Studies by Bouchard et al. showed that transfusions of plasma factor V to control gastrointestinal bleeding allowed for hemostatic competence in a patient completely devoid of plasma and platelet factor V (Bouchard, Brummel Ziedins et al.). Endocytosis of factor V by the patient’s platelets following plasma administration was time-dependent. The functional significance of his acquired platelet-derived factor V was studied subsequent to plasma administration in a whole blood clotting assay at a point in time when no detectable plasma-derived factor V could be observed. When the
patient’s platelets were initially maximally activated with PAR1 and 4, clot formation and thrombin generation in a tissue factor-based clotting assay were observed. Thus, despite a complete absence of detectable plasma factor V, and approximately 7% (0.11 nM) normal levels of platelet-derived factor V/Va confers hemostatic competence in this individual. In a second study (Bouchard, Chapin et al.), no thrombin generation was observed in platelet poor plasma obtained from factor V deficient patients; however, appreciable amounts of thrombin were generated with the use of their platelet rich plasma. Thrombin generation in the platelet rich plasma could be completely inhibited by both an anti-factor V antibody and APC. In addition, washed platelets from the factor V deficient patients contained measurable levels of the functional factor V antigen (Duckers, Simioni et al. 2010; Castoldi, Duckers et al. 2011). Taken together, these observations support the notion that platelet-derived factor V is the more hemostatically relevant cofactor pool.

1.5 Structure/Function Relationships of Factor V/Va

Factor V, is a multidomain protein (A1-A2-B-A3-C1-C2) that circulates in plasma in a single chain, inactive form. The activation of factor V can be catalyzed by thrombin (Esmon 1979; Nesheim and Mann 1979; Nesheim, Myrmel et al. 1979; Dahlback 1980; Monkovic and Tracy 1990), the physiological activator of factor V, and other proteases that include factor Xa (Smith and Hanahan 1976; Foster, Nesheim et al. 1983), plasmin (Lee and Mann 1989), meizothrombin (Tans, Nicolaes et al. 1994), elastase (Camire, Kalafatis et al. 1998), cathepsin G (Turkington 1993; Allen and Tracy
1995), calpain (Rodgers, Cong et al. 1987), and factor Xla (Whelihan, Orfeo et al. 2010), which will result in the production of a cofactor with variable amounts of cofactor activity. Factor V can also be activated by monocyte-associated (Tracy, Rohrbach et al. 1983), platelet-associated (Kane and Majerus 1982), and neutrophil-associated proteases (Oates and Salem 1987). The degree to which these cellular activators contribute to factor V activation in physiological events is unknown.

The activation of factor V to Va by thrombin occurs via limited proteolysis at Arg709, Arg1018, and Arg1545 (Esmon 1979) (Figure 1.5). Cleavage of factor V by thrombin occurs in a stepwise manner with initial cleavage at Arg709 releasing the N-terminally-derived 105 kDa heavy chain fragment, and a 280 kDa fragment consisting of the light chain and the B domain. Cleavage of the 280 kDa fragment at Arg1018 releases the 71 kDa B domain, and produces a 220 kDa intermediate. Lastly, cleavage at Arg1545 of the 220 kDa fragment releases the remainder of the B domain (150 kDa) and the light chain (74/71 kDa). The active cofactor, factor Va, is composed of a heavy chain (amino acids 1-709) and light chain (amino acids 1545-2196) component which are non-covalently linked by a Ca\textsuperscript{2+} ion. The heavy chain (~105 kDa) is comprised of the A1-A2 domains, while domains A3-C1-C2 form the light chain (~74 kDa). Based on the available amino acid sequences of factor V from various species, the A and C domains are highly conserved amongst species, including the various species listed in the NCBI protein data base (http://www.ncbi.nlm.nih.gov). The factor V A domains share 40% homology with the copper transport plasma protein, ceruloplasmin (Ortel, Takahashi et al. 1984; Koschinsky, Funk et al. 1986), while the C domains belong to the discoidin
Factor V is activated by thrombin via three cleavages. An initial cleavage at Arg709 will produce a heavy chain fragment of 105 kDa, and another fragment of 280 kDa. Another cleavage at Arg1018 will remove the 71 kDa B domain fragment leaving a large 220 kDa fragment. Finally, cleavage at Arg1545 will produce another B domain fragment (150 kDa) and a light chain fragment of 74 kDa. The three cleavages, collectively, result in the release of the B domain and yield the activated form of the procofactor, factor Va comprised of heavy (105 kDa) and light chain (74 kDa) components that are noncovalently linked by a calcium ion.
protein family from the slime mold *Dictyostelium discoideum* (Jenny, Pittman et al. 1987). The coagulation protein, factor VIII, that has a domain arrangement identical to that of factor V, shares 40% sequence identity in both the A and C domains, while the B domains in both proteins are only 14% homologous (Kane and Davie 1988). Unlike the A and C domains, the B domain is poorly conserved amongst the studied factor V species (Nicolaes and Dahlback 2002; Mann and Kalafatis 2003), and possesses unique tandem repeats, the function of which remains to be studied. Release of the B domain appears to result in the removal of the conformational constraints that inhibit factor V activity. Insight into this observation was made using a partially B-domainless mutant form of factor V lacking residues 811-1491 (FV des811-1491, FV-DT), that was observed to have partial, constitutive activity compared to thrombin-activated factor V (Kane, Devore-Carter et al. 1990; Keller, Ortel et al. 1995). Further studies have identified a region within the B domain that is important for factor V activation. The region is highly basic, and deletion of this region yields a factor V derivative with cofactor properties in the absence of proteolysis with thrombin, suggesting that the full length B domain is not responsible for the maintenance of an inactive state, but rather the inhibition of activity is brought upon by specific B domain sequences, some of which remain unidentified (Zhu, Toso et al. 2007).

1.5.1 Factor Va Interaction with Membranes

Several protein-protein interactions and protein-membrane interactions are essential for factor Va function. The binding of factor Va to membrane surfaces is crucial for its function given the membrane dependence of the prothrombinase complex, the
binding of factor Va to Xa, and the inactivation of factor Va by APC. Studies with synthetic phospholipid vesicles (Kim, Quinn-Allen et al. 2000; Nicolaes, Villoutreix et al. 2000) and activated platelets (Tracy and Mann 1983) have provided substantial information concerning factor Va membrane interactions, and have collectively suggested a role for the factor V light chain in phospholipid binding. In 1983, Tracy et al. showed that the factor V light chain mediates prothrombinase complex assembly on activated platelets (Tracy and Mann 1983). In their studies, the 74 kDa component of factor V dissociated from the heavy chain component with the use of EDTA was observed to associate with the platelet surface.

The binding of factor Va to synthetic phospholipid vesicles has also been shown to occur via the light chain of factor V. Phospholipids composed of 25% phosphotidylserine (PS) and 75% phosphotidylcholine (PC) allow for favorable binding of factor Va with a dissociation constant of $10^{-9}$ M (Izumi, Kim et al. 2001). Unlike membrane binding of the vitamin K-dependent coagulation proteins, factor Va binding to phospholipids is not calcium-dependent (Higgins and Mann 1983; Krishnaswamy and Mann 1988). Studies performed using lipophilic probes, mutagenesis studies, as well as X-ray crystallography have shown that factor Va membrane binding sites are localized within the A3, C1 and C2 domains of the light chain (Kalafatis, Rand et al. 1994; Kim, Quinn-Allen et al. 2000; Adams, Hockin et al. 2004; Peng, Quinn-Allen et al. 2005).

The phospholipid binding site in the A3 domain was identified, and characterized by Kalafatis et al. by proteolysis of a phospholipid-bound isolated form of the light chain (Kalafatis, Jenny et al. 1990). The regions protected from proteolytic cleavage covered
the A3 domain, and were observed to interact with phospholipid vesicles directly. Collectively, their findings indicated that a 99-amino acid segment, featuring residues 1667-1765 in human factor V, localized within the central part of the A3 domain is involved in membrane binding. Although the sites within the region that directly interacted with the membrane surfaces were undefined, hydropathy plots suggested that two hydrophobic regions, 1716-1727 and 1741-1748, are involved in the interaction of the light chain with phospholipid vesicles.

Studies on the contribution of the factor V C1 domain to membrane binding were initially conducted by Saleh et al. using a molecular model of the C1 domain that was constructed based on the factor V C2 domain structure. Two residues of the C1 domain (Tyr1956/Leu1957) located at the predicted apex of a β-hairpin loop analogous to the previously identified Trp2063/Trp2064 membrane binding sites of the C2 domain were mutated by alanine-scanning mutagenesis, and screened for phospholipid binding and prothrombin activity. The mutant Y1956A/L1957A interacted weakly with phospholipid vesicles using fluorescent resonance energy transfer (FRET), and its binding affinity was observed to decrease by 12-fold in comparison to the native protein. In addition, the Y1956A/L1957A mutant was found to markedly decrease prothrombinase activity on phospholipid vesicles. A second mutant (R2023A/R2027A) was also observed to have decreased binding to phospholipid vesicles and was demonstrated to impair procoagulant activity. Ultimately, the study showed that two pairs of amino acids—Y1956/L1957 and R2023A/R2027A within the C1 domain contribute to the binding of factor V to membranes. A later study by Peng et al. showed that the mutation of the proposed C1
membrane binding sites (Y1956/L1957) in combination with mutations in the previously identified C2 membrane binding sites (W2063/W2064) results in a four-fold decrease in thrombin generation (Peng, Quinn-Allen et al. 2005).

The C2 domain of factor V, which lies in tandem with the C1 domain, has also been shown to contain determinants of membrane binding (Ortel, Devore-Carter et al. 1992; Gilbert, Novakovic et al. 2012). Studies with anti-factor V antibodies directed toward the C2 domain have been shown to alter both function and membrane binding (Ortel, Moore et al. 1998). In addition, the deletion of the C2 domain has been observed to result in the loss of the PS binding potential of factor Va (Ortel, Devore-Carter et al. 1992). The crystal structure of the factor V C2 domain revealed hydrophobic residues (Trp2063 and Trp2064) at the apex of three β-hairpin loops of a beta barrel motif corresponding to the C2 domain that were suggested to line a groove also containing polar residues, that can be inserted into membranes (Macedo-Ribeiro, Bode et al. 1999). The factor V model based on the bovine factor Vα crystal structure (Adams, Hockin et al. 2004) with a modeled A2 domain from ceruloplasmin (Orban, Kalafatis et al. 2005) confirmed these data and identified a similar membrane binding site in the C1 domain. The bovine factor Vα structure also predicted that the membrane binding site within the A3 domain is positioned away from the C domains.

Other studies have demonstrated a role for N-linked glycosylation of the light chain in modulation of factor Va membrane binding. Asn2181 in the C2 domain, which is within close proximity of the membrane binding region, is differentially glycosylated. By sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), this differential
glycosylation gives rise to two factor Va light chain isoforms, factor Va\textsubscript{1} (71 kDa) and factor Va\textsubscript{2} (74 kDa) (Kim, Ortel et al. 1999; Nicolaes, Villoutreix et al. 1999). Factor Va\textsubscript{1}, the glycosylated form, binds phospholipids with a much lower affinity than factor Va\textsubscript{2}, and therefore has lower cofactor activity in prothrombinase (Rosing, Bakker et al. 1993; Kim, Ortel et al. 1999; Nicolaes, Villoutreix et al. 1999). Collectively, these studies have provided both mechanistic and structural evidence for a role of the factor V light chain in membrane binding.

1.5.2 Interaction of Factor Va with Factor Xa

Aside from enhancing the catalysis of the factor Xa-mediated cleavage of prothrombin (Krishnaswamy 1990), factor Va serves as part of the receptor for factor Xa in the prothrombinase complex, forming a high affinity complex with the protein. Upon binding to the platelet membrane surface, both the heavy and light chains of factor Va contribute to binding factor Xa (Kalafatis and Beck 2002). Several studies have demonstrated the localization of factor Xa binding sites in factor Va. Based on peptide inhibition studies, a number of factor Xa binding sites have been identified in the heavy chain of factor V, including residues 311-325, 323-331, and 493-506 (Heeb, Kojima et al. 1996; Kojima, Heeb et al. 1998; Kalafatis and Beck 2002). In addition, when site-directed mutagenesis was used to create recombinant factor V constructs, residues 467, 511, and 653 in the heavy chain were identified (Steen, Villoutreix et al. 2002). It has also been observed that in the absence of a specific heavy chain region (Asp683-Arg709), factor Xa has reduced cofactor activity and the binding between factor Xa and
prothrombin becomes impaired also suggesting that part of the heavy chain of factor V is important for optimal interaction between both proteins (Bakker, Tans et al. 1994). This binding interaction between both factor Va and Xa requires the presence of a membrane surface ($K_d \sim 3$ nM), without which the binding interactions decrease by three orders of magnitude and are tremendously weaker ($K_d \sim 1$ µM) (Bloom, Nesheim et al. 1979; Tracy, Peterson et al. 1979; Pusey, Mayer et al. 1982; Higgins and Mann 1983; Monkovic and Tracy 1990). To date, only a single factor Xa binding site has been identified in the light chain of factor V at His 1683 (Steen, Villoutreix et al. 2002).

Factor Xa also plays a role in cleaving factor V and similar to thrombin, can cleave factor V at Arg 709, 1018, and 1545 to form an active cofactor molecule (Monkovic and Tracy 1990; Keller, Ortel et al. 1995; Thorelli, Kaufman et al. 1997). However, this cleavage occurs at a rate that is two orders of magnitude less than factor V cleavage with thrombin because the enzyme-substrate ratio (1:10) required for efficient catalysis is not met. At high factor Xa concentrations, both factor V and factor Va, can be cleaved at Arg1765 within the A3 domain of the light chain (Tracy, Nesheim et al. 1983; Odegaard and Mann 1987; Thorelli, Kaufman et al. 1997) (Figure 1.6). This cleavage gives rise to an N-terminally derived 30 kDa fragment and a C-terminally derived 48/46 kDa fragment in both human (Thorelli, Kaufman et al. 1997) and bovine forms of factor V (Tracy, Nesheim et al. 1983; Odegaard and Mann 1987).
Figure 1. 6 Cleavage of factor Va by factor Xa.

Factor Xa cleaves the light chain of human factor Va at Arg1765, generating an N-terminally derived 30 kDa fragment (A3), and a 48/46 kDa fragment (A3, C1 and C2), due to differential glycosylation, that is derived from the C-terminus (Asn2181).
1.6 The Factor V Region Mediating its Endocytosis by Megakaryocytes

Despite knowing a great deal regarding factor Va’s structural features important for its function in Prothombinase, little is known about the factor V domains important for its interactions with the megakaryocyte two receptor system and its endocytosis. Of the two megakaryocyte receptors known to participate in factor V endocytosis, only LRP-1 is well-characterized. LRP-1 is composed of an 85 kDa membrane spanning C-terminal region that is noncovalently associated with a 515 kDa N-terminal extracellular region (Herz, Kowal et al. 1990). LRP-1 binds to its ligands via its extracellular domain, whilst its cytoplasmic domain binds to adaptor proteins that serve as a link between LRP-1 and other membrane proteins (Gotthardt, Trommsdorff et al. 2000). Within the extracellular domain are four ligand binding domains termed clusters I, II, II, and IV (Willnow, Orth et al. 1994). The LRP-1 ligand binding domains are composed of arrays of cysteine-rich, calcium-binding regions termed complement-type repeats. Studies have also shown that lysine side chains are implicated in ligand binding to LRP-1 (Lenting, Neels et al. 1999; Bovenschen, Boertjes et al. 2003). The binding of all ligands to LRP-1 is inhibited by receptor associated protein (RAP), an endoplasmic reticulum chaperone protein that prevents improper folding of LRP-1 prior to transport to the cell surface. One LRP-1 ligand, factor VIII, which displays an overall amino acid identity of 30% to factor V, possesses two high-affinity LRP-1 binding domains within its A2 domain (R484-F509) and A3 domain (E1811-K1818) that include the lysine residues at positions 493, 496, and 499, and positions 1813 and 1818 respectively (Saenko, Yakhyaev et al. 1999; Sarafanov, Ananyeva et al. 2001; Sarafanov, Makogonenko et al. 2006; Sarafanov, Makogonenko et
al. 2007). More recently, the C1 domain residues Lys2092 and Phe2093 of factor VIII have been found to be important for endocytic uptake by LRP1 (Meems, van den Biggelaar et al. 2011). As little information pertaining to factor V’s LRP-1 binding site could be ascertained based on its known homology to other ligands, the primary amino acid sequences in the A and C domains of factor VIII and factor V were compared since both share 40% identity in these regions. Of all the factor VIII regions/sites involved in LRP-1 binding, only the region within the A3 domain (E1811-K1818) was found to have some of the same residues as the corresponding region in the A3 domain of factor V. However, a peptide mimicking this region was observed to have no effect on endocytosis of factor V by megakaryocytes (data not shown).

Experiments were subsequently performed using various factor V molecules and monoclonal antibodies to identify regions of factor V important for its endocytosis by megakaryocytes (Bouchard et al., unpublished observation). Initially, the ability of various human and bovine factor V and Va molecules, and isolated factor V domains to inhibit endocytosis of 125I-labeled factor V by megakaryocytes was studied using the megakaryocyte-like CMK cell line. Both thrombin-activated human and bovine factor Va and the intact procofactors, which display high sequence identity within their heavy (84%) and light (86%) chains with a less identical B-domain (59%) (Guinto and Esmon 1984) inhibited 125I-factor V endocytosis by megakaryocytes when present at a 50-fold molar excess (Table 1). Similarly, a 50-fold molar excess of isolated human factor V light chain inhibited 125I-factor V endocytosis by ~85%. In contrast, human factor V heavy chain had little effect on 125I-factor V endocytosis (~20% inhibition). B-domainless factor
V also substantially inhibited $^{125}$I-factor endocytosis. These observations suggest that factor V binding and/or endocytosis is mediated primarily by the light chain, and confirm that the B-domain is not involved. Similar experiments were performed using recombinant factor VIII. Consistent with a role for LRP-1 in factor V endocytosis, factor VIII inhibited $^{125}$I-factor V uptake to the same degree as unlabeled factor V (~100%) when both were present at a 25-fold molar excess. However, at its plasma concentration (1 nM) (Hoyer 1981), inhibition was not observed (data not shown).

In subsequent experiments, the ability of various anti-factor V antibodies to inhibit $^{125}$I-labeled factor V endocytosis was determined. Consistent with the results of the studies performed with various factor V molecules (see Table 1), anti-factor V light chain antibodies, E9, #2, and #5, inhibited factor V endocytosis by megakaryocytes by greater than 80% (Figure 1.7). One of these antibodies, E9, inhibited $^{125}$I-factor V endocytosis to the same extent as unlabeled factor V. Another anti-factor V light chain antibody (#9), as well as an anti-factor V heavy chain antibody (#17) had little or no effect on factor V endocytosis ($\leq 20\%$ inhibition). This latter observation is consistent with the inability of isolated factor V heavy chain to inhibit $^{125}$I-factor V endocytosis.

1.7 Clinical Relevance of Characterizing the Megakaryocyte Receptor System Mediating Factor V Endocytosis

As the platelet-derived pool of factor V represents the more physiologically relevant cofactor, understanding the cellular events mediating factor V endocytosis by megakaryocytes would advance studies aimed at developing forms of therapy that function primarily in maintaining a balance in hemostasis, which is especially crucial in
Table 1. Inhibition of factor V endocytosis by various human and bovine factor V molecules and factor VIII.

<table>
<thead>
<tr>
<th>Competitor</th>
<th>% $^{125}$I-Factor V Endocytosed $^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>50X human factor V</td>
<td>0.2</td>
</tr>
<tr>
<td>50X human factor Va</td>
<td>1.8</td>
</tr>
<tr>
<td>50X bovine factor V</td>
<td>0.0</td>
</tr>
<tr>
<td>50X bovine factor Va</td>
<td>7.7</td>
</tr>
<tr>
<td>50X human factor V light chain</td>
<td>16.2</td>
</tr>
<tr>
<td>50X human factor V heavy chain</td>
<td>80.6</td>
</tr>
<tr>
<td>50X B-domainless factor V</td>
<td>14.3</td>
</tr>
<tr>
<td>25X human factor V</td>
<td>0.5</td>
</tr>
<tr>
<td>25X human factor VIII</td>
<td>0.9</td>
</tr>
</tbody>
</table>

$^1$Cells in serum-free medium were incubated in the presence of 50-fold molar excesses of intact human or bovine factor V and thrombin-activated factor Va; isolated human factor V heavy or light chain; or a recombinant, partially B-domainless human factor V construct. Molar excesses (25-fold) of human factor VIII and human factor V were also utilized. Following a 1 hr incubation (37°C), $^{125}$I labeled factor V (10 nM) was added (1 hr, 37°C). To remove excess, as well as bound $^{125}$I-factor V, cells were washed extensively by centrifugation (250 x g, 7 min) followed by resuspension in 20 mM Hepes, 0.15 M NaCl, pH 7.4. The radioactivity associated with the dry cell pellet was assessed using a $\gamma$-counter.
Figure 1. 7 Inhibition of factor V endocytosis by megakaryocytes with various anti-factor V antibodies.

$^{125}$I-factor V (5 nM) was preincubated (1 hr, 37°C) with anti-factor V light chain (LC) (E9, #2, #5 and #9) or heavy chain (HC) (#17) antibodies (1.0 µM) prior to the addition of CMK cells. Excess and cell surface-associated $^{125}$I-factor V were removed, and the radioactivity associated with the dry cell pellets assessed as described in Table 1. The data are expressed as the percent cpm associated with the cells in the presence of antibody as compared to the cpm associated with the cells in the absence of antibody. The effect of incubation with a 50-fold molar excess of unlabeled, human factor V (FV) was used as a positive control.
the prevention of cardiovascular events. Given the importance of platelet factor V in blood coagulation, anticoagulant drugs could be developed that target the mechanism by which plasma factor V is internalized by megakaryocytes. Anticoagulant drugs function by way of multiple mechanisms, that include inhibition of enzymes involved in the addition of carboxyglutamic acid to the vitamin K-dependent coagulation proteins (Warfarin), targeting serine protease that are involved in blood coagulation for activation (Heparin), and blocking receptors that are crucial for platelet adhesion, aggregation, and fibrinogen binding (Clopidogrel). In a similar manner, drugs that target the megakaryocytes receptors involved in endocytosing factor V could prevent factor V internalization, and thus the formation of the more procoagulant form of factor V, platelet-factor V. It would be expected that the megakaryocytes would continue to fragments into platelets therefore intact platelets would still be preserved in whole blood; however, the absence of platelet factor V in the platelets would significantly reduce the efficiency of blood coagulation as factor V enhances the catalytic rate of prothrombin activation in the prothrombinase complex, and the ratio of platelet to plasma factor V at the site of injury far exceeds that of plasma to platelet factor V.

Additionally, the megakaryocyte endocytic receptor system could be exploited to develop ways to introduce factor VIII into the platelets of individuals with hemophilia A due the structural similarities between factor VIII and V. In individuals with hemophilia A, which is characterized by a deficiency in factor VIII, who are on replacement factor VIII therapy, the production of neutralizing factor VIII antibodies presents complications. Therefore, extensive work is being done that includes studies ranging from gene therapy
to drug development against potential inhibitors of factor VIII in an attempt to regulate factor VIII levels in these patients. Since factor VIII is homologous to factor V, and furthermore serves as an LRP-1 ligand, the identification of the receptor binding regions in the light chain of factor V could allow for the manipulation of factor VIII such that it can be endocytosed by megakaryocytes, and ultimately stored in platelets. This would lead to the maintenance of intact factor VIII in platelets, and contribute to balance in hemostasis in individuals with hemophilia A.

Summary

Studies aimed at understanding the mechanism by which factor V is internalized by megakaryocytes are important given the significance of platelet factor V in maintaining normal hemostasis, and the fact that this cellular process could be manipulated to develop potential therapies aimed at maintaining normal hemostasis. Presently, the endocytic mechanism is not fully characterized, and key questions including the identity of the regions of factor V that are involved in interacting with the megakaryocyte receptors remain to be addressed. The goal of the studies detailed in this thesis was to epitope map anti-factor V antibodies found to inhibit internalization of factor V by megakaryocytes. In Chapter 2, conventional approaches for epitope mapping antibodies will be described. Chapter 3 will define the approaches utilized in the current study, as well as the results of these experiments. Finally, future approaches to unequivocally identify these antibody epitopes are detailed.
CHAPTER TWO: Introduction to Epitope Mapping
Epitope mapping is a key step in antibody characterization, particularly in instances where that antibody serves as a research tool or is used in therapeutic strategies. It can provide substantial information regarding protein and ligand recognition for understanding structure-function relationships important for biological processes. The identification of antigen epitopes can aid in the design of vaccines for the treatment of viral diseases. Antibodies can shed light on causes of disease found to have a neutralizing potency, as certain diseases are a result of antibodies elicited by microorganisms and unrelated proteins. Functionally important residues on proteins can also be identified through epitope mapping of antibodies that are known to effect protein function. Antibodies that have defined binding sites can also serve in determining the topology of transmembrane proteins and protein orientation with respect to intracellular structures. Furthermore, antibodies are important for the medical diagnosis of certain diseases, including HIV and Hashimoto’s disease (Zaletel 2007; Kalus, Wilkemeyer et al. 2011). The focus of this thesis is epitope mapping studies of monoclonal antibodies to define a receptor binding site(s) within the coagulation protein, factor V.

2.1 Molecular Structure of Antibodies

Antibodies (150 kDa) are comprised of four polypeptide chains, two heavy (H) and two light (L) chains of 50 kDa and 25 kDa, respectively (Figure 2.1a). The structure of the heavy chain determines which immunoglobulin (IgG) class an antibody will fall into- IgE, IgM, IgA, IgD, or IgG-with the later isoform being the most prevalent in serum. IgG is also the most widely utilized antibody isoform in biological applications.
Figure 2.1 Structural characteristics of antibodies

(a) Antibodies are composed of four polypeptide chains (two identical heavy or H chains and two identical light or L chains). The two heavy chains are covalently linked to the two light chains through disulfide bridges. Each chain contains N-terminal variable (V) and C-terminal constant (C) regions. Each H chain is divided into a specific number of immunoglobulin domains depending on the antibody isotype. In the case of IgG, each heavy chain contains a V_H, C_H1, C_H2, and C_H3 domains, while each L chain contains a single variable and constant domain (V_L and C_L). The V_L and C_L domains are disulfide linked to the V_H and C_H1 domains forming the Fab domain of an antibody that is linked to the Fc domain through a hinge region that is formed by the noncovalent association of the remaining constant domain of the heavy chain (C_H2 and C_H3). 

(b) The building blocks of antibodies are the immunoglobulin domains, comprised of a pair of antiparallel β-sheets held in place by three loops (CDR1, CDR2, and CDR3) on each extremity that adopt a unique conformation termed the immunoglobulin fold. 

(c) Each heavy and light chain variable region possesses three CDR regions, namely V_HCDR1, V_HCDR2, V_HCDR3, and V_LCDR1, V_LCDR2, V_LCDR3 that form the antigen binding site of an antibody. 

(d) The crystal structure of hen egg lysozyme (HEL) antibody-antigen complex. Lysozyme (yellow) interacts with residues (red) at the interface of the antibody and antigen complex. This figure was used with permission from Springer Science + Business Media, and a minor change was made to the original image (Sundberg 2009).
The IgG light and heavy chains contain repeating, independent immunoglobulin domains of approximately 110 amino acid residues in length that fall into one of two categories, variable (V) or constant (C). Each heavy chain is divided into an N-terminal variable domain (VH) followed by three constant domains (CH1, CH2, and CH3). Conversely, each light chain is composed of a single variable (VL) and constant domain (CL) that are covalently linked with the VH and CH1 domains through disulfide bridges. Both the heavy chains are also linked via disulfide linkages.

In 1959, Rodney Porter showed using papain that IgG can be cleaved into three fragments: two antigen binding fragments termed Fab (F corresponds to fragment, and ab to antigen binding) (Porter 1973), which consist of the N-terminal half of the heavy chain (VH and CH1), and the entire light chain (VL and CL), and the Fc fragment (fragment crystallizable) that contains both C-terminal heavy chain halves (CH2 and CH3). The Fab fragments are linked via a flexible hinge region to the Fc fragment. The N-terminal variable regions of the Fab fragments mediate antigen binding, and the central paradigm is that the unique structural arrangement of the immunoglobulin domain of the variable region accounts for the high degree of specificity exhibited by antibodies.

The immunoglobulin domains, which serve as antibody building blocks, each have a common structure (Figure 2.1b). The structure of an immunoglobulin domain consists of a pair of beta sheets organized in antiparallel fashion that form a central hydrophobic core with three loops that lie at one end of the structure forming a binding surface. The loop contains a variety of amino acids that are responsible for the generation of diverse antibody structures with distinct interaction surfaces that are highly specific.
The three loops are referred to as the complementarity determining regions (CDRs) or the hypervariable regions. From the N-terminal end of either the \( V_L \) or \( V_H \), the regions are called CDR1, CDR2, and CDR3, and of all the CDRs, CDR3 is the most variable. The variable regions (\( V_H \) and \( V_C \)) are each composed of two immunoglobulin domains and therefore have a total of six loops or CDRs (\( V_H\)CDR1, \( V_H\)CDR2, \( V_H\)CDR3 and \( V_L\)CDR1, \( V_L\)CDR2, \( V_L\)CDR3) that come together to form an antigen binding site or paratope (Figure 2. 1c). IgGs contain two antigen binding sites and therefore can bind two epitopes. It is the combinatorial association between the CDRs on the \( V_L \) and \( V_H \) and the three dimensional structure formed by these regions that allows for specific antibody recognition and antigen binding. The first direct evidence of the CDRs forming the antigen binding site and being important for specificity came from structural analysis of antibodies with hapten ligands, including phosphorylcholine and vitamin K\(_1\) (Haselkorn, Friedman et al. 1974; Padlan, Davies et al. 1976). Figure 2. 1d highlights the involvement of CDR’s at the interface of lysoyme in complex with the Fab fragment of a lysozyme antibody (Amit, Mariuzza et al. 1986).

The same principles involved in the binding of substrates to enzymes also apply to the binding of antigens to antibodies. Antibody-antigen binding involves noncovalent interactions, including hydrogen bonds, van der Waals interactions, electrostatic interactions, and hydrophobic interactions that allow for strong, specific, and reversible binding. These interactions together with overall surface complementarity (Rini, Schulze-Gahmen et al. 1992) determine antibody affinity, and most antibodies have affinities for their specific antigens in ranges between \( 10^{-7}\)\( \text{M} \) to \( 10^{11}\)\( \text{M} \) (Kranz, Herron et al. 1981;
Kranz, Herron et al. 1982; Foote and Eisen 1995) regardless of the nature of the antibody’s epitope. Aside from proteins, antigens that bind antibodies can also include polysaccharides, carbohydrates, nucleic acids and lipids, however proteins are the only antibody binding partners that can evoke an immune response. Studies have shown that the topology of the antigen binding site of an antibody can vary significantly depending on antigen size. Small antigens typically bind in the cleft of the antigen binding site, whilst macromolecules interface with the extended surfaces in the binding site of the antibody (Jones and Thornton 1996; MacCallum, Martin et al. 1996). Although antibodies are highly specific towards antigens, it is not unusual for them to cross-react with molecules that are structurally similar, but unrelated (Berzofsky and Schechter 1981). An example of cross-reactivity is that of a mouse monoclonal IgE antibody against the hapten 2, 4–dinitrophenol that binds with high affinity ($K_d$-20 nM) (Eshhar, Ofarim et al. 1980), which has also been observed to bind other small molecules with varying affinity, including a structurally unrelated protein, Trx-shear 3 (Varga, Kalchschmid et al. 1991).

The epitopes on antigens, also known as the antigenic determinant, that antibodies recognize and bind to are comprised of a small number of amino acids. The antigenic determinant as a whole, however, is made up of a greater number of amino acid residues besides those involved in direct binding. Epitopes fall under one of two categories: linear (continuous) or conformational (discontinuous) epitopes (Sela 1969). Linear epitopes are composed of adjacent amino acid residues in a polypeptide chain that are presented to the antibody regardless of whether or not the protein is in its native state. Therefore, even
with the application of proteolytic enzymes to produce protein fragments, a linear epitope is highly likely to remain intact and detectable by an antibody though the interactions between the antibody and the linear antigen fragment tend to be weaker as it does not retain the conformation present in the folded protein. In contrast, discontinuous epitopes are highly dependent on protein conformation and are formed by the spatial organization of amino acids formed as a result of protein folding. When an antibody that recognizes conformational epitopes is presented with its antigen partner in an unfolded state, it is highly unlikely that the epitope will be detected. There are exceptions however, as ~10% of antibodies that target conformational epitopes are able to bind fragments from a target antigen because linear epitopes can in fact comprise a large portion of a discontinuous epitope (Barlow, Edwards et al. 1986). The binding affinity of antibodies to such epitope fragments is typically low considering that such epitope fragments are part of a more complex whole.

The discovery of monoclonal and polyclonal antibodies has made it possible to design antibodies against specific antigens, which can be used as specific tools in biomedical studies. Monoclonal antibodies, which are produced from a single clone of B cells, are highly specific and detect a single epitope, whereas polyclonal antibodies can bind to a variety of epitopes. Although, both antibody types are extensively used for research purposes, epitope mapping studies are primarily performed using monoclonals due to their high degree of specificity. The conventional methods that are used in epitope mapping will be described in the succeeding parts of this chapter.
2.2 Epitope Mapping Methods

There are a number of different methods described in several reviews that can be utilized to epitope map antibodies (Johne, Gadnell et al. 1993; Kustanovich and Zvi 1996; Megy, Bertho et al. 2006). The approach one decides to employ is largely dependent on the nature of the antibody epitope, therefore it is essential to distinguish between linear and conformational epitopes as an initial step. One of the simplest ways to determine whether an antibody recognizes a linear epitope versus a conformational epitope is by Western blotting. If the antibody is unable to bind after the protein has been denatured using SDS, and analyzed under both reducing and nonreducing conditions, it is highly likely that the antibody epitope is conformational-dependent. Furthermore, under both reducing and nonreducing conditions, it can be determined if the epitope is dependent on a formed disulfide bond. Epitopes that are defined from a structural standpoint typically feature the contact amino acid residues at the interface of the antigen-antibody complex, and are therefore generally comprised of a large number of residues. In contrast, epitopes that are identified using a functional-based method are comparatively smaller and primarily consist of residues that play a direct role in antibody binding. Neither approach is selective as both can be utilized in epitope mapping antibodies with epitopes of a linear or conformational nature, however, not every structural- or functional- based method can be used to identify both epitope types. Finally, it is important to note that linear epitopes are easier to map relative to conformational epitopes given that the complete analysis of epitopes of this nature must be done within the framework of the native antigen structure.
2.3 Structural Approaches

2.3.1 X-ray Crystallography

Crystallographic analysis is a structural approach to epitope mapping that allows for direct visualization of the amino acids involved in interacting with an antibody. While these types of data would be unequivocal, this method is not the most practical and very few epitopes have been mapped this way. Antibody-antigen complexes typically reveal on average between 15-20 amino acid contact residues (Amit, Mariuzza et al. 1986), whereas the conventional functional epitope mapping approaches generally identify fewer important contact residues. The crystal structure of lysozyme in complex with a Fab fragment from a lysozyme antibody was generated in 1986 (Amit, Mariuzza et al. 1986). The interface between the antibody and antigen was observed to be tightly packed with 16 lysozyme residues and 17 antibody residues that were closely associated (see Figure 2.1d). Van der Waals and hydrogen bonding interactions were primarily involved in the interaction, and the CDR3 region in the $V_{\text{Heavy chain}}$ region was found to contribute significantly to the formation of the complementary antibody-antigen complex, which further illustrates the importance of CDRs in generating distinct functional antibody binding sites. To date, there exists a limited number of available antigen-antibody structures.

2.3.2 Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) is another structural approach that is complementary to X-ray crystallography (Zvi, Kustanovich et al. 1995; Megy, Bertho et
al. 2006). Unlike crystallography, NMR is performed in solution so crystal generation is not an issue, however antigen size is a limiting factor and the method is therefore better suited to peptide antigens and unsuitable for the study of complex epitopes. This method is primarily used to characterize the interaction between the antigen-antibody immunocomplex, and typically a priori information regarding the reactivity of the antigen or peptide with the antibody is required. To define the amino acids of the antigen that are in contact with the antibody, small peptides against the antibody of interest are synthesized with various isotopes (\(^{13}\)C, \(^{2}\)H, or \(^{15}\)N), and used to assemble an immunocomplex that is then analyzed by NMR. The epitope of anti-gp120, a human immunodeficiency virus (HIV) antibody, was mapped by NMR using a 24 amino acid peptide (RP135), that corresponded to an antigenic determinant of the virus glycoprotein gp120 (Zvi, Kustanovich et al. 1995). The complex of RP135 with the anti-gp120HIV neutralizing antibody, which cross-reacts with the peptide, featured 14 contact residues as part of the antigenic determinant recognized by the antibody that was further confirmed by competition binding with truncated RP135 peptides.

2.3.3 Computational Methods

Another structure-based epitope mapping approach is the use of computational epitope prediction tools that are based on the knowledge of a protein’s three-dimensional structure and/or sequence. These bioinformatics tools typically target structural properties and protein characteristics that could be used to identify potential epitope regions within proteins. The first attempts at epitope prediction based on protein structure began in 1984
when temperature factors, a crystallographic parameter, were found to correlate with epitopes in lysozyme, the tobacco mosaic virus, and myoglobin (Westhof, Altschuh et al. 1984). Thornton and colleagues also found that areas of high protrusion indexes in 3D structures corresponded to protein epitopes in myohaemerythrin and lysozyme (Thornton, Edwards et al. 1986). Additionally, a relationship between epitopes and solvent accessibility as well as flexibility of the antigen determining region was established (Novotny, Handschumacher et al. 1986). These findings gave rise to the web-based tools that allow for the prediction and visualization of epitopes in the primary and 3D structure of proteins.

An example of an epitope prediction tool that can be described as a structure-based function recognition method is one originally designed by Delisi et al. that utilizes all-atom molecular dynamic simulation in monitoring protein motions on a timescale that reveals interactions and conformations that could be important for function and recognition (Ma, Wolfson et al. 2001; Glazer, Radmer et al. 2009; Kamberaj and van der Vaart 2009). This de novo prediction method has been utilized in predicting epitopes in a number of drug-based studies (Lerner, Bowman et al. 2007; Glazer, Radmer et al. 2009). Using molecular dynamics, the epitope of H5N1 hemagglutinin 1046T was predicted based on molecular dynamics simulations on monoclonal antibody variable fragment 8H5Fv (Mulyanto and Saleh 2011). Another example of an epitope prediction tool is one that combines protein dynamics and energetics to determine low intensity, energetic interaction networks in the protein structure (Scarabelli, Morra et al. 2010). The idea is that epitopes may correspond to regions that interact with other parts of the protein via
low intensity energy interactions, which triggers conformational changes within the protein.

This method requires the use of protein antigens for which crystal structures are available either in isolation or in complex with an antibody so that the validity of the epitope prediction can be verified using the corresponding structure of the antibody-antigen complex. Additionally, these in silico approaches are typically combined with in vitro approaches to verify the predicted epitopes. A number of other prediction methods that utilize other epitope features in prediction including solvent accessibility, and spatial distribution also exist. As the available X-ray crystallographic structures of antigen-antibody complexes in the protein data bank (PDB) continue to grow, it is anticipated that there would be an increased utilization of 3D structure-based prediction methods.

2.4 Functional Approaches

The functional epitope mapping approaches can be divided into four major categories 1) competition methods 2) protein/antigen modification 3) fragmentation methods and 4) use of synthetic peptides. These methods primarily entail the introduction of a variable into the immunocomplex, which is then followed by testing for the antigen function.

2.4.1 Competitive Binding to the Antigen
The basic principle behind this method is to determine whether two antibodies would bind an antigen at the same time, which would imply that they have different epitopes, or compete with each other for antigen binding. These types of studies are typically performed using the antibody of interest and a well-characterized antibody with a known antigen binding site. Alternatively, a ligand that is known to bind the antigen could be used. In the event that the antibodies compete with each other for binding, one can approximate the epitope of the antibody of interest considering it overlaps with that of the competitor. Experiments using this approach are performed using a radio- or enzyme-labeled form of the antibody, an unlabeled form of the antibody of interest, and the antigen immobilized onto a solid support (Tzartos, Rand et al. 1981; Thanh, Man et al. 1991). Antibodies that have similar epitopes will compete and the labeled antibody will be displaced from the immobilized antigen. A simpler method based on this concept is the use of Ouchterlone gel-diffusion plates, which are used to monitor the binding of an antibody to a ligand and produce a thin white line that indicates formation of the immune complex. In the event that the competing antibodies recognize the epitope, no signal would be formed. Competition studies can also be performed using a Biacore approach. Biacore is a biosensor based on surface plasmon resonance, which utilizes an optical method to measure binding interactions in real time (Johne, Gadnell et al. 1993; Clement, Boquet et al. 2002; Wassaf, Kuang et al. 2006). These competitive approaches are applicable to both linear and nonlinear epitopes and cannot distinguish between either type.
Taylor et al. characterized and epitope-mapped monoclonal antibodies (NS1, NS1, NS5, CS6, CS8, and CS9) against p22\textsuperscript{phox}, a subunit of the integral membrane protein flavocytochrome b (Cyt b) that forms the catalytic core of the NADPH enzyme complex (Taylor, Burritt et al. 2004). The antibodies were observed to detect p22\textsuperscript{phox} by western blotting, and to identify their epitopes, peptides were generated using a phage display library. Of the six antibodies, three (NS2, NS5, and CS9) were observed to react with peptides corresponding to distinct regions of p22\textsuperscript{phox} while CS6 and CS8 were found to bind a region previously characterized as the epitope of a discontinuous Cyt b monoclonal antibody, 44.1. Competition studies coupled with FRET were performed using these antibodies and 44.1, a previously characterized Cyt b antibody. Both CS6 and CS8 were observed to inhibit binding in the presence of 44.1 suggesting that all three antibodies recognize overlapping epitopes as indicated by phage display. CS9, NS1, and 44.1 were found to bind simultaneously and FRET indicated that their epitopes are within close proximity to the 44.1 epitope, whilst NS5 was observed to bind far away. Furthermore, the effect of the characterized monoclonal antibodies on the catalytic activity of the NADPH enzyme complex was examined and CS9, NS1, NS5, CS6 and CS8 were found to inhibit superoxide formation. These antibodies, therefore, are presented as a valuable set of probes for further investigation of cytochrome b function in superoxide production.
2.4.2 Protein Modification

This approach is especially useful for the identification of epitopes of a conformational nature that are difficult to map with simpler techniques. A range of techniques are employed in modifying antigens, including: protection from selective chemical alteration by antibody binding, site-directed mutagenesis, polymerase chain reaction (PCR)-random mutagenesis, NMR-hydrogen deuterium exchange, recombinant deoxyribonucleic acid (DNA) methods, and homolog scanning. Several of these techniques are described below.

2.4.2.1 Site-Directed Mutagenesis

The use of site-directed mutagenesis entails either prior knowledge of an epitope spanning region or scanning the primary sequence of an antibody and predicting regions that could potentially be involved in antibody binding. Alternatively, potential binding regions could be selected randomly from a primary sequence. Site-directed mutagenesis can be performed in multiple ways. One example features the use of recombinant complementary DNA (cDNA) to express antigen variants that have specific mutations. cDNAs of varying length containing an amino acid codon that differs from that in the native DNA sequence can be synthesized to produce protein variants that are different from the native protein. The reactivity of the antigen variants with antibodies can then be determined using a variety of assays. Mutations that have huge effects on binding would suggest that the antibody recognition site is possibly compromised. Alternatively, mutations can be introduced by PCR mutagenesis. The expressed proteins can then be tested for cross-reactivity with the antibodies to screen for epitope-negative mutants. If
properly designed, site-directed mutagenesis can allow for the identification of amino acids within the primary sequence of an antigen (Alexander, Alexander et al. 1992; Mengwasser, Bush et al. 2005). For antigens with a known crystal structure, site-directed mutagenesis can aid in confirming the specific residues that are involved in antibody binding (Vidali, Hidestrand et al. 2004). It is important to note that this technique is laborious, and would be facilitated by prior knowledge of an epitope.

2.4.2.2 Differential Chemical Modification

The location of an epitope can also be mapped using differential chemical modification techniques. Chemical modification entails the use of a chemical reagent that targets specific amino acid side chains within a protein (see Table 2 for a list of protein modifying reagents) (Figure 2.2). Amino acid residues commonly targeted for protein modification include lysine, arginine, tyrosine, tryptophan, aspartic acid, and glutamic acid. Following modification, mass spectrophotometers are routinely used to analyze modified protein or peptide samples. In the event of successful modification; an antigen can then be screened for its ability to bind the antibody of interest. A number of criteria must be met to optimize mapping of an epitope using this method: 1) chemical modification must not alter protein conformation; 2) surface exposed amino acid side chains should be targeted for modification; 3) the modifying reagent must be specific for one amino acid or a group of amino acids; 4) the modified groups must be able to withstand the conditions required for mass spectrometry analysis (extremely acidic or basic). Failure of the antigen to bind to the antibody would suggest that its epitope is
Table 2. Reagents for the chemical modification of proteins

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Specificity/Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic anhydride</td>
<td>Lysine, e-amino groups, tyrosine hydroxyl; preferred reaction is at lysine; pH 8 or greater; reaction can be “driven” to α-amino groups at pH less than 6.5. Avoid nucleophilic buffers such as Tris; hydrolysis of the reagent is an issue above pH 9.5. Acetic anhydride has been used for trace labeling in the study of protein conformation, and more recently the deuterated derivative has been used in proteomics for differential isotope tagging.</td>
</tr>
<tr>
<td>N-Acetylimidazole (l-acetylimidazole)</td>
<td>Tyrosine hydroxyl groups, lysine e-amino groups, transient reaction at histidine; neutral pH.</td>
</tr>
<tr>
<td>Bromoacetamide (2-bromoacetamide)</td>
<td>Cysteine, reaction with active site histidine residues, also reaction with lysine, methionine, and possibly carboxylic acids. Reaction at pH 5–9, but reaction with methionine at pH 3.0. Reaction rate below pH 7.5 is usually slow as the modification of cysteine requires thiolate anion ($pK_a$ for cysteine is 8.7. Reaction is slower than iodoacetamide. A neutral reagent.</td>
</tr>
<tr>
<td>Bromoacetic acid (2-bromoacetic acid)</td>
<td>Reaction parameters similar to bromoacetamide except bromoacetic acid is a charged reagent at pH greater than 4 ($pK_a$ is 2.7 at 25°C). Amide and acid derivatives can show different reaction patterns.</td>
</tr>
<tr>
<td>Bromoethylamine</td>
<td>Modification of sulfhydryl groups; conversion of cysteine to lysine analogue (2-bromoacetic acid); reaction with cysteine at alkaline pH (see bromoacetamide). Reaction is reasonably specific for cysteine with possible modification at the amino-terminal α-amino group and histidine.</td>
</tr>
<tr>
<td>N-Bromosuccinimide</td>
<td>Modification of tryptophan with some oxidative side reactions; pH 4–6.</td>
</tr>
<tr>
<td>2,3-Butanedione (diacetyl)</td>
<td>Modification of arginine residues; reversible reaction with the product stabilized by the presence of borate; reaction at alkaline pH.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Specificity/Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Iminothiolane (Traut’s reagent)</td>
<td>Insertion of a sulfhydryl group into a protein via modification of a lysine residue. This reaction can be used for other amino functions and can be useful for matrix building.</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>Reaction is faster than with bromo- or chloro derivatives. Reaction characteristics similar to bromoacetamide. As with bromoacetamide, iodoacetamide is neutral.</td>
</tr>
<tr>
<td>Iodoacetic acid</td>
<td>See bromoacetic acid for reaction conditions. As with bromoacetic acid, reagent is charged at pH greater than 5 ($pK_a$ is 3.12 at 25°C).</td>
</tr>
<tr>
<td>Methyl acemidate</td>
<td>Modification of amino groups. Imido esters are the functional groups for a number of cross-linking agents such as dimethylureimidate. One of the more interesting imido esters is methyl picolinimidate. Reaction at pH 8–10.</td>
</tr>
<tr>
<td>Methyl methane thioulsulfonate</td>
<td>Methyl methanethiosulfonate is one of a group of alkyl methanethiosulfonate derivatives which reversibly modify cysteine residues in proteins. Reaction occurs at slightly alkaline pH (pH 7.5).</td>
</tr>
<tr>
<td>2-Nitrophenylsulfonyl chloride (α-nitrophenyl-sulfonyl chloride)</td>
<td>Modification of tryptophan residues in proteins; reaction occurs at acid pH; modified tryptophan can be converted to the 2-thiotorphophan derivatives. This modification has been used to purify tryptophan peptides from protein hydrolysates. An analogue, 2-(trifluoromethyl)-benzenesulfonyl chloride has been developed for use in mass spectrometry.</td>
</tr>
<tr>
<td>Phenylglyoxal</td>
<td>Modification of arginine residues in proteins; reaction accelerated in the presence of bicarbonate buffers; reaction at alkaline pH. $\beta$-Hydroxyphenylglyoxal and $\beta$-nitrophenoxyglyoxal are useful derivates.</td>
</tr>
<tr>
<td>Sodium sulfite</td>
<td>Oxidative sulfotolysis to cleave disulfide bonds; conversion of cysteine to S-sulfocysteine.</td>
</tr>
</tbody>
</table>

This table was reproduced with permission from Springer Science + Business Media. (Hoyer 1981).
Figure 2.1 General approach for differential chemical modification of antigens coupled with mass spectrometry

See text for details. This figure was reproduced with permission from Springer Science + Business Media. (Dhungana, Fessler et al. 2009).
likely to contain a modified residue or residues. In this event, the antigen-antibody complex can then be subjected to chemical modification alongside the free antigen, and both the eluted and free antigen can be analyzed by mass spectrometry. The antigen regions involved in antibody binding should in theory be shielded and therefore reactivity with the modifying reagent should be markedly diminished. This method is commonly referred to as protection from chemical alteration by antibody binding.

2.4.2.3 Hydrogen/Deuterium Exchange

Another modification technique that bears strong similarity to modification with chemical reagents is hydrogen/deuterium exchange (H/D exchange) (Figure 2.3). This method, unlike chemical modification, is not specific towards one type or group of amino acids. Rather, the entire antigen is targeted for modification as the hydrogens on the backbone amide on the protein backbone are the groups targeted for exchange with deuterium, though occasional exchanges have been observed at some side chain amino acids. However, exchanges other than those occurring at the backbone amide hydrogens are excluded during analysis. There are a number of factors to consider that affect H/D exchange rate, namely hydrogen bonding type, solvent accessibility, and protein primary structure. Solvent accessibility is a key factor to consider in H/D exchange as the rate of exchange is typically slower at the protected buried amines than the rate of exchange of the bonds that are solvent exposed (Milne, Mayne et al. 1998). The kinetics of exchange at shielded regions versus solvent exposed regions coupled with mass spectrometry can used to study protein-protein interactions, including antigen-antibody interactions (Hoofnagle, Resing et al. 2003; Busenlehner and Armstrong 2005). In epitope mapping
Figure 2. 2 General approach for characterizing conformational and discontinuous epitopes by H/D exchange and mass spectrometry.

Antigen and antibody are individually labeled with deuterium by incubating in D₂O. Immune complex is formed using deuterated antigen and antibody. Back exchange is performed on the free antigen and the immune complex. Following quenching of the exchange reaction, the antigen is proteolytically cleaved using immobilized pepsin at pH 2.25. This figure was used with permission from Springer Science + Business Media. (Dhungana, Fessler et al. 2009).
an antigen, the degree of H/D exchange on the free antigen is compared alongside that of
the corresponding antigen-antibody complex. H/D exchange is carried out by incubating
both the free antigen and antigen-antibody individually in D₂O to allow for the exchange
of the backbone amide hydrogens with deuterium at physiological pH to maintain protein
conformation. Both the deuterated antigen and antibody are then allowed to form an
immune complex and subjected to back exchange so that the deuterons can be substituted
for hydrogens, with the exception of those involved in binding and are protected by the
antibody. The free antigen is also subjected to back exchange in preparation for
comparison with the antigen-antibody complex. The exchange reaction is quenched at
acidic pH values (pH 2-3) at low temperatures (0-4°C) to minimize back exchange
reactions. For analysis, both the free antigen and antigen-antibody complex samples are
subjected to proteolysis with pepsin (or another protease that functions at low pH values)
and mass spectrometry is used to analyze the digested samples. Tandem mass
spectrometry (MS/MS) can be used to identify any preserved deuterons in the sample. If
desired, mass information can also be obtained using the intact protein.

Using H/D exchange followed by matrix-assisted laser desorption/ionization
(MALDI), Baerga-Ortiz et al. identified the epitope of a monoclonal antibody against
human thrombin (Baerga-Ortiz, Hughes et al. 2002). The epitope region, located on the
protein surface, was found to retain deuterium in the presence of the antibody compared
to thrombin in the absence of antibody. This discontinuous epitope was found to be
comprised of two peptides that were localized to exosite I on thrombin and a second site
that overlapped with the thrombomodulin binding site.
2.4.3 Antigen Fragmentation Methods

Epitope mapping can also be achieved by employing sequence-dependent methods that involve the use of antigen fragments. Fragments may take the form of peptide sequences that are generated by limited proteolysis, or cDNA fragmentation. This is a relatively simplified and straightforward approach to detecting epitopes; however, it applies largely to continuous epitopes (Jemerson and Blankenfeld 1989) depending on the fragmentation method utilized though information pertaining to epitopes of a discontinuous nature can also be obtained if the epitope is comprised of regions that are largely continuous.

2.4.3.1 Limited Proteolysis

Epitope mapping by limited proteolysis can be performed using two different approaches: 1) epitope excision, or 2) epitope extraction, which target either the free antigen or the antigen-antibody complex for proteolysis, respectively. During epitope excision (Figure 2.4), the antigen is bound to an immobilized antibody and subjected to proteolysis. In this case, the antibody plays a shielding role given that antibodies in general are relatively robust and resistant to proteases (Parham 1983). The parts of the noncovalently-bound antigen that are protected by the antibody are digested by proteases more slowly than the areas that are readily accessible. This results in shielding of the region(s) where the antigen is localized. Consequently, the peptides that constitute the antibody-binding site are generated at a relatively slower rate during the digestion of the immunocomplex relative to the digestion of the free antigen. The distinction in the
Figure 2. 3 Epitope excision.

See text for details. This figure was reproduced with permission from Springer Science + Business Media. (Dhungana, Williams et al. 2009).
kinetics of antigen digestion can allow for the assessment of the peptides corresponding to the antigen epitope, and the ultimate identification of the epitope in its entirety (Jemmerson and Paterson 1986; Jemmerson and Blankenfeld 1989). The kinetic profile of antigen digestion derived either in the presence or absence of antibody can be generated by monitoring the molecular weight of the antigen by mass spectrometry at various time points, and MS/MS can be employed to determine the amino acid composition of the obtained peptides. For mass spectrometry analysis, the antibody-bound peptides are obtained by separating the unbound peptides from the bound peptides. Alternatively, antibody-bound peptides can be analyzed directly; however, the physical requirements associated with mass spectrometry prevent the use of the antigen-bound antibody samples.

Contrarily, MALDI methods allow for direct placement of the immobilized antibody onto a MALDI plate (Parker, Papac et al. 1996; Peter and Tomer 2001; Parker and Tomer 2002). The bound peptide fragment can be eluted in situ by the addition of an acidic MALDI matrix. This method provides a straightforward and clean approach for sample analysis that is relatively rapid. The success of epitope mapping by this principle is attributed to two factors 1) the resistance of antibodies to proteolysis, especially the antigen-binding domains 2) the high $K_d$ of the immunocomplex which allows for minimal nonspecific interactions.

In contrast to epitope excision in which the immunocomplex is initially formed and subsequently targeted for digestion, epitope extraction (Figure 2.5) features the utilization of free antigen that is digested in solution and reacted with the antibody. This
**Figure 2. Epitope extraction.**

See text for details. This figure was reproduced with permission from Springer Science + Business Media. (Dhungana, Williams et al. 2009).
method is highly dependent on a high affinity between the antibody and antigen. When the digested sample is applied to a column of immobilized antibody, the peptide containing the epitope would bind to the antibody and remain retained on the column. With both techniques, it is important to analyze both the eluted peptides and the unbound peptides present in the wash buffer in order to determine the parts of the antigen that are not interacting with the antibody.

Antibody purity is a concern in the described epitope mapping methods involving limited proteolysis. Some antibodies are not always available as pure samples, a result of being obtained from crude sources that include ascites or cell supernatants, and can interfere with mass spectrometry analysis and/or the immobilization of the antibody to microbeads. These challenges were addressed by Peter and Tomer, who optimized the epitope excision method by introducing indirect immunoabsorption in which an anti-Fc antibody can be used as a secondary antibody to capture the Fc region of the primary antibody (Peter and Tomer 2001). With this approach the antibody of interest can be enriched from a crude mixture without prior purification or immobilization of the proteins contained in the solution. Secondly, since the capture antibody recognizes the Fc portion of the primary antibody, this facilitates a desirable presentation of the primary antibody in which the antigen binding regions are exposed to the solution. Finally, the indirectly immobilized antibody is stabilized by cross-linking the primary antibody to the secondary antibody using a lysine specific cross-linker. Cross-linking also reduces the complexity of sample analysis, as derivatized lysines cannot be cleaved by lysine-specific proteases, and therefore limits the number of peptides that will be generated upon
proteolysis of the immunocomplex (Peter and Tomer 2001).

2.4.3.2 Chemical Fragmentation

An alternative to limited proteolysis is chemical fragmentation. A number of chemicals exist that target specific amino acid residues within a protein for cleavage, including cyanogen bromide (methionine residues) (Houghten and Li 1983), O-iodosobenzoic acid (tryptophan residues and tyrosines to a lesser extent) (Mahoney and Hermodson 1979), and 2-nitro-s-thiocyanatebenzoic acid (cysteine residues) (Stark 1977). Following chemical fragmentation, experiments are performed to analyze the reactivity of the antigen fragments with the antibody. The method is analogous to that of limited proteolysis in that fragmentation can be performed in the presence or absence of antibody. It is important to note that the conditions for chemical fragmentation are usually denaturing, and therefore are not ideal for discontinuous epitopes, but can be used to identify continuous epitopes.

2.4.3.3 cDNA Fragmentation

If the antigen of interest was derived recombinantly, many other methods for generating antigen fragments are available. Gene-targeted random epitope libraries consisting of diverse peptides can be generated using DNA clones encoding the target antigen (Nguyen and Morris 1993). Random digests of the recombinant cDNAs can be performed with DNase 1, followed by cloning and expression of the cDNA fragments in bacteria to generate random epitope libraries that are subsequently screened with
antibodies by immunoblotting. Phage display methodology is another gene-targeted approach for epitope mapping that also entails the use of a library of epitopes (Wang and Yu 2004). Unlike other random peptide libraries generated by bacterial expression, a rod-shaped virus of bacteria known as filamentous bacteriophage are utilized in displaying the library, and are believed to provide a better environment for folding thereby promoting the display of conformational epitopes. For antigen fragment or peptide display, mainly the phage gIII and GVIII genes encoding coat proteins have been targeted for genetic modification. Phage libraries are prepared by cloning small fragments of DNA encoding the antigen of interest into a phage expression vector consisting of a coding sequence for the phage coat protein allowing the protein to be displayed on bacteriophage. By utilizing a collection of gene fragments, the generated library is not largely diverse, but rather is of limited complexity and presents the native peptide sequence, thus, increasing the effectiveness of the method. Selection of the epitope presenting phage is accomplished by panning methods that are enriched for affinity selection. Any displayed peptides that are observed to bind to an antibody can be identified by sequencing the encoding insert in the genome of the phage. This approach has the advantage of being able to identify, not only linear, but also discontinuous epitopes. In theory, critical residues involved in antibody binding can be identified and mimotopes, which mimic discontinuous epitope structures, can also be discerned (Wang, Du Plessis et al. 1995; Wang and Yu 2004). This method was employed in epitope mapping complement component C9, a globular membrane inserting protein (Stanley and Herz 1987).
2.4.4 Synthetic Peptide-based Methods

The use of synthetic peptides that mimic parts of an antigen is an alternative approach to epitope mapping that is relatively rapid and practical for linear epitopes. If the primary structure of the antigen is known, the protein sequence can be scanned to generate overlapping peptides (PEPSCAN) (He, Zhou et al. 2005). The resulting peptide library can then be probed for binding to the respective antibody by ELISA or any solid phase binding platform. Furthermore, as the peptides can be synthesized automatically, minimal labor from the user is required.

In contrast, if peptide ligands are to be identified without prior knowledge of the antigen of interest then a combinatorial approach would have to be employed where arrays of individual peptides sequences are generated randomly (Houghten, Pinilla et al. 1991). This is a de novo approach to peptide synthesis (also known as the SPOTS technique) that entails the synthesis of peptide mixtures with randomized positions that are defined from a statistical standpoint using a certain set of amino acids. To limit the large number of potential peptides that would be generated given the combinatorial nature of this approach, peptides of shorter length are favored, and a limited number of positions within each sequence have defined amino acids. The generated peptides are spotted onto a planar support (microchip) and potential epitopes are subsequently identified by detection of the antibody by a fluorophore-labeled secondary antibody and immunofluorescence microscopy.

In rare cases where antigens are difficult to purify, synthetic peptide-based methods are highly beneficial. One major limitation of this approach is that it is not
useful in the identification of conformational epitopes, which is the category that many protein epitopes fall under. As with every method, each of the aforementioned epitope mapping techniques has its limitations, requires a certain amount of effort, and involves a degree of risk depending on the rate of failure. Often multiple techniques are employed in epitope mapping to ensure that the observations made are unequivocal.

To date, a number of attempts have been made to epitope map antibodies. With the ever increasing need for understanding structure-function relationships, protein-protein interactions, and vaccine development amongst other research goals, novel epitope mapping approaches are likely to be introduced and the existing methods will be optimized. Additional information, including detailed backgrounds, materials and applications, and stepwise protocols for each approach can be found elsewhere (Reineke and Schutkowski 2009).

**Summary**

In this study, epitope mapping approaches were undertaken to define the receptor binding region(s) in factor V that allow for its binding to and endocytosis by megakaryocytes. Previous studies indicate that the light chain of factor V mediates its endocytosis. A monoclonal antibody, E9, directed toward the light chain of factor V has also been observed to inhibit factor V binding and endocytosis by megakaryocytes suggesting that the factor V light chain region(s) recognized by this antibody is/are likely to be involved in receptor binding. Thus, epitope mapping studies of E9 to identify the
amino acid regions within the factor V antigen targeted by this antibody were performed and are outlined in Chapter 3.
CHAPTER THREE: Studies to Identify the Factor V Residues Mediating its Endocytosis to Form the Unique Platelet-derived Cofactor Molecule
3.1 MATERIALS AND METHODS

3.1.1 Materials Iodine-125 and the Western Lightening chemiluminescence reagent were obtained from PerkinElmer, Inc (Waltham, MA). Iodogen tubes were from Thermo Scientific (Rockford, IL). Hirudin was from Calbiochem (San Diego, CA). Vesicles comprised of 75% phosphotidylcholine/25% phosphatidylserine (PC/PS) were generously provided by Dr Kenneth Mann (University of Vermont, Burlington, VT). Tris-glycine gels (4-12% and 10% gradient gels) were from Invitrogen (Carlsbad, CA). Simplastin was purchased from Trinity Biotech (Berkley Heights, NJ). Apiezon Oil was from J.B Biddle Co (Blue Bell, PA). Asp-N from Pseudomonas fragi, recombinantly prepared Trypsin Gold (mass spectrometry grade), and ProteaseMAX, were purchased from Promega (Madison, WI).

3.1.2 Proteins and Antibodies Human factor V was purified from pooled plasma by immunoaffinity chromatography and characterized as described previously (Nesheim, Katzmann et al. 1981). Human thrombin and factor Xa were purchased from Haematologic Technologies, Inc. (Essex Junction, VT). Bovine serum albumin (BSA) was obtained from MP Biomedicals (Solon, OH). Mouse monoclonal antibodies directed towards human factor V (E9 and anti-factor V #2, #17, and #9) were obtained from the Monoclonal Antibody Core Facility (Department of Biochemistry, University of Vermont, Burlington, VT, USA). Nonspecific mouse IgG (Reagent Grade) was from Sigma (St Louis, MO). Horse anti-mouse IgG coupled to horseradish peroxidase was from Jackson ImmunoResearch Laboratories, Inc (Westgrove, PA).
3.1.3 Cell Culture Reagents The megakaryocyte-like cell line, CMK, was obtained from DSMZ (Braunschweig, Germany). Fetal bovine serum (FBS), 100X penicillin (10,000 U/mL)/streptomycin (10,000 µg/mL) solution and 100X L-glutamine (200 mM) were all purchased from Invitrogen.

3.1.4 CMK Cell Culture. The megakaryocyte-like cell line, CMK, originally derived from a megakaryoblastic leukemia patient, was cultured as described previously (Taniguchi, London et al. 1999). Cells were maintained in growth medium consisting of RPMI-1640 media supplemented with 10% FBS, 2 mM L-glutamine, and 100 U/mL penicillin/100 µg/mL streptomycin (CMK complete media). The cells were subcultured once a week (day 7) by centrifugation (250 x g, 8 min) and resuspension (1:40) in CMK complete media, and maintained at 37°C in an atmosphere of 5% CO₂ and 99% humidity. The cells were utilized for experiments when they had reached a plateau in their growth (days 5 and 7) prior to replenishing their growth medium. Prior to experimental use, the cells were washed twice by centrifugation followed by resuspension with 5mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 0.14M NaCl, 2.7mM KCl, 12 mM NaHCO₃, 0.42mM NaH₂PO₄, 1mM MgCl₂, pH 7.4 containing 5mM dextrose and 0.35% bovine serum albumin, (Ca²⁺-free HTA buffer). Viability was assessed by trypan blue exclusion.

3.1.5 Factor V Iodination. Factor V was radiolabeled as described using the Iodogen transfer technique (Monkovic and Tracy 1990). Briefly, iodine-125 in its sodium bound
state (Na$^{125}$I) was activated in an Iodogen precoated tube by incubation for 6 min at 4°C. Following activation, factor V (0.5 mg) was added to the free iodine and incubated for 9 min at 4°C. The radiolabeled factor V (125I-factor V) was separated from free iodine using gel filtration chromatography, and trifluoroacetic acid precipitation was used to determine the percentage of radiolabeled protein. A γ-counter (Cobra II Auto-Gamma Counter, Canberra-Packard, Canada, IL) was utilized for radioactivity detection. Subsequent to radiolabeling, the protein was analyzed by SDS-PAGE (5000 cpm/lane) and phosphorimaging. Its activation to factor Va was assessed by incubation of 125I-factor V with thrombin overtime followed by SDS-PAGE and autoradiography as noted in a previously described protocol (Suzuki, Dahlback et al. 1982).

3.1.6 SDS-PAGE and Phosphorimaging. Proteins diluted 1:5 in 312.5mM Tris, 25mM ethylenediaminetetraacetic acid (EDTA), 10% SDS, 50% glycerol, 0.05% bromophenol blue, pH 6.8 (5X SPB) were reduced with β-mercaptoethanol BME (5%), and separated by SDS-PAGE using 4-12% or 10% polyacrylamide gels following the method of Laemmli (Laemmli 1970). For phosphorimaging, gels were fixed with 9% acetic acid and 18% methanol, subsequently treated with 5% glycerol, and dried under vacuum at 65°C. The dried gel was then exposed overnight onto a phosphorimaging screen that was subsequently analyzed using a Molecular Imager FX operating through the Quantity One analysis software v.4.6.3.

3.1.7 Effect of Anti-factor V Antibodies on 125I-Factor V Binding to CMK Cells. To determine the ability of the factor V light chain antibodies (E9 or anti-factor V #2) to
inhibit factor V binding to megakaryocytes, an equilibrium binding method (Bouchard, Meisler et al. 2008) was utilized. CMK cells were washed two times by centrifugation (250 x g, 8 min) followed by resuspension in Ca\(^{2+}\)-free HTA buffer prewarmed to 37°C. Prior to initiating binding, the cells (2x10\(^6\) cells/mL) were equilibrated for 10 min at 37°C and \(^{125}\)I-factor V was preincubated with molar excesses of E9 (1 µM) or anti-factor V #2 (1 µM) for 30 min. Time-dependent binding of 30 nM \(^{125}\)I-factor V to the cells was initiated by incubating the cells with \(^{125}\)I-factor V (-/+ E9 or anti-factor V #2). Aliquots (250 µL) were collected over time (0-25 min), and layered over an Apiezon A oil/n-butylphthalate mixture (1:9, v/v; 500 µL) that was subsequently subjected to centrifugation in order to separate the cell bound \(^{125}\)I-factor V from the free \(^{125}\)I-factor V. The amount of bound factor V was determined by counting the amount of radioactivity associated with the cell pellets using a γ-counter. The radioactivity associated with the supernatants was also determined to ensure the consistency of the aliquot collecting. Specific binding was determined in experiments performed with under steady state conditions using 50-fold molar excess of unlabeled factor V to determine nonspecific binding, which was subtracted from total binding. For experiments in which the ability of E9 to displace CMK cell surface bound \(^{125}\)I-factor V (30 nM) was tested, binding was monitored from 0 to 10 min at which time the reaction mixture was split in two, and E9 (0.1 µM) was added to one reaction mixture. In the second reaction mixture, Ca\(^{2+}\)-free HTA served as the control. Aliquots were removed from both mixtures over time (15-25 min), and bound \(^{125}\)I detected as described above.

In other experiments, varying concentrations of E9 (0, 0.01, 0.02, 0.05, 0.1, 0.2,
0.5, 1.0, 2.0 µM) were incubated with 30 nM $^{125}$I-factor V for 30 min at 37°C. Binding was initiated by the addition of CMK cells (2x$10^6$ cells/mL) in Ca$^{2+}$-free HTA, and the samples were incubated for an additional 30 min. Bound $^{125}$I-factor V was detected as described above.

### 3.1.8 Effects of E9 and Anti-factor V #2 on Factor V Cofactor Activity.

Factor V cofactor activity was measured using a plasma-based clotting assay. A standard curve was generated by making dilutions (1:4 to 1:250) of a normal human plasma pool in 20 mM HEPES, 150 mM NaCl, pH 7.4, containing 5 mM CaCl$_2$, and 0.1% PEG (HBS/Ca$^{2+}$/PEG) Each dilution was mixed with an equal volume of plasma made factor V-deficient by anti-factor V immunoadsorption, and clot formation was initiated by the addition of Simplastin, which contains relipidated tissue factor and Ca$^{2+}$. The factor V activity of each dilution versus the clot time was plotted on a semi-logarithmic scale with the assumption that normal human plasma contains 1 U factor V/mL (7-10 µg factor V/mL). To assess the function of factor V in the presence of E9 and anti-factor V #2, a clotting assay was performed using factor V (1:10 dilution) that was preincubated with 30-fold molar excesses of the antibodies. A non-specific mouse IgG in addition to factor V light (anti-factor V #9) and heavy (anti-factor V #17) chain antibodies known to not affect prothrombinase complex assembly were utilized as controls. The equation generated with the standard curve was used to extrapolate the factor V activity in the presence of the antibodies.
3.1.9 Western Blotting of Factor V. For epitope characterization of the factor V antibodies, samples of factor V and Va were separated by SDS-PAGE using 4-12% or 10% gels under reducing or non-reducing conditions and transferred onto a nitrocellulose membrane according to the method of Towbin et al. (Towbin, Staehelin et al. 1979). Briefly, nonspecific binding sites were blocked with 5% nonfat dry milk in 20 mM Tris, 0.15 M NaCl, pH 7.4 (TBS) containing 0.05% Tween 20 (TBS-T) overnight at 4°C. Factor V and Va were probed with each of the factor V light chain antibodies, E9, anti-factor V #9, and #2 (5 µg/mL each), in TBS-T for 1hr at ambient temperature. Following three washes with TBS-T, bound antibody was detected using a horse anti-mouse IgG secondary (0.2 µg/mL) conjugated to horseradish peroxidase, followed by chemiluminescence.

3.1.10 Factor Xa Proteolysis of Factor Va. Factor Va was subjected to proteolysis by factor Xa as previously described (Tracy, Nesheim et al. 1983). Factor V (1µM) containing trace $^{125}$I-factor V (5000 cpm/µL) in HBS/Ca$^{2+}$/PEG was activated with 20 nM thrombin (10min, 37°C) as described by Suzuki et al. (Suzuki, Dahlback et al. 1982). Hirudin (30 nM) was subsequently added in order to inhibit further thrombin activity. Factor Va (60 nM) was incubated with 20 µM PC/PS vesicles for 15 min at 37°C. Following incubation, 52 nM Xa was added and aliquots removed over time (0-60 min). The reaction was quenched by the addition of 5X SPB to a final concentration of 1X. To analyze cleavage of factor V, the collected samples were analyzed by SDS-PAGE and autoradiography as described. In some experiments, factor Va was pre-incubated with 0.1
µM E9, anti-factor V #2, a non-specific mouse antibody, anti-factor V #9, or anti-factor V #17 for 30 min prior to cleavage of factor Va with factor Xa. Densitometric analyses were performed using the Quantity One software v.4.6.3.

3.1.11 Immunoprecipitation of Factor Xa-Cleaved Factor Va by E9. $^{125}$I-factor Va was cleaved by factor Xa as described above. E9-coupled to sepharose, prepared as previously described (Katzmann, Nesheim et al. 1981; Nesheim, Katzmann et al. 1981), was equilibrated with 20 mM HEPES, 0.15 M NaCl, pH 7.4 containing 5 mM Ca$^{2+}$ (HBS/Ca$^{2+}$) by centrifugation (3X, 2000 x g, 3 min), followed by resuspension. Factor Xa-cleaved $^{125}$I-factor Va was incubated with the immobilized E9 for 2 hours at 4°C with gentle rocking. Unbound protein was removed by centrifugation as described above. The resin was washed with HBS/ Ca$^{2+}$ or 20 mM Tris, 1.65 mM NH$_4$Cl, 1 mM benzamidine, pH 7.4 (high salt buffer) until the OD ≤ 0.01. Bound protein was eluted with 62.5mM Tris, 5mM EDTA, 2% SDS, 10% glycerol, 0.01% bromophenol blue, pH 6.8 (1X SPB). Additional EDTA (2.5 mM) was added to ensure that the Ca$^{2+}$ was chelated and to promote the dissociation of the heavy and light chain. The eluted samples were analyzed by SDS-PAGE and autoradiography as described. In some reactions, PC/PS vesicles were disrupted by the addition of 10% nonyl phenoxypolyethoxylethanol-40 (NP-40), and 1% Triton X-100 (2 min, 37°C) prior to the addition of immobilized E9.

3.1.12 Solid Phase Binding Assay. A solid phase binding assay for competition experiments was designed using 96-well microtiter plates. The factor V light chain
monoclonal antibody, E9 (33.3 nM), in 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4 (PBS), was immobilized onto the microwells by incubation at 4°C overnight. The plates were washed three times with PBS, and blocked with 5% BSA in PBS for 1 hr at 37°C. To confirm that the immobilized E9 could interact with factor V, the binding of varying concentrations of $^{125}\text{I}$-factor V (0, 15.2, 30.3, 60.6, 90.9, 121.2, 151.5, and 227.3 nM) to E9 was measured following a 2 hr incubation at RT with gentle shaking. The unbound proteins were removed by aspiration and the wells were washed with PBS three times. Bound material was eluted with 1N NaOH, and aliquots of the eluted samples were counted using a $\gamma$-counter. To confirm the specificity of E9 in the assay, $^{125}\text{I}$-prothrombin was substituted for $^{125}\text{I}$-factor V. As the amount of protein bound in a solid phase assay is known to increase proportionally with the amount of IgG coated on the wells, varying concentrations (0, 3.3, 6.7, 13.3, 20.0, 26.7, and 33.3 nM) of E9 were also used to coat the wells to determine an optimal concentration of E9 for the assay.

For the competition assay, initial experiments were performed to determine the amounts of factor V needed to displace E9-bound $^{125}\text{I}$-factor V by using a fixed concentration of $^{125}\text{I}$-factor V (15.2 nM), and unlabeled single chain factor V at various concentrations (0, 3.8, 7.6, 30.3, 90.9, 151.5, 303.0, and 454.5 nM) as a competitor. To assess the ability of various forms of unlabeled factor V (factor Va, factor Xa-cleaved factor Va, and proteolytic fragments of factor V) to displace $^{125}\text{I}$-factor V, $^{125}\text{I}$-factor V (15.2 nM) and 10-fold molar excess of each competitor were used.
3.1.13 Proteolysis of Factor V with Asp-N and Trypsin. $^{125}$I-Factor V was digested with Asp-N or trypsin following the in-solution digestion method described per the manufacturer’s instructions. Prior to digestion with Asp-N, $^{125}$I-factor V was subjected to reduction and alkylation by incubation with dithiothreitol (DTT) for 20 min (50-60°C), followed by incubation with 15 mM iodoacetamide (15 min, ambient temperature, in the dark). The digestion enhancer, ProteaseMAX surfactant (1%), was added to solubilize the protein and facilitate digestion. Lastly, Asp-N was added to a final protease:protein ratio of 1:20 (w/w) and the sample was incubated for 18 hr. The protease was quenched either by heating or with the use of an endoproteinase specific inhibitor. In digestions performed with trypsin, $^{125}$I-factor V was incubated with trypsin under non-denaturing conditions at in a final protease:protein ratio of 1:20 (w/w) for 1 hr at 37°C. The reaction was terminated with the use of a serine protease inhibitor cocktail. For analysis, the digested proteins were separated by SDS-PAGE (4-12% gradient gels), and silver-stained by the method of Samson et al. (Sammons, Adams et al. 1981).
3.2 RESULTS

3.2.1 The Effect of Various Factor V Light Chain Antibodies on $^{125}$I-factor V Binding to CMK Cells

As previous studies have demonstrated that factor V endocytosis by megakaryocytes is inhibited by a number of monoclonal antibodies directed against the human factor V light chain (anti-factor V #2, E9, and anti-factor V #5), subsequent analyses were performed to confirm that these antibodies blocked factor V binding as well. Time-dependent binding of factor V to the megakaryocyte-like cell line, CMK, was studied in the absence of Ca$^{2+}$, which is not required for binding of factor V to the cell surface but absolutely necessary for the clathrin-mediated endocytosis of factor V in a minimally altered system (Bouchard, Meisler et al. 2008). Previous observations demonstrate that factor V binding to CMK cells under this condition is mediated by a two receptor system, is specific, time- and concentration-dependent, reversible, and saturable (Bouchard, Meisler et al. 2008). To determine the effects of E9 and anti-factor V #2 on time-dependent binding, a plasma concentration of $^{125}$I-factor V (30 nM) was treated with 10-fold molar excesses of the antibodies, and binding to the CMK cell surface was assessed. Compared to binding in the absence of antibody (Figure 3.1, blue diamonds), binding of $^{125}$I-factor V to the CMK cell surface in the presence of E9 was inhibited by ~75% (Figure 3.1, red squares). In contrast, despite having previously shown to inhibit factor V endocytosis, anti-factor V #2 had no effect on $^{125}$I-factor V binding (Figure 3.1, green circles). As an additional control, time-dependent binding was monitored in the presence of a nonspecific mouse IgG, which resulted in a binding curve nearly identical to that observed in the absence of antibody (data
Figure 3. Effect of monoclonal factor V light chain antibodies on factor V binding.

Time-dependent binding of factor V was monitored in the absence or presence of IgG (E9 or anti-factor V #2). Factor V (30 nM) was pre-incubated with buffer or 10-fold molar excesses of IgG in Ca²⁺-free HTA (30 min). Binding was initiated by the addition of CMK cells (2x10⁶ cells/mL). Each point represents the mean ± standard deviation (SD) of two experiments performed in duplicate. Curves were fit by eye and drawn using Microsoft PowerPoint.
3.2.2 E9 is a Potent Inhibitor of Factor V Binding to the CMK Cell Surface

The inhibition of factor V binding to the CMK cell surface by E9 was further investigated by monitoring the binding of $^{125}$I-factor V in the presence of varying concentrations of E9. For these experiments, binding of $^{125}$I-factor V at each E9 concentration was determined at 30 min, by which time the binding of factor V to the megakaryocyte cell surface is typically at steady state. Under these conditions, the data indicate that $^{125}$I-factor V binding decreases steadily with increasing concentrations of E9, and inhibition was nearly complete at 0.1 µM E9 (Figure 3.2).

The effect of E9 on factor V binding to CMK cells was further characterized to determine if it inhibited binding to one or both receptors by assessing its ability to displace $^{125}$I-factor V from the CMK cell surface. As shown in Figure 3.3, following the addition of E9, a decrease in binding was observed with ~70% of the cell surface bound factor V dissociated by 25 min as compared to binding in the absence of E9.

3.2.3 Western Blotting Analyses of Factor V using Various Anti-Factor V Light Chain Antibodies

To determine the nature of the epitope recognized by E9 and anti-factor V #2, western blotting analyses were performed using intact and thrombin-activated factor V under reducing conditions (Figure 3.4). Another anti-factor V antibody, #9, which is used routinely in the laboratory to detect the factor V light chain by western blotting was
Figure 3. 2 Inhibition of factor V binding to the megakaryocyte cell surface by E9.

$^{125}$I-factor V (30 nM) was pre-incubated with varying concentrations of E9 (0, 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 µM) prior to the addition of CMK cells. After 30 mins, binding was assessed by collecting equivalent aliquots (150 µL) from each sample, separating the cell-surface bound protein from the unbound protein by centrifugation through oil as described, and determining the amount of radioactivity associated with the cell pellets. Nonspecific binding was estimated in a parallel reaction in the presence of a 50-fold molar excess of unlabeled factor V. Specific binding was determined by subtracting nonspecific binding from total binding. The data represent the mean ± SD of the specific binding from two experiments performed in duplicate. The curve was fit by eye and drawn using Microsoft PowerPoint.
Figure 3. 3 Displacement of factor V from the megakaryocyte cell surface with E9.

$I^125$-factor V (30 nM) binding to the CMK ($2 \times 10^6$ cells/mL) cell surface was monitored over time in the absence of IgG. At 10 mins following the initiation of binding, the reaction was evenly divided and added to either a $Ca^{2+}$-free HTA control or 1.5 µM E9. Binding was further monitored by the collection of aliquots at 15, 20, and 25 min. Each data point represents the mean ± SD of two experiments performed in duplicate. The dashed line denotes ~70% inhibition of binding.
Figure 3. 4 Western blotting of intact and thrombin-activated factor V with various anti-factor V light chain antibodies.

Factor V (1 µM) was activated to factor Va by thrombin (2 U/mL, 10 min, 37°C). Thrombin activity was quenched with hirudin (30 nM). Factor V and factor Va samples were diluted 1:5 in 5X SPB and subjected to SDS-PAGE under reducing conditions on 4-12% gels. Resolved proteins were transferred onto a nitrocellulose membrane, and subjected to western blotting as described. Single chain factor V and factor Va were probed using anti-factor V light chain antibodies, #2 (Panel A), #9 (Panel B), and E9 (Panel C). The numbers on the left indicate the migration of the molecular weight markers. NOTE: The data shown in panel A) were obtained from an experiment performed at a different time compared to those shown in Panels B) and C).
included as a control. Both anti-factor V #9 and #2 recognize intact factor V and the factor V light chain. Similar results were obtained under non-reducing conditions (data not shown) These data suggest that these antibodies are directed against a linear epitope within the factor V light chain and suggest that the epitopes are not conformation specific. The anti-factor V antibody, E9, in contrast, did not recognize either factor V or Va. As no reactivity was observed under non-reducing conditions either, these data suggest that E9 is directed against a discontinuous and/or conformation-dependent epitope.

3.2.4 Factor V Cofactor Activity is Not Perturbed by E9

To examine factor V function in the presence of E9, a plasma clotting assay was performed. Factor V was used at 1/10 its normal plasma concentration, which results in an average clot time of 30 sec. In the presence and absence of E9, clot times in the range of 29-30 sec were observed, as were the clot times obtained in the presence of control antibodies. These results suggest that E9 does not affect factor Va binding to PC/PS vesicles or its cofactor activity in Prothrombinase.

3.2.5 E9 Does Not Affect Cleavage of Factor Va by Factor Xa at Arg1765 of the Light Chain

Previous studies have demonstrated that the light chain of bovine and human factor V is cleaved by factor Xa at Arg1765 into two fragments of ~30 and 48/46 kDa (residues 1546-1765 and 1766–2196, respectively) (Tracy, Nesheim et al. 1983; Odegaard and Mann 1987; Thorelli, Kaufman et al. 1997) (Figure 3. 5A). In an attempt to
utilize factor V light chain fragments in epitope mapping studies, initial experiments were performed to examine the cleavage pattern of human factor Va with factor Xa under conditions previously described for the bovine molecule (Tracy, Nesheim et al. 1983). The proteolysis of thrombin-activated $^{125}$I-factor V with factor Xa resulted in the generation of two light chain fragments of 48/46 and 30 kDa (Figure 3. 5). In addition, cleavage at Arg1765 was observed to occur at a relatively faster rate in the presence of PC/PS vesicles as compared to cleavage performed in the absence of a membrane surface (data not shown). To test the ability of the factor V light chain antibodies to detect the factor Xa-generated products of factor Va, western blotting analysis was performed with anti-factor V #2, but not E9, as this antibody does not react with factor V under denaturing conditions. In comparison to anti-factor V #9, which detects the 48/46 kDa fragment, neither the 30 nor the 48/46 kDa cleavage products were detected by anti-factor V #2 (Figure 3. 6) suggesting that its epitope encompasses Arg1765 and that this antibody may inhibit cleavage of factor Va by factor Xa.

To assess this possibility, additional experiments were performed to determine whether the factor Xa cleavage site (Arg1765) within the light chain region of factor V is protected by the association of anti-factor V #2 with the light chain. $^{125}$I-factor V was subjected to proteolysis with factor Xa in the absence or presence of factor V antibodies, including anti-factor V #2, #9, #17, and E9 (Figure 3. 7A). The presence of anti-factor V #2 inhibited factor Va cleavage by factor Xa. After a 10 min incubation in the presence of #2, minimal amounts of the 48/46 kDa cleavage products were present. Unlike anti-factor V #2, E9 did not affect the cleavage of the factor V light chain and behaved similarly to
Figure 3. 5 Factor Va cleavage by factor Xa on PC/PS vesicles.

Panel A) Factor Xa cleaves factor Va at Arg1765, resulting in the production of an N-terminally-derived 30 kDa fragment, and a differentially glycosylated 48/46 kDa fragment derived from the C-terminal end of the factor V light chain. Panel B) Proteolysis of thrombin-activated factor Va by factor Xa was monitored over time. ¹²⁵I-Factor Va (60 nM) was pre-incubated on 20 µM PC/PS vesicles for 15 min, and following the addition of factor Xa (52 nM), aliquots were collected and quenched in 5X SPB at 4°C. The proteins were separated by SDS-PAGE (5000 cpm/lane) and analyzed by autoradiography as described.
Figure 3.6 Anti-factor V #2 does not detect factor Xa-catalyzed cleavage products of factor V by western blotting

Thrombin-activated factor Va was cleaved at Arg1765 by factor Xa as described. Intact factor Va light chain and light chain cleavage products were probed by western blotting with anti-factor V #9 and #2. The numbers in the center indicate the migration of the molecular weight markers. The positions of the intact light chain and the 48/46 kDa fragments are indicated.
Figure 3. E9 does not affect cleavage of the factor Va light chain at Arg1765.

**Panel A)** Cleavage of $^{125}$I-factor Va bound to synthetic phospholipid vesicles by factor Xa in the presence and absence of antibodies was assessed at 0 min (black bars) and 10 min (grey bars) by autoradiography as described. The light chain (FV LC), heavy chain (FV HC), and B domain of thrombin-activated factor Va are indicated, as are the 48/46 kD and 30 kD factor Xa cleavage products. The migration of the molecular weight markers is indicated on the left of each panel. The red box highlights the results obtained in the presence of E9 and anti-factor V #2. **Panel B)** Densitometric analyses of the protein bands visualized by phosphorimaging was performed as described. The bars indicate the mean ± SD of two experiments performed in triplicate.
the control antibodies (a non-specific mouse IgG, anti-factor V #9 and #17). Densitometry analysis of the phosphorimaging data (Figure 3. 7B) indicated that similar to the cleavage of the factor V light chain in the presence of a factor V heavy chain antibody, and a nonspecific mouse antibody, only ~40% of the factor V light chain was retained in the presence of E9 at 10 min. In contrast, ~80% of the light chain was retained in the presence of anti-factor V #2. These data collectively suggest that the E9 recognition site/epitope on the factor Va light chain is not localized around Arg1765.

3.2.6 Binding of Factor Xa-Cleaved Fragments of Factor V to E9

$^{125}$I-factor Va (Figure 3. 8A, lane 1a) was cleaved with factor Xa (Figure 3.8A, lane 2) and immunoprecipitated with E9 to determine which light chain fragment E9 binds. Both the 48/46 and 30 kDa cleavage products were present in the starting material (Figure 3. 8A, lane 2), and each elution (Figure 3. 8A, lanes 4 and 5). Not unexpectedly, the B domain (150 kDa) was not retained on the column and was present in the flowthrough (Figure 3. 8A, lane 3). These data suggest that E9 is binding to an epitope comprised of residues from both the 30 kDa or the 48/46 kDa fragment that are within close proximity when the protein is in its native conformation. Alternatively, it can be hypothesized that the region detected by E9 may be localized within either the 30 kDa or the 48/46 kDa fragment, and that these fragments are noncovalently associated. To test this hypothesis, high salt buffer was used in performing washes after immunoprecipitation with E9 to disrupt any noncovalent interactions contributing to the association between the cleavage products. However, both fragments still appeared in the...
Figure 3. 8 Immunoprecipitation of factor Xa-cleaved factor Va with E9.

$^{125}$I-factor Va was cleaved with factor Xa, and the proteolytic products immunoprecipitated with E9. **Panel A** ) Lanes 1a (lighter exposure), and 1b illustrate thrombin-activated factor V prior to cleavage with factor Xa (lane 2). Lane 3 depicts the flowthrough and lanes 4 and 5 depict the products pulled down by E9. In this particular experiment, washes were performed using HBS/Ca$^{2+}$. **Panel B** ) To disrupt potential noncovalent associations, high salt washes were performed. The lanes are numbered identical to panel A.
elution (Figure 3. 8B, lanes 4 and 5).

As PC/PS vesicles provided a membrane surface for factor Va prior to factor Xa cleavage and incubation with immobilized E9, it was also hypothesized both the 48/46 and 30 kDa fragments were retained on the vesicle surface following factor Xa cleavage. Thus, upon immunoprecipitation, both fragments would be isolated as E9 does not affect factor Va’s interaction with the vesicle surface. However, disruption of the vesicles with detergents prior to the addition of E9 resulted in an elution pattern identical to that obtained when the reaction contained intact PC/PS vesicles (data not shown).

As the heavy chain (105 kDa) fragment of factor V also undergoes cleavage, though at a slower rate compared to the light chain, the cleaved factor Va samples were also subjected to western blotting analysis using a factor V antibody directed towards its heavy chain, anti-factor V #17, to determine if either of the observed products were heavy chain- derived. By western blotting analysis neither the 48/46 nor the 30 kDa fragments were detected by the antibody. Thus, it appears that both of these products are specific to the light chain (data not shown).

3.2.7 Development of a Solid Phase Binding Assay to Probe Interactions Between E9 and Factor V.

A solid phase assay was developed in order study the ability of factor V fragments generated by proteolysis to displace intact factor V from E9. Initial experiments were performed to identify appropriate concentrations of E9 and unlabeled factor V to use. Binding of increasing concentrations of $^{125}$I-factor V to a fixed concentration of immobilized E9 was specific, concentration-dependent, and saturable (Figure 3. 9A). The
specificity of E9 was confirmed with the use of $^{125}$I-prothrombin, and linear, non-saturable binding was observed. In order to determine an appropriate concentration of E9 for the assay, the amount of antibody bound to the plate was varied and its binding to a fixed concentration of $^{125}$I-factor V determined (Figure 3. 9B). Under these conditions, $^{125}$I-factor V binding was saturable and dependent upon the concentration of E9. Based on the results of these experiments, E9 and $^{125}$I-factor V were used at concentrations of 3.33 nM, and 15.2 nM, respectively.

Preliminary experiments were performed using unlabeled single chain factor V to test its ability to displace $^{125}$I-factor V from immobilized E9, and to determine optimal concentrations of competitors that result in displacement for utilization in subsequent experiments. Incubation of $^{125}$I-factor V bound to immobilized E9 with varying concentrations of single chain factor V resulted in a steady, concentration-dependent decrease in bound $^{125}$I-factor V that correlated with the concentration of unlabeled factor V (Figure 3. 10). At concentrations of unlabeled single chain factor V $\geq$151.1 nM, no significant difference was observed with respect to the amount of $^{125}$I-factor V bound. Based on these data, ten-fold excesses of competitors were utilized in subsequent competition assays.
Figure 3. 9 Titration of factor V and E9 in a solid phase binding assay.

$^{125}$I-factor V and E9 were varied to determine appropriate concentrations to utilize for the assay. **Panel A**) Immobilized E9 (151.5 nM) was incubated with various concentrations of radiolabeled factor V (0-230 nM) (blue line). Binding specificity was confirmed with the use of varying concentrations of $^{125}$I-prothrombin (red line). **Panel B**) A fixed amount of $^{125}$I-factor V (15.2 nM) was incubated with varying concentrations of E9 (0, 3.3, 6.7, 13.3, 20.0, 26.7, and 33.3 nM). The data points represent mean ± SD of two experiments performed in duplicate. Curves were fit by eye and drawn using Microsoft PowerPoint.
Figure 3. 10 Displacement of E9-bound factor V with unlabeled single chain factor V.

Panel A) Schematic of the competitive solid phase binding assay. See text for details
Panel B) The ability of single chain factor V to displace E9-bound $^{125}$I-factor V was assessed using a fixed concentration of $^{125}$I-factor V (15.2 nM) and E9 (3.33 nM), and varying concentrations of single chain factor V. $^{125}$I-factor V binding was monitored as described. Bars represent mean ± SD of two experiments performed in duplicate. Curves were fit by eye and drawn using Microsoft PowerPoint.
3.2.8 Displacement of E9-bound $^{125}$I-factor V with Various Factor V Proteolytic Fragments

This highly sensitive assay was subsequently used to identify the ability of various factor V proteolytic fragments to compete with $^{125}$I-factor V for E9 binding. In initial experiments, the ability of factor V, thrombin-activated factor V, and factor Xa-cleaved factor Va to displace $^{125}$I-factor V bound to immobilized E9 was assessed. Each competitor resulted in an ~75-85% decrease in bound $^{125}$I-factor V suggesting that the E9 epitope is not altered/destroyed by these cleavages (Figure 3.11). This observation is consistent with the immuprecipitation data indicating that E9 bound both intact factor Va and factor Xa-cleaved factor Va. Factor Xa-cleaved factor Va was observed to have the most substantial effect on bound $^{125}$I-factor V as ~86% of the protein was displaced. These data strongly suggest that cleavage results in presentation of the epitope in a more favorable conformation for E9 binding.

Additional experiments were performed using fragments of factor V generated with the proteases, Asp-N or trypsin, by limited proteolysis under non-denaturing conditions. Treatment of factor V with each of these proteases resulted in an increase in the appearance of lower molecular weight proteins followed by a decrease over time as illustrated by SDS-PAGE and silver staining (Figures 3.12A and 3.13A). Neither the Asp-N- (Figure 3.12B) nor the trypsin- (Figure 3.13 B) derived factor V fragments displaced bound $^{125}$I-factor V in the solid phase binding assay. Collectively, the data suggest one or both of the following scenarios: 1) the E9 epitope is not retained in factor V fragments generated with Asp-N or trypsin due to cleavages by the proteases 2) the generated
Figure 3. 11 Displacement of E9-bound factor V by factor Va and factor Xa-cleaved factor Va.

The ability of various forms of factor V to compete with $^{125}$I-factor V bound to immobilized E9 was examined using a solid phase binding assay. Ten-fold molar excesses of single chain factor V (black bar), factor Va (blue bar), and factor Xa-cleaved factor Va (green bar) were incubated with a fixed concentration of $^{125}$I-factor V bound to immobilized E9 for 2 hr at ambient temperature. Bound protein was eluted and detected as described. The red bar depicts binding of $^{125}$I-factor V in the absence of an added competitor. Bars represent mean ± SD of an experiment performed twice in duplicate.
Figure 3. 12 Effect of Asp-N-derived factor V fragments on factor V binding to E9.

Factor V was subjected to partial proteolysis with Asp-N, under non denaturing conditions, at a final protease to protein ratio of 1:20. Aliquots were collect over time (0-18 hr), resolved by SDS-PAGE and silver stained (Panel A) as described. The arrows to the right of the panel depict various factor V species. Panel B) Factor V fragments generated with Asp-N at a ten-fold molar excess were incubated with $^{125}$I-factor V bound to immobilized E9 for 2 hr at ambient temperature. Displacement was assessed as described. Data represent mean ± SD of two experiments performed in duplicate. The data are depicted as follows: No added factor V (red bar), single chain factor V (black bar), and Asp-N derived factor V fragments (blue bars).
Figure A: SDS-PAGE gel showing the time course of Factor V (FV) proteolysis with Asp-N protease. The gel lanes are labeled 0 (time control), 30, 60, 90, and 180 minutes (mins). Arrows indicate the positions of the FV single chain (330 kDa) and FV B domain (150 kDa).

Figure B: Bar chart showing the time course of labeled Factor V bound (cpm) in the presence of Asp-N protease. The labels include 'No added FV', '151.5 nM unlabeled FV', and various time points (30, 60, 90, 180 min).
Figure 3. 13 Effect of trypsin-derived factor V fragments on factor V binding to E9.

Factor V was subjected to partial proteolysis with trypsin, under non denaturing conditions, at a final protease to protein ratio of 1:20. Similar to proteolysis performed with Asp-N, aliquots were collected over time (0-60 min), resolved by SDS-PAGE, and silver stained (Panel A) as described. The arrow to the right illustrates the single chain factor V species. Panel B) Displacement experiments with the trypsin-generated factor V fragments were performed as described for Asp-N in Figure 3. 13. Data represent mean ± SD of two experiments performed in duplicate. The data are depicted as follows: No added factor V (red bar), single chain factor V (black bar), and trypsin-derived factor V fragments (green bars)
fragments are not maintaining favorable conformations due to the small sizes of the generated peptides.
DISCUSSION

Factor V binding to and endocytosis by the megakaryocyte two receptor system to form the unique platelet-derived cofactor molecule appears to be mediated by the factor V light chain. In this study, the role of the light chain in factor V endocytosis by megakaryocytes was further addressed, with the primary focus being to specifically identify the amino acid regions within the factor V light chain. The monoclonal anti-factor V light chain antibody, E9, which inhibits both the endocytosis and binding of factor V by megakaryocytes, also displaced bound $^{125}$I-factor V by ~70%. Based on the two receptor model for factor V endocytosis this suggests that E9 is inhibiting the interaction of factor V with the unknown factor V receptor or both receptors. To differentiate among these scenarios, a protein that antagonized LRP-1/ligand interactions, RAP, (Herz, Goldstein et al. 1991; Bu and Rennke 1996; Andersen, Christensen et al. 2000) can be utilized in $^{125}$I-factor V displacement studies. If E9 is affecting binding to the factor V receptor alone, then addition of RAP will inhibit factor V binding to LRP-1 and increase the level of $^{125}$I-factor V displacement. If E9 is affecting binding to both receptors, no difference in displacement would be observed in the presence of E9 alone versus E9 with added RAP.

To date, several phospholipid binding sites within the light chain of factor V have been identified and include: residues 1667-1765 (A3 domain) (Kalafatis, Jenny et al. 1990), Tyr1956, Leu1957, Arg2023, and Arg2027 (C1 domain) (Saleh, Peng et al. 2004), and the Trp2063 and Trp2064 (C2 domain) (Adams, Hockin et al. 2004). Adams et al. solved the crystal structure of the bovine APC-inactivated factor Vα, factor Vαi, which
corroborated some existing data on the membrane binding sites of the factor V light chain by showing that the C2 domain featured a common beta barrel structure with three long loops protruding from one end, two of which contain water-exposed hydrophobic residues that are believed to penetrate membranes. The fact that E9 did not affect factor V cofactor activity in a plasma-based clotting assay suggests that the factor Xa sites mediating its binding to factor Xa, and the lipid membrane surface are likely to be excluded from the epitope.

In addition, Steen et al. identified a His1683 residue localized within the A3 domain of the factor V light chain that is implicated in factor Xa binding (Steen, Villoutreix et al. 2002). Under certain conditions, factor Va bound to PC/PS vesicles or the platelet surface is cleaved by factor Xa forming light chain fragments of 48/46 (residues 1766-2196, A3, C1 and C2 domains) and 30 kDa (residues 1546-1765, A3 and C1 domain) (Tracy, Nesheim et al. 1983; Odegaard and Mann 1987; Thorelli, Kaufman et al. 1997). In the presence of E9, cleavage was unaffected. In comparison, cleavage was reduced in the presence of anti-factor V #2. Furthermore, it is unlikely that the E9 epitope is localized around the factor Xa cleavage. This was confirmed in subsequent experiments using a competitive, solid phase assay and immunoprecipitation, which suggested that the E9 epitope encompasses both fragments or is localized on one of the light chain fragments and these fragments are noncovalently-associated. Indeed, Tracy et al. observed that factor Xa-cleaved factor Va retains its activity in the Prothrombinase complex assembled on platelets (Tracy, Nesheim et al. 1983) suggesting that the fragments remain associated on the membrane surface. A similar observation was also
made by Thorelli et al. using synthetic phospholipid vesicles. In this study, attempts to disrupt noncovalent interactions between the fragments had no effect on E9 recognition.

Further antigen fragmentation using Asp-N and trypsin coupled with the solid phase assay was also attempted. These experiments were performed under limited proteolysis and non-denaturing conditions to generate longer polypeptides that may retain their native conformation. However, the peptides produced with Asp-N and trypsin did not compete with $^{125}$I-factor V for E9 binding suggesting that proteolysis is destroying the epitope by cleavage or the resulting peptides are not retaining their native forms.

In conclusion, this study describes the first attempt at epitope mapping an antibody, E9, that inhibits the binding and endocytosis of factor V by megakaryocytes. The combined observations suggest that E9’s epitope is conformational, specific, and extremely labile. While no definitive conclusions can be made regarding the regions of factor V that form E9’s epitope of factor V that form E9’s epitope, these efforts have resulted in the production of a solid phase binding assay that can be used in future studies.
FUTURE DIRECTIONS

The major objective of this study was to epitope map E9, a factor V light chain antibody, which has been observed to inhibit factor V binding to and endocytosis by megakaryocytes. This study has shown that the E9 epitope is conformation-dependent/discontinuous and extremely labile. However, further studies failed to define what region(s) of the factor V light chain is/are recognized by E9.

In experiments using factor Xa–cleaved factor Va, E9 immunoprecipitated both the 48/46 and 30 kDa fragments suggesting that the light chain fragments are noncovalently associated or that E9’s epitope comprises both fragments. However, it is likely that the products are noncovalently associated as was shown by Tracy et al. in the bovine system (Tracy, Nesheim et al. 1983; Thorelli, Kaufman et al. 1997). To confirm this, the light chain antibody, anti-factor V #9, which detects the 48/46 kDa light chain fragment could be employed in a modification of the solid phase binding assay where wells coated with anti-factor V #9 versus E9 could be used to detect cleaved $^{125}\text{I}$-factor Va. Following extensive washing to remove unbound fragments, phosphorimaging can be used to detect that which remains bound to the antibody. The detection of the isolated 48/46 kDa fragment only by anti-factor V #9 would suggest that the fragments are not noncovalently-associated. This would imply that E9 recognizes both fragments. Alternatively, in the event that the fragments are noncovalently associated, further experiments would have to be performed to separate the fragments. In studies examining the proteolysis of factor Va by factor Xa, Odegaard et al. describe the 48/46 and 30 kDa fragments as being “strongly noncovalently associated,” and used high performance
liquid chromatography (HPLC) to isolate each fragment (Odegaard and Mann 1987). As it is clear that E9’s epitope is not only conformation-dependent, but extremely labile, another approach would have to be explored for our purposes.

A competitive solid phase assay was developed in this study that can be exploited to further address the localization of the E9 epitope, and for future epitope mapping studies of other factor V antibodies (e.g., anti-factor V #5). In this competitive assay, ten-fold molar excesses of the competitors were utilized. Future experiments should be performed with >ten-fold excess of competitor as the polypeptides that were generated may have lower binding affinities relative to the intact protein. Additionally, the use of other proteases, such as Lys C or chymotrypsin to generate fragments of factor V may yield useful information. Alternatively, the solid phase assay could be utilized in experiments designed to identify the E9 epitope by epitope excision as described in Chapter 2. As a control, duplicate experiments could be performed using radiolabeled factor V to confirm retention of factor V fragments on the antibody.

The presented data indicate that anti-factor V #2, which was previously observed to inhibit factor V endocytosis, had no effect on factor V binding to megakaryocytes. The original hybridoma clone of the anti-factor V #2 antibody could be obtained and used to produce a new batch of the antibody. Its effect on binding and endocytosis could then be tested to determine if the newly prepared antibody behaves as expected. The fact that the antibody was observed to inhibit cleavage by factor Xa could suggest that its epitope spans Arg1765. Interestingly, the region (DEKKSWYYEKKSSSWR/LTSEMKKKSHFMHANGMIY), surrounding this
cleavage site (R/L) features a marked presence of lysine residues (shown in blue), which have been shown to mediate the binding of other ligands to LRP-1 (Bovenschen, Boertjes et al. 2003; Ranganathan, Cao et al. 2011). Furthermore, there is a high degree of sequence identity in the amino acids surrounding the factor Xa cleavage site in addition to conservation of lysine residues between the human and bovine forms of factor Va, both of which inhibit factor V endocytosis. Therefore, if possible, further studies with this antibody may be useful to identify what regions of factor V mediate its endocytosis by megakaryocytes. Furthermore, anti-factor V #2 is directed toward a nonconformational/linear epitope, which should be easier to map compared to conformational/nonlinear epitopes.

In studies not presented in this thesis, the arginine-specific reagent, phenylglyoxal, was used in a single attempt to modify factor V for a protein modification approach. Mass spectrometry analysis was unable to identify any modified Arg residues indicating that factor V was not modified by this reagent. Due to time constraints, this approach was abandoned. Future experiments could focus on optimizing the conditions for protein labeling with this reagent, and the solid phase binding assay developed in this study could be used to analyze the reactivity of the modified protein with E9. Additionally, other modifying reagents, specified in Chapter 2, could be employed in the event that modification with phenylglyoxal is ultimately unsuccessful. In addition, H/D exchange, which targets the backbone amide hydrogens for modification could also be performed.

If the experiments described above are not successful, then alternative approaches
would have to be employed. Though time intensive, X-ray crystallography could be used to determine the structure of the immunocomplex of the isolated light chain of factor V (Nesheim, Katzmann et al. 1981) and the Fab fragment of E9. Alternatively, fragments of the factor V light chain could be crystallized with the antibody in the event that we are able to generate fragments of the factor V light chain that can be isolated under non-denaturing conditions. If successful, this approach would allow for the visualization of the contacting residues at the interface of the immunocomplex, which would include the E9 epitope.

Alternatively, bioinformatics could be performed as a number of computational tools for epitope mapping exist. When the three dimensional structure of the antigen is known, the epitope could potentially be predicted computationally. The 2.8 Å crystal structure of APC-inactivated bovine factor Va was solved by Adams et al. (Adams, Hockin et al. 2004), and is readily available in the Protein Data Bank (PDB ID: 1SDD).

Epitope mapping antibodies that target conformational epitopes is a challenging and time consuming endeavor. Extensive work was done in epitope mapping the E9 antibody, and although additional work remains to be done, the data presented in this study present a solid framework that can be used for future studies aimed at defining this antibody’s epitope. Knowledge of the E9 epitope would provide information regarding the cellular process responsible for the generation of platelet-derived factor V, and would specifically contribute to the understanding of the mechanism of the megakaryocyte two receptor system. Furthermore, given that E9 is utilized primarily in isolating factor V
from both human and bovine plasma, defining its epitope would prove useful for other biochemical applications.
REFERENCES


" Blood 106((Suppl. 1): 506a, #1779.).


Ranganathan, S., C. Cao, et al. (2011). "Molecular basis for the interaction of low density lipoprotein receptor-related protein 1 (LRP1) with integrin alphaMbeta2: 120


